

# Site-specific cleavage of chromosomes *in vitro* through Cre–lox recombination

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## ABSTRACT

Site-specific recombination systems are useful tools for chromosome engineering *in vivo* and site-specific DNA cleavage methods have applications in genome analysis and gene isolation. Here, we report a new method to fragment chromosomes *in vitro* using the Cre–lox site-specific recombination system. Two lox sites were targeted into the 5.7 Mb chromosome I of *Schizosaccharomyces pombe*. *In vitro* recombination between chromosomal lox sites and exogenously provided lox oligonucleotides ‘cleaved’ the chromosome at the defined lox sequences. Site-specific cleavage of lox sites in the tobacco genome was also demonstrated. This recombination-based cleavage method provides a novel approach for structural and functional analyses of eukaryotic chromosomes as it allows direct isolation of chromosome regions that correspond to phenotypes revealed through Cre–lox mediated chromosome rearrangements *in vivo*. Moreover, recombination with end-labeled lox oligonucleotides would permit the specific end-labeling of chromosome segments to facilitate the long range mapping of chromosomes.

## INTRODUCTION

Site-specific recombinases are a group of enzymes that catalyze recombination at specific target sequences (1,2). Of those, the Cre–lox, FLP–FRT and R–Rs systems from bacteriophage P1, *Saccharomyces cerevisiae* and *Zygosaccharomyces rouxii*, respectively, have received considerable attention for the manipulation of heterologous genomes *in vivo*. These systems are strikingly similar as a single protein, Cre, FLP or R, is sufficient to catalyze recombination between corresponding lox, FRT or Rs sites that are 31–34 bp in length. In addition to the numerous reports of excision, inversion and targeted integration of transgenes in various organisms (see 3–5 for reviews), intra- or inter-chromosomal recombination resulting in the rearrangement of large segments of DNA has also been reported in yeast (6), *Drosophila* (7–10) and plants (11,12). Site-specific chromosomal rearrangements could provide unprecedented opportunities for genome analysis since the recombination break-points are molecularly tagged and can be precisely defined. In some

organisms, the pre-selection of breakpoints is possible through the homologous integration of recombination sites. We envision that if the recombination sequences can be specifically cleaved *in vitro*, it could enable the direct isolation of chromosomal DNA flanked by recombination sequences, such as regions defined by deletions and inversions generated by site-specific recombination *in vivo*.

Here, we describe that the Cre–lox system can essentially ‘cleave’ a chromosome at defined lox sites through *in vitro* recombination with exogenously provided recombination sites. The specific cleavage of lox sites that have been targeted into chromosome I of *Schizosaccharomyces pombe* generated chromosomal segments of predicted sizes. Efficient cleavage of a lox sequence in the tobacco genome was also observed. The cleavage method described here not only provides a novel approach for structural and functional analysis of chromosomes but also permits the single site labeling of a eukaryotic genome via recombination with labeled lox oligonucleotides. End-labeled DNA substrates thus produced would facilitate the physical mapping of chromosome regions adjacent to the lox sites.

## MATERIALS AND METHODS

### Cre purification

Cre was purified from *Escherichia coli* harboring a cre-expression plasmid, pMQ23, as described (13) with the following modifications. After streptomycin sulfate/ammonium sulfate fractionation, the resulting pellet was resuspended in PGE (0.01 M NaPO<sub>4</sub>, pH 6.8, 10% glycerol and 1 mM EDTA), dialyzed against 2 l PGE for 3 h with a buffer change and subjected to purification through hydroxyapatite, heparin, phosphocellulose and Sephadex G-75 gel-filtration chromatography.

### Plasmids

Standard recombinant DNA techniques were used throughout (14). pMQ23 has cre fused to the λ P<sub>L</sub> promoter under the control of the λ C<sup>ts</sup> repressor. pMQ6 contains a lox sequence in the ClaI site of the ade2::URA3 gene-disruption vector pDS23 (15). pMQ73a was made by ligating a lox–URA3 fragment into a portion of a PCR-amplified swi4 gene (16) and then inserting the swi4 flanked-lox–URA3 fragment into pUC19. Details of plasmid constructs are available upon request.

