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Site-specific recombination of asymmetric *lox* sites mediated by a heterotetrameric Cre recombinase complex

Talia Saraf-Levy,[a] Stephen W. Santoro,[b] Hanne Volpin,[a] Tzvika Kushnirsky,[a] Yoram Eyal,[a] Peter G. Schultz,[c] David Gidoni[a] and Nir Carmi[a,*]

[a]*Institute of Plant Sciences, A.R.O, The Volcani Center, PO Box 6, Bet-Dagan 50250, Israel*
[b]*Department of Molecular and Cellular Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA*
[c]*Department of Chemistry and The Skaggs Institute for Chemical Biology, The Scripps Research Institute, SR202, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA*

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Abstract—Previous reports have demonstrated that new Cre recombinase specificities can be developed for symmetrically designed *lox* mutants through directed evolution. The development of Cre variants that allow the recombination of true asymmetric *lox* mutant sites has not yet been addressed, however. In the present study, we demonstrate that a mixture of two different site-specific Cre recombinase molecules (wt Cre and a mutant Cre) catalyzes efficient recombination between two asymmetric *lox* sites in vitro, presumably via formation of a functionally active heterotetrameric complex. The results may broaden the application of site-specific recombination in basic and applied research, including the custom-design of recombinases for natural, asymmetric, and *lox*-related target sequences present in the genome. Future applications may potentially include genomic manipulations, for example, site-specific integrations, deletions or substitutions within precise regions of the genomes of mammalians and other organisms.

1. Introduction

Site-specific recombination systems mediate the control of a large variety of critical biological functions in nature through the precise excision, inversion or integration of defined DNA units. Such functions include: control of transcription during development; control of plasmid copy number; resolution of multimeric phage genomes and plasmids into monomeric forms; and integration or excision of viral genomes into or from the host chromosome, respectively.[1–5] Site-specific recombination systems function through specific interactions of recombinase enzymes (of the integrase family) with their cognate DNA target sequences and provide an experimental model for studying the protein–DNA and protein–protein interactions involved in recombination. There are two major integrase families: the serine integrases and the tyrosine integrases.[6,7] The latter group of the λ integrase family of tyrosine recombinases, which carry out conservative site-specific recombination reactions, includes the prototypical λ phage Int protein, bacterial XerC/D,[4] Cre from phage P1,[8] and FLP from yeast.[9] Cre recombinase from bacteriophage P1 is perhaps the simplest and best-characterized recombinase system.[8,10] Cre, a 38.5-kDa protein, catalyzes the recombination of two identical 34-bp sites termed *loxP* (Fig. 1). Like the *FRT*, the target site of FLP,[9] the *loxP* site is palindromic, comprising two identical 13-bp inverted repeats surrounding an 8-bp asymmetric spacer region that confers directionality to the site and hence to the recombination reaction.[10–13] Each identical 13-bp palindrome half-site is a binding site for a Cre monomer and recombination occurs within the 8-bp spacer region. Cre catalyzes recombination between two *loxP* sites, which are brought together in an antiparallel fashion through protein–protein interactions between four Cre monomers. The recombination reaction proceeds through a square-planar Holliday junction intermediate.[14–21]

Cre mediates precise recombination, both in vitro and in vivo, of linear, circular or supercoiled DNA substrates.[8] Depending on the relative orientation of two intramolecularly positioned *loxP* sites, recombination result in either inversion or excision of the intervening

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* Corresponding author. Tel.: +972 3 968 3771; fax: +972 3 966 9583; e-mail: nircarmi@volcani.agri.gov.il

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Figure 1. Symmetric and asymmetric *lox* sites. The symmetric *loxP* site is the cognate site for Cre recombinase; the symmetric *lox* M7 site is a variant previously described;[32] *loxP*-M7 and M7-*loxP* are chimeric asymmetric variants comprising one 13-bp half-site from each of *loxP* and *lox* M7. Nucleotides that differ between the variants are denoted: letters in bold correspond to nucleotides of the wild-type site (*loxP*); underlined letters correspond to nucleotides of the *lox* M7 variant site; gray letters correspond to nucleotides within the spacer region. Arrows indicate cleavage sites.

