

Methodology article

Mutant loxP vectors for selectable marker recycle and conditional knock-outs

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Abstract

Background: Gene disruption by targeted integration of transfected constructs becomes increasingly popular for studies of gene function. The chicken B cell line DT40 has been widely used as a model for gene knock-outs due to its high targeted integration activity. Disruption of multiple genes and complementation of the phenotypes is, however, restricted by the number of available selectable marker genes. It is therefore highly desirable to recycle the selectable markers using a site-specific recombination system like Cre/loxP.

Results: We constructed three plasmid vectors (neoR, puroR and bsr), which carry selectable marker genes flanked by two different mutant loxP sites. After stable transfection, the marker genes can be excised from the genome by transient induction of Cre recombinase expression. This excision converts the two mutant loxP sites to an inactive double-mutant loxP. Furthermore we constructed a versatile expression vector to clone cDNA expression cassettes between mutant loxP sites. This vector can also be used to design knock-out constructs in which the floxed marker gene is combined with a cDNA expression cassette. This construct enables gene knock-out and complementation in a single step. Gene expression can subsequently be terminated by the Cre mediated deletion of the cDNA expression cassette. This strategy is powerful for analyzing essential genes, whose disruption brings lethality to the mutant cell.

Conclusions: Mutant loxP vectors have been developed for the recycle of selectable markers and conditional gene knock-out approaches. As the marker and the cDNA expression cassettes are driven by the universally active and evolutionary conserved β -actin promoter, they can be used for the selection of stable transfectants in a wide range of cell lines.

Background

Defined mutations can be introduced into embryonic stem (ES) cells by homologous recombination and the effect of the mutation can be studied in animals derived from these cells. Many genes are however essential for

embryonic development and a homozygous deletion result in non-viable embryos. Gene disruption in a cell line can be an alternative to the production of a knock-out animal. The chicken B cell line DT40 is popular for these studies due to its high ratios of targeted to random inte-

gration [1]. DT40 mutants have been used in the field of B cell antigen receptor signaling, cell cycle regulation, apoptosis, histon acetylation, homologous recombination and DNA repair [2]. Although mating of DT40 clones is not possible, multiple mutations can be accumulated in the same cell clone by the step-wise transfection of knock-out constructs. This approach holds great promise for the analysis of genetic networks, but it is currently limited by the inherent consumption of one selectable marker for each introduced mutation.

The Cre-loxP recombination system of bacteriophage P1 has been employed for genetic manipulation of higher eukaryotic cells [3]. Cre recombinase recognizes a 34 base pair (bp) Cre recombination signal sequence called loxP, and can delete genes which are flanked by two loxP sites in the same direction. The mutant loxP system, which is composed of two different mutant loxP sites (loxP_RE and loxP_LE), was first utilized for genetic manipulation of tobacco [4]. This mutant loxP system has been applied to mouse ES cell for site-specific insertion mutagenesis [5]. While the standard loxP site has a 13 bp palindrome on both ends, 5 bp of the palindromic sequences are mutated in loxP_RE and loxP_LE (Figure 1a). Cre recombinase can still recognize the mutant loxP sites and delete the intervening region. During this process loxP_RE and loxP_LE are converted to a new loxP_RE+LE site, which is poorly recognized by Cre [5]. This has the advantage that genetic instability associated with recutting of the remnant loxP_RE+LE site is minimized.

Recombination of loxP sites can be achieved by transient transfection of a Cre expression plasmid [6]. Alternatively Cre recombination can be controlled by stable transfection of an inducible Cre recombinase construct. A tightly regulated Cre recombinase, MerCreMer is designed as a fusion protein of Cre and the hormone-binding domains (HBDs) of the mutated estrogen receptor (Mer) [7]. This Cre recombinase is active in the presence of the Mer ligand, 4-hydroxy tamoxifen, but sequestered in an inactive form by heat shock proteins in the absence of the ligand. MerCreMer functions efficiently in DT40 cells [8] as well as in mammalian cell lines.

