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A randomized library approach to identifying functional *lox* site domains for the Cre recombinase

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ABSTRACT

The bacteriophage P1 Cre/*loxP* site-specific recombination system is a useful tool in a number of genetic engineering processes. The Cre recombinase has been shown to act on DNA sequences that vary considerably from that of its bacteriophage recognition sequence, *loxP*. However, little is known about the sequence requirements for functional *lox*-like sequences. In this study, we have implemented a randomized library approach to identify the sequence characteristics of functional *lox* site domains. We created a randomized spacer library and a randomized arm library, and then tested them for recombination *in vivo* and *in vitro*. Results from the spacer library show that, while there is great plasticity, identity between spacer pairs is the most important factor influencing function, especially in *in vitro* reactions. The presence of one completely randomized arm in a functional *loxP* recombination reaction revealed that only three wild-type *loxP* arms are necessary for successful recombination in Cre-expressing bacteria, and that there are nucleotide preferences at the first three and last three positions of the randomized arm for the most efficiently recombined sequences. Finally, we found that *in vitro* Cre recombination reactions are much more stringent for evaluating which sequences can support efficient recombination compared to the 294-CRE system.

INTRODUCTION

The bacteriophage P1 Cre/*loxP* site-specific recombination system is widely used as a genetic engineering tool (1,2) due to its well-defined recognition sequence, lack of any necessary co-factors and efficacy in both bacterial and eukaryotic systems. Since its discovery, Cre/*loxP* has been applied to temporal and spatial gene activation/deactivation (3–5), site-specific genomic integration and deletion (6–10) as well as the construction of libraries (11) and cloning strategies (12). Interest in expanding and improving the types of reactions that the Cre recombinase can facilitate has led to the discovery of numerous functional recognition sequences for Cre; however, no sequence characteristics have been defined to evaluate what constitutes a functional site other than its need to bind the recombinase and the role spacer compatibility plays in efficient recombination events (16,19,22).

The Cre recombinase acts on a 34-bp sequence known as *loxP* (13,14). This recognition sequence consists of two 13-bp palindromic arms separated by an 8 bp spacer region (14,15). In a site-specific recombination reaction two *loxP* sites are brought together, each arm binding one recombinase monomer (16), while strand exchange takes place within the spacer regions (17). *LoxP* sequences are maintained following recombination events. This characteristic is responsible for the reversible nature Cre/*loxP* reactions.

Successful site-specific recombination has been shown to occur between sites with sequences having varying degrees of similarity to *loxP* (8,9,15,18–21). Beginning with the description of the *Escherichia coli loxB* site (15,18), several endogenous genomic sequences from different organisms have been discovered that can serve as substrates for Cre-mediated site-specific recombination (9,20,21). Sauer (20) first identified endogenous cryptic *lox* sites in *Saccharomyces cerevisiae*. These cryptic sites contained as few as 14 out of 34 bases in common with *loxP*, and illustrated the apparent importance of the TATA sequence adjacent to the spacer region in functional sites. Later, other functional genomic *lox* sites were reported for yeast, human and mouse (9,21). Together, the description of these functional sites demonstrates flexibility in sequence recognition by the Cre recombinase.

The search for endogenous *lox* sequences within genomes for use in genomic engineering and gene therapy has been a driving force in the discovery of alternative functional *lox* sites. However, diversification and manipulation of *lox* site behavior has also identified many alternative substrates for Cre. Albert *et al*. (8) were the

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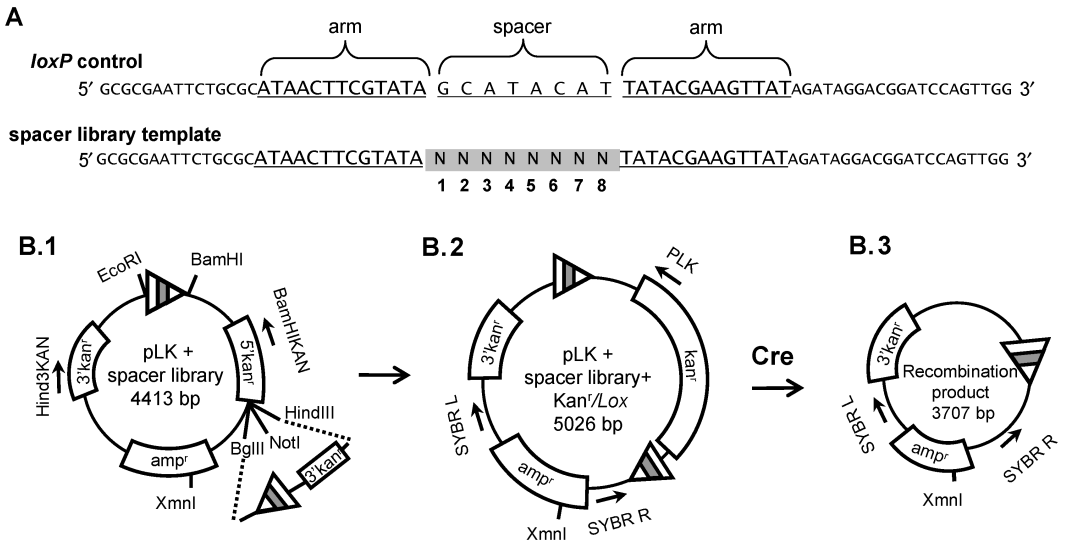

Figure 1. Constructs for spacer library. (**A**) Sequence of the template oligonucleotides used to create either the *loxP* control library site or the spacer library site are shown. The position of each base in the spacer is numbered 1–8. N is any nucleotide (A, T, G or C). *LoxP* sequences are underlined. (**B.1**) The pLK plasmid with the spacer library cloned as an EcoRI/BamHI fragment (segmented triangle, degenerate segment filled-in) followed by PCR with primers Hind3KAN and BamHIKAN (arrows) to form kanr/*lox* fragment. (**B.2**) The final spacer library with reiterated *lox* library sites (segmented triangles) and complete kanamycin resistance marker after subcloning of the PCR product at BglII/HindIII is depicted. Arrows represent the positions of the PLK sequencing primer and SYBRL, R primers for PCR detection of recombination. (**B.3**) Cre-mediated site-specific recombination results in the deletion of the kanamycin resistance marker. amp^r = gene for β-lactamase, kan^r = kanamycin resistance marker, *lacZ* = gene encoding β-galactosidase.

first to screen for arm mutations that could facilitate stable Cre/*loxP* integration reactions; while mutation studies of the spacer (19,22) documented the effects of single and double base mutations on recombination efficiency. Recently, two different surveys of partially randomized spacer libraries (NNNTANNN) (23,24) added to the growing list of spacer sequences that are proficient in recombination.

