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Analysis of spacer regions derived from intramolecular recombination between heterologous loxP sites

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Abstract

In Cre–loxP recombination system, Cre recombinase binds cooperatively to two 13 bp inverted repeats in a 34 bp loxP and catalyzes strand exchange in the 8 bp spacer region. Up to date, spacer sequences within the recombined loxP sites derived from two loxP sties that have different 8 bp spacer regions have never been analyzed. In the present study, we analyzed the spacer sequences within the recombined products, resulted from intramolecular recombination between heterologous loxP sites including M2, M3, M7, M11, and 2272 *in vivo* and *in vitro*. From the analyses, it was found that loxP sites with aberrant 8 bp spacers can be generated from Cre-mediated recombination between heterologous loxP sites at significantly high frequency, proposing the possibility that recombination between heterologous loxP sites would have not undergone typical formula of Cre–loxP recombination.

Keywords: Cre–loxP recombination; Heterologous loxP site; Spacer region; Aberrant spacer

Cre recombinase, a member of the integrase family, is a 38 kDa protein from bacteriophage P1 and catalyzes precise site-specific recombination between two loxP sites [1]. The loxP is a 34 bp DNA sequence composed of a central asymmetric 8 bp spacer region flanked with two identical 13 bp inverted symmetric region [2]. Cre monomers bind cooperatively to two 13 bp inverted repeats in a loxP and catalyze strand exchange in the 8 bp spacer region. An asymmetric spacer region determines the orientation of a loxP site and directionality of recombination events, either excision or inversion. Recombination between two loxP sites placed in the same orientation on the same DNA molecule results in the excision of the DNA segment intervening two loxP sites, whereas the inversion of DNA segment in recombination between the opposite-oriented two loxP sites [3,4]. With respect to the cleavage in loxP, cleaved sites are apart from 6 bp (core or exchange region) within a 8 bp spacer region. That is, DNA is cleaved at the sites between the first and second bases within a spacer of each strand, and then 6 bp core (position 3′–3) of each strand of spacer are reciprocally exchanged each other. The resulting recombinant products become to have the bases different from the parental spacers at position 4′ and 4. Sequence homology within 6 bp cores is required for efficient strand exchange but it does not seem to be strict requisition because the productive recombination events were actually observed between loxP sites with heterologous spacers in a variety of degree [3,5–7].

The mutant loxP sites with the altered spacer have been extensively studied to elucidate the role of nucleotide sequence of spacer region or to identify the one that undergoes recombination with the same mutant loxP but not other mutant loxP or wild type loxP site (wt loxP) [5,6,8]. These studies have examined intramolecular recombination efficiency between mutant loxPs bearing altered spacers as well as between wt loxP and mutant loxP, without any analyses for spacer sequence left in individual products after recombination.

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Here, to examine whether intramolecular recombination between heterologous lox sites would result in the expected recombination products as in homologous recombination, we analyzed the sequences of spacers within the recombinants resulted from recombination between heterologous mutant loxP sites including M2, M3, M7, M11, and 2272 in a Cre-expressing bacteria and *in vitro*. We found that loxP sites with aberrant 8 bp spacers in recombination products are generated at significantly high frequency, regardless of a degree of base-mismatch between two different loxP sites. Our results may give new insights into recombination events between loxP sites that do not share homology in spacer region.

Materials and methods

Construction of plasmids. To examine intramolecular recombination, the construction of pFB2 was started by introducing the *lacZ′* gene into the 3′ end of loxP wt of pRGB [9]. The *lacZ′* gene was amplified from pGEM-3Zf (+) using GalF-1 primer (5′-GAC TAG TAA TTC ACT GGC CGT CGT TTT AC) and GalF-2 primer (5′-GCA CGC GTT TAT CTC CAT TCG CCA TTC-3′). PCR product of 200 bp was digested by SpeI and MluI and ligated with pRGB vector treated with same restriction enzymes. Then homologous and heterologous pairs of wild type loxP (wt loxP), M2, M3, M7, and 2272 were inserted to position of loxP site 1 and loxP site 2 in pFB2 plasmid in the same orientation. In cloning loxP sequences into loxP site 1, the PCR products of 400 bp, which were amplified using five 5′ primers composed of NheI recognition site and respective loxP sequences (5′-CTA *GCT AGC* ATA ACT TCG TAT A—spacer region (8 bp)—TAT ACG AAG TTA T-3′) and a 3′ primer with NotI recognition site (5′-GAG TCA TTC TCG ACT *TGC GGC CGC* TTT TAT TTC CA GCT TGG-3′), were digested with NheI and NotI, and then ligated into same restriction enzyme-treated pFB2. For the successive cloning of loxP sequences into loxP site 2 on pFB2, a 5′ primer (5′-CTG GTA AAC *CAT ATG* AAT TTT C-3′) and five 3′ primer with respective loxP sequences (5′-G*AC TAG TAT* AAC TTC GTA TA—spacer region (8 bp)—TAT ACG AAG TTA T-3′) were used for amplification of 120 bp DNA fragments containing NdeI and SpeI recognition sites. The PCR product treated with NdeI and SpeI, was inserted into pFB2, resulting in the final pFB2 vectors containing two loxP sites in the same orientation.

