Mutually exclusive recombination of wild-type and mutant *loxP* sites *in vivo* facilitates transposon-mediated deletions from both ends of genomic DNA in PACs

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ABSTRACT

Recombination of wild-type and mutant *loxP* sites mediated by wild-type Cre protein was analyzed *in vivo* using a sensitive phage P1 transduction assay. Contrary to some earlier reports, recombination between *loxP* sites was found to be highly specific: a *loxP* site recombined *in vivo* only with another of identical sequence, with no crossover recombination either between a wild-type and mutant site; or between two different mutant sites tested. Mutant *loxP* sites of identical sequence recombined as efficiently as wild-type. The highly specific and efficient recombination of mutant *loxP* sites *in vivo* helped in developing a procedure to progressively truncate DNA from either end of large genomic inserts in P1-derived artificial chromosomes (PACs) using transposons that carry either a wild-type or mutant *loxP* sequence. PAC libraries of human DNA were constructed with inserts flanked by a wild-type and one of the two mutant *loxP* sites, and deletions from both ends generated in clones using newly constructed wild-type and mutant *loxP* transposons. Analysis of the results provides new insight into the very large co-integrates formed during P1 transduction of plasmids with *loxP* sites: a model with tri- and possibly multimeric co-integrates comprising the PAC plasmid, phage DNA, and transposon plasmid(s) as intermediates in the cell appears best to fit the data. The ability to truncate a large piece of DNA from both ends is likely to facilitate functionally mapping gene boundaries more efficiently, and make available precisely trimmed genes in their chromosomal contexts for therapeutic applications.

INTRODUCTION

Large insert DNA clones propagated in bacteria or yeast have played a pivotal role in sequencing and localizing genes on a physical map of the human genome (1–4). Unfortunately, these resources could not be used directly to pursue functional studies in human cells as they lacked mammalian cell-responsive control or reporter elements. Much effort has therefore gone into either retrofitting clones from such libraries to make them amenable to analysis in mammalian cells (5–12) or alternatively, reconstruct genomic libraries in shuttle vectors that can be propagated in both bacterial and human cells (13–17). Progressive deletions from an end of DNA inserts in bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PACs) using a *loxP* transposon have been described previously (18,19) and used in mapping genetic markers on a physical map of the chromosome (19). The ability to truncate genomic DNA from both ends should greatly facilitate mapping transcription regulatory sequences that sometimes operate over large distances, define gene boundaries and make available precisely trimmed genes in their chromosomal contexts for numerous applications. The adaptability of the deletion mapping procedure to truncate DNA from both ends using wild-type and mutant *loxP* transposons was therefore explored.

Cre-recombination of wild-type and several single base substitution mutants have shown that a *loxP* site recombines only with its identical copy barring a few exceptions (20). The recombination can tolerate base changes in the 8 bp spacer region, including double base substitutions, but identical pairs were required for the reaction *in vitro* (21). Recent studies in cells, however, arrived at a different conclusion: a tagged wild-type Cre protein recombined a wild-type *loxP* site containing plasmid to wild-type and mutant *loxP* 511 sites flanking insert DNA in a BAC with equal efficiency (10). Another report analyzing several *loxP* mutant sites described cross-recombination between wild-type and mutant ones, including the two used here, to be in the range of 1–12% (22). Although several

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BAC libraries with wild-type and mutant *loxP* 511 sites flanking genomic DNA have become available (23,24), truncation of insert DNA from both ends has not been reported. Effort was therefore directed first on resolving the dilemma over recombining wild-type and mutant *loxP* sites with wild-type Cre protein. The results were then used to develop a procedure to delete large insert DNA from both ends in several PAC clones.

MATERIALS AND METHODS

Construction of pJCPAC-Mam2

The oligodeoxyribonucleotides d (GGCCGCATAA CTTTCGT-ATAATGTGTACTATACGAAGTTATGTTTAAACGC) and d (GGCCGCGTTTAAACATAA CTTTCGTATAGTACACAT-TATACGAAGTTATGC) were annealed to create the mutant *loxP**-1 site.