DNA. When *loxP* sites are positioned on two different molecules, intermolecular recombination results in integration.[10,11] Due to its simplicity and versatility, Cre has proven to be a valuable tool for the manipulation of transgenes and chromosomal DNA that harbor artificially positioned *loxP* sites in various eukaryotic cells and organisms.[22–29] In order to broaden the potential targets and utility of Cre-based recombination, random and directed mutations coupled with in vitro selection techniques have been applied to isolate Cre mutants that recognize variant *lox* sites.[30–32] The identities of base pairs 2–7 on each side of *loxP* (Fig. 1) were previously demonstrated to be of particular importance for Cre recognition.[33] A *loxP* variant termed *lox* M7, which harbors symmetrical mutations within base pairs 5-7 on each side of the *loxP* site, was found to be unrecognizable by wt Cre.[32] Using a directed evolution strategy, two Cre variants that recognize *lox* M7, termed CM1 (corresponding to C2(+)#1) and CM2 (corresponding to C2(+/−)#4), were selected from libraries of Cre variants generated by targeted random mutagenesis.[32] One mutant, CM1, was derived using positive selection pressure for recombination of the *lox* M7 site. A second mutant, CM2, was generated using both positive selection pressure for recombination of the *lox* M7 site and negative selection pressure against recombination of the wild-type *loxP* site. CM1 exhibits relaxed substrate specificity in vitro and in vivo, catalyzing recombination of both *lox* M7 and *loxP* substrates with approximately equal efficiency. In contrast, CM2 recombines the *lox* M7 site both in vivo and in vitro approximately 40-fold more efficiently than the wild-type *loxP* site.[32] Recent studies based on crystal structural analyses have revealed that water molecules mediate the binding specificities of CM1 and CM2, and that the relaxed substrate specificity of CM1 results from more versatile protein–DNA interactions.[34]

While previous reports demonstrate that new Cre target-site specificities can be developed for symmetrical *lox* variants, the development of Cre mutants that catalyze the recombination of true asymmetric *lox* variant sites has not been addressed. In the present study, we demonstrate that a combination of two different Cre recombinase variants catalyzes recombination in vitro between asymmetric *lox* sites at efficiencies comparable to that of wt Cre on *loxP* sites. The results suggest that wt Cre and Cre variants can interact and function as a heterotetrameric unit to catalyze asymmetric *lox* site recombination.

2. Results and discussion

2.1. Design of an in vitro recombination assay for asymmetric *lox* variant sites

We approached the question of asymmetric recombination by employing modified Cre recombinase versions recently developed for different *lox*-related site specificities.[32] The experimental design consisted of an in vitro recombination assay which allowed us to monitor the efficiency of recombination obtained using one or a combination of two site-specific Cre-related recombinases of asymmetric *lox* site substrates. Specifically, a 4-kb linear double-stranded DNA molecule containing a pair of chimeric asymmetric sites, termed *loxP*-M7 and M7-*loxP*, served as the substrates (Fig. 1). Each asymmetric site was composed of a wild-type *loxP* half-site and *lox* M7[32] half-site (Fig. 1). Each member of the pair of *lox* sites was positioned in direct orientation relative to the other, flanking an intervening ~1-kb DNA fragment (Fig. 2b). A recombination event was expected to excise the intervening DNA fragment, resulting in a 3-kb linear fragment and a ~1-kb circular DNA molecule (Fig. 2a). The activities of purified recombinant wt Cre (specific for *loxP*), CM1 (equally specific for *loxP* and *lox* M7[32]), and CM2 (displaying a 40-fold higher specificity for *lox* M7 than *loxP*;[32]) enzymes and an equal mixture of wt Cre and CM2 were assayed for the ability to recombine the four combinations and positions of asymmetric *loxP*-M7 and M7-*loxP* DNA chimeric substrates, displayed in Figure 2b.

2.2. Recombination of asymmetric *lox* sites in vitro

The recombination activities of wt Cre, CM1, CM2, and the wt Cre–CM2 mixture were first assayed at concentrations ranging from 15 to 2000 nM with 1 nM substrate of *loxP*, *lox* M7 (controls) and on the four combinations of *loxP*-M7 and M7-*loxP* DNA asymmetric substrates (shown in Fig. 2b) in a reaction time of 90 min (Fig. 3). Wild-type Cre exhibited K_D of 29 nM (Table 1) for *loxP*, consistent with the previously reported 28 nM.[32] The CM2 exhibited K_D of 187 nM for *lox* M7 (Table 1) which is approximately the K_D (220 nM) that was previously reported.[32] Wild-type Cre exhibited measurable activity (~10%) with the asymmetric DNA substrates only at 1000 nM enzyme concentrations (Figs. 3c–f). For the rate experiments, we chose to use 200 nM enzyme concentration because higher concentrations of CM2 in the mixture could increase the rate, but results in nonspecific recombination of CM2 on the asymmetric site (Figs. 3c–f). In this