Cre-mediated recombination. In vivo recombination was carried out in Cre-expressing BS1365 bacterial cell which was kindly provided by Dr. B. Sauer (Stowers Institute for Medical Research, USA). BS1365 bacterial cells were transformed with pFB2 for recombination, plated on ampicillin (100 μg/ml)/kanamycin (50 μg/ml)-containing LB agar and then incubated at 37 °C overnight to allow plasmids to recombine two loxP sites during the cell growth. For *in vitro* recombination, the purified Cre (New England Biolabs) was used. Recombination reaction, performed with 300 ng pFB2 plasmid, 5 U Cre, 33 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.5 in 50 μl reaction mixture for 1 h at 37 °C, was terminated by the incubation for 10 min at 70 °C.

Analysis of recombinants. BS1365 colonies with recombined plasmids were grown in broth culture at 37 °C overnight for plasmids preparation. An aliquot of plasmids isolated from BS1365 cells were used to transform *Escherichia coli* JM109 for the detection of clones with 3.4 kbp recombinant. Transformed JM109 cells were incubated on LB agar containing ampicillin (100 μg/ml), IPTG (Isopropyl β-D-1-thiogalactopyranoside, 1 mM), and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 20 μg/ml) at 37 °C overnight. From blue-colored colonies, plasmids were isolated and linearized with NcoI before loaded to agarose gel electrophoresis. An aliquot of plasmids were subjected to DNA sequence analysis. *In vitro* recombination analyses, 2 μl of recombination reaction mixtures were used as a template for PCR to amplify DNA covering recombined loxP sites (30 cycles of 40 s/94 °C, 40 s/52 °C, and 30 s/72 °C). The used primers are as follows: S1 (5′-CAA CGT GAA AAA ATT ATT ATT CGC-3′), GalF-4 (TCT CCA TTC GCC ATT CAG GCT G), Seq (5′-CGG TGA CGG TGA TAA TTC ACC-3′), and R3D8VL3′ (ACT GTT GAA CAG ACT CTG-3′). The PCR products were cloned into pGEMT-easy vector (Promega), followed by blue colony selection and sequence analysis of plasmids from blue colonies.

Results

Design of plasmid for the recombination assay between two loxP sites

When two loxP sites are placed in the same orientation on the same DNA molecule, intramolecular recombination results in the excision of the DNA segment intervening two loxP sites [3,4]. A series of plasmids for the detection of recombination event was constructed by employing wt loxP and five mutant loxP sites including M2, M3, M7, M11, and 2272 that contain the altered bases within their spacer region (Fig. 1 and 2A) [6,8]. These mutant loxPs have been reported to recombine each other with a variety of efficiencies, although they do not share complete homology over their spacer region [6]. Two mutant loxPs were cloned into pFB2 vector in all of possible combinations, resulting in thirty-six pFB2-loxP1/loxP2 constructs (Fig. 2A). pFB2-loxP1/loxP2 of 5.3 kbp was designed so that upon recombination, *lac* promoter and *lacZ′* structural gene are adjoined by the excision of 1.9 kb DNA fragment intervening two loxPs, leading to the expression of β-galactosidase from 3.4 kb recombinant plasmid under the IPTG induction (Fig. 2A).