Previous studies have expanded the list of *lox* sites with which the Cre recombinase is able to recombine without pursuing a full-scale randomized approach to the 34bp sequence. Here, we report on the first randomized sequence studies aimed at defining functional *lox* sequences. We have created a randomized spacer library and a randomized arm library, both of which have been tested for functionality *in vivo* in Cre-expressing bacteria and in *in vitro* reactions. The results of these studies indicate that Cre is very flexible in the sequences it can recombine. We show that *lox* sites with matching spacers recombine more effectively than sites with non-matching spacers and that the central 'TA' is not required for efficient recombination. Our results also indicate that there is a sequence bias in functional arms and that the mode of recombination (*in vivo* or *in vitro*) is important when evaluating site function.

MATERIALS AND METHODS

Constructing a randomized spacer library

The spacer library plasmid backbone (pLK) was engineered from the previously described pPG3-*loxR*/*loxP* plasmid (23) modified to replace its *loxP* site with a HindIII–NotI–BglII linker at its XbaI site.

Two separate syntheses of the library template (5′ GCGCGAATTCTGCGCATAACTTCGTATANNN NNNNNTATACGAAGTTATAGATAGGACGGATC CAGTTGG 3′) were done in order to compensate for non-random base frequencies in the oligonucleotide manufacturing process. PCR was used to create double-stranded DNA from the templates using primers EcoRI 5′ CCAACTGGATCCGTCCTATCT 3′ and BamHI 5′ GCGCGAATTCTGCGCATAACT 3′. Each template PCR product pool was separately cloned into pLK as an EcoRI/BamHI fragment (Figure 1B.1). Clones were propagated in *E. coli* strain DH5α under ampicillin selection. Inserts were confirmed by loss of the SacI restriction site.

Library clones were chosen at random to go through reiteration of their *lox* site. PCR with primers BamHI KAN 5′ GATTTTGAGACACAACGTGGCT 3′ and Hind3 KAN 5′ GCGCGCAAGCTTTTGCCATTCTC ACCGGA 3′ was used to amplify the region of the pLK + library plasmid that contained the *lox* spacer library sequence and the 3′ end of the kan^r gene. PCR products were ligated as BamHI/HindIII fragments (613 bp) into their parent clones (Figure 1B.1). pLK + library + Kan/*Lox* clones (Figure 1B.2) were then propagated in DH5α under kanamycin selection.

The pLK + *LoxP*^{2} control plasmid was constructed using the same cloning strategy starting with the template primer 5′ GCGCGAATTCTGCGCATAACTTCGTATA GCATACATTATACGAAGTTATAGATAGGACGG ATCCAGTTGG 3′.

Assaying a randomized spacer library for site-specific recombination

In vivo recombination. Plasmid DNA was prepared from pLK + library + Kan/*Lox* clones, transformed into

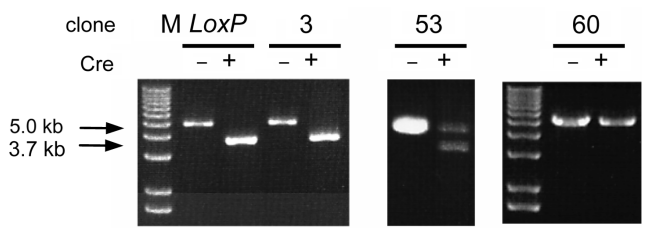

Figure 2. Examples of spacer library *in vivo* recombination. All spacer library constructs were grown in DH5α (−Cre) and 294-CRE (+Cre) bacterial strains. XmnI restriction digests of plasmid DNAs isolated from DH5α and 294-CRE overnight bacterial cultures were visualized by agarose gel electrophoresis. Examples of each type of recombination product are pictured. The *loxP* control and clone #3 show complete recombination. Clone #53 exhibits both parental and deletion products, while no recombination products can be visualized for clone #60. Full-length plasmids were 5026 bp in size, while recombination resulted in a 3707 bp product. M = marker, *LoxP* = pLK + *LoxP*2 control plasmid.

294-CRE *E. coli* (25) and grown at 37°C under ampicillin selection. Following overnight growth, plasmid DNA was isolated, digested with XmnI and its size determined by agarose gel electrophoresis (Figure 2).

In vitro recombination. pLK + library + Kan/*Lox* clone DNA was isolated by QIAprep spin miniprep column (Qiagen) and eluted in EB buffer (10 mM Tris–HCl, pH 8.5). Cre reactions were performed with 75 ng of plasmid DNA, 1U of MBP-Cre extract and 3 µl of 10 × Cre buffer (500 mM Tris–HCl, pH 7.5; 330 mM NaCl and 110 mM $MgCl_2$) in a 30 µl total volume. Reactions were incubated at 37°C for 15 min and then transferred for storage at −20°C.

Recombination was detected through high-speed PCR utilizing iProof High-Fidelity DNA Polymerase (BioRad). A standard 25 µl PCR mix was used containing 2 ng of template DNA taken directly from *in vitro* Cre reactions. The primers used were pLK SYBR R 5′ GAGATAGGG TTGAGTGTTGTTCC 3′ and pLK SYBR L 5′ GACCT ACACCGAACTGAGATACC 3′. Cycling conditions included an initial denaturation at 98°C for 30 s, 17 cycles of 98°C for 10 s, 67°C for 10 s and 72°C for 2 min followed by a final extension of 72°C for 4 min.

PCR samples were pre-incubated with SYBR Green I nucleic acid stain and visualized by agarose gel electrophoresis. Gels were scanned via a Storm PhosphorImager system (Molecular Dynamics) and analyzed with ImageQuant 5.1 software.

