Asymmetry of Shufflon-specific Recombination Sites in Plasmid R64 Inhibits Recombination between Direct sfx Sequences*

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The shufflon of plasmid R64 consists of four DNA segments separated and flanked by seven sfx recombination sites. Rci-mediated recombination between any inverted sfx sequences causes inversion of the DNA segments independently or in groups. The R64 shufflon selects one of seven pilV genes encoding type IV pilus adhesins, in which the N-terminal region is constant, while the C-terminal regions are variable. The R64 sfx sequences are asymmetric. The sfx central region and right arm sequences are conserved, but left arm sequences are not. Here we constructed a symmetric sfx sequence, in which the sfx left arm sequence was changed to the inverted repeat of the right arm sequence and made artificial shufflon segments carrying symmetric sfx sequences in inverted or direct orientations. The symmetric sfx sequence exhibited the highest inversion frequency in a shufflon segment flanked by two inverted sfx sequences. Rci-dependent deletion of a shufflon segment flanked by two direct symmetric sfx sequences was observed, suggesting that asymmetry of R64 sfx sequences inhibits recombination between direct sfx sequences. In addition, intermolecular recombination between symmetric sfx sequences was also observed. The extra C-terminal domain of Rci was shown to be essential for inversion of the R64 shufflon using asymmetric sfx sequences but not essential for recombination using symmetric sfx sequences, suggesting that the Rci C-terminal segment helps the binding of Rci to asymmetric sfx sequences. Rci protein lacking the C-terminal domain bound to both arms of symmetric sfx sequence but only to the right arm of asymmetric sfx sequence.

Conservative site-specific recombination plays a key role in creating biological diversity in prokaryotes (1, 2). Site-specific recombination systems are classified into two distinctive groups, integration-excision and inversion systems, according to the relative location of the two recombination sites. When the recombination sites lie on different DNA molecules, recombination between them results in integration or fusion of two DNA molecules. When the sites lie on the same DNA in direct orientation, recombination between them results in excision or resolution of the DNA segment. Integration and excision of λ phage into and out of the Escherichia coli chromosome may be one of the best examples of this mechanism (3). When the recombination sites lie on the same DNA in the inverted orientation, recombination between them results in inversion of the DNA segment. In many bacterial pathogens, antigenic variation by site-specific recombination is important to escape from host immune systems. In plasmid conjugation and phage infection, DNA inversion by site-specific recombination is used to enlarge recipient and host specificity, respectively.

While most site-specific recombination systems consist of two recombination sites and the cognate recombinase gene, several multiple DNA inversion systems containing more than two recombination sites as well as the recombinase gene have been reported after the discovery of shufflon in IncI1 plasmid R64 (4, 5). The R64 shufflon is located at the 3′ end of the pilV gene and consists of four invertible DNA segments, which are separated and flanked by seven shufflon-specific recombination sites, sfx sites (Fig. 1AB). The rci gene, located adjacent to shufflon region, encodes a site-specific recombinase of the inte-grase family (6). Rci-mediated recombination between any two sfx sites in inverted orientation results in inversion of the shufflon segments independently or in groups. Shufflon recombination selects one of seven different pilV genes encoding adhesins in which the N-terminal region is constant, while the C-terminal region is variable. If recombination occurs between direct sfx sites within a single shufflon region, the DNA segment(s) flanked by them will be deleted. If recombination occurs between sfx sites on different DNA molecules, plasmids with a complicated shufflon will be produced. However, no such recombination has been observed during our experiments. The pilV gene is the last in the pil operon encoding a type IV pilus called the thin pilus of plasmid R64 (7). PilV adhesins, most likely located at the tip of thin pilus, determine the recipient specificity in R64 liquid matings via recognition of lipopolysaccharide of recipient cells (8). The C-terminal segments of PilV adhesins were demonstrated to specifically bind to lipopolysaccharide of recipient cells (9).

