Using an in vivo phagemid system to identify non-compatible *loxP* sequences☆

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Abstract The site-specific recombination system of bacteriophage P1 is composed of the Cre recombinase that recognizes a 34-bp *loxP* site. The Cre/*loxP* system has been extensively used to manipulate eukaryotic genomes for functional genomic investigations. The creation of additional heterologous *loxP* sequences potentially expands the utility of this system, but only if these *loxP* sequences do not recombine with one another. We have developed a stringent in vivo assay to examine the degree of recombination between all combinations of each previously published heterologous *loxP* sequence. As expected, homologous *loxP* sequences efficiently underwent Cre-mediated recombination. However, many of the heterologous *loxP* pairs were able to support recombination with rates varying from 5 to 100%. Some of these *loxP* sequences have previously been reported to be non-compatible with one another. Our study also confirmed other heterologous *loxP* pairs that had previously been shown to be non-compatible, as well as defined additional combinations that could be used in designing new recombination vectors.

Key words: Cre; *LoxP*; Recombination; Phagemid; Functional genomics

1. Introduction

Bacteriophage P1 contains a site-specific recombination system responsible for genome partitioning after replication [1]. This system is composed of a 38-kDa Cre recombinase which mediates recombination between two 34-bp *loxP* sites and does not require any accessory factors. The *loxP* site contains two 13-bp inverted repeats flanking an asymmetric 8-bp core region that gives the *loxP* site an apparent directionality. Cre-mediated recombination between two aligned *loxP* sites will excise the intervening DNA, while recombination between inverted *loxP* sites should result in the inversion of that same DNA [2]. Crystal structures reveal that each 13-bp inverted repeat is individually bound by a Cre monomer and recombination involves the formation of a Holliday junction [3,4]. Strand exchange begins after single-strand (ss) nicks are produced at a specific site within each 8-bp core by conserved tyrosines on two Cre subunits, generating nucleophiles that complete the exchange of one set of strands. The Holliday intermediate is resolved to the recombinant products after cleavage and exchange of the remaining pair of DNA strands by the second pair of Cre subunits.

The Cre/*loxP* recombination system has proven to be an important tool for in vivo manipulation of eukaryotic genomes [5,6]. Gene activation and deactivation strategies in mammalian cells [7–10] and transgenic mice [11–13] have been developed by expressing Cre in a temporally and/or spatially controlled fashion. However, further exploitation of this system has been limited by the availability of only one *loxP* sequence. To this end, studies have identified hetero-specific *loxP* sequences that recombine efficiently with the identical *loxP* mutant and not with the original wild-type (WT) *loxP* sequence (see Fig. 1): 511 *loxP* contains a C to T transition at position 2 of the core region [14], 2272 *loxP* contains a C to G transversion at position 2 and an A to C transversion at position 7 [15], 5171 *loxP* contains a C to T transition at position 2 and a T to C transition at position 4 [15], and FAS *loxP* is a naturally occurring sequence near the *FAS1* gene in *Saccharomyces cerevisiae* [16]. The combination of WT *loxP* and 511 *loxP* has been successfully used for a double-reciprocal crossover reaction to transfer a DNA segment or gene to a predetermined target site [7,17,18] and has facilitated the creation of phagemid antibody libraries [19,20]. Recent reports, however, have suggested recombination can occur between WT and 511 *loxP* sequences [7,15].

In order to expand the utility of the Cre/*loxP* system for future functional genomic studies and to verify that each *loxP* sequence does not recombine with any other published *loxP* sequence, we devised an assay to study recombination between all possible combinations of the five heterologous *loxP* sites in vivo. Results show that when homologous *loxP* sequences are present, recombination occurs at nearly 100% for all five sequences. However, one third of the chimeric constructs containing different *loxP* sequences were able to support recombination above 5%, with one heterologous pair nearly 100% efficient. We also observed differences in recombination rates between different *loxP* sequences depending on their relative orientation to one another. These results impact the design of recombination vectors in which more than one *loxP* sequence is used.

2. Materials and methods

2.1. Bacterial strains

DH5αF′ (Gibco BRL, Rockville, MD, USA): F′/*end*A1 *hsd*R17

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Abbreviations: GFP, green fluorescent protein

PII: S0014-5793(01)02806-X

(r_K-mK^+) *sup*E44 *thi*-1 *rec*A1 *gyr*A (Na1^r) *rel*A1 Δ (*lac*ZYA-*arg*F) U169 *deo*R (Φ80*dlac*Δ(*lac*Z)M15).