The oligodeoxyribonucleotides d (GGCCGCATAA CTTTCGTATAAAGTATCCTATACGAAGTTATGTTTAAACGC) and d (GGCCGCGTTTAAACATAA CTTTCGTATAGGATACTTTATACGAAGTTATGC) were annealed to create the mutant *loxP**-2 site. The two sites *loxP**-1 and -2 refer to mutant *loxP* sites 5171 and 2272, respectively, described earlier as better 'exclusive' mutants showing efficient recombination *in vitro* using Cre-containing mammalian cell extracts (21).

A PmeI site was built into each oligodeoxyribonucleotide, and NotI overhangs were generated upon annealing. The dephosphorylated oligodeoxyribonucleotides were ligated into the unique NotI site in pJCPAC-Mam1 [see Figure 1 of Ref. (16)]. The two vectors with a wild-type and one of the two different mutant *loxP* sites (*loxP**-1 or *loxP**-2) in the same orientation and flanking the BamHI site were named pJCPAC-Mam2A and pJCPAC-Mam2B, respectively. New libraries of human DNA isolated from a foreskin fibroblast cell line (Viomed, Minnetonka, MN) were made in these vectors. Details of the library will be described elsewhere.

Construction of the transposon plasmids pTnloxPwt, pTnloxP*-1 and pTnloxP*-2

The markerless transposon plasmid pTnMarkerless2 described previously (25) served as the starting point of pTnloxPwt. The plasmid DNA was linearized at the unique BglIII site, filled in with Klenow polymerase, and ligated to the blunt-ended fragment Epstein-Barr nuclear antigen-origin of replication P (EBNA-ori P) used earlier (16). The resulting plasmid is 11 kb in size. Transpositions of pTnloxPwt were selected by P1 headful packaging as described in detail elsewhere (25). All plasmids were propagated in NS3516 cells (laqI^q) to prevent activation of the transposase gene.

The transposon plasmid pTnSpliceTerminator (AT) (P. K. Chatterjee, unpublished data) served as the starting point for both mutant *loxP* site-containing plasmids. The rabbit B-globin 3' terminal exon splice acceptor cassette was removed by NotI digestion, and into it was ligated a *loxP**-tetracycline resistance gene cassette excised from plasmid pZT344loxP*Tet (J. S. Coren, unpublished data). The two mutant *loxP**Tn plasmids contain the chloramphenicol resistance gene and a PGKpuromycin resistance gene on one side of the mutant *loxP* site, and the tetracycline resistance gene on

the other side. The location of the tetracycline resistance gene with respect to the mutant *loxP* site ensures tetracycline resistance only in inversions generated from the transposition events. Both deletions as well as inversions with pTnloxP*-1 and pTnloxP*-2 were therefore selected using the chloramphenicol resistance marker.

Generation of nested deletions in individual PAC clones

The clones with large inserts were identified in the pilot libraries generated in pJCPAC-Mam2A and pJCPAC-Mam2B by field inversion gel electrophoresis (FIGE) after NotI digestion. Nested deletions using pTnloxPwt were generated in several clones as described previously (25). Deletions with pTnloxP*-1 and pTnloxP*-2 that contain an antibiotic resistance marker to score for transpositions were generated as described previously (26).

End-sequencing of PAC deletion clones

Typically, miniprep DNA was isolated from 60 clones picked randomly from the several hundred to a thousand member PAC deletion library. Of these, ~60% were of unique size on FIGE. The DNA of 20 clones from each deletion series was sequenced directly using a transposon-end primer (19) and Big Dye Terminator chemistry on an ABI-3100 AVANT genetic analyzer. The primer extended products were purified using Magnesil (Promega Corporation) according to the manufacturer's procedures as described previously (27). New primers used to sequence the newly created ends of deletions from the mutant *loxP* side of insert DNA already trimmed from the wild-type *loxP*-end are listed below:

- Seq 11 d (CTTCCATGTCTGGCAGAATGC)
- Seq 12 d (GTTTCATCATGCCGTCTGTGATG)
- Seq 13 d (CGCTGGCGATTTCAGGTTTCATC)
- Seq 14 d (CAAGGCGACAAGGTGCTGATG)

RESULTS

Construction of PAC clones with human DNA inserts flanked by wild-type and mutant *loxP* site

The PAC cloning vector pJCPAC-Mam1 described previously (15) was linearized at its unique NotI site and ligated to two versions of a mutant *loxP* site to generate pJCPAC-Mam2A and pJCPAC-Mam2B as described in Materials and Methods. PAC libraries of 80–140 kb size-selected human genomic DNA were constructed in these shuttle vectors and details will be reported elsewhere (J. S. Coren, manuscript in preparation).