In our previous study (10), R64 Rci protein was overexpressed and purified. In vitro shufflon inversion was observed in a reaction mixture that contained purified Rci protein and supercoiled DNA of a plasmid carrying a shufflon segment. When a DNA fragment carrying an sfx sequence was incubated with Rci protein, specific cleavages were introduced into both strands of DNA in the form of 5′-protruding 7-bp staggered cut at the sfx site (downward and upward arrows in Fig. 1B), sug-
deletion was observed, suggesting that asymmetry of R64
by two symmetric
sions in a shufflon segment flanked by two
were made. The symmetric
sequences, both right and left arms of asymmetric
sequences are highly conserved among various R64
sequences, which function as recombinase-binding elements
were protected (11). The sequence of the
left arm strongly
affected the frequency of shufflon inversion. A model was pro-
posed in which one molecule of Rci binds to the
right arm in a sequence-specific manner and the second molecule of Rci
binds to the sfx left arm in a non-sequence-specific manner (11)
(see Fig. 6A). The extra C-terminal domain of Rci protein was postulated to be involved in its binding to the sfx left arm.

In the present study, a symmetric sfx site, in which the sfx left arm sequence was changed to the inverted repeat of the right
arm sequence, was constructed and artificial shufflon segments carrying symmetric sfx site in inverted or direct orientations were made. The symmetric sfx site exhibited the highest inversion frequency in a shufflon segment flanked by two sfx sites in inverted orientation. In addition, for a shufflon segment flanked by two symmetric sfx sites in direct orientation, rci-dependent deletion was observed, suggesting that asymmetry of R64 sfx sites inhibits recombination between direct sfx sites.

**EXPERIMENTAL PROCEDURES**

**Bacteria, Plasmids, and Growth Conditions—**E. coli NF83
*recA56 Δ(lac-proAB) rpsl thi ara 80d lacZΔM15, JM109
*recA1 Δ(lac-proAB) endA1 gyrA96 thi hsdR17 supE44 relA1 F’
*traD36 proAB lacPΔZΔM15, and DH5α supE44 ΔlacU169 80d
*lacZΔM15 hsdR17 recA1 endA1 gyrA96 thi relA1 were used (13, 14).
*pUC18, pUC118, pACYC177, and pTK219 were used as vectors (13–15).
PINV-101, pINV109, and pKK098, in which the
rci gene was cloned into pACYC177, were described previously
(11).

Luria-Bertani (LB) medium was prepared as described previ-
ously (14). Solid media contained 1.5% agar. Antibiotics were added to liquid or solid media at the following concentrations:
ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and kana-
mycin, 50 μg/ml.

**Construction of Plasmids—**All recombinant plasmids were constructed as described previously (14). The Scal site of
*pACYC177 was converted to an EcoRI site to give pACYC177E.
To construct the rci+ plasmid pKK097, a 1313-bp R64
sequence (nucleotide numbers 17,608 to 18,920 of the sequence
under GenBank™ accession number AB027308, the same sequence as pKK098) was inserted into the KpnI-BamHI site of
*pTK219 carrying pSC101 rep. The rci gene in pKK097 and
pKK098 was converted to an rci-lacZ fusion gene. Comparison of
β-galactosidase activity between E. coli cells harboring the
resultant plasmids suggested that Rci expression in pKK098 is
5-fold higher than that in pKK097.

A 2115-bp R64 sequence (nucleotide numbers 15,509–17,623) carrying the entire shufflon region was inserted into the
AccI-SphI site of pUC18 to give pAG105. pINV- and pDEL-
series plasmids carrying artificial shufflon segments were con-
structed as described previously (11). The oligonucleotides cor-
responding to both strands of sfxaa201 were annealed and inserted into one or both ends of an artificial shufflon segment
in the inverted or direct orientations as described in the legend
to Fig. 1C. sfxaa101 and sfxaa201 were inserted into the PstI site
of pUC18 to give pASYM101 and pSYM201, respectively.
*sfxaa201 was inserted into the BamHI-HindIII site of
*pACYC184 to give pSYM210. Isolation of rci mutants will be described elsewhere.

**In Vivo and in Vitro Recombination Assays—**For in vivo
recombination assays, pINV- or pDEL-series plasmids carrying various sfx sequences were introduced into E. coli cells harboring the rci+ plasmid pKK098 or pKK097, and the cells were grown at 37 °C. At intervals, plasmid DNA was extracted by the alkaline extraction method (14). Plasmid DNA was analyzed by electrophoresis on a 0.7% agarose gel before or after EcoRI digestion. The *in vivo* inversion frequency is indicated as inversions per generation (11), while the initial *in vivo* deletion fre-
quency is indicated as deletions per generation.