We have constructed three plasmid vectors containing selectable marker cassettes flanked by mutant loxP sites. Following integration into the genome of DT40 cell, the marker cassettes are efficiently deleted, if MerCreMer is induced by 4-hydroxy tamoxifen. Another vector containing a cDNA expression cassette flanked by loxP sites should be useful for conditional knock-outs.

Results

Vectors containing mutant loxP flanked marker cassettes

Mutant loxP vectors, pLoxNeo, pLoxPuro and pLoxBsr, are designed respectively to have neomycin resistance (neoR), puromycin resistance (puroR), blasticidin S resistance (bsr) genes located between the loxP_RE and loxP_LE (Figure 1b). All resistant markers are driven by the chicken β -actin promoter. Transcription of the pLoxNeo, pLoxPuro and pLoxBsr is terminated by an artificial polyA signal, the SV40 polyA signal and the HSV tk polyA signal, respectively. These mutant loxP-flanked selectable marker genes can be used as *Bam*HI cassettes in knock-out construct and expression constructs. Knock-out constructs can be generated by cloning sequences from the 5' and the 3' end of the target locus into the multiple cloning sites (MCS_1 and MCS_2) of the mutant loxP vectors. An alternative strategy is to first clone the 5' and 3' target locus sequences into the vector, and then insert the selectable marker cassette into the *Bam*HI-compatible sites (*Bam*HI [GGATCC], *Bgl*II [AGATCT] or *Bcl*I [TGATCA]).

A primer derived from the marker gene can be used with a primer derived from a sequence of the target locus which was not included in the knock-out construct to screen for targeted integration events. The sequences of a suitable primer for each marker gene are given in Table 1I. These primers worked most reliable among a couple of primers tested for PCR amplifications. Several attempts to find primers suitable for PCR amplification in the direction upstream of the β -actin promoter were unsuccessful. Attempts to amplify through the β -actin promoter also failed. We suspect that this is due to the extremely GC-rich sequence of β -actin promoter.

Recombination efficiency of mutant loxP sites

The chicken B cell line DT40 was used to test the recombination efficiency of mutant loxP sites after chromosomal integration (Figure 2a). First a DT40 subclone, of which is transgenic for the viral oncogene v-myb and has a high gene conversion activity (unpublished results) was stably transfected with the inducible MerCreMer plasmid vector [7]. This results in a cell clone called DT40 Cre 1. The MerCreMer mRNA is constitutively expressed in DT40 Cre 1, but the protein is inactive in the absence of tamoxifen due to the fused HBD.

Both alleles of activation induced cytidine deaminase (AID [9,10]) gene were disrupted in DT40 Cre 1 by knockout constructs which are derived from pLoxPuro and pLoxBsr. The phenotype of the AID $-/-$ mutant cell line will be described separately. To induce and verify Cre mediated excision of the marker cassettes, the AID $-/-$ mutant was cultured in the presence of 4-hydroxy tamoxifen, and recombination events between loxP_RE