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## Strains

*Nicotiana tabacum* transgenic lines ntCB7 and ntCB34 were described previously (11,17). *S.pombe* strains were constructed as follows: for MQade2, linear pMQ6 (*ade2::lox-URA3* disruption vector) was transformed into Sp223 (*h<sup>-</sup> leu1.32 ura4.294 ade6.216*) and Ura<sup>+</sup> deep-red *ade2<sup>-</sup>ade6<sup>-</sup>* colonies were confirmed by Southern blotting. For MQswi4, JS5 (*h<sup>+</sup> leu1.32 ura4.294*) was transformed with linear pMQ73 (*swi4::lox-URA3* disruption vector) and Ura<sup>+</sup> transformants were screened by polymerase chain reaction (PCR) and confirmed by Southern analysis. MQswi4 was mated to MQade2 to generate MQswi4ade2, which contains *swi4::lox* and *ade2::lox* transgenes as confirmed by Southern analysis.

## In vitro recombination reaction

Chromosome-size DNA from *S.pombe* was prepared as described (18) except microbeads were used. Microbeads ( $5 \times 10^7$  genome equivalents in 50  $\mu$ l) and *lox2* DNA (5 ng) were pre-incubated for 1.5 h at 50°C to allow diffusion of *lox2* into microbeads. The *lox2* sequence is from the annealing of two oligonucleotides:

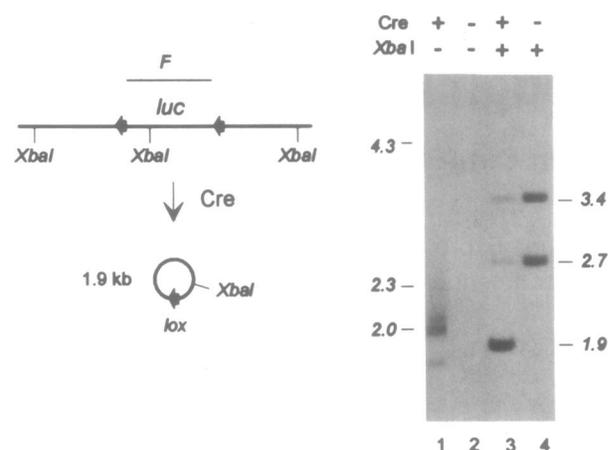
5'-GATAACTTCGTATAGCATACATTATACGAAGTTAT-3' and  
5'-CTAGATAACTTCGTATAATGTATGCTATACGAAGTTAT-CTGCA-3'.

Reactions with 150 ng Cre were incubated for 1 h at 37°C in 100  $\mu$ l of 50 mM Tris-HCl (pH 7.5), 33 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.1 mg/ml BSA and terminated with 1/10 vol of ESP (18) for 1 h at 50°C. Plant DNA was isolated as previously described (11). Cre reactions with plant DNA were similar to the ones described above except that pre-incubation with *lox2* was omitted, reaction time was extended to over night (~16 h), and the reactions were terminated by heating the samples at 75°C for 5 min.