Rci protein was purified and the *in vitro* recombination assay was performed as described previously (10) with a slight modi-
fication. Reaction mixtures containing 32 mM Tris-HCl (pH 7.6), 15 mM NaCl, 80 mM KCl, 4 mM spermidine, 0.7 mM EDTA,
22% glycerol, 38.5 mM supercoiled pSYM201 DNA, and 68 nM Rci protein were incubated at 37 °C. After termination of the reaction by the addition of SDS, the product DNA was collected by ethanol precipitation and subjected to electrophoresis on a 0.7% agarose gel.

**DNase I Footprint Analyses—**Rci wt and Rci292 proteins were purified and DNase I footprint analyses were per-
formed, as described previously (10, 11). Varying amounts of Rci wt or Rci292 protein and 5’ end-labeled DNA fragments (0.1 pmol) were mixed in a 100-μl buffer (50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 200 mM KCl, 5 mM spermidine, 0.3 mg/ml bovine serum albumin, 11% glycerol, and 5 μg of sonicated salmon testis DNA). Then 0.5 unit of DNase I was added and incubated at 30 °C for 7 min. Following phenol extraction and ethanol precipitation, samples were analyzed by electrophoresis on a sequencing gel. The cleavage pattern was visualized by autoradiography.

**RESULTS**

**Construction and Activity of Symmetric sfx Sequence—**In our
previous study (11), we found that the R64 sfx sequences are
asymmetric and that the sfx left arm sequences greatly affect
inversion frequency. To examine the significance of asymmetry of sfx sequences in the R64 shufflon, we constructed a symmetric sfxaa201 sequence, in which the sfx left arm sequence was changed to the inverted repeat of the right arm sequence (Fig. 1B). The sfxaa201 sequence was inserted into tester plasmids

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2 The abbreviation used is: wt, wild type.
Symmetric sfx Sequence in Plasmid R64 Shufflon

FIGURE 1. A, the structure of the R64 shufflon. At the top, a restriction map of this region is shown. The four shufflon segments A–D and seven sfx sequences 1–7 are indicated by bars and filled triangles, respectively. The horizontal arrows indicate the pilV segments encoding the N-terminal constant and C-terminal variable regions and the rci coding sequence. Rci-mediated recombination between any inverted sfx sites results in inversion of shufflon segments independently or in groups and switches the C-terminal segments encoding the N-terminal constant and C-terminal variable regions and the rci coding sequence. The horizontal arrows indicate the site of DNA crossover in the top and bottom strands, respectively, during shufflon recombination. C, Rci-mediated DNA inversion and deletion in various plasmids carrying asymmetric and symmetric sfx sequences. Structures of pINV-series and pDEL-series plasmids are described. In these figures, sfx central region and right or left arm conserved sequences are indicated by boldface type. The numbers at the right correspond to those in A. sfx sequences are classified into four types, sfxa, sfxb, sfxc, and sfxd, according to the differences in the three nucleotides in the right arm. Downward and upward arrows indicate the site of DNA crossover in the top and bottom strands, respectively, during shufflon recombination. The in vivo inversion or deletion frequencies were calculated from the results of Fig. 2 and similar experiments and are indicated as inversions or deletions per generation, respectively, in the presence of pk098 or pk097.

FIGURE 2. Activity of symmetric sfx sequences. Plasmid DNA was introduced into E. coli cells harboring (A) pk098 or (B) pk097. Plasmid DNA was extracted from cells grown overnight and electrophoresed on a 0.7% agarose gel before (A) or after (B) EcoRI digestion. Lanes: 1, only pk098 (A) or pk097 (B); 2, only pIN101 without pk098 or pk097; 3, pIN101; 4, pIN109; 5, pINV201; 6, pINV202; 7, pDEL301; 8, pDEL302; M, molecular length markers. Arrow, DNA band from rci+ plasmid; open arrowhead, DNA band from original plasmid; filled arrowhead, DNA band from plasmid with inversion; stippled arrowhead, DNA band from plasmid with deletion.

