Reproducible doxycycline-inducible transgene expression at specific loci generated by Cre-recombinase mediated cassette exchange

Ee Tsin Wong[1,2], John L Kolman[2], Yao-Cheng Li[2], Larry D Mesner[3], Wolfgang Hillen[4], Christian Berens[4] and Geoffrey M Wahl[2,*]

[1]Division of Biological Sciences, University of California at San Diego, La Jolla, CA, USA, [2]The Salk Institute for Biological Studies, La Jolla, CA, USA, [3]Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia, USA and [4]Department of Microbiology, University of Erlangen-Nuremberg, Erlangen, Germany

ABSTRACT

Comparative analysis of mutants using transfection is complicated by clones exhibiting variable levels of gene expression due to copy number differences and genomic position effects. Recombinase-mediated cassette exchange (RMCE) can overcome these problems by introducing the target gene into pre-determined chromosomal loci, but recombination between the available recombinase targeting sites can reduce the efficiency of targeted integration. We developed a new LoxP site (designated L3), which when used with the original LoxP site (designated L2), allows highly efficient and directional replacement of chromosomal DNA with incoming DNA. A total of six independent LoxP integration sites introduced either by homologous recombination or retroviral delivery were analyzed; 70–80% of the clones analyzed in hamster and human cells were correct recombinants. We combined the RMCE strategy with a new, tightly regulated tetracycline induction system to produce a robust, highly reliable system for inducible transgene expression. We observed stable inducible expression for over 1 month, with uniform expression in the cell population and between clones derived from the same integration site. This system described should find significant applications for studies requiring high level and regulated transgene expression and for determining the effects of various stresses or oncogenic conditions *in vivo* and *in vitro*.

INTRODUCTION

Many studies require the ability to compare the functional consequences of introducing mutations into genes of interest. Rigorous comparative analyses require similar gene expression levels in each stable cell clone. Comparison between mutant genes is difficult using the common strategy of deriving stable cell lines as gene expression level is unpredictable due to variable copies of integrated DNA combined with position effects on gene expression. All of these confounding variables can be eliminated if each transgenic clone is derived by independently introducing the gene of interest into a pre-determined genomic locus (1).

Gene targeting by homologous recombination is widely applied in mouse embryonic stem cells (ES cells) and occasionally in other cell types to produce accurately modified loci (2). However, it is a sophisticated and time consuming procedure that can be compromised by the low recombination efficiency of some loci. Alternatively, the RMCE strategy uses bacterial and yeast DNA recombinases to target any DNA sequence into a pre-determined genomic locus that was previously modified to contain the appropriate recombinase recognition sequences (3). For example, the widely used bacteriophage P1 Cre/LoxP recombination system utilizes LoxP sites consisting of two 13 bp inverted repeats and a central 8 bp spacer sequence. The central 8 bp sequence within each LoxP site determines if a pair of LoxP sites is compatible for recombination by Cre (4). LoxP sites containing the same 8 bp core sequences recombine, but LoxP sites differing by one or more base pairs exhibit reduced or no recombination (4–6). The success of RMCE relies on the use of two heterospecific LoxP sites (LoxP sites of different sequences) that will not recombine with each other. Thus, flanking a donor gene of interest with heterospecific sites enables it to precisely replace

genomic DNA flanked by identical sequences upon expression of Cre-recombinase. However, the previously published LoxP sites (L2) and LoxP511 (L1) (4) are not entirely heterospecific when tested in mammalian cells, and are consequently not ideal for RMCE (1,6). For example, deletion of the intervening sequence occurs when L2 and L1 are placed as direct repeats on the same DNA, while inversion occurs if they are placed as inverted repeats (1). Due to the wide usage of RMCE, the development of new LoxP sites with greater specificity for directional exchange is clearly needed.

RMCE can also facilitate structure-function analyses by ensuring reproducible expression of different mutant alleles from the same locus. However, the biological significance of such studies can be compromised if the expression level does not reflect that present physiologically. For that, a reliable inducible system with low basal expression and reproducible ligand-dependent induction is required. Tight regulation of gene expression is particularly important if, e.g., the gene of interest is toxic, or induces cell cycle arrest or apoptosis. The tetracycline (tet) regulatable system has proven especially valuable as it uses an inexpensive inducer (doxycycline or dox), has high dynamic range, low background in un-induced state and can be used *in vivo* and *in vitro* (7). Regulation in this system involves highly specific interaction between the Tet repressor (TetR) and Tet operator (*tetO*) DNA sequence (Tet response element or TRE). In the original Tet-Off system, the DNA binding domain of TetR was fused with the potent herpes simplex virus VP16 transactivation domain to form the tetracycline responsive transactivator (tTA) (8). tTA in the absence of doxycycline (dox) binds *tetO* to initiate transcription. The Tet-On system was later developed due to its wider application *in vivo* (e.g. for gene therapy and in transgenic animals) (7,9). Random mutagenesis of TetR generated a new transactivator (rtTA), which binds and transactivates gene expression in the presence of dox. Improved versions of rtTA have been developed to give tighter gene expression, increased sensitivity towards the inducer, enhanced stability and expression in mammalian cells, and more uniform transgene expression in the induced cells (10,11).

We incorporated the Cre/LoxP and Tet-On systems into one integrated system to enable tightly regulated induction of gene expression at reproducible levels between experiments and in different clones of mammalian cells. A new LoxP site (L3) was developed to minimize unwanted intrachromosomal recombination between heterospecific LoxP sites. When tested in two different cell lines and at six independent integration sites, incoming DNA was correctly targeted at high efficiencies. Expression of the reporter gene, luciferase-green fluorescence protein fusion (LucGFP), was uniformly induced across most of the RMCE clones derived from the same integration site. Such a highly efficient gene targeting approach in combination with predictable and reproducible gene expression should find wide application *in vitro* and *in vivo*.

MATERIALS AND METHODS

Construction of L3

L3 was made from two oligonucleotides that contained 16 complementary (**bold**) 3-prime residues (5′-GGA *CTC GAG* ATA ACT TCG **TAT AAA GTC TCC TAT A** and 5′-CCT ATC GAT ATA ACT TCG T**AT AGG AGA CTT TAT A**). The oligos were made duplex by 10 cycles of PCR at an annealing temperature of 42°C. The result was cloned into pCR2.1 using TOPO cloning (Invitrogen) and confirmed by sequencing. The specificity of L3 derives from an internal non-repetitive 8 bp sequence (underlined) that deviates from wild-type at three positions (A<u>T</u>GT<u>A</u>T<u>G</u>C).

Plasmids construction

Naming of the wild-type and LoxP511 sites are according to previously published data (12). pL1L2 and L1HyTK2L were gifts of S. Fiering (1). pL32L was made by substituting L1, bounded by XhoI and NcoI in plasmid L1HyTK2L with L3 from pCR2.1-L3, bounded by XhoI (oligonucleotide restriction site in *italics* above) and BspLU11 I within pCR2.1. pL3L3 was made from pL32L. L3 was removed with XhoI and PvuII and re-inserted in the position of 2L using SalI and SbfI blunted with T4 DNA polymerase.

L3HyTK2L was constructed by replacing L1 in L1HyTK2L with L3 from pL32L by AhdI and ClaI digestion. The L3HyTK2L cassette was cloned into a retrovirus backbone by inserting L3HyTK2L restricted with NotI and XbaI into pCFB-EGSH (Stratagene) digested with the same enzymes, generating RV-L3HyTK2L.

To facilitate insertion of genes into the inducible L3-2L exchange vector, we constructed L3-TRE-MCSpolyA-2L by cloning the fragment containing seven *tetO* sites, multiple cloning sites and a polyadenylation signal derived from XhoI and SapI/Klenow treated pTRE2 (BD Bioscience) into L3HyTK2L previously digested with XhoI and PshAI. The exchange plasmid, L3-TRE-LucGFP-2L (pLi028), was derived by cloning a BglII-NotI fragment containing LucGFP from pLuciferase-EGFP (gift from D. Buscher) into BamHI-NotI sites of L3-TRE-MCSpolyA-2L.

A bicistronic transregulator-expressing cassette was obtained by amplifying the TetR(B/E)-KRAB (tTR or Tet-transrepressor) gene by standard PCR using the primers 5′-(B/E)-BamHI and 3′-(B/E)-BglII, followed by restriction with BamHI and BglII and ligation with BamHI-restricted and dephosphorylated pWHE120(sM2), yielding pWHE124. The polio-virus IRES element was amplified with 5′-P-IRES-SmaI and 3′-P-IRES-SmaI from pCMV-KRAB-rtTA (13), restricted with SmaI and inserted into likewise-digested and dephosphorylated pWHE124, yielding pWHE125-P. The plasmid pWHE134 containing the tricistronic transregulator-cassette with rtTA2^S-M2, tTR and a neomycin selection marker separated by two IRES elements was constructed by restricting pWHE125-P with EcoRI and HpaI and ligating the fragment encoding the regulatory cassette with pIRESneo (BD Bioscience) containing the selection marker. pIRESneo had previously been restricted with BamHI, the 5′ overhangs filled-in with T4 DNA polymerase, and then restricted with EcoRI. All primer and plasmid sequences are available upon request.

Recombination assay by transient transfection

The 293 HEK cells were cotransfected by electroporation with 2 µg of LoxP test plasmid and either 18 µg of GFP expressing plasmid or 18 µg of Cre-expressing plasmid, pOG231 (14). Extra-chromosomal DNA was harvested by Hirt extraction

(15) 48 h post-transfection and examined by Southern hybridization after digestion with SspI or NdeI. The probe used was a 426 bp NcoI-SacII fragment corresponding to the hygromycin resistance gene common to all the LoxP plasmids.