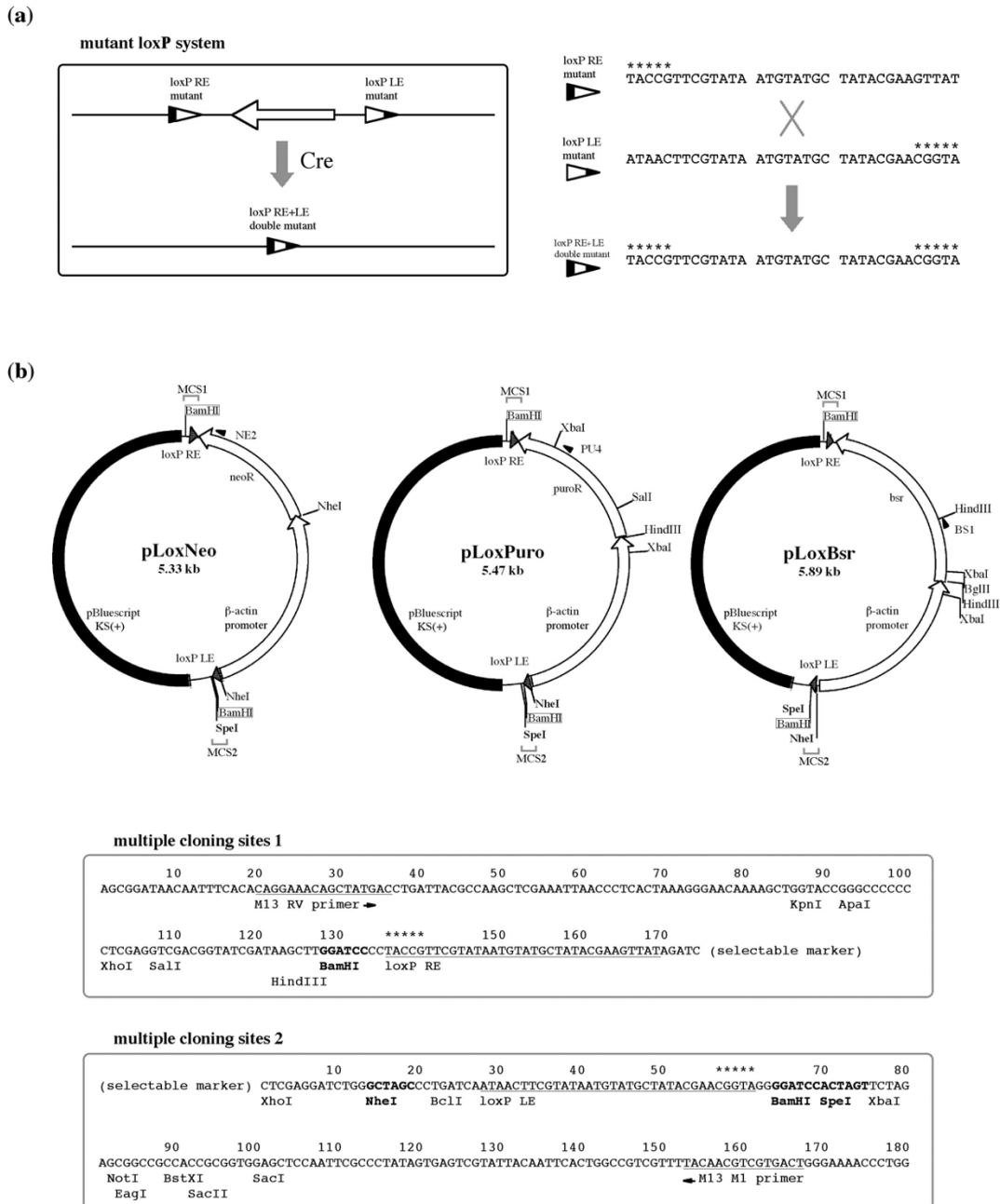


Figure 1

Mutant loxP sites and mutant loxP vectors. (a) The scheme of mutant loxP system is shown with sites for the rearrangement of mutant loxP sites. Nucleotide changes in mutant loxP sequences are stressed by stars. (b) Maps and positions of multiple cloning sites in the mutant loxP vectors, pLoxNeo, pLoxPuro and pLoxBsr. Each of the mutant loxP-flanked selectable marker genes can be utilized as a BamHI cassette. pLoxNeo, pLoxPuro and pLoxBsr have unique SpeI sites outside of mutant loxP-flanked region. pLoxPuro and pLoxBsr have unique NheI sites inside of mutant loxP-flanked region. These SpeI and NheI sites are important for the combination of the mutant loxP vectors with pExpress (Figure 3). Location of the PCR primers specific for the selectable marker genes (NE2, PU4, BSI) are shown by triangles.