Analysis of recombination of wild-type and mutant *loxP* site-containing plasmids *in vivo* using phage P1 transduction

Several studies have analyzed recombination between wild-type and mutant *loxP* sites with Cre protein using both non-native systems in cells (10,20,22) and *in vitro* protocols with mammalian cell extracts containing Cre protein (21), with mixed results. Certain mutant *loxP* sites such as *loxP* 511 and *loxP* 512, as well 5171 and 2272 (referred here as *loxP**-1 and -2, respectively), produced conflicting results in these studies. Success in creating deletions specific to

Figure 1. (A) A schematic representation of P1 transduction of Tn plasmids. The phage DNA circularizes upon entering the cell by Cre recombination of its terminally redundant *loxP* sites and forms co-integrates with the transposon plasmid if the *loxP* site carried by it is also wild-type. Co-integrate DNA is cleaved at the 'pac site', and packaging occurs in the direction shown by the thin arrow adjacent to the 'pac site'. The transduced plasmid is recovered when the phage containing Tn plasmid DNA infects new NS3516 cells. Mutant *loxP* site-containing Tn plasmids pTnloxP*-1 and -2 fail to form co-integrates with the wild-type *loxP* site-containing phage DNA under identical conditions. The recovery of the mutant *loxP* site containing transposon plasmids, if any, is taken as a measure of cross-recombination activity between mutant and wild-type *loxP* sites. (B) New transposon plasmids containing wild-type or mutant *loxP* sites. A schematic representation of the transposon plasmids constructed for bi-directional deletions is shown. The transposon plasmid pTnloxPwt has no antibiotic resistance conferring marker within the transposing part of the DNA marked by the small rectangular boxes. The wild-type and mutant *loxP* sites are indicated by bold and broken arrows, respectively.

each end of insert DNA in BACs and PACs in a controlled manner requires that there be no leakage or crossover recombination between wild-type and mutant *loxP* sites. The gel-based assay using ethidium bromide stain to detect product DNA bands of recombination (21) allows at best only a 20-fold

difference in signal to be registered. It was therefore essential to test the recombination cross-reactivity by an assay with greater sensitivity and broader range, and that also used conditions specifically developed for making progressive deletions from one end of insert DNA (18). Recombination

analysis was therefore extended to include transduction using phage P1.

The linear DNA within viable phage P1 is flanked by two copies of wild-type *loxP* sequence. P1 is also known to transduce with high efficiency a second plasmid with a wild-type *loxP* site (28–30). Its ability to transduce the Tn plasmids that carry either wild-type or mutant *loxP* sites was therefore tested to evaluate the recombination cross-reactivity between these sites (for illustration see Figure 1A). The three Tn plasmid-containing strains #17 with wild-type *loxP*, #18 with mutant *loxP**-1 and #19 with mutant *loxP**-2 were each infected with P1 vir phage, and the cell lysates were used to infect fresh NS3516 cells using procedures described previously (26). The NS3516 cells containing the phage-transduced Tn plasmids were selected in Luria–Bertani (LB) plates containing antibiotics specific to the Tn plasmid. The results shown in the top three rows of Table 1 clearly demonstrate that phage P1 vir is capable of transducing only the wild-type *loxP* site containing plasmid #17. The cross-recombination between the wild-type *loxP* site in P1 and either *loxP**-1 in #18 or *loxP**-2 in #19, that would have had to occur for their recovery, was found to be at least 1000-fold lower. Using a larger cell sample and multiple plates to compare relative efficiencies of transduction, we estimate that wild-type *loxP* sites recombine with one another at least 10 000-fold more efficiently than with either of the two mutant sites under these native *in vivo* conditions of transient Cre protein expression during P1 infection.