Cell culture and construction of stable cell lines

Both HeLa and chinese hamster ovary (CHO) cells were cultured in DMEM (Cellgro) supplemented with 5% fetal bovine serum (Atlanta biologicals) and 1× non-essential amino acids (Invitrogen) in a humidified atmosphere containing 7% CO_2 at 37°C.

The HeLa cell line M2K was generated by stably transfecting the cell line HRM2 (16) with pCMV-TetR(B/E)-KRAB (13) and pPUR (BD Bioscience). Details will be published elsewhere (Schätz, Knott, Hillen and Berens; manuscript in preparation). To generate the cell line M2PK, $\sim 5 \times 10^5$ cells were transfected with 2 μg PvuII-linearized pWHE134 using Lipofectamine (Invitrogen). Twenty-four hours later, cells were seeded into 15 cm plates and selected with 800 μg/ml G418 (Invitrogen) for at least two weeks. Positive clones stably producing the transfected regulators were identified by transient transfection of 0.1 μg pUHC13-3 (8) and 0.6 μg pUHD16-1 for normalization of transfection efficiency, and 1.3 μg pWH802 as non-specific DNA. Clone with the highest induction of luciferase activity after incubation with 1 μg/ml dox for 24 h was chosen. Cells were routinely maintained in medium containing 400 μg/ml G418.

For retroviral delivery of L3HyTK2L into CHO, HeLa M2K and M2PK cells, $\sim 5 \times 10^5$ cells were infected with culture supernatant containing RV-L3HyTK2L (as described by manufacturer). Resistant clones were selected in 400 μg/ml hygromycin B (Calbiochem) for CHO and 100 μg/ml for HeLa cells.

L3HyTK2L was also integrated into the dihydrofolate reductase gene (DHFR) locus of DR-8 (a derivative of CHOK1) by homologous recombination using the strategy previously described, generating the stable clone 146-111 (17). For dox inducibility, pWHE134 was stably integrated into 146-111 by an approach described above, generating stable line 111-134.

### Cre recombination and selection for RMCE derivatives

A total of 30 μg of exchange plasmid and 3 μg of pOG231 were electroporated into $\sim 4 \times 10^6$ cells at 250 V 1500 μF (Hybaid). Approximately 1×10^5 cells were re-plated onto 15 cm tissue culture plate 4 days after electroporation. Ganciclovir (Moravek Biochemicals) was added the next day to a final concentration of 2 μM. The drug-containing medium was washed off and replaced with fresh medium after 3 days. Colonies were picked between 7 to 10 days after transferring into fresh medium.

PCR screening

Clones isolated from ganciclovir selection were screened for recombinants by PCR for loss of HyTK gene and gain of specific recombination junctions. A total of 5 ng of genomic DNA was added to a PCR mix containing 0.2 μM primers, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$ and 0.05 U/μl AmpliTaq Gold polymerase (Applied Biosystems). Amplification was performed once at 94°C for 10 min, followed by 40 cycles at 94°C for 30 s, 57°C for 30 s and 72°C for 30 s, followed by a final extension step at 72°C for 10 min. Refer to Figure 1 in results section for the position of primer pairs used. The same strategy was employed for HeLa and CHO cells. Primer sequences are available upon request.

Reporter assays

Cells grown on 35 mm dishes were washed once with phosphate-buffered saline (PBS) and lysed in 200 μl lysis buffer [50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, 1× complete protease inhibitor mix (Roche
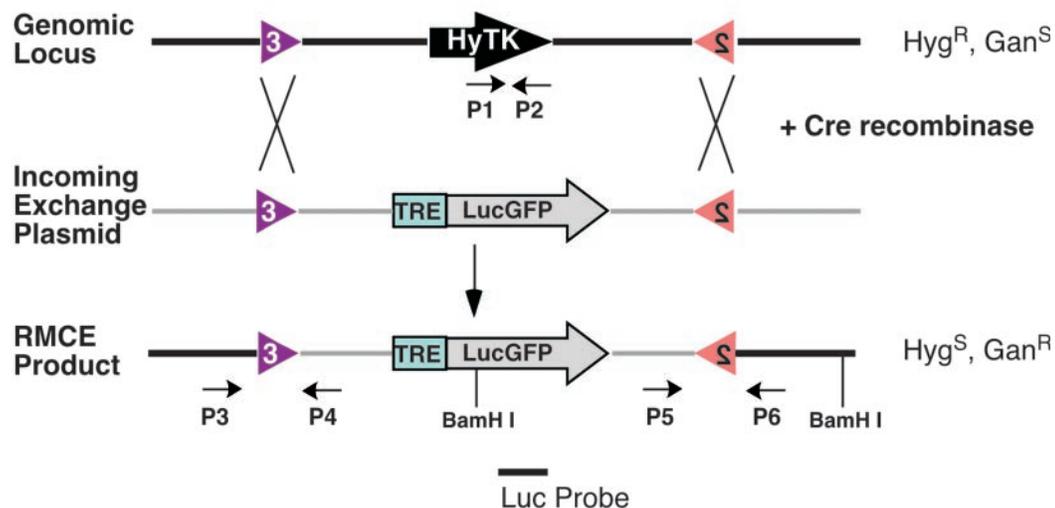
Figure 1. Principle of RMCE and screening strategy for recombinants. The positive-negative selection marker HyTK that confers resistance to hygromycin (Hyg[R]) and sensitivity to ganciclovir (Gan[S]) is flanked by a pair of heterospecific LoxP sites placed in inverted orientation (inverted triangles) and integrated as a single copy into a genomic site. In the presence of Cre-recombinase and incoming DNA harboring the same pair of LoxP sites, exchange will replace the existing HyTK with DNA on the exchange plasmid. Cells that have successfully undergone RMCE can be selected based on resistance to ganciclovir. The tetracycline inducible LucGFP (TRE-LucGFP) is used as the reporter to characterize uniformity of gene expression between clones after integration into the genome. Also shown are the positions of BamHI site and Luc probe used for Southern blot and primer pairs (P1–P6) used for screening of recombinants.
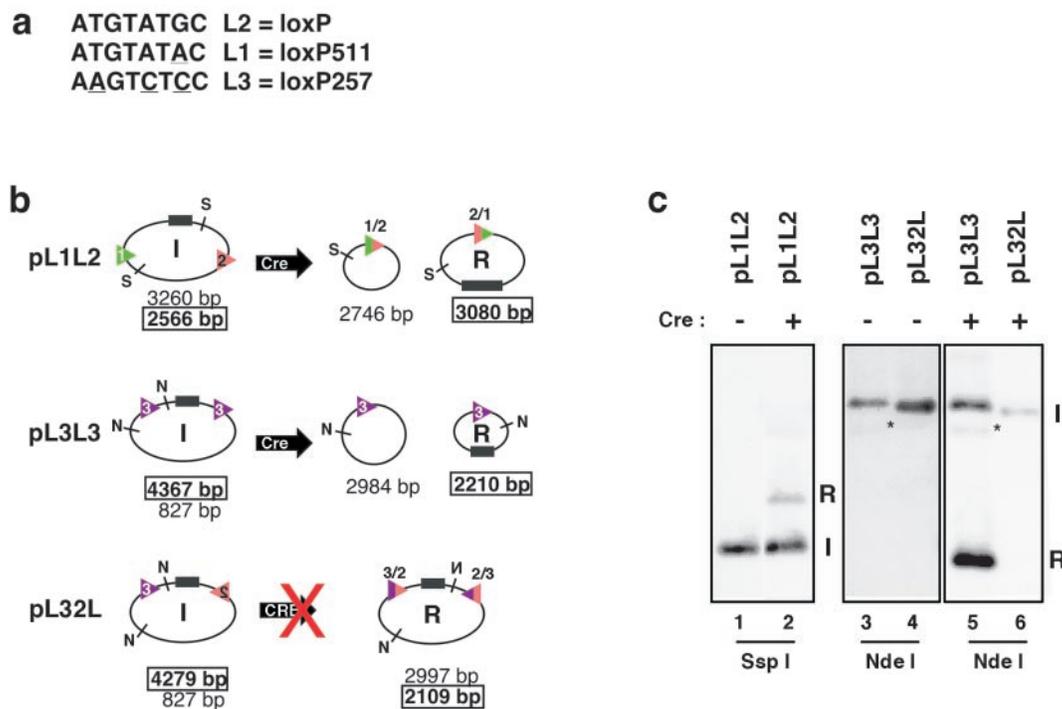
Figure 2. Construction and characterization of new LoxP site for RMCE. (**a**) Sequence of the 8 bp spacer found within the LoxP site. LoxP and LoxP511 are named according to previously published (12). Recombination occurs efficiently between LoxP sites with identical spacer sequence. Changes to the sequence within the 8 bp spacer generates LoxP sites that recombine with themselves but not with other LoxP sites. Underlined base(s) represents the base change that differs from the original L2 sequence. (**b**) A schematic of the three test plasmids (or I = input DNA), pL1L2, pL3L3 and pL32L and the possible products of RMCE (R = recombinant). Shown are the positions of the LoxP sites, the restriction sites used for the Southern blot analysis (S = Ssp I, N = Nde I), and the target sequence for the probe (black box). The sizes of the predicted restriction fragments are listed below. The boxed fragment corresponds to that detected by the probe. Note that only the L3L3 intramolecular recombination would be predicted to occur if L1, L2 and L3 do not recombine with each other. (**c**) Southern blot analysis of test plasmids transfected into 293 cells in the absence or presence of a Cre-expressing plasmid [pOG231, (14)]. Bands were detected using a probe to the hygromycin sequence common to the test plasmids. Unrecombined, input plasmids are marked with 'I'; products of Cre recombination are marked with 'R'. Note the presence of a non-specific band in lanes 3 and 5 marked with an asterisk.

