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Inactivation of the *SGS1* and *EXO1* Genes Synergistically Stimulates Plasmid Integration in Yeast

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Summary

Different procedures used for targeted genetic manipulations are based on homologous recombination between chromosomal and exogenous DNA. A double-strand break (DSB) present on the plasmid molecule stimulates and directs plasmid integration to the homologous sequence in the yeast genome and we wondered whether this process could be further enhanced in selected DNA repair mutants. In order to compare the results obtained in different yeast strains, the efficiency of transformation with a linear integrative plasmid was compared to that obtained with a circular replicative plasmid. With respect to the wild type, the relative efficiency of transformation was increased in the *sgs1* and *exo1* mutants and decreased in the *rad1* and *srs2* mutants. Inactivation of the *SGS1* or *EXO1* gene stimulated plasmid integration 4- to 5-fold, while 15-fold increase was observed in the double *sgs1 exo1* mutant. This result indicates that the two proteins participate in different cellular processes that limit plasmid integration in the wild type yeast. Southern blot analysis of 20 transformants obtained in the double mutant confirmed that they occurred by homologous integration to the target sequence. Homologues to both *EXO1* and *SGS1* genes have been found in other organisms and we suggest that their inactivation may also lead to enhanced gene targeting.

Key words: gene targeting, *SGS1*, *EXO1*, *RAD1*, *SRS2*, plasmid integration

Introduction

Initial studies of the yeast *Saccharomyces cerevisiae* transformation pointed out that the integration of exogenous circular DNA occurs by recombination with the homologous sequence present in the genome (*1*). A double-strand break (DSB) introduced on the plasmid molecule prior to transformation stimulates and targets integration process to the chromosomal homologous region (*2*) and these observations have led to the elaboration of the DSB model for genetic recombination (*3*). Linear DNA fragments introduced by transformation may participate in another type of homologous recombination, where the resident allele is directly replaced by the allele present on the transforming fragment (*4*). Both plasmid integration and gene replacement, with different experimental modifications, are today routinely used to introduce any desired genetic alteration in the yeast genome. However, gene targeting technologies are less efficient in other organisms, from fungi (*5*) to mammals (*6*), where the large majority of integration events usually occur by illegitimate recombination. This presents the major obstacle for genetic manipulations in fundamental and applied research and a lot of effort is being made to overcome this problem (*7*). Development of procedures that would stimulate homologous recombination and/or suppress the illegitimate integration was proposed as a way to improve the efficiency of gene targeting (*6*).

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More than 100 gene products are involved in DNA repair and replication in yeast and a subset of these genes participate in different pathways of homologous and illegitimate recombination (*8,9*). The genes assigned to the *RAD52* epistasis group occupy the central position in homologous recombination, including three homologues to the bacterial *recA* gene expressed in vegetative cells. The step preceding the strand exchange is processing of the DSBs and formation of long 3′ single-stranded ends that initiate recombination. In spite of numerous genetic and biochemical studies, the molecular sequence of events and the exact role of several nucleases involved in this process are still not known (*10*). Another poorly characterized step is resolution of the recombination intermediate and the activity equivalent to that of the bacterial RuvC protein, which cuts Holliday junction (HJ) intermediates, has not been identified in yeast yet. Formation and appropriate resolution of HJs could be the critical point in gene targeting, since it has been shown that more than 95 % of repair events do not result in integration of the linearized replicative plasmid (*11*). In other words, channelling of the recombination process to the pathway that gives crossover products could significantly increase plasmid integration.

Recently Ira *et al.* (*12*) have shown that deletions of the *SGS1* and *SRS2* genes increase the proportion of DSB repair events which give crossover products in the yeast vegetative cells and it was proposed that these two helicases suppress crossovers by distinct mechanisms. Other DNA repair mutants tested in their study did not influence crossover frequency, including the *exo1* and *rad1* mutants. However, the study of plasmid gap repair in different nuclease mutants revealed increased crossover frequency in the *exo1*, but not in the *rad1* mutant (*13*). Both *rad1* and *srs2* mutants were isolated in a screen designed to detect mutations that decrease plasmid-chromosome recombination (*14*).

