DNA Repair 22 (2014) 12–23

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

Sgs1 and Exo1 suppress targeted chromosome duplication during ends-in and ends-out gene targeting

Anamarija Štafa[a,b,*], Marina Miklenić[a], Bojan Žunar[a], Berislav Lisnić[a,1], Lorraine S. Symington[b], Ivan-Krešimir Svetec[a,*]

[a] Laboratory for Biology and Microbial Genetics, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Zagreb 10000, Croatia
[b] Department of Microbiology and Immunology, Columbia University Medical Center, New York, NY 10032, USA

ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 5 July 2014
Accepted 9 July 2014

Keywords:
Gene targeting
Ends-in
Ends-out
Homologous recombination
Break-induced replication
Whole chromosome duplication

ABSTRACT

Gene targeting is extremely efficient in the yeast *Saccharomyces cerevisiae*. It is performed by transformation with a linear, non-