

# Use of gap repair in fission yeast to obtain novel alleles of specific genes

Corwin F. Kostrub\*, Elissa P. Lei and Tamar Enoch

Department of Genetics, Harvard Medical School, Warren Alpert Building, 200 Longwood Avenue, Boston, MA 02115, USA

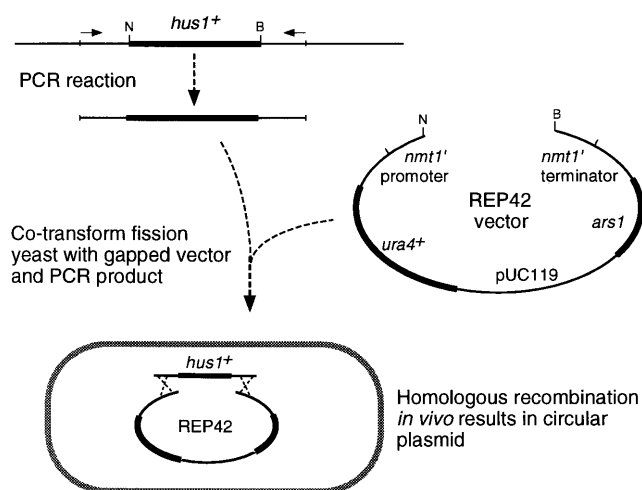
Received July 8, 1998; Revised and Accepted August 26, 1998

## ABSTRACT

We have adapted a method for making libraries of mutations in any specific gene for use in the fission yeast *Schizosaccharomyces pombe*. This elegant and simple method consists of PCR amplification of the gene of interest, followed by co-transformation of fission yeast with the PCR fragment and a linearized plasmid vector prepared such that the ends of the vector share DNA sequence with the ends of the PCR fragment. Homologous recombination between the vector and the PCR fragment occurs at a high frequency and results in a collection of yeast transformants, most harboring a mutated allele of the original gene within the vector of choice. This library can then be screened or selected for phenotypes of interest.

Using classical genetic screens in model organisms such as yeast, one can easily identify genes involved in a particular process. However, mutagenizing yeast and screening or selecting for the appropriate phenotype often results in the acquisition of only one allele of a gene, or only complete loss of function alleles of a gene. It is often useful to obtain additional alleles of a particular gene of interest, such as conditional temperature-sensitive alleles or alleles with novel phenotypes. With these goals in mind, we have adapted a method of generating new alleles of any known gene for use in the fission yeast *Schizosaccharomyces pombe*.

Gene-localized mutations have been made using the method of gap repair in the budding yeast *Saccharomyces cerevisiae* (1), but the feasibility of this technique in the fission yeast *S.pombe* was untested. Here we describe the successful application of this technique in the fission yeast. Gap repair relies on the ability of yeast to repair gapped DNA sequences *in vivo* by homologous recombination. This phenomenon has previously been used in *S.pombe* to clone mutations from the chromosomal genome onto a plasmid in order to sequence the mutant allele (2). By co-transforming the yeast with a linearized vector containing a selectable marker and an autonomously replicating sequence, along with a DNA fragment that spans the gap in the vector, homologous recombination between the vector and the DNA fragment insert *in vivo* results in a circular plasmid containing the DNA fragment, which can efficiently propagate (Fig. 1). We have found that this reaction occurs efficiently in *S.pombe*. If the DNA fragments are generated by mutagenic PCR, the transformation results in a collection of fission yeast, each harboring a repaired plasmid containing a potentially mutant form of the DNA



**Figure 1.** Schematic overview of the method of gap-repair. For our pilot experiment, we PCR-amplified the *hus1*<sup>+</sup> gene using pREP42-*hus1*<sup>+</sup> (6) as a template and co-transformed this PCR product with a linearized pREP42 vector (7,8) into a strain deleted for the *hus1*<sup>+</sup> gene (6). The ends of the PCR product share sequence with the ends of the linearized pREP42 vector. Homologous recombination *in vivo* results in a repaired circular plasmid that directs expression of the *hus1*<sup>+</sup> gene from the *nmt1*' thiamine-repressible promoter.

fragment. The complexity of the resulting library is only limited by the complexity of the original collection of mutant PCR products. This collection of mutants can then be screened directly for phenotypes of interest. After mutants with interesting phenotypes are identified, determining their actual nucleotide sequence is very simple since they exist on plasmids which can readily be recovered in *Escherichia coli*.

