PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2008, 35(6): 650~660
www.pibb.ac.cn

A Model Study: Simulation of Marker Gene Deletion and Target Gene Replacement Mediated by Cre/*lox* System in Cell Line*

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Abstract The incorporation of site-specific recombination systems can help to overcome bottlenecks in livestock transgenic technology. For evaluating the efficiency of Cre/*lox* mediated DNA recombination in embryos and somatic cells, a working model was established using rat mammary carcinoma cell line SHZ-88, aimed at creation of and use repeatedly of selected "friendly loci" in transgenic livestock. An integration vector pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P, which red fluorescence gene *DsRed* served as the first target gene and green fluorescence gene GFP as marker gene, was constructed for introduction of acceptor loci in genome. At the same time a replacement vector pT-*lox*2272-neo-*lox*P in which Neo coding sequence served as the second target gene was also constructed for replacing *DsRed* gene. Transgenic cell clones were produced by electroporating SHZ-88 cell with the integration vector. Cells from three transgenic clones selected randomly were further amplified and were then co-electroporated with the replacement vector as well as *cre* gene. Analysis of the expression patterns of DsRed and GFP indicated that among the 1 070 cell colonies the efficiency on marker GFP deletion was 91.1% and the efficiency on gene replacement was 29.3%. Molecular analysis by PCR and Southern blotting confirmed that the color patterns as expressed by cell colonies could represent the actual molecular events. This working model mediated by Cre/*lox* system should be useful for the improvement of the present animal transgenic technology.

Key words *lox*2272, *lox*P, Cre, marker gene deletion, target gene replacement

Animal transgenic technology holds considerable promise for livestock industries as well as has important uses for biomedicine industries[1, 2]. However, a quarter of a century has passed since the first successes in the production of "supper mouse" and of mice expressing a human gene in their mammary tissue[3, 4], the applications of transgenic animal technology have not yet had the impact on industry as predicted by many people at that time. The main obstacles to prevent the progress of this important technology have been the low efficiency in production of transgenic livestock and of the insertion of foreign genes at a random fashion in genome. To circumvent the difficulties in mass production of transgenic livestock, methods of high efficiency and low cost were tried by many investigators[5~9]. However, all these methods have their inherent limitations and have not yet been ready for use in commercial livestock production.

Almost at the beginning, some authority authors had foreseen the problems in the application of transgenic technology to livestock and had envisaged that one possible way to produce transgenic livestock efficiently might be to screen and use repeatedly so called "friendly gene loci"[10]. However, mainly for quick returns nobody had ever tried seriously this strategy. More recently, several investigators have re-emphasized on the importance of creating genomic acceptor sites for foreign genes by insertion of pairs of hetero-specific *lox* sites to appropriate gene loci[11]. Cre/*lox* site-specific recombination system is a high

*This work was supported by a grant from Hi-Tech Research and Development Program of China(2002AA206311 and 2006AA02Z148).
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Received: January 29, 2008 Accepted: March 10, 2008

efficient tool for gene insertion/excision [12]. With the mediation of Cre recombinase any intervening sequence between two *lox* sites in the same orientation can be excised [13]. In recent years, several hetero-specific *lox* sites have been identified and tested [14, 15], such as *lox*511, *lox*5171 and *lox*2272. By flanking a pairs of hetero-specific *lox* sites to a foreign gene, it can be replaced by another gene flanked by the same pairs of *lox* sites after its insertion into the mammalian genome, namely "Recombinase Mediated Cassette Exchange" (RMCE) [16]. This system has been successfully used to modify precisely mice stem cell[17~19] and several types of somatic cells [20].

With the advent in site-specific recombination it is now quite possible that by combining use of the available technologies, for example, *in vitro* fertilization, somatic cloning with Cre/*lox* system, "friendly loci" or foreign gene acceptor sites could be identified and used repeatedly in commercial practice. Aimed at finding "friendly loci" to be used in animal mammary bioreactor, we have designed and constructed a set of vectors which contain two expression cassettes: one for selection of positive transgenic embryos and the other for exchange target gene by RMCE after the first target gene has expressed efficiently at a "friendly locus". In this paper we report an examination of its function in deletion of marker gene and in exchange of target genes using a simulated method.

1 Materials and methods

1.1 Construction of integration vector

The integration vector pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P, containing both of the red and green fluorescence protein coding sequences, flanked by a pairs of *lox*2272/*lox*P and a pairs of *lox*P/*lox*P respectively, was constructed by removing required elements from intermediate vectors of pT-*lox*P-GFP-*lox*P (kept in the authors' laboratory), pDsRed1-1 (Clontech, USA) and pEGFP-N1 (Clontech) and then cloned into vector pTE-*lox*2272-BIB (kept in the authors' laboratory) in sequential.

1.2 Construction of replacement vector

The replacement vector pT-*lox*2272-neo-*lox*P was constructed in two steps: firstly to create the intermediate plasmid pTE-*lox*2272-neo-*lox*P by recombining of the plasmid pTE-*lox*2272-BIB-*lox*P-GFP-*lox*P (constructed in this experiment) with plasmid p-TN containing Neo coding sequence (kept in the authors' laboratory), secondly restriction sites *Eco*47 Ⅲ and *Mlu* Ⅰ were deleted by PCR amplification and re-cloning into plasmid pMD18-T (Takara, China).

