

Minimal cross-recombination between wild-type and loxP511 sites *in vivo* facilitates truncating both ends of large DNA inserts in pBACe3.6 and related vectors

Leighcraft A. Shakes¹, Douglas M. Garland^{1,2}, Deepak K. Srivastava¹, Ken R. Harewood¹ and Pradeep K. Chatterjee^{1,*}

¹Julius L. Chambers Biomedical/Biotechnology Research Institute and ²Department of Biology, North Carolina Central University, 1801 Fayetteville Street, Durham, NC 27707, USA

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ABSTRACT

Contrary to several earlier reports, we find that cross-recombination between wild-type and the mutant loxP511 sites is <0.5% of that between two wild-type sites if Cre protein is expressed by phage P1 during an infection. The finding enabled us to develop a procedure to truncate DNA progressively from both ends of large genomic inserts flanked by these two loxP sites in pBACe3.6 and related vectors with transposons carrying either a wild-type or a loxP511 sequence. Newly constructed loxP511 transposons contained either a kanamycin resistance gene or no marker. Insert DNA ends in deletions were sequenced with primers unique to each transposon-end remaining after the respective recombination. End-sequencing 223 deletions confirmed that the low level of cross-recombination, observed between those sites during the P1 transductions, does not complicate the procedure: truncations from the unintended end of genomic inserts did not occur. Multiple BACs pooled together could also be processed in a single tube to make end-deletions. This deletion technology, utilizing the very minimal cross-recombination between the mutant and wild-type loxP sites of most BAC clones in the public domain and a heterologous one inserted as a transposon, should facilitate functionally mapping long-range gene regulatory sequences and help to isolate genes with defined functional boundaries in numerous projects including those of therapeutic interest.

INTRODUCTION

A comparison of genomic sequences indicates much of the DNA that is highly conserved between human and mouse do not actually code for proteins (1), but is thought to be involved in regulating gene expression (2,3). Part of this conserved non-coding DNA comprises *Cis*-acting sequences, sometimes located far away from the coding region of a gene, regulating transcription (2–5). Identifying these functionally presents a major challenge because traditional approaches have addressed only short range interactions between DNA sequence modules in small plasmids.

Localizing regulatory sequences distal to a gene functionally requires that the gene and its regulators be housed in large insert clones such as BACs or PACs (6–8). Libraries of clones with large inserts and high coverage of human, mouse and rat DNA exist (9–11), and the availability of a set of BAC clones spanning the entire human genome has set the stage for serious functional mapping projects (12). Procedures capable of truncating large genomic inserts in clones from these libraries should facilitate such effort (13–19).

Methods that progressively delete DNA from one end of genomic inserts in BACs and PACs have been described, and used to localize genetic markers on a physical map of the chromosome (20,21). Cre recombination of a randomly transposed loxP site to insert DNA with one endogenous to the clone generates a deletion series. The resulting truncations are recovered by packaging in a phage P1, with the size of clones limited to 110 kb. Despite this size limitation, the ease of scanning 100 kb of conserved non-coding DNA for gene regulatory sequences quickly is a distinct advantage, and the procedure was used recently to functionally identify three new enhancer sequences 27 kb upstream of the Nkx2-5 gene in transgenic mice (3).

*To whom correspondence should be addressed. Tel: +1 919 530 7017; Fax: +1 919 530 7998; Email: pchatterjee@nccu.edu

Because regulatory elements can sometimes be within introns or located downstream of a gene, it would be desirable to be able to truncate BAC inserts from both ends. Fortunately cloning vehicles, such as the pBACe3.6 and pTARBAC1 or pTARBAC2 vectors, used for the newer resource libraries contain the insert DNA flanked by two different loxP sites (22). An arrangement such as this might have been considered ideal for deleting DNA from both ends with transposons carrying the different loxP sites had results from several subsequent studies not discouraged developing such strategies: cross-recombination between wild-type and the mutant loxP511 site that flanked DNA inserts in these clones, have been reported to range from 5 to 100% in a variety of settings (19,23–26).

A recent study using a pair of different loxP site mutants suggests the earlier findings might have been influenced by excessive and/or sustained levels of Cre protein from constitutive expression, during recombination (27). Cross-recombination between wild-type and loxP511 sites was therefore, reinvestigated using transient expression of Cre protein which occurs during phage P1 transduction. We now report that the leakage in recombination between these two sites was found to be not more than 0.5% of that between two wild-type sites. The results enabled us to progressively delete both ends of genomic inserts in pBACe3.6 and related vectors using transposons that carry either a wild-type or a mutant loxP511 site.