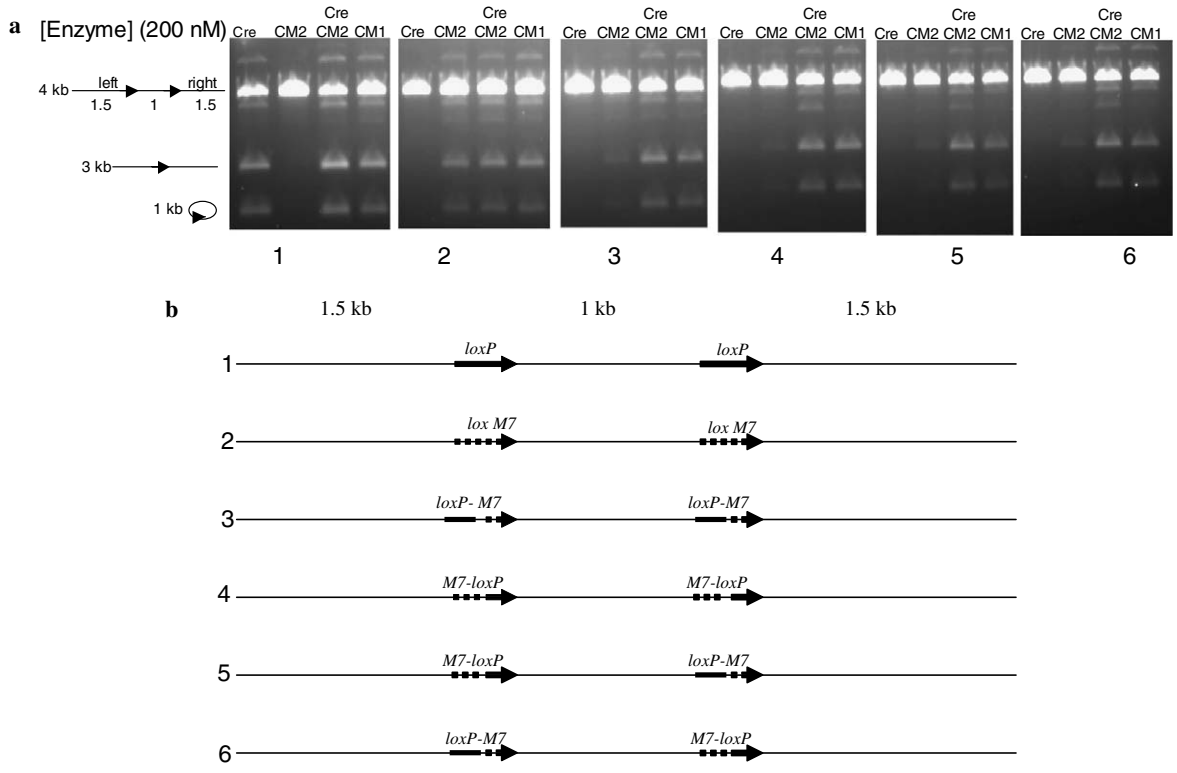

Figure 2. Recombination on asymmetric substrates in vitro. (a) Gel electrophoresis representation of recombination by the Cre variants, wt Cre, CM1, CM2 (200 nM), and a mixture of wt Cre and CM2 (100 nM each) on the various *lox* substrates indicated in (b). Schematics refer to: the linear substrate (4 kb); the linear plasmid recombination product (3 kb); and the circular excised insert recombination product (~1-kb). (b) Schemes of linear DNA substrates harboring *lox*P (1), loxM7 mutant (2), and the asymmetric chimeric substrates composed of half-site *lox*P and half-site *lox* M7 in four different orientations (3–6).

context, we point out that Cre binding at subsaturation is not necessarily a rate-limiting step.[37] CM2 exhibited measurable but inefficient activity, recombining approximately 5–10% of the asymmetric substrates within the reaction period, when present at a concentration of 200 nM (Figs. 2a, 3c–f). In contrast, the catalytic efficiencies of CM1 and the wt Cre–CM2 mixture on the asymmetric sites were significantly higher (Figs. 2a, 3c–f). When present at a concentration of 200 nM, CM1 and the wt Cre–CM2 mixture catalyzed recombination of approximately 35% of the asymmetric substrate in 90 min (Figs. 3c–f).

In order to compare the rates at which wt Cre, CM2, and the wt Cre–CM2 mixture approach equilibrium in recombining the asymmetric DNA substrates, a reaction containing 200 nM enzyme and 1 nM DNA substrate was allowed to proceed for 90 min. Aliquots were removed and quenched periodically during the reaction period (Fig. 4). While CM2 recombination proceeded at a rate of only 0.0030–0.0052 min^{-1} (Table 1), reaching approximately 10% recombination in 90 min on the asymmetric substrates, the wt Cre–CM2 mixture exhibited significantly higher recombination rates of 0.020–0.026 min^{-1} on the asymmetric substrates (Table 1) and reached recombination extents of approximately 25% after 20 min and 35% after 90 min (Fig. 4c–f). Recombination of the asymmetric substrates by wt Cre was not observed (Fig. 4c–f). The rate and the K_D of the wt Cre–CM2 mixture on the asymmetric substrates are probably not affected from the four different asymmetric *lox* variants that were tested (Figs. 2a, 3, 4 and Table 1).

