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An Expedient Reliable Double Fluorescent Reporter System for ϕC31 Integrase Function Evaluation*

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Abstract A reporter system for ϕC31 integrase was developed in NIH3T3 cells. The reporter plasmid coding green fluorescent protein (GFP) coupled with red fluorescent protein (RFP) was co-transfected with the plasmid coding ϕC31 integrase, to show the activity of integrase in the cells. Fluorescence activated cell sorter (FACS) was used to measure the proportion of the cells containing red and green fluorescence. The increment of green cells was positively related to the increase in the transfection with plasmid coding ϕC31 integrase. Approximately 90% of green cells were observed under a ratio of [plasmid-ϕC31-integrase]/[reporter plasmid] at 10∶1. This suggests that the ϕC31 integrase reporter system provides a probe for the function of ϕC31 integrase in cells.

Key words ϕC31 integrase, reporter system, sequence-directed recombination, NIH3T3 cells
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The bacteriophage ϕC31 integrase is a powerful genetic tool for gene therapy, genome manipulation and transgenesis [1]. It is encoded by a phage of streptomyces soil bacteria[2]. The enzyme is a member of the serine catalyzed site-specific recombinases. This serine integrase mediates sequence-directed recombination between a bacterial attachment site (*attB*) and a phage attachment site (*attP*). Recombination results in generation of two hybrid sites, *attL* and *attR*, and the reaction is therefore unidirectional[3]. In the presence of ϕC31 integrase, a DNA sequence flanked by two same oriented *attB* and *attP* sites will be cut[4~6]. Another feature of the ϕC31 system is that the integrase solely mediates integration[7, 8], which distinguishes it from most other commonly used systems, such as Cre/loxP or FLP/FRT, where the recombinase can catalyze both the integration and the excision reactions.

For evaluating the function of ϕC31 integrase, this recombination event has been used to delete DNA sequence in mammalian cells (NIH3T3, ES cells, 293 cells), and alter some gene expression *in vivo*[4~6]. However, lacZ (β-galactosidase) or human placental alkaline phosphatase (PLAP) was usually as the reporter gene which would cause many inconvenience, such as time consuming for specimens, difficulty for detection in living cells. The present study sought to combine the power of the ϕC31 integrase system with the red[9~11] and green[12] fluorescent proteins to create a fluorescent binary switch that can be detected in living cells expediently.

1 Materials and methods

1.1 Reagents and materials

The plasmid CMV-Int (ϕC31 integrase expression plasmid) and pBCPB$^+$ were kindly given by Dr. M.P. Calos[13]. A plasmid that expresses the red fluorescent

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protein (RFP), ROSA26mT/mG was kindly provided by Dr. Liqun Luo [11]. The plasmid EGFP-N1 and MD19-T were purchased from Clonetech and Takara Biotechnology respectively. All restriction enzymes were purchased from New England Biolabs. The plasmids were amplified in a DH10B strain (Invitrogen) of *Escherichia coli*.

1.2 Construction of ϕC31 integrase reporter plasmid

The ϕC31 reporter vector was constructed as follows: Firstly, a 2.6 kb mT fragment (loxp-mT-pA-loxp) was PCR-amplified from the plasmid ROSA26mT/mG, with the oligonucleotide primers of *Cla* Ⅰ-mT1 (28 bp, CGCatcgatGTGCTGGTTATTGTGCTGT) and *Pvu* Ⅰ-mT2 (25 bp, AAAcgatcgCCTGTCCGTTCGCTTT). The mT amplicon was purified and then ligated into pMD19-T vector to create pT-mT. Next, for construction of pBmTP, the mT amplicon that was already inserted in pT-mT was dual-digested by *Cla* Ⅰ and *Pvu* Ⅰ, and it was then ligated into pBCPB$^+$ digested by the same two enzymes. Finally, a 3.5 kb fragment (attB-loxp-mT-pA-loxp-attP) cut from pBmTP by *Bsr*B Ⅰ was inserted into the *Sma* Ⅰ restriction site of pEGFP-N1 to generate ϕC31 reporter vector, pmT/EGFP.

