JOURNAL OF BIOSCIENCE AND BIOENGINEERING
Vol. 106, No. 6, 563–567. 2008
DOI: 10.1263/jbb.106.563



# Construction and Characterization of Single-Gene Chromosomes in *Saccharomyces cerevisiae*

Kazuo Yamagishi,[1] Minetaka Sugiyama,[1] Yoshinobu Kaneko,[1] Masafumi Nishizawa,[2] and Satoshi Harashima[1]*

*Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita-shi, Osaka 565-0871, Japan*[1] *and Department of Microbiology and Immunology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160-8582, Japan*[2]

Received 3 July 2008/Accepted 18 August 2008

**Based on a previously developed PCR-mediated chromosome splitting method, a genome engineering technique was developed in haploid *Saccharomyces cerevisiae* for incorporating any desired chromosomal region into a chromosome that carries a single gene. Based on the viability of cells carrying an essential gene in such a construct, close physical proximity of two telomeres and a centromere does not appear to compromise gene function. Spontaneous loss of constructed single-gene chromosomes during vegetative growth was high (0.2–0.4 per cell division), suggesting the possibility of creating novel cells carrying single-gene chromosomes derived from various chromosomal regions in a variety of combinations by exploiting combinatorial loss.**

[**Key words:** budding yeast, Cre-loxP system, chromosome instability, chromosome loss, chromosome splitting, mini-chromosome]

On-going and more recent developments in gene manipulation technology have facilitated important research contributions in the biological sciences and their application in biotechnology. Chromosome manipulation technology as an outgrowth of gene manipulation technology has provided opportunities for creating novel strains of organisms with a variety of genomic constitutions (1–3). Chromosome manipulation technology includes methods for breaking and splitting a chromosome, deleting or inverting chromosomal segments, and for fusing non-homologous chromosomes. A simple and rapid chromosome splitting technique called PCR-mediated chromosome splitting (PCS) that we recently developed has made it possible to manipulate chromosome on a large scale (4).

The PCS method splits chromosomes by introducing two PCR fragments, one containing a target sequence for splitting, a selective marker and a telomere-seed sequence, while the other contains a target sequence, a centromere and a telomere-seed sequence. These two PCR fragments can be prepared easily by performing two PCRs, thus making it possible to split chromosomes very rapidly compared to traditional methods, one of which we developed (5), and which requires cloning of the target sequence to facilitate homologous recombination in yeast. This technique was further improved for use with yeast artificial chromosomes (YAC) to generate new YACs from endogenous YACs previously introduced into yeast by transformation, by adding autonomously replicating sequences such as *H4ARS*, into splitting DNA fragments (6). Here we designate this improved method the ARS-PCS method.

In the present study, we used the ARS-PCS method to create extremely short (about 7 kb) mini-chromosomes by spitting native chromosomes and then characterized the behavior of such mini-chromosomes. Single-gene chromosomes were generated by this approach and were found to be mitotically unstable, suggesting that this technology may not only be useful for revealing the function of presently uncharacterized chromosomal regions, but also for constructing genetically novel strains of organisms.

## MATERIALS AND METHODS

**Strains, media, and growth conditions** *Saccharomyces cerevisiae* strains harboring mini-chromosomes were derived from FY833 (*MAT***a** *ura3-52 his3*Δ*200 leu2*Δ*1 lys2*Δ*202 trp1*Δ*63*) by splitting at appropriate sites (4, 6). *S. cerevisiae* strain SCGLC7L and SCGLC7+ARS were constructed by splitting chromosome V upstream of *GLC7* at position 432,081 (http://yeastgenome.org/) using the PCS or ARS-PCS method, respectively. *S. cerevisiae* strain SCGLC7LR was constructed by splitting chromosome V in SCGLC7L+ARS downstream of *GLC7* at position 434,220. *S. cerevisiae* strain SCGLC7LR-M was constructed by removing *CgHIS3* by use of the Cre-loxP system in SCGLC7LR. *S. cerevisiae* strain SCSIW14L was constructed by splitting chromosome XIV downstream (575,776) of *SIW14* and strain SCSIW14LR was constructed by further splitting of upstream (574,027) of *SIW14* in SCSIW14L. *S. cerevisiae* strain SCSTE2L was constructed by splitting chromosome VI downstream (84,123) of *STE2* and strain SCSTE2LR was constructed by further splitting of upstream (81,828) of *STE2* in SCSTE2L Yeast strains were grown according to standard procedures either in glucose-based nutrient-rich medium (YPD) or in appropriate selective media.

**Plasmid construction** Plasmids used as templates for PCS

* Corresponding author. e-mail: harashima@bio.eng.osaka-u.ac.jp
phone: +81-(0)6-6879-7420 fax: +81-(0)6-6879-7421

and the Cre recombinase-expressing plasmid, pSH47, have been described (4, 7). pUG6-CEN-ARS which was used as a template for the ARS-PCS method was constructed by amplifying the entire sequence of *H4ARS* and cloning it into the *Hin*dIII site in p3121 (4, 7), the resultant construct was designated pUG6-CEN-ARS. pRS316-GLC7 was constructed by amplifying upstream of *GLC7* at position 432,081 to downstream of *GLC7* at position 434,220 and cloning it into the *Sma*I site in centromere-type plasmid with *URA3* marker, pRS316 (8).

**Chromosome splitting and removal of selective marker** Chromosome splitting was performed according to the PCS (4) and ARS-PCS methods (2). Yeast strains harboring mini-chromosomes were derived from FY833. Cre-loxP recombination was used as needed by transforming the strain with the Cre recombinase-expressing plasmid, pSH47 (4, 7). Transformants were cultivated in nutrient-rich galactose medium over 12 hours and spread onto YPD plates. Colonies were replicated to -Ura and -His selective media, and those found to be auxotrophic for both uracil and histidine were selected (4).