Diagnostics)] and incubated for 30 min at 4°C. A total of 20 μl of cell lysate was then mixed with 100 μl of luciferase reagent [20 mM Tricine, 1.07 mM $MgCO_3$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 μM coenzyme A (USBiological), 530 μM ATP and 470 μM luciferin (Molecular Probes)] before reading on the luminometer (Lumat LB9507). Luciferase activity was normalized to total protein content (BioRad Laboratories). GFP fluorescence was analyzed by passing single cell suspensions in PBS through the FACScan (Becton Dickenson).

Determination of population doublings

The population doubling is calculated based on the formula 3.34 ($\log N_i$-$\log N_f$), where N_i is the initial cell count and N_f is the final cell count.

RESULTS

Designing a new LoxP site

The success of directional RMCE relies on the use of two heterospecific LoxP sites ideally exhibiting no recombination with each other. The natural LoxP (L2) site and the mutant LoxP511 (L1) are commonly used (1,12), but they differ by only 1 bp in the central 8 bp spacer region. A plasmid bearing L1 and L2 sites (pL1L2) can recombine illegitimately when cotransfected with the Cre-recombinase plasmid (compare Lane 1 and Lane 2 in Figure 2c). As such recombination reduces the effectiveness of RMCE, we designed a new LoxP site (designated LoxP257 or L3; see Figure 2a) that differs from the original LoxP site by 3 nt. A plasmid carrying a pair of L3 sites (pL3L3) recombines efficiently with itself in the presence of Cre-recombinase (compare lanes 3 and 5 in Figure 2c). More importantly, no recombination was detected in a plasmid containing L3 and the inverse L2 (2L) site (pL32L) in the presence of Cre (compare lanes 4 and 6). Thus, the newly designed L3 site paired with L2 is more ideal for RMCE than is the pair L1 and L2.

Highly efficient RMCE by L3-2L after integration into the genome

We next perform a vigorous test for the efficiency of cassette exchange mediated by L3 and L2. L3 and L2 are integrated into the genome of CHO and HeLa cells by homologous recombination or retroviral delivery. CHO was chosen because it is a commonly used cell line for the expression of recombinant proteins of pharmaceutical value (18–20) and because of our ongoing studies of DNA replication control in the DHFR locus. HeLa was chosen because it is commonly used for analyses of a variety of biological processes and is

readily transfected. We made use of a chimeric hygromycin phosphotransferase-thymidine kinase (HyTK) gene for both positive and negative selections (1). The HyTK fusion gene, which confers resistance to hygromycin and sensitivity to ganciclovir, was flanked by L3 and L2 (L3HyTK2L). This cassette was first inserted into the DHFR locus in CHO cells by homologous recombination. The DHFR locus was chosen due to our interest in its replication domain and because it can be amplified to high levels in the presence of methotrexate (21), which may be used to enhance production of proteins of biological interest (21,22). Also, robust procedures are available for homologous recombination between a cosmid carrying the 3′ half of the DHFR coding sequence with DR-8 (a DHFR-deficient CHO cell line hemizygous for a 3′ truncated DHFR gene) (23,24) (Figure 3a). This reconstructs the full-length DHFR gene, which is readily identified by growth of cells in medium lacking thymidine, hypoxanthine and glycine (17). We modified the targeting vector to include the L3HyTK2L cassette downstream of the DHFR gene. Southern blot analysis using probes spanning different regions of the DHFR locus confirmed the reconstruction of the DHFR gene and a probe for HyTK confirmed the integration of the L3HyTK2L cassette (Figure 3b), this clone is now named 146-111.

We next used retroviruses to insert single copies of the L3HyTK2L cassette into HeLa (Figure 3c) or CHO cells genome (data not shown). Southern blot analysis using a probe against HyTK demonstrated single copy integration at random sites in the genome of different clones (Figure 3d). Four HeLa clones and one CHO (CHO #4) clone exhibiting high and stable expression of HyTK [assayed by western blot for TK expression, (data not shown); polyclonal antibody against TK kindly provided by Ian R Wickersham] were used for subsequent recombination experiments.

To test the efficiency of recombination at the genomic site, plasmid DNA containing L3 and L2 sites was electroporated with a Cre-expressing plasmid (14) into the previously constructed CHO and HeLa parental lines containing L3HyTK2L cassette (L3-2L parent). LoxP sites, L3 and L2, were placed as inverted repeats to eliminate the possibility of recombination and deletion of the selection gene. Recombinants were selected based on resistance to ganciclovir after the replacement of the TK gene (Figure 1). A clone that is resistant to ganciclovir can either lose the TK gene by RMCE or has retained TK but silenced its expression or mutationally inactivated it. We identified recombinants that underwent RMCE using PCR to identify clones that lost the TK gene and gained the predicted recombinant junctions (Figure 1 and Table 1). Single copy integration of the incoming DNA was also confirmed by Southern blot analysis (Supplementary Data).

The overall frequency of correct recombination was 81% for CHO and 69% for HeLa cells (Table 1). The efficiency of recombination does not appear to be affected by the size of the incoming DNA, as fragments between the lengths of 100 to 4000 bp recombined with similar frequencies (Table 1). All clones that were negative for recombination junctions were positive for HyTK by PCR, suggesting that they arise due to silencing of the TK gene. No clone was tested positive for inversion between LoxP sites or illegitimate deletion of the TK sequence (Table 1).

## Uniform and stable doxycycline-inducible expression of transgene by RMCE clones

It has been previously reported that gene expression is uniform between RMCE clones derived from the same parental line (1,25). Since it is often essential to regulate the level of gene expression, we further tested the uniformity and stability in gene expression by comparing gene induction in RMCE clones containing an integrated copy of L3-TRE-LucGFP-2L. The Tet-On system we used has been modified as follows to enable stringent on-off regulation. The transactivator (rtTA) is a modified bacterial TetR protein fused to VP16 transcriptional activation domain (rtTA2^S-M2). It activates gene transcription by binding to TRE in the presence of dox (10,11); in the absence of dox, a Tet-transrepressor (tTR), TetR(B/E)-KRAB, binds TRE and actively represses gene expression (13).

We tested two ways of generating rtTA and tTR expressing stable cell lines. In the first system, rtTA and tTR were linked by an IRES element in a single plasmid (pWHE134) and were transcriptionally controlled by one promoter (single plasmid Tet-On system exemplified by the M2PK HeLa and CHO 111-134 in Figure 4a). In the second system, rtTA and tTR genes were on separate plasmids and their expression were driven from two independent promoters (two plasmid Tet-On system in HeLa cell line M2K in Figure 5a).

Gene expression was induced at a saturating dose of dox (2 µg/ml) for 72 h followed by a luciferase assay to determine the maximum induction attained by each RMCE clone. To compare uniformity in gene expression between clones, cells were induced at a non-saturating dose of dox followed by single cell analysis for GFP fluorescence using flow cytometry. We first tested the dose response to dox by titrating the concentration of dox and measuring the mean fluorescence units at 3 days (Figure 4b and c) after induction. Flow cytometric analysis showed uniform GFP expression in the induced populations and the fluorescence increased in a dose-dependent manner in CHO 111-134 cells from 12.5 to 200 ng/ml. A similar titration experiment was performed for HeLa cells (data not shown). Dox used between 50 and 100 ng/ml for CHO 111-134 and HeLa cells, respectively, fell within the linear range of induction and was chosen to test the uniformity in gene expression in the RMCE clones.

The single plasmid Tet-On system in HeLa cells (M2PK in Figure 4) generates low basal luciferase activity in the un-induced state and high level of luciferase activity at high dox concentration in all the four RMCE clones tested (Figure 4d). The fold increase in luciferase activity ranged from 3×10^4- to 7×10^4-fold over the un-induced state in these clones. Three of four RMCE clones exhibited greater than 90% GFP expression while one exhibited greater than 70% expression after induction with sub-maximal dose of dox (Figure 4e). Three clones had mean GFP fluorescence ranging between 90–110 and one clone had a mean GFP fluorescence of about 70. These data show that within single clones, individual cells exhibited similar expression levels, though we did detect some inter-clonal variability.