In the work presented here we analyzed the effect of the null mutations in the *RAD1*, *EXO1*, *SRS2* and *SGS1* genes on plasmid integration. Integration was decreased in the *rad1* and *srs2* strains, but increased in the *exo1* and *sgs1* mutants. These two mutations acted synergistically in promoting integrative transformation resulting in 15-fold increase with respect to the wild type strain.

Material and Methods

Yeast strains and plasmids

The list of yeast strains used in this work is presented in Table 1. The double mutant ES1 *sgs1 exo1* was constructed by crossing of FF1727 with YOR033c and random spore analysis. The genotype of the strain ES1 was determined by complementation analysis and Southern blotting. The plasmid pAB218-7 (6.6 kb), containing the yeast *URA3* and *CYC1* genes, was used in plasmid integration assay after the restriction with *Eco*RI that creates a DSB in the 1.7 kb *CYC1* region (*15*). Replicative plasmid pLS42 (6.5 kb), containing the yeast *URA3* gene (*16*), was used as a control for transformation efficiency. Standard media and procedures were used for cultivation of bacterial and yeast strains and DNA manipulations (*17,18*).

Yeast transformation

Yeast cells were transformed by a modified spheroplast procedure (*19*) with minor modifications. Briefly, the transforming DNA was added to the spheroplast suspension and after the addition of polyethylene glycol, the suspension was mixed with 75 mL of regenerating agar without uracil and poured in three Petri dishes. A mass of 60 ng of the plasmid pAB218-7 cut with *Eco*RI and 0.5 ng of the plasmid pLS42 were used for transformation and no carrier DNA was added. Transformation efficiency obtained with replicative plasmid was typically $3–4 \cdot 10^5$ transformants per μg of DNA. Each experiment was performed in triplicate and was repeated three to five times for each strain.

Molecular analysis of transformants

Southern blot analysis was performed as already described (*20*). Genomic DNA was cut with *Pvu* II and hybridized to the labelled pBR322.

Results

Integration of linearized plasmid molecules into the yeast genome occurs by homologous recombination and the aim of this study was to investigate whether the efficiency of this process may be enhanced in some DNA repair mutants. In order to compare the results obtained

Table 1. Yeast strains

Strain	Relevant genotype	Source
FF18733	*MAT***a** *leu2–3,112 trp1–289 ura3–52 his7–2 lys1–1*	F. Fabre*
FF18744	*MAT***a** *leu2–3,112 trp1–289 ura3–52 his7–2 lys1–1 srs2::LEU2*	F. Fabre*
FF1727	*MAT***a** *leu2–3,112 trp1–289 ura3–52 his7–2 lys1–1 sgs1::LEU2*	F. Fabre*
FF1481	*MAT***a** *leu2–3,112 trp1–289 ura3–52 his7–2 lys1–1 rad1::LEU2*	F. Fabre*
BY4742	*MATα leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0*	EUROSCARF**
Y11809	*MATα leu2Δ0 ura3Δ0 his3Δ1 lys2Δ0 exo1::kanMX4*	EUROSCARF**
ES1	*MAT***a** *leu2–3,112 ura3–52 his7–2 lys1–1 exo1::kanMX4 sgs1::LEU2*	This study

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** http://web.uni-frankfurt.de/fb15/mikro/euroscarf

in different transformation experiments, yeast strains were transformed with integrative plasmid pAB218-7 cut with the restriction enzyme *Eco*R I (Fig. 1A) and replicative plasmid pLS42, used as a control. For each sample the efficiency of transformation was expressed as the number of transformants per μg of plasmid DNA. For each strain, the relative efficiency of transformation (RET) was calculated as the ratio between transformation efficiencies obtained with integrative and replicative plasmid.

The DNA repair mutants used in this study were derived from different wild type strains, FF18733 and BY4742 and we first determined the RETs in these two strains. The results presented in Table 2 indicated that the differences in the genetic background did not influence significantly the RET. Therefore, the data obtained in these eight experiments were pooled together and the mean value was used for comparison with the mutant strains.