BS1365 (kind gift from Dr. Brian Sauer): BS591 F′ kan (BS591: *rec*A1 *end*A1 *gyr*A96 *thi*-1 Δ *lac*U169 *sup*E44 *hsd*R17 [lambda *imm*434 *nin*5 X1-cre]).

2.2. LoxP/green fluorescent protein (GFP) constructs

GFP [21] was amplified with primers (see Section 2.3) that appended appropriate *loxP* sites 5′ and 3′ of the GFP encoding sequence. The primers incorporated different restriction sites 5′ to each *loxP* sequence for identification, as well as the restriction sites used for cloning (*Hin*dIII and *Eco*RI) (see Figs. 1 and 2). In addition, the 3′ primers inserted a stop codon immediately after the GFP encoding sequence. The *loxP* sequences (except for 511-I *loxP*) are orientated such that potential ATG start sites are located on the opposite strand. The PCR products were cloned into the multiple cloning site of pUC119 (ATCC # 37461) inactivating the alpha peptide fragment of β-galactosidase (*lac*Z′), but leaving the start codon of *lac*Z′ in frame with GFP, allowing translation of GFP upon induction with isopropyl-1-thio-β-D-galactoside (IPTG). This construct also contains both the M13 origin of replication, allowing incorporation into filamentous phagemid particles in the presence of M13K07 helper phage (NEB, Beverly, MA, USA), and the colE1 origin of replication, allowing double-strand (ds) DNA to be amplified. The *loxP*/GFP plasmids were transfected into DH5αF′ cells and fluorescent colonies containing the appropriate *loxP* sites, as determined by restriction digests, were used to generate phagemid after helper phage infection as described [22]. *LoxP*/GFP constructs that have not had an opportunity to recombine are denoted as 1° while constructs that have had an opportunity to recombine are denoted as 2° (see Section 2.4).

2.3. Primers

WT *loxP*/GFP-5′: CCC AAG CTT CGC TAG CAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT ATC CAT GAG TAA AGG AGA AGA ACT T

WT *loxP*/GFP-3′: GGA ATT CGC TAG CGA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT TTG TAT AGT TCA TCC ATG CC

511 *loxP*/GFP-5′: CCC AAG CTT CCT GCA GAT AAC TTC GTA TAG TAT ACA TTA TAC GAA GTT ATC CAT GAG CAA AGG

511 *loxP*/GFP-3′: GGA ATT CCT GCA GGA TAA CTT CGT ATA ATG TAT ACT ATA CGA AGT TAT TTG TAG AGC TCA TCC

511-I *loxP*/GFP-5′: CCC AAG CTT CGG ATC CAT AAC TTC GTA TAA TGT ATA CTA TAC GAA GTT ATC CAT GAG TAA AGG

511-I *loxP*/GFP-3′: GGA ATT CGG ATC CGA TAA CTT CGT ATA GTA TAC ATT ATA CGA AGT TAT TTG TAT AGT TCA TCC

2272 *loxP*/GFP-5′: CCC AAG CTT CAG ATC TAT AAC TTC GTA TAG GAT ACC TTA TAC GAA GTT ATC CAT GAG TAA AGG

2272 *loxP*/GFP-3′: GGA ATT CAG ATC TGA TAA CTT CGT ATA AGG TAT CCT ATA CGA AGT TAT TTG TAT AGT TCA TCC

5171 *loxP*/GFP-5′: CCC AAG CTT CCC GCG GAT AAC TTC GTA TAG TAC ACA TTA TAC GAA GTT ATC CAT GAG TAA AGG

5171 *loxP*/GFP-3′: GGA ATT CCC GCG GGA TAA CTT CGT ATA ATG TGT ACT ATA CGA AGT TAT TTG TAT AGT TCA TCC

FAS *loxP*/GFP-5′: CCC AAG CTT CAC GCG TAC AAC TTC GTA TAT ACC TTT CTA TAC GAA GTT GTC CAT GAG TAA AGG

FAS *loxP*/GFP-3′: GGA ATT CAC GCG TGA CAA CTT CGT ATA GAA AGG TAT ATA CGA AGT TGT TAT TTG TAT AGT TCA TCC

2.4. Cre in vivo recombination assay

BS1365 cells (constitutively expressing Cre recombinase) were grown to an OD_{600} of 0.5 at 37°C and infected with *loxP*/GFP 1° phagemid. Infected cells were allowed to recombine overnight at 37°C in the presence of ampicillin. An aliquot of the overnight culture that had undergone recombination was diluted and grown to midlog at 37°C and then infected with M13K07 helper phage. Cells were then grown overnight at 37°C with ampicillin and kanamycin in order to harvest *loxP*/GFP 2° phagemid. Recombination was detected by infecting DH5αF′ cells at OD_{600} of 0.5 with the various *loxP*/GFP 2° phagemids. The cells were plated on 2×YT agar containing 100 μg/ml ampicillin, 1 mM IPTG and 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) at dilutions that allowed individual colonies to be counted. 2° transductions into DHαF′ were performed to avoid possible heterogeneity from the recombination reactions and to utilize alpha complementation of *lac*Z′ which is not possible with BS1365.

