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Cre Stoplight: A Red/Green Fluorescent Reporter of Cre Recombinase Expression in Living Cells

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ABSTRACT

The Cre/lox system is a powerful genetic tool with which to manipulate the genome. Here, we describe the development of a simple reporter system for Cre recombinase, called the Cre Stoplight. In the absence of Cre, the red fluorescent protein is expressed; when Cre catalyzes a recombination event, the green fluorescent protein is produced. Testing this system in transiently transfected cells showed that it produced robust signals (90% of the cells converted from red to green) when equal amounts of the plasmids encoding Cre recombinase and the Cre Stoplight were used. A 1:100 ratio of enzyme to reporter plasmid produced similar results, and a 1:10 000 ratio was necessary to significantly reduce the number of cells converting to green (1%).

INTRODUCTION

The Cre/lox system is a powerful genetic tool for manipulating the genome and controlling gene expression. The Cre recombinase enzyme mediates the specific recombination between two loxP (locus of crossing over) sites in the genome. This recombination has been used to delete genes in specific cell types in mice, recombine plasmids in living cells, and alter/recombine sequences in vitro (12–14). In the present study, we sought to combine the power of the Cre/lox system with the red (3,8,9) and green (2,10) fluorescent proteins to create a fluorescent binary switch that can be detected in living cells.

MATERIALS AND METHODS

Our idea was to create a construct that expressed the red fluorescent protein (DsRed®; Clontech Laboratories,

Palo Alto, CA, USA) in the absence of Cre recombinase and the enhanced green fluorescent protein (EGFP; Clontech Laboratories), following a Cre-mediated recombination event. To create this, we used plasmids recently produced by Invitrogen (Carlsbad, CA, USA) as a part of the Echo™ cloning system (Figure 1). By replacing the Neo gene of pcDNA3.1E with the sequence encoding EGFP and using pUni/V5-His-TOPO to introduce the DsRed coding sequence, it was possible to create a construct in which the cytomegalovirus (CMV) promoter was separated from DsRed by a loxH site (6). Downstream of DsRed, a loxP site is followed by a sequence encoding EGFP. In the case of Cre-mediated recombination at the loxH/loxP sites, the DsRed sequence is deleted and EGFP is produced.

Briefly, the constructs were built as follows. An EcoRI synthetic linker was ligated into the PmeI/SmaI-digested pcDNA3.1E. The coding sequence of EGFP was then inserted into this site as an EcoRI fragment, creating the plasmid pYY9. The DsRed coding sequence was amplified from pDsRed1-N1 (Clontech Laboratories) with primers 5'-AGTCGAGGCCGCTACAGGAACAGGTGGTG-3' and 5'-GCC-ACCATGGT GCGCTCCTCCAAGAAC-3'. This product was subcloned into pUni/V5-His-TOPO to create pRY1. The SmaI site of this plasmid was then converted into a NotI site with a synthetic linker, creating pYY10. Cre-mediated recombination was used to combine plasmids pYY9 and pYY10, giving rise to pYY11. A NotI digest of the plasmid, followed by ligation, was used to drop the pUni/V5-His-TOPO backbone, creating the Cre Stoplight.

HEK 293 cells (5) were cultured in 2 mL DMEM with 10% FCS and Earl's salts in 35-mm, glass-bottom microplates (MatTek, Ashland, MA, USA). The cells were transiently transfected with varying ratios of a Cre recombinase expression plasmid (pBS-185; Invitrogen) approximately 48 h after plating, using LIPOFECTAMINE™ 2000 (Invitrogen). Total DNA was kept constant at 1 µg. To examine the Cre Stoplight in the context of genomic integration, cells were co-transfected with a 1:1 ratio of Cre Stoplight and

Table 1. Relative Amounts of Red and Green Fluorescent Cells, Following Transfection with Different Ratios of pBS185 to Cre Stoplight DNA (1:1, 1:100, or 1:10 000)