Constructing an arm library

Inverse PCR was used to remove a KpnI site at 623 bp from the pSV-β-Galactosidase Control Vector (Promega) (primers: KpnI invers1 5′ GTACCGGTGGGTGAAGA CCAG 3′ and KpnI invers2 5′GAGACCGCCACGGC TTACGGC 3′) (Figure 4A.1). A linker (annealed primers NBKH5′ 5′ CATGGTTCGAACTGGTACCA 3′ and NBKH3′ 5′ AGCTTGGTACCAGTTCGAAC 3′) was inserted after digesting pSV-β-Gal with NcoI and HindIII. pSV-β-Gal(–KpnI + linker) was modified at its BamHI/PstI sites with a BamHI–*Lox*–NotI–NsiI fragment that was later removed by BamHI/NotI digest and replaced with annealed primers Bam*LoxP*XbaNot5 5′ GATCCA TAACTTCGTATAATGTATGCTATACGAAGTTAT TCTAGAGC 3′ and Bam*LoxP*XbaNot3 5′ GGCCGCT CTAGAATAACTTCGTATAGCATACATTATACGA AGTTATG 3′ to form the *loxP* site. PCR with primers KpnI3X 5′ CAGGTACCATAACTTCGTATAGC 3′ and BstBI 5′ CCCTGTCCT TCGAACTCGAG 3′ was used to create double-stranded DNA from the arm library template oligonucleotide 5′ CAGGTACCATAACTTCG TATAGCATACATNNNNNNNNNNNNCTCGAGT TCGAAGGACAGGG 3′. This PCR product was ligated into pSV-β-Gal(−KpnI + linker) + *loxP* as a BstBI/KpnI fragment to create pSV-β-Gal + *LoxP* + arm library (Figure 4A.1) and then transformed either into 294-CRE or DH5α *E. coli*.

The control *loxP* site was added as annealed oligos BstbXho*LoxP*Kpn3 5′CATAACTTCGTATAGCATAC ATTATACGAAGTTATCTCGAGTT 3′ and BstbXho LoxPKpn5 5′CGAACTCGAGATAACTTCGTATAAT GTATGCTATACGAAGTTATGGTAC 3′ to create the control plasmid pSV-β-Gal + $LoxP^2$.

Assaying a randomized arm library for site-specific recombination

In vivo recombination. The final ligation of the arm library PCR product (described above) was transformed into 294-CRE ultra-competent cells and then plated on LB agar containing ampicillin and X-gal (5-Bromo-4-chloro-3-indolyl β-D-galactoside). Colonies were picked based on blue/white color selection after overnight growth at 37°C. These colonies were then used to inoculate overnight cultures from which plasmid DNA was isolated. Following isolation, plasmid DNA was digested with XhoI and its size determined by agarose gel electrophoresis (Figure 5).

In vitro recombination. To assay recombination for arm library clones *in vitro*, the *lacZ*/*loxP* fragment lost as a result of recombination was added back to randomly chosen clones (Figure 4A.3). The *loxP*/*lacZ* 3.7 kb fragment was isolated from pSV-β-Gal + *loxP* by HindIII/XbaI restriction digest. After treatment with Klenow, the *lacZ*/*loxP* fragment was blunt-end ligated into arm library clones filled-in following NdeI digest. Reconstructed clones were propagated in DH5α. Plasmid DNA from reconstructed clones was isolated via QIAprep spin miniprep column (Qiagen) and eluted in EB buffer (10 mM Tris–HCl, pH 8.5). *In vitro* reactions consisted of 0.1 µg of DNA, 1 U of MBP-Cre extract and 3 µl of 10 × Cre buffer (500 mM Tris–HCl, pH 7.5; 330 mM NaCl and 110 mM $MgCl_2$) in a 30 µl total volume. Reactions were incubated at 37°C for 15 min and then transferred for storage at −20°C. Recombination was detected by blue/white color screen in DH5α following transformation of 2 µl of the *in vitro* reaction.

Sequencing

All spacer library clones were sequenced with primers PLK 5′ TAAATGAGCATCCATGTTGG 3′ and M13F-pUC(−40) 5′ GTTTTCCCAGTCACGAC 3′. All arm library clones and reconstructed arm library clones were

sequenced with the pSVβ 5′ CGACTGGAAAGCGG CAGTG 3′ primer. The arm library *loxP* site was sequenced with the pQEPromotor 5′ CCCGAAAAG TGCCACCTG 3′ primer. The pMAL-Cre expression plasmid was sequenced with primers pTYB11 #5 5′ GG TCGAAATCAGTGCGTTCG 3′, pTYB11 #4 5′ CGAG TTGATAGCTGGCTGGT 3′ and pTYB11 #3 5′ CGAACGCACTGATTTCGACC 3′.

Library sequence analysis

Sequence pools from both the spacer and arm libraries were subject to χ^2 analysis: $\chi^2 = \sum \frac{(O-E)^2}{E}$ where O is the observed nucleotide value and E is the expected nucleotide value.

Purification of Cre recombinase

Active Cre recombinase was purified through a maltose-binding protein (MBP) tag previously described by Kolb and Siddel (26). The *cre* gene was PCR amplified from the pMC-Cre plasmid (27) with primers Cre ATG 5′ ATGT CCAATTTACTGACCGTACACC 3′ and Cre2 5′ GGT GGTCTCGAGCTAATCGCCATCTTCCAGCAGGCG 3′, digested with XhoI and cloned in frame into the pMAL –c2x vector (New England Biolabs) digested with SalI. pMAL-Cre was then transformed into *E. coli* K12 ER2508 (New England Biolabs) for expression.

Three hours after induction of a 1 l culture, bacteria were harvested by centrifugation and resuspended in 80 ml of column buffer (20 mM Tris–HCl, pH 7.4; 400 mM NaCl and 1 mM EDTA) and then frozen at −20°C. Cells were lysed by 3 cycles of freeze/thaw using an ice water bath. MBP-Cre was purified by affinity chromatography on an amylose resin column following New England Biolabs' product manual (pMAL Protein Fusion and Purification System Instruction Manual V. 5.1). Storage buffer (100% glycerol; 1 mM EDTA and 30 mM Tris–HCl, pH 7.5) was added on a 1:1 ratio to aliquots followed by storage at −20°C.

For our purposes, 1 U of purified recombinase is defined as the amount of enzyme necessary for maximum recombination after a 15 min incubation at 37°C of either 75 ng of spacer library control (pLK + $LoxP^2$) or 0.1 μg of the arm library control plasmid (pSV-β-Gal + $LoxP^2$) in a 30 μl total reaction volume.

RESULTS

In vivo recombination of a spacer library

What are the sequence requirements for functional *lox* spacers? In order to investigate what constitutes a functional spacer sequence, we created two randomized spacer libraries from template oligonucleotides consisting of two *loxP* arms separated by an 8 (N) randomized spacer region (Figure 1A). Since it was already known that the most efficient recombination events usually take place between *lox* sites with matching spacer sequences (22), we reiterated the library sites in order to test the function between matching spacer pairs (Figure 1B).

