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Repeated Chromosome Splitting Targeted to δ Sequences in *Saccharomyces cerevisiae*

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We have previously developed a chromosome-splitting technique based on homologous recombination in *Saccharomyces cerevisiae*. To facilitate chromosome splitting at multiple sites, we focused on the δ sequences that are distributed in more than 200 copies throughout the yeast genome. We constructed a new chromosome-splitting vector harboring the *YFLWdelta4* sequence and the *hisG-URA3-hisG* cassette, and transformed yeast cells with this vector. The karyotype analysis of transformants showed that chromosomes XIV, III, and IV, or other chromosomes are split. After the excision of the *URA3* gene, the transformant with split chromosome IV was subsequently transformed with the same vector. Karyotype analysis revealed that further splitting occurred at chromosome X, the split chromosome IV, or other chromosomes. These results indicate that δ sequences are efficient target sites for repeated chromosome splitting at multiple sites with a single vector.

[**Key words:** chromosome engineering, δ repeated sequence, yeast, genome]

Chromosome engineering techniques, including those for breaking a chromosome, for deleting or inverting chromosome segments and for fusing two nonhomologous chromosomes (1–3), are useful not only for studying the organization of eukaryotic genome but also for the improvement of industrially important strains. We have already developed a one-step chromosome splitting method in a haploid yeast cell (Fig. 1) (2), and succeeded in creating a yeast strain having 21 chromosomes by this method (4). However, the current methods that we have used in chromosome splitting are time-consuming and hamper high-throughput performance of chromosome splitting supposing that one intends to split chromosomes many times. One of these time-consuming processes is cloning of the target sequence required for initial step of chromosome splitting into a chromosome-splitting vector prior to each transformation. The chromosome-splitting technique will have a wider variety of applications if this process is circumvented. Thus, we focused on the δ direct repeat sequences that are found at both ends of the yeast transposon, Ty (5). Each δ sequence and its homologues are distributed throughout the yeast genome and are present in more than 200 copies (http://www.yeastgenome.org/), making them a potential target site for repeated chromosome splitting at multiple sites. To test this idea, we cloned the *YFLWdelta4* sequence as an *Eco*RI-*Sal*I fragment by PCR using a cosmid library of yeast chromosome VI (a gift of Y. Murakami) as a template and primers 5′-CTCGAATTCTGTTGGAATAAAAATCAACT-3′ and 5′-CTCGTCGACTGTAGAGAATGTGGATTTTG-3′, corresponding to the sequences from nucleotides 143534 to 143553 and 143865 to 143846 of chromosome VI (underlined), respectively (http://www.yeastgenome.org/), since *YFLWdelta4* shares high homology (>90%) with other δ se-

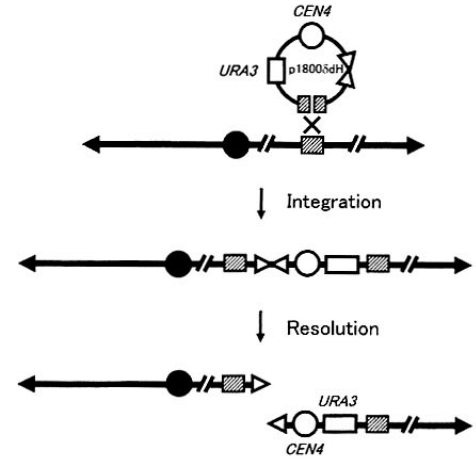

FIG. 1. A schematic diagram of chromosome splitting. This diagram is illustrated for the case of chromosome splitting with plasmid p1800δdH. Integration of the chromosome-splitting vector p1800δdH followed by telomere resolution results in splitting of a chromosome at the target site into two monocentric chromosomes. The open triangles, open circles, closed circles, open boxes, and hatched boxes indicate the Tr sequences, *CEN4*s, original centromeres, *hisG-URA3-hisG* markers, and δ sequences, respectively.

