Cre reconstitution allows for DNA recombination selectively in dual-marker-expressing cells in transgenic mice

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ABSTRACT

Cre/LoxP-based DNA recombination has been used to introduce desired DNA rearrangements in various organisms, having for example, greatly assisted genetic analyses in mice. For most applications, single gene promoters are used to drive Cre production for conditional gene activation/inactivation or lineage-tracing experiments. Such a manipulation introduces Cre in all cells in which the utilized promoter is active. To overcome the limited selectivity of single promoters for cell-type-specific recombination, we have explored the ‘dual promoter combinatorial control’ of Cre activity, so that Cre activity could be restricted to cells that express dual protein markers. We efficiently reconstituted Cre activity from two modified, inactive Cre fragments. Cre re-association was greatly enhanced by fusing the Cre fragments separately to peptides that can form a tight antiparallel leucine zipper. The co-expressed Cre fusion fragments showed substantial activity in cultured cells. As proof of principle of the utility of this technique in vivo for manipulating genes specifically in dual-marker-positive cells, we expressed each inactive Cre fragments in transgenic mice via individual promoters. Result showed the effective reconstitution of Cre activates LoxP recombination in the co-expressing cells.

INTRODUCTION

The Cre/LoxP system utilizes P1 bacteriophage Cre recombinase to catalyze recombination between tandem LoxP DNA sequences (1,2). This system has been widely used in multiple organisms, including yeast (3), plants (4–7) and animals (8–14). The Cre/LoxP technology is particularly useful for mammalian genetics, because it allows the analyses of essential genes in specific organs by gene inactivation (8–15) or controlled ectopic gene expression (16,17). When combined with visible marker proteins, Cre-LoxP-based gene activation allows for cell marking and cell lineage analyses in living animals (17).

Specific gene promoters are usually utilized to drive Cre expression in desired tissues. Thus, the promoter specificity limits where Cre can be expressed. To this end, most available promoters drive gene expression in multiple cell types. This deficiency has greatly limited our ability to manipulate genes within specific cells, such as stem cells that can only be identified by their expression of several molecular markers (18–20). An approach that introduces Cre exclusively to cells that express more than one protein marker would facilitate our understanding of the function and fate of specific cells in vivo.

Active protein can be reconstituted from peptide fragments of corresponding molecules. For some proteins, fragmented peptides can directly re-associate to restore activity (21–23). In other scenarios, assisted protein reconstitution is required. In this latter case, protein can be cleaved to two inactive fragments. Each fragment was then fused to one of a pair of interacting protein motifs respectively. The interacting motifs could bring the protein fragments to proximity to facilitate efficient reassembly (23–30). Both the above schemes have been explored for Cre activity reconstitution (31,32). In one report, cDNA molecules were designed to produce two inactive Cre halves in same cells. This approach, combined with improving protein translation from Cre cDNA (33), was reported to reconstitute 32.5% of wild-type Cre activity (32). In the other case, cDNA molecules were constructed to produce two inactive Cre moieties connected with FK506-binding protein (FKBP12) and FKBP12-rapycin-associated-protein (FRP), respectively. Because the interaction between FKBP12 and FRP was FK506...
dependent, Cre activity could be restored only when both moieties and FK506 were present (31,34). This method restores ~3–4% Cre activity (31). The usefulness of these two systems in animal models has not been reported.

We attempted to reconstitute Cre in mouse cells that express two protein markers. Because we could obtain less than 2% Cre activity using the published Cre fragment complementation process (32), we utilized assisted-Cre reassembly for this purpose. The Cre open reading frame (ORF) was cleaved into two cDNA fragments, each encoding an inactive Cre peptide. Each cDNA fragment was then fused to an ORF for one of two peptides that could form antiparallel leucine zippers (35). This leucine zipper was artificially designed and has been reported to effectively assist protein reconstitution in vitro and in vivo, and these peptides do not seem to interfere with normal cellular functions (25,28,35). When these two modified Cre fragments were co-expressed in tissue culture, ~30% Cre activity could be restored, an 8-fold improvement over previously published methods (31,32). When expressed in the pancreatic tissue of transgenic mice from individual promoters, the inactive Cre fragments effectively induce LoxP-based recombination. This approach opens the possibility to study gene function or perform lineage labeling in cells that express dual protein markers in animal models.