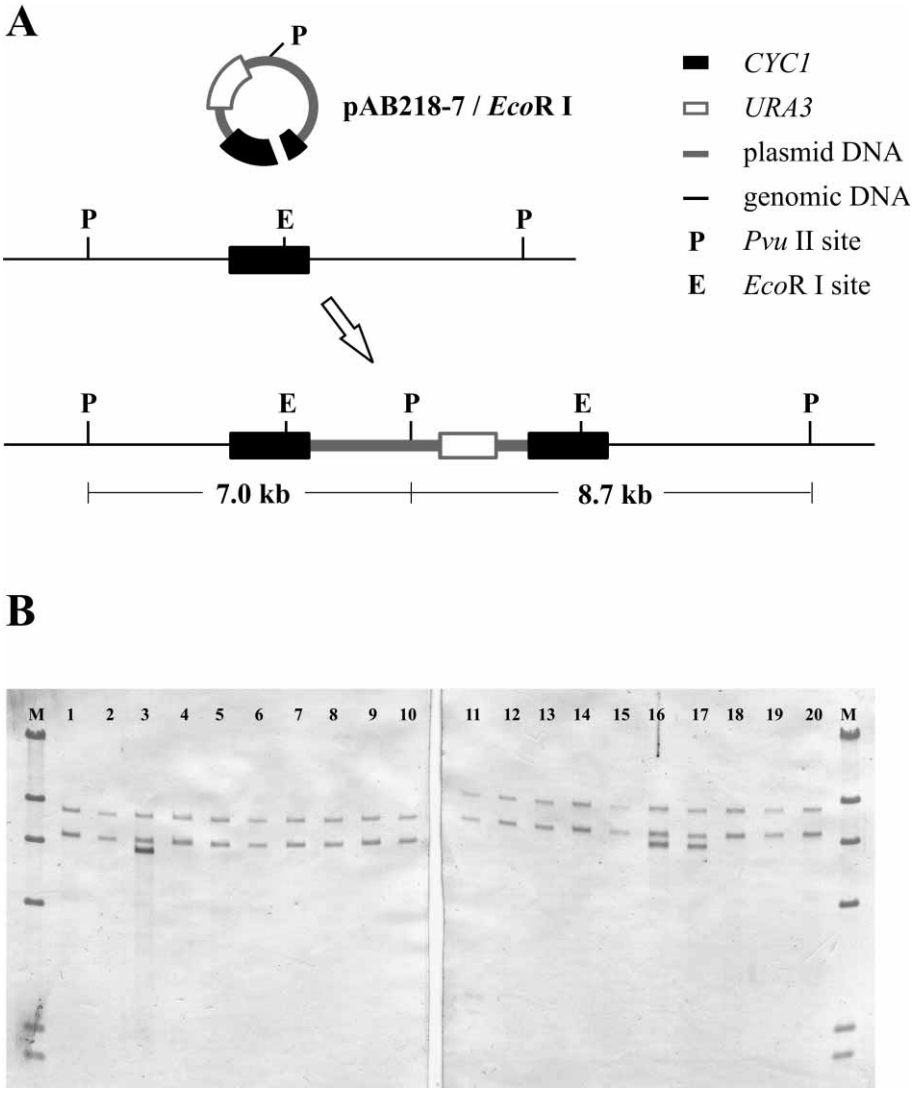

Fig. 1. Molecular analysis of transformants obtained in the *sgs1 exo1* strain. **A.** Schematic presentation of plasmid integration targeted to the *CYC1* region by a DSB introduced with *Eco*R I. Position of restriction sites for *Pvu* II, used to digest the genomic DNA of transformants, is indicated. **B.** Southern blot analysis of 20 transformants. Lanes 3, 16, 17 multiple integration. M, size standard. Genomic DNA was hybridized to the labeled vector sequence

Table 2. RET indicates the ratio between transformation efficiencies obtained with integrative plasmid pAB218-7 cut with *Eco*R I and replicative plasmid pLS42