Fifty-five clones (40 from group one and 15 from group two) were randomly chosen for reiteration. Following reiteration, clones were sequenced at each *lox* site and then passaged through 294-CRE *E. coli* cells, which constitutively express Cre recombinase. Successful site-specific recombination events resulted in the deletion of the kanamycin resistance gene (Figure 1B). Restriction digests with XmnI linearized both parental (5026 bp) and recombined plasmids (3707 bp). Clones exhibited either complete recombination, a mixture of parental and recombined plasmids, or no recombination product at all (Figures 2 and 3). In addition, two of the clones (#41 & #205) had an unknown product along with their recombination product (data not shown).

Sequencing revealed that not all clones contained *lox* sites with matching spacers (Figure 3). A subset of unmatched spacers was cloned for comparison to the matched spacer pairs. The remaining unmatched pairs were the result of the cloning process and were also included for comparison.

All clones with full-length matching spacers (33/33) recombined to completion with no parental plasmid detected. Many clones with unmatched spacers also recombined to completion (12/21) or had some visible recombination product (6/21). Clone #23 contained shortened 7 bp matching spacers and no observable recombination product. Overall, this *in vivo* assay for recombination appears to differentiate, in some cases, between matched and unmatched spacer pairs. It does not provide information on the efficiency of recombination between the various spacer pairs, which recombined to completion *in vivo*.

Sequence analysis of spacer library clones

χ^2 analysis of all unique library sequences indicated that there were positions in both template libraries that were not randomly represented in terms of nucleotide frequency. Instead of each nucleotide having a frequency of 25% at each of the 8 positions of the spacer, positions 2, 3, 5, 7 and 8 in the first library all had significantly unequal base distribution (Supplementary Figure 1). The second library contained only one position (6), which had statistically unequal nucleotide distribution. This means that these libraries do not represent statistically random sequence populations. The non-random base distributions could be a result of the oligonucleotide manufacturing process and/or the manipulations (PCR, cloning and propagation in *E. coli*) necessary to clone the library oligonucleotide (28).

In vitro recombination of a randomized spacer library

The *in vivo* analysis of the spacer library did not demonstrate any differences in recombination based on nucleotide composition at specific positions within the spacer. Therefore, we assayed for recombination *in vitro* in an attempt to better distinguish functional diversity due to variation in spacer sequences. All spacer library clones were tested for function *in vitro* using purified Cre recombinase. Standard reactions were allowed to proceed for 15 min at 37°C. Recombination was detected by PCR followed by SYBR Green I staining of the PCR product and quantitation of band intensities on an agarose gel.

clone	5′ *lox* site spacer	3′ *lox* site spacer	*in vivo* products	% recombination *in vitro*
LoxP	GCATACAT	same	F	70.4 ± 2.0
39	GCAGTGGT	same	F	54.7 ± 10.0
33	TCTGTGGC	same	F	48.1 ± 5.1
9	TTGTTCGG	same	F	47.1 ± 10.3
28	CCGTTGCG	same	F	45.2 ± 2.8
14	ACTAAAGC	same	F	37.9 ± 2.6
29	AGAATCAT	same	F	34.0 ± 10.3
592	GGACGCGG	same	F	33.1 ± 3.5
64	GCGGTTTA	same	F	27.6 ± 6.0
63	AGGAGGTA	same	F	24.5 ± 7.3
37	CAGACGGC	same	F	22.7 ± 2.6
25	CGGATTGG	same	F	19.5 ± 10.6
4	TTGCTGAG	same	F	18.1 ± 16.9
10	GCATGTCT	same	F	17.8 ± 5.3
412	ACGCGATC	same	F	17.7 ± 7.6
7	AGGAGGCG	same	F	16.9 ± 13.4
512	GGGTGGCG	same	F	16.3 ± 1.6
17	TGTGGGCG	same	F	7.5 ± 4.2
6	GGGCCGTG	same	F	6.2 ± 1.1
21	GGCAATAG	same	F	5.9 ± 8.3
3	TTCCGGTG	same	F	1.7 ± 1.6
23	TAGTGCG*	same	N	0.0 ± 0.0
42	TGTGGGCG	TGTTGGCG	F	22.0 ± 3.7
20	CTACACGG	GGGCAGGT	F	18.9 ± 2.7
57	GTAGGAGG	TTGAAAGC	F	14.1 ± 5.8
56	AGTGCCGG	GGGTGGCG	M	12.6 ± 6.9
55	GTCGCCTG	CGGGTAGG	F	5.2 ± 1.1
38	AGGCGTGC	ATCAGCGC	F	2.8 ± 0.7
8	TATTCTTC	GCCTGTTT	F	2.6 ± 0.1
53	TTTGTGCG	GGGGCACT	M	2.4 ± 1.1
49	CCGCGGG*	GTCGCCTG	F	2.4 ± 1.8
51	GGGTGGCG	AGTGCCGG	N	1.6 ± 1.1
41	GTGAGCGA	ACGCGATC	M	1.2 ± 1.1
47	TACCGTGG	GCGCGGGG	M	0.9 ± 0.2
60	TGGAATAA	GCGGGCGG	N	0.8 ± 0.6
30	TGGGATGG	CTTAAAAT	F	0.6 ± 0.8
48	GGGGCACT	TTTGTGCG	F	0.6 ± 0.4
59	GGACGCGG	GTGGGCTG	M	0.4 ± 0.6
24	CAGCGGCT	TCTATCAG	F	0.3 ± 0.5
19	GGCCTACT	CCATGTAT	N	0.0 ± 0.0
492	CGGGTAGG	CCGCGGG*	F	0.0 ± 0.0
206	GTCATGTA	same	F	56.7 ± 2.8
270	CCAGAATG	same	F	53.7 ± 1.9
271	CAAATCAT	same	F	52.3 ± 3.7
268	GCAGGATT	same	F	51.9 ± 4.2
267	GTATACTT	same	F	50.7 ± 3.1
207	TCTTCAAT	same	F	45.7 ± 6.2
269	TTCGAACT	same	F	42.9 ± 10.1
265	TAAGTATC	same	F	39.6 ± 8.9
202	GTGTGCAA	same	F	32.6 ± 9.0
203	CGTGACGC	same	F	32.2 ± 2.1
208	TAAAAGCC	same	F	23.4 ± 4.7
266	CATAAATG	same	F	13.1 ± 4.0
204	GAACGGTC	same	F	9.1 ± 4.2
201	CAGGAAAG	TACCACAT	F	4.6 ± 2.3
205	CTAATCTA	GGATTAAA	M	2.6 ± 0.5

Figure 3. Spacer library sequences. The sequences of spacers for clones from the first and second library template oligonucleotides are shown in descending order of percent *in vitro* recombination. Clones from the second library template oligonucleotide are given below the dotted line. Spacer sequences for the *lox* sites 5′ to the kanamycin resistance marker and 3′ of the kanamycin marker are given. For clones with matching *lox* spacers only the 5′ site sequence is shown. For clones with unmatched spacers the sequence of both sites is shown. Spacers containing only 7 bases are marked with asterisks (*). Spacer sequences that appear more than once are underlined. The type of recombination products observed for *in vivo* reactions is noted in the middle column. All clones displayed either full recombination (F), a mixture of parental and recombination products (M) or no recombination products (N). The average percent recombination for *in vitro* Cre reactions is shown for each clone. The average and SD were calculated from three separate *in vitro* experiments.