carrying an artificial shufflon segment (Fig. 1C). pIN101 and pIN202 carried one or two sfxaa201 sequences at one or both ends of the artificial shufflon segment in direct orientation, respectively.

pIN101, pIN109, pIN201, and pIN202 plasmids were introduced into E. coli cells harboring the rci+ plasmid pk098 (P15A ori), and the transformed cells were grown overnight. Plasmid DNA was extracted from the cells and analyzed by agarose gel electrophoresis (Fig. 2A). While only monomer bands of pIN101 or pIN109 as well as pk098 were observed (Fig. 2A, lanes 3 and 4), many DNA bands of pIN201 and pIN202 were observed in the presence of pk098 (lanes 5 and 6). From the estimation of the sizes of multiple DNA bands it was shown that they corresponded to multimers of pIN201 and pIN202, suggesting the occurrence of intermolecular recombination using sfxaa201. When plIN20 series DNAs were digested with EcoRI, two sets of DNA bands, from the original and shufflon-inverted forms of plasmids, were observed (data not shown). The densities of the original and inverted bands were similar even 3 h after the introduction of pIN201 and pIN202 into E. coli cells harboring pk098, indicating high recombination activity of sfxaa201. PlIN20 series plasmids were introduced into E. coli cells harboring pk097, in which rci was cloned into a low copy number plasmid pTK219 (pSC101 rep) (Fig. 2B). Rci activity from pk097 was ~10-fold less than that from pk098 for the inversion of pIN101 and pIN109 (Fig. 1C), probably according to the difference in their copy number. Under these conditions, the inversion frequency of pIN201 and pIN202 was 20–30-fold higher than that of pIN109, which exhibited the highest activity among R64 sfx sequences.
Symmetric sfx Sequence in Plasmid R64 Shufflon

Deletion of the Shufflon Segment Flanked by Symmetric sfx Sequences in Direct Orientation—pDEL301 and pDEL302 were introduced into E. coli cells harboring pKK097 or pKK098. Multimers of pDEL301 and pDEL302 were also produced (Fig. 2A, lanes 7 and 8). EcoRI digestion of plasmid DNAs suggested the formation of short DNAs (Fig. 2B, lanes 7 and 8). Restriction enzyme analysis and sequencing of small plasmid DNAs indicated that small plasmids are produced by the recombination between sfxaa201 and sfxa112 of pDEL301, or between sfxaa201 and sfxa112 of pDEL302, resulting in the deletion of the shufflon segment. Small plasmids carrying sfxa112 and sfxaa201 were produced from pDEL301 and pDEL302, respectively. The rate of shufflon deletion in pDEL301 was 7–15-fold higher than that of pDEL301 (Fig. 1C). These results indicated that Rci-dependent deletion of the shufflon segment flanked by symmetric sfx sequences in the direct orientation is possible, which has never been observed in the R64 shufflon. These findings suggest that asymmetry of R64 sfx sequences inhibits recombination between direct sfx sequences.

Intermolecular Recombination between Symmetric sfx Sequences—In the experiments in Fig. 2A, plasmid multimer formation by the intermolecular recombination between symmetric sfx sequences was suggested. To confirm this, a pUC18 derivative plasmid pSYM201 carrying one sfxaa201 sequence was constructed. When the control plasmid pASYM101 carrying one asymmetric sfxa101 sequence was introduced into E. coli cells harboring pKK098, only the original monomer plasmid DNA was detected (Fig. 3A, lane 5). For pSYM201 carrying symmetric sfxaa201, a series of multimer bands were formed in the presence of rci (lane 8). The multimerization of pSYM201 DNA was confirmed by the finding that only the monomer band was recovered when the mixture of multimers was digested with EcoRI or HindIII (data not shown). The multimer level reached more than 10mer. Even-numbered multimers were more frequent than odd-numbered multimers. No multimer formation was observed in the absence of rci (lane 7). Octamer DNA of pSYM201 was isolated by transformation (lane 9). When it was introduced into cells harboring pKK098, many multimer bands were again formed (lane 11). These results indicate the occurrence of in vivo intermolecular recombination between symmetric sfx sequences, which has never been observed in shufflon recombination of R64.