Strain	Relative efficiency of transformation (RET)				Mean value ± standard deviation	Mean value for both strains ± standard deviation
	1	2	3	4		
FF18733	0.002921	0.004323	0.002564	0.001762	0.002892±0.001070	0.002841±0.001137
BY4742	0.0014756	0.002309	0.004692	0.002681	0.002789±0.001365	

Different DNA nucleases and helicases participate in the process of homologous recombination in yeast. We decided to analyze the role of null mutations in the genes encoding two helicases, Srs2 and Sgs1 and two nucleases, Rad1 and Exo1 on plasmid integration. Interestingly, while deletion of the *RAD1* gene, encoding the subunit of the ss-endonuclease, decreased plasmid integration, integrative transformation was stimulated by deletion of the *EXO1* gene. Mutations in the genes encoding two DNA helicases, Srs2 and Sgs1 also had opposite effects on plasmid integration (Fig. 2). In comparison with the wild type strains, the RET increased more than 4-fold in the *exo1* and *sgs1* mutants. In order to find out whether plasmid integration can be further stimulated we constructed the double *sgs1 exo1* mutant and here more than 15-fold increase in the RET was observed (Fig. 2).

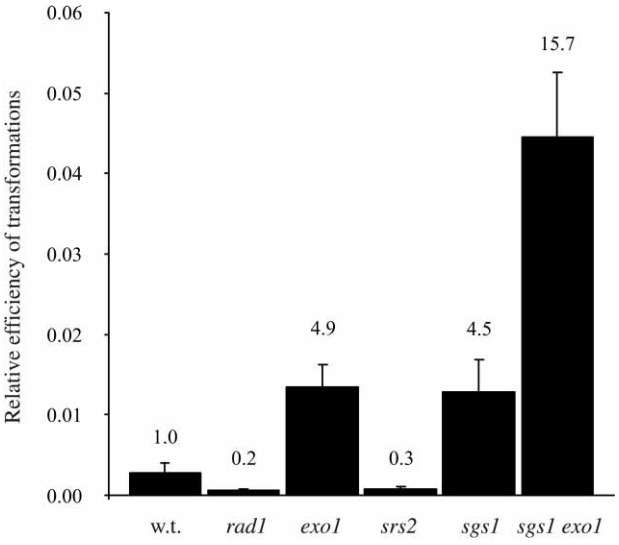

Fig. 2. Relative efficiency of transformation in different yeast strains. Increase in RET in comparison with the wild type (w.t.) is indicated, together with standard deviations (vertical bars)

Integration of the exogenous DNA in the yeast genome by illegitimate recombination has also been observed (*20,21*). Although this process occurs with very low efficiency in the wild type yeast, it was possible that the high increase in integrative transformation observed in the *sgs1 exo1* double mutant was due to the increased illegitimate integrations. In order to rule out this possibility, we performed the Southern blot analysis of 20 independent transformants. They all arose by DSB-targeted homologous integration in the *CYC1* region (Fig. 1B) and three transformants contained multiple plasmid integrations. In our previous study we found out that, using the same transformation procedure, multiple integrations occur with the frequency of 10–20 % (22).

Discussion

The possibilities for gene manipulations in a number of organisms are limited by the lack of efficient gene targeting procedures. This is certainly not the case in the yeast *Saccharomyces cerevisiae*, where the large majority of integration events occur by recombination between homologous sequences present in exogenous and genomic DNA. However, even here, the efficiency of integrative transformation is much lower than the efficiency of transformation obtained with replicative plasmids. In the experimental system described in this work, the yield of transformants per μg of DNA in the wild type strain was more than 300-fold lower with linearized integrative plasmid pAB218-7 than with the replicative plasmid pLS42 (Table 2), although the two plasmids are of comparable sizes. This is not surprising, since integrative transformation requires efficient search for homology in the genome followed by the initiation of the recombination process. Moreover, only those recombination events giving rise to crossover products result in plasmid integration, while the repair of DSBs present on plasmid molecules is rarely associated with reciprocal recombination (*11*). Finally, linear DNA molecules used for gene targeting may be subjected to degradation by different nucleases present in the cell decreasing the chance for successful integration. In other words, although gene targeting is routinely used for gene manipulations in yeast, it seems reasonable to speculate that the efficiency of this process could be further enhanced.

