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Recombination System Based on Cre α Complementation and Leucine Zipper Fusions

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Abstract In a previous study, a recombination system based on the α complementation of cre recombinase and protein transduction was established. This system relied on the transient expression of the inactive, self-excisable C-terminal (β) and the transduction of the N-terminal (α) cre fragments to cells as a purified protein. This recombination system potentially results in a less invasive and more controllable cre recombination in mammalian cells. In this study, we have employed a more efficient complementation triggering sequence using more than only the overlapping amino acids to help the α and β fragments reassociate. In order to increase the association efficiency of the complementing fragments of cre recombinase, we chose to use a fusion of cre fragments to a self-heterodimerizing pair of proteins to trigger their binding and thus increase the efficiency of the restored enzymatic activity. For this purpose, the leucine zipper motifs (bJun and bFos) of the AP-1 transcription were fused to cre fragments (α and β, respectively). This resulted in an increased reassociation efficiency of the fragments and a two times more efficient recombination system compared with the previous study.

Keywords α complementation · Cre recombinase · Leucine zipper motif · Protein transduction · Reassociation efficiency

Introduction

Cre recombinase (cre) has been used as a simple and efficient tool to engineer the genomes of experimental animals [1]. However, the lack of temporal control over its activity limits its usefulness [2]. The expression of cre in the early developmental stages of a mouse embryo with a floxed vital gene results in the inactivation of that gene and early embryonic death, making it impossible to study the role of genes at later stages [3]. Prolonged expression of cre at a high level can lead to its interactions with loxP-like sequences in the

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mammalian genome, resulting in chromosomal rearrangements. This phenomenon is known as the toxic effect of permanent cre expression [4–6]. An ideal cre recombination system is expected to have control over the temporal and tissue-specific expression of active protein in each cell in order that it does not result in unwanted chromosomal rearrangements.

The idea of splitting cre into reassociable fragments was developed in an attempt to control the time of cre activation [7, 8]. The general idea of such research has been to introduce the inactive fragments of cre to cells that could then reassociate and restore the enzymatic activity. The goal of this study is to explore new methods of using cre as a tool of gene excision. For this purpose, the α-complementation strategy [7] and protein transduction has been used. We developed a new recombination system using cre α-complementation and protein transduction [9]. This strategy included the introduction of cre to cells by both gene transfection and protein transduction methods (Fig. 1a). Cre was divided into its N and C terminals (α and β, respectively). The relative enzymatic activity of the fragmented cre was quantified and compared to the wild-type enzyme. The cell permeation abilities of fragments were further characterized with fluorescent labeling.

In this study, we improved the efficiency of our previous research by using interacting fusion proteins that would help with fragment reassociation. Dimerizing fragments such as FKBP12 and FRB had previously been used for this purpose [8]. In this method, the cre enzymatic activity was restored by the addition of rapamycin, a ligand that brings FKBP12 and FRB together and consequently allows the N and C terminal fragments of cre to come into close proximity. To improve our previous system, we chose to use the leucine zipper family of