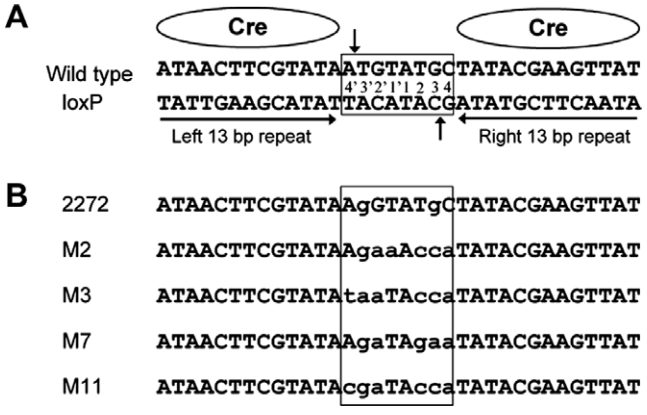

Fig. 1. Structure of a wild type loxP site and mutant loxP sites. Sequence of wild type loxP expressed in double stranded DNA (A) and mutant loxP sites containing the altered bases within spacer region expressed in single-stranded DNA (B). Two 13 bp-inverted repeat regions recognized by Cre are indicated by horizontal arrows. The 8 bp spacer regions are boxed and lower case letters indicate nucleotides that differ from the wild type loxP spacer sequence. Nucleotide positions are numbered starting from the center of the spacer with and without prime (′) on the left and right, respectively. Vertical arrows indicate the cleavage sites and to the scissile phosphodiester bonds are 6 bp apart.

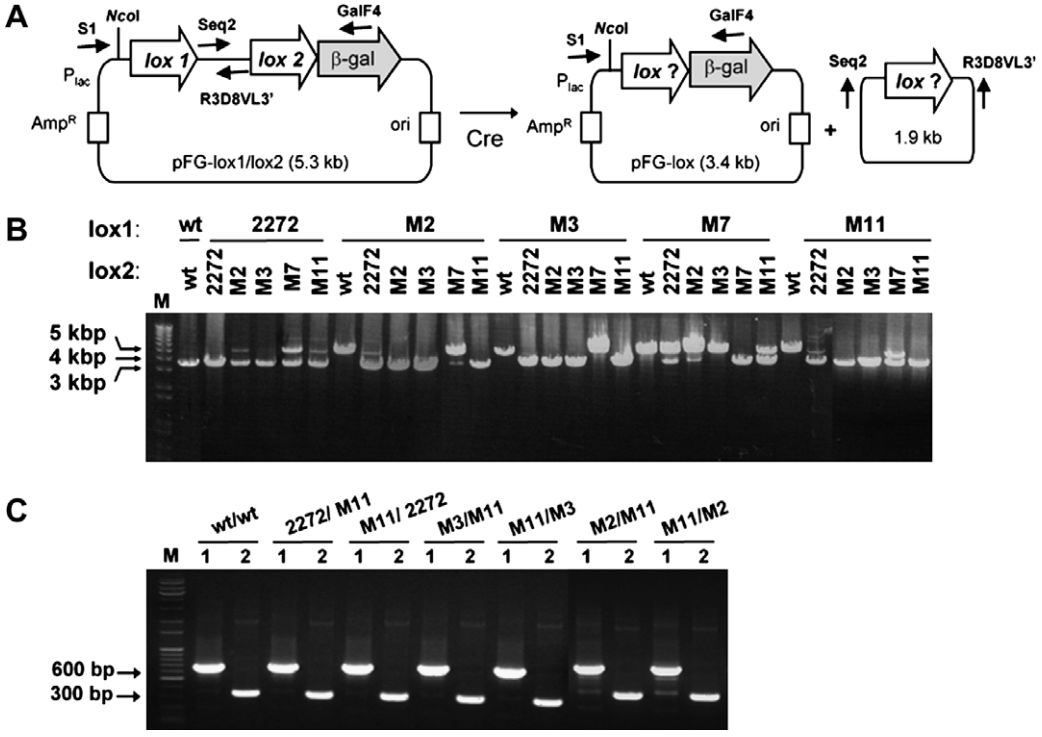

Fig. 2. Cre-mediated recombination between two loxP sites. (A) Schematic diagram representing plasmid structure and its recombined products. Two loxP sites are in a directly repeated orientation so that recombination between the loxP sites would excise the intervening DNA fragment from the plasmids. (B) Restriction analysis of plasmids isolated from *E. coli* BS1365. Lane M contains DNA size marker and the other lanes contains linearized plasmids following recombination of pFB2-loxP1/loxP2s with a distinct pair of loxP sites. (C) Analysis of PCR products amplified from *in vitro* recombination of five plasmids. '1' and '2' indicate 620 bp amplified from 3.4 kbp DNA and 330 bp amplified from 1.9 kbp DNA, respectively.