Plasmid integration in different nuclease mutants has already been studied by Symington *et al.* (*13*). In *rad1* and *mre11* mutants a decrease in plasmid integration was observed, while deletion of the *EXO1* gene resulted in 2.2-fold increase. This stimulation of plasmid integration was not attributed to the greater stability of linear DNA in the mutant, but rather to the inactivation of the mismatch repair system, which was shown to decrease recombination between sequences that are not perfectly homologous (*22,23*). In another study, Ira *et al.* (*12*) analyzed the frequency of crossovers associated with chromosomal DSB repair in the *sgs1* and *srs2* mutants. Deletion of either of the two helicases resulted in 2- to 3-fold increase in the crossover frequency. They proposed that these two helicases act in different steps of the recombination process that result in noncrossover products. The main goal of the present study was to establish whether inactivation of these genes would increase plasmid integration already observed in the *exo1* mutant.

Results obtained with nuclease mutants, *rad1* and *exo1*, are similar to those obtained by Symington *et al.* (*13*). However, while we observed 5-fold increase in plasmid integration, plasmid integration in the *exo1* mutant was stimulated only 2.2-fold in their assay. The reduction in plasmid integration in the *rad1* mutant was also more pronounced in the results reported here. These differences could be due to different factors, like genetic background of the strains or different recombination substrates used in these studies. They followed integration of the plasmid containing the 238 bp gap in the middle of the *MET17* region (2.6 kb), while our plasmid contained a DSB in the *CYC1* region (1.7 kb). It should also be noted that the high concentration of carrier DNA used by Symington *et al.* (*13*) in lithium acetate transformation protocol could influence the results, especially when nuclease mutants are compared to the wild type strain. In our experiments yeast spheroplasts were transformed in the absence of carrier DNA and this could explain more pronounced effects observed in both nuclease mutants.

The proportion of DSB repair events associated with crossover was shown to be elevated in both *srs2* and *sgs1* mutants (*12*), but the results presented in Fig. 2 indicate that the absence of Sgs1 or Srs2 helicase have opposite effects on plasmid integration. While inactivation of the *SGS1* gene stimulated integration more than 4--fold in comparison with the wild type, a 3-fold reduction of the RET was observed in the *srs2* mutant. *srs2* mutants are sensitive to UV and ionizing radiation (*24*) and have decreased capacity to repair DSB induced *in vivo* on a centromeric plasmid (*14*) or in the ectopic position in the genome (*25*). It has been proposed that toxic recombination intermediates are formed in the absence of the Srs2 protein (*26*) and this could also explain a decrease in plasmid integration observed in our plasmid integration assay in the *srs2* mutant. On the other hand, inactivation of the *SGS1* gene could influence only the resolution of the intermediate, favouring formation of the crossover products. Consequently, repair of the DSB present on the plasmid molecule was channelled to the pathway resulting in reciprocal recombination, thus enhancing integrative transformation in the *sgs1* mutant.

Finally, the most interesting result emerging from our study is the synergistic effect of mutations in the genes *SGS1* and *EXO1* in stimulation of plasmid integration. Inactivation of either gene promotes integration 4- to 5-fold, while 15-fold increase in RET was observed in the double mutant. This observation indicates that the two proteins are involved in different processes that limit plasmid integration in the wild type yeast. However, our findings may also have practical implications on the development of gene targeting strategies. Namely, homologues of these two genes have been detected in higher organisms, including mammals, and their inactivation may result in increased gene targeting like in the experiments described here. An increase in the proportion of DSB repair events that occur by homologous recombination has already been reported in chicken (*27*) and human (*28*) cell lines mutated in the *SGS1* homologue (*Blm–/–*). However, since *Blm–/–* cells exhibit genomic instability, temporary inactivation of *SGS1* and *EXO1* homologues by the interfering RNA (iRNA) technology (*29*) could be a particularly convenient way to stimulate targeted integrations.