1.3 Cell culture and electroporation

SHZ-88 cells (Cell Bank, The Chinese Academy of Sciences, Shanghai) were cultured at 37℃ in 5% CO_2 in RPMI1640 (Gibco, USA) medium plus 10% FCS(Hyclone, USA), 100 U/ml penicillin (Gibco) and 100 mg/L streptomycin(Gibco).

For producing of cell colonies carrying integration vector, SHZ-88 cells (1×10^7/0.8 ml in PBS) were electroporated at 200 V and 960 μF with 50 μg of ApaLI-digested pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P. At 48 h after culture, the cell colonies showing both red and green color under a fluorescence microscope (Nikon, Japan) were picked and expanded for the subsequent experiments.

For assay of the efficiencies of Cre mediated DNA sequence deletion and replacement, the above picked cell colonies，which always showed both red and green color even after 5 ～6 passages，were co-electroporated at 200 V and 960 μF with 20 μg of the replacement vector pT-*lox*2272-neo-*lox*P and 10 μg of Cre expression vector pBS185 (Gibco) in their circular forms. At 36 h after electroporation, the cells were selected with 400 g/L G418 (Gibco) for 7 days, and then observed and scored under the fluorescence microscope. Through comparing the proportions of different colored cell clones, the efficiencies of Cre mediated DNA sequence deletion and replacement were calculated.

1.4 Assay by PCR and Southern blots

Primer G1 (Forward 5′ TAGCGCTACTAGTCT-CGG 3′) and G2 (Reverse5′ CTCTCCCATATGGTC-GAC 3′) were used in PCR reactions for assay of the insertion of the integration vector in the genomes of the green and red transgenic clones, for assay of the red clones resulted from marker deletion and for assay of the colorless clones resulted from gene replacement plus deletion. The conditions of reaction with primer G1 and G2 were: 94℃ 40 s, 58℃ 30 s, 72℃ 4 min, 30 cycles. Primer P1(Forward 5′ GAGTGATGAGGTTC-GCAAGA 3′) and P2 (Reverse 5′ TAGCGCCGTAA-ATCAATCGA 3′) were used in PCR assay for the integration of Cre coding sequence in the genomes of the red clones resulted from marker deletion and the colorless clones resulted from gene replacement plus deletion. The conditions of reaction with primer P1

and P2 were: 94℃ 40 s, 60℃ 30 s, 72℃ 1 min, 30 cycles.

For Southern blots, Primer R1 (Forward 5′ TGCGCTCCTCCAAGAACGTC 3′) and R2 (Reverse 5′ AGCTTCAGGGCCTTGTGGAT 3′) were used for synthesis of probes specific to pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P and conditions of reaction were: 94℃ 40 s, 56℃ 30 s, 72℃ 40 s, 30 cycles; Primer N1 (Forward 5′ GGATCGGCCATTGAACAAGA 3′) and N2 (Reverse 5′ CAGAAGAACTCGTCAAGAAG 3′) were also used for the synthesis of probe specific to pT-*lox*2272-neo-*lox*P and conditions of reaction were: 94℃ 40 s, 54℃ 30 s, 72℃ 40 s, 30 cycles. Six micrograms of genomic DNA was digested with appropriate restriction enzymes, electrophoresed on a 0.9% agarose gel (Sigma, USA) and blotted onto a nylon membrane (Amersham, UK). Southern blots were performed using a DIG DNA Labeling and (RNA blot) Detection Kit Ⅰ(Roche, Ger).

1.5 Data analysis

Student' t-test was used to analyze data and $P < 0.05$ was considered significant statistically.

2 Results

2.1 Experiment design

For the easiness of observation of marker gene deletion and target gene replacement, vector pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P was constructed and used as a working model to create imitated "friendly loci" in the genome of cells, instead of using the more complex original mammary tissue specific expression vector without a visualized phenotype. At the same time vector pT-*lox*2272-neo-*lox*P was also constructed for use in RMCE to replace the red fluorescence gene *DsRed* with *neo* gene. To introduce Cre recombinase into cells, a commercial expression vector PBS185 was used in the electroporation experiments together with plasmid pT-*lox*2272-neo-*lox*P. In theory, the outcomes of these experiments should be as follows: upon the integration of pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P transgenic cells showing both of red and green color would be produced along with colorless none-transgenic cells. The transgenic single cells could then be amplified in culture to single clones. However, upon the introduction of pT-*lox*2272-neo-*lox*P and pBS185, under the mediation of Cre enzyme, the green fluorescence gene GFP should be deleted by the recombination of the two wild type *lox*P sites leaving one wild type *lox*P site in the location. At the same time, the red fluorescence gene sequence flanked by a *lox*2272 site and a wild type *lox*P site should be replaced by *neo* gene flanked by the same combination of hetero-specific *lox* sites in the replacement vector. Since marker gene GFP was flanked by a pair of wild type *lox*P sites in the process of gene replacement, the extreme 3' end *lox*P site could well be used to react with the *lox*P site in the replacement vector instead of the upstream *lox*P site. As a result, GFP sequence would be replaced together with DsRed sequence. These predicted results are illustrated schematically in Figure 1.