In addition, the 511-I/511 *loxP*/GFP and WT/511 *loxP*/GFP dsDNA 1° constructs were transfected into BS1365 and grown in 1 ml of 2×YT containing 100 μg/ml ampicillin overnight at 37°C. The *loxP*/GFP 2° dsDNA was isolated using Qiagen spin columns (Qiagen, Valencia, CA, USA) and then used to transfect DH5αF′ cells and plated on 2×YT agar containing 100 μg/ml ampicillin, 1 mM IPTG and 20 μg/ml X-gal.

The resulting 2° colonies from either phagemid transduction or dsDNA transfection were visualized after excitation at 488 nm. Recombination that excised GFP gave blue, non-fluorescent colonies. Recombination that inverted GFP resulted in white, non-fluorescent colonies and was confirmed by diagnostic PCR. Colonies containing phagemid DNA that did not recombine were white and fluorescent after excitation at 488 nm. For 511-1/511, both excision and inversion rates were used to calculate the overall recombination percentage for a given pair of *loxP* sites (Table 1). Values shown are the averages of at least three independent trials with standard deviations indicated.

3. Results

An in vivo assay was developed to test the level of recombination between the five published *loxP* sequences in a prokaryotic system (Fig. 1). GFP flanked by either homologous or heterologous *loxP* sites was cloned into and disrupted the frame of the *lac*Z′ reporter gene (Fig. 2). Removal of the GFP by Cre-mediated recombination will leave a single *loxP* site and allow the *lac*Z′ fragment to be translated. Therefore, colonies containing DNA that had not undergone recombination will remain white in the presence of X-gal and show fluorescence after excitation at 488 nm (Fig. 2A,B, top), whereas colonies in which recombination has excised GFP will be blue and non-fluorescent (Fig. 2A, bottom), and colonies in which recombination has inverted GFP will produce white colonies which are non-fluorescent (Fig. 2B, bottom). Constructs are named according to the *loxP* sequence present 5′ and 3′ of GFP; e.g. WT *loxP*/GFP contains the WT *loxP* sequence at both locations while WT/511 *loxP*/GFP has the WT *loxP* sequence at the 5′ site and the 511 *loxP* sequence at the 3′ site. In all, 21 combinations were constructed that

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                                12345678
WT LoxP     ATAACTTCGTATAGCATACATTATACGAAGTTAT (Nhe1)
511 LoxP    ATAACTTCGTATAGTATACATTATACGAAGTTAT (Pst1)
511-I LoxP  ATAACTTCGTATAATGTATACTATACGAAGTTAT (BamH1)
FAS LoxP    ACAACTTCGTATATACCTTTCTATACGAAGTTGT (Mlu1)
2272 LoxP   ATAACTTCGTATAGGATACCTTATACGAAGTTAT (Bgl2)
5171 LoxP   ATAACTTCGTATAGTACACATTATACGAAGTTAT (Sac2)
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Fig. 1. *LoxP* sequences. The sequence of each *loxP* tested is shown and labeled on the left. 511-I *loxP* contains an inverted core region sequence compared to the 511 *loxP* orientation. The 13-bp inverted repeats are underlined with the 8-bp asymmetric core region numbered with potential ATG start sites located on the bottom, non-coding strand (except for 511-I). The restriction site used to identify each *loxP* sequence is in parentheses to the right of the respective sequence. The FAS *loxP* sequence contains one pair of recombinatorial neutral mutations in the 13-bp inverted repeats [24].