Conclusions

Different factors can influence the efficiency of procedures used for targeted genetic manipulations. Here we demonstrate that the reciprocal recombination between exogenous DNA and chromosomal homology can be strongly enhanced in some yeast DNA-repair mutants. Our results may encourage similar studies in other organisms, where inefficient gene targeting presents the major obstacle for genetic manipulations.

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References

1. A. Hinnen, J.B. Hicks, G.R. Fink, Transformation of yeast, *Proc. Natl. Acad. Sci. USA, 75* (1978) 1929–1933.
2. T.L. Orr-Weaver, J.W. Szostak, R.J. Rothstein, Yeast transformation: A model system for the study of recombination, *Proc. Natl. Acad. Sci. USA, 78* (1981) 6354–6358.
3. J.W. Szostak, T.L. Orr-Weaver, R.J. Rothstein, F.W. Stahl, The double-strand-break repair model for recombination, *Cell, 33* (1983) 25–35.
4. R.J. Rothstein, One-step gene disruption in yeast, *Methods Enzymol. 101* (1983) 202–211.
5. U. Klinner, B. Schäfer, Genetic aspects of targeted insertion mutagenesis in yeasts, *FEMS Microbiol. Rev. 28* (2004) 201–223.
6. K.M. Vasquez, K. Marburger, Z. Intody, J.H. Wilson, Manipulating the mammalian genome by homologous recombination, *Proc. Natl. Acad. Sci. USA, 98* (2001) 8403–8410.
7. M.R. Capecchi, Choose your target, *Nat. Genet. 26* (2000) 159–161.
8. F. Pâques, J.E. Haber, Multiple pathways of recombination induced by double-strand breaks in *Saccharomyces cerevisiae*, *Microbiol. Mol. Biol. Rev. 63* (1999) 349–404.
9. Y. Aylon, M. Kupiec, New insight into the mechanism of homologous recombination in yeast, *Mutat. Res. 566* (2004) 231–248.
10. L.S. Symington, Role of *RAD52* epistasis group genes in homologous recombination and double-strand break repair, *Microbiol. Mol. Biol. Rev. 66* (2002) 630–670.
11. Z. Zgaga, K. Gjuračić, I.K. Svetec, P.T. Mitrikeski, S. Gregorić: Plasmid Integration in Yeast: Conceptions and Misconceptions. In: *Current Studies of Biotechnology, Vol. II*, Z. Kniewald (Ed.), Zagreb, Croatia (2001) pp. 135–142.
12. G. Ira, A. Malkova, G. Liberi, M. Foiani, J.E. Haber, Srs2 and Sgs1-Top3 suppress crossovers during double-strand break repair in yeast, *Cell, 115* (2003) 401–411.
13. L.S. Symington, L.E. Kang, S. Moreau, Alteration of gene conversion tract length and associated crossing over during plasmid gap repair in nuclease-deficient strains of *Saccharomyces cerevisiae*, *Nucleic Acids Res. 28* (2000) 4649–4656.
14. M. Elias-Arnanz, A.A. Firmenich, P. Berg, *Saccharomyces cerevisiae* mutants defective in plasmid-chromosome recombination, *Mol. Gen. Genet. 252* (1996) 530–538.
15. I.K. Svetec, B. Lisnić, Z. Zgaga, A 110 bp palindrome stimulates plasmid integration in yeast, *Period. Biol. 104* (2002) 421–424.
16. Z. Zgaga, Transformation of *Saccharomyces cerevisiae* with UV-irradiated single-stranded plasmid, *Mutat. Res. 263* (1991) 211–215.
17. J. Sambrook, D.W. Russell: *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (2001).
18. F. Sherman, G.R. Fink, J.B. Hicks: *Methods in Yeast Genetics: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1986).
19. Z. Zgaga, R. Chanet, M. Radman, F. Fabre, Mismatch-stimulated plasmid integration in yeast, *Curr. Genet. 19* (1991) 329–332.
20. K. Gjuračić, Z. Zgaga, Illegitimate integration of single--stranded DNA in *Saccharomyces cerevisiae*, *Mol. Gen. Genet. 253* (1996) 173–181.
21. R.H. Schiestl, T.D. Petes, Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*, *Proc. Natl. Acad. Sci. USA, 88* (1991) 7585–7589.
22. P. Koren, I.K. Svetec, P.T. Mitrikeski, Z. Zgaga, Influence of homology size and polymorphism on plasmid integration in the yeast *CYC1* DNA region, *Curr. Genet. 37* (2000) 292–297.