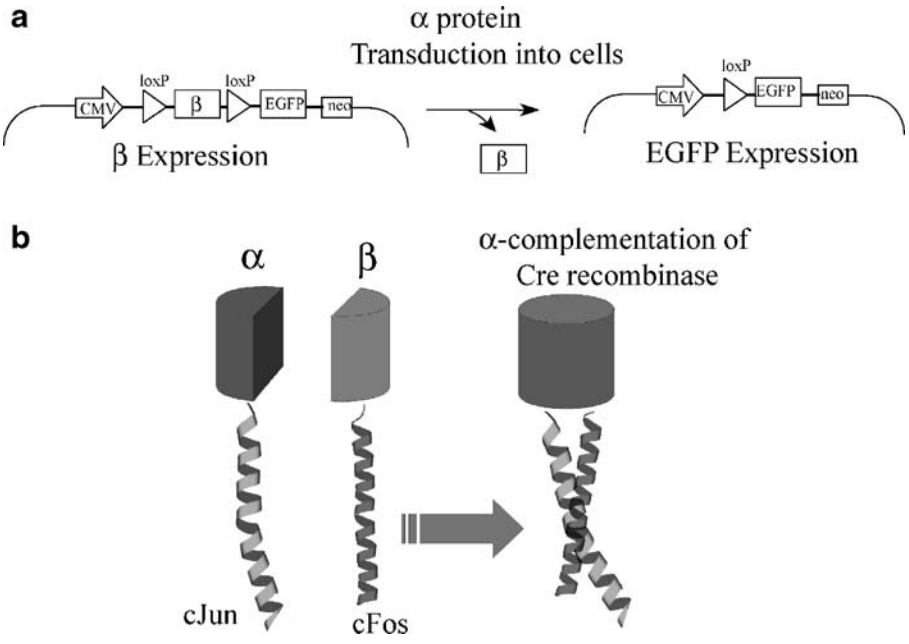

Fig. 1 The improved cre recombination system based on cre α complementation and leucine zipper fusions. **a** The general strategy of cre α complementation. Cells were transfected with a fragment expressing floxed β. At the desired time of Cre activation, α protein was added to cells resulting in enzymatic activity in cells with both fragments. As a result of this activity, the β sequence was deleted from the genome and no longer expressed. **b** The α and β fragments were fused to the basic region-leucine zipper (bZIP) family of transcription regulatory proteins. Heterodimerizable bZIP domains of Jun and Fos triggered the reassociation of α and β fragments fused to them, respectively

AP-1 transcription regulatory proteins. These proto-oncogenes encode α helices containing a DNA-binding domain with clustered basic amino acids (b) and an adjacent leucine zipper motif (Zip), responsible for the dimerization of the transcription factor [10]. The association between c-Jun and c-Fos is required for binding to DNA [11]. Jun proteins have the ability to form dimers (Jun–Jun) that are stable. In contrast, Fos proteins cannot form stable homodimers [12]. In a previous study, a library of antiparallel leucine zippers fused with randomized fragments to either Glutamic acid (Glu) or Lysine (Lys) was tested to help with the reconstitution of cre fragments [13]. The investigators used the artificially designed leucine zipper [14] to assist cre reconstitution. When co-expressed in tissue culture, the two modified cre fragments restored about 30% and 12% cre activity with or without the fusion to NLS, respectively. The high affinity of these proteins together with their ability to independently form heterodimers made them attractive candidates for our purpose.

In this study, the wild-type bZip domains of Jun and Fos [15] were fused to the C-terminal of α and N-terminal of β, respectively (Fig. 1b), and the interaction between the fusion proteins and their cell permeation ability was studied.

Materials and Methods

Plasmid Construction

pCMV-α-bJun

Sequences encoding Jun 257–318 (bJun) and Fos 118–210 (bFos) were fused to sequences encoding the α and β fragments, respectively. The bZIP domains and cre fragments were connected by linker peptides (KQKVMNH in bFos-β and RSIAT in α-bJun). bJun was amplified from the cDNA library of HeLa cells. The linker RSIAT was added at its 5′ end by polymerase chain reaction. α fragment lacking the stop codon was amplified from pBluescript-α [9]. RSIAT-bJun and the new α fragment without stop codon (NSC) were both cloned into *Eco*RV-digested pBluescriptII to make pBluescript-(RSIAT-bJun) and pBluecript-α (NSC). After confirming the integrity of the inserts by capillary sequencing, α (NSC) was digested with *Kpn*I and *Sma*I. This fragment was inserted into pBluescript-(RSIAT-bJun) already digested with *Sal*I followed by blunting and then digested with *Kpn*I. Resulting plasmid was named pBluescript-α-bJun. In order to make pCMV-α-bJun, the α-bJun fragment was separated from pBluescript-α-bJun by *Sal*I and *Sma*I double digestion. This fragment was cloned into pcDNA3.1(+) already digested with *Apa*I, blunted and digested with *Xho*I.