Table I: Useful PCR primers

Primer	(vector)	Length	Sequence
NE2	(pMloxNeo)	30 mer	GGCTGACCGCTTCCTCGTGCTTTACGGTAT
PU4	(pMloxPuro)	29 mer	CAGCGCCCGACCGAAAGGAGCGCACGACC
BS1	(pMloxBsr)	33 mer	CGATTGAAGAACTCATTCCACTCAAATATACCC
forward	(pExpress)	30 mer	TTATTGTGCTGTCTCATCATTTTGGCAAAG
reverse	(pExpress)	30 mer	CTCTACAAATGTGGTATGGCTGATTATGAT

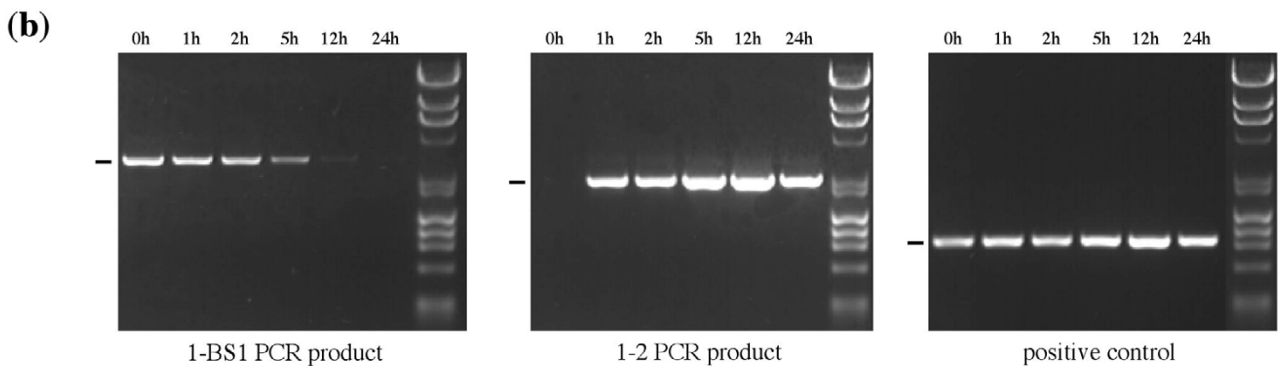
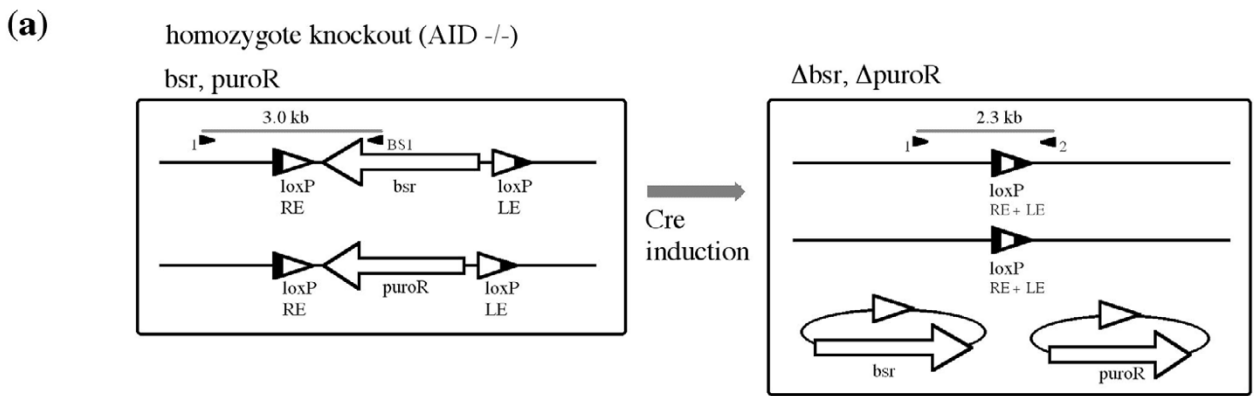