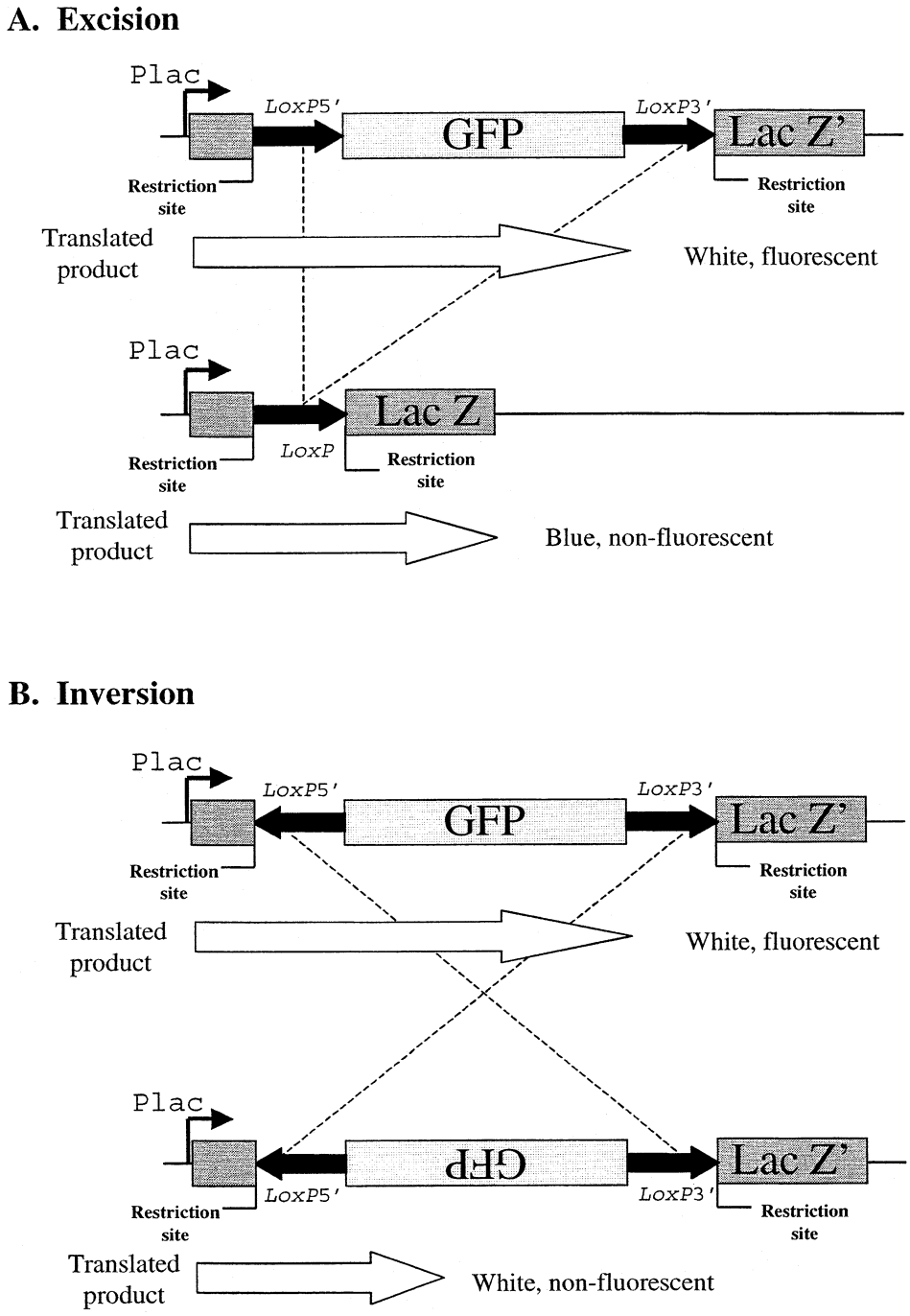

Fig. 2. Cre recombination assay. GFP (light gray box) flanked by the 5′ and 3′ *loxP* sites (solid arrows showing the directionality of the 8-bp core sequence) was cloned into the pUC1119 polylinker disrupting the translation of the *lac*Z′ fragment (dark gray box). The *lac* promoter is designated with an angled arrow and the expected translation product is shown as an open arrow beneath. The 2° colony phenotype is shown to the right of the translated product. A: Excision recombination product. Cre recombination between compatible *loxP* sites excises the GFP gene (dashed lines) and allows the *lac*Z′ fragment to be translated from the *lac* promoter. B: Inversion recombination product. Cre recombination between inverted *loxP* sites inverts the GFP gene (dashed lines), but still does not allow translation of the *lac*Z′ fragment as a stop codon in frame with the *lac* promoter is located 131 nucleotides into the reverse complement of GFP.

paired the five *loxP* sequences with one another, as well as an inverted 511 *loxP* sequence (511-I). All constructs were verified by restriction analysis using the enzymes indicated in Fig. 1 (data not shown). Filamentous phagemids containing each construct were then individually transduced into BS1365. Phagemid particles containing *loxP*/GFP DNA were then produced from the Cre cells after recombination and used to transduce DH5αF′ in order to observe the recombinatorial outcome. *LoxP*/GFP constructs that have not had the opportunity to recombine are denoted 1° and constructs that have been passaged through BS1365 are denoted 2°.