Generating progressive deletions from the mutant *loxP* end of DNA inserts with a transposon containing the same mutant *loxP* site

New transposon plasmids pTnloxP*-1 and pTnloxP*-2 (shown schematically in Figure 1B) were constructed as described in Materials and Methods and were tested initially on several

clones from the genomic libraries constructed in pJCPAC-Mam2A and pJCPAC-Mam2B. Detailed analyses on two clones JCPAC-9 and JCPAC-13, with 120 kb and 140 kb DNA inserts, respectively, are presented here. Thus, plasmids pTnloxP*-1 and pTnloxP*-2 were introduced into JCPAC-13 and JCPAC-9, respectively, and transposition was induced with isopropyl- β -D-thiogalactopyranoside as described previously (26). Transduction of the resulting deletions with P1 phage was considered not to be a hurdle as the PAC vectors contained a wild-type *loxP* site in addition to the mutant one. The results are shown in Figure 2, and schematic illustrations are provided in Figure 3A [see also Figure 1 of Refs (18,25)]. Because the starting PAC clones have a NotI site only at one end of the insert, digestion of their DNA with NotI enzyme produce no separate vector band (lanes 2 and 15). Deletions generated in JCPAC-9 and JCPAC-13 with pTnloxP*-2 and pTnloxP*-1 exchange one of the NotI sites in the PAC vector with one from the transposon (Figure 3A). Thus, no separate vector DNA band is seen in deletions either (lanes 3–11 and 16–26, Figure 2).

A set of deletions from either PAC was sequenced directly using a transposon-end-based primer (19). All deletions produced sequence reads that scored between 95 and 99% identity exclusively with one strand of the genomic DNA insert by BLAST analysis: JCPAC 9 and 13 deletions mapped to human chromosome 1 and 4, respectively. The end sequences also matched an order consistent with the size of the deletion clone in the array (data not shown). Taken together, the results therefore validate that deletions generated with the mutant *loxP* transposons truncate exclusively from the mutant *loxP* end of the insert DNA in the PAC clone. Deletions were made in four additional PACs with inserts mapping to chromosomes 10, 15 and 19 from the two libraries (a pair from each of the two libraries with inserts flanked by wild-type and either *loxP**1 or *loxP**2) with identical results.

Table 1. P1 transduction of plasmids carrying wild type and/or mutant *loxP* sites

Clone	Size (kb)	Marker	<i>LoxP</i> site	Transduction with phage P1	Transformed clones	After P1 transduction		
						kan	cam	tet
#17	12	camR	wt <i>loxP</i> only	++++				
#18	10	tetR	<i>loxP</i> *-1 only	0				
#19	10	tetR	<i>loxP</i> *-2 only	0				
#2	85	kanR	wt <i>loxP</i> only	++++	#2/17	++++	++++	N/A
					#2/18	++++	N/A	0
					#2/19	++++	N/A	0
#5	120	kanR	wt <i>loxP</i> only	0	#5/17	0	++++	N/A
					#5/18	0	N/A	0
					#5/19	0	N/A	0
#8	60	kanR	wt plus <i>loxP</i> *-2	++++	#8/17	++++	++++	N/A
					#8/18	++++	N/A	0
					#8/19	++++	N/A	++++
#9	120	kanR	wt plus <i>loxP</i> *-2	0	#9/17	0	++++	N/A
					#9/18	0	N/A	0
					#9/19	0	N/A	++
#12	60	kanR	wt plus <i>loxP</i> *-1	++++	#12/17	++++	++++	N/A
					#12/18	++++	N/A	++++
					#12/19	++++	N/A	0
#13	140	kanR	wt plus <i>loxP</i> *-1	0	#13/17	0	++++	N/A
					#13/18	0	N/A	++
					#13/19	0	N/A	0

0, 0–3 colonies/plate; ++, 50–100 colonies/plate; and +++, 500–1000 colonies/plate.