2.2 Production of transgenic cells

For assay of the efficiencies of Cre mediated DNA sequence deletion and replacement, acceptor sites were created by electroporating SHZ-88 cells with linear pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P DNA. As showed in Figure1, this construct contains two fluorescence genes: a red fluorescence protein gene flanked by a *lox*2272 site and a wild type *lox*P site, and a green fluorescence gene flanked by two wild type *lox*P sites. Theoretically, transgenic cell clones should show both of red and green fluorescence color under the fluorescence microscope while none-transgenic cells should be colorless. In our experiment about 50% cells showed both red and green fluorescence color under the fluorescence microscope after electroporation and culture for recovery. This could largely attribute to the transient expression of *DsRed* and GFP genes because both of them preceded by the strong CMV promoter. However, following growth in culture most cells lost fluorescence color but a few fluorescent cell clones formed. When these cell clones were examined under the fluorescence microscope, all cells in a single clone maintained uniformly their newly acquired properties i.e. showing both of red and green fluorescent color. These clones represented the original transgenic single cells that had the inputting DNA construct copy or copies integrated into their genomes. These clones were then used for the experiments of marker gene deletion and target gene replacement. One of the cell clones is shown in Figure 2. These results confirmed that the predicted acceptor loci had been created in groups of cells.

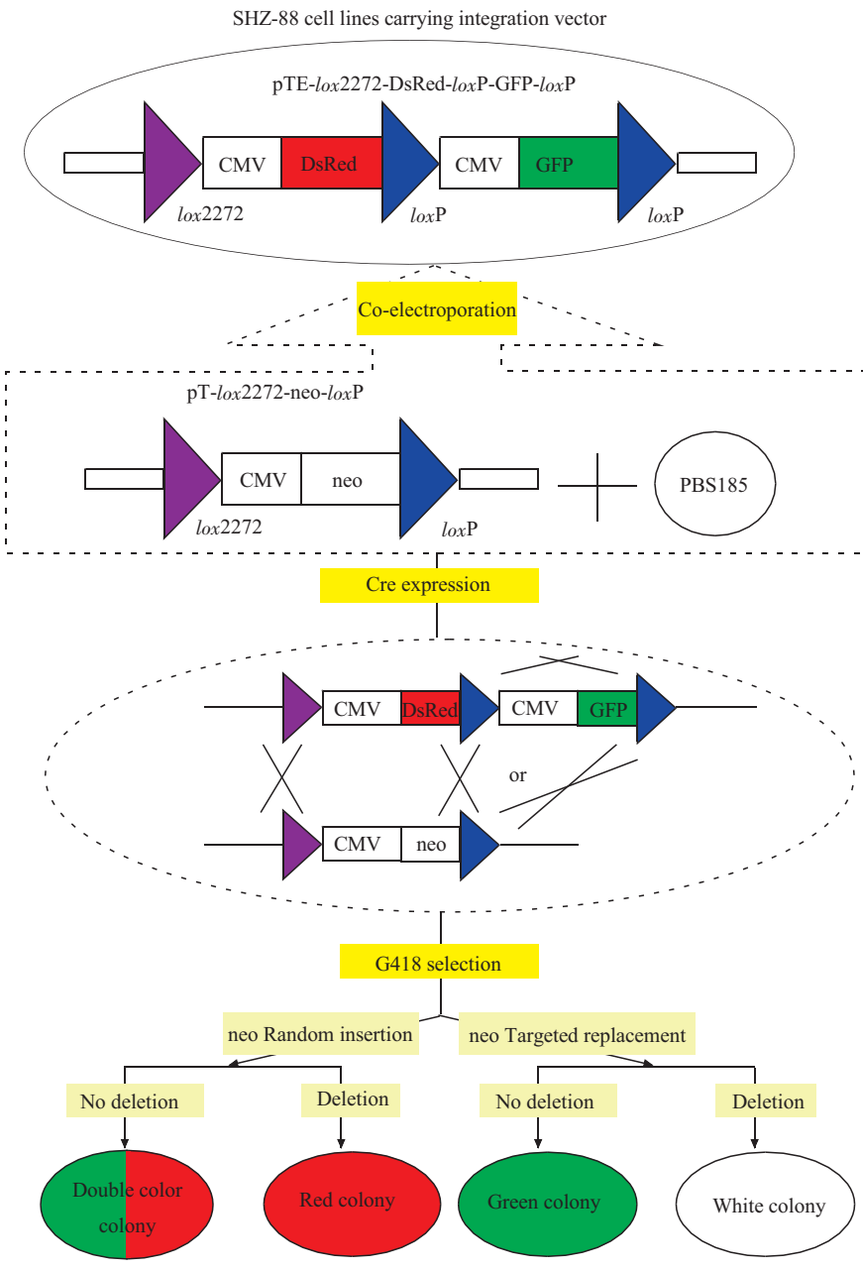

Fig. 1　Schematic representation of marker gene deletion and target gene replacement as explained in text

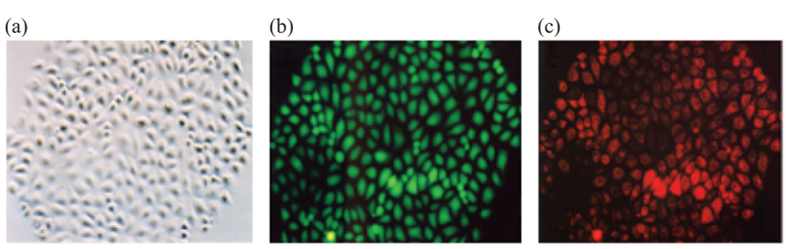

Fig. 2　A transgenic cell clone under the fluorescence microscope (10×10)

The same cell clone is observed under a fluorescence microscope at bright field (a), fluorescence fields with the wavelength of 488 nm for GFP detection (b) and of 558 nm for DsRed detection(c).