MATERIALS AND METHODS

The oligodeoxyribonucleotides, d (GGCCGCTAATAACTTCGTATAGTATACATTATACGAAGTTATTGTTTAAACC) and d (GGCCGGTTTAAACAATAACTTCGTATAATGTACTATACGAAGTTATTAGC), were annealed by heating equimolar quantities in 10 mM Tris-HCl, pH 8.0, buffer containing 1 mM EDTA and 10 mM NaCl to 70°C and slow cooling to room temperature. The duplex oligo contains the mutant loxP511 sequence along with a PmeI site, and was ligated to plasmid DNA linearized with NotI enzyme.

Construction of Tn10 minitransposons with mutant loxP511 sites

The transposon plasmid pTnloxP*-1 (27), was the starting point for all mutant loxP511 site-containing plasmids. The gene for chloramphenicol resistance located near one of the 70 bp inverted repeat ends was removed by digesting the pTnloxP*-1 DNA to completion with PvuII, gel purifying the largest fragment, and performing a partial digest with ScaI. The largest fragment was again gel purified, and the two blunt ends created by the PvuII and ScaI enzymes ligated with high concentration T4 DNA ligase. Transformed colonies were selected on LB agar plates with ampicillin. The gene for ampicillin resistance is located outside the 70 bp inverted repeat ends of the transposon.

Next the sequences for tetracycline resistance gene and the mutant loxP*-1 site were removed by NotI digestion, and it was ligated with a duplex oligonucleotide with NotI compatible ends that contained the mutant loxP511 sequence and a PmeI restriction site (shown above). Plasmids acquiring the loxP511 oligonucleotide were screened for by digesting

clone DNA with PmeI enzyme. Both orientations of the loxP511 site-inserted transposon were isolated. They were named pTnLoxP511(A)markerless 1 and pTnLoxP511(B)markerless 1. They are designated markerless because they do not contain any antibiotic resistance markers within the 70 bp inverted repeat ends of the transposon to select for transpositions of the loxP511 site into target DNA [see discussion (28)].

An additional oligonucleotide duplex for anchoring a sequencing primer (Seq 25) was inserted into the unique AscI site of pTnLoxP511(B)markerless 1 to generate pTnLoxP511(B)markerless 2. The plasmid was linearized with AscI and the duplex obtained by hybridizing the following oligonucleotides M13C and M13D was ligated as described above.

M13C: d (CGCGCCTAGTAAAACGACGGCCAGTAGTCGTGACTGGGAAAACCTGTTTAAACC); M13D: d (CGCGGGTTTAAACAGGTTTTCCAGTCACGACTACTGGCCGTCGTTTTACTAGG).

A RSV neomycin resistance gene cassette, coding for kanamycin resistance in bacteria, was excised from pTnBAC/loxP (14) with AscI, and inserted into the AscI site of pTnLoxP511(B)markerless 1 (Figure 1). The resulting transposon

Wt & loxP511 Transposon plasmids

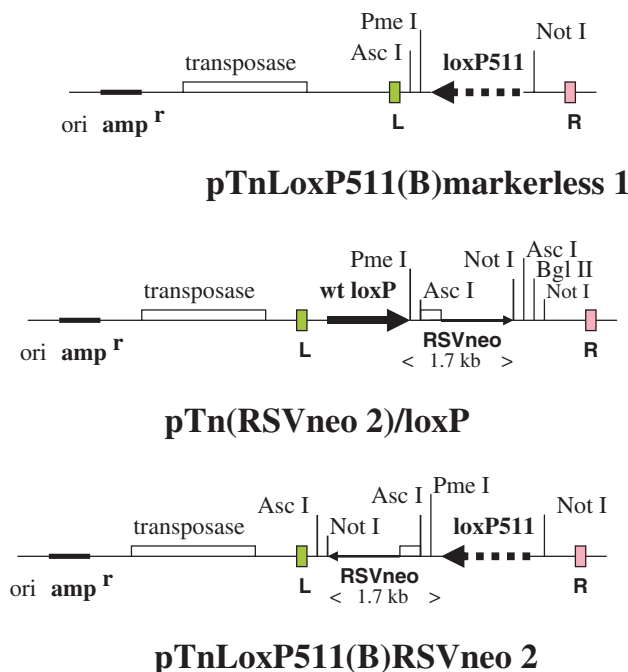


Figure 1. New transposon plasmids containing wild-type and loxP511 sites. A schematic representation of transposon plasmids used for deleting both ends of insert DNA cloned in the pBACe3.6 and related vectors pTARBAC1 and pTARBAC2. The transposon plasmid pTnLoxP511(B)markerless 1, shown in the top panel, has no antibiotic resistance marker within the transposing part marked by the small rectangular boxes colored pink or green. Bottom panel shows a kanamycin resistance gene-containing variant, pTnLoxP511(B)RSVneo 2. The middle panel shows a wild-type loxP transposon plasmid with kanamycin resistance gene. Wild-type loxP site is shown with the thick continuous arrow, while the mutant loxP511 sites are indicated by bold broken arrows. The pink end R remains in deletion clones generated with the wild-type loxP transposons, while the green end L is left behind in deletions with the loxP511 transposons.

plasmids are named pTnLoxP511(B)RSVneo 1 or pTnLoxP511(B)RSVneo 2 to distinguish the two orientations of the RSVneo cassette. Only pTnLoxP511(B)RSVneo 2 is discussed here.