Figure 2. Progressive deletions from the mutant *loxP* end of genomic DNA insert generated with pTnloxP*-1 and pTnloxP*-2. DNA isolated from deletion clones generated with either pTnloxP*-1 or pTnloxP*-2 in clones JCPAC-13 and -9, respectively, were analyzed by PFGE after digestion with NotI enzyme. Lanes 2 and 15 show the starting JCPAC-9 and -13, respectively. Lanes 1 and 14 contain 5 kb ladder as standard size.

Closer inspection of lanes 9 and 10 of Figure 2 reveals DNA at the position of the 5 kb marker band, reminiscent of that from transposon plasmid DNA (19). Further analysis of these two deletion clones revealed that they were both ampicillin and tetracycline resistant, indicating that the mutant *loxP* transposon plasmid was indeed transduced by phage P1. This is contrary to what one might predict from the results discussed in the above section, and is addressed below.

A mutant *loxP* plasmid is efficiently transduced with phage P1 if a second plasmid in the cell has both a wild-type and the same mutant *loxP* site

It is plausible that the recovery of mutant *loxP* plasmids by P1 phage seen in lanes 9 and 10 of Figure 2 is due to a piggy-backing phenomenon. The following experiment tests the hypothesis. Unlike the experiments described above, the P1 transduction of mutant *loxP* plasmids was conducted this time around in the presence of a second large plasmid containing, in addition to a wild-type *loxP* site, (i) a mutant *loxP* site of the same kind, (ii) a mutant *loxP* site of a different kind and (iii) a mutant *loxP* site of the same kind but not as part of the same DNA molecule. The transposase gene was kept repressed in these experiments, and characterization of plasmid DNA isolated from transductions confirmed that no transpositions occurred. The results are summarized in Table 1.

Three different types of PAC plasmids were used: Clones 2 and 5 contained only a wild-type *loxP* sequence while clones 8, 9 and 12, 13 carried a wild-type and one of the two mutant *loxP* sites flanking the insert DNA (for descriptions see Table 1). The first member of each pair (2, 8 and 12) was less than P1 headful size, while the second member (5, 9 and 13) is >110 kb. Phage P1's ability to transduce each of these PAC plasmids was tested (Figure 1A). The results in Table 1, column 5 are in accordance with those described earlier. Namely, plasmids smaller than the capacity of a P1 head are efficiently transduced, while those larger than this limit size are not (18).

The ability of the three pairs of PAC plasmids to piggy-back the Tn plasmids #17, 18 and 19 upon transduction with phage P1 was next tested. Each Tn plasmid contains either a wild-type or a mutant *loxP* site and was transformed into each PAC clone. The transformed cells were selected on LB agar plates containing either kanamycin plus chloramphenicol (#17) or kanamycin plus tetracycline (#18 and #19). Single transformed colonies were expanded for experiments. Table 1 summarizes results of three independent experiments using different single colonies.

Although P1 could not transduce a mutant *loxP* plasmid directly (see Results), it can do so efficiently if there is another plasmid in the cell that contains both a wild-type and the same mutant *loxP* site (illustrated in Figure 5). This is seen for both 8/19 and 12/18 in Table 1. The cross-recombination between *loxP**-1 and -2 is non-existent, as shown by the results of 8/18 and 12/19. Piggy-backing requires not only the same mutant site on both PAC and Tn plasmid but occurs even when the PAC is too large to be transduced itself (compare results of 9/19 and 13/18 with 8/19 and 12/18). The efficiency is lower in that case. The exquisite recombination specificities of mutant and wild-type *loxP* sites seen with the native *in vivo* conditions used here are contrary to earlier reports (10,22).

The requirement that the mutant and wild-type *loxP* sites be part of the same molecule (see above) is demonstrated by the fact that PAC clones 2 and 5 with wild-type *loxP* are incapable of helping P1 phage transduce mutant *loxP* plasmids 18 or 19 (see results of 2/18 and 2/19), despite both 18 and 19 existing in the cell at 50–100 copies owing to their pBR322 origin of replication.