Isolation of the DNA containing the recombined loxP sites

After induction of recombination in BS1365 bacterial cells, pFB2-loxP1/loxP2 plasmids were isolated from individual broth cultures of BS1365 colonies and digested with restriction enzyme to ensure the 3.4 kbp recombinants (Fig. 2B). The NcoI-digestion shows that recombination efficiency between homologous two mutant loxPs was equivalent to the pair of wt loxPs, based on the appearance of only 3.4 kbp excision products generated from 5.3 kbp substrates. In the cases of recombination between heterologous loxP sites, the recombined plasmids of 3.4 kbp were revealed in a variety of degree, depending on a combination of two loxP sites, ranged from 0% to 100% (Fig. 2B). The counterparts, circular recombinant products of 1.9 kbp, were not able to be seen on agarose gel because they would be incapable to replicate in bacterial cells due to lacking an origin and diluted out during population growth in BS1365. Therefore, excision products of 3.4 kbp containing replication origin would occupy the majority in the plasmid population.

When aliquots of plasmids purified from BS1365 cells were transformed into *E. coli* JM109 cells which do not express Cre and serve alpha-complementation to isolate individual recombinant clones, the frequency of blue colonies to total colonies was corresponding to the ratio of 3.4 kbp DNA- to 5.3 kbp DNA-intensity on a agarose gel. As expected, plasmids isolated from culture of blue JM109 colonies showed all 3.4 kbp of DNA size, whereas 5.3 kbp of non-recombined parental DNA size in plasmids isolated from white colonies (data not shown). Recombination efficiencies between M2, M3, and M11 were comparable to those observed in a previous report [6]. From our results, recombination efficiencies estimated by intensity of 3.4 kbp DNA compared to 5.3 kbp DNA and frequency of blue colonies, were not significantly affected by relative position of two loxPs in pFB2-loxP1/loxP2 plasmids (Fig. 2B and Table 1).

Through *in vitro* recombination, we tried to isolate the 1.9 kbp counterpart to replicable 3.4 kbp recombinant. However, 3.4 and 1.9 kbp circular DNAs could not be isolated from the agarose gel due to very low recombination efficiency less than 5% in even maximum concentration of Cre, except some cases of recombination, for examples 35% in wt x wt, 33% in 2272×2272, 28% in $M2 \times M2$, and 33% in $M7 \times M7$ (data not shown). Then, we performed PCR (Fig. 2C), in which representative seven plasmids (pFB2-wt/wt, -2272/M11, -M11/2272, -M3/M11, -M11/M3, -M11/M2, and -M11/M2) reacted with Cre were chosen for template DNA. As expected, 620 bp DNA by S1—GalF4 primer set and 330 bp DNA by Seq2—R3D8VL3 primer set were amplified (Fig. 2A and C) and

Table 1
Recombination efficiency between two loxP sites in BS1365 bacterial cells

loxP 1	loxP 2					
	wt	2272	M2	M3	M7	M11
wt	100.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
2272	0.0 ± 0.0	100 ± 0.0	73.8 ± 2.0	100.0 ± 0.0	33.0 ± 0.4	78.1 ± 6.7
M2	0.0 ± 0.0	83.7 ± 0.9	87.6 ± 1.1	100.0 ± 0.0	10.9 ± 2.5	100.0 ± 0.0
M3	0.0 ± 0.0	98.5 ± 0.3	100.0 ± 0.0	46.6 ± 1.0	0.0 ± 0.0	100.0 ± 0.0
M7	0.0 ± 0.0	47.6 ± 1.3	6.6 ± 0.3	0.0 ± 0.0	100.0 ± 0.0	62.3 ± 1.7
M11	0.0 ± 0.00	76.2 ± 4.2	100.0 ± 0.0	100.0 ± 0.0	57.2 ± 0.5	100.0 ± 0.0

cloned into pGEMT-easy vector for sequence analysis of loxP regions.