As a test of our in vivo Cre recombination system, we transduced either DH5αF′ or BS1365 with WT *loxP*/GFP 1° phagemid. Transduced cells were allowed to 'recombine' overnight in the presence (BS1365) or absence (DH5αF′) of Cre recombinase before 2° phagemids were produced. The

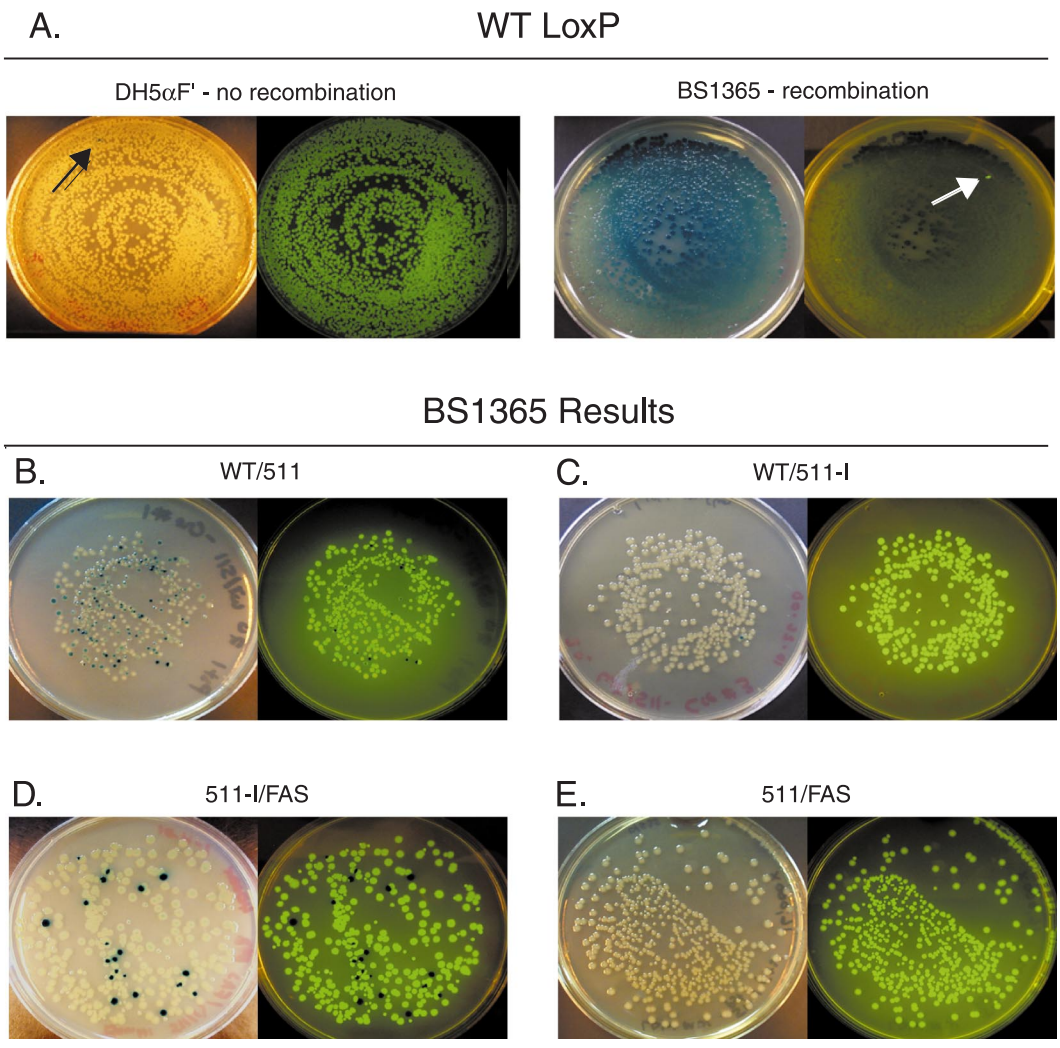

Fig. 3. Representative recombination results. A: 2° colonies containing WT *loxP*/GFP that were originally transduced into either DH5αF′ (left) that do not or BS1365 (right) that do express Cre recombinase for the 1° infection. Arrows mark the one blue and one fluorescent colonies that resulted from DH5αF′ or BS1365 incubations, respectively. 2° colonies obtained with (B) WT/511 *loxP*/GFP, (C) WT/511-I *loxP*/GFP, (D) 511-I/FAS *loxP*/GFP or (E) 511/FAS *loxP*/GFP that were allowed to recombine in BS1365. The left side of each panel is the 2° plate in normal light and the right side is the same plate after excitation at 488 nm.