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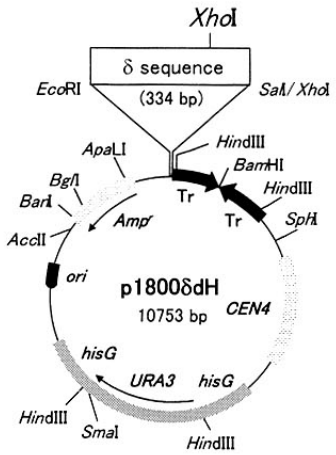

FIG. 2. Structure of chromosome-splitting vector p1800δdH for targeting δ sequences. The δ sequence indicates the *YFLWdelta4* sequence 334 bp in size. The regions marked *Amp*^{r} and *ori* and the thin lines indicate the DNA fragments originating from pBR322. The regions marked *URA3* and *CEN4* are genes originating from *S. cerevisiae*. The inner arc with an arrowhead indicates the transcription direction of genes. The Tr sequence (arrow) is derived from the end of the extrachromosomal rDNA of *Tetrahymena*. The *hisG-URA3-hisG* marker confers Ura$^+$ phenotype, but recombination between the repeated *hisG* sequences results in the loss of the *URA3* marker (8). Growth on 5-FOA was used to select clones that showed rescued Ura$^-$ phenotype.

quences, as determined by results of database analysis (data not shown). After confirming the successful amplification of the *YFLWdelta4* sequence by DNA sequencing, the *YFLWdelta4* sequence was used to construct the chromosome-splitting vector p1800δdH which harbors the *hisG-URA3-hisG* cassette as a reusable selection marker (Fig. 2).

Prior to the transformation of yeast, plasmid p1800δdH was linearized by cutting the *Xho*I site in the δ sequence (Fig. 2). The linearized vector was introduced into strain W303-1A (*MAT***a** *leu2-3, 112 trp1-1 ura3-1 ade2-1 his3-11, 15 can1-100*) by the lithium acetate method (6), and some of resulting uracil prototrophic (Ura$^+$) transformants were analyzed of their chromosome structure by pulsed-field gel electrophoresis (PFGE) and Southern blotting. Chromosome splitting occurred in approximately 20% of Ura$^+$ transformants. The splitting of the δ sequence by transformation with plasmid p1800δdH is summarized in Fig. 1. The results are shown in Fig. 3A. In lane 2, the band corresponding to the intact chromosome XIV (800 kb) was not detected, but a new band corresponding to a 210-kb fragment was observed and the intensity of the band corresponding to chromosome V (600 kb) was enhanced, suggesting that chromosome XIV was split into a 210-kb fragment and a 600-kb fragment. Similarly, in lane 3, while the band corresponding to the intact chromosome III (360 kb) was not detected, two new bands corresponding to 200-kb and 170-kb fragments were observed, suggesting that chromosome III was split. Moreover, in lane 4, the band corresponding to the intact chromosome IV (1640 kb) was absent, whereas two new bands corresponding to 1000-kb and 660-kb fragments were observed, suggesting that chromosome IV was split. In lanes 5 to 7, no apparent alterations in the electrophoresis patterns were observed, but new bands corresponding to170-kb (lane 5), 110-kb (lane 6), and 60-kb (lane 7) fragments, which are smaller than chromosome I (240 kb), were detected.

To confirm that the new bands are a result of chromosome splitting, we carried out Southern analysis with a *URA3* probe (Fig. 3B), since one of the split chromosome fragments should contain the *URA3* gene derived from the chromosome-splitting vector (Fig. 1). In the parental strain (lane 1), the signal generated by the *URA3* probe was detected at a position corresponding to the intact chromosome V since strain W303-1A harbors the *ura3-1* locus in chromosome V, which should hybridize with the *URA3* probe. In lane 2, the intensity of the signal corresponding to chromosome V (600 kb) was enhanced. By Southern analysis using the *CEN4* probe, the signal was not detected at the position of the intact chromosome V in lane 1, but observed at a position corresponding to chromosome V in lane 2 (data not shown), suggesting that the 600-kb fragment derived from chromosome XIV overlaps with chromosome V since one of the split chromosome fragments should hybridize with the *CEN4* probe (Fig. 1). In lanes 3 to 7, the hybridization signals were detected at positions corresponding to 200-kb (lane 3), 660-kb (lane 4), 170-kb (lane 5), 110-kb (lane 6), and 60-kb (lane 7) fragments. These results suggest that chromosome splitting occurred in these transformants and that the splitting sites were selected randomly. Based on the disappearance of intact chromosomes XIV, III, and IV and from the estimated sizes of the chromosome fragments newly generated in lanes 2 to 4 (Fig. 3A), chromosomes XIV, III, and IV were highly likely to split in respective transformants. With respect to other transformants, it was not possible to determine which chromosome was split. One possibility is that large chromosomes (>900 kb) are split at the δ site located at the chromosome end in these transformants since elimination of relatively short segments (60–220 kb) from large ones may not result in detectable alterations in the PFGE profiles of large chromosomes.