**CHEF gel electrophoresis and Southern hybridization** Chromosomal DNA from *S. cerevisiae* embedded in agarose plugs was prepared essentially as described previously (9). Chromosomal DNA was separated in a 1% agarose gel using a CHEF-DRIII system (Bio-Rad Laboratories, Hercules, CA, USA).

Southern transfer of DNA, hybridization, washing, and detection were performed using the ECL system according to the manufacturer's instructions (GE Healthcare, Buckinghamshire, UK). Probe DNA was amplified by PCR and labeled using the ECL direct nucleic acid labeling system.

**Calculation of mitotic loss of mini-chromosomes** Because single-gene chromosomes carry the *CgHIS3* marker inserted upon splitting by PCS, rate of loss was evaluated by counting colonies that appeared on the histidine selective and non-selective plates ($His^+$ and $His^-$), respectively. Overnight cultures of each strain were used to inoculate synthetic complete (SC) without uracil medium and cells were then grown for 2 h. A portion of the cultures was diluted and spread onto uracil selective plates to measure initial cell number. Cultures were grown for an additional two generations and diluted and spread onto uracil selective plates to estimate final cell number. Colonies on uracil selective plates were counted and replicated to histidine selective plates. Colonies on histidine selective plates were counted and loss rates of mini-chromosome were calculated according to the following equation.

$$\text{Loss rate} = 1 - (F/I)^{1/N}$$

where F and I equal the number of colonies on selective and YPD medium, respectively. N equals $\text{Log}_2(A_f/A_i)$. $A_i$ equals the initial $OD_{600}$ value and $A_f$ equals the final $OD_{600}$ value (10). More than 1000 colonies were counted in every case and loss rates were measured at least 3 times per strain.

## RESULTS

**Construction of single-gene chromosomes** The ARS added PCS method which we call the ARS-PCS method is needed to construct single-gene chromosomes because generally, such small fragments do not harbor ARS elements (4, 6, 11). To apply the ARS-PCS method for creating of a single-gene chromosome, we constructed a new vector containing an *H4ARS* sequence from a *CEN4*-containing vector, p3121 (7). The resultant plasmid, designated pUG6-CEN4-ARS, was used as template for the first PCR in the ARS-PCS method (Fig. 1A).

To demonstrate the utility of pUG6-CEN4-ARS as a PCR template for the ARS-PCS method, we employed *GLC7* as a model gene to generate a single-gene chromosome as there are no ARSs near the *GLC7* locus on chromosome V (11–13). First, chromosome V was split 1 kb upstream of *GLC7* with the ARS-PCS method by introducing two PCR fragments into host strain FY833 (see Materials and Methods). One carried the *CgLEU2* marker and a telomere-seed sequence, and the other harbored *CEN4*, *H4ARS*, and a telomere-seed sequence. One of the resultant $Leu^+$ transformants was designated SCGLC7L+ARS (Fig. 1A). To evaluate the effect of *H4ARS*, splitting was also performed using the original PCS method not employing ARS (4). The resultant strain was designated SCGLC7L. Based on the 10 transformants analyzed by PFGE and Southern analysis (data not shown), both methods yielded 100% splitting of chromosomeV upstream of *GLC7*, indicating that because *GLC7* is essential, expression of the gene on the split chromosome was sufficient to yield viable transformants.

Using these strains, we subsequently attempted splitting chromosome V 500 bp downstream of *GLC7* with traditional PCS method (Fig. 1B). SCGLC7L and SCGLC7L+ARS were introduced with two PCR fragments, one carried the *CgHIS3* marker, telomere-seed sequence and recombination sequence, and the other harbored *CEN4*, telomere-seed sequence and recombination sequence. $His^+$ transformants were obtained from both SCGLC7L and SCGLC7L+ARS. Four of 10 SCGLC7L+ARS transformants had mini-chromosomes of the expected size (about 9 kb; Fig. 1B, lanes 1, 3, 4 and 8) by PFGE/Southern analysis, while two were unexpectedly longer, at about 11 kb (Fig. 1B, lanes 7 and 9). However, lanes 3, 4 and 8 in Fig. 1B showed both chromosome V band and expected small band, indicating that *GLC7* is remained in chromosome V. The copy number of the new mini-chromosome in three transformants appeared to increase, perhaps to compensate for its instability (Fig 1B, lanes 1, 7 and 9) (14). In contrast to the ARS-PCS method, no transformants with mini-chromosomes were obtained using the conventional PCS method without an ARS element (data not shown), indicating that the ARS-PCS method can effectively convert different chromosomal regions into single-gene chromosomes. The strain with expected *GLC7* mini-chromosome in Fig. 1B, lane 1, was designated SCGLC7LR and further analyzed.

**Structural analysis of a single-gene chromosome of unexpected length** The unexpectedly large single-gene chromosome (11 kb) was analyzed as it appeared in two of the 6 transformants (Fig. 1B). One possible explanation for the difference in chromosome size was that telomere length is different in those single-gene chromosomes because telomere length has been observed to fluctuate in *S. cerevisiae* (15). Alternatively, it is possible that some unexpected sequence was inserted into the single-gene chromosome. To distinguish these two possibilities, telomere length in these two types of mini-chromosomes was analyzed. Chromosomal DNA was extracted and genomic Southern blotting was performed using *CgHIS3* as a probe. The results indicated that telomere length was not significantly different between the 9 and 11 kb mini-chromosomes (data not shown).

To investigate the second possibility, we eliminated the marker by loxP-mediated recombination because the loxP