1.3 Cell transient transfections and fluorescence analysis

NIH 3T3 cells were cultured in DMEM (Gibco BRL) with 10% newborn calf serum (Hyclone), 50 U/ml of penicillin, and 50 g/L of streptomycin (Gibco BRL) at 37℃ with 5% CO_2. NIH3T3 cells were plated in 25 cm^2 culture dish and then transiently transfected with a variety of proportions of pmT/EGFP and pCMV-Int: 1∶0, 1∶100, 1∶10, 1∶1, 10∶1, and 100∶1, using Lipofectamine 2000 (Invitrogen) according to manufacturer's instruction. The pmT/EGFP plasmid was kept constant at 200 ng. All of the plasmids for transfection were purified using Qiagen Plasmid Kit (Qiagen). At 24 h after transfection, approximately 1/3 of the cells were used to measure the ratio of red and green fluorescent by fluorescence activated cell sorter (FACS, Becton-Dickinson FACS Calibur, USA), and the other 2/3 were separated equally into two components. At 48 h and 72 h after transfection, cells were harvested respectively for the measurement of the ratio of red and green fluorescence by FACS analysis. At least 100 000 cells were determined. The red and green fluorescent images were then imaged 24, 48, and 72 h after transfection.

1.4 Cell stably transfection in genome and fluorescence analysis

To examine the ϕC31 integrase reporter plasmid in the context of genomic integration, cells were transfected with pmT/EGFP that was linearized by *Apa*L Ⅰ and selected in growth medium containing G418 (Sigma-Aldrich) 600 mg/L, and maintained in growth medium containing G418 300 mg/L for 14 days to get single colonies. Afterwards, pCMV-Int was transfected using Lipofectamine 2000. 48 h later, cells were collected for measurement of the ratio of red and green fluorescent by FACS analysis. The red and green fluorescent images were also captured.

2 Results

2.1 Analysis of the efficiency of this reporter system

To test the efficiency of this reporter system, NIH3T3 cells were transiently transfected with a variety of proportions of pmT/EGFP and pCMV-Int. A bright red fluorescent was produced in NIH3T3 cells when there was absent of ϕC31 integrase, while enhanced green fluorescent visualized following a ϕC31 integrase-mediated recombination event (Figure 1). The results are shown in Figure 2 and Figure 3.

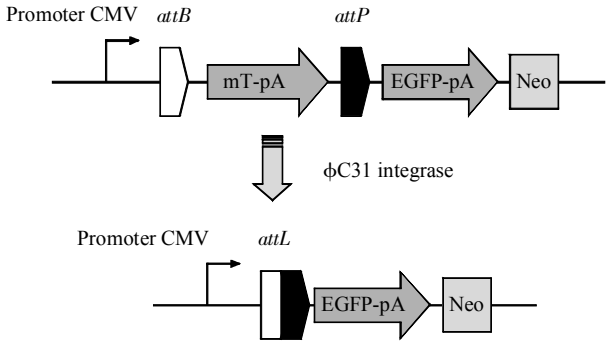

Fig. 1 Schematic representation of ϕC31 integrase-mediated recombination of *attB* and *attP*-flanked reporter gene

The pmT/EGFP vector contains a CMV promoter followed by the mT cassette and SV40 polyA (pA) signal between *attB* and *attP* sites. Downstream in this vector, a promoter-less EGFP gene followed by a pA serves as a reporter gene for detection of ϕC31 integrase mediated removal of the mTd Tomato cassette. In the presence of the ϕC31 integrase, the *attB* and *attP*-flanked mTd Tomato cassette can be excised and result in generation of a hybrid sites, *attL*, followed by transcription and expression of EGFP. N-terminal membrane tagged version of tdTomato, mT; Bacterial attachment site, *attB*; Phage attachment site, *attP*; Simian virus 40 polyadenylation site, pA; neomycin resistance gene, Neo.