Figure 2

Quantitation of mutant loxP recombination following the induction of Cre recombinase. (a) Configuration of the AID locus in an AID $-/-$ DT40 mutant before and after Cre recombinase induction. The cell clone stably expresses a transfected MerCreMer construct. PCR primers for detecting mutant-loxP recombination are shown together with the sizes of the resulting PCR products. (b) Time course of mutant loxP recombination. The cells were cultured for the indicated time in the presence of 0.05 mM 4-hydroxy tamoxifen for inducible-Cre activation. Crude extract of 500 cells were utilized for PCR reaction. The region downstream of loxP_LE, which is not affected by recombination, was amplified as a positive control.

and loxP_LE were quantified by PCR (Figure 2b). In the absence of tamoxifen, recombination products between loxP_RE and loxP_LE were not detected. Because of the tight MerCreMer control, it was possible to work with this cell line over a period of weeks without the occurrence of recombination events. One hour after tamoxifen induction, mutant loxP recombination products were easily detected. PCR products derived from the unarranged locus were only faintly present 12 hour after tamoxifen addition and undetectable 24 hour after tamoxifen addition. This indicates that deletion of mutant loxP cassettes in virtually all cells of the culture required only one day of exposure to active Cre. Excision of the mutant loxP flanked marker cassettes were also confirmed in loci other than AID. Several different DT40 knock-out mutants were cultured overnight with tamoxifen for Cre induction, and were then subcloned by limiting dilution. The frequency of subclones which have deleted the selectable markers ranged from 60% to 100% depending on the targeted locus (data not shown).

Expression cassette vector designed for conditional knock-out

An expression vector pExpress was designed in which the expression of a cloned cDNA is controlled by the chicken β -actin promoter and a SV40 poly A signal (Figure 3a). The coding sequence of a cDNA can be inserted into unique *HindIII*, *NheI*, *EcoRV*, *BglII*, *NcoI*, *SmaI* sites. A variety of restriction sites can be chosen for cloning of the cDNA, because 1) the *NheI* site is compatible with *XbaI*, *SpeI* and *AvrII* sites, 2) the *BglII* site is compatible with *BamHI* and *BclI* sites and 3) *EcoRV* and *SmaI* digests generate blunt ends. The forward and reverse primers (Table 11) can be utilized for sequencing and PCR amplification of the inserted cDNA.

The pExpress cDNA expression cassette can be combined with the selectable marker cassettes of the mutant loxP vectors. pLoxNeo, pLoxPuro and pLoxBsr have unique *SpeI* sites outside of the mutant loxP-flanked region (Figure 1b). pLoxPuro and pLoxBsr have unique *NheI* sites inside of the mutant loxP-flanked region (Figure 1b). The *SpeI* and *NheI* are compatible with each other, and are for the insertion of the cDNA-expression *SpeI* cassettes (Figure 3b). Although the *NheI* site is not unique in pLoxNeo, a *NheI*-partial digest allows insertion of the cDNA expression cassette as a *SpeI* fragment. Cloning of the cDNA expression cassette into the *SpeI* site of a mutant loxP vector generates an expression vector, in which only the selectable marker is flanked by mutant loxP sites. If the cDNA expression cassette is cloned into the *NheI* site of a mutant loxP vector, both of selectable marker and expression cassette are contained in the region flanked by mutant loxP sites. This second construct is useful if one intends to remove the cDNA ex-

pression cassette at a later time together with the marker cassette.

The combination of a marker gene and an expression cassette can be inserted as a *BamHI* cassette between the 5' and 3' target gene sequence of a knock-out construct (Figure 3b). It is also possible to design knockout/complementation constructs, in which the cDNA expression cassette is derived from the target gene. After target integration of this construct, the expressed cDNA should complement the gene disruption. Subsequent deletion of the expression cassette by Cre induction will lead to a complete gene knock-out.

Discussion

We have developed mutant loxP vectors for selectable marker recycling and conditional knock-outs.