The rate and the K_D of the wt Cre–CM2 mixture, in 200 nM enzyme mix, on the asymmetric substrates exhibited approximately the rate and K_D of CM2 on *lox* M7, however, it exhibited slower rate and higher K_D when compared to wt Cre on *loxP* (see Table 1). As the concentration of CM2 within the wt Cre–CM2 mixture was lower than its K_D (Ref. 32, Table 1), this indicates that the rate-limiting factor of the wt Cre–CM2 mixture on asymmetric substrates is probably CM2. The K_D of wt Cre–CM2 mixture on *lox* M7 or on *loxP* was approximately twice the K_D of CM2 on *lox* M7, or the K_D of the wt Cre on *loxP* (Table 1), therefore interference between wt Cre and CM2 in the mixture is not likely. There was no significant difference among the Hill coefficient values that were measured with the various Cre types tested on their cognate substrates (Table 1). However, this result may not be accurate when $[E_{50}]$ is low.

The results support two major conclusions: (1) A Cre-related recombinase with relaxed substrate specificity that functions equally efficiently on *loxP* and *lox* M7 substrates (CM1) can as efficiently catalyze recombination of the corresponding chimeric asym-

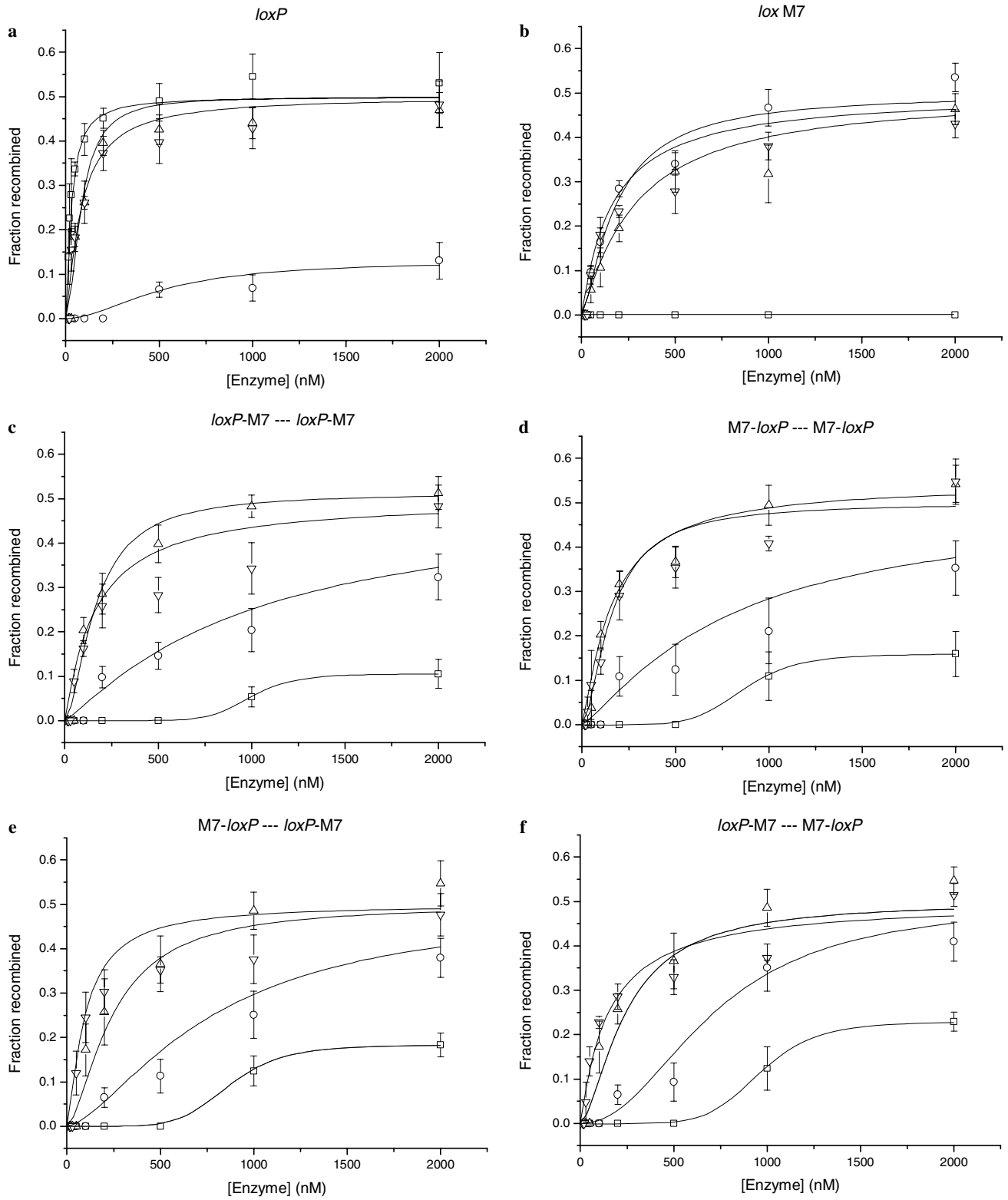

Figure 3. Recombination activity as a function of enzyme concentrations. The substrates (1 nM) were incubated with a range of concentrations (15–2000 nM) of Cre variants [wt Cre (square), CM2 (circle), a mixture of CM2 and wt Cre (triangle), and CM1 (triangle upside down)] for 90 min. Reaction products were separated by electrophoresis on 1% agarose gels and quantified as described in the experimental procedures. Recombination activity was calculated as a ratio of the combined intensities of the ~1 and 3 kb products over the intensity of the total substrate in the reaction (1 + 3 + 4 kb).

metric substrate. (2) A combination of two different Cre variants (wt and CM2) possessing selective binding specificities for their respective cognate *lox* half-sites (*loxP* or *lox* M7, respectively) can efficiently catalyze recombination of the chimeric *loxP*-M7, M7-*loxP* asymmetric substrates.