To demonstrate the usefulness of the same chromosome-splitting plasmid, p1800δdH, for successive splitting, we then chose transformant no. 3 (Fig. 3A, lane 4) for further study. After confirming the successful split of chromosome IV in transformant no. 3 by Southern blotting using two open reading frames, *YDL237w* and *YDR540c*, as probes, which should hybridize with the left-hand and right-hand fragments of chromosome IV, respectively (data not shown), we screened clones showing the uracil auxotrophic (Ura$^-$) phenotype due to the loss of the *URA3* gene by plating cells of transformant no. 3 on 5-FOA medium. The elimination of the *URA3* gene was confirmed by Southern hybridization analysis (Fig. 3A, lane 8; Fig. 3B, lane 8).

Then, plasmid p1800δdH that was digested with *Xho*I as described above was introduced into the Ura$^-$ clone of transformant no. 3 and karyotype patterns of desired transformants (approximately 4% of Ura$^+$ transformants) were shown in Fig. 3A (lanes 9–14). In lane 9, the band corresponding to the intact chromosome X (770 kb) was absent, but a new band corresponding to 500 kb appeared and the intensity of the band corresponding to chromosome VI (280 kb) was enhanced, suggesting that chromosome X was split into a 500-kb fragment and a 280-kb fragment. Similarly, in lane