**MATERIALS AND METHODS**

**DNA constructs and transgenic mouse production**

For cDNA encoding the fusion of leucine zipper-forming peptides with Cre moieties (Figure 1), overlapping DNA oligos were synthesized and PCR-amplified with nlsCre cDNA [with a nuclear localization signal (NLS) present in Cre’s n-terminus] as template (36). One final cDNA ORF (called nCre) encodes a protein with N-terminal half of Cre fused with N-peptide at Cre C-terminus (with a NLS at its n-terminus, Figure 1). The olligos utilized were: X5, Nz1, Nz2, Nz3 and Nz4 (Table 1). Anther cDNA ORF (called nCre) encodes a protein with N-terminal half of Cre fused with N-peptide at Cre C-terminus (with a NLS at its n-terminus, Figure 1). The DNA oligos used were: N3, Cz1, Cz2, Cz3 and Cz4 (Table 1). To add an extra nuclear localization signal coding sequence to cCre in its 5’ end (to produce nlcCre), we utilized the following oligos: Nlc, N3, Cz1, Cz2, Cz3 and Cz4 (Table 1). PCR fragments were cloned into the pBluescriptKSII vector to produce pYW415, pYW429 and pYW418, respectively. The Xhol–NotI fragments from these constructs were ligated into the corresponding sites of the pCIG-expression vector, containing the CMV-chicken-β actin promoter to drive gene expression, to produce pYW427, pYW443 and pYW425 (37). For CMV-stop-GFP, an EcoRI–SpeI fragment (contains a Poly A signal) from PBS302 (38) was ligated into the EcoRI–SpeI sites of pGreenLatern-1 to produce pYW421 (39). As control for Cre activity assay, the full-length Cre (which could form antiparallel leucine zippers (35). This leucine zipper was artificially designed and has been reported to effectively assist protein reconstitution in vitro and in vivo, and these peptides do not seem to interfere with normal cellular functions (25,28,35). When these two modified Cre fragments were co-expressed in tissue culture, ~30% Cre activity could be restored, an 8-fold improvement over previously published methods (31,32). When expressed in the pancreatic tissue of transgenic mice from individual promoters, the inactive Cre fragments effectively induce LoxP-based recombination. This approach opens the possibility to study gene function or perform lineage labeling in cells that express dual protein markers in animal models.

Figure 1. A diagram of the half-Cre molecules and the interacting peptide sequences. (A) The Cre molecule was designed to be cleaved into two molecules between two glycine residues (amino acid residues 190–191, as numbered in X03453). The N-terminal half was fused with one of a pair peptides that form a leucine zipper (N-peptide), whereas the C-terminal half was fused with the other peptide (C-peptide). (B) The C- and N-peptide sequences.

| Primer name | Primer sequence |
|-------------|-----------------|
| T3          | gcggccgatgtttaaatcaagatgtttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
For transgene constructs, PCR-amplified SV40 polyA sequences from pGreen/lenti1 were inserted into the Smal site of pBluescript KSII, producing pGD103 (oligonucleotides utilized: pA1, pA2; Table 1). The XhoI–NotI (filled-in)–digested nCre or nlcCre fragments were inserted into the XhoI–EcoRV site of pGD103, producing YW452 and YW451, respectively. Finally, XhoI (filled-in)–NotI fragments from YW452 and YW451 were inserted downstream of the murine Pdx1 promoter (Smal/NotI-restricted plasmid #571; gift from C. Wright). Inserts were released with Sall–NotI for transgenic animal production in the Shared ES Cell/Transgenic Animal Resources in Vanderbilt Medical Center. nCre<sup>gr</sup> and nlcCre<sup>gr</sup> genotyping was with ng1 and cga oligonucleotides (Table 1). R26R-EYFP (R26YFP) and Z/AP reporter animals genotyping, and alkaline phosphatase detection was by published methods (36,41). All mouse care, handling and crosses followed IACUC protocol M/03/354 (Gu), approved by the Animal Welfare Committee of Vanderbilt Medical Center.