Construction of a Tn10 minitransposon with wild-type loxP sites

The kanamycin resistance gene-containing wild-type loxP transposon plasmid, pTn(RSVneo 2)/loxP, was constructed by introducing a RSV neomycin gene cassette into the previously described pTnMarkerless2 transposon plasmid (28). It differs from pTnBAC/loxP (14) in that all sequences related to the chloramphenicol resistance gene were removed from the latter, in order to reduce recombination between the chloramphenicol resistance gene in BACs and those sequences in the pTnBAC/loxP plasmid (14) [see discussion (28)]. The RSV neomycin gene cassette was excised from pTnBAC/loxP with *AscI*, the DNA fragment purified from an agarose gel, and ligated to pTnMarkerless2 linearized also with *AscI*. Plasmids containing both orientations of the RSV neo gene cassette were isolated, pTn(RSVneo 1)/loxP and pTn(RSVneo 2)/loxP, and only the latter orientation used here and shown in Figure 1.

Generating deletions from the loxP511 end of inserts in pBACe3.6 vector-derived clones

The following 10 clones from the human and mouse BAC libraries were obtained from BACPAC resources, CHORI (Oakland, CA): RP11-219A15, RP11-158M20,

RP11-434D2, RP23-209O22, RP23-92L23, RP23-366M16, RP23-101N20, RP23-124B2, RP23-444K15, RP23-130D16. The genomic inserts in these clones are flanked by a wild-type and a mutant loxP511 site in the BAC vector pBACe3.6 (9–11,22).

Nested deletions using pTnLoxP511(B)markerless 1, pTnLoxP511(B)markerless 2 and pTnLoxP511(B)RSVneo 2 from the loxP511 end were generated in each of the above clones as previously described (28,29).

Processing multiple BAC clones to make end-deletions

BACs were also pooled together and processed together in a single tube to make end-deletions. Each BAC clone was transformed separately with the transposon plasmid, and the transformed colonies grown to saturation before pooling.

End-sequencing of BAC deletion clones

Typically, miniprep DNA was isolated from 60 clones picked randomly from the several hundred member BAC deletion library. Deletion clones were passed through an ampicillin sensitivity screen (20) if the transposon plasmid was recovered in more than 20% of the deletions. Approximately half the clones from the first round deletion series analyzed on FIGE were of unique size. Deletions arising from intra-insert recombinations independent of loxP-Cre, seen only when using markerless transposons of either type, were weeded out after FIGE analysis (lane 1 of Figure 2 shows an e.g.). These are identified by their BAC vector DNA fragment