	% of Total Fluorescent Cells		
	Red	Green	Both
24 h			
1:1	11	89	0
1:100	64	90	54
1:10 000	84	17	1
48 h			
1:1	33	86	19
1:100	81	72	53
1:10 000	99	1	1
72 h			
1:1	18	97	15
1:100	85	41	26
1:10 000	100	<1	<1

Images were collected 24, 48, and 72 h after transfection with filters and exposure times that ensured that there was no overlap between the signals generated by the DsRed and EGFP proteins.

pcDNA3.1E and subsequently maintained on G418 selection for 25 days.

The cells were imaged with an Axiovert S100 TV inverted microscope fitted with Ludl filter wheels (Carl Zeiss, Thornwood, NY, USA) in the epifluorescence excitation and emission paths, and a cooled charge-coupled device (CCD) camera (MicroMAX™; Roper Scientific, Trenton, NJ, USA) was used to collect the images. Specific excitation and emission filters and a common dichroic element were used to isolate the signals of the two different fluorescent proteins (HQ FITC and Texas Red® excitation and emission filters, and FITC/Texas Red V3 dichroic; Chroma Technology, Brattleboro, VT, USA). The filter wheels and camera were controlled by IPLabs software (Scanalytics, Fairfax, VA, USA). Sets of the red and green fluorescent images were collected to analyze the relative percentage of cells that had converted from red to green fluorescence. The images were analyzed and prepared for publication with IPLabs and Adobe® InDesign® soft-

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Figure 1. Schematic diagram of the Cre Stoplight. A CMV enhancer and promoter (CMV) are separated from the EGFP coding region by a *loxH* site, the red fluorescent protein coding region (DsRed), a BGH polyadenylation site (BGH poly A) (4), the terminator for T7 RNA polymerase (TT; Tphi, GenBank® accession no. NC001604), and *loxP*. In the presence of the Cre recombinase enzyme, the region between the *loxH* and *loxP* sites is excised, and EGFP is expressed instead of DsRed. Simian virus 40 (SV40) polyadenylation site, SV40 poly A; ampicillin resistance, AMP.

ware. Manipulations of the images were confined to merging the gray-scale images of the red and green fluorescent proteins to create RGB color files for Figures 2 and 4, adjusting the brightness/contrast of the final printouts to match most closely what we saw through the microscope and adding lettering and a scale bar.

RESULTS

The Cre Stoplight should produce only DsRed protein unless Cre recombinase is present. When we tested the Cre Stoplight plasmid alone in HEK 293 cells, there were some unexpected properties of the DsRed signal that set some constraints on the use of this reporter. Cells transfected with the Cre Stoplight reporter produced a bright red fluorescence in 1–2 days (Figure 2A), but this signal developed much more slowly than the signal produced by EGFP under similar conditions. Moreover, some of the red cells had a very weak green fluorescent signal in addition to the red signal. At 24 h, this produced cells with a green-to-red signal ratio of 1:30, which decreased to 1:100 or less by 48 h. These results can be explained by the recent observation that the DsRed protein matures quite slowly and passes through a green intermediate state (3,9). Because the DsRed signal matures slowly and the weak green signal is seen after 24 h, this reporter construct has a limited temporal resolution and appears to provide an accurate readout only after 48 h.

Forty-eight hours after co-transfection with pBS185 (the Cre recombinase

Figure 2. Expression of EGFP and DsRed in HEK 293 cells 48 h after transfection with various ratios of pBS185 (Cre recombinase expression plasmid) to Cre Stoplight. Digital images of the green and red signals were collected separately and then combined to generate a pseudo-colored image in which cells that express both proteins are yellow. (A) Cells transfected with only the Cre Stoplight expressed almost exclusively red fluorescent protein. (B) A strong green signal appeared in 87% of the cells transfected with a 1:1 ratio of pBS185 to Cre Stoplight, while in cultures transfected with a ratio of 1:100, this dropped to 72% (C). Indeed, even in cultures transfected with a 1:10 000 ratio of pBS185 to Cre Stoplight, there was a small (approximately 1%) number of bright green cells (D). Scale bar = 50 μ m.