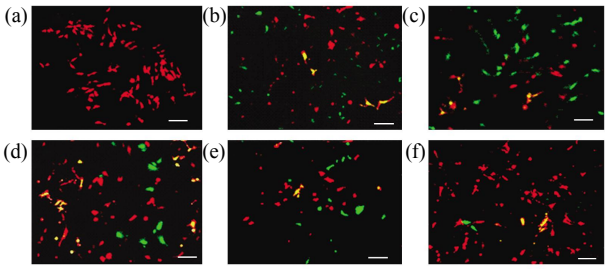

Fig. 2　Expression of EGFP and Td Tomato in NIH3T3 cells 48 h after transfection with various ratios of pCMV-Int to pmT/EGFP vector

Manipulations of the images were confined to merge the gray-scale images of the red and green fluorescent proteins to create RGB color files in which cells that express both proteins are yellow. Cells transfected with the pmT/EGFP only express almost exclusively red fluorescent protein (a). A strong green signal appears in 85% of the fluorescent cells transfected with a 1∶100 ratio of pmT/EGFP to pCMV-Int (b), and a similar result approximately 90% is obtained when transfected with a ratio of 1∶10 (c), green signal drops to 68% with the transfected ratio of 1∶1 (d). 34% of cells shows green signal when transfected with a ratio of 10∶1 (e), while only 5% in a ratio of 100∶1(f). The scale bars represent 50 μm.

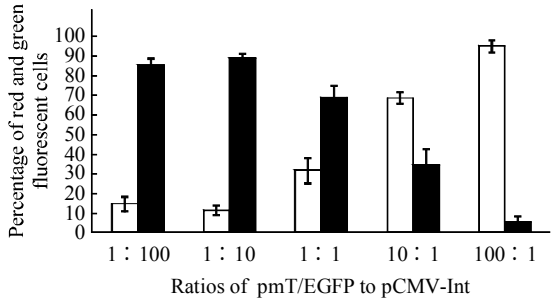

Fig. 3　Relative amounts of red and green fluorescent cells 72 hours after transfection with a variety of ratios of pmT/EGFP to pCMV-Int

□: Percentage of red fluorescent cells; ■: Percentage of green fluorescent cells. n = 3.

2.2　The deletion of this reporter system in genome

To test whether this reporter system could be used in the context of the genome, we maintained transfected cells under G418 selection for 14 days and reached single colonies, followed by enlarging and transiently transfecting with pCMV-Int. FACS analysis showed that roughly 60% of cells were capable of converting from red to green 48 h later (Figure 4). FACS offers a more quantitative way to detect the signal alternation generated by the pmT/EGFP.

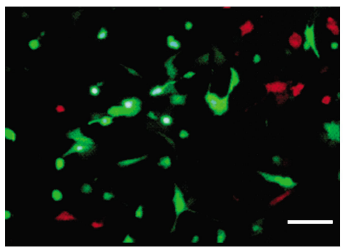

Fig. 4　Cells carrying the pmT/EGFP as a transgene can be converted from red to green by transient expression of pCMV-Int

Imaged here are cells that were resistant to the conversion (upper right, red) and the majority that expressed only EGFP (green) two days after transfection with pCMV-Int. The scale bars represent 100 μm.

3　Discussion

To evaluate the function of ϕC31 integrase, a set of dual reporter vectors were constructed[5,6]. In these vectors, the LacZ gene or PLAP is under the control of a eukaryotic promoter but its expression is inhibited by an intervening stop cassette that is flanked by *attB* and *attP*, consisting of a selectable marker followed by polyadenylation signals. When there is presence of ϕC31 integrase, the recombination will remove the stop cassette allowing expression of LacZ. However, it would be somewhat difficult to interpret the absence of reporter expression when using this approach: Are the cells actually transfected, or is the promoter sufficient to drive reporter expression in that particular cell type, and is the level of reporter expression sufficient for detection? Moreover, although the bacterial LacZ gene has been widely used as a sensitive and reliable indicator, the detection of β-galactosidase activity requires fixation or hypotonic permeabilization of cells, precluding all subsequent studies if viable, physically unmanipulated cells are needed. Therefore, usage of red and green fluorescence may solve the above problems. Although similar research about Cre Stoplight that converted red fluorescence to green fluorescence to detect Cre recombinase was reported previously[14], there were some unexpected properties of the DsRed signal. Firstly, a bright red fluorescent signal developed much more slowly than the signal produced by EGFP under similar conditions. Moreover, some of the red cells revealed a very weak green fluorescent signal in addition to the red signal. DsRed protein matures quite slowly and passes through a green intermediate state. Due to a slow

