Site-specific cleavage of chromosomes \textit{in vitro} through Cre–\textit{lox} recombination

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ABSTRACT

Site-specific recombination systems are useful tools for chromosome engineering \textit{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 \textit{in vitro} using the Cre–\textit{lox} site-specific recombination system. Two \textit{lox} sites were targeted into the 5.7 Mb chromosome I of \textit{Schizosaccharomyces pombe}. \textit{In vitro} recombination between chromosomal \textit{lox} sites and exogenously provided \textit{lox} oligonucleotides ‘cleaved’ the chromosome at the defined \textit{lox} sequences. Site-specific cleavage of \textit{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–\textit{lox} mediated chromosome rearrangements \textit{in vivo}. Moreover, recombination with end-labeled \textit{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–\textit{lox}, FLP–FRT and R–R\textsubscript{s} systems from bacteriophage P1, \textit{Saccharomyces cerevisiae} and \textit{Zygosaccharomyces rouxii}, respectively, have received considerable attention for the manipulation of heterologous genomes \textit{in vivo}. These systems are strikingly similar as a single protein, Cre, FLP or R, is sufficient to catalyze recombination between corresponding \textit{lox}, FRT or R\textsubscript{s} 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), \textit{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 \textit{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 \textit{in vivo}.

Here, we describe that the Cre–\textit{lox} system can essentially ‘cleave’ a chromosome at defined \textit{lox} sites through \textit{in vitro} recombination with exogenously provided recombination sites. The specific cleavage of \textit{lox} sites that have been targeted into chromosome I of \textit{Schizosaccharomyces pombe} generated chromosomal segments of predicted sizes. Efficient cleavage of a \textit{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 \textit{lox} oligonucleotides. End-labeled DNA substrates thus produced would facilitate the physical mapping of chromosome regions adjacent to the \textit{lox} sites.

MATERIALS AND METHODS

Cre purification

Cre was purified from \textit{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\textsubscript{4}, 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 \textlambda\textsubscript{P} promoter under the control of the \textlambda\textsubscript{C} repressor. pMQ6 contains a \textit{lox} sequence in the \textit{ClaI} site of the \textit{ade2::URA3} gene-disruption vector pDS23 (15).

pMQ73a was made by ligating a \textit{lox}–\textit{URA3} fragment into a portion of a PCR-amplified \textit{swi4} gene (16) and then inserting the \textit{swi4} flanked-\textit{lox}–\textit{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+ leu1.32 ura4.294 ade6.216) and Ura+ deep-red ade2+ade6– colonies were confirmed by Southern blotting. For MQswi4, J5S (h+ leu1.32 ura4.294) was transformed with linear pMQ73 (swi4::lox–URA3 disruption vector) and Ura+ 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 x 10^7 genome equivalents in 50 μl) 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′-GATAACGTCGTATAGCATACATTATACGAAGTTAT-3′ and 5′-CTAGATAACCTCGTATAATGTATGCTATACGAAGTTAT-3′.

Reactions with 150 ng Cre were incubated for 1 h at 37°C in 100 μl of 50 mM Tris–HCl (pH 7.5), 33 mM NaCl, 5 mM MgCl2, 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 x 10^6 amphiidiploid 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 XhoI (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 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 XhoI 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 XhoI 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 XhoI, 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.

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 DRII 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.

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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 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 was resolved under these fractionation conditions.

The 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 e 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.

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.
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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REFERENCES