Generating deletions from both ends of insert DNA in two PAC clones

Having established that recombination between wild-type and mutant *loxP* sites by Cre protein is mutually exclusive, attention was refocused on generating bi-directional deletions of insert DNA in the PAC clones. Deletions from the wild-type *loxP* end of the genomic inserts in JCPAC-9 and -13 were made first for the following reason: transpositions of markerless transposons such as pTnloxPwt are selected by the ability of the P1 head to package both *loxP* sites from the deleted co-integrate and requires a larger than the P1 headful length (110 kb) starting PAC clone (25). Deletion libraries were generated in JCPAC-9 and -13 with pTnloxPwt using procedures identical to those described previously (25,26). A NotI digest of several clones from each deletion series is shown in Figure 4A. As shown schematically in Figure 3B, a deletion using pTnloxPwt introduces a NotI site in the clone such that the insert DNA is now flanked on either side by a NotI site.

Figure 3. (A) Schematic representations of deletions and inversions formed with pTnloxP*-1 and -2 in JCPAC-13 and -9. The transposon is shown as the triangle with the locations of NotI sites indicated. Note that inversions are isolated only if the starting PAC plasmid is <110 kb. (B) Schematic representations of sequential deletions generated first with pTnloxPwt and then with either pTnloxP*-1 or -2 in JCPAC-13 and -9, respectively. Note that all of the EBNA, ori P and PGKpuromycin gene cassettes are replenished after the bi-directional deletions so as to render the deletion clone effective in mammalian cells.

Thus, NotI digest of deletion clone DNA generates a 20 kb vector DNA band not seen in digests of starting PAC clones or any intra-insert deletions generated by illegitimate recombinations [for discussion see (25)]. Deletion-end sequencing of several clones and BLAST analysis revealed homology of all deletions from the same PAC clone to only one strand of DNA, and were arrayed according to their sizes.

The largest clone from each series was chosen for creating bi-directional deletions, and the results are shown in Figure 4B. Thus, clones 1 and 77 derived from JCPAC clones 9 and 13, respectively, were subjected to the nested deletion procedure

this time using the respective mutant *loxP* transposons. Plasmids pTnloxP*-1 and -2 were introduced into deletion clones 77 and 1, respectively, and deletions were generated using procedures identical to those described previously (26). NotI digests of DNA from several clones from either series is shown in Figure 4B. The locations of NotI and *loxP* sites in PAC and transposon (shown in Figures 1B and 3B) predict no change in the vector DNA band between starting and deletion clones.

A sizable fraction of isolated clones in the second step were inversions generated by transpositions of the mutant *loxP* site

Figure 4. (A) Deletions from the wild-type *loxP* end generated with pTnloxPwt in JCPAC-9 and -13. NotI digests of deletion clone DNA analyzed by FIGE. Lanes 1 and 17 contain starting JCPAC-9 and -13 DNA, respectively. Lane 16 shows a 5 kb ladder. (B) Deletions from the mutant *loxP* site end of deletion clones 1 and 77 generated with pTnloxP*-2 or -1, respectively. Deletion clones 1 and 77 were obtained earlier from JCPAC-9 and -13, respectively, by deleting with pTnloxPwt. FIGE analysis of NotI digested clone DNA. Lanes 1 and 18 contain the starting deletion clones 1 and 77, and lane 17 shows a 5 kb ladder.

in an orientation opposite to that in the PAC. Inversions were isolated this time around because the starting deletion clones 77 and 1 are less than P1 headful size. Such clones were tetracycline resistant (see Figure 3A) and are easily identified.

Sequencing ends of insert DNA truncated at both termini with transposon-based primers

While the first round of deletions from either the wild-type or mutant *loxP* end can be sequenced with transposon primers described previously (19), ends of inserts deleted from both ends required new primers from transposon regions that are unique to the newly created end. Thus, primers from within the chloramphenicol resistance gene specific to mutant *loxP* transposons were used to sequence the new ends of insert DNA and are described in Materials and Methods. All chloramphenicol-related sequences are deleted from pTnloxPwt used for truncating the other end.