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expression plasmid), there was a bright green fluorescence that could not be confused with the signal produced by the Cre Stoplight alone. The large separation between the dim green signal produced by the DsRed and the bright one produced by the EGFP (5- to 10-

fold difference as measured by the CCD camera) made it possible to image only the EGFP-containing cells by adjusting the collection times and instrument sensitivity. While the presence of Cre recombinase produced a bright green signal, there was also often a strong red fluorescence in many cells. There are two possible explanations for this. The Cre-mediated recombination of the Cre Stoplight may not occur instantaneously, so the DsRed signal might represent the initial transcripts produced by the unaltered Cre Stoplight plasmid. Alternatively, it is possible that not all of the Cre Stoplight plasmids in a cell are converted by the Cre recombinase.

To test the efficiency of this reporter system, HEK 293 cells were transiently transfected with four different pBS185 to Cre Stoplight ratios: 0:1, 1:1, 1:100, and 1:10000. The cells were then imaged 24, 48, and 72 h after transfection (Table 1). Cells transfected with the Cre Stoplight alone remained red throughout the time period (Figure 2A). Cells co-transfected with the pBS185 and Cre Stoplight plasmids at ratios of 1:1 (Figure 2B) and 1:100 (Figure 2C) converted to green roughly 90% of the time. Indeed, the ratio had to be reduced to 1:10000 (Figure 2D) to see a large drop in the efficiency of red to green conversion.

Fluorescence-activated cell sorting (FACS) offers a more quantitative way to look at the signals generated by the Cre Stoplight. To calibrate the instrument, we sorted HEK 293 cells transiently transfected with an EGFP expression plasmid (Figure 3A). This was easily distinguished from the bright red signal produced in cells transfected with the Cre Stoplight alone (Figure 3B). Co-transfection of the Cre Stoplight and pBS185 appeared to convert 94% of the bright red fluorescent cells so that they exceeded the threshold for green detection (Figure 3C; vertical line).

To test whether this reporter system could be used in the context of the genome, we maintained transfected cells under selection for 25 days. Roughly half continued to express the DsRed protein. Transient transfection with pBS185 revealed that many of the cells were capable of converting from red to green (Figure 4). However, without generating

clonal lines, it is impossible to determine the exact efficiency of this process. Moreover, we did find a very small number of spontaneous conversions to green in the absence of pBS185.

DISCUSSION

In the context of transiently transfected cells or transgenic animals with conditional genomic alterations, it is often first necessary to determine whether the cells have been successfully transfected with the Cre construct or if Cre is being expressed in the correct cell types at the correct time. One existing construct for detecting Cre expression involves separating a strong promoter element from *lacZ* with a transcription/translation stop sequence flanked by *loxP* sites (15). A drawback to this approach is that the absence of reporter expression can be difficult to interpret: are the cells actually transfected, is the promoter sufficient to drive reporter expression in that particular cell type, and is the level of reporter expression sufficient for detection? Several Cre reporter systems have been built to address this problem. All of these systems use Cre-mediated recombination of two *loxP* sites to replace one reporter with another. However, the reporters used to date have largely been histochemical markers, such as *lacZ* and human placental alkaline phosphatase (7) or *lacZ* and CAT (1). One system employs GFP, but the second, conditional reporter is *lacZ*

Figure 3. The Cre-mediated conversion of the Cre Stoplight from red to green can be quantified using FACS. HEK 293 cells transfected with EGFP alone show a strong green fluorescence signal (A). Cells transfected with the Cre Stoplight alone show a strong red fluorescence signal (B) that is largely converted to a green fluorescence by pBS185 (C).