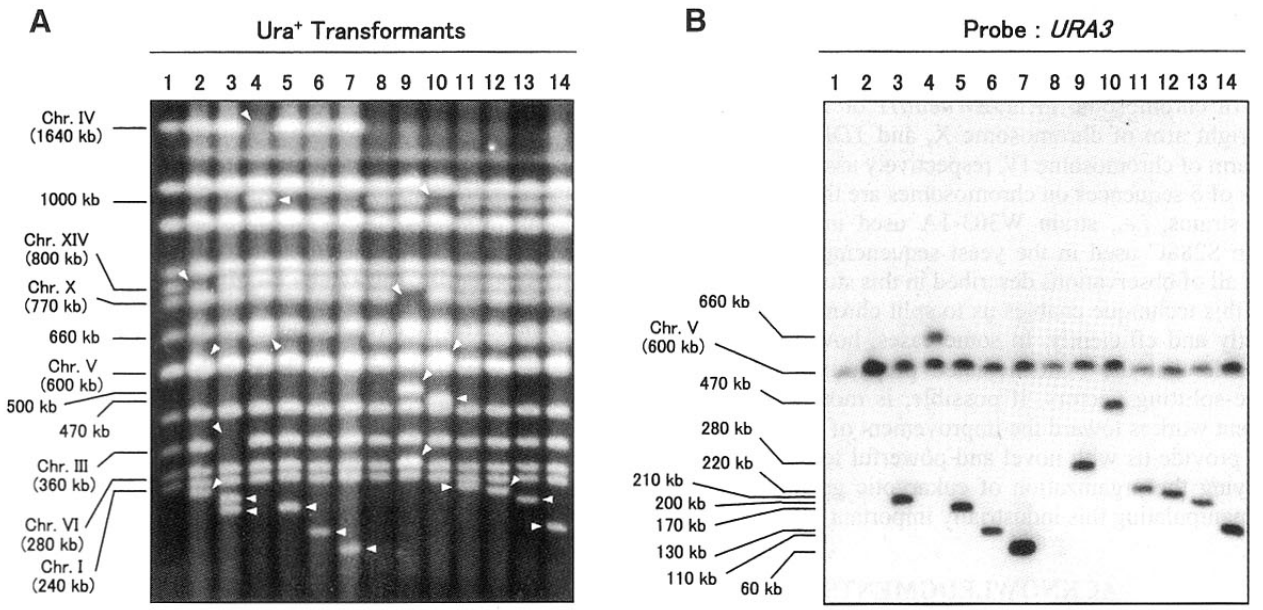

FIG. 3. Electrophoretic karyotyping and Southern analysis of Ura$^+$ transformants. (A) Electrophoretic karyotypes of Ura$^+$ transformants by PFGE. Chromosomal DNAs of *S. cerevisiae* embedded in agarose plugs were prepared as described previously (2). Chromosomal DNAs were separated on a 1% gel by CHEF electrophoresis using the application of the CHEF DR III system (Bio-Rad Laboratories, Richmond, CA, USA) with a 60-s pulse for 15 h, followed by a 90-s pulse for 9 h, at 6 V/cm, in 0.5× TBE buffer at 14°C. DNA was visualized by staining with ethidium bromide. DNA samples were obtained from the following strains: the parental strain, W303-1A (lane 1), Ura$^+$ transformants nos. 1 to 6 of strain W303-1A (lanes 2–7), the Ura$^-$ clone obtained from transformant no. 3 (lane 8), and Ura$^+$ transformants nos. 7 to 12 of the Ura$^-$ clone obtained from transformant no. 3 (lanes 9–14). Arrowheads indicate the lost and newly generated chromosomal fragments detected by staining. The positions of chromosomes I, III, IV, V, VI, X and XIV, and the sizes of the new chromosomal fragments observed in lanes 4, 9, and 10 are indicated on the left-hand side of the panel. (B) Southern hybridization analysis of chromosomes resolved by PFGE. Southern hybridization was performed with a 1.2-kbp *URA3* probe. Probe labeling and detection were carried out using the ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Hybridization signals were detected on an ECL film (Amersham Pharmacia Biotech). Lanes 1 to 14 contain DNAs corresponding to those in lanes 1 to 14 in panel A. The estimated sizes of signals observed are indicated on the left-hand side of the panel.

10, the 1000-kb fragment derived from chromosome IV disappeared whereas a new band corresponding to a 470-kb fragment appeared and the intensity of the band corresponding to chromosome V (600 kb) was enhanced, suggesting that the 1000-kb fragment derived from chromosome IV was split. In other lanes (lanes 11–14), no apparent alterations in the electrophoresis patterns were observed, but new bands corresponding to 220-kb (lane 11), 210-kb (lane 12), 200-kb (lane 13), and 130-kb (lane 14) fragments, which are smaller than chromosome I (240 kb), were detected. Southern analysis using a *URA3* probe revealed that the hybridization signal was detected at a position corresponding to chromosome VI (280 kb) in lane 9, but not in lane 8 (parental strain) (Fig. 3B). In lane 10, the hybridization signal using *URA3* probe was detected at a position corresponding to the new band (470 kb). In addition, the hybridization signal generated by the *CEN4* probe was detected at a position corresponding to chromosome V (data not shown), suggesting that the 600-kb fragment derived from the 1000-kb fragment overlaps with chromosome V. In lanes 11 to 14, the hybridization signals were detected at positions corresponding to 220-kb (lane 11), 210-kb (lane 12), 200-kb (lane 13), and 130-kb (lane 14) fragments. These results indicate that chromosome splitting occurs successively at multiple sites in these transformants. It should be mentioned that the reason transformants harboring chromosome VI split at the *YFLWdelta4* locus were not observed was because the splitting of chromosome VI at the *YFLWdelta4* locus generates two unstable fragments, one that is acentric and another that is dicentric, due to the direction of the *YFLWdelta4* sequence in plasmid p1800δdH.

The original chromosome-splitting method that we have reported requires cloning of the target sequences into a chromosome-splitting vector such as pCSV1 (2) prior to each transformation. This is a time-consuming and laborious process. In addition, the number of available selection markers limits repeated splitting. To solve these problems, we constructed the new chromosome-splitting vector p1800δdH carrying a δ sequence which is a representative repeated sequence found in more than 200 copies of yeast chromosomes as the target site and a *hisG-URA3-hisG* cassette which is often used as a reusable marker. We then repeatedly transformed yeast cells with this vector and results presented here demonstrated that δ sequences can be used as target sites for repeatedly splitting chromosomes at multiple sites. Although we did not detect simultaneous chromosome splitting in individual transformants as reported by Sakai *et al.* (7) in which they used δ sequences as target sites for integrating an expression vector, the issue of simultaneous splitting could be overcome by carrying out successive transformations using the same splitting vector, as shown in this study. Based upon the information of Sacchromyces Genome Database (http://www.yeastgenome.org/) and the observed sizes of chromosome fragments in lanes 4, 9, and 10,

chromosome IV (lane 4), X (lane 9), and the 1000 kb fragment derived from chromosome IV (lane 10) were likely to be split at the *YDRWdelta19* or *YDRWdelta20* on the right arm of chromosome IV, *YJRWdelta11* or *YJRWdelta12* on the right arm of chromosome X, and *YDLCdelta1* on the left arm of chromosome IV, respectively assuming that positions of δ sequences on chromosomes are the same between two strains, *i.e.*, strain W303-1A used in this study and strain S288C used in the yeast sequencing project. Along with all of observations described in this study, we conclude that this technique enables us to split chromosomes conveniently and efficiently. In some cases, however, it is conceivable that the PCR-mediated preparation of chromosome-splitting vectors, if possible, is more efficient. Our current work is toward the improvement of this method that will provide us with novel and powerful tools not only for studying the organization of eukaryotic genomes but also for manipulating this industrially important microorganism.

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