DISCUSSION

The ability to truncate large pieces of DNA from both ends in a controlled manner should facilitate mapping gene regulatory sequences that act over large distances so as to be able to functionally define their boundaries and make available the entire genes in their chromosomal contexts for numerous applications. The highly specific recombination demonstrated by wild-type and the two mutant *loxP* sites with wild-type Cre protein under native *in vivo* conditions might become useful in the design of sequence selective tags and/or molecular switches in certain applications. Using a phage P1 transduction assay, to evaluate relative recombination efficiencies between wild-type and either of the two *loxP* mutant sites, a much higher degree of specificity has been demonstrated than was possible earlier with the gel-based assay (21). Also, the piggybacking of mutant *loxP* plasmids during P1 transduction offers a unique way to measure cross-recombination between two different mutant *loxP* sites without having to construct new phage carrying those sites (see Results). The differences between the results obtained *in vivo* here and those reported earlier (10,22) might be related to the transient versus constitutively expressed Cre protein available for the recombinations. *LoxP* site promiscuity does appear to increase with the level and persistence of Cre protein.

Recombining a pair of either wild-type or the two mutant *loxP* sites with similar efficiency by Cre protein expressed during P1 infection is not surprising. The most sensitive sites on DNA with respect to Cre protein recognition, base pairs 2 and 6, lie within the 13 bp inverted repeats of the *loxP* site (31), and these are invariant in all the *loxP* sites tested here. The mutations are in only the 8 bp spacer region.

The selectivity between wild-type and mutant *loxP* sites is also not surprising as this region is thought to require complete base pairing during crossover (21). This is consistent also with efficiencies of homologous recombination being highly sensitive to mutations in the region of homology between partners: the 8 base spacer of *loxP* apparently serves a similar role to the spacer region in the FLP/FRT system and presumed Holliday-like junctions would otherwise contain disruption of base pairing (32).

Figure 5. A schematic diagram showing possible multi-plasmid co-integrates as intermediates during transduction of mutant *loxP* site-containing plasmids by phage P1. The terms '*cre*' stands for the gene encoding Cre protein and is indicated by the black dot, '*pac* site' represents a sequence recognized by the packaging machinery of P1, where a double strand cut is made and packaging of DNA starts. The thin arrow adjacent to the '*pac* site' indicates the direction of packaging.

The results of Table 1 demonstrate that phage P1's ability to transduce a mutant *loxP* plasmid depends upon a third plasmid having both a wild-type and an identical mutant *loxP* site. Such a scenario suggests that multi-plasmid co-integrates are bona fide intermediates in the cell. A schematic representation of such intermediates likely to participate in the transduction process is shown in Figure 5. Packaging starts at the *pac* site and goes clockwise (indicated by the thin arrow) till the P1 head is full. Note that the DNA piece BAKJ in the final co-integrate is phage DNA and is lost upon packaging. If the DNA piece HI (genomic insert) is small as in PAC clones 2, 8 and 12, the second wild-type *loxP* site (between I and J) is able to fit comfortably inside the P1 head [see Figure 1 of Ref. (18)]. Cre protein is expressed immediately upon entry of the linear DNA into the cell upon infection, and helps recombine the DNAs between the two mutant and wild-type *loxP* sites. Both plasmids are therefore regenerated, and both antibiotic resistance markers are expressed as seen for clones 8/19 and 12/18 in Table 1.

If the DNA piece HI is very large as in PAC clones 5, 9 and 13, the second wild-type *loxP* site (between I and J) is unable to fit in the P1 head. Upon infecting fresh NS3516 cells, the DNA inside the P1 head is now unable to salvage the starting PAC clones 5, 9 and 13 [see Figure 1 of Ref. (18)]. However, the two mutant *loxP* sites recombine to regenerate, albeit at 10- to 20-fold lower efficiency, the mutant *loxP* plasmids as seen for clones 9/19 and 13/18 in Table 1. Note that the newly

expressed Cre protein would not be available in this case as the gene encoding it is lost while packaging of the DNA in the P1 head, and the occasional recombination of mutant *loxP* sites most likely is mediated by cellular recombinases.

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