2.3 Marker gene deletion and target gene replacement in cells

As described in the previous section of experiment design, red fluorescent protein gene was used as imitated target gene that could be replaced by another target gene (in this experiment is *neo* gene) while green fluorescent protein gene was designed to serve as marker that should be deleted after selection of transgenic embryos (in the present experiment is transgenic cells). For examining the efficiencies of Cre/*lox* system in these two roles, three transgenic single clones, which produced both of red and green fluorescence color, were empirically picked up and designated as clone A1, clone A2 and clone A3. The above three clones were amplified by culture to enough cell numbers, three cell samples were taken from each of the three clones and then all samples were co-electroporated by the replacement vector pT-*lox*2272-neo-*lox*P and Cre expression vector pBS185. All together nine co-electroporation experiments were carried out and for each of the three clones three repeats were set up on independent days. Electroporated cells grew very well under the selection of G418 and a lot of cells clones were observed. Observing under the fluorescence microscope, three types of cell clones were found: colorless clones (Type1), red clones (Type2), double colored clones (Type3). The representative images for three types of cell clones were shown in Figure 3. These results signified that two types of Cre mediated reactions had occurred accurately, leading to marker gene deleted and target gene replaced in part of the cells. This is in good agreement with predicted results in experiment designs, according to which two wild type *lox*P sites would recombine by themselves and delete the intervening GFP whereas the neo gene flanked by a *lox*2272 site and a wild type *lox*P site would replace the intervening sequence between the *lox*2272 site and one of the *lox*P sites.

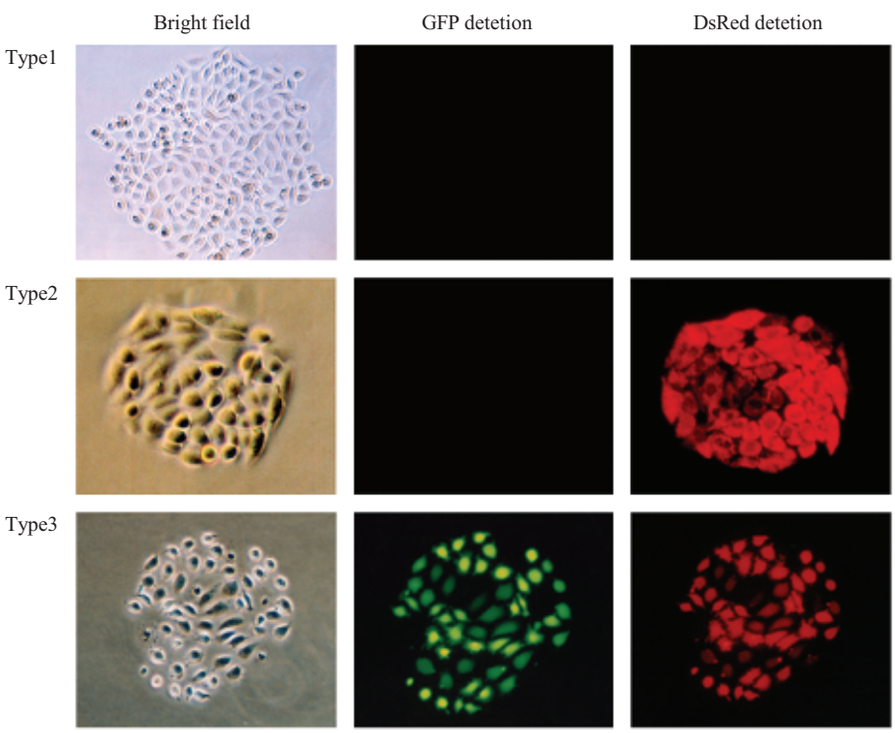

Fig. 3 The representative cell clones observed under the fluorescence microscope(10×20)after co-electroporation

Type1: A clone showing colorless resulted from GFP gene deletion and *DsRed* gene replacement; Type2: A clone showing red color resulted from green GFP deletion; Type3: A clone showing both of red and green colors came from none Cre mediated reaction.

The efficiencies of the two types of Cre/*lox* mediated reactions were estimated by comparing the proportions of different colored cell clones. The efficiency of marker gene deletion was calculated as the percentage of colorless clones (Type1) plus red color clones (Type2) in total clones while the

efficiency of target gene replacement was calculated as the percentage of colorless clones (Type1) in the total. These results are presented in the following Table 1. Data analysis showed that among the total observed 1 070 cell clones, colorless clones accounted for 29.3% indicating about one third *DsRed* gene were replaced by *neo* gene. However, red color clones plus colorless clones were the majority, accounting for 91.1% of the total clones signifying that deletion of the GFP gene was more efficient than that of gene replacement. Of course these could be attributed to an added effects of two site specific recombination reactions: one between the two wild type *lox*P sites and the other happened in the process of gene replacement mediated by two *lox*2272 sites in genome and in replacement vector respectively as well as by the *lox*P site in the replacement vector and the far 3' end wild type *lox*P site of the integration vector in genome. However, there were still about 10% clones remaining red & green colors signifying Cre/*lox* mediated reaction was not performed. The above efficiencies were compared among the clones originated from clone A1, clone A2 and clone A3 because each group of clones resulted from a single cell having a unique integration site. However, the differences of efficiency in Cre/*lox* mediated reaction were not significant statistically though the absolute figure appeared different.