PCR reactions can be made mutagenic by several methods (3). Two well-described approaches are (i) altering the ratio of available deoxynucleotides added to the PCR reaction and (ii) the addition of a sub-optimal cation, often Mn<sup>2+</sup>, to the reaction. One advantage of these approaches is that the level of mutagenesis can be controlled, either by how severely the ratio of nucleotides is skewed or by the concentration of Mn<sup>2+</sup> added. The error rate of *Taq* polymerase under standard conditions may be sufficient to obtain a complex mixture of mutant PCR products, for example when amplifying very large genes or amplifying for many cycles (4). Which ever method is used, it is wise to create the collection of mutant PCR products by pooling together several independent PCR reactions rather than to perform one large PCR reaction to

\*To whom correspondence should be addressed. Tel: +1 617 432 0288; Fax: +1 617 432 7663; Email: kostrub@rascal.med.harvard.edu

minimize the possibility of an early mutagenic event 'jackpotting' the entire pool.

A very efficient transformation is required to obtain a large library of colonies to screen. For this purpose, we recommend the high-efficiency protocol of Okazaki *et al.* (5). For the gap repair transformations, we used 100 ng (0.02 pmol) of gapped vector and 500 ng (0.8 pmol) of PCR product. To test the efficiency of the gap repair reaction, we co-transformed fission yeast deleted for the *hus1*<sup>+</sup> gene with a linearized REP42 plasmid (cut with *NdeI* and *BamHI*) and a DNA fragment containing the *hus1*<sup>+</sup> gene flanked by REP42 vector sequence generated by a non-mutagenic PCR reaction (Fig. 1). In this instance, the gap repair recombination allows expression of the *hus1*<sup>+</sup> gene from the repaired plasmid and rescues the HU-sensitive phenotype of the *hus1*Δ strain. Therefore, the proportion of total transformants that were HU resistant gives the proportion of colonies resulting from proper gap-repair reactions. Typical results are listed in Table 1.

**Table 1.** Efficiency of gap repair and mutagenesis

Phenotype	Number of cells	Percentage of total
<b>Non-mutagenic PCR</b>		
Total cells counted	554	100
HU sensitive	75	14
HU resistant (express <i>hus1</i> <sup>+</sup> )	479	<b>86</b>
<b>Mutagenic PCR</b>		
Total cells counted	425	100
HU sensitive	194	46
HU resistant	231	<b>54</b>

Using this assay, we found that ~86% of the transformants expressed the *hus1*<sup>+</sup> gene. Our gap repair transformations resulted in  $\sim 8 \times 10^4$  colonies/μg of gapped vector (a control transformation using uncut vector yielded  $\sim 1 \times 10^5$  colonies/μg), indicating that gap repair occurs efficiently. In these experiments, the vector and PCR fragment overlap by ~600 bp on either side, but we have used overlaps as short as 200 bp without reducing efficiency. Although we have not sequenced the sites of recombination to assess the precision of the recombination events, we have performed gap repair using only the 3' half of a gene, such that recombination would occur within the coding region. We found that ~70% of the resulting colonies expressed functional protein. It is unclear whether the remaining colonies resulted from imprecise recombination or recombination with other homologous sequences in the genome. Transforming yeast with gapped vector alone resulted in a background of colonies, often one third the number obtained using vector plus insert. Because the vector shares extensive sequence with the *nmt1*<sup>+</sup> genomic locus, this background may be due to recombination with those sequences.