Table 1. Hill coefficient, K_D, and rates of approach to equilibrium

DNA substrates	Rate at [enzyme] (200 nM)			Enzyme affinity for substrate (K_D)				Hill coefficient			
	Cre (min^{-1})	Cre+CM2 (min^{-1})	CM2 (min^{-1})	Cre (nM)	Cre+CM2 (nM)	CM2 (nM)	CM1 (nM)	Cre (h)	Cre+CM2 (h)	CM2 (h)	CM1 (h)
loxP—*loxP*	0.038 ± 0.0035	0.032 ± 0.0023	0	29 ± 2.45	67 ± 13		82 ± 15	1.25 ± 0.17	1.68 ± 0.37		1.2 ± 0.27
*lox*M7—*lox*M7	0	0.01 ± 0.00086	0.025 ± 0.003		347 ± 47	187 ± 25	165 ± 46		1.1 ± 0.19	1.36 ± 0.21	1.0 ± 0.13
loxP-M7—*loxP*-M7	0	0.020 ± 0.00128	0.003 ± 0.00042		164 ± 19	635 ± 123	182 ± 52		1.66 ± 0.33	1.5 ± 0.13	1.05 ± 0.16
M7-*lox*P—M7-*lox*P	0	0.026 ± 0.00411	0.004 ± 0.00074		160 ± 21	803 ± 114	188 ± 27		1.62 ± 0.37	1.6 ± 0.29	1.3 ± 0.2
M7-*lox*P—*lox*P-M7	0	0.024 ± 0.0022	0.005 ± 0.00163		224 ± 32	718 ± 44	100 ± 17		1.6 ± 0.29	1.52 ± 0.25	1.23 ± 0.12
loxP-M7—M7-*lox*P	0	0.025 ± 0.0028	0.004 ± 0.001		243 ± 23	716 ± 64	151 ± 35		1.59 ± 0.26	2.16 ± 0.38	1.03 ± 0.17

2.3. Cre variants and the formation of a presumably heterotetrameric structure

The present study demonstrates the feasibility for site-specific recombination of asymmetric *lox* sites by a combination of two different Cre variants possessing selective binding specificities for their respective cognate half-site on the site. The results presented here strongly suggest that recombination in this system is catalyzed by a heterotetrameric assembly of the two Cre variants (wild-type Cre and the CM2 mutant). This conclusion is consistent with the fact that the DNA binding and catalytic domains within Cre reside in two distinct and independent locations on the protein.[19,35,36] This arrangement permits the formation of a recombination synapse involving two asymmetric *lox* sites aligned in an antiparallel orientation and each bound by a wt Cre–CM2 heterodimer (Fig. 5). The recombination synapse involving two of each Cre variant monomer and two asymmetric *lox* sites can then go on to form a Holliday junction intermediate, followed by resolution to complete the recombination reaction.