In CHO 111-134, all five clones derived by RMCE at the DHFR locus showed high luciferase activities upon induction at 2 µg/ml dox whereas the expression of luciferase was close to background in the absence of dox (Figure 4d). The fold
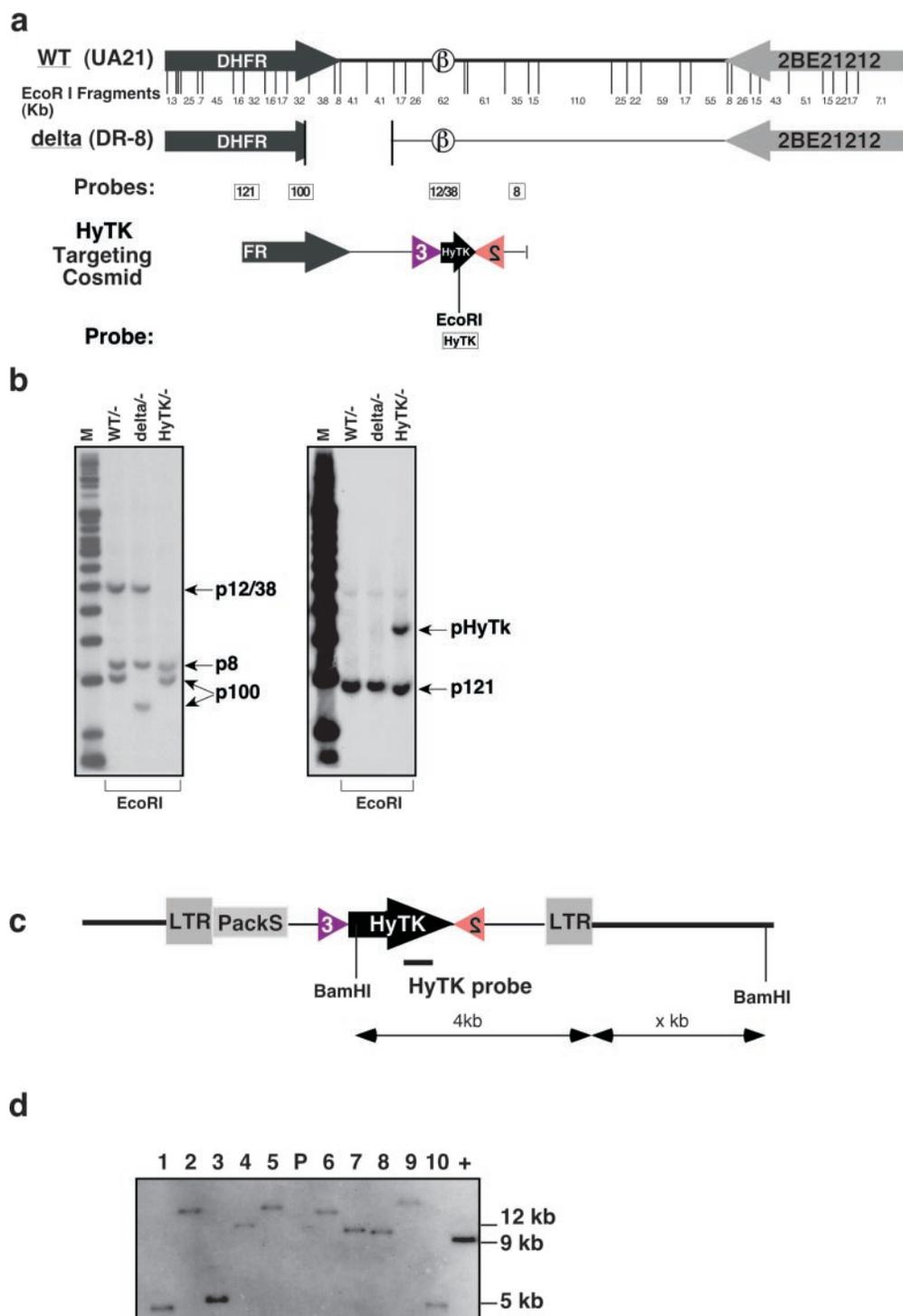
Figure 3. Integration and analysis of L3-2L introduced into the genomes of CHO and HeLa cells. (**a**) Engineering L3-2L into DR-8 cell line by homologous recombination using the strategy described by Kalejta *et al*., (17). The diagram shows the genomic structure at the DHFR locus (wild-type), sizes of the fragments generated by EcoRI digestion and the positions of the probes used for Southern blot. Targeting cosmid DNA containing the 3′ half of the DHFR gene is used to reconstruct the entire gene in DR-8 cell line that contains truncation at the 3′ end of the DHFR gene. The L3 and L2 sites are placed in inverted orientation and flanking the HyTK positive-negative selectable marker gene. The entire cassette was inserted downstream of DHFR gene after homologous recombination. (**b**) Southern blot of genomic DNA derived from CHO cells hemizygous for wild-type DHFR [WT/- or UA21 (17)], 3′ truncated DHFR [delta/- or DR-8 (17)] and HyTK reconstituted cells (HyTK/- or 146-111). DNA was restricted with EcoRI before blotting and the positions of the bands generated by each probe are indicated with an arrow. Probe 121 marks the 5′ flank of the cosmid DNA, probe 100 tests for reconstruction at the 3′ end of the DHFR gene, probe 12/38 indicates the presence of *ori*-beta (β), probe eight marks the 3′ flank of the cosmid DNA, and probe HyTK indicates the insertion of HyTK into the genome. M, molecular weight marker. (**c**) Schematic diagram of retrovirus RV-L3HyTK2L. L3 and L2 are placed in inverted orientation flanking HyTK gene. Also shown is the position of the BamHI site and the probe used for Southern blot. Upon integration into the genome, the expected size of the fragment generated by BamHI is 4 + x kb. (**d**) Southern analysis to determine the copy number of integrated HyTK gene in the HeLa cell clones (1 to 10) derived after infection with RV-L3HyTK2L. Genomic DNA was restricted with BamHI and probed against HyTK gene. Positive control (+) is original retrovirus DNA used to generate the stable line. P, parental HeLa.

Table 1. RMCE efficiency in cell lines tested

Parental line	L3-2L clones	RMCE exchange plasmid	Cre plasmid pOG231 (μg)	PCR for junction (no. +ve/no. tested)	PCR for HyTK gene (no. +ve/no. tested)	% Recombination
CHO	4	10μg L3-SV40bGal-2L	1	5/5	0/5	100
		30μg L3-SV40bGal-2L	3	5/5	0/5	100
		60μg L3-SV40bGal-2L	6	5/5	0/5	100
		0μg	3	0/3	3/3	0
		10μg L3-SV40bGal-2L	0	0/3	3/3	0
		30μg L3-IndbGal-2L	3	6/12	6/12	50
CHO	146-111	30μg L3 DHFR 207 bp 2L	3	8/11	3/11	73
		30μg L3 DHFR 109 bp 2L	3	12/12	0/12	100
		30μg L3 DHFR 101 bp 2L	3	11/12	1/12	92
		30μg L3 DHFR 2400 bp 2L	3	8/12	4/12	67
		30μg L3 DHFR 2299 bp 2L	3	12/12	0/12	100
		30μg L3 DHFR 2291 bp 2L	3	8/10	2/10	73
		30μg L3-TRE-LucGFP-2L	3	7/12	5/12	58
Overall frequencies in CHO						81
HeLa	M2K2	30μg L3-TRE-LucGFP-2L	3	4/7	3/7	57
	M2K3	30μg L3-TRE-LucGFP-2L	3	6/8	2/8	67
	M2PK7	30μg L3-TRE-LucGFP-2L	3	5/8	3/8	63
	M2PK10	30μg L3-TRE-LucGFP-2L	3	3/3	0/3	100
Overall frequencies in HeLa						69

Two different loci were tested in CHO and four independent sites were tested in HeLa. The L3-2L site in CHO #4 was generated by retrovirus integration. LoxP sites in CHO 146-111 were introduced by homologous recombination into the DHFR locus. All RMCE clones were screened by PCR and scored for positive RMCE junctions (P3 + P4 and P5 + P6) and positive for TK (P1 + P2) using primer pairs indicated in Figure 1. The percentage of successful recombinants is expressed as the number of positive junctions over the total number of clones tested.

increase in luciferase activity range from 6×10^3 to 4×10^4. As shown in Figure 4e, five out of five RMCE clones were greater than 92% GFP positive after induction at sub-maximal dose. Clone 1 had the highest mean GFP fluorescence (236) while clone 3 showed the lowest mean GFP fluorescence (94). Other RMCE clones had mean GFP fluorescence 105, 107 and 138, respectively. These results indicated that the reporter gene expression in these RMCE clones derived from the L3HyTK2L CHO parental cell line had substantial inducibility and uniformity.

In the two plasmid Tet-On system (M2K HeLa cells in Figure 5), basal luciferase activity was relatively low and uniform across the four clones analyzed. The maximum fold induction in luciferase activity ranged from 1×10^4 to 3×10^4 in the clones analyzed (Figure 5b). Three of the four RMCE clones tested show greater than 80% green cells after induction and exhibited mean GFP fluorescence between 100 and 110 (Figure 5c). One of the clones showed greater than 50% green cells and mean GFP fluorescence of 86 upon induction.

The RMCE approach enables genes to be independently introduced into a single genomic locus. Comparison between genes obtained from independent RMCE experiments is possible if the level of gene expression is preserved in clones obtained from independent RMCE experiments. Using the same L3-2L parent (HeLa M2K), we compared GFP expression in clones obtained from two independent RMCE experiments performed at the same genomic locus (Figure 5d). Seven out of ten clones obtained in the second RMCE experiment had similar mean GFP fluorescence level as 3 out of 4 of the clones obtained in the first RMCE experiment.

We evaluated whether the inducible phenotype was stable over time using one representative RMCE clone from M2PK and one from M2K HeLa. These clones were continuously passaged for 1 month before analysis. The basal GFP level remained low, and dox addition produced robust GFP induction when assayed 1 month later. Uniformity of expression in M2PK #1 was very high over this time period (i.e. 90% of cells exhibited GFP fluorescence after 1 month, compared to 92% originally). (Figure 6). However, the mean GFP fluorescence and the percentage of GFP expressing cells after induction declined slightly in HeLa M2K #3 (Figure 6).