To produce a homozygous cell clone, two selectable markers are normally required for the disruption of both alleles, and one further selectable marker is needed for phenotype complementation. Since three markers are consumed for each knock-out study, disruption of multiple genes is restricted by the number of available selectable marker.

Recycle of the mutant loxP marker cassettes solves this problem and enables the addition of an unlimited number of mutations to a cell clone. Genetic instability is supposed to be low even after multiple rounds of selectable marker excision because the mutant loxP sites are inactivated after recombination. The excision efficiency of mutant loxP sites after overnight Cre induction ranged between 60–100% when different loci were tested. This variation is likely due to chromosomal context of the integration site. This excision efficiency may be easily improved by longer exposure of cells to the active Cre-recombinase.

Cre-mediated marker excision has previously been used for animal and cell line knock-outs. Mice carrying loxP flanked genes have for example been crossed with Cre-transgenic mice which express Cre in a cell-type-specific or inducible manner [11,12]. Tetracycline-regulated Cre expression was used for a conditional RAD51 knock-out in DT40 cells [13] and another inducible Cre, CreER, which carries a HBD of mutated human oestrogen receptor was used to excise markers after targeting of the HPRT locus [14]. The advantage of MerCreMer, described here, is in its tight control in addition to a high level of induction [15]. In contrast to MerCreMer, Cre carrying a single HBD has leaky recombination activity even in the absence of the ligand (unpublished result). Other advantages of our vectors are the use of mutant loxP sites, the presence of multiple convenient restric-