## RESULTS

To test Cre-mediated 'cleavage' at *lox* sites in a complex genome, we used two previously described *Nicotiana tabacum* lines (11,17), each containing a hemizygous *lox*-containing transgene. As the haploid complement of this tobacco species is estimated to be ~4200–4600 Mb (19), the cleavage target is present in ~8800 Mb of nuclear DNA. The first line, ntCB7, harbors a luciferase (*luc*) gene flanked by *lox* sites in the same orientation. Previously, we have shown that Cre-mediated recombination *in vivo* caused efficient excision of the *luc* fragment (17). Figure 1 shows this same reaction *in vitro*. DNA ( $1 \times 10^6$  amphidiploid equivalents) from this plant line was treated with Cre recombinase, fractionated by gel electrophoresis, and the excision product was detected with a radiolabeled *luc* probe. The product released from Cre treatment has the electrophoretic mobility of a circular molecule which was converted to the expected 1.9 kb linear fragment upon subsequent treatment with endonuclease *Xba*I (Fig. 1, lanes 1 and 3). A comparison of the hybridization intensity of the 1.9 kb product to the 3.4 and 2.7 kb fragments representing unexcised DNA (Fig. 1, lane 4) suggests that the *in vitro* excision reaction approached completion for the two closely linked sites.

Although intrachromosomal excision reactions can be used to isolate defined segments of DNA, the recombination efficiency would depend on the distance between two recombination sites. In addition, excision would occur only when two recombination

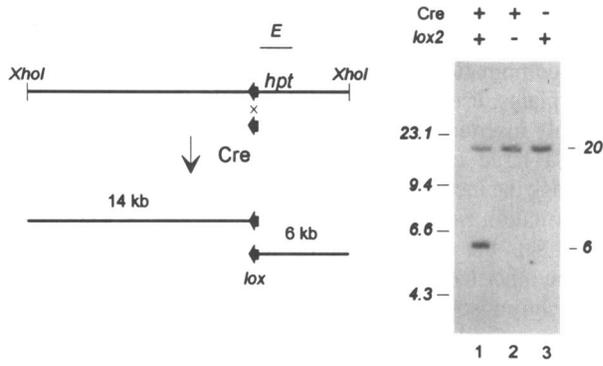


**Figure 1.** *In vitro* excision of a *luc* gene from the tobacco genome. The *luc* gene is flanked by *lox* sites (arrowheads) of the same orientation. DNA was treated with (+) or without (-) Cre and *Xba*I as indicated, fractionated in a 1% agarose gel and hybridized with *luc* DNA shown as probe F. Numbers to the left and right of the blot indicate size markers and predicted sizes in kb.

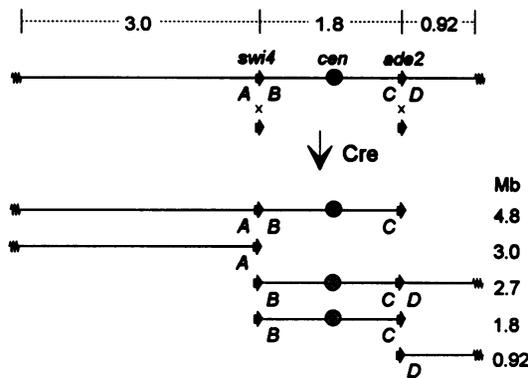
sites are in the same orientation. We envision that a more general approach for isolating DNA segments bound by recombination sites would be to cleave the chromosome at the *lox* sites through intermolecular recombination with exogenously provided *lox* sequences. Figure 2 illustrates this approach on tobacco line ntCB34, which harbors a hemizygous *lox* site. Previous work showed that this site is present on a 20 kb *Xho*I fragment (11). The Cre-mediated cleavage of the *lox* sequence would produce two smaller fragments, the sizes of which depend on the location of the *lox* site on the *Xho*I fragment. DNA from this plant was treated with Cre along with an oligonucleotide containing the 34 bp *lox* sequence, here referred to as *lox2*. The DNA was then cleaved with endonuclease *Xho*I, fractionated by gel electrophoresis and analyzed by Southern blotting. Recombination between *lox2* and the chromosomal *lox* site indeed cleaved the 20 kb fragment into two smaller pieces, of which the 6 kb fragment hybridized to the *hpt* probe (Fig. 2, lane 1). This cleavage of the 20 kb fragment is strictly dependent on the provision of both Cre and *lox2* (Fig. 2, lanes 2 and 3).