Spacer sequences in the products derived from in vivo recombination

When sequences of spacer regions in the *in vivo* recombined plasmids of 3.4 kbp were analyzed, we found aberrant recombined spacer regions that are difficult to consider as the products passed through a typical Cre–loxP recombination pathway (Table 2). In the mechanism of Cre–wt loxP recombination accepted without doubt, the strand cleavages and exchanges at positions flanking 6 bp core duplex generate the two separate products containing different bases from the original spacers at position 4′ and 4 (Fig. 3). Enough interestingly, unexpected spacers which still keep the unchanged bases at position 4′ or 4, were found in recombined plasmids at a significantly high frequency (Table 2). A representative is recombination between 2272 at loxP1 position and M11 at loxP2 position (2272 × M11) (Table 2 and Fig. 3B). According to typical recombination pathway, 2272 × M11 is supposed to exclusively produce the recombined spacers of AGGTATCA and AGATACCA in an equal frequency (Fig. 3B and Supplementary Table 1). However, parental aberrant products containing the sequences that are considered to be a part of two parental spacers participated in recombination, CGATACCA and AGGTATCC were often detected as 70% (7 of 10 clones) and 10% (1 of 10 colons), respectively. The expected spacer of AGGTATCA was detected to be only 20% (2 of 10 colons) (Table 2). Similar observations for these unexpected spacer sequences were made in recombination with plasmids containing different pair of spacers. Examples of parental aberrant spacers are as marked by superscript 'a' in Table 2. Moreover interestingly, the non-parental aberrant spacers that have a third base but not either a parental or correctly recombined spacer sequence were also observed in recombined products. The examples are marked by bold and superscript 'b' in Table 2.

Table 2
Analyses of spacer sequences on *in vivo* recombined plasmids

loxP 1	loxP 2				
	2272	M2	M3	M7	M11
2272					AGGTATCA (2)
	AGGTATCC (10)	AGGTATCA (1)	AGGTATCA (1)	AGATAGAA (10)	**AGGTATCC**[a] **(1)**
		AGAAACCA (9)	**TAATACCA**[a] **(9)**		**CGATACCA**[a] **(7)**
M2	AGGTATCC (3)				
	AGAAACCA (3)		AAATACCA (1)	AGATAGAA (10)	AGATACCA (1)
	AGAAACCC (3)	AGAAACCA (10)	AGAAACCA (9)		AGAAACCA (9)
	AGGTTTCC[b] **(1)**				
M3	TGGTATCC (1)	TAATACCA (4)			
	TAATACCC (6)	TGAAACCA (5)	TAATACCA (10)	No recombination[c]	TAATACCA (4)
	AGGTATCC[a] **(3)**	**TGATACCA**[b] **(1)**			TGATACCA (6)
M7	AGGTATCC (6)				
	AGATAGAA[a] **(2)**	AGAAACCA (10)	No recombination[c]	AGATAGAA (10)	AGATACCA (2)
	AGATACCT[b] **(1)**				**CGATACCA**[a] **(8)**
	AGATACCC[b] **(1)**				
M11	CGATACCC (1)				
	CGATACCA[a] **(1)**	CGATACCA (7)	**CGATACCA**[a] **(10)**	CGATAGAA (8)	CGATACCA (10)
	AGGTATCC[a] **(8)**	CGAAACCA (3)		CGATACCA (2)	

The numbers in bracket indicates the number for the spacer sequences found after *in vivo* intramolecular recombination between two loxP sites.
[a] Unexpected parental spacer.
[b] Unexpected non-parental spacer.
[c] Sequences could not be analyzed due to no recombination between two loxP sites.