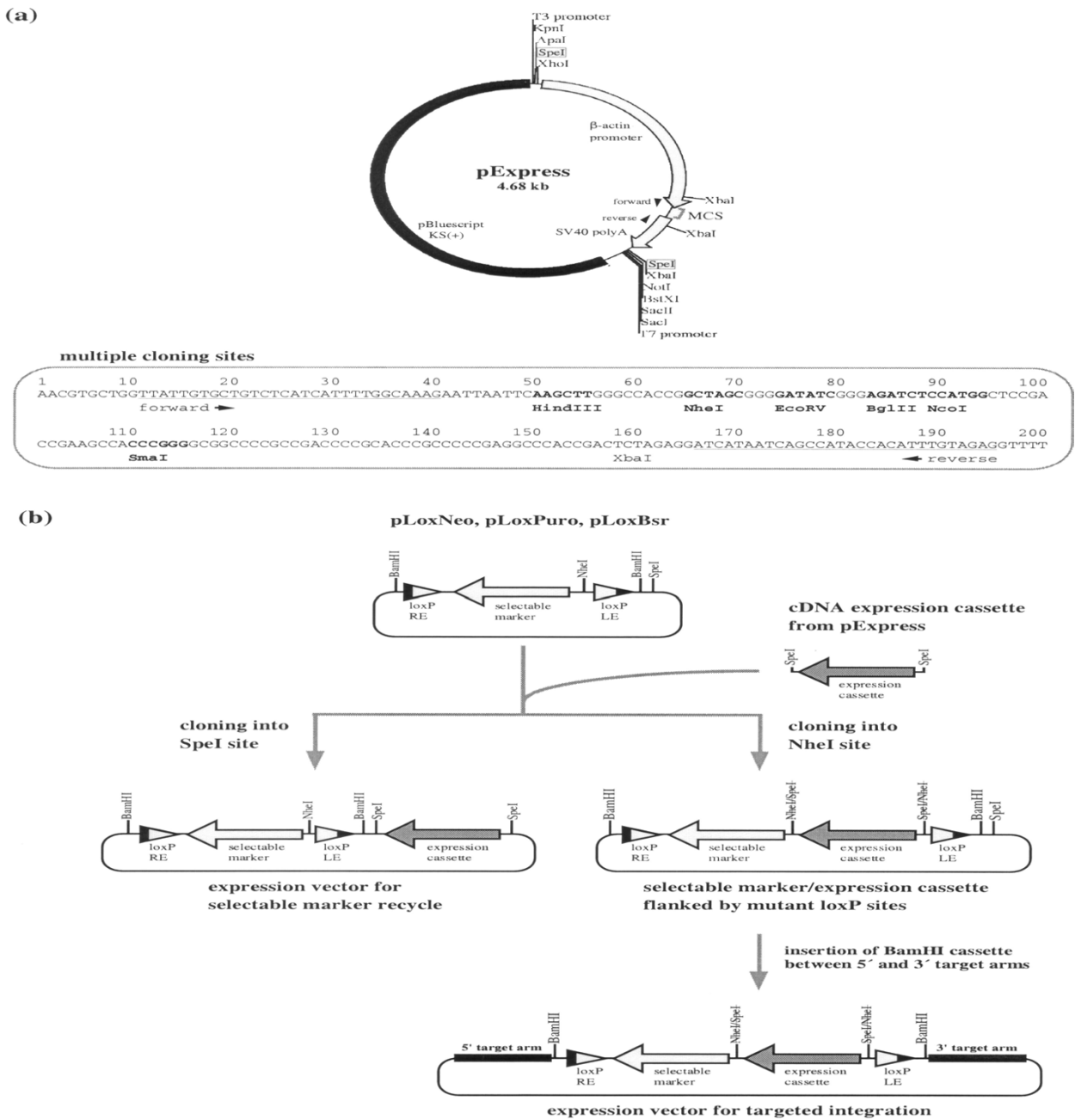


Figure 3

Design of the cDNA expression vector and the conditional expression vectors. (a) Map and multiple cloning sites of expression vector pExpress. After cloning of the cDNA coding sequence into pExpress, the expression cassette can be excised as a SpeI cassette. Location of forward and reverse primers are shown by triangles. (b) Insertion of the cDNA expression cassette into the mutant loxP vectors. Cloning of the expression cassette (SpeI cassette) into the SpeI site of mutant loxP vectors (pLoxNeo, pLoxPuro and pLoxBsr) produces an expression vector whose selectable marker can be recycled. Cloning of the expression cassette (SpeI cassette) into the NheI site of mutant loxP vectors (pLoxPuro and pLoxBsr) produces a vector in which a selectable marker and an expression cassette is flanked by mutant loxP sites.

tion sites flanking the marker cassettes and the use of the strong and insertion site independent β -actin promoter.

The expression vector pExpress offers high flexibility and many options when combined with the mutant loxP vectors. First, any one of three selectable marker genes

(neoR, puroR or bsr) can be chosen. Second, the cDNA expression cassette can be located either inside or outside of mutant loxP-flanked region. Finally, expression constructs can be designed for either random or targeted integration.

The transfection of the cDNA expression cassette combined with a targeting vector enables its insertion as a single copy into a defined chromosomal location. This should produce transfectants with similar expression levels, as there is no variation due to differences in copy numbers and insertion sites. The defined expression level in independent transfectants will be advantageous for studies in which the effect of amino acid alterations is analyzed by genetic complementation. Targeted integration of an expression cassette is also preferable over random integration, if the subsequent excision is intended. This is due to the fact that the excision of a single copy is easier to predict and to verify than the excision of multiple randomly integrated copies.

Conclusions

The mutant loxP system will further facilitate genetic manipulation of chicken B cell line DT40. The mutant loxP vectors have now been tested for a year, and have been shown to function reliably in different chromosomal contexts. All genes in the vectors are driven by the chicken β -actin promoter, which is also strong in mammalian cells including mouse ES cells. The vector cassettes should therefore be also useful for reverse genetics of mammalian cells.