Monitoring population doubling by Tet-regulated H2BGFP expression

The highly inducible nature of this system suggested potential applications for transiently marking individual cells to 'pulse label' them. As one example, we previously generated a very stable histone H2BGFP fusion protein that is readily incorporated into chromatin to generate brilliantly fluorescent chromosomes and nuclei (26). We reasoned that if the H2BGFP gene was induced for several cell doublings to uniformly label the chromatin in each cell, and it was then turned off, the fluorescence of the cell would be diminished by a factor of two each time the cell divided since nucleosomes segregated randomly when DNA is replicated (27). Thus, fluorescence microscopy or flow cytometry could be used to determine how many times a cell doubled by merely measuring its fluorescence intensity relative to that at the beginning of the experiment.

Figure 7 shows a flow cytometric analysis of the fluorescence intensity of HeLa cells followed for seven population doublings. The decrease in fluorescence per population doubling is precisely that expected for a 2-fold decrease per cell division. Importantly, there was no decrease in fluorescence observed in cells that were held in quiescence by thymidine block (Figure 7a). This shows that the decreased fluorescence required entry into and progression through S-phase, and was not the result of H2BGFP degradation.

DISCUSSION

We have combined the powers of RMCE and regulated gene expression into a single highly efficient system that should facilitate structure-function studies, enable production of biopharmaceuticals, or provide a means of tracking cell proliferation in real time. The Cre/LoxP recombination system is preferred for site-specific recombination because it exhibits higher efficiency of recombination over the Flp/FRT system in specific cell types such as mouse embryonic stem cells and primary cells (28–31). However, the system we describe could as easily be implemented using engineered Flp recombination target sequences designed not to recombine with each other. We first engineered a new LoxP site to minimize intra-chromosomal exchanges that generate deletions, and to max-imize exchanges between the chromosomal and plasmid donor LoxP targets. To minimize the need for drug selection markers, we introduced into the chromosomal target a HyTK fusion gene. This enables positive selection to identify clones containing the LoxP target, and then counterselection using gancyclovir to select for loss of the chromosomal target and concomitant replacement with the donor plasmid. This is especially valuable for situations in which introduction of extraneous transcriptional control sequences needs to be avoided. We also used an optimized Tet-On system to enable robust controlled expression of the donated sequences. This should allow for analyses of phenotypic effects of the intro-duced gene when it is expressed at normal, sub- or supra-physiologic levels.

We tested the system by first analyzing the stringency of recombination with the newly characterized L3 LoxP site. A pair of heterospecific recombination sites that do not recom-bine with each other is necessary for efficient RMCE (6). When used with the original LoxP site (L2), the first LoxP variant (LoxP511 or L1) that was generated greatly enhanced the frequencies of exogenous gene integration when coupled with positive selection (12). However, using negative selection as a means to enrich for recombinants, the efficiencies were markedly different (1,6). Deletion of intervening sequences without replacement occurred when L2 and L1 were placed as direct repeats, suggesting interaction between L1 and L2 (6). When placed as inverted repeats, inversion of the intervening sequence resulted in an equal proportion of clones in either
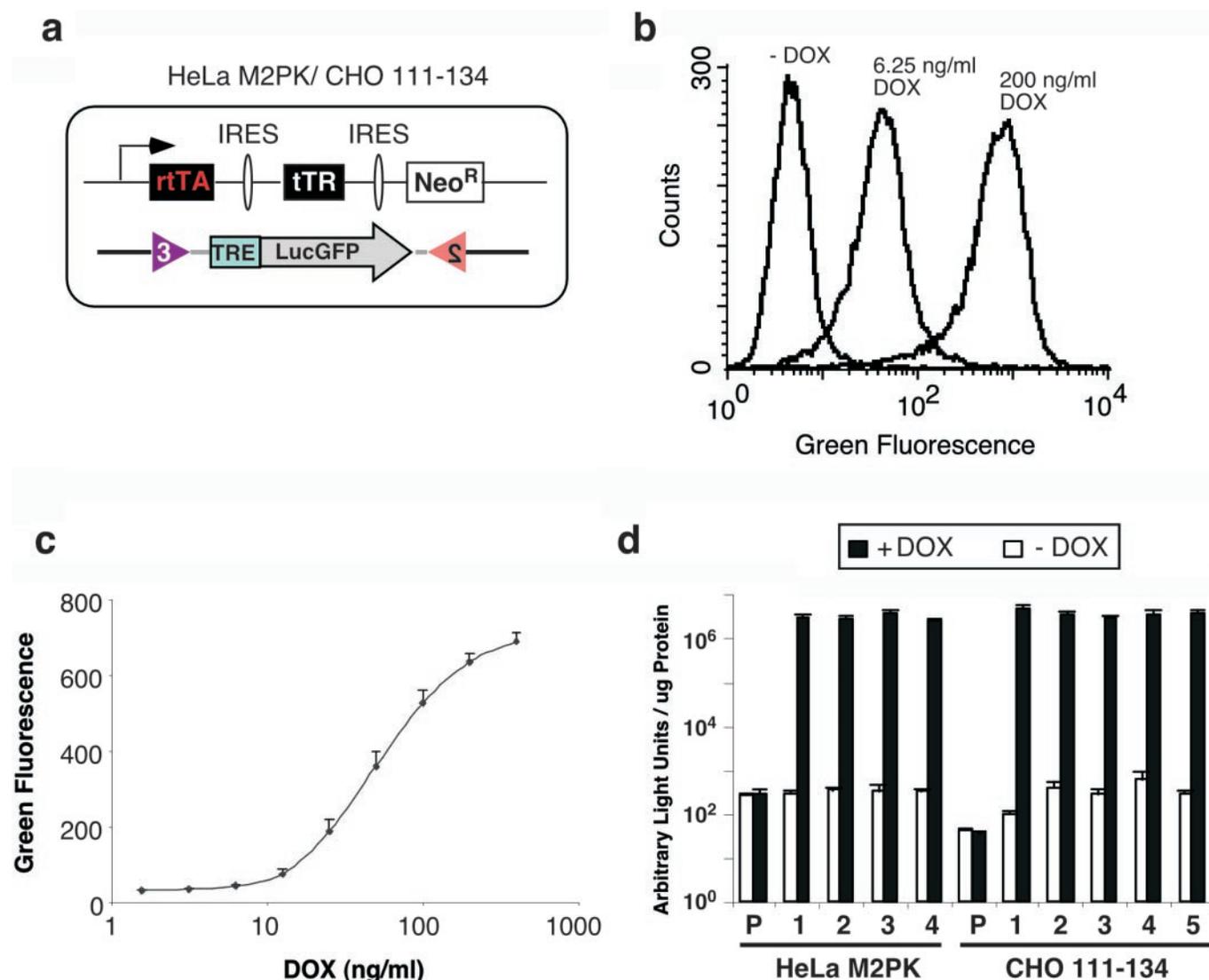
e
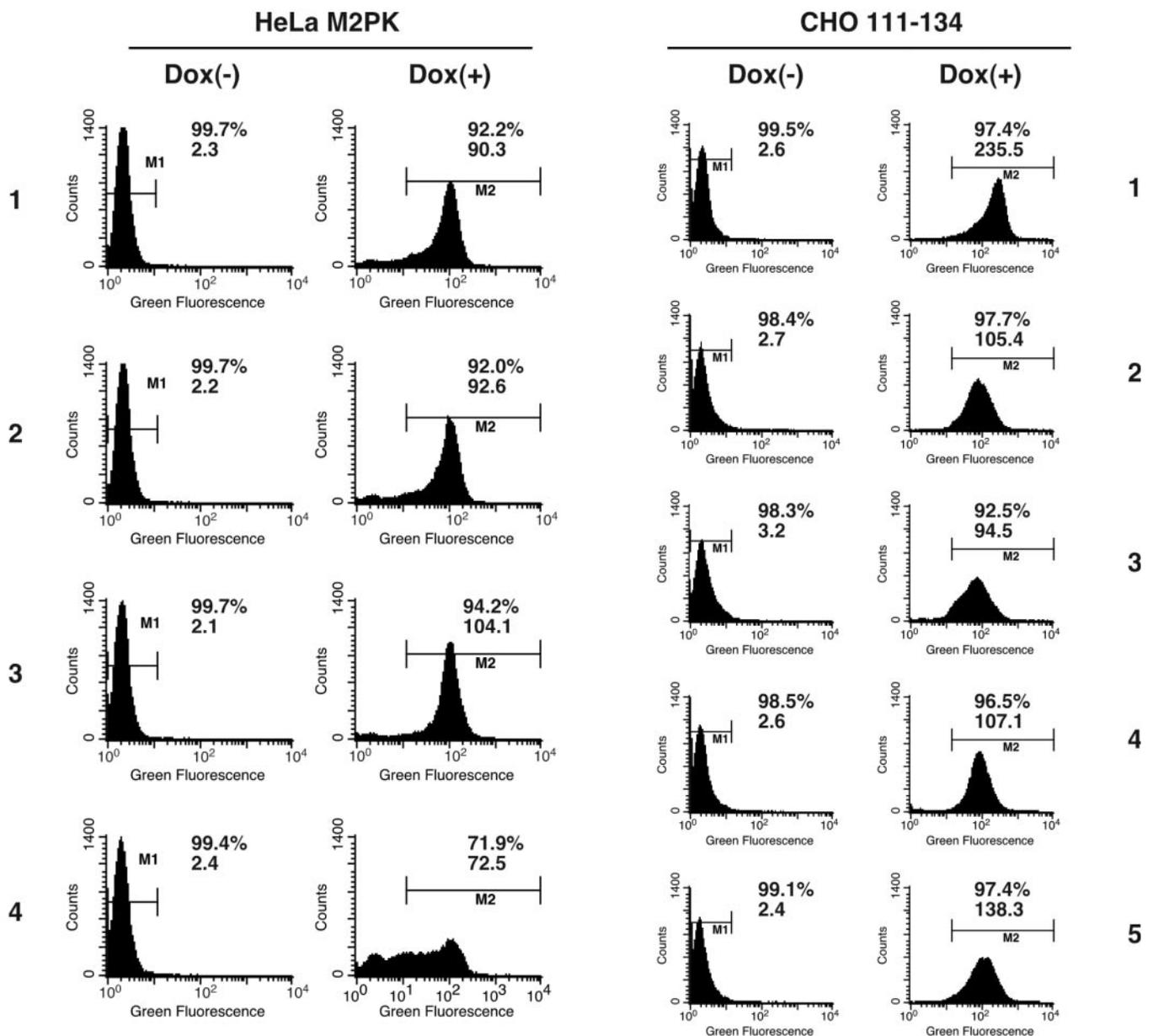
Figure 4. GFP induction by single plasmid Tet-On CHO 111-134 and HeLa M2PK RMCE clones. The RMCE clones were named after the L3-2L parent that they were derived from. (**a**) Schematic diagram showing the various genes that were incorporated to make up the RMCE clones. In CHO 111-134 and HeLa M2PK, rtTA and tTR genes are linked by IRES and transcriptionally driven by one promoter. Neo^R codes for neomycin/G418 resistance gene. The L3-TRE-LucGFP-2L cassette is inserted as single copy into the genome by RMCE as described in Figure 1. (**b**) Histogram plot showing green fluorescence exhibited by CHO 111-134 after induction with increasing concentration of dox for 72 h. (**c**) Graph shows the mean fluorescence units exhibited by CHO 111-134 after induction with increasing dox concentrations for 72 h. Data are obtained from two independent experiments. (**d**) Normalized luciferase activity to show maximum gene induction in cells before and after incubation with 2 µg/ml dox for 72 h. Data represents mean and standard error of three independent experiments. Parental lines (P) do not have luciferase gene inserted into their genomes. (**e**) Histogram plots of GFP expression by RMCE derivatives before (−) and after (+) dox induction in one representative experiment. HeLa and CHO cells were induced with 0.1 and 0.05 µg/ml dox, respectively for 72 h before harvesting for flow cytometry. Each population was gated (M1 and M2) and the percentage of the gated cells and their corresponding mean GFP fluorescence are indicated in each plot.