As the results with CM1 illustrate, site-specific recombination of asymmetric target sites can be facilitated by a single Cre-recombinase variant. CM1, a variant of Cre with relaxed substrate specificity that functions equally efficiently with the *loxP* and *lox* M7 substrates,[32] also rapidly recombines the asymmetric *loxP*-M7 substrate when present at a concentration of 200 nM (see Figs. 3c–f). In contrast, CM2, a recombinase with switched substrate specificity that exhibits ~40-fold higher recombination efficiency with *lox* M7 than *loxP* substrate,[32] was close to approaching the catalytic rate of CM1 on the asymmetric substrates only when present at the higher 2000 nM enzyme concentration (Figs. 3c–f), indicating the loss of specificity of CM2 at high concentrations. Comparably, wt Cre reached only 20% recombination on the asymmetric substrates at 2000 nM (Figs. 3c–f), indicating that wt Cre becomes promiscuous in vitro at higher concentration as observed with the *lox* AT mutant.[37] Nevertheless, Cre mutants with higher specificity could be selected for asymmetric *lox* recombination via a heterotetrameric complex. Although recombination of a nonpalindromic *lox* mutant has been previously reported[31] the mutations described were within the 'tolerant' region of *loxP* in nucleotides 11and 13.[33] Additionally, wt Cre was shown to facilitate recombination on certain asymmetric *lox* mutants, however, by shifting the equilibrium to integration over excision.[38,39] In the present study, site-specific recombination by a single promiscuous Cre mutant or a mixture of two specific Cre variants on a nonpalindromic site containing mutated nucleotides within the 'intolerant' region of *loxP* in nucleotides 2–7, which are critical for Cre binding, was demonstrated.

Interestingly, both the wt Cre–CM2 mixture and CM1 demonstrated comparable catalytic efficiencies, suggesting that the heterotetrameric assembly of Cre variants imposes no penalty on recombination efficiency. Because Cre variants with relaxed specificity, such as CM1, could in principle recognize nontargeted