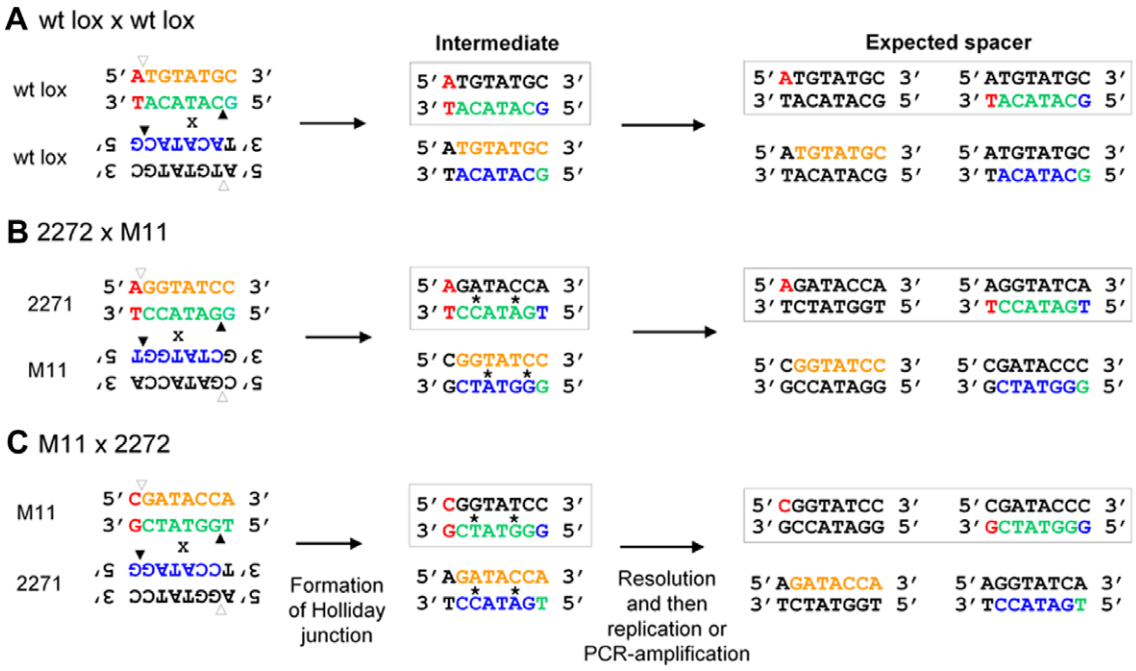

Fig. 3. Cre-mediated recombination pathway and recombination products. (A) Well-known favored anti-parallel synapsis between direct repeated two wt loxP sites. (B) Recombination through synapses between mutant loxP 2272 and M11 and (C) between M11 and 2272. Positions for strand exchanges between top (open arrowheads) and bottom strands (filled arrowheads) are apart from 6 bp. The mismatched base pairs in reaction intermediates are marked with an asterisk (*). Theoretical spacer sequences expected on 3.4 kbp plasmids are boxed and the spacers expected on 1.9 kbp plasmids are expressed without box.

Spacer sequences in the products derived from in vitro recombination

As it was difficult to obtain directly the recombined products of 3.4 and 1.9 kbp from *in vitro* recombination, we amplified the DNA regions covering recombined loxP sites by PCR in which seven representative recombination reaction mixtures are used as templates (Fig. 2C). *In vitro* recombination between homologous wt loxPs (wt × wt) as well as heterologous mutant loxPs (M2 × M11 and M11 × M2) that have resulted in all of the expected spacer sequences on 3.4 kbp DNA derived from *in vivo* recombination (Table 2 and Supplementary Table 1), showed also the all expected recombined spacers in the DNAs amplified from 1.9 kbp as well as 3.4 kbp recombinants (Table 3 and Supplementary Table 1). In contrast, loxPs in 3.4 kbp recombinants from 2272 × M11 and M11 × 2272 were found to be most unexpected aberrant spacers (Table 3, Fig. 3B and C), as in the case of *in vivo* recombination (Table 2).

Although aberrant spacers on 3.4 kbp recombinants derived from *in vivo* and *in vitro* recombination were not revealed as same sequences and same frequency, it was demonstrated that a pair of loxPs producing aberrant spacers *in vivo* recombination also tends to generate them *in vitro* (for example, 2272 × M11 and M2 × M11 in

Table 3
Analyses of spacer sequences on *in vitro* recombined plasmids

DNA	Recombination						
	wt × wt	2272 × M11	M11 × 2272	M3 × M11	M11 × M3	M2 × M11	M11 × M2
3.4 kbp	ATGTATGC (10)	**AGGTATCC[a] (3)**	**AGGTATCC[a] (2)**	TAATACCA (10)	**CGATACCA[a] (10)**		
		CGATACCA[a] (1)	**CGATACCA[a] (2)**			AGAAACCA (2)	CGATACCA (6)
		AGGTATCG[b] (2)	**TGGTATCC (2)**			AGATACCA (8)	CGAAACCA (4)
		CGATACCC[b] (2)	**CGATACCT[b] (4)**				
1.9 kbp	ATGTATGC (10)		**AGATACCC[a] (1)**	CGATACCA (10)	TAATACCA (10)	CGAAACCA (10)	
		AGGTATCC[a] (1)	**AGGTATCC[a] (1)**				
		CGATACCA[a] (3)	**CGATACCA[a] (1)**				AGAAACCA (9)
		TGGTATCC[b] (3)	**CGATATCA[a] (1)**				AGATACCA (1)
		CGATACCT[b] (3)	**GGATACCA[b] (1)**				
			AGGTATCG[b] (5)				

The numbers in bracket indicates the number of the spacer sequences observed after *in vitro* intramolecular recombination between two loxP sites.
[a] Unexpected parental spacer.
[b] Unexpected non-parental spacer.