Figure 3 contains the averaged results of three separate *in vitro* recombination experiments.

The amount of Cre used in these reactions was titrated to give maximum recombination for the *loxP* control (pLK + $LoxP^2$). Due to the reversible nature of the Cre/*loxP* system, there is less than 100% recombination for an *in vitro* deletion reaction irrespective of reaction time (29,30). The 70.4% maximal recombination achieved here mirrors previously published data for *in vitro* Cre reactions (29,30).

Compared to the recombination results in the 294-CRE bacterial system, the *in vitro* system appears to be more stringent in terms of the types of recombination events allowed and the amount of recombination product produced. For example, whereas the range of average recombination *in vitro* for the set of matching spacer clones was 56.7–1.7%, these clones were indistinguishable from one another *in vivo*. Non-matching spacer clones that had functioned *in vivo* did not necessarily function well *in vitro*; 7 of 12 clones that showed full recombination *in vivo* had <3.0% recombination under our *in vitro* conditions.

Therefore, not all clones that appeared to be as functional as the wild-type *loxP* control *in vivo* were equivalent when compared *in vitro*. The *loxP* control averaged 70.4% *in vitro* recombination while the best library clone averaged 56.7%. Of the clones with full-length matching spacers (33), the group averaged 30.5% *in vitro* recombination, while clones with non-matching spacers averaged 4.6%.

These results suggest that *in vitro* Cre reactions are much less permissive in the types of spacer pairs that can undergo successful recombination compared to *in vivo* 294-CRE reactions. Matching spacer pairs are favored in recombination events, and while *in vivo* it is difficult to see any difference in recombination across sequences, the *in vitro* environment clearly discriminates between spacer sequences. Further analysis of these sequences is hampered by the small number of clones sampled.

In vivo recombination of an arm library

Our second basic question addressed what arm sequences the Cre recombinase can use to facilitate site-specific recombination events. Cre is known to bind cooperatively to its recognition sequence (31,32), and its contact positions within the arms have been mapped (33,34). By randomizing only one arm out of the four that make-up the Cre/*lox* recombination complex, we could study what minimal functional sequences Cre can co-operatively bind and recombine.

In order to easily screen large numbers of randomized arm library sites, we constructed a plasmid in which color selection could be used to visualize recombination events in a bacterial system. A template oligonucleotide containing one *loxP* arm plus the *loxP* spacer followed by a 13 N randomized region was used to create the arm library (Figure 4B). Once the template was made double stranded through PCR, it was cloned into the β-galactosidase reporter construct pSV-β-Gal + *LoxP*. This reporter construct had been modified with a *loxP* site at the

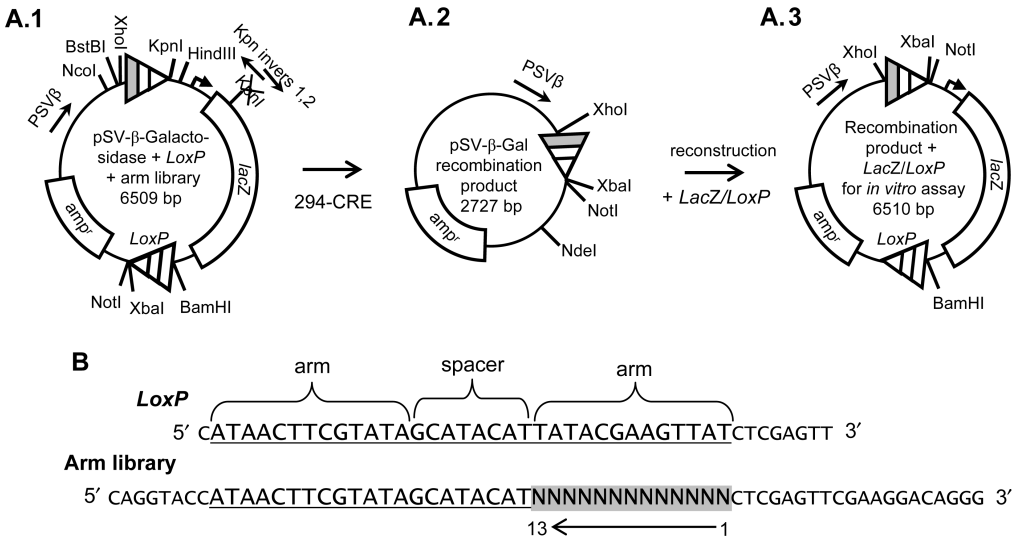

Figure 4. Arm library construction. (**A.1**) Plasmid pSV-β-Galactosidase with a *loxP* site at NotI/BamHI (segmented triangle) and the arm library (segmented triangle, degenerate arm shaded) PCR product cloned within the added NcoI/HindIII linker as a BstBI/KpnI fragment. Arrows depict the positions of primers for inverse PCR (Kpn invers 1 and 2) and sequencing (PSVβ). (**A.2**) The result of Cre-mediated site-specific recombination is a 2727 bp plasmid containing the library arm within its *lox* site. Selected recombination products were chosen to be rebuilt by adding back the *lac*Z/*loxP* segment lost to recombination. (**A.3**) The resulting construct was then used in *in vitro* recombination reactions. (**B**) Sequences of the control *loxP* library site and the arm library template are shown. *LoxP* sequences are underlined. The degenerate arm sequence is shaded and a number is given to each base. Position 1 starts with the 3′ nucleotide of the arm and continues towards the 5′ end in order to position 13. amp^r = gene for β-lactamase, *lac*Z = gene encoding β-galactosidase.

3′ end of the *lac*Z coding region. With the arm library site in place at the 5′ end, the *lac*Z gene was flanked by *lox* sites (Figure 4A.1). Following transformation into 294-CRE cells, any site-specific recombination would result in removal of *lac*Z and loss of β-galactosidase activity (Figure 4A.2).