mature of the DsRed signal and interference of weak green signal, until 48 h post transfection can they observe the fluorescent binary switch. To avoid this problem, the current study applies a new DsRed variant, Td Tomato as the red reporter gene, which can enhance brightness and photostability. For instance, it has six times the brightness of DsRed.T3, eight times the brightness of mRFP1, and two times the brightness of tdRFP. In addition, tdTomato is over 10 times more photostable than mRFP1[10]. The $t_{0.5}$ of Td Tomato for maturation at 37℃ is 1h, but 10 h for DsRed[9]. The Td Tomato is less toxicity of molecule than DsRed, and has been used to produce transgenic mouse as a red fluorescent marker[11].

Combining the ϕC31 integrase fluorescent alternation system and ϕC31 integrase, we observed the cells converted from red to green in transient or stably transfections, giving the evidence that mTd Tomato sequence flanked by two same oriented *attB* and *attP* sites was cut, and thus the expression of EGFP gene can be driven by CMV promoter. Comparing to the dim green signal produced by the DsRed, we did not observe the similar phenomenon produced by the Td Tomato. However, a bright green signal was produced in the presence of ϕC31 integrase, and there was also often red fluorescence in many cells. Two possible explanations were proposed for the above phenomenon. One is that the ϕC31 integrase-mediated recombination of the pmT/EGFP may not occur instantaneously, so the red signal might represent the initial transcripts produced by the unaltered pmT/EGFP plasmid. Alternatively, it is likely that not all of the pmT/EGFP plasmids in single cells are converted by the ϕC31 integrase.

In the studies of ϕC31 integrase to catalyze the genomic integration, one study show that the largest increase in number of G418-resistant colonies was obtained with the 1∶1 ratio of ϕC31 integrase to the donor *attB* plasmid[15]. However, our study showed that the most amount of cells converted red to green with a ratio of pmT/EGFP to pCMV-Int in 1∶10. The role of integration or deletion, transient or stably transfection, toxity of various amount plasmids and different type of cells might cause these diverse results. Moreover, the ϕC31 integrase could mediate the deletion of reporter plasmid in the context of genome. Meanwhile, this fluorescent binary switch can be detected in living cells expediently and quantitatively by FACS.

Overall, the ϕC31 integrase fluorescent alternation system we constructed in this study offers the advantages of live cell sorting and quantitative measurements of the two proteins to evaluate the function of ϕC31 integrase. In addition, this reporter system could be used in the context of the genome, indicating that it may be used to produce a "reporter" mouse strain. So this system may also be useful to produce transgenic mouse as "reporter" mice for ϕC31 integrase. We can construct the ϕC31 integrase targeting reporter constructs, *via* homologous recombination in embryonic stem cells, into a ubiquitously expressed genomic site. Therefore, the ϕC31 integrase reporter mouse, which could be a fluorescent binary switch when the ϕC31 integrase is present, will be a useful tool for lineage-tracing studies.

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一种快捷可靠评价链霉菌噬菌体 ϕC31 整合酶功能的双荧光报告系统 *

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摘要　在 NIH3T3 细胞中构建了一种链霉菌噬菌体 ϕC31 整合酶报告系统．该报告载体同时编码红色荧光蛋白和绿色荧光蛋白，与编码 ϕC31 整合酶的载体共转染可以反映 ϕC31 整合酶的活性．细胞中从红色荧光到绿色荧光的变化和百分比的变化可经流式细胞仪检出．随着转染中 ϕC31 整合酶表达载体的比例升高，表达绿色荧光的细胞比例上升．ϕC31 整合酶表达载体和报告系统载体比例在 10∶1 时，可达最高约 90%的红绿荧光转变率．这表明该 ϕC31 整合酶报告系统提供了一种在细胞中快捷可靠的评价 ϕC31 整合酶功能的方法．

关键词　ϕC31 整合酶，报告系统，位点特异性重组，NIH3T3 细胞系
学科分类号　Q28　　**DOI:** 10.3724/SP.J.1206.2008.00698

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