Cre activity assay
A reporter plasmid (YW421) expressing eGFP Cre dependently was used to assay Cre activity. Reporter YW421, plus cCre or nCre plasmids (or both), and mCherry-producing plasmid (42) were co-transfected into HK293 cells using calcium-phosphate-based technique. After 14–16 h, transfected cells were analyzed by flow cytometry for fluorescence expression. The percentage of red cells that express eGFP was plotted against the Cre plasmid(s) concentration. For most transfections, 0.2 μg YW421, 0.1 μg mCherry and 0.1–2 ng Cre-expressing plasmids were utilized for each well of 12-well dishes. With bigger wells, the plasmid amount was scaled-up proportional to the well area. To ensure that the Cre activity comparisons were made in a linear range, a standard curve was constructed varying the concentration of Cre-producing plasmid, measuring the output green/red ratio. Reconstituted Cre activity was calculated against this standard curve. All assays used a minimum of triplicate samples. All assays utilized nlsCre as a control.

Immunofluorescence/immunohistochemistry
Established protocols were used. Briefly, tissues were fixed in 4% paraformaldehyde overnight at 4°C, or 4 h at room temperature, and prepared as frozen sections. Frozen sections conserve GFP fluorescence. Primary antibodies used were: guinea pig anti-insulin and guinea pig anti-glucagon (Dako, Carpinteria, CA, USA); rabbit anti-ss, guinea pig anti-PP (In Vitrogen, Carlsbad, CA, USA); rabbit anti-amylase, biotinylated Dolichos biflorus agglutinin (DBA, Sigma, St Louis, MO, USA). Secondary antibodies used were: Cy3-conjugated donkey anti-rabbit IgG, Cy3-conjugated donkey anti-guinea pig IgG, Cy3-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA, USA). Alkaline phosphatase staining followed reported protocols (43). All antibodies utilized 1:1000 dilutions.

Microscopy
For whole mount YFP fluorescence, tissues were dissected and fixed in 4% paraformaldehyde (overnight, 4°C), washed and mounted in PBS in chambers on glass slides. Samples were observed using either inverted fluorescence microscope (for regular observations) or confocal microscopy (for high quality pictures). Confocal imaging was also utilized to observe immunofluorescence-stained samples. Typically, 0.4 μm optical z-sections were taken for thick samples. A maximum of two adjacent optical sections were stacked and projected to produce a high quality picture for each figure.

RESULTS
Creating inactive Cre fragments for reconstitution
Reconstituting Cre activity from two inactive peptide fragments could benefit from a pair of interacting protein motifs to bring Cre fragments to proximity for refolding. Additionally, Cre ORF needs to be cleaved at a specific site so that the encoded Cre fragments will be inactive, yet are able to reassemble into an active molecule when brought together.

We considered several criteria in choosing interacting protein motifs to assist in Cre reconstitution, including high affinity, high specificity, and lack of dominant-negative effects in living cells. The reported pair of antiparallel, heterodimer leucine zipper-forming peptides (Figure 1, named as N- and C-peptide) fit this profile (44,45). These peptides were shown to effectively assist protein folding both in vitro and in vivo with no detectable dominant negative effects in living animals (24,25,44,45). To choose the best point to separate Cre into two portions, we examined the Cre 3D structure for the residues and secondary structures that are crucial for its activity (4,32,46). We choose to generate open reading frames (ORF) which encodes Cre amino acid residues 1–190 and 191–343, separated between two glycine residues at 190 and 191 (Figure 1). The flexibility of the peptide bond between glycine and other amino acid residues makes it more likely to tolerate addition of extra peptides without disrupting the secondary and tertiary structure of Cre. In addition, these two glycine residues are localized between two β-sheets and are expected to point away from the DNA elements during recombination (46). Therefore, connecting leucine zippers with each half of the Cre protein at this position is expected to minimally interfere with Cre function.

We derived three cDNA ORFs that encode three Cre fragments, nCre, cCre and nlcCre, for Cre reconstitution (Figure 1A). nCre was a fusion between the N-peptide to the N-terminal half Cre. We included the SV40 large T antigen NLS in the N-terminus of this molecule. cCre was a fusion between the C-peptide to the C-terminal half Cre (Figure 1B). nlcCre also contains a SV40 large T antigen NLS in its n-terminus, otherwise, it is identical to cCre (Figure 1). The presence of an NLS in both N- and C-terminal half Cre molecules respectively is likely to restrict both molecules in the nucleus and allow for
efficient interaction. In order to express these expected
protein fragments, the ORFs were put under the control
of the CMV-β actin promoter in the pCIG vector.

Leucine zipper-forming peptides in Cre fragments
assist Cre reconstitution

The substrate for testing reconstituted Cre activity was a
reporter plasmid that produces eGFP in a Cre-dependent
manner (Figure 2A). The reporter plasmid was transfected
into HEK293 cells in large excess (see Materials and
methods section), together with Cre-producing plasmids.