Hundreds of colonies were screened by blue/white color selection via seeding of transformations on LB/X-gal agar plates. After overnight growth at 37°C, ~87.9% of colonies were stained dark blue, 3.5% were white with blue centers (white w/blue) while 8.6% were white only (data not shown). The pSV-β-Gal + *LoxP*[2] control in 294-CRE resulted in white colonies only, while blue-stained colonies were observed for both control and arm library constructs in Cre− DH5α *E. coli*.

It was assumed that blue colonies would contain only unrecombined, non-functional arm library sites, while white colonies would be the result of successful site-specific recombination between functional *lox* sites. White colonies with blue centers were unexpected, but could be explained by a slower rate of recombination, relative to the phenotypically white colonies, for the cells that seeded these colonies.

Upon examination of plasmid DNA from each type of colony (Figure 5), it appeared that all colonies contained DNA that had undergone successful recombination events. Preliminary analysis showed that all plasmid DNAs linearized with XhoI were the size of the deletion product. However, full-length parental product could be visualized for blue colonies after overloading the restriction samples on an agarose gel (data not shown). Retransforming blue colony DNA into DH5α (−Cre) cells resulted in a 1:75 ratio of blue to white colored colonies, pointing to a small population of unrecombined plasmid DNA present after ~48 h of growth in 294-CRE.

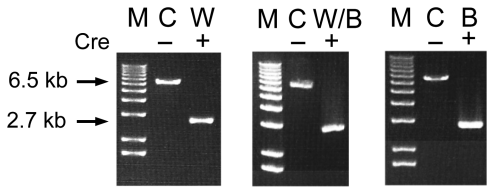

Figure 5. *In vivo* recombination. Arm library clones were chosen at random from 294-CRE colonies that resulted from the transformation of the pSV-β-Gal + *LoxP* + arm library ligation. Examples of XhoI restriction digests of plasmid DNAs visualized by agarose gel electrophoresis are shown. Full-length constructs were 6509 bp, while site-specific recombination resulted in a 2727 bp product. M = marker, C = control −Cre, W = white colony, W/B = white colony with blue center, B = blue colony.

It was unclear whether blue clones had single or multiple library constructs present within a single clone. If so, this could account for the continued presence of a full length, non-functional plasmid. To further investigate, two blue clones (#3 and #5) were chosen randomly for re-transformation of their plasmid DNA into DH5α followed by isolation and sequencing of resulting white and blue colony DNAs. Plasmids from these DH5α colonies were the expected sizes (blue colonies ~6 kb, white ~2.7 kb, data not shown). Sequencing revealed that both blue and white colonies were derived from the same arm library construct. It was concluded that each original 294-CRE blue clone contained a single arm library construct.

Sequencing was also used to identify clones, which might have multiple library constructs present. Background sequence seen on the sequence chromatograms only at the 13 base randomized arm region could be a result of more than one sequence present within a clone. Only 2 blue clones (#16 and #18) out of 37, along with 2 white with blue center clones (#5 and #12) out of 15, had strong evidence from their sequence chromatograms to suggest there might be multiple arm library constructs present in each clone. Evidence of unrecombined products could also be seen in sequence chromatograms, as slightly more than half (20 of 37) of the blue clones had strong background sequence at the recombination junction.

Finally, growth time could play a role in accumulation of recombination products and help explain the observed β-galactosidase activity in both blue and white with blue center colonies. If so, serial culturing of blue 294-CRE clones over several days would result in total loss of β-galactosidase activity. To test this, 10 blue colonies were chosen at random for extended serial culture. Following 5 days of continuous growth culture, colonies no longer stained positive for β-galactosidase when plated on LB agar containing X-gal (data not shown). The mechanism by which small numbers of unrecombined plasmids persist over time in the presence of the recombinase is unknown.

Sequence analysis of arm library clones

In total, 93 clones were sequenced (Figure 6). Forty-one sequences were from white colonies, 37 were from blue and 15 were from white colonies with blue centers. A random sequence population was derived from the ratios of blue:white:white w/blue colonies (~37:4:1) observed upon plating of the library (Supplementary Figure 2). χ^2 analysis of the random library sequence population revealed again that the base distributions at several positions of the library template oligonucleotide were not statistically 25% for each nucleotide (Supplementary Figure 3). Positions 1, 2, 4, 5, 8 and 9 had significantly skewed base frequencies. Again, these nucleotide frequencies could be due to the manufacture of the oligonucleotide and/or the cloning process.

Despite the non-random nature of the arm library template, it is still possible to analyze the sequences from the efficiently recombined white clones to the overall library population from a statistical standpoint. In order to compare the arm sequences of white clones to that of the random library population, the frequencies for the expected (E) base distribution in the χ^2 analysis [$\chi^2 = \Sigma((O-E)^2/E)$] were taken directly from the random sequence population calculated above.

χ^2 analysis on all 41 white clone arm sequences (Supplementary Figure 3) showed statistically significant nucleotide changes at 6 arm positions. Positions 1, 2, 3, 11, 12 and 13, translating into the first 3 and last 3 arm positions, were statistically different from the random set population. Comparison of the nucleotide frequencies at these 6 positions shows a trend towards the sequence 'ATA' at both ends of the library arm for white colony clones (Supplementary Figure 4). This 'ATA' sequence is also present in the wild-type *loxP* site. While 5 of the 6 biased positions are known contacts for the recombinase, position 3 has not been previously identified as an important position for functional arm sequences. These data indicate that there are preferences at the ends of the arm for efficiently recombined *lox* sites.

Though the total number of sequences in the white colonies with blue centers group is low for statistical analysis, it too shows significant nucleotide distribution changes at positions 11 and 12 (Supplementary Figure 3). Comparison of the nucleotide frequencies at these two positions shows a trend towards the sequence 'AT' (Supplementary Figure 5). This, along with the simple calculation of the average number of conserved bases (Figure 6) for each colony type group, points to a continuum for the number of conserved bases in an arm relative to its probable function. The more bases conserved overall, and the more bases conserved at the ends of the arm, the more likely an arm is to recombine efficiently in the 294-CRE system.

Arm library *in vitro* recombination

Several blue and white clones were chosen at random to be tested in *in vitro* Cre reactions. The process of adding back the *lacZ* gene plus the *loxP* site resulted in a construct (Figure 4A.3) that was almost identical to the pSV-β-Galactosidase + *LoxP* + arm library construct before it underwent recombination (Figure 4A.1). *In vitro* reactions were carried out as described for the spacer library except the amount of Cre input was titrated for maximum recombination of 0.1 μg of the arm library control, pSV-β-Gal + $LoxP^2$. Recombination was detected via color selection following transformation of 2 μl of the *in vitro* reaction into DH5α cells and plating on LB/X-gal agar.