Table 1 Efficiencies of Cre/*lox* mediated sequence deletion and replacement[1)]

Cell clone	Batch of electroporation	Type 1	Type 2	Type 3	Total	Deletion %	Replace %
A1	1st	34	73	13	120	89.2	28.3
	2nd	41	82	8	131	93.9	31.3
	3th	35	77	10	122	91.8	28.7
A2	1st	23	59	14	96	85.4	24.0
	2nd	30	71	11	112	89.3	26.8
	3th	21	65	12	98	87.8	21.4
A3	1st	41	77	9	127	92.9	32.3
	2nd	46	83	5	134	96.2	34.3
	3th	48	74	8	130	93.8	36.9

[1)]Cells of three clones (A1, A2 and A3) were co-electroporated with the replacement vector pT-*lox*2272-neo-*lox*P and the Cre expression vector pBS185 in three repeats for each of the clones in similar conditions. Type1: a clone showing colorless at both wave lengths 488 nm and 558 nm resulted from GFP gene deletion and DsRed1 gene replacement; Type2: a clone showing red color resulted from green GFP deletion; Type3: a clone showing both of red and green colors came from none Cre mediated reaction.

$$\text{Deletion}/\% = \frac{\text{Number of Type1 and Type2}}{\text{Number of Total}} \times 100; \quad \text{Replace}/\% = \frac{\text{Number of Type1}}{\text{Number of Total}} \times 100$$

2.4 Synthesis of specific primers for molecular detection

Four pairs specific primers were synthesized for molecular detection of recombinant events: Primer G1 and G2 were for detection of vector pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P integration in cell genomes, for detection of GFP gene deletion as well as for detection of the replacement of *DsRed* gene by *neo* gene. Because these two primers were positioned outside *lox*2272 site at 5' end and the wild type *lox*P site at the extreme 3' end, the lengths of PCR amplified fragments would tell the recombination events at molecular level. Primer R1 and R2 were specific to DsRed coding sequence and PCR amplification by this pair of primers would produce a 500 bp probe , which was designated as DsRed probe. Primer N1 and N2 were specific to Neo coding sequence and PCR amplification would produce a 780 bp probe, designated as Neo probe. The relative positions of the above primers and DNA probes amplified by them are illustrated schematically in Figure 4. Primer P1 and P2 were specific to *cre* gene sequence and PCR amplification with this pair of primers would produce a 761 bp fragment.

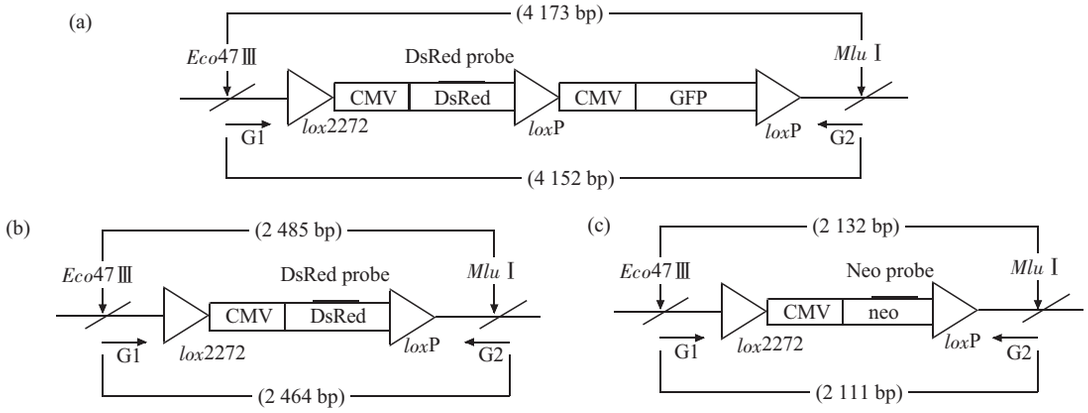

Fig. 4　Schematic illustration of positions of synthesized specific primers and probes

(a) For detection of vector pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P integration in genomes of the transgenic cells, a specific fragment of 4 152 bp should be produced by PCR with primer G1 and G2, and after the genomic DNA was double digested with *Eco*47Ⅲ plus *Mlu* Ⅰ a specific fragment of 4 173 bp should be hybridized by Southern blots with DsRed probe. (b) For detection of GFP gene deletion in genomes of the red clones , a specific fragment of 2 464 bp should be produced by PCR with primer G1 and G2, and after the genomic DNA was double digested with *Eco*47Ⅲ plus *Mlu* Ⅰ a specific fragment of 2 485 bp should be hybridized by Southern blots with DsRed probe. (c) For detection of GFP gene deletion and *DsRed* replacement with *neo* gene in genomes of the colorless clones, a specific fragment of 2 111 bp should be produced by PCR with primer G1 and G2 , and after the genomic DNA was double digested with *Eco*47Ⅲ plus *Mlu* Ⅰ a specific fragment of 2 132 bp should be hybridized by Southern blots with Neo probe.