To obtain a collection of *hus1*<sup>+</sup> mutants, we made our PCR step mutagenic by adding 50 μM MnCl<sub>2</sub> to the reactions. We tested the effectiveness of our mutagenesis by repeating the previous gap repair transformation using *hus1*<sup>+</sup> fragments generated with our mutagenic PCR conditions. Again, the proportion of HU-resistant colonies was counted (Table 1). By comparing the proportion of HU-resistant colonies resulting from the mutagenic PCR reaction to the efficiency of gap repair found using non-mutagenic PCR, we estimate that our mutagenesis resulted in an ~37% loss of function rate, showing that our PCR reaction was indeed mutagenic.

We have used our gap repair method to obtain dominant alleles of the *hus1*<sup>+</sup> gene. To accomplish this, we performed the gap

repair transformation into a wild-type fission yeast strain using the mutagenized *hus1*<sup>+</sup> PCR products. This transformation resulted in a large number of colonies (~20 000) of wild-type fission yeast, each potentially containing a plasmid expressing a mutant version of the *hus1*<sup>+</sup> gene. Our gapped vector (REP42) includes a thiamine repressible promoter (*nmt1*<sup>'</sup>) used to control the expression of our *hus1*<sup>+</sup> mutants (Fig. 1). To screen for dominant alleles, we replica plated the colonies to media lacking thiamine to induce the promoter and screened for interesting phenotypes. The plasmids from chosen colonies were rescued in *E.coli* for re-testing and sequencing. By screening only 20 000 colonies, we obtained 23 HU-sensitive colonies and six colonies inviable in the absence of thiamine (the two phenotypes for which we screened). DNA sequencing showed that each of the mutants contains multiple nucleotide changes, resulting in at least one amino acid change per mutant and several amino acid changes in most mutants. We conclude that our level of mutagenesis was excessive, because the existence of multiple amino acid changes in many mutants complicates structure–function analysis. However, the dominant alleles of our gene have proved to be useful for genetic studies, and we are currently analyzing the phenotypes in greater detail.

The success of the *hus1*<sup>+</sup> screen illustrates the value of the gap repair method to obtain novel alleles of known genes. Our group has used gap repair to screen for new alleles of several genes, and we believe it is a broadly applicable technique. One limitation we have encountered is that the gap repair transformation does not occur efficiently in mutant strains with abnormal recombination for obvious reasons, but this limitation should not be relevant to most applications of the technique. As a result of the fission yeast genome project, large numbers of genes with unknown function are being identified. Large-scale targeted disruptions of these genes of unknown function could be made, in the hope that the phenotypes of the disruptions will help identify the functions of the genes. However, these disruptions would be complete loss of function alleles of the genes. It may also be useful to be able to obtain other types of alleles of the genes, particularly in the case of essential genes. The technique described here makes these types of screens very feasible.

## ACKNOWLEDGEMENTS

We thank Gladys Reimundo and Maria Sanchez for technical assistance, Matt Endrizzi and Sidong Huang of Bill Dietrich's laboratory for their sequencing expertise, and Carolyn Chapman, Sarah Evans, Musetta Leung and Elspeth Stewart for helpful comments on the manuscript. C.F.K. was supported by a Howard Hughes Predoctoral Fellowship. Work in T.E.'s laboratory is supported by a grant from the National Institutes of Health (GM50015).

## REFERENCES

- Muhlrad,D., Hunter,R. and Parker,R. (1992) *Yeast*, **8**, 79–82.
- Carr,A.M., MacNeill,S.A., Hayles,J. and Nurse,P. (1989) *Mol. Gen. Genet.*, **218**, 41–49.
- Leung,D.W., Chen,E. and Goeddel,D.V. (1989) *Technique*, **1**, 11–15.
- Tindall,K.R. and Kunkel,T.A. (1988) *Biochemistry*, **27**, 6008–6013.
- Okazaki,K., Okazaki,N., Kume,K., Jinno,S., Tanaka,K. and Okayama,H. (1990) *Nucleic Acids Res.*, **18**, 6485–6489.
- Kostrub,C.F., Al-Khodairy,F., Ghazizadeh,H., Carr,A.M. and Enoch,T. (1997) *Mol. Gen. Genet.*, **254**, 389–399.
- Maundrell,K. (1993) *Gene*, **123**, 127–130.
- Basi,G., Schmid,E. and Maundrell,K. (1993) *Gene*, **123**, 131–136.