Deleting From the loxP511 end of DNA insert in pBACe3.6

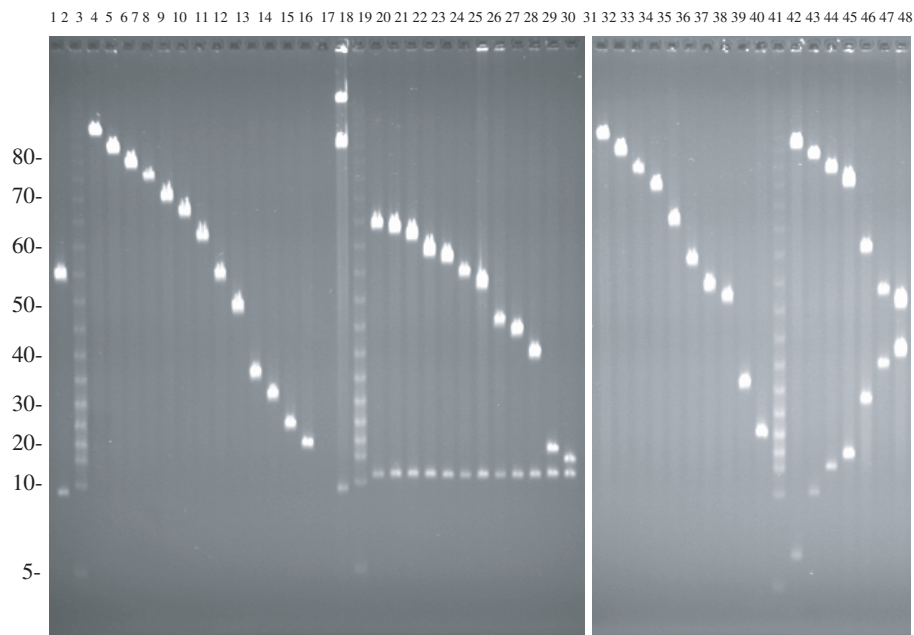


Figure 2. FIGE analysis of *NotI* digested DNA isolated from progressive truncations made from one or both ends of insert DNA. Left panel: DNA isolated from deletion clones generated with either pTnLoxP511(B)markerless 1 (lanes 3–15) or pTnLoxP511(B)RSVneo 2 (lanes 19–30) in BAC clone RP23-444K15 and RP11-219A15, respectively, were analyzed by FIGE. Lane 17 contains the DNA from starting BAC clone RP23-444K15, while lanes 2, 18 and 41 contain 5 kb DNA ladders. Right panel: Second round deletions made with pTn(RSVneo 2)/loxP from the wild-type loxP end of insert DNA from the deletion clone shown in lanes 3 and 31. Lanes 32–40 show deletions, while 42–48 show inversions. Deletions with pTnLoxP511(B)RSVneo 2 (lanes 19–30) produce a 10.5 kb BAC vector DNA fragment, while those with pTnLoxP511(B)markerless 1 (lanes 3–15) produce no BAC vector band because one of the *NotI* sites is lost as a result of the deletion (Figure 3). Intra-insert deletions independent of *LoxP*-*Cre* recombination produce the same sized BAC vector band as starting BAC (lane 1).

identical in size to starting BACs (28). DNA from 20 clones of each deleted series was sequenced directly using a transposon-end primer (20) and Big dye terminator chemistry on an ABI-3100 AVANT genetic analyzer. Primer extended products were purified using Magnesil (Promega Corporation) according to procedures supplied by the manufacturer and described in (27,28). Primers for sequencing newly created ends of deletions from the loxP511 side of insert DNA, after trimming the wild-type end, have been developed. These are listed below:

Sequencing primers

Seq 8: d (GCAGTGTGACCGTGTGCTTCTCAAATGC); Seq 21: d (GATCGGCGCGCCATGATC); Seq 25: d (GCCAGTAGTCGTGACTG); Neo 8: d (GTTTTCCCTTG-TCCAGATAGC); Neo 11: d (CTGAGTGCTTGC GGCA-GCG); Neo 12: d (GAATCGTTTTCCGGGACG); Neo 16: d (GATTCATGCTGGAGTTCTTCGCC).

RESULTS

Truncating DNA inserts from both ends using loxP transposons critically depends on the degree of leakage in recombination between the two loxP sites flanking the insert. Because varying degrees of recombination cross-reactivity between these sites was observed in previous studies (19,23–26), it was important to reinvestigate this under the P1 transduction conditions that are actually used in our deletion procedure (27).

Estimating recombination cross-reactivity between wild-type and mutant loxP511 sites using phage P1 transduction

Several mutant loxP sites have been tested both for their recombination efficiency and exclusivity under different Cre protein-exposure conditions (23–27). Phage P1 expresses Cre early during an infection and efficiently transduces a wild-type loxP plasmid with high fidelity (30–32). The ability of the wild-type loxP site in phage P1 to transduce the mutant loxP511 plasmid was used as an indicator of recombination cross-reactivity between these sites (27). Results in Table 1 indicated that although P1 was able to efficiently transduce the plasmid with a wild-type site, it could recover the mutant loxP511 plasmid only very inefficiently (compare #17 with #21). Unlike the results with two other mutant loxP sites described earlier (27), inhibition of transduction of the

loxP511 mutant was incomplete. Comparing parallel transductions we find that the loxP511 plasmid is transduced 0.1–0.5% as efficiently as the wild-type. This level of recombination cross-reactivity is significantly less than that which was reported earlier in several *in vivo* and *in vitro* analyses (19,23–26). The exclusivity between the two sites appears high, although not absolute as reported originally (33). Consistent with previous findings (27), phage P1 was able to efficiently transduce the loxP511 plasmid if another carrying both a wild-type and the loxP511 site exists in the cell (rows 5, 6, Table 1). Phage P1 transduced the loxP511 plasmid in the presence of BAC deletions, #39 and #45, generated from BAC RP23-444K15 by truncating from the wild-type loxP end with a markerless wild-type loxP transposon (28).