direction (1). This is not desirable, especially when directionality of the integrant is important. We solved the problem of recombination between L2 and L1 by generating a new LoxP site, LoxP257 (L3), which was designed based on findings from *in vitro* recombination assays (5). L3 differs from L2

by three bases within the 8 bp spacer sequence. When used in combination with L2 and L3 generated recombinants at high frequency, suggesting the feasibility of using L3 and L2 for RMCE. The finding was consistent with a previous study showing that a modified LoxP sequence (LoxP2272)
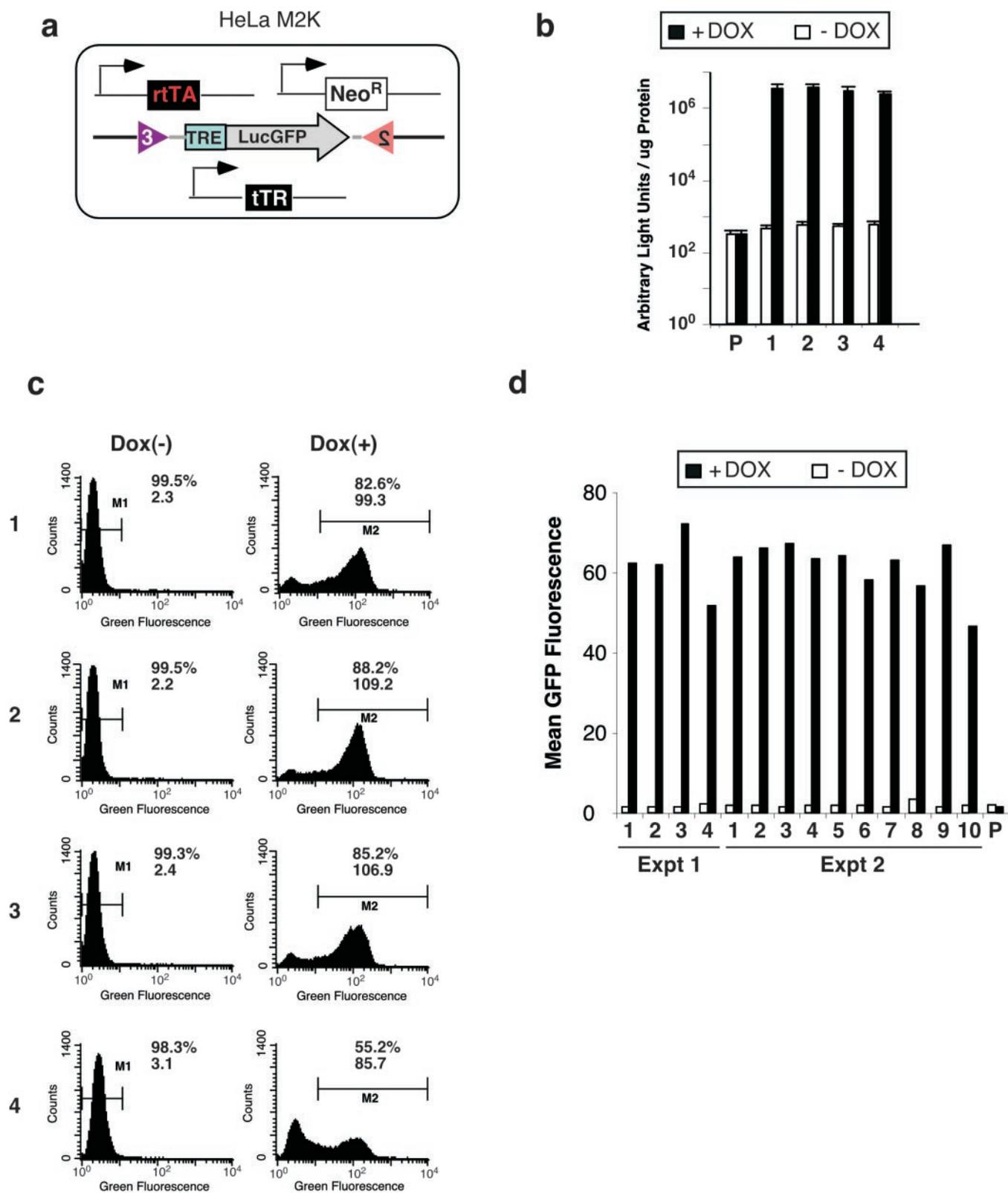
Figure 5. Reporter gene induction by two plasmids Tet-On stable HeLa M2K RMCE clones. (**a**) Schematic diagram showing the various genes that were incorporated to make up M2K RMCE clones. Two independent promoters are used to drive the rtTA and tTR genes. The reporter gene LucGFP is inserted into the genome by RMCE. (**b**) Normalized luciferase activity to show maximum gene induction in cells before and after incubation with 2 μg/ml dox for 72 h. Data shown are mean with standard error obtained from three independent experiments. Parental line (P) do not express the luciferase gene. (**c**) Histogram plots of GFP expression by M2K RMCE derivatives before (−) and after (+) dox induction at 0.1 μg/ml for 72 h. Cells were harvested for flow cytometry and each population was gated (M1 and M2) with the corresponding percentage of gated cells and mean GFP fluorescence shown in each plot. (**d**) Graph shows mean GFP fluorescence of RMCE clones obtained from two independent RMCE experiments. Cells were either un-induced (−) or treated with 0.1 μg/ml dox for 72 h.
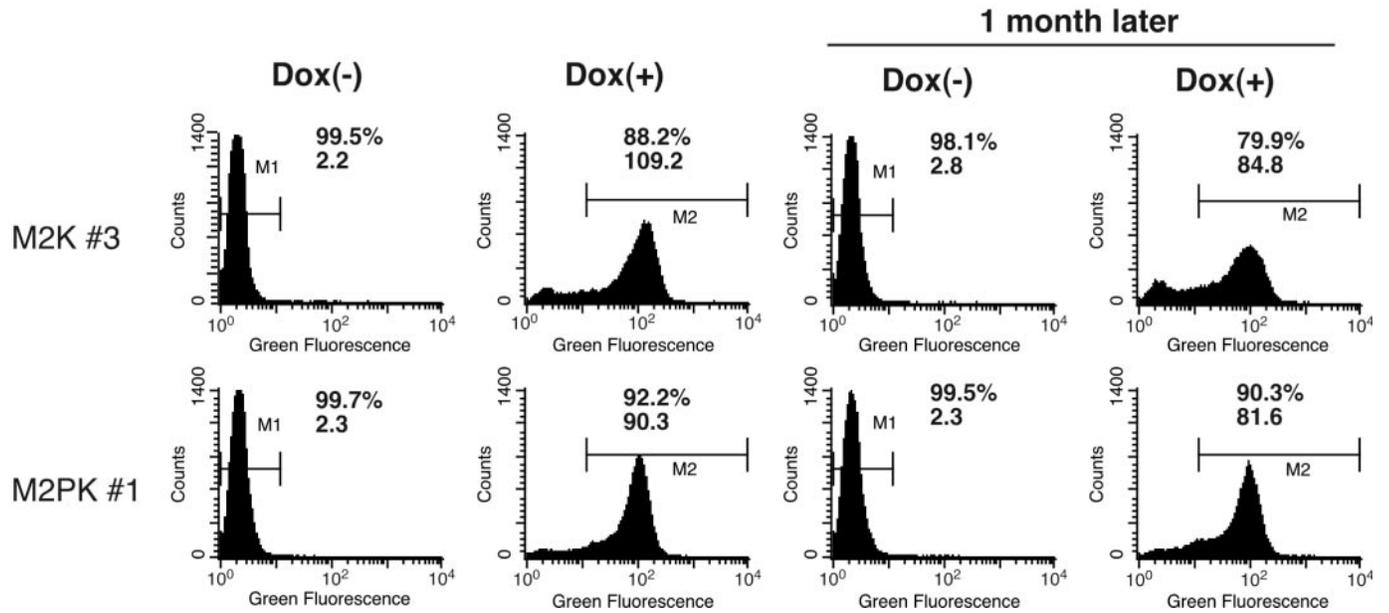
Figure 6. Stability of gene induction at the LoxP loci. Histogram plots showing GFP induction in M2K #3 and M2PK #1 before and after 1 month of continuous passage. Experiments were performed twice and data shown are from one experiment.
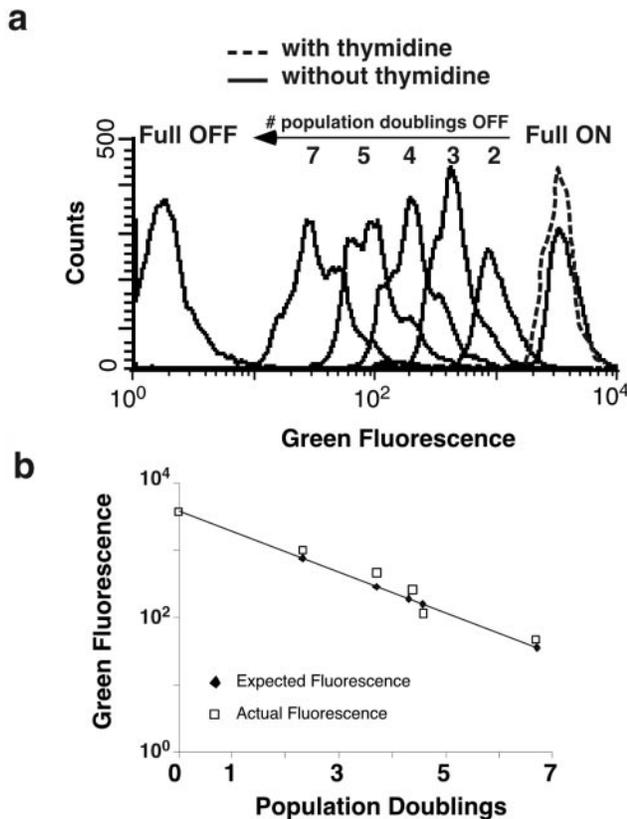
Figure 7. Monitoring cell doubling using inducible H2BGFP. HeLa cells stably expressing TRE-H2BGFP were generated by L3-2L mediated RMCE. Cells were either not induced (− dox) or induced with 2 μg/ml dox (+ dox) for 3 days to achieve maximum expression before washing out the dox in the presence or absence of 2 mM thymidine. Cells were harvested each day after the removal of dox and their fluorescence monitored by flow cytometry (Actual fluorescence). The expected fluorescence is the theoretical fluorescence value assuming 50% decay after each population doubling. Experiments were performed twice and data from one experiment are shown as a histogram in (**a**) and as a graph in (**b**).