The above demonstration that site-specific recombination can cleave chromosomal DNA at defined *lox* sites supports the possibility of fractionating large *lox*-flanked chromosome segments from the rest of the genome. However, a higher eukaryotic genome with a *lox*-flanked chromosome segment of a known distance is currently not available. Thus, we tested the recombinase-mediated cleavage reactions on the fission yeast *S.pombe*. The 14 Mb haploid genome of *S.pombe* is well characterized and amenable to the engineering of *lox* sites into known loci. Through homologous recombination, a *lox* site was inserted into *ade2* or *swi4* of the 5.7 Mb chromosome I to yield strain MQade2 or MQswi4, respectively. From a mating of MQswi4 and MQade2, both the *swi4::lox* and the *ade2::lox* loci were introduced into chromosome I to form strain MQswi4ade2. Physical and genetic maps (20,21) suggest that *ade2* is between *mei2* and *rad1*, which are 1.0 and 0.7 Mb, respectively, from the right telomere. The *swi4* locus is ~3 Mb from the left telomere (Fig. 3).

Large DNA embedded in microbeads was prepared from MQade2 and MQswi4ade2, treated with Cre recombinase in the



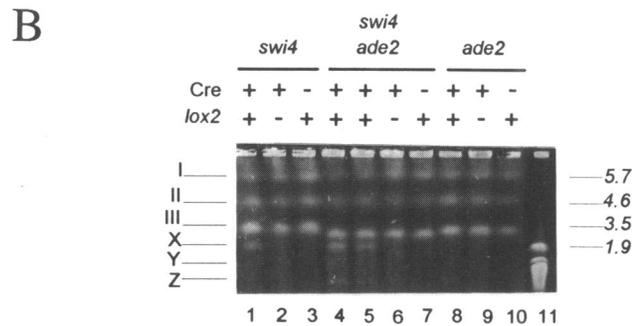
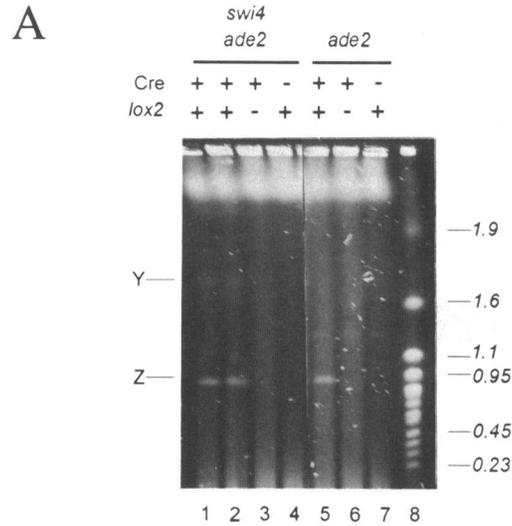
**Figure 2.** Cre-mediated cleavage of tobacco DNA at a defined *lox* site (arrowhead). DNA was treated with (+) or without (-) Cre and *lox2* as indicated, followed by cleavage with *XhoI*, gel electrophoresis in 1% agarose and hybridization with *hpt* DNA shown as probe E. Numbers to the left and right of the blot indicate size markers and predicted sizes in kb.



**Figure 3.** Schematic diagram of Cre-*lox* recombination between a *lox* oligonucleotide and one or both chromosomal *lox* sites placed in *S.pombe* chromosome I. Relevant genetic markers are *ade2*, *swi4*, centromere (*cen*) and *lox* sites (arrowheads). Strains MQade2, MQswi4 and MQswi4ade2 harbor a *lox* site in *ade2*, *swi4* and in both genes, respectively. Cleavage of MQade2 DNA yields 4.8 and 0.92 Mb products; of MQswi4 DNA yields 3.0 and 2.7 Mb products; and of MQswi4ade2 DNA at one or both *lox* sites yields all of the products shown. A, B, C and D represent hybridization probes.