When pSYM210 carrying one symmetric sfxaa201 sequence in pACYC184 was introduced into E. coli cells harboring pKK097, a series of multimer bands were also formed (Fig. 3B, lane 5). When pSYM201 and pSYM210 were simultaneously present in rci E. coli, a mixture of pSYM201 and pSYM210 multimers were formed (lane 6). Fusion plasmids containing one copy of pSYM210 and one to five copies of pSYM201, respectively, Arrow, DNA band from rci plasmid pKK097. C, in vitro intermolecular recombination between symmetric sfxaa201 sequences. pSYM201 DNA was incubated with purified Rci protein for the indicated period and electrophoresed on a 0.7% agarose gel.

The C-terminal Multimer Formation Domain of Rci Is Not Required for Recombination Using Symmetric sfx Sequences—In our previous work (11), it was postulated that the extra C-terminal domain of Rci is involved in the non-sequence-specific binding of Rci to the left arm of asymmetric sfx sequences. To examine this postulation, two missense mutants, Rci F297S and Y271F, of the active center tyrosine was also constructed. A mutant, Rci Y271F, of the active center tyrosine was also constructed. pAG105 contained a fixed R64 shufflon region carrying seven symmetric sfx sequences, which has never been observed in shufflon recombination of R64. When pSYM210 carrying one symmetric sfxaa201 sequence in pACYC184 was introduced into E. coli cells harboring pKK097, a series of multimer bands were also formed (Fig. 3B, lane 5). When pSYM201 and pSYM210 were simultaneously present in rci E. coli, a mixture of pSYM201 and pSYM210 multimers were formed (lane 6). Fusion plasmids containing one copy of pSYM210 and one to five copies of pSYM201 were recovered by transformation (lanes 7–11). When these plasmids were introduced into rci E. coli, a mixture of pSYM201 and pSYM210 multimers were again produced (data not shown). These results indicate that fusion and resolution of plasmids using symmetric sfx sequences are possible.

In vitro recombination assay was performed using supercoiled pSYM201 DNA and purified Rci protein (Fig. 3C). A closed circular monomer band of pSYM201 DNA and a small amount of open circular band were seen at the beginning of the reaction. A dimer band appeared after 5 min and increased in amount during incubation. At the late stages of incubation a tetramer band also appeared. These results indicate the occurrence of in vitro intermolecular recombination between symmetric sfx sequences.

The C-terminal Multimer Formation Domain of Rci Is Not Required for Recombination Using Symmetric sfx Sequences—In our previous work (11), it was postulated that the extra C-terminal domain of Rci is involved in the non-sequence-specific binding of Rci to the left arm of asymmetric sfx sequences. To examine this postulation, two missense mutants, Rci F297S and L326H, and two deletion mutants, Rci D284am and L344am, in the Rci C-terminal region were constructed. A mutant, Rci Y271F, of the active center tyrosine was also constructed. pAG105 contained a fixed R64 shufflon region carrying seven asymmetric sfx sequences. Many DNA bands were produced from pAG105 by multiple inversions in the presence of the wild-type Rci (Fig. 4A, lane 3). In contrast, all of the five Rci mutants were inactive for shufflon inversions in pAG105 (Fig. 4A, lanes 4–8), indicating that the Rci C-terminal domain is
essential for the R64 shufflon inversions using asymmetric sfx sequences. For inversion between symmetric sfxaa201 sequence and asymmetric sfxa112 sequence of pINV201, only the Rci L326H mutant exhibited low activity among Rci C-terminal mutants (Fig. 4B). For deletion between sfxaa201 and sfxa112 sequences of pDEL301, Rci C-terminal mutants exhibited no recombination activity (Fig. 4C).

For inversion between the two symmetric sfxaa201 sequences of pINV202, Rci D284am, F297S, L326H, and L344am mutants were active, whereas their activities were lower than that of the wild-type Rci (Fig. 4D). For deletion between two symmetric sfxaa201 sequences of pDEL302, Rci D284am, F297S, L326H, and L344am mutants exhibited one-third the level of activity as the wild-type Rci (Fig. 4E), indicating that the Rci C-terminal domain is not required for recombination using symmetric sfx sequences.