An mCherry-producing plasmid (42) was co-transfected as
a control for cell transfection efficiency, with the green/red
fluorescence ratio providing an index for Cre activity.

To determine whether assisted-Cre reconstitution
would be feasible in vivo, we used a Pdx1 promoter to drive nCre
and nleCre expression in transgenic mice (Pdx1-nCre
and Pdx1-nleCre). The Pdx1 promoter is well characterized,
with expression restricted to all cells in the pancreas, as
well as posterior foregut cells of the duodenum and antral
stomach (47,48). Four Pdx1-nCre (nCre<sup>β1−4</sup>) and six
Pdx1-nleCre (nleCre<sup>β1−6</sup>) independent transgenic mouse
lines were derived (Figure 3A).

We first crossed the nleCre<sup>β1</sup> line with all four nCre<sup>β</sup>
lines to determine whether Cre activity could be reconstituted,
using the Z/AP reporter allele's Cre-dependent
activation of alkaline phosphatase (43). Two lines, nCre<sup>β1</sup>
and nCre<sup>β3</sup>, when combined with nleCre<sup>β1</sup>, showed AP
activity in ~5% of pancreatic cells in newborn animals
(data not shown). The nCre<sup>β2</sup> and nCre<sup>β6</sup> lines showed no
detectable recombination when combined with nleCre<sup>β1</sup>
and Z/AP. Semi-quantitative RT-PCR verified that these
two latter lines express nearly undetectable levels of nCre
mRNA (data not shown), and were sacrificed. As expected,
neither nleCre<sup>β</sup> nor nCre<sup>β</sup> alone could induce
Z/AP recombination (data not shown).

We next utilized nCre<sup>β1</sup> to determine which of the six
nleCre<sup>β</sup> transgenic lines gave the highest recombination
efficiency (Figure 3B). We switched to the R26R-EYFP
mouse line (41) for this experiment because of the
convenience of observing YFP fluorescence as a reporter
for Cre activity (Figure 3). Neonatal nCre<sup>β1</sup>; nlleCre<sup>β2</sup>
and nleCre<sup>β3</sup>; R26R-EYFP animals had 31.2±4.1 (n = 4) and 22.6±2.9% (n = 3)
pancreatic cells recombined respectively, whereas,
nleCre<sup>β1</sup>; nleCre<sup>β3</sup>, nlleCre<sup>β4</sup> and nlleCre<sup>β6</sup> mouse lines showed
3−17% pancreatic cells recombined when they were
combined with nCre<sup>β1</sup> and R26R-EYFP (data not shown).
Our subsequent characterization used the
nCre<sup>β1</sup> and nleCre<sup>β2</sup> transgenic lines.

Cre activity is detected in early embryonic stages

We next assessed whether there was a time dependency to
the reconstitution of Cre activity as compared to the
activity of the Pdx1 promoter, by assessing YFP expression
in nCre<sup>β1</sup>; nleCre<sup>β2</sup>; R26R-EYFP pancreata at several stages of embryogenesis. Robust YFP expression
in the pancreatic region was observed at E13.0 (8.1±3.1% of
all pancreatic cells counted in four pancreatic buds,
Figure 4A–C), but not at E11.5 (data not shown). The
percentage of labeled cells increased gradually during embryogenesis, so that at E15.5 and E17.5,
~16.4±2.9% or 26.2±3.7% (n = 4), respectively, of pancreatic cells
expressed YFP (Figure 4D and E and data not shown).
From birth to 2-month-old adults, the overall percentage of YFP<sup>+</sup>
pancreatic cells remained relatively stable (data not shown), consistent with the idea that the bulk of the
pancreatic mass comprises exocrine tissues that express
only a low level of Pdx1, and as such might not reach the
Cre threshold for recombination of the reporter allele.