pCMV-bFos-β

The bFos fragment was amplified from HeLa cells cDNA together with the KQKVMNH linker at its 3′ end. The amplified fragment was inserted into pBluescript II to make pBluescript-(bFos-KQKVMNH). After confirming the sequence, this fragment was separated from pBluescript-(bFos-KQKVMNH) by first digesting with *Bam*HI, blunting, and then digesting with *Sac*I. Then, it was inserted into pBluescript-β [9], which was already digested with *Xho*I, blunted and digested with *Sac*I. Resulting plasmid was named pBluescript-bFos-β. In order to make pCMV-bFos-β, the bFos-β fragment was separated from pBluescript-bFos-β by *Bst*XI digestion, followed by blunting and then *Hin*dIII digestion. This fragment was inserted into pcDNA3.1(+) already digested with *Nhe*I, blunted and digested with *Hind*III.

pCMV-bFos-β[EGFP]

To develop the α recombination system, β fragment in β[EGFP] [9] was exchanged to bFos-β. The plasmid, pEGFP-loxP-MCS-loxP, which was constructed in our previous experiment to make β[EGFP], was digested with *Hin*dIII, followed by blunting. The bFos-β fragment was separated from pBluescript-bFos-β by digesting with *Hin*dIII and *Bst*XI, followed by blunting both ends of the fragment. Then bFos-β was cloned into pEGFP-loxP-MCS-loxP to make pCMV-bFos-β[EGFP].

pET-NHis-α-bJun

The α-bJun fragment was separated from pBluescript-α-bJun by first *Hin*cII digestion followed by blunting and then *Sma*I digestion. This fragment substituted α in pET-NHis-α [9] in which α was removed from the vector by *Nco*I digestion followed by blunting and *Eco*RV digestion.

pET-NHis-bFos-β

The $FL_1β$ fragment was separated from pBluescript-bFos-β by *Eco*T*22*I digestion, followed by blunting and then *Hin*dIII digestion. This fragment substituted α in pET-NHis-α vector [9], which was already removed from the vector by *Nco*I digestion, followed by blunting and then *Hin*dIII digestion.

Expression and Purification of Proteins

Escherichia coli BL21(DE3) was transformed with the pET-NHis-α-bJun and pET-NHis-bFos-β expression vectors. Both α-bJun and bFos-β were expressed and purified with the following protocol. Transformed cells were cultured in Luria–Bertani medium with 100 μg/ml ampicillin at 37 °C until the absorbance at 600 nm reached 0.7. Protein expression was induced by adding isopropyl-beta-D-thiogalactopyranoside at the final concentration of 0.5 mM and continuing incubation at 37 °C for three more hours. Cells were harvested by centrifugation and lysed by Bug Buster. Since α-bJun and bFos-β were expressed in the insoluble fractions, after lysing the cells and centrifugation, the pellets were collected and re-suspended in denaturing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 4 M urea). The samples were rotated for 1 h at 4 °C before centrifugation. The supernatants were incubated with Ni-NTA agarose gel for 45 min at 4 °C in Poly-Prep chromatography columns. After discarding the flow through, the gels were washed four times with ten bed volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 4 M urea, 10 mM imidazole). Each protein was eluted with elution buffer (same buffer as wash buffer but with 200 mM imidazole) and refolded by dialyzing against phosphate-buffered saline (PBS) containing 0.5 M L(+)-arginine hydrochloride (Wako). Protein concentrations were measured using the Dye Reagent Concentrate protein assay (Bio-Rad).

Protein Labeling

Purified α-bJun protein was incubated with Oregon Green 488 maleimide (FMP) for 2 h per manufacturer's instructions. The un-reacted dye was removed by dialyzing the proteins to their storage buffer. The labeled protein was added to cells at the final concentration of

2 μM. After 7 h of incubation, cells were trypsinized and transferred to glass bottomed dishes (Iwaki).