Tables 2 and 3). Frequent appearance of aberrant spacers does not seem to be caused from an error by Taq polymerase, because all of the spacers amplified from recombinants derived from wt × wt demonstrated only expected spacer sequence. An evidence of reciprocal strand exchanges of Cre–loxP system could be provided as CGAAACCA on 3.4 kbp and AGATACCA on 1.9 kbp in M11 × M2.

Discussion

We provide the evidence that aberrant spacer sequences from recombination between heterologous loxP sites are generated in a significantly high frequency. These aberrant recombinants fall into two classes: the first was the parental spacers containing the sequences that are considered to be a part of two parental spacers participated in recombination. The second was non-parental spacers, unlikely to be originated from a part of two parental spacers. No matter what aberrant spacers happen, frequency of aberrant spacers was not accord with the degree of base-mismatch between two spacer regions (Tables 2 and 3). Besides, most of aberrant spacer sequences are indicated by the bases given at position 4′ and 4 within a spacer.

At present, it is difficult to explain how these aberrant spacers were generated through the Cre–loxP recombination. *In vivo* recombination, explanation for the aberrant products which lack the distinct rule of base change may be somewhat applicable to the proposal by Aranda et al. [10]. That is, resolution of Holliday junction intermediates that contain unstable heteroduplex (Fig. 3B and C) due to non-identical sequences in 6 bp exchange regions might collide with replication machinery of plasmid, resulting in generation of aberrant spacer products. However, these aberrant spacers from *in vivo* and *in vitro* recombination are not allowed to consider simply them as a result of errors during replication of a plasmid or errors during PCR, because base changes into aberrant spacers were largely shown at position 4 and 4′. More remarkably, M11 × M3 showed always only one aberrant sequence (CGATACCA) both *in vivo* and *in vitro* (Tables 2 and 3). Even though appearance of aberrant spacers cannot be fully understood at this study, it could be suggested that recombination between some heterologous loxP sites may not follow a typical Cre–loxP recombination pathway.

From data for structure of Cre–loxP synaptic complex by atomic force microscopy, X-ray crystallographic and biochemical analyses, it has been strongly proposed that wild type spacer regions are aligned in an anti-parallel fashion in a synaptic complex, by virtue of spacer bending in the same direction in two wt loxP sites, and so favoring to undergo productive strand exchange without large structural rearrangements of two loxP sites over parallel fashion (Fig. 3A) [4,11,12]. Cre induces an asymmetric DNA bend in spacer region of wt loxP sites and the bending depends on a different conformation of the spacer regions achieved by their nucleotide sequence [11–13]. Sadowski et al. have proposed that in the Cre–wt loxP synaptic complex, the 8 bp spacer region is kinked immediately next to the top strand cleavage site in the DNA duplex, that is, kink between the T3′ and G2′ bases and then cleavage between the A4′ and T3′ base [11]. When well-designed spacer mutants such as loxS (GCATATGC) [12] and lox4 (GTGTATGT) [14] have been employed for biochemical assays to investigate Cre–loxP recombination mechanisms, unlike in the Cre–wt loxP structures, Cre–loxS synaptic complex demonstrated that the kink was at T3–G4, 5 bp apart from the cleavage site. Though synaptic configurations of mutant spacers used in our study have not been described yet, the conformation of the spacer in the Cre-mutant loxP synapses might be asymmetric due to different bending positions each other in spacers. Therefore, it may be suggested that Cre-mutant loxP synapses increase the feasibility for induction of the strand cleavages by Cre at imprecise positions on loxP sites.

Although the mechanism for the generation of aberrant spacers is not be described at this study, our data would be noteworthy in aspect of suggesting the possibility for non-typical recombination between heterologous loxP sites which do not share complete homology of spacer region. Further experimental work is needed to elucidate the mechanism for the generation of aberrant spacers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.08.145.

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