We examined YFP expression in Pdx1-producing cells
at corresponding ages (Figure 4G–I) using the Pdx1-Cre<sup>β8</sup>
animals (39). Robust YFP expression was observed
in more than half of the Pdx1<sup>−</sup> pancreatic cells at E10.5.
After E13.0, most, if not all, of the Pdx1<sup>−</sup>
pancreatic cells had turned on YFP expression. These results demonstrate that substantial Cre activity could be restored in early
embryonic stages. Yet a delayed recombination and a
mosaic pattern are likely to result using this Cre
reconstitution approach, as opposed to the conventional
Cre molecule.
Figure 2. Assisted Cre reconstitution restores substantial Cre activity in cell culture. (A) The structure of the Cre reporter. EGFP could be expressed only after Cre-mediated excision of the stop signal. (B1–B3) One example of Cre activity assays. (B1) EGFP expression (green) reported detectable Cre activity. (B3) mCherry expression (magenta) indicates transfected cells. (B2) Merge of B1 and B3. (C) An example of flow cytometry analysis for Cre activity (0.5 ng pCIG-Cre plasmid/well). In the four quadrants (R1–R4), R1 represents untransfected cells; R2, transfected cells without detectable Cre activity; R3, transfected cells with active Cre; R4, cells with active Cre yet have lost mCherry expression. The percentage of each cell type is labeled. The ratio of R3/(R2+R3) is used as Cre activity index. (D) The plot of GFP+/mCherry+ cells [=R3/(R2+R3)] versus the amount of Cre-expressing plasmid. (E) Four assays for the relative Cre activity reconstituted from various Cre fragments. The four DNA samples used were (labeled below each dot plot): no Cre control, pCIG-nCre + pCIG-nlcCre, pCIG-nCre + pCIG-nlcCre, pCIG-nCre + Ubc-nlcCre (all plasmids were transfected at 2 ng/well). Note the low GFP background when no Cre activity is present. (F) Relative Cre activity restoration when different Cre fragments are utilized for complementation. (G) Relative Cre activity with a combination of 1 ng of pCIG-nCre with various amount of pCIG-nlcCre.
The presence of two reporter alleles substantially increases cell-labeling efficiency

One potential application for this Cre reconstitution approach is to mark and study the lineage of progenitor/stem cells that express two protein markers. It is possible that a higher percentage of cell labeling would be observed in the presence of two reporter alleles. We therefore analyzed nCre[g]; nlcCre[g]; R26R-EYFP/R26R-EYFP embryos. Surprisingly, 63.2 ± 5.4% (n = 4) of pancreatic cells express YFP at E17.5, more than double that of the nCre[g]; nlcCre[g]; R26R-EYFP littermates (26.5 ± 4.9%, n = 3). This result demonstrates that the presence of two floxed reporter alleles substantially increases the chance of introducing DNA recombination (in the presence of a given amount of Cre), such that more complete lineage labeling would be obtained in the presence of two reporter alleles. At present, we do not understand why this increased recombination occur with the presence of two floxed alleles and it remains to be seen whether this same result holds for the other reporter alleles that are commonly in use in lineage tracing experiments in vivo.

Reconstituted Cre induces recombination in all pancreatic cell types

Effective application of Cre reconstitution requires Cre to be restored in a cell context-independent manner. We therefore examined whether all pancreatic cell types could be labeled with Cre reporter expression in neonatal and adult nCre[g]; nlcCre[g]; R26R-EYFP pancreata. The vertebrate pancreas contains two exocrine cell types, the pancreatic duct and acinar cells, and four major endocrine cell types, α, β, δ and PP cells. The pancreatic duct cells can be recognized by their specific expression of an epitope that binds to the DBA lectin, whereas the acinar cells, α, β, δ and PP cells can be recognized by their expression of amylase (amy), glucagon (glc), insulin (ins), somatostatin (SS) and pancreatic polypeptide (43), respectively. The acinar cells are derived from pancreatic progenitors that continuously express high levels of Pdx1 (36). In differentiated acinar cells, a low level of Pdx1 expression is maintained (47). The β and δ cells are also derived from Pdx1+ progenitors and they maintain a high level of Pdx1 expression throughout life. On the contrary, the pancreatic duct, α and PP cells only transiently express Pdx1 during their differentiation. If nCre and nlcCre can reassemble in a cell type independent fashion, we expect that all pancreatic cell types can be labeled with Cre reporter expression, and a larger proportion of acinar, β and δ cells should be labeled, than that of duct, α and PP cells. Indeed, 28.1 ± 4.8%, 32.5 ± 3.7% and 21.7 ± 6.1% acinar, β and δ cells in neonatal nCre[g]; nlcCre[g]; R26YFP pancreas expressed YFP (n = 4, Figure 5A–C). Whereas only 8.3 ± 4.8%, 4.4 ± 2.3% and 12.5 ± 3.7% duct, α and PP cells expressed YFP at the same age (n = 4, Figure 5D–F).