Gene Transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (*v*/*v*) fetal bovine serum in 24-well plates. The plasmids pcDNA-Cre, pcDNA-α, pcDNA-β, pCMV-α-bJun, pcCMV-bFos-β, DsRed[EGFP], and pCMV-FL_1β[EGFP] were transfected into mammalian cells. Transfections were performed 24 h after seeding the cells by applying 0.4 μg of DNA and 1.5 μl of Fugene 6 (Roche) to every well.

Results and Discussion

Confirmation of the Triggering Effect of bZip Domains on Cre Fragment Reassociation

To confirm the triggering effect of bZip domains on cre fragment reassociation, cells were transfected with different combinations of α and β fragments with or without bZip fusions (Fig. 2a), in addition to the DsRed[EGFP] reporter plasmid.

The number of enhanced green fluorescent protein (EGFP) expressing cells was counted to quantify the recombination activity of each pair (Fig. 2b). When driven by separate promoters, the combined expression of α and β did not result in a higher EGFP expression relative to the negative control. In addition, little activity was observed for the combination of α and bFos-β. Interestingly, the combination of α-bJun and β resulted in a recombination activity 9.6 times higher than the α and β pair. However, the highest activity was observed in the cells transfected with the plasmids containing α-bJun and bFos-β, which resulted in 42 times higher activity than the unmodified α and β. This was equal to 23% of the wild-type cre activity. This activity is higher than what Xu et al. [13] reported for cre fragment reassociation without fusion to NLS, which was 12% cre activity. This result suggests that bFos and bJun heterodimerization, as expected, could increase the efficiency of fragmented cre enzymatic activity by triggering the proximation of the fragments. It was also understood that bZip fragments did not have any inhibitory effect on the activity of the reassociated protein even though they were fused to the N terminus of β.

Confirming the Cell Transducibility of the Fusion Proteins

Since the α-bJun can form homodimers with increased size of the fragment (monomer size, 33 kD), we assessed whether the fusion of α fragments to bJun changed its cell permeation ability.

The purified α-bJun protein was labeled with Oregon Green Maleimide and incubated with COS7 cells. Figure 3 shows that the α-bJun fragment still has a high cell transduction capacity. Because the cell permeation ability of α fragment is still very high, we decided to use this fragment in the form of protein in our recombination system.

Regulation of Improved α-Complementation System

COS7 cells were transiently transfected by β[EGFP] or pCMV-bFos-β[EGFP]. The α-bJun protein was added to the transfected cells at final concentrations ranging from 0.5 to 2 μM. After 7 h of incubation, the medium was exchanged for fresh medium. Twenty-four hours