2° phagemids from both strains were then separately transduced into DH5αF′ (Fig. 3A). The pair of plates on the left show that an insignificant amount of recombination occurs in the absence of the Cre recombinase while the pair of plates to the right demonstrate virtually complete recombination with the same construct in the presence of Cre. The ability to observe the one blue (DH5αF′, Fig. 3A, arrow far left plate) and one fluorescent colonies (BS1365, Fig. 3A, arrow far right plate) demonstrates the high level of sensitivity of our in vivo system.

Having demonstrated that our system is capable of detecting recombination events and that recombination is mediated solely by Cre recombinase, we examined the recombination rates of all the *loxP*/GFP constructs. Representative 2° colonies are shown in Fig. 3B–E and Table 1 shows the level of recombination observed for all of the constructs tested. As expected, constructs containing the same *loxP* sequences 5′ and 3′ of GFP efficiently underwent Cre-mediated recombination (>99%). Evaluating the degree of recombination between WT *loxP* with the other *loxP* sequences reveals that some combinations are compatible with one another to varying extents (Table 1, WT column). For example, WT *loxP* and 511 *loxP* recombined at a level of approximately 10% (Fig. 3B), while WT *loxP* and the inverted orientation of 511, 511-I *loxP*, recombined at the much reduced level of 1.4% (Fig. 3C). Additionally, WT *loxP* paired with 5171 *loxP* gave a recombination rate of nearly 6%. A negligible amount of recombination was observed between WT *loxP* and either FAS *loxP* or 2272 *loxP*. The remaining *loxP* constructs also identified several additional pairs of sequences that could recombine with one another. The only heterologous sequence (other than WT *loxP*) which 511-I was unable to recombine with was 2272 *loxP* (Table 1, 511-I column). Once again, the relative orientation of 511 *loxP* appeared to influence whether recombination could occur in some cases. Thus, FAS *loxP* could recombine with 511-I *loxP* at approximately 6% (Fig. 3D), but was unable to recombine when 511 *loxP* was used instead (Fig. 3E). However, 2272 *loxP* recombined with both 511-I and 511 *loxP* at minimal levels (<2%), while recombination was above 50% for both when paired with 5171 *loxP*.