Generating deletions from the loxP511 end of insert DNA in pBACe3.6

Because the 511 and wild-type loxP sequences cross-recombine only slightly, insert DNA flanked by these can be deleted from both ends using transposons carrying those sites. Verifying authenticity of the newly created ends after each round of deletion should compensate for the minor leakiness. Transposons were constructed with the two types of loxP sites such that a different end of the Tn10 was left behind after deletion formation at each end of insert DNA. Unique primers were designed into each transposon-end remaining after the recombination.

Two classes of transposons were constructed carrying the loxP511 site: (i) markerless and (ii) with kanamycin resistance gene as marker. Only transposons of the ‘B’ series of both classes, with the loxP511 oriented as shown in Figure 1, were used in this study. The ‘A’ series of both classes had the loxP511 site in the opposite orientation. As noted earlier (28), transposition of a markerless loxP transposon is selected through P1-headful packaging and not by the transfer of an antibiotic resistance gene into the target DNA. This necessitates the starting BAC to be larger than P1-headful size. Depending on which end of the genomic insert is deleted first, the markerless transposon could carry a wild-type or a mutant loxP511 site. Markerless transposons with wild-type loxP sites have been previously described (28).

The transposon plasmid pTnLoxP511(B)markerless 1 was transformed into each of the 10 BACs listed in Materials and Methods. Transformed colonies were selected on LB agar plates containing chloramphenicol plus ampicillin. Colonies

Table 1. P1 transduction of plasmids carrying wild type and/or loxP511 sites.

Clone	Size (kb)	Marker	LoxP site	After transduction with phage P1	
				kan	cam
#17	12	camR	Wild-type loxP only	N/A	++++ ^a
#21	8	kanR	loxP511 only	+ ^b	N/A
#39	40	camR	Wild-type plus loxP511	N/A	++++ ^a
#45	60	camR	Wild-type plus loxP511	N/A	++++ ^a
Transformed clones					
#39/21				++++ ^a	++++ ^a
#45/21				++++ ^a	++++ ^a
Wt loxP	ATAACTTCGTATA GCATACAT TATACGAAGTTAT				
loxP511	ATAACTTCGTATA GTATACAT TATACGAAGTTAT				

^a++++, ~1000 colonies/plate.

^b+, ~5 colonies/plate.

from a plate were pooled and processed for making end-deletions as described earlier for wild-type loxP transposon-transformed BACs (28,34). BAC deletions were selected on plates that contained chloramphenicol only. Miniprep DNA isolated from deletion clones were digested with NotI enzyme before analysis by FIGE (29). Lanes 3–15 of Figure 2 shows DNA isolated from clones deleted from the loxP511 end of insert in BAC RP23-444K15. Lane 1 displays an intra-insert deletion independent of loxP-Cre (28), with the vector DNA fragment size identical (8.8 kb) to starting BAC (lane 17).

Deletions from the loxP511 end were also made with pTnLoxP511(B)RSVneo 2 (Figure 1) in a subset of the BAC clones. Lanes 19–30 Figure 2, shows NotI digested DNA from a set of these deletions with the BAC vector fragment now at 10.5 kb.

Specificity of loxP-Cre recombinations during deletion formation: using size of pBACe3.6 vector DNA fragment with NotI as a diagnostic

The two Not I sites in pBACe3.6 vector are located asymmetrically with respect to the loxP sites: the NotI sites at positions 2,849 and 11 583 are 1.978 and 0.142 kb in front of the wild-type and mutant loxP511 sites, respectively (35) (Figure 3). Transposons pTnLoxP511(B)RSVneo 2 and pTn(RSVneo 2)/loxP would substitute the BAC vector NotI site with one from the transposon after recombining the respective loxP sites (Figures 1 and 3). Because the NotI sites in both transposons are 1.7 kb away from their respective loxP sites, they would yield BAC vector DNA NotI-fragments of different size in clones deleted from the wild-type or the mutant loxP511 ends of insert: recombinations with either the mutant 511 or wild-type loxP sites in the BAC would produce a 10.5 or 8.4 kb

vector DNA fragment with NotI, respectively, (lanes 19–30 and 16–30 in Figures 2 and 4, respectively). Note that fragments of 10.5 and 8.4 kb did not appear together during a deletion experiment with either of these transposons, demonstrating the exclusivity of the recombination reactions. It corroborates the selectivity observed during the P1 transductions described above. Deletion-end-sequencing with Tn-based primers also support the conclusion.

Deletions from the wild-type loxP end of insert DNA with pTnMarkerless2, described earlier (28), yield the expected vector DNA NotI-fragment of 6.8 kb (lanes 1–13, Figure 4). Intra-insert deletions independent of LoXP-Cre recombination are isolated only when using markerless transposons (28), because the probability of isolating one in conjunction with a transposon insertion is low, and can be easily identified by their BAC vector DNA fragment with NotI: it is the same size as the starting BAC, as shown in lane 1 of Figure 2. Note that markerless transposons cannot be used for second round deletions because the starting clone would be less than P1-headful size (28).