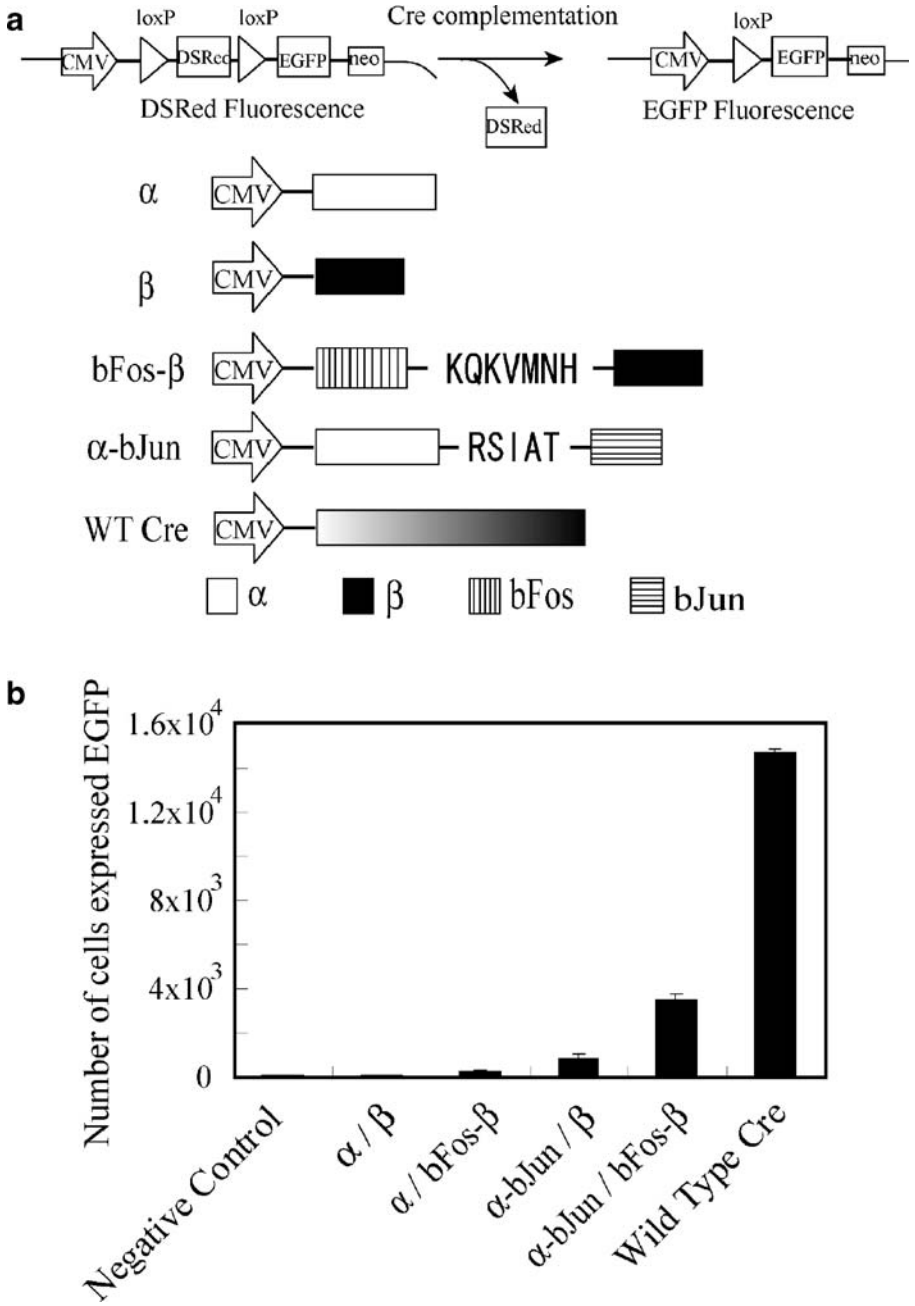

Fig. 2 Comparison between the recombination activity of fragmented cre before and after fusion to bZip fragments. **a** Schematic drawing of reporter system and the constructs used for transfection. **b** COS7 cells were seeded on 24-well plates (1×10^5 cells in each well). They were transfected with the DsRed[EGFP] reporter plasmid and plasmids containing cre fragments with the different combinations as mentioned in the figure. The number of EGFP-expressing cells to quantify the activity of the modified fragmented cre was counted

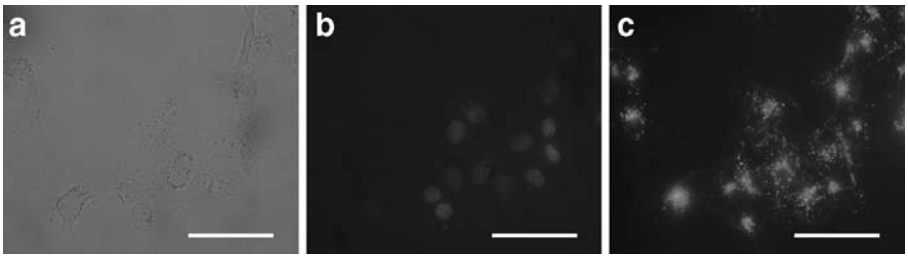

Fig. 3 The transduction test of α-bJun protein. The protein was labeled with Oregon Green maleimide and incubated with cells for 7 h. Cells were trypsinized and seeded on new plates and observed through fluorescent microscopy. The **b** and **c** images show the result of Hoechst and Oregon Green labeling, respectively. The *scale bars* represent 80 μm

after the addition of the proteins, cells were observed with fluorescence microscopy, and the number of EGFP-expressing cells was counted.