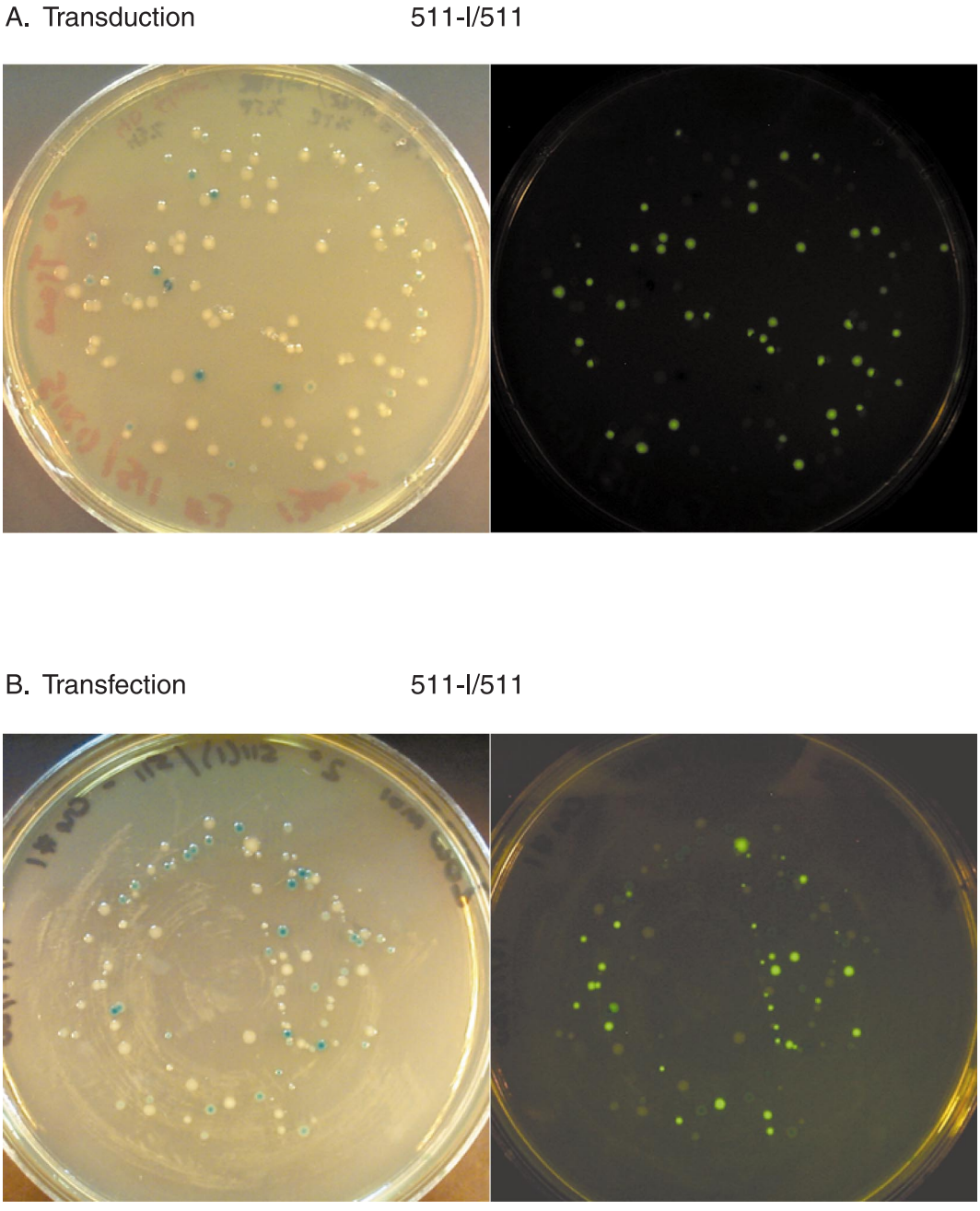

Fig. 4. 511-I/511 *loxP*/GFP recombination. 2° colonies obtained after (A) phagemid transduction or (B) dsDNA transfection into BS1365. The left side of each panel is the 2° plate in normal light and the right side is the same plate after excitation at 488 nm.

Table 1
LoxP recombination summary

3′ lox site	5′ lox site					
	WT	511-I	511	FAS	2272	5171
WT	99.6 (±0.7)					
511-I	1.4 (±1.6)	99.2 (±1.9)				
511	10.3 (±1.4)	*75.3 (±9.3)	99.8 (±0.3)			
FAS	0.2 (±0.3)	5.7 (±3.1)	0.0 (±0.0)	99.4 (±0.3)		
2272	0.5 (±0.4)	0.3 (±0.4)	1.6 (±0.3)	1.7 (±0.8)	99.7 (±0.5)	
5171	5.7 (±2.7)	77.7 (±5.1)	99.9 (±0.1)	4.8 (±0.8)	0.1 (±0.1)	99.5 (±0.9)

The 5′ *loxP* sequence is indicated on the top row and the 3′ *loxP* sequence on the left column. The mean of at least three independent trials is shown with the standard deviation in parentheses. The asterisk denotes recombination rates from both excision and inversion events.

Of the remaining heterologous *loxP* constructs, only FAS *loxP* paired with 5171 *loxP* gave a detectable recombination rate of roughly 5% (Table 1, FAS column).

As a final test, we also determined the level of recombination between 511-I *loxP* and 511 *loxP*. The *loxP* sequences in this construct are identical to one another but are inversely orientated, which should give rise to an efficient inversion reaction during Cre-mediated recombination (Fig. 2B) [2]. As indicated in Table 1, overall recombination occurred at a relatively high level of 75%. However, excised recombination products were observed in addition to the expected inverted products (Fig. 4A, blue and non-fluorescent, white colonies, respectively). Excision occurred at a rate of $43 \pm 8\%$ and inversion at a rate of $32 \pm 6\%$. Restriction analysis was done to verify that the 2° DNA was derived from the 511-I/511 *loxP*/GFP 1° construct and that each type of 2° colony contained the expected recombination product as indicated by its phenotype on the plate (data not shown).

The probability that these results were, in part, due to the use of phagemid (and its ssDNA component) was examined by using transfection rather than transduction to deliver the *loxP*/GFP constructs into bacterial cells. The 511-I/511 *loxP*/GFP 1° dsDNA was transfected into BS1365 cells and the purified 2° dsDNA was used to transfect DH5αF′ cells. Similar results to those with transductions were obtained (Fig. 4B); that is, overall recombination was efficient ($59 \pm 2\%$) with both excision ($24 \pm 2\%$) and inversion ($34 \pm 3\%$) products present. We also determined that the growth rates of cells containing the excised, inverted, or normal GFP constructs were identical (data not shown).