with two base changes from L2 also enabled high efficiency exchange (6). Other than having a high inter-molecular recombination efficiency, the probability of selecting a recombinant clone among the ganciclovir resistant clones is increased when TK expression from the L3HyTK2L parent line is high and stable. Using this criterion for the selection of the L3HyTK2L cell line, we observed high efficiency, accurate RMCE in two different mammalian cell lines at six independent genomic loci. We also observed little size dependence on RMCE frequency or accuracy. The high frequency of correctly recombined clones expedites screening for desirable clones.

We used homologous recombination to introduce L3HyTK2L into several single copy loci, including the intergenic region of the CHO DHFR locus. Our data demonstrate that the DHFR locus was favorable for RMCE and for tightly regulated gene induction. Recombination occurred at very high efficiency (50–100%) and we readily obtained clones exhibiting robust inducible expression (up to 10^4-fold by luciferase, and >100-fold by GFP analyses). This locus offers a number of advantages for other studies. For example, it can be readily amplified using selection for methotrexate-resistance (21). As CHO cells are often used to produce biopharmaceuticals, the combination of amplification and highly regulated expression could provide substantial advantages for reducing production costs, enabling stable maintenance of transgenes, and obtaining very high level expression of potentially toxic proteins. In studies to be presented elsewhere, we found the L2 and L3 sites to be useful for generating genetically engineered ES cells, mouse embryo fibroblasts, and mice with different p53 point mutations or fusion genes with high efficiency (F. Toledo, C. Lee and G.M. Wahl, manuscript in preparation).

Using a strategy similar to that employed for the delivery of Flp/FRT tagged sites into the genome of mammalian cells (25,32), we integrated the LoxP sites at single copy into random positions in the genome of HeLa and CHO cells by retroviral delivery (Figure 3c). An advantage of the retroviral

system is that it ensures delivery of the entire transgene at high efficiency and at single copy (Figure 3d) into the genome. Active transcription sites are the preferred retroviral integration sites (33,34). Consistent with this, we were able to select retroviral integrations at high efficiency, an advantage over gene targeting by homologous recombination.

An important advantage of RMCE approach that is valuable for structure-function studies and production of recombinant proteins of pharmaceutical value is the ability to repeatedly obtain clones exhibiting predictable and uniform gene expression at high frequencies (1,25,35). We combined that with the improved version of the Tet-On inducible system to evaluate the reproducibility of gene induction at each locus (11). By single cell analysis, GFP was uniformly expressed within the population and it was induced in a dose-responsive manner. The improved rtTA (rtTA2^s-M2) demonstrated many superior properties over the original rtTA. Beside the ability to induce graded transgene expression, induction exhibited a greater dynamic range, higher sensitivity to dox and faster induction rate (within 24 h, data not shown) compared to the original rtTA system (Figure 4b and c) (11,36). Recombinants derived from the same L3-2L parental line have the reporter gene inserted at the same genomic locus. As expected, the majority of the RMCE clones obtained in one experiment showed similar levels of expression upon induction (Figures 4 and 5). The uniformity in gene expression was also found between clones obtained from independent RMCE experiments (Figure 5d). Hence, clones that reproducibly induce different transgenes can be readily obtained through multiple rounds of RMCE.

Maintaining stable transgene expression is as important as high transgene expression. Since transgene expression may be subjected to silencing, especially when viral promoter is used to drive gene expression (37,38) and when expression is not maintained under selective pressure (39). We measured the stability of expression in HeLa cells after at least 30 population doublings in culture. We observed that the level of inducibility, and the fraction of cells expressing the transgene, was stable over the time period studied when transcription of rtTA and tTR were coupled to that of the neomycin resistance gene by the IRES elements and by maintaining the culture under G418 selection. Hence, the use of IRES to mediate coexpression of multiple genes will gain popularity in both *in vitro* and *in vivo* applications (40,41).

The tight and reversible gene expression regulated by the Tet-On system was further demonstrated by switching H2BGFP expression on and then off to monitor cell doubling (Figure 7) as a function of fluorescence units. H2BGFP is incorporated into the chromatin after synthesis, and tracks with nucleosomes that are segregated randomly to daughter cells during each division (27). Being a stable protein, H2BGFP fluorescence should approximately halve with each division if its expression is prevented. Consistent with this prediction, after H2BGFP expression was turned off for different numbers of population doublings, flow cytometric analysis revealed the expected 2-fold decay per population doubling (Figure 7). Hence, the inducible expression of H2BGFP can be used to measure population doublings within a defined time frame. For example, as stem cells are expected to divide fewer times than transit amplifying cells, the retention of H2BGFP fluorescence should be able to be used as a marker to localize putative stem cells *in situ* and as a means to isolate them by fluorescence based cell sorting (42–44).