On average, a total of 878 colonies were counted for each reconstructed clone encompassing three replicates of their *in vitro* reactions. The *loxP* control reaction reached a recombination level of $66.0 \pm 3.1\%$. Overall, there was a striking difference in recombination between constructs from white versus blue colonies (Figure 6). No reconstructed blue clone achieved >0.6% recombination *in vitro* on average, while a full range of total recombination (1.4–54.6%) was observed for white reconstructed clones.

These *in vitro* results are more in line with what was predicted for white and blue clones. Clearly, blue clone arm sequences do not support effective recombination *in vitro*, and as might be expected, average fewer conserved *loxP* bases (2.9, Figure 6). Supporting the idea that clones from white colonies with blue centers have characteristics that are part of a continuum between blue and white clones, their average number of conserved bases (4.9) falls between that of the blue and white clones. On average, white clone arms have almost twice as many conserved (both contact, 4.2 and non-contact, 5.4) bases compared to the blue clones.

It has already been noted that white clone arms tend to have conserved 'ATA' motifs at each end. Drawing precise conclusions about base conservation or preference in relation to percent recombination *in vitro* is precluded by

	White colony clones	contact bases	*loxP* bases	% *in vitro* recombination
LoxP	ATAACTTCGTATA	9	13	66.0 ± 3.1
1	ACTATGTCGCACT	4	6	27.4 ± 5.2
2	GAAAGGGGCTATA	5	6	50.3 ± 5.4
3	CTGCAATCTCATT	4	5	17.8 ± 1.9
4	GCACCAGCCTATC	5	6	10.9 ± 3.6
5	CAAAATCCGGAAA	4	7	7.7 ± 2.7
6	CTAGCCACAATAA	4	5	11.2 ± 3.8
7	ACAGAAACGCGTT	3	5	23.6 ± 5.0
8	AACAAGTCATATA	7	9	54.6 ± 3.9
10	CAAGTTTCAATAA	2	5	18.3 ± 2.8
11	AACCGATCCCCTC	3	4	1.4 ± 1.1
12	AAAACCCCCATAA	5	6	
14	CACTGGCCGTATC	4	5	
15	TTACTTGAACCAC	1	3	
16	CACCATGTGTATT	3	5	
17	TGGATCCGAAATA	4	4	
18	ATCACGATGACTT	5	6	
19	AATATATCAACTG	4	5	
20	AAACACGCCAACA	4	5	
21	TTGCCACGGCTAA	3	4	
22	CTCACTACCAAAA	6	7	
24	ATAACACACCAAT	5	6	
26	AACACCACCGATA	7	7	
27	AAGCAAGCCATTA	4	4	
28	CAACACAAAAATA	3	4	
29	TCTCCACACATAG	1	1	
30	TAAAAACGACATA	4	5	
31	ATAAACCCACTCA	5	6	
32	ATCGCAGTCTACG	5	5	
33	CTAAGGACGCATA	6	8	
35	ATGGGCCCAGATA	6	6	
37	ACAACCTCCCTTA	6	8	
38	CAAACACCCGGCA	4	5	
39	CCACCTACAGAGA	4	6	
40	TGAACGCAAGAAA	4	5	
42	ATACTTACACAAT	4	6	
43	AAACAATCCCATG	4	6	
44	ACAGCCTCCGGAGT	4	7	
45	AAAGCTCCTACTA	5	7	
46	AACCACAGTATAA	2	2	
47	AAAGCGCCCGGTA	5	6	
48	TTAGATAGCGAGA	3	5	
	Average	4.2	5.4	

	Blue colony clones	contact bases	*loxP* bases	% *in vitro* recombination
LoxP	ATAACTTCGTATA	9	13	66.0 ± 3.1
2	CGGAAGTCACTCA	3	4	
3	AACCTGAGCTAAC	3	3	0.5 ± 0.3
5	ACCGCAACCAAAC	4	4	0.2 ± 0.2
6	ATGTATAACACGG	2	3	0.2 ± 0.2
7	CAACACCACCGGC	0	1	
8	CCAGGAACGTGCG	2	4	0.6 ± 0.3
9	CCCAACACTACCG	2	2	0.2 ± 0.2
11	TGCAAAACCAGCA	3	3	0.1 ± 0.1
12	CATACTCGACGCG	2	3	0.0 ± 0.0
13	CCAAAACCAACGG	2	3	0.0 ± 0.0
14	CCGCGGAATTGCA	2	2	
15	ACTCCGCGACGAG	2	2	0.0 ± 0.0
16	CTGCCTGTGAAGA	4	6	
17	TATCATGTCCCCG	0	1	
18	AATCACGGCGCTC	2	2	
19	GGAAACACCTACC	4	5	
20	TACCCGCCAACGC	2	2	
21	ATTCCAGCCCCGC	4	4	
22	CCCTAAAAGCACG	1	2	
23	CAATCACACAGAA	2	3	
24	CACGCGCCAAGAA	3	3	
25	CCCCACTAGCCGT	0	2	
26	AATGGACGCAGCC	1	1	
27	GTGTACCCGTGGC	2	3	
29	AACACGCGGGATT	5	6	
30	AGACCGCCCAAAC	4	5	
34	GATAAACCCCCAA	3	3	
37	GATACTCTGCTCA	3	5	
39	TCCAAATAAACAA	2	3	
40	CCACTGGGCACCA	1	2	
42	CTAGGCATTGCAG	1	2	
43	TACCAAAGCCAGA	2	2	
45	ACACCAACCACAC	3	4	
47	ACTGGCGCGGCCC	1	2	
48	CACACACGAAAGG	3	3	
49	CGCACACACGGCA	3	3	
53	AACAAACCTCTAC	3	3	
	Average	2.3	2.9	

	White colony w/blue center clones	contact bases	*loxP* bases
LoxP	ATAACTTCGTATA	9	13
1	ACAACCACCGTTC	5	6
2	CAAGCGCAAAATA	4	5
3	AAAACCACGTATC	7	9
4	CACCCACCAAAGG	3	3
5	CACACCCTCCAGA	4	4
6	TAGCGCACAGACA	3	3
7	TAAGCCTATAATG	3	5
8	ATCGGCACGCCCA	4	5
9	TCTCGTAACCACA	2	3
10	ACAATAACGCGAA	4	6
11	CACGATCCCAACA	3	4
12	CGCGCTACAGAAA	4	5
13	ATAACATCCATCC	5	7
14	ACCTAGTAAAATT	3	4
15	TAAAGTCTCAATG	3	5
	Average	3.8	4.9

Figure 6. Arm library sequences. The sequence of the randomized arm for each clone's arm library site is shown. As written, these sequences are the reverse complement of the randomized oligonucleotide detailed in Figure 4B. Clones are grouped by colony color. Bases in common with *loxP* are in green. Contact bases are in red. The number of contacts and bases in common with *loxP* is calculated to the right. Percent *in vitro* recombination is shown for clones that were reconstructed. The percent recombination is calculated based on three separate *in vitro* experiments.

the small overall sample size. However, it is interesting to note that the two best performing arms tested (White #2 and #8) maintained a conserved 'TATA' sequence next to the spacer.