Because pancreatic β cells maintain high levels of Pdx1 expression in postnatal animals, we expected that Cre activity would be maintained in these cells in the nCre[g]; nlcCre[g]; R26YFP animals and the labeling index of the β cells should increase over age. Indeed, the percentage of...
YFP$^+$ β cells increased to ~62% in 2-month-old pancreata (Figure 5G). On the contrary, the labeling indices of cells that do not express detectable levels of Pdx1 (e.g. the duct and α cells) in postnatal pancreas did not increase, even though these labeled cells were still present in 2-month-old pancreas (Figure 5H and I).

**DISCUSSION**

The Cre/LoxP-based DNA recombination has revolutionized mammalian genetic analysis (14,17). With tissue-specific Cre production or temporally controlled Cre activity (10), this system allowed for precise gene manipulation in spatially and temporally desirable manners, thus revealing gene functions and cell lineages during animal development and tissue regeneration. One challenge for this system is obtaining proper promoters to drive Cre expression with the appropriate cell-type specificity. This is a critical issue for studying progenitor or stem cells, which usually need multiple markers for identification. Here we report the successful reconstitution of Cre activity from modified, inactive Cre fragments in both cell culture as well as in transgenic mice. By modifying this approach such that the expression of the individual inactive Cre fragments is driven by separate promoters, with a defined overlap of expression, such a system could prove extremely versatile in allowing controlled DNA rearrangements (gene inactivation, ectopic gene expression and lineage labeling) in highly spatially or temporally defined cell populations.

Previous Cre activity assays introduced Cre-expressing constructs to cells that carry one copy of a reporter gene, which expresses LacZ upon LoxP-based recombination (31,32,34). Because only one single event is required for turning on LacZ expression, it is not clear whether this approach can accurately determine Cre activity (49). LoxP-based chromosomal integration of extra chromosomal circular DNA could be used for a more accurate Cre activity assay, yet this latter approach is time consuming and proper cell selection is required (49). We devised a straightforward Cre activity assay by providing a large excess of Cre reporter that could be co-transfected with Cre-expressing plasmids. Within a reasonable Cre concentration range, the percentage of cells that turns on reporter expression displayed a linear correlation with the amount of Cre-producing plasmid transfected, demonstrating that this approach could be used to assay for Cre activity. With fluorescent protein reporters and flow cytometry, this Cre assay was rapid, being accomplished within two days. Using this assay, we showed that unassisted Cre reconstitution could only recover <2% Cre activity, whereas assisted Cre reassembly recovers nearly 30% Cre activity, demonstrating the key importance of our protein-interaction-motif-based modification. Furthermore, we compared this assisted-Cre reconstitution approach with the unassisted Cre complementation method (32) side by side in tissue culture. Results showed that the former protocol allowed for Cre recovery at least 10-fold more efficiently than the latter scheme. These analyses suggest that assisted-Cre reconstitution is more desirable for introducing widespread DNA recombination within specific tissues, whereas the simple Cre-complementation approach (32) might be more useful when recombination in a small number of cells is desired, for example, for mosaic analyses or clonal cell lineage tracing.

The reconstituted Cre activity is sufficient to induce LoxP-based DNA recombination in both tissue culture and in transgenic mice. However, a mosaic recombination pattern was observed, i.e. not all cells that are expected to express both Cre moieties undergo recombination in transgenic mouse. There are two possible explanations for this finding. First, the reconstituted Cre activity is too low to induce recombination in all Pdx1-expressing cells. Second, the Pdx1 promoter-controlled transgene expression is of a mosaic pattern per se. Because the Pdx1 promoter used in this study rarely expresses transgene in mosaic pattern (36,50,51), and multiple independent mouse lines tested in this study all produce a similar recombination pattern, this latter possibility is an unlikely scenario.

This Cre-complementation technique could potentially be utilized for loss of gene function analyses, ectopic gene expression and cell lineage analyses. Although the low Cre activity recovery is not ideal for inactivating gene function, generation of mosaic pattern is advantageous in assessing cell clonality for lineage tracing. It could also allow for detailed analyses of the mutant cell behaviors if
loss or gain of gene function in a subset of cells avoids the lethality caused by a more widespread ectopic gene expression or loss of gene activity. Results obtained from such analysis could shed light on whether a gene exerts its function in a cell autonomous or non-autonomous fashion.

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