In conclusion, the LoxP/Cre recombination system offers reproducible and directional DNA integration into specific loci in the genome at high frequency. The single plasmid Tet-On system, which ensures stable expression of the transactivator and transrepressor, allows reproducible and graded induction of transgenes to a similar level in the RMCE clones derived from single and independent recombination experiments.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

REFERENCES

1. Feng,Y.Q., Seibler,J., Alami,R., Eisen,A., Westerman,K.A., Leboulch,P., Fiering,S. and Bouhassira,E.E. (1999) Site-specific chromosomal integration in mammalian cells: highly efficient CRE recombinase-mediated cassette exchange. *J. Mol. Biol.*, **292**, 779–785.
2. Capecchi,M.R. (1989) Altering the genome by homologous recombination. *Science*, **244**, 1288–1292.
3. Baer,A. and Bode,J. (2001) Coping with kinetic and thermodynamic barriers: RMCE, an efficient strategy for the targeted integration of transgenes. *Curr. Opin. Biotechnol.*, **12**, 473–480.
4. Hoess,R.H., Wierzbicki,A. and Abremski,K. (1986) The role of the loxP spacer region in P1 site-specific recombination. *Nucleic Acids Res.*, **14**, 2287–2300.
5. Lee,G. and Saito,I. (1998) Role of nucleotide sequences of loxP spacer region in Cre-mediated recombination. *Gene*, **216**, 55–65.
6. Kolb,A.F. (2001) Selection-marker-free modification of the murine beta-casein gene using a lox2272 [correction of lox2722] site. *Anal. Biochem.*, **290**, 260–271.
7. Bockamp,E., Maringer,M., Spangenberg,C., Fees,S., Fraser,S., Eshkind,L., Oesch,F. and Zabel,B. (2002) Of mice and models: improved animal models for biomedical research. *Physiol. Genomics.*, **11**, 115–132.
8. Gossen,M. and Bujard,H. (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl Acad. Sci. USA*, **89**, 5547–5551.
9. Gossen,M., Freundlieb,S., Bender,G., Muller,G., Hillen,W. and Bujard,H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science*, **268**, 1766–1769.
10. Baron,U., Gossen,M. and Bujard,H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Res.*, **25**, 2723–2729.
11. Urlinger,S., Baron,U., Thellmann,M., Hasan,M.T., Bujard,H. and Hillen,W. (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc. Natl Acad. Sci. USA*, **97**, 7963–7968.

12. Bouhassira,E.E., Westerman,K. and Leboulch,P. (1997) Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. *Blood*, **90**, 3332–3344.

13. Forster,K., Helbl,V., Lederer,T., Urlinger,S., Wittenburg,N. and Hillen,W. (1999) Tetracycline-inducible expression systems with reduced basal activity in mammalian cells. *Nucleic Acids Res*., **27**, 708–710.

14. O'Gorman,S., Dagenais,N.A., Qian,M. and Marchuk,Y. (1997) Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc. Natl Acad. Sci. USA*, **94**, 14602–14607.

15. Hirt,B. (1967) Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol*., **26**, 365–369.

16. Knott,A., Garke,K., Urlinger,S., Guthmann,J., Muller,Y., Thellmann,M. and Hillen,W. (2002) Tetracycline-dependent gene regulation: combinations of transregulators yield a variety of expression windows. *Biotechniques*, **32**, 796798, 800 passim.

17. Kalejta,R.F., Li,X., Mesner,L.D., Dijkwel,P.A., Lin,H.B. and Hamlin,J.L. (1998) Distal sequences, but not ori-beta/OBR-1, are essential for initiation of DNA replication in the Chinese hamster DHFR origin. *Mol. Cell*., **2**, 797–806.

18. Zang,M., Trautmann,H., Gandor,C., Messi,F., Asselbergs,F., Leist,C., Fiechter,A. and Reiser,J. (1995) Production of recombinant proteins in Chinese hamster ovary cells using a protein-free cell culture medium. *Biotechnology (NY)*, **13**, 389–392.

19. Fussenegger,M., Schlatter,S., Datwyler,D., Mazur,X. and Bailey,J.E. (1998) Controlled proliferation by multigene metabolic engineering enhances the productivity of Chinese hamster ovary cells. *Nat. Biotechnol*., **16**, 468–472.

20. Warner,T.G. (1999) Enhancing therapeutic glycoprotein production in Chinese hamster ovary cells by metabolic engineering endogenous gene control with antisense DNA and gene targeting. *Glycobiology*, **9**, 841–850.

21. Alt,F.W., Kellems,R.E., Bertino,J.R. and Schimke,R.T. (1978) Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem*., **253**, 1357–1370.

22. Kellems,R.E. (1991) Gene amplification in mammalian cells: strategies for protein production. *Curr. Opin. Biotechnol*., **2**, 723–729.

23. Jin,Y., Yie,T.A. and Carothers,A.M. (1995) Non-random deletions at the dihydrofolate reductase locus of Chinese hamster ovary cells induced by alpha-particles simulating radon. *Carcinogenesis*, **16**, 1981–1991.

24. Urlaub,G. and Chasin,L.A. (1980) Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. *Proc. Natl Acad. Sci. USA*, **77**, 4216–4220.

25. Schubeler,D., Maass,K. and Bode,J. (1998) Retargeting of retroviral integration sites for the predictable expression of transgenes and the analysis of *cis*-acting sequences. *Biochemistry*, **37**, 11907–11914.

26. Kanda,T., Sullivan,K.F. and Wahl,G.M. (1998) Histone-GFP fusion protein enables sensitive analysis of chromosome dynamics in living mammalian cells. *Curr. Biol*., **8**, 377–385.

27. Burhans,W.C., Vassilev,L.T., Wu,J., Sogo,J.M., Nallaseth,F.S. and DePamphilis,M.L. (1991) Emetine allows identification of origins of mammalian DNA replication by imbalanced DNA synthesis, not through conservative nucleosome segregation. *EMBO. J*., **10**, 4351–4360.

28. Andreas,S., Schwenk,F., Kuter-Luks,B., Faust,N. and Kuhn,R. (2002) Enhanced efficiency through nuclear localization signal fusion on phage PhiC31-integrase: activity comparison with Cre and FLPe recombinase in mammalian cells. *Nucleic Acids Res*., **30**, 2299–2306.

29. Ringrose,L., Lounnas,V., Ehrlich,L., Buchholz,F., Wade,R. and Stewart,A.F. (1998) Comparative kinetic analysis of FLP and cre recombinases: mathematical models for DNA binding and recombination. *J. Mol. Biol*., **284**, 363–384.

30. Westerman,K.A. and Leboulch,P. (1996) Reversible immortalization of mammalian cells mediated by retroviral transfer and site-specific recombination. *Proc. Natl Acad. Sci. USA*, **93**, 8971–8976.

31. Seibler,J., Schubeler,D., Fiering,S., Groudine,M. and Bode,J. (1998) DNA cassette exchange in ES cells mediated by Flp recombinase: an efficient strategy for repeated modification of tagged loci by marker-free constructs. *Biochemistry*, **37**, 6229–6234.

32. Wirth,D. and Hauser,H. (2004) Flp-mediated integration of expression cassettes into FRT-tagged chromosomal loci in mammalian cells. *Methods Mol. Biol*., **267**, 467–476.

33. Scherdin,U., Rhodes,K. and Breindl,M. (1990) Transcriptionally active genome regions are preferred targets for retrovirus integration. *J. Virol*., **64**, 907–912.

34. Schroder,A.R., Shinn,P., Chen,H., Berry,C., Ecker,J.R. and Bushman,F. (2002) HIV-1 integration in the human genome favors active genes and local hotspots. *Cell*, **110**, 521–529.

35. Fukushige,S. and Sauer,B. (1992) Genomic targeting with a positive-selection lox integration vector allows highly reproducible gene expression in mammalian cells. *Proc. Natl Acad. Sci. USA*, **89**, 7905–7909.

36. Kringstein,A.M., Rossi,F.M., Hofmann,A. and Blau,H.M. (1998) Graded transcriptional response to different concentrations of a single transactivator. *Proc. Natl Acad. Sci. USA*, **95**, 13670–13675.

37. Teschendorf,C., Warrington,K.H.,Jr, Siemann,D.W. and Muzyczka,N. (2002) Comparison of the EF-1 alpha and the CMV promoter for engineering stable tumor cell lines using recombinant adeno-associated virus. *Anticancer Res*., **22**, 3325–3330.

38. Paterna,J.C., Moccetti,T., Mura,A., Feldon,J. and Bueler,H. (2000) Influence of promoter and WHV post-transcriptional regulatory element on AAV-mediated transgene expression in the rat brain. *Gene. Ther*., **7**, 1304–1311.

39. Barnes,L.M., Bentley,C.M. and Dickson,A.J. (2003) Stability of protein production from recombinant mammalian cells. *Biotechnol. Bioeng*., **81**, 631–639.

40. Wong,E.T., Ngoi,S.M. and Lee,C.G. (2002) Improved co-expression of multiple genes in vectors containing internal ribosome entry sites (IRESes) from human genes. *Gene. Ther*., **9**, 337–344.

41. Salucci,V., Scarito,A., Aurisicchio,L., Lamartina,S., Nicolaus,G., Giampaoli,S., Gonzalez-Paz,O., Toniatti,C., Bujard,H., Hillen,W. *et al.* (2002) Tight control of gene expression by a helper-dependent adenovirus vector carrying the rtTA2(s)-M2 tetracycline transactivator and repressor system. *Gene. Ther*., **9**, 1415–1421.

42. Tumbar,T., Guasch,G., Greco,V., Blanpain,C., Lowry,W.E., Rendl,M. and Fuchs,E. (2004) Defining the epithelial stem cell niche in skin. *Science*, **303**, 359–363.

43. Welm,B.E., Tepera,S.B., Venezia,T., Graubert,T.A., Rosen,J.M. and Goodell,M.A. (2002) Sca-1(pos) cells in the mouse mammary gland represent an enriched progenitor cell population. *Dev. Biol*., **245**, 42–56.

44. Clarke,R.B., Spence,K., Anderson,E., Howell,A., Okano,H. and Potten,C.S. (2005) A putative human breast stem cell population is enriched for steroid receptor-positive cells. *Dev. Biol*., **277**, 443–456.