Effects of the four Rci C-terminal mutations on various shufflon recombinations using symmetric or asymmetric sfx sequences are summarized in Fig. 4G. When these results were combined, it may be concluded that the C-terminal multimer...
formation domain of Rci is not required for recombination using symmetric sfx sequences.

Role of the Rci C-terminal Multimer Formation Domain in Binding to Asymmetric and Symmetric sfx Sequences—Previ-ously, specific binding of Rci to R64 sfx sequences was demonstrated (10, 11). To examine the role of the Rci C-terminal domain in its binding to asymmetric and symmetric sfx sequences, DNase I footprint analyses were performed (Fig. 5). Intact Rci (Rci wt) and Rci292, carrying N-terminal 292 amino acids of Rci, were purified. Rci wt protected both arms of asymmetric sfxa101 sequence from DNase I cleavage as was found previously (11) (Fig. 5A). Rci292 protected only the right arm of sfxa101, whereas strong enhancement was noted for the left arm of sfxa101 by Rci292 binding (dots in Fig. 5B). These results suggest that the Rci C-terminal domain plays a key role for its binding to both arms of asymmetric sfx sequences. In con-trast, Rci wt and Rci292 protected both arms of symmetric sfxaa201 sequence (Fig. 5, C and D). It seems that Rci292 bound to symmetric sfxaa201 sequence more tightly than Rci wt. We have also purified Rci310 and found similar protection patterns of sfxa101 and sfxaa201 as Rci292 (data not shown). These results suggest that Rci can bind to the conserved 12-bp arm sequences of symmetric sfx sequences by itself even without the C-terminal domain.

DISCUSSION

Different levels of complexity are known for various conserva-tive site-specific recombination systems using integrase family recombines. The λ Int system is rather complex (3). Int and IHF proteins are required for λ integration, while the Xis protein is additionally required for excision. The attB site is ~21 bp long, while the attP site, containing attP core site, is ~243 bp long. The attB and attP core sites consist of 7-bp central regions flanked by core-type Int-binding inverted-repeat sequences. The attP site also contains arm-type Int-, IHF-, and Xis-binding sites.

In contrast, only the Cre protein and two 34-bp loxP sites are required for the P1 Cre-loxP system (16). The loxP site consists of a 6-bp central region flanked by 14-bp Cre-binding inverted-repeat sequences. The loxP central region is asymmetric and determines its orientation. Cre promotes intramolecular excision and inversion reactions as well as intermolecular integration reactions. Van Duyne and co-workers (12, 17–19) analyzed three kinds of Cre-DNA reaction intermediates by x-ray crys-
tallography using modified \textit{loxP} DNA substrates. In the first complex, two Cre molecules bind to each arm of \textit{lox} DNA, which induces a keen kink at the \textit{lox} site. Two Cre-\textit{lox} complexes assemble and form a synaptic complex. In the second complex, the OH groups of the active center tyrosine in two Cre molecules bind to the 3' end phosphate of recombining strands of \textit{lox} DNA, forming a phosphodiester bond. In the third complex, four Cre molecules bind to the Holliday junction DNA at the \textit{lox} site. The three complexes exhibit a pseudo-4-fold symmetry. Later Cre-DNA reaction intermediates containing \textit{loxP} site in which the central region is asymmetric were analyzed (20, 21). Among four Cre molecules in the complexes, two Cre molecules exhibit “cleaving” (cleavage-component) subunit conformation, and the other two Cre molecules exhibit “non-cleaving” subunit conformation. A switch between cleaving and non-cleaving conformations regulates sequential cleaving and rejoining reactions of top and bottom strands. The \textit{loxP} top strand was shown to be exchanged at first. Introduction of C to G mutation into the left arm of \textit{loxP} DNA site resulted in misorientation of \textit{loxP} DNA in the complex.