2.5　Molecular detection of acceptor sites in genome of cell

A genomic DNA sample was isolated from amplified cells originated from clone A3, which was a transgenic cell clone showing both of red and green fluorescent colors and was used as template for PCR amplifying specific DNA fragment primed by primer G1 and G2. As shown in Figure 5a, a fragment of 4 152 bp was detected. For further classification of vector integration, genomic DNA was double digested with *Eco*47Ⅲ plus *Mlu* Ⅰ and Southern blotted with the DsRed probe. As indicated in Figure 5b, a specific fragment of 4 173 bp was hybridized. Thus molecular detection data were consistent with the fluorescent color reactions and indicated that pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P had integrated into the genomes of the transgenic cells. Therefore they were suitable materials for assay of the efficiencies of Cre/*lox* mediated site-specific recombination in designed conditions.

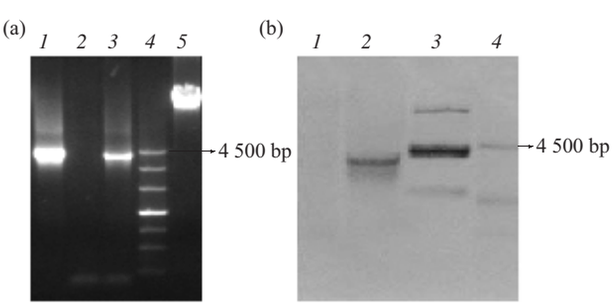

Fig. 5　PCR and Southern blot detection of transgenic cell clone

(a) Lane *1* is positive control; lane *2* is negative control; lane *3* is the amplified fragment from genomic DNA; lane *4* is DNA marker; lane *5* is the size of isolated genomic DNA. (b) Lane *1* is negative control; lane *2* is Southern blotted fragment; lane *3* is positive control; lane *4* is DNA marker.

2.6　Molecular detection of marker gene deletion

The same method was used to detect the deletion of marker gene GFP. DNA samples from two cell clones showing red fluorescent color without green fluorescent color after co-electroporation with pT-*lox*2272-neo-*lox*P and pBS185 were used as templates for PCR using primer G1 and G2. The specific DNA band of 2 464 bp was detected in samples (Figure 6a) and this was the expected size when GFP sequence had been deleted. With the DsRed probe the 2 485 bp *Eco*47Ⅲ/*Mlu* Ⅰ fragment was also detected in Southern blotting (Figure 6b). These evidences signified that the deletion of GFP sequence had happened. For examination of the status of vector pBS185 sequence in genomes of the two clones, the expected 761 bp fragment was checked by amplification using primer P1 and P2. Results indicated that this specific fragment could only be amplified from one of the clones but not from the other one (Figure 6c). These data indicated that the GFP sequence in one cell clone was deleted by the mediation of Cre protein produced from the integrated gene copies while in the other clone it was mediated by Cre protein of transient expressed in cytoplasm.

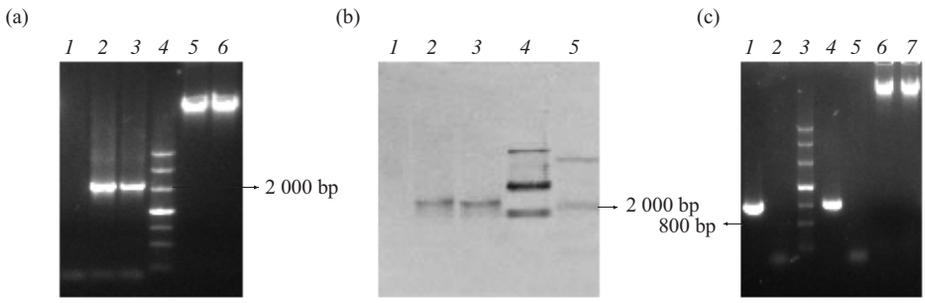

Fig. 6　Detection of the deletion of GFP in red cells by PCR and Southern blots

(a) Lane *1* is negative control; lane *2*, *3* is the amplified 2 464 bp fragment; lane *4* is DNA marker; lane *5*, *6* is genomic DNA. (b) Lane*1* is negative control; lane *2*, *3* is southern blot detected the 2 485 bp *Eco*47 Ⅲ/*Mlu* Ⅰ fragment; lane *4* is positive control; lane *5* is DNA marker. (c) Lane *1*, *2* are positive and negative control respectively; lane *3* is DNA marker; lane *4* is amplified 761 bp Cre gene fragment; lane *5* is the result of amplification for Cre gene insertion; lane *6*, *7* is genomic DNA.