Figure 4. Cells carrying the Cre Stoplight as a transgene can be converted from red to green by transient expression of Cre recombinase. Imaged here are cells that were resistant to the conversion (upper left, red) and the majority that expressed only EGFP (green) three days after transfection with pBS1185. Scale bar = 20 μ m.

(11). While useful, all of these constructs require the fixation and staining of the cells or tissue to be analyzed. There is also a potential difficulty in interpreting the results in that the signals produced by these systems cannot be detected simultaneously.

The two fluorescent proteins produced by the Cre Stoplight construct offer the advantages of live-cell sorting and obtaining quantitative measurements of the two proteins for comparison and establishment of detection thresholds. One can envision how the signals produced by this switch could be used to detect events that occur either very quickly in the cell or produce only low levels of expression. The ability to rapidly sort cells based on this signal could, in turn, make it possible to use the signal as a tool for separating and comparing cells that have responded differently.

REFERENCES

1. Araki, K., M. Araki, J. Miyazaki, and P. Vassalli. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of cre recombinase. *Proc. Natl. Acad. Sci. USA* 92:160-164.
2. Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263:802-805.
3. Fradkov, A.F., Y. Chen, L. Ding, E.V. Barsova, M.V. Matz, and S.A. Lukyanov. 2000. Novel fluorescent protein from discosoma coral and its mutants possesses a unique far-red fluorescence. *FEBS Lett.* 479:127-130.
4. Goodwin, E.C. and F.M. Rottman. 1992. The 3'-flanking sequence of the bovine growth hormone gene contains novel elements required for efficient and accurate polyadenylation. *J. Biol. Chem.* 267:16330-16334.
5. Graham, F.L. and J. Smiley. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-74.
6. Liu, Q., M.Z. Li, D. Leibham, D. Cortez, and S.J. Elledge. 1998. The univector plasmid-fusion system, a method for rapid construction of recombinant DNA without restriction enzymes. *Curr. Biol.* 8:1300-1309.
7. Lobe, C.G., K.E. Koop, W. Kreppner, H. Lomeli, M. Gertsenstein, and A. Nagy. 1999. Z/AP, a double reporter for cre-mediated recombination. *Dev. Biol.* 208:281-292.
8. Lukyanov, K.A., A.F. Fradkov, N.G. Gurskaya, M.V. Matz, Y.A. Labas, A.P. Savitsky, M.L. Markelov, A.G. Zaraisky et al. 2000. Natural animal coloration can be determined by a nonfluorescent green fluorescent protein homolog. *J. Biol. Chem.* 275:25879-25882.
9. Matz, M.V., A.F. Fradkov, Y.A. Labas, A.P. Savitsky, A.G. Zaraisky, M.L. Markelov, and S.A. Lukyanov. 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. *Nat. Biotechnol.* 17:969-973.
10. Prasher, D.C., V.K. Eckenrode, W.W. Ward, F.G. Prendergast, and M.J. Cormier. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111:229-233.
11. Sato, M., Y. Yasuoka, H. Kodama, T. Watanabe, J.I. Miyazaki, and M. Kimura. 2000. New approach to cell lineage analysis in mammals using the cre-loxP system. *Mol. Reprod. Dev.* 56:34-44.
12. Sauer, B. 1998. Inducible gene targeting in mice using the cre/lox system. *Methods* 14:381-392.
13. Sauer, B. 1993. Manipulation of transgenes by site-specific recombination: use of cre recombinase. *Methods Enzymol.* 225:890-900.
14. Sauer, B. and N. Henderson. 1989. Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. *Nucleic Acids Res.* 17:147-161.
15. Tsien, J.Z., D.F. Chen, D. Gerber, C. Tom, E.H. Mercer, D.J. Anderson, M. Mayford, E.R. Kandel et al. 1996. Subregion- and cell type-restricted gene knockout in mouse brain. *Cell* 87:1317-1326.

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