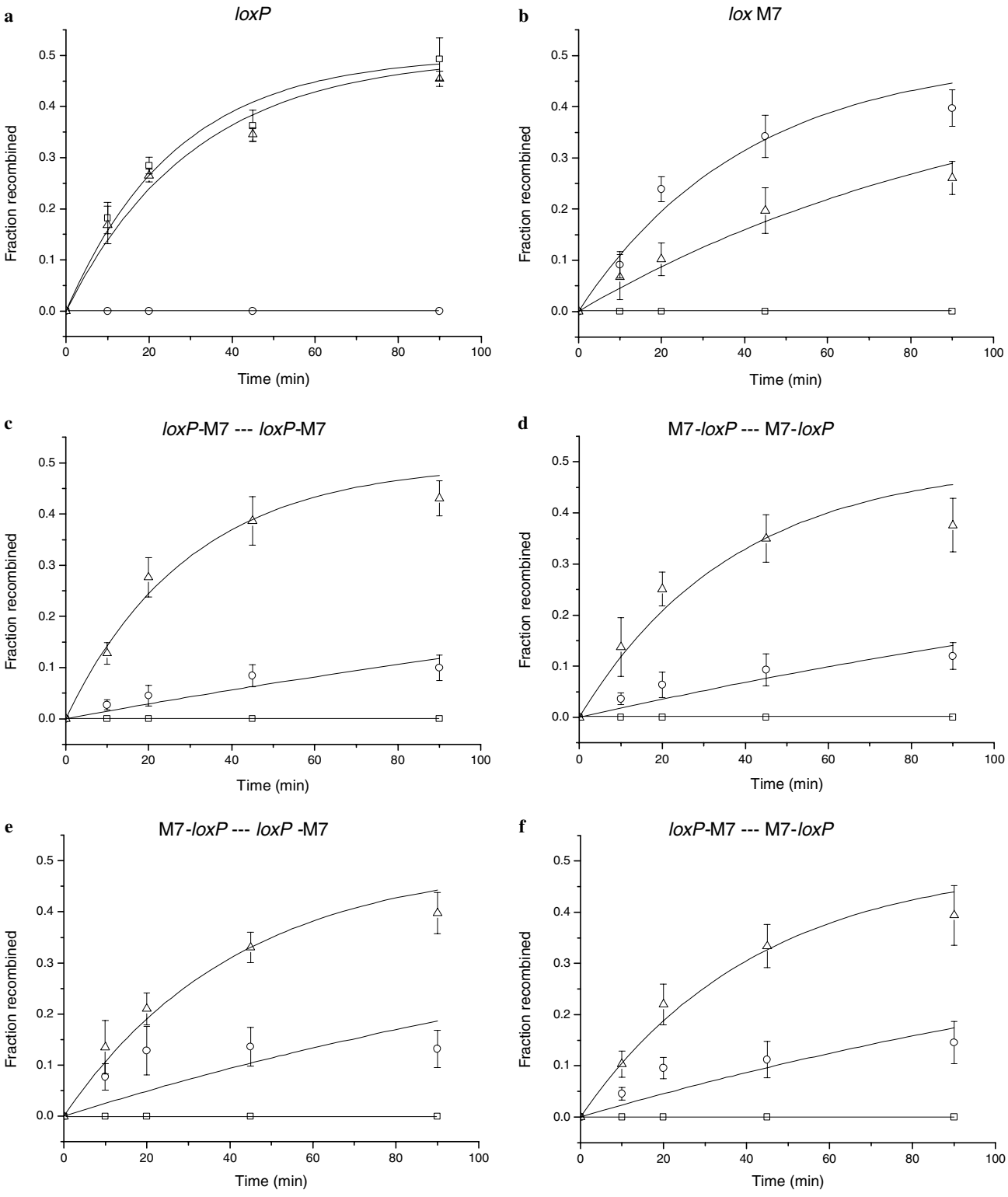

Figure 4. Recombination activity as a function of reaction time. The substrates (1 nM) were incubated for 10, 20, 45 or 90 min with wt Cre (square), CM2 (circle), and a mixture of CM2 and wt Cre (triangle) at total enzyme concentration of 200 nM. Reaction products were separated by electrophoresis on 1% agarose gels and quantified as described in the experimental procedures. Recombination activity was calculated as a ratio of the combined intensities of the ~1 and 3 kb products over the intensity of the total substrate in the reaction (1 + 3 + 4 kb).

lox-related sites, such variants could result in off-target effects. In contrast, the use of two different highly specific Cre variants permits the targeting of asymmetric sites with minimal risk of off-target activities.

2.4. Natural asymmetric *lox*-like sites

We employed a bioinformatics approach in order to evaluate the approximate number of asymmetric *lox*-like

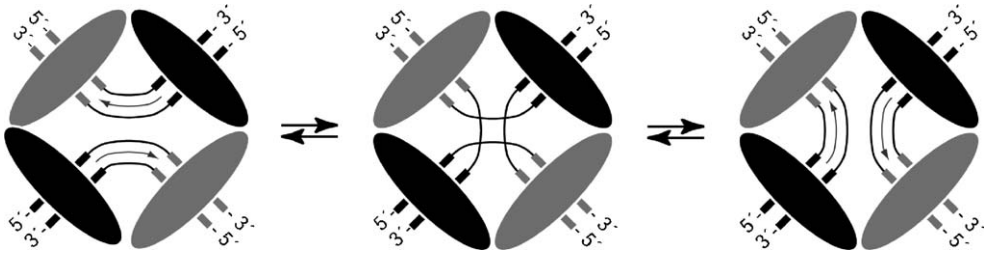

Figure 5. Schematic model of recombination by a heterotetrameric Cre assembly. Black- and gray-shaded ellipses represent wt Cre and CM2 monomers, respectively, each bound to a *lox* half-site. Black-shaded strands represent the *loxP* half-site and the gray-shaded strands represent the *lox* M7 half-site of *loxP*-M7, respectively.

Table 2. Computational screen for asymmetric versus symmetric *lox*-like sites within the genome of human, mouse, and rat

	Right side					
	8	9	10	11	12	13
Left side						
Human						
8	243/1*	46	4	2	0	0
9	37	11	1	0	0	0
10	3	0	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
Mouse						
8	181	36	3	1	0	0
9	39	2	2	0	0	0
10	5	3	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0
Rat						
8	145	31	6	1	0	0
9	28	5	0	0	0	0
10	6	1	0	0	0	0
11	0	0	0	0	0	0
12	0	0	0	0	0	0
13	0	0	0	0	0	0/2*

Numbers correspond to base identity of *loxP* in left or right side of the 13 inverted repeats.
Asterisk numbers represent symmetric sites. Right and left corresponds to *loxP* right and left sides relative to the spacer.

recombination targets in vivo, within several mammalian genomes, including human, rat, and mouse (Table 2). Wt Cre has been shown to tolerate changes within the *lox′* 8 bp spacer region.[13] Computational screen in human, rat, and mouse for potential *lox*-like sites was performed, based on the 33 tolerable spacers described in,[13] revealing several hundred asymmetric *lox*-like sites in each genome (Table 2). Interestingly, a couple of symmetric *lox*-like sites of 13 bp palindrome were found in the rat genome (Table 2).