A new wild-type loxP transposon pTn(RSVneo 2)/loxP was used to make deletions from the wild-type end of insert DNA, producing the expected 8.4 kb BAC vector DNA fragment with NotI (lanes 16–30, Figure 4).

Truncating insert DNA from both ends sequentially in the same BAC

Trimming either end of insert DNA in pBACe3.6 derived clones was readily achieved using transposons specific for the respective ends. Both ends in the same clone can be deleted similarly except that the second round of deletions must necessarily be performed with a resistance gene marker-containing

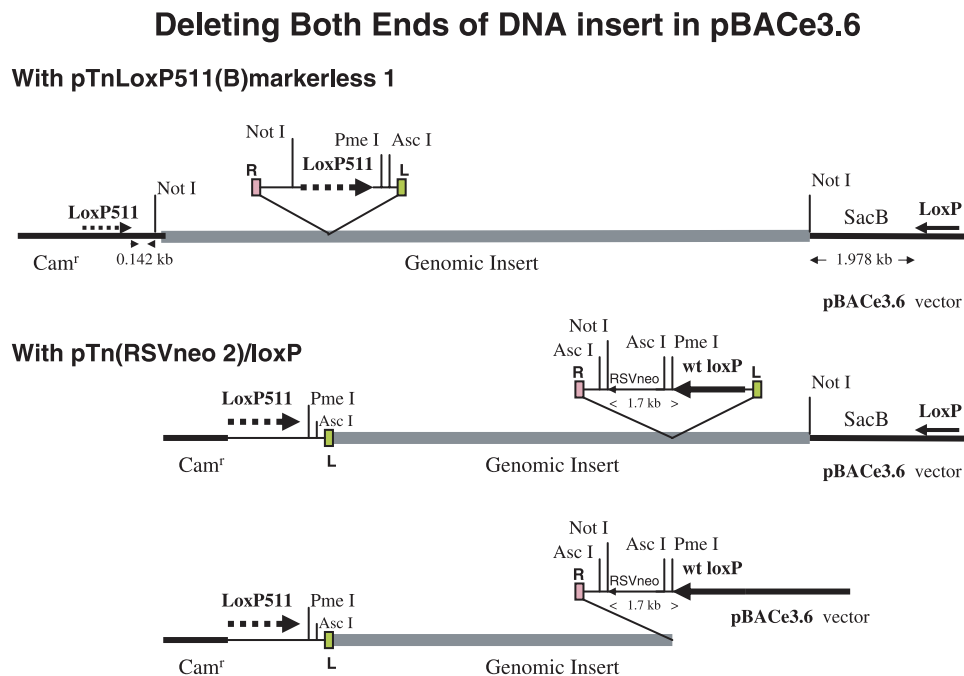


Figure 3. Schematic representation of deletions made sequentially from the (i) mutant loxP511 end of genomic DNA insert generated with pTnLoxP511(B)markerless 1 (top panel) and (ii) wild-type loxP end with pTn(RSVneo 2)/loxP (bottom panel). The transposon is shown as the triangle with the locations of NotI sites indicated. Note that Cre mediated deletions generated with the loxP511 sites would substitute a NotI with a PmeI site (top panel). Deletions using the wild-type loxP sites would retain the NotI site as shown in the bottom panel.