23. A. Datta, A. Adjiri, L. New, G.F. Crouse, S. Jinks-Robertson, Mitotic crossovers between diverged sequences are regulated by mismatch repair proteins in *Saccharomyces cerevisae*, *Mol. Cell. Biol. 16* (1996) 1085–1093.

24. A. Aboussekhra, R. Chanet, Z. Zgaga, C. Cassier-Chauvat, M. Heude, F. Fabre, *RADH*, a gene of *Saccharomyces cerevisiae* encoding a putative DNA helicase involved in DNA repair. Characteristics of radH mutants and sequence of the gene, *Nucleic Acids Res. 17* (1989) 7211–7219.

25. Y. Aylon, B. Liefshitz, G. Bitan-Banin, M. Kupiec, Molecular dissection of mitotic recombination in the yeast *Saccharomyces cerevisiae*, *Mol. Cell. Biol. 23* (2003) 1403–1417.

26. A. Aboussekhra, R. Chanet, A. Adjiri, F. Fabre, Semidominant suppressors of Srs2 helicase mutations of *Saccharomyces cervisiae* map in the *RAD51* gene, whose sequence predicts a protein with similarities to procaryotic RecA proteins, *Moll. Cell. Biol. 12* (1992) 3224–3234.

27. W. Wang, M. Seki, Y. Narita, E. Sonoda, S. Takeda, K. Yamada, T. Masuko, T. Katada, T. Enomoto, Possible association of BLM in decreasing DNA double strand breaks during DNA replication, *EMBO J. 19* (2000) 3428–3435.

28. R. Onclercq-Delic, P. Calsou, C. Delteil, B. Salles, D. Papadopoulo, M. Amor-Gueret, Possible anti-recombinogenic role of Bloom's syndrome helicase in double-strand break processing, *Nucleic Acids Res. 31* (2003) 6272–6282.

29. C.C. Mello, D. Conte, Revealing the world of RNA interference, *Nature, 431* (2004) 338–342.

Inaktivacija gena *SGS1* i *EXO1* sinergistički stimulira integraciju plazmida u kvascu

Sažetak

Metode koje se primjenjuju za uvođenje ciljanih genetičkih promjena osnivaju se na procesu homologne rekombinacije između kromosomske i egzogene DNA. Dvolančani lom na plazmidnoj molekuli stimulira i usmjerava integraciju plazmida u homolognu sekvenciju u kvaščevom genomu, pa je u ovom radu istraženo može li se učestalost tog procesa dodatno pospješiti inaktivacijom pojedinih gena odgovornih za popravak DNA. Da bi se usporedili rezultati dobiveni u različitim sojevima kvasca, međusobno su uspoređene uspješnosti transformacije postignute lineariziranim integrativnim i kružnim replikativnim plazmidom. U usporedbi s divljim tipom, relativna uspješnost transformacije povećana je u mutantima *sgs1* i *exo1*, a smanjena u *rad1* i *srs2*. Inaktivacija gena *SGS1* i *EXO1* stimulirala je integraciju plazmida od 4 do 5 puta, dok je u dvostrukom mutantu *(sgs1 exo1)* uočeno povećanje od 15 puta. Ovaj rezultat pokazuje da spomenuti proteini sudjeluju u različitim staničnim procesima koji ograničavaju integraciju plazmida. Analiza 20 transformanata dvostrukoga mutanta, metodom po Southernu, pokazala je da su oni nastali homolognom integracijom plazmidne molekule u ciljanu regiju. Homolozi gena *EXO1* i *SGS1* pronađeni su i u drugim organizmima pa bi njihova inaktivacija također mogla povećati uspješnost uvođenja ciljanih genetičkih promjena.