Materials and Methods

Oligo nucleotides

ML1 GATCCCCTACCGTTCGTATAGCATAACATTATACGAAGTTATTGATCAGGGCTAGCG

ML2 AATTCGCTAGCCCTGATCAATAACTTCGTATAATGTATGCTATACGAACGGTAGGG

ML3 CTAGCCCAGATCTATAACTTCGTATAGCATAACATTATACGAACGGTAGGGGATCCA

ML4 AGCTTGATCCCCTACCGTTCGTATAATGTATGCTATACGAAGTTATAGATCTGGG

LK5 AGCTTGGGCCACCGGCTAGCGGGGATATCGGGAGATCTC

LK6 CATGGAGATCTCCCGATATCCCCGCTAGCCGGTGGCCCA

LK9 CGGACTAGTGGCTCGAGGGAGATCTGGA

LK10 CTAGTCCAGATCTCCCTCGAGCCACTAGTCCGGGCC

Primer 1 [AI1] GTTCTGTGCACCAGAGGGCTGAACAGTCA

Primer 2 [AI13] CTCCTTTCTTGGCTGGGTGAGAGGTCATA

Linker preparation

Linker oligo-nucleotides were designed to minimize internal secondary structure. Linkers were prepared as follows: Oligo-nucleotides were dissolved into TE at a final concentration of 100 pmol/ μ l. 40 μ l of each oligo-nucleotide solution was combined, and incubated at 70°C for 5 minutes. 20 μ l of 5 \times annealing buffer (5 \times annealing buffer: 0.5 M Tris HCl pH 7.5, 0.35 M MgCl₂) was added to the combined oligo-nucleotide solution and was incubated at 70°C for 5 minutes. After slowly cooling down to room temperature for more than one hour, 10 pmol of the annealed linker was ligated with 100 ng of plasmid which had been digested by the appropriate restriction enzymes without alkali-phosphatase treatment. Under this condition, linker ligation was detected in more than 90% of bacterial transformants. The sequence of linker was confirmed by sequencing of plasmid clones. As expected, linker concatemers were not observed in the plasmid clones.

Construction of mutant loxP vectors

The linker ML1-ML2 was cloned between *Bam*HI and *Eco*RI sites of pBluescript KS (+). The resulting plasmid was digested with *Nhe*I and *Hind*III and was ligated with the linker ML3-ML4. The plasmid resulting from this ligation was digested with *Bgl*II, and was ligated with the *Bam*HI cassette of β -actin NeomycinR, β -actin PuromycinR, β -actin blasticidinR [16] to produce pLoxNeo, pLoxPuro and pLoxBsr, respectively. The full-length sequences of the cassettes are available [<http://genetics.hpi.uni-hamburg.de/dt40Reagents.html>].

Construction of pExpress

The *Bam*HI-*Xho*I fragment of β -actin PuromycinR [16] was cloned between the *Bam*HI and the *Xho*I sites of pBluescript KS (+). The resulting plasmid was digested with *Hind*III and *Nco*I, and was ligated with linker LK5-LK6 to produce plasmid bEX5. pBluescript KS (+) was digested with *Apa*I and *Spe*I, and ligated with linker LK9-LK10. The plasmid resulting from this ligation was digested by *Xho*I and *Bgl*II, and ligated with the *Xho*I-*Bam*HI fragment of bEX5 to produce pExpress.

Cre induction and PCR analysis

Medium containing 0.05 mM 4-hydroxy tamoxifen (H7904, SIGMA) was used for Cre induction. Crude cell

extract for PCR was prepared from AID $-/-$ DT40 cells after Cre induction as follows: The cells were once washed with PBS, and were suspended into $1 \times$ PCR buffer with 1 mg/ml proteinase K and 0.5% Tween 20. This solution was incubated for 45 min at 56°C for proteinase K-mediated proteolysis, followed by a 10 min incubation at 95°C to inactivate the proteinase K. Crude extract corresponding to 500 cells was used for each PCR reaction. PCR amplification was performed with Expand Long Template PCR System (Roche): 2 min initial incubation at 93°C , 30 cycles consisting of 93°C for 10 sec, 65°C for 30 sec and 68°C for 5 min with cycle elongation of 20 sec per cycle, and a final 5 min elongation step at 68°C .

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