4. Discussion

We have developed an in vivo system to determine the degree of recombination between the five published heterologous *loxP* sequences. When the same *loxP* sequence was present at both sites, recombination was greater than 99% for each homologous pair (Table 1) and was dependent upon Cre recombinase (Fig. 3A). Our results show that not only was recombination between many of the different *loxP* sequences possible (Table 1), but that the relative orientation of the sequences also seemed to determine if recombination could occur (Table 1, 511-I vs. 511 columns and Fig. 3B–E). In the case of the 511-I/511 *loxP*/GFP construct, excision in addition to the expected inversion reaction product was observed (Fig. 4A). This excision, however, was not due to ssDNA present during the transductions used to introduce the constructs into the Cre expressing cells since transfection gave similar results (Fig. 4B). A phagemid system was chosen for this study as it closely mimics current methods used to create very large phagemid antibody libraries [19,20] and identification of non-compatible *loxP* sequences is a prerequisite to maintaining diversity within these libraries.

Our results agree with other published studies that indicate that WT/FAS, WT/2272 and 2272/5171 pairings are non-compatible [15,16]. In addition, we also identify other sets of non-compatible *loxP* sites that could be used together (Table 1). Among the heterologous *loxP* pairings that had not been previously characterized, the most striking is the degree of recombination between either orientation of 511 *loxP* and 5171 *loxP* (78% and 100% for 511-I and 511, respectively). However, it is unclear as to why no inversion reactions were detected between 511-I and 5171 *loxP* sequences (data not shown). It is intriguing to speculate whether the sequence conservation at positions 2, 5, and 7 of the 8-bp core region in each of these different *loxP* sequences somehow facilitates this excision reaction (Fig. 1). We also observed recombination between WT *loxP* and 5171 *loxP* (6%), which had previously been reported to be non-compatible with one another [15].

Our results also differ in some respects to previous studies that have examined recombination between the WT and 511 *loxP* sequences. For example, Hoess et al. did not observe recombination between WT *loxP* and 511 *loxP* [14]. In contrast, two other studies both reported a low degree of recombination between these two heterologous sequences [7,15]. The in vivo results presented here support those previous findings that WT *loxP* can recombine with 511 *loxP* to a limited, but measurable degree. The cross-reactivity between WT *loxP* and 511 *loxP* that we observed was not due to the use of phagemid since the same level of heterologous recombination was obtained after transfections (data not shown). Other reports have used the WT *loxP*/511 *loxP* heterologous pairing to facilitate a double-crossover recombination reaction. These studies used either the inverted orientation of 511 *loxP* [17], which we show allows a very low level of recombination with WT *loxP*, or were simply screening for the emergence of a new cell line after gene replacement using transient Cre recombinase expression [18], in which unexpected recombination would not be seen. The combination of WT *loxP* and 511 *loxP* has also been used to generate large phagemid antibody libraries in a recombinatorial fashion: one used the 511-I *loxP* orientation [20], while the other used 511 *loxP* and has displayed some background level of deletions [19], although it is unclear whether these deletions are due to heterologous recombination between the *loxP* sites or spontaneous eviction from the ssDNA phage genome.

Many possibilities could account for the apparent disparities between the results presented in this work with those obtained in other studies. The format of our experiments has allowed us to examine thousands of different recombination events for each pair of *loxP* sites. In this respect, our results differ from those previously reported which have tended to look only for specific events in small trial numbers. Additionally and perhaps more importantly, by carrying out prolonged incubations of the *loxP*/GFP constructs in cells containing high concentrations of the Cre recombinase, our experiments represent a very stringent examination of lox site compatibility which probably amplifies low levels of recombination occurring between disparate sequences in the absence of strict sequence homology. This possibility is supported by a recent report demonstrating that persistent high-level Cre expression leads to gross chromosomal rearrangements in the absence of exogenous *loxP* sites [23]. Therefore, it is possible that the heterologous pairs of *loxP* sequences, that we demonstrate are capable of very low levels of recombination, might not have been observed in previous studies. Our assay, therefore, by being more stringent, has identified those *loxP* sequences that should be non-compatible with one another in a variety of conditions for future applications. If, however, *loxP* sequences which we have identified as capable of recombining with one another at low levels need to be used, we suggest limiting the time and/or amount of Cre recombinase expression.

Given the growing importance of the Cre/*loxP* system for the elucidation of gene function, more elaborate schemes to activate or deactivate genes, as well as allowing selectable markers to be recycled for subsequent re-use require the availability of sets of non-compatible *loxP* sites. Integrating multiple non-compatible *loxP* sites into a genome at defined locations allows the subsequent Cre-mediated introduction of a transgene construct to different chromosomal locations by simply specifying the corresponding *loxP* sites on the targeting vector. The expanded utility of these techniques and the creation of multiple targeting vectors can have a tremendous impact on the growing field of functional genomics. Our study identifies several pairs of non-compatible heterologous *loxP* sequences that can be used to create different targeting vectors containing two or more *loxP* sites for manipulation.

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