presence of *lox2* and then fractionated by pulsed field gel electrophoresis. Under conditions that resolve fragments <2 Mb, a band of ~0.92 Mb (band Z) was seen from MQade2 and MQswi4ade2 (Fig. 4A, lanes 1, 2 and 5). The appearance of band Z depended on both Cre and *lox2* and the product size falls within the estimated range of distance from *ade2* to the right telomere (21). With MQswi4ade2, an additional fragment (band Y) was present and only in reactions with both Cre and *lox2*. Band Y is ~1.8 Mb and agrees with the estimated distance between *swi4* and *ade2* (21). The staining intensity of band Y is less than that of band Z and is likely due to the lower probability of cleavage at both *lox* sites. The other predicted cleavage products with MQade2 and MQswi4ade2 DNA are >2 Mb and were not resolved under these fractionation conditions.

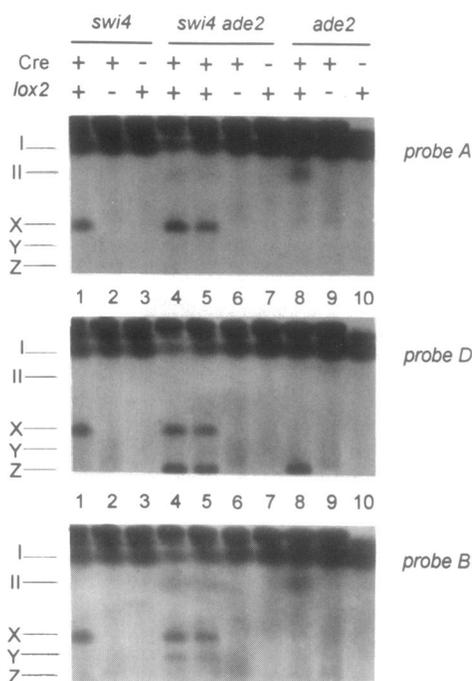
To detect the cleavage products >2 Mb, large DNA from MQade2, MQswi4ade2 and MQswi4 was subjected to Cre-*lox*-mediated cleavage and fractionated under conditions that separate the three *S.pombe* chromosomes. These conditions, however, do



**Figure 4.** Cre-*lox* mediated cleavage of *S.pombe* chromosome I at *ade2* and/or *swi4*. MQade2 (labeled *ade2*), MQswi4 (labeled *swi4*) and MQswi4ade2 (labeled *swi4ade2*) DNA was treated with (+) or without (-) Cre and *lox2* as indicated. Mobility of chromosomes I, II and III, and size markers are indicated in Mb. Cre reaction products were fractionated in 0.8% agarose in a BioRad CHEF DR11 apparatus with (A) 150 V, switch time ramp from 80 to 400 s, 40 h or (B) 70 V, switch time ramp from 20 to 30 min, 48 h.

not give good resolution for the smaller size fragments. A band of ~3 Mb (Fig. 4B, band X) was produced from MQswi4 DNA in the presence of Cre and *lox2* (lane 1), but not from reactions omitting either (lanes 2-3). The product size is consistent with the estimated distance between *swi4* and either the left telomere (3 Mb) or the right telomere (2.7 Mb) (21). DNA to either the left (Fig. 3, probe A) or the right (Fig. 3, probes B, C and D) of *swi4::lox* hybridized to band X (Fig. 5, data not shown for probe C). Hence, band X represents the co-migration of both chromosome 'arms' split at the *swi4* locus.