On the other hand, Rci and HU proteins are required for the R64 shufflon system (10, 22). R64 \textit{sfx} sites consist of conserved 7-bp central region and 12-bp right arm sequences and a non-conserved 12-bp left arm sequence, indicating that R64 \textit{sfx} sequences are asymmetric. Only intramolecular inversions have been observed in the R64 shufflon. In the present study, a symmetric \textit{sfxaa201} sequence was shown to exhibit the highest inversion frequency. When two \textit{sfxaa201} sequences are located in direct orientation, Rci-mediated deletion of a DNA segment flanked by them was observed. In addition, Rci-mediated intermolecular recombination using the \textit{sfxaa201} sequence was observed, whereas intermolecular recombination never occurs in the R64 shufflon system. These results strongly suggest that asymmetry of the R64 \textit{sfx} sites inhibits intramolecular recombination between direct \textit{sfx} sites and intermolecular recombination. Asymmetry of \textit{sfx} arm sequences may emphasize the differences in cleaving and non-cleaving conformations of Rci subunits.

In many site-specific recombination systems, the length of spacer sequence in recombination site may be important. Deletion of one nucleotide from the \textit{loxP} and \textit{attP} sites dramatically reduced the recombination activity (23, 24). Symmetric recombination sites with one nucleotide deletion (\textit{sfxaa231}, CCACGTA-CCGGGTGCACATTCGGTACGTGG) and one nucleotide insertion (\textit{sfxaa232}, CCACGTA-CCGGGTGCGATCAGTGG) were constructed. The inversion frequency of plINV231 or plINV232 carrying \textit{sfxaa231} or \textit{sfxaa232} at one site and \textit{sfxa112} at the other site was 0.0018 and 0.0025, respectively, in the presence of pKK098. plINV231 and plINV232 did not produce multimer bands. These results indicate that the length of spacer sequence of symmetric \textit{sfx} sequence should be seven base pairs to exhibit high inversion activity and intermolecular recombination.

Most site-specific recombinases in the integrase family consist of a DNA-binding domain and a catalytic domain, which contains conserved box I and box II sequences. Phase integrases carry an extra N-terminal domain, which binds to the \textit{attP} arm-type binding sites. The C termini of most recombinases are located shortly after the box II sequences. In contrast, Rci carries an extra C-terminal multimer formation domain that is essential for recombination in the R64 shufflon using asymmetric \textit{sfx} sites. Here, Rci protein lacking the C-terminal multimer formation domain was shown to promote recombination using symmetric \textit{sfx} sites. These situations can be explained as shown in Fig. 6. One molecule of Rci initially binds to the conserved right arm of asymmetric \textit{sfx} sequences in a sequence-specific manner. Then the C-terminal multimer formation domain of the Rci molecule bound to the \textit{sfx} right arm recruits the second molecule of Rci, which binds to the \textit{sfx} left arm in a non-sequence-specific manner (Fig. 6A). For this mechanism, the Rci C-terminal domain is essential, and only the intramolecular inversion reaction is permitted. In contrast, Rci molecules can bind to both arms of the symmetric \textit{sfx} sequences by itself in a sequence-specific manner (Fig. 6B). For this mechanism, the Rci C-terminal domain is not essential and intramolecular inversion, and deletion reactions as well as intermolecular reactions are possible.

Recently, several multiple inversion systems using integrase family recombinases have been reported in \textit{Mycoplasma pulmonis}, \textit{Mycobacterium bovis}, and \textit{Bacteroides fragilis} (25–28). These systems control antigenic variations of surface proteins.

**FIGURE 6.** Role of the Rci C-terminal domain in recombination using asymmetric (A) and symmetric (B) \textit{sfx} sequences. The DNA-binding and catalytic domains of Rci are collectively represented by \textit{Y}. The active center tyrosine is indicated by \textit{Y} in the circle. One molecule of Rci binds to the conserved right arm of the asymmetric \textit{sfx} sequence, and then another Rci molecule is recruited to its left arm with the help of the multimer formation domain. In this case, only inversion of shufflon segments is possible. B, Rci molecules (even without the multimer formation domain) bind to both arms of symmetric \textit{sfx} sequences. In this case, both inversion and deletion of shufflon segments are possible.
and capsular polysaccharides as well as type I restriction specificities. Most of recombination sites of these multiple inversion systems appear to be asymmetric (29). Further studies on the recognition of sfx sequences by Rci may contribute to understanding the shufflon systems.

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