DISCUSSION

We have demonstrated the utility of a randomized library approach to the understanding of the nature of the functional domains of the Cre recombinase recognition sequence, as well as the distinct differences in function of *lox* sequences between *in vivo* and *in vitro* environments. Although this study did not test a large sampling of all sequences possible in each library (spacer = 4^8 = 65 536 and arm = 4^{13} = 67 108 864), its results are significant. By electing to randomize whole sections of the *lox* site, we have observed a great range of functional sequences, function tied to recombination environment

and the first unbiased, systematic evaluation of *lox* arm sequences.

Two previous studies have concentrated on the sequence of the spacer region and its role in recombination efficiency. Lee and Saito (22) undertook the task of creating single and double-base mutations of the spacer and noted variability in the recombination efficiency across mutants. Several of their observations are supported by our work such as low or undetectable recombination *in vitro* between unmatched spacers, variable recombination between like mutant spacers, and no mutant spacer with equal or greater recombination efficiency than the wild-type *loxP* spacer.

Our study is, however, in conflict with a published report from Missirlis *et al.* (24). While we agree that mutant spacers and sites with mismatched spacers do recombine, our observations do not substantiate Missirlis *et al.*'s conclusion that a G-rich spacer is the most favored for recombination or that self-recombination is not significantly greater than recombination with other spacers. Though we cannot rule out the possibility of base preferences within the spacer for efficiently recombined sites, G-rich spacers were not the most effective in our data set.

We observed frequency imbalances in our libraries that were the result of the oligonucleotide manufacturing process and/or the cloning of the library. These biases were not from clonal selection since all clones in our study were chosen at random for further manipulation. By sequencing only successful recombination products, Missirlis *et al.* leave their study open to the possibility that their analyzed pool of products did not originate from a truly random population. Without published analysis of the pool of clones created from the PCR and ligation of the input oligonucleotides, it is unclear whether their observations are the result of nucleotide frequency biases present in the library clones or true base preferences of the Cre recombinase.

Second, we believe our observations support the general conclusion that recombination between matching spacers is more efficient *in vitro* and more likely to proceed to completion *in vivo* than recombination between mismatched spacer pairs. Based on our observations, we believe that Missirlis *et al.*'s conclusions based on equating efficiency of recombination with the number of times a recombination event appears in a shotgun library *in vitro* experiment could be misleading. This could easily be determined by testing Missirlis *et al.*'s spacer sequences in traditional *in vitro* and *in vivo* assays.

In our data set, the non-matching spacer clone (#42) with the highest percent recombination *in vitro* (22.0%) contained sites with homologous spacers with the exception of one position. The remaining unmatched spacer clones all had <20% recombination *in vitro*, compared to 21 of 33 matching spacer clones with >20% recombination. All matched spacer pairs recombined to completion *in vivo* compared to 12 of 21 unmatched.

This is the first study to report on a fully randomized (8N) spacer library. Results from this study show there to be great sequence plasticity for successfully recombined spacers. In past work, importance was given to the role of the central 'TA' dinucleotide as critical for recombination (19,22). Our work demonstrates that a central 'TA' is not necessary to achieve >45% recombination *in vitro* or complete recombination in 294-CRE. Also, complete identity through the central six bases is not required for recombination as previously thought (22). Unfortunately, our sample set was too small to statistically evaluate for base preferences in efficiently recombined spacers.

Results from both spacer and arm libraries suggest that recombination in the 294-CRE strain of *E. coli* is robust compared to *in vitro* recombinase reactions. Whether recombination is aided by replication and/or endogenous *E. coli* factors is unclear. This is an important issue to consider when testing for the function of new *lox* sites or libraries, as recombination in 294-CRE may not transfer well to the *in vitro* environment. It should also be noted that recombination efficiency in other *in vivo* systems might differ significantly from both the *E. coli* and *in vitro* systems tested here.

Though our results from the spacer library study were not unexpected, the fact that our arm library demonstrated effective recombination with the presence of one randomized arm was surprising. It appears that an arm sequence with as few as one conserved *loxP* base can comprise 1 of the 4 *lox* arm sequences necessary for recombination in 294-CRE, and as few as 4 of 13 conserved bases were sufficient for recombination *in vitro*. This could be explained by Cre's cooperative binding properties, where three bound recombinase monomers might be able to recruit the final subunit to the complex leading to recombination in the absence of a fourth arm sequence that has any resemblance to *loxP*.

Our arm library testing system proved useful in the screening of hundreds of clones at once and in identifying the minimal arm sequences required for recombination. Despite not working with a truly random library, statistics reveal nucleotide preferences at 6 out of 13 arm positions for efficiently recombined clones. The 'ATA' motif at both ends of the arm was overrepresented in sequences from efficiently recombined clones. Though other studies have looked at the effects of base mutations on the arms (35,36), Sauer (20) was the first to speculate on the importance the 'TATA' sequence flanking the spacer region. Ours is the first study to find evidence of base preferences for functional arm sequences and disproves the notion that bases at the outer end of the arm are not important for efficient recombination.

Clearly, the results from both libraries must be interpreted with the fact that they were tested in the context of the wild-type *loxP* sequence. The arm library in particular identifies the weakest arm sequences that can be tolerated in an otherwise wild-type reaction. Mutations in the *loxP* arm or spacer coupled to the fully randomized arm might exert greater selective pressure to conserve *loxP* bases in the library arm.

In the future, we plan to continue to test the function of various *lox* site libraries. How mutations in the spacer and in both arms combine to affect function has not been explored. Information from this study and future work would be of use in the expansion of the library of

functional *lox* sequences, the search for functional endogenous *lox* sequences in genomes, finding non-compatible *lox* site pairs, and the creation of sites capable of stable recombination events.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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