With MQade2 DNA, the 0.92 Mb band was again visible with ethidium bromide staining (Fig. 4B, lane 8, band Z). This band hybridized to DNA on the right (Fig. 5, probe D) but not the left side (Fig. 5, probes A and B, probe C blot not shown) of *ade2::lox*. The predicted size of the other product from cleavage at *ade2::lox* is 4.8 Mb. This product would co-migrate with chromosome II (Fig. 4B, lane 8) and could only be observed by Southern blotting. A band with the mobility of chromosome II was detected by probes



**Figure 5.** Southern blot analysis of the Cre-*lox* cleavage products from MQ*ade2* (labeled *ade2*), MQ*swi4* (labeled *swi4*) and MQ*swi4ade2* (labeled *swi4ade2*) DNA shown in Figure 4B. Hybridization probes A, B, C and D represent DNA on one side of the *lox* sequence as depicted in Figure 3. The blot with probe C (not shown) gave an identical pattern as the blot with probe B.

A, B (Fig. 5) or C (not shown), but not by probe D, and only in lanes where Cre and *lox2* were included in the reaction. This confirms that a Cre-generated chromosome I fragment representing DNA on the left side of *ade2::lox* co-migrated with chromosome II.

With MQ*swi4ade2* DNA, in addition to bands Y and Z seen in Figure 4A, band X was also present after treatment with Cre and *lox2* (Fig. 4B, lanes 4 and 5). Band Y is barely detectable by ethidium bromide staining and as in Figure 4A, the lower abundance of band Y is likely due to the lower probability of cleavage at both chromosomal *lox* sites. This interpretation is supported by the presence of a faint band of 4.8 Mb detected by Southern blotting with probes A and B (Fig. 5, lanes 4 and 5), representing DNA cleaved at *ade2* but not at *swi4*. Band Y hybridized to DNA within (probe B, lanes 4 and 5; probe C, not shown) but not outside (probes A and D, lanes 4 and 5) the intervening region, supporting the interpretation that it is the chromosome segment between *swi4* and *ade2*.

## DISCUSSION

Various techniques have been described for the single-site cleavage of genomic DNA. Specific binding of a repressor to a chromosomally placed recognition site (22) or the formation of a DNA triple-helix (23–26) can protect a single restriction site from methylation prior to endonuclease cleavage at the non-methylated site. Direct cleavage of chromosomally placed recognition sites has also been achieved with intron-encoded endonucleases (27,28),  $\lambda$  terminase (29), or with a DNA cleavage agent attached to the Cap protein (30). Like all other methods that are not based on the formation of a triple-helix, Cre-mediated

site-specific cleavage requires prior introduction of a recognition site. In some systems, site-selection is possible through homologous recombination into known loci (31,32). In others, such as in higher plants, useful targets can be selected from collections of randomly inserted recognition sites in the genome (3–5,29). To amass a large collection of *lox*-transgenic lines, we would encourage the inclusion of the 34 bp *lox* sequence in routine gene transformation experiments.

What sets the recombinase-mediated cleavage method apart from the other techniques is that the same recognition sites that flank a chromosome segment will mark the intervening DNA for both *in vivo* and *in vitro* manipulations. Since the intervening DNA need not be flanked by recombination sites of the same orientation, the DNA representing either inversions or deletions caused by site-specific recombination *in vivo* can be cleaved by intermolecular recombination *in vitro*. In addition, cleavage products have defined ends, the *lox* sequences. Subsequent recombination reactions *in vitro* can integrate the cleavage product into another *lox*-containing DNA fragment, such as a yeast (YAC) or bacterial (BAC) artificial chromosome vector, or a chromosome of another organism.

The Cre-*lox* *in vitro* recombination reaction can also yield products that are end-labeled. The *lox* oligonucleotide can be chemically synthesized such that the label is on only one end of the *lox* sequence. When used as a substrate for recombination with chromosomal *lox* sites, these end-labeled sequences would be incorporated into the chromosomal fragment. Fragments that are end-labeled can facilitate physical analysis of the region surrounding the *lox* site, such as through partial digestion of the DNA fragment by rare-cutting endonucleases. In principle, it should be possible to compile chromosome maps through the analysis of a large collection of *lox*-transgenic lines, with each line harboring a *lox* site in a different chromosomal location.

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