At each concentration of α-bJun protein, the recombination activity was compared between the cells transfected with either floxed β or floxed bFos-β (Fig. 4). In the case of bFos-β, the recombination efficiency was at least two times higher than the unmodified β. Although it is lower than we expected from the transient test result (Fig. 4), our new system is a considerably more efficient system than our previous recombination system in which the α and β had not been fused to bZip domain proteins.

The lack of a considerable increase in recombination activity with the increase in α-bJun concentration may be explained by the homodimerization of the protein and its effect on the cell permeability of α. It was suspected that the lack of recombination activity with high concentration of α-bJun protein could be due to the homodimerization of this protein that

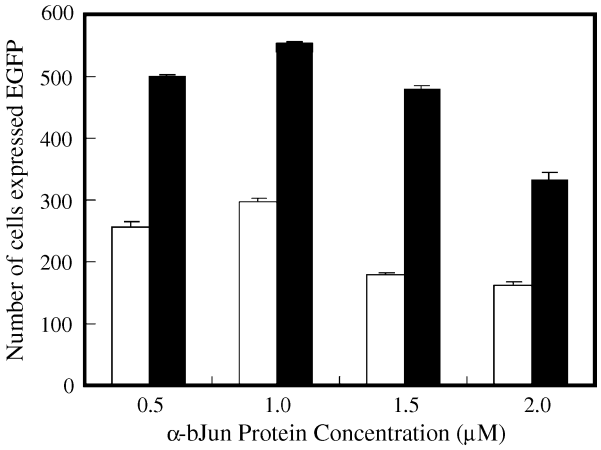

Fig. 4 Induction of recombination by addition of α-bJun protein to cells. COS7 cells were transiently transfected with either β[EGFP] or pCMV- bFos-β[EGFP]. Twenty-four hours after transfection, they were incubated with α-bJun protein for 7 h at final concentrations from 0.5 to 2 μM. The results were determined by counting EGFP-expressing cells 24 h after the addition of proteins. *White bars* The recombination resulted from β[EGFP] plasmid and α-bJun protein; *black bars* the recombination resulted from pCMV-bFos-β[EGFP] plasmid and α-bJun protein; each number is the average of the data from three experiments

makes it inaccessible for bFos-β molecules. It might be feasible to switch the bZip fragments and fuse α to bFos and vise versa. Lower concentrations of bJun, which can exist in cells if it is transiently expressed, can make it less readily homodizerable. There is also the possibility that by using bJun as a gene in cells, it could heterodimerize with bFos immediately after expression. Trying alternative interacting protein candidates that would not homodimerize and yet need to ligand for heterodimerization could also increase the efficiency of this system.

Conclusion

The efficiency of the recombination system based on α complementation was improved by fusing cre fragments to the bZip domain of AP-1 transcription factor. bZip proteins, which are comprised of bJun and bFos, were fused to the C-terminus of α and the N-terminus of β, respectively, together with two linker peptides. Transfection with a combination of plasmids containing α-bJun and bFos-β genes, resulted in 42 times greater recombination efficiency than the combination of unmodified α and β. The new fusion fragments were used to develop our recombination system based on the α complementation method. bFos-β was sent to cells as a floxed gene. The bFos-β protein that was expressed in cells regained enzymatic activity upon the addition of α-bJun-purified protein to cells. This new system was evaluated as two times more efficient than the previous system. Fragmentation of cre recombinase has made it possible to take advantage of both gene and protein therapies, thus enabling researchers to limit the recombination within a specific site by using tissue-specific promoters to drive the expression of an inactive fragment of cre. In addition, the recombination initiation can be in the control of the experimenter by using the purified other inactive protein. The present research has great potential as a highly efficient recombination system suitable for a range of applications.

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