2.7　Molecular detection of target gene replacement

According to experiment designs if marker gene GFP was deleted and the first target gene *DsRed* was replaced by the second target *neo* gene, cell clones should be colorless, and at the same time GFP and DsRed sequences should be disappeared from molecular detection. Moreover, *neo* gene sequence should be detected in the original place occupied by *DsRed* by PCR amplification and Southern blot. To prove these events did happen, genomic DNA from two colorless cell clones were subjected to PCR amplification using primer G1 and G2. As shown in Figure 7, the expected fragments of 2 111 bp were amplified from both of the two cell samples(Figure 7a) and at the same time the 2 132 bp *Eco*47 Ⅲ/*Mlu* Ⅰ fragment could also be detected by Southern blot with the Neo probe (Figure 7b). Considering together with the phenotypes of the cells, these data supported the conclusions that indeed the marker gene GFP was deleted from the integration site and the first target gene *DsRed* was replaced by another target gene, the *neo* gene, in the colorless cells. As in the previous section, the status of *cre* gene carried by vector pBS185 was also checked by PCR amplification using primer P1 and P2, the specific 761 bp fragment was detected from PCR amplification of the two genomic DNA samples (Figure 7c) indicating that copy or copies of vector pBS185 has integrated into the genomes of these two clones and Cre protein was produced by constant expression of the gene in cells rather than transient expression.

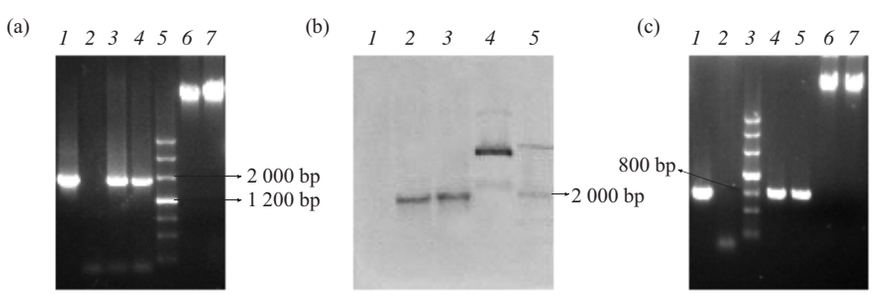

Fig. 7　Detection of gene replacement in colorless cells by PCR and Southern blot

(a) Lane *1*, *2* are positive and negative controls respectively; lane *3*, *4* is the amplified 2 111 bp fragment; lane *5* is DNA marker; lane *6*, *7* is genomic DNA. (b) Lane *1* is negative control; lane *2*, *3* is Southern blot detected the 2 132 bp fragment; lane *4* is positive control; lane *5* is DNA marker. (c) Lane *1* is positive control; lane *2* is negative control; lane *3* is DNA marker; lane *4*, *5* is the amplified 761 bp fragment; lane *6*, *7* is genomic DNA.

3　Discussion

Transgenic livestock has great potentials in biomedical industries and agriculture[1, 2]. However, the dream of use widely transgenic livestock in commercial production will not come to true unless transgenic efficiency could be increased substantially and “position effect” of transgenes expression could

be overcome. The selection of "friendly loci" among numerous integration loci, as some authority investigators[10] suggested many years ago, is apparently a good and practical strategy because many useful transgenic individuals of farm animal bearing friendly insertion loci have been produced over the years[21]. To better explore this possibility two novel methods must be established: firstly, to produce large numbers of transgenic founder animals with low cost; secondly, to use of the selected loci repeatedly. In pursuing this strategy, we have suggested a route as illustrated schematically in Figure 8 and developed a set of vectors. The main features of our vectors are that it could serve to select transgenic embryos through *in vitro* fertilization whereby to save recipients; it can serve to remove marker gene at early embryonic stages

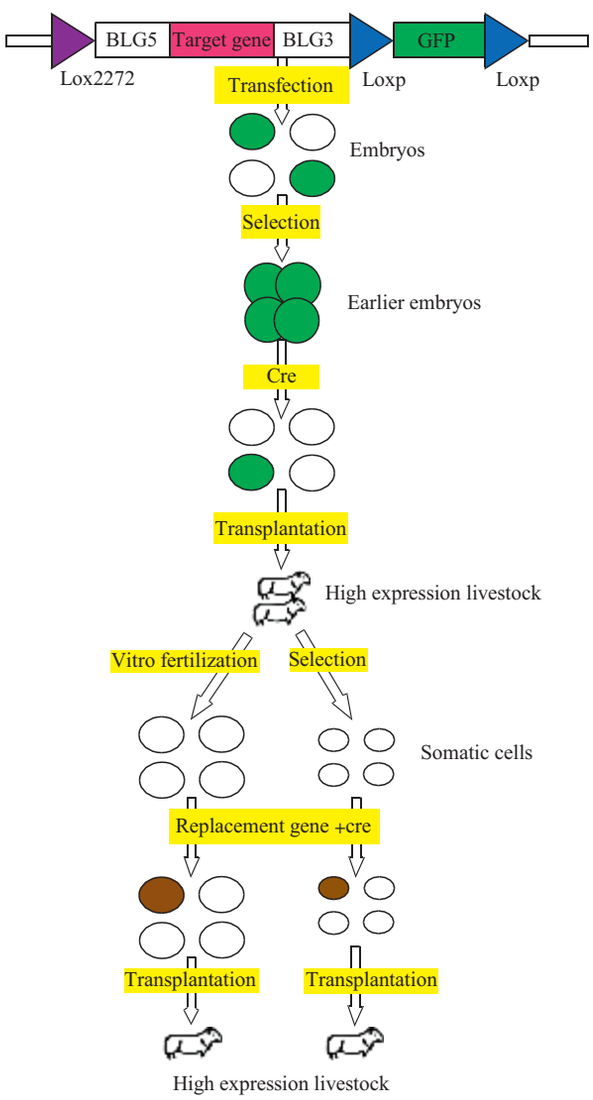

Fig. 8　Schematic presentation of selection and use of "friendly loci"