3. Conclusions

We have demonstrated the ability of facilitating site-specific recombination by two Cre variants on an asymmetric site via a heterotetrameric complex. The results with the powerful techniques of directed molecular evolution provide the basis for custom-development of pairs of Cre variants for precise recombination within plant or animal genomes. Site-specific integration into human *lox*-like sites, using a selected single Cre variant, was already demonstrated to be efficient,[30] however, the approach presented here is predicted to be of particular utility in broadening the prospects for genetic manipulation of eukaryotic genomes. Such technology could enable the precise integration, deletion or replacement of specific genes and genomic DNA segments. The ability to site-specifically insert transgenes may enable us to reduce or overcome the possibility of insertional mutations and the cause of, for example, cancer development,[40] which has been reported to result from random integration. Pseudo *lox* sites that facilitate efficient recombination by wt Cre have already been shown within the human[41] and mouse genomes.[42,43] Optimal expression of Cre recombinase is required for specific integration[43,44] which is essential for gene therapy treatments; therefore, broadening the numbers of potential integration sites (Table 2) may contribute to achieving efficient and harmless integration. Potential inhibition of HIV replication by site-specific recombination was reported as a result of inserting *loxP* into HIV within the nonessential U3 region of the LTR.[45] Asymmetric *lox*-related sequences were found within the HIV LTR region and the *lox*-LTR spacer could replace the wt spacer in a successful recombination reaction[46] The results presented here may suggest a potential strategy for AIDS treatment which is based on directed evolution of Cre recombinases via formation of heterotetrameric complexes designed for site-specific excision of asymmetric *lox*-like sites present within the long terminal repeats (LTR).

4. Experimental

4.1. Construction of wild-type and mutant *loxP* substrates

The wild-type *loxP* site and mutant *lox* M7 site substrate constructs were described previously.[32] In this work, *loxP*, *lox* M7, and the four combinations of two chimeric asymmetric *lox* variants, *loxP*-M7 and M7-*loxP*, were designed to contain one half-site identical to *loxP* and a second half-site identical to *lox* M7 (Figs. 1 and 2b). The substrate plasmids were created by cloning the various *lox* sites (synthesized as oligonucleotides) flanking a ~1-kb spacer of the *BamHI-EcoRI* fragment

of the *nptII* gene[47] into a Bluescript vector at the *XhoI*/*PstI* restriction sites. All *lox* sites were cloned in a direct orientation (Fig. 2b).

4.2. Expression and purification of recombinant Cre variants

Expression and purification of recombinant wild-type and mutant Cre enzymes from bacterial expression vectors harboring wt Cre and the Cre mutants CM1 [C2(+)#1] and CM2 [C2(+/−)#4] were performed as described.[32]

4.3. In vitro analysis of recombinase activity

In vitro analysis of recombinase activity was performed essentially as described.[32] Recombination assays were conducted under conditions of variable reaction time or enzyme concentration using three separate repetitions. Recombination products were separated by gel electrophoresis containing 0.5 μg/ml ethidium bromide and quantified by digital imaging system of Alpha Innotech Flourchem 8800.

Concentration-dependent assays were performed at enzyme concentrations of 15–2000 nM on each substrate (Fig. 3) for a fixed reaction time of 90 min in a reaction buffer containing 300 mM NaCl, 20 mM Tris (pH 7.5), and 1 mM EDTA in a final volume of 40 μL. All reactions were carried out at 37 °C and stopped by incubation at 70 °C for 10 min.

Data were fit to the Hill equation: $F = F_{eq}[E^h]/([E_{50}]^h+[E]^h)$, where F is a fraction recombined at enzyme concentration [E], F_{eq} = fraction recombined at equilibrium, and $[E_{50}]$ = enzyme concentration for 50% recombination. Time–course experiments were allowed to proceed for 10–90 min, with 200 nM enzyme concentrations and 1 nM of each substrate (Fig. 4). Reaction products were analyzed on a 1% agarose gel containing 0.5 μg/μL ethidium bromide. Data were fit to the equation: $F = F_{eq}(1 - e^{-kt})$; F = fraction recombined at time t, F_{eq} = maximal fraction recombined at $t = \infty$, and k = observed rate constant.

4.4. Computational screen

Human, mouse, and rat chromosomes were searched for perfect matches to the 33 tolerant spacers described in Ref. 13 From each perfect hit, 13 nucleotides upstream and downstream to the spacer were retrieved and analyzed for identity to the *loxP* sequence. Sequences showing 8 or more nucleotides identical to the *loxP* sequence both upstream and downstream from the spacer were scored for their palindromic characteristics. The score ranges from 0 to 13 based upon the number of nucleotides within the 13 nucleotides upstream from the spacer that were reverse complements to the nucleotides within the 13 nucleotides downstream from the spacer. Since the prerequisite for the palindromic score were at least 8 nucleotides identical to the *lox* sequence on both sides of the spacer, the palindromic score cannot be below 3.

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