Deleting From the Wt-loxP End of DNA insert in pBACe3.6

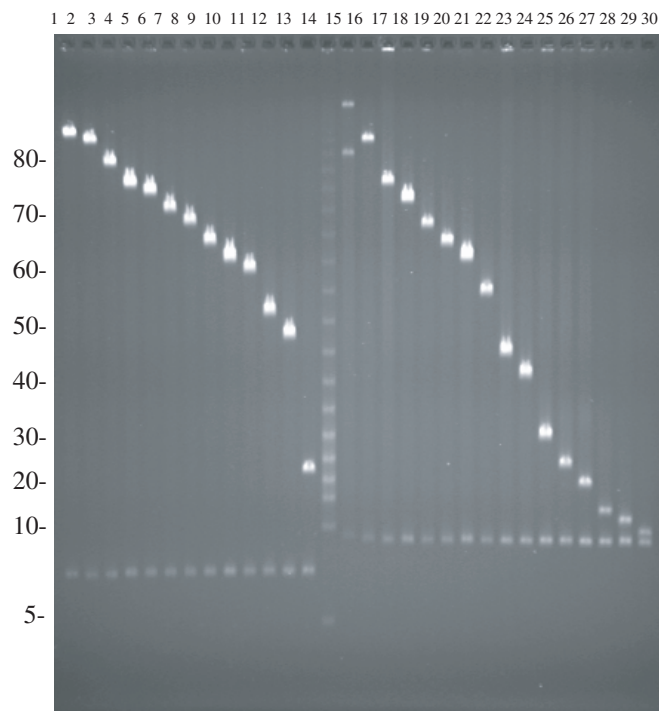


Figure 4. FAGE analysis of NotI digested DNA isolated from deletions made from the wild-type loxP end. Left panel: DNA isolated from deletion clones generated with pTnMarkerless2 (28) in BAC RP23-92L23 is shown in lanes 1–13. Lanes 16–30 show DNA from first round deletions generated with pTn(RSVneo 2)/loxP in clone RP23-444K15. Lane 15 contains DNA from starting BAC RP23-444K15. Lane 14 shows a 5 kb DNA ladder. The size of BAC vector DNA fragment is 6.8 and 8.4 kb with pTnMarkerless2 and pTn(RSVneo 2)/loxP, respectively. The starting BAC produces a 8.8 kb BAC vector band (lane 15).

transposon. Preference as to which end of insert DNA, the wild-type or the loxP511, will be truncated first dictates the choice of markerless transposon: pTnMarkerless2 [with wild-type loxP (28)] or pTnLoxP511(B)markerless 1 (Figure 1).

A second round of truncations with pTn(RSVneo 2)/loxP was made with the largest clone in the deletion series obtained with pTnLoxP511(B)markerless 1 (shown in lanes 3 and 31 of Figure 2). NotI digested DNA isolated from clones obtained in the second round of deletions is shown in lanes 32–40, Figure 2. Because the starting clone here was <110 kb, both deletions and inversions were isolated in about equal numbers (14,29). Inversions are shown in lanes 42–48 (Figure 2).

Similar truncations from both ends were also made by deleting first from the wild-type loxP end of inserts with pTnMarkerless2, and then using pTnLoxP511(B)RSVneo 2 to trim the other end of a clone isolated in the first round deletions (data not shown).

Processing a mixture of BAC clones transformed with transposon plasmid to increase throughput of making end-deletions

Multiple BACs can be processed together as a mixture in a single tube to make end-deletions. Because clones tend to

have different growth characteristics, this is best achieved by transforming each clone separately with the desired transposon plasmid and growing them to saturation before pooling. As many as 32 BACs were transformed in parallel. Aliquots from nine such transformed BAC cultures were pooled, and processed for deletions exactly as described earlier (29). For simplicity of sequencing downstream, BACs in a pool were transformed with the same Tn-plasmid. Each deletion was sequenced with primers specific to both ends of the insert DNA remaining after the deletion i.e. one from the transposon, the other specific to the opposite unaltered end of insert DNA (SP6 or T7). The latter sequence helped identify the parent from which the deletion was obtained. Analysis of the results indicates although each of the nine BACs is represented in deletions, the distribution is quite skewed: deletions from clone RP23-444K15 dominate 4-fold over those from RP11-219A15, RP23-209O22 or RP23-92L23, although all four were equally efficient in producing deletions when processed individually.

Sequencing ends of deletions with primers from the wild-type or loxP511 transposons

Transposon-ends marked R (pink) and L (green) shown in Figures 1, 3 and 5 remain after deleting from the wild-type or loxP511 end of insert DNA, respectively. Note that the opposite orientation of the loxP511 site in transposons of the ‘A’ series would have left behind the R (pink) end in a deletion, and this would have interfered during end-sequencing of clones after a second round of deletions with the wild-type loxP transposon. Because both ends of all transposons described here share the same 70 nt, primer Seq 1 (20) located within it can sequence the new insert DNA end generated by all of them. However, Seq 1 loses its uniqueness after deletions are made from the other end. Primers unique to each end of the transposons described here are listed in Materials and Methods. Although both orientations of the loxP511 site and the RSV neomycin gene cassette were constructed in transposons, and analyzed, only those conforming to the desired uniqueness of sequencing primers are presented in Figure 5 for clarity.

The 600 base reads obtained from each deletion-end could BLAST to sequences in the database at the 95–99% homology level. The sequential order of bands in FAGE of the NotI digested DNA from deletion clones corresponded with the order of sequence homology on the chromosome, and BLASTed to only one strand of DNA.