by adding Cre protein; it can serve to replace target genes in embryo or somatic cell after identification of "friendly loci". In the present study emphasis was concentrated on evaluation of the efficiencies of Cre/*lox* system in marker gene GFP deletion and the imitated target gene *DsRed* replacement. Using cell line instead of embryo in experiment is mainly for the easiness in manipulation and straightforward in observation of results. In the simulated conditions biases may arise in results and their explanations. We do hope that this simulation could reflect, to some extent, the real events happening in embryo and in somatic cells.

According to the presence of different fluorescent color, cell clones were classified into three categories: colorless were regarded as clones in which marker gene were deleted and target gene were replaced; red color were regarded as clones with marker gene deleted but target gene remaining while the appearance in both of red and green colors were regarded as Cre mediated reaction never happened. Theoretically there should be green clones, namely clones in which *DsRed* gene was replaced without GFP gene deletion. However it had never been observed any green clone in our experiments. One possible reason for this could be that the cell numbers used in the experiments were not great enough so that the chance for the appearance of green clone was too low. Another reason could be that the green GFP gene was subjected to two recombination reactions consisting of a deletion reaction between the two flanking wild type *lox*P sites and of a replacement reaction mediated by *lox*2272 and the extreme 3' end *lox*P site so that the events of replacement without deletion were extremely rare. Except to screen them on purpose otherwise they will never being found.

The overall efficiency of gene replacement mediated by two pairs of hetero-specific *lox*P sites (*lox*2272 and wild type *lox*P) was about 30%. If this proportion sustained in embryo or somatic cell it should be good enough for repeated use of selected loci in practice. With an overall efficiency of marker deletion around 90% Cre/*lox*P site-specific recombination is a highly effective in deleting marker gene indicating for saving recipients selection and transplantation of transgenic embryo is practical in production of livestock.

In this experiment the introduction of Cre recombinase was accomplished by co-electroporation

of Cre coding gene with the replacement vector. It is interesting to note that among the four clones characterized only three of them integrated pBS185 sequence in their genomes whereas the other clone expressed Cre transiently. However when data on recombination were compared, the differences among the four clones were not significant statistically. This fact indicated that in practice transient expression of *cre* gene or direct supply Cre protein is not only possible but also desirable since the integration of copies of *cre* gene in genome should be avoided in most cases.

In conclusion， it is possible to realize site specific expression foreign genes in livestock by introducing Cre/*lox* system to selected “friendly gene loci”. Towards this aim, we have proved in different experiments the following key facts: Firstly, positive transgenic embryo could be distinguished from none-transgenic and transgenic mosaic by naked eyes using marker gene GFP. This means that a lot more transgenic founder animals could be produced with the same amount of money, which will in turn enhance the chance for identifying “friendly loci” in genome. Secondly, with the combination use of homo-specific and hetero-specific *lox* sites, it is possible that marker genes could be deleted and target genes could be replaced in one enzymatic reaction in early embryo or somatic cell. Thirdly, Cre protein can be introduced into cells or blastomeres by transient expression of *cre* gene or direct provision of Cre protein.

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一种利用 Cre/*lox*P 系统进行标记基因删除与靶基因置换的细胞模型研究 *

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摘要 Cre/*lox* 系统可以介导 DNA 的定点插入和定点删除，可利用其实现转基因动物中“友好位点”的重复利用及标记基因的有效删除．为直观地评估该系统介导的以上两种重组反应的效果，通过标记基因并利用大鼠乳腺癌细胞系 SHZ-88 进行了模型研究．首先构建了两个载体：a．整合载体 pTE-*lox*2272-DsRed-*lox*P-GFP-*lox*P，含有红色荧光标记基因 *DsRed* 和绿色荧光标记基因 GFP；b．置换载体 pT-*lox*2272-neo-*lox*P，含有筛选标记基因 *neo*，用以置换 *DsRed* 基因．然后，用整合载体转染 SHZ-88 细胞，并随机挑取了 3 个同时表达 DsRed 和 GFP 的稳定整合细胞克隆．随后用置换载体和 Cre 表达载体 PBS185 对以上每个克隆分别进行了 3 次共转染，通过 G418 筛选并扩增培养后，总共获得 1 070 个克隆．通过分析标记基因 DsRed 和 GFP 在这些克隆中的表达情况：Cre 介导的删除效率为 91.1%，定点置换效率为 29.3%．最后对部分克隆进行了 PCR 和 DNA 印迹鉴定，分子鉴定结果与发光的表型状况一致．这一方法为 Cre/*lox* 系统在转基因家畜生产中的进一步应用提供了实验依据．

关键词 *lox*2272，*lox*P，Cre，标记基因删除，靶基因置换

学科分类号 Q5

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* 国家高技术研究发展计划资助项目(863)(2002AA206311 和 2006AA02Z148).

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收稿日期：2008-01-29，接受日期：2008-03-10