DISCUSSION

Functionally localizing gene regulatory sequences that operate over large distances using BAC transgenics requires procedures to systematically delete DNA at either end of inserts. Both gap-deletions as well as truncations from one end of the reporter gene-tagged BAC insert have been made either by a targeted deletion strategy using homologous recombination, or using loxP transposons (2–5). We believe the loxP transposon deletion strategy has several advantages over homology-based targeted deletion methods. Induction of recombination often triggers intra-insert DNA rearrangements outside of the regions of homology actually targeted: mammalian DNA is known to be recombinogenic, and is

Location of Transposon based Sequencing primers

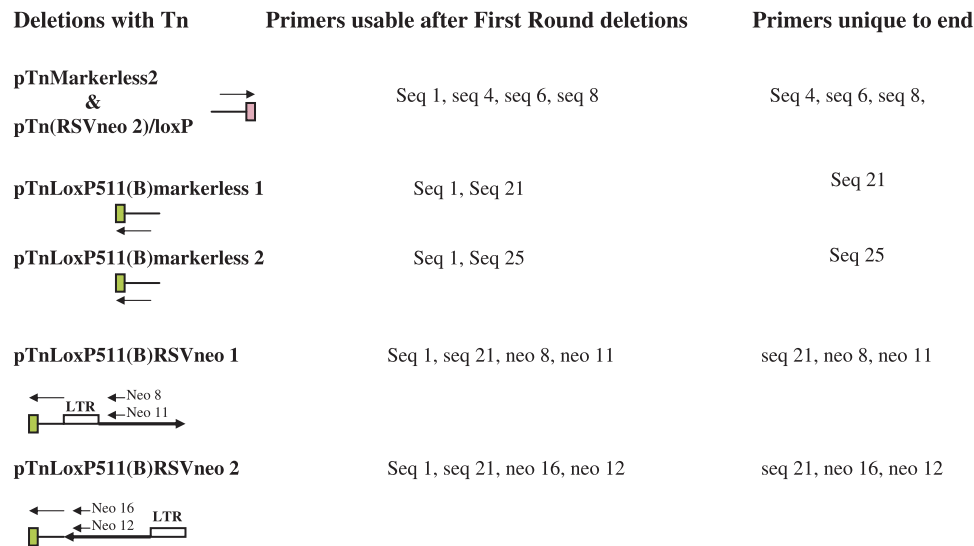


Figure 5. A schematic diagram showing sequencing primers usable after first and second round deletions. Pink rectangles represent the R-end of Tn10 remaining in deletions generated with wild-type transposons such as pTnMarkerless2 (28), and pTn(RSVneo 2)/loxP. Green rectangles indicate the L-end of Tn10 remaining in deletion clones made with any of the loxP511 transposons described here.

usually kept intact by propagating them in host strains that are rendered highly recombination deficient (6,7,36). Occasionally, such intra-insert DNA deletions and/or rearrangements can become excessively more frequent than the desirable targeted deletion (P. K. Chatterjee, unpublished data).

A second, more important, advantage offered by the loxP transposon-end-deletion procedure is that an entire array of deletions from a particular end of the BAC insert is obtained in a single experiment. This library of truncated BAC-GFP deletions can be made without the need to construct new targeting vectors each time.

The illustrations in Figures 1 and 3 indicate that sequences ahead of a loxP site are retained in the deleted clone. This characteristic can be used to bring regulatory sequences that are far away, or from another gene, to the near vicinity of the gene of interest during truncations. Exogenous sequences, inserted at suitable sites in the loxP transposons, can be easily engineered into a BAC clone to regulate a gene in novel ways.

Studies requiring genes with well defined functional boundaries are likely to benefit from the procedures described here. They allow isolating a gene free of interfering regulatory elements of an adjacent gene existing in the same BAC clone.

The end-deletion technology can be adapted to handle multiple BACs simultaneously. However, results indicate the yield of deletions can be skewed unless BACs with similar growth characteristics are used when pooling.

Recombination of loxP sites with Cre protein has been widely used in both bacterial and mammalian systems (19,23,24,26,37–41). The potential for using wild-type and mutant loxP sites in conjunction is even greater. However, the results from a number of recent studies suggest the exclusivity between the wild-type and mutant loxP511 sites might be compromised by the level and persistence of Cre protein (19,23–26). Results presented here and those from an earlier

study (27) indicate that cross-recombination between wild-type and mutant loxP sites can be reduced if the Cre protein is transiently expressed as in a phage P1 infection. The differences in specificity observed between results presented here and those reported by other investigators earlier might be related to the different ways by which loxP sites are exposed to Cre protein *in vivo*. Although removal of host cell factors and mutations in transposases relax target site specificity during Tn10 and yeast Ty1 insertions (42), there is yet no evidence to suggest that additional phage encoded and/or induced factors during a P1 infection alters fidelity of Cre recombinase. Thus in the absence of evidence for enhanced specificity conferred by phage P1 encoded proteins other than Cre, in either bacterial or mammalian systems (43,44), these results and previous work by others (24,26) appear to support the idea that high levels and/or persistent exposure to Cre protein might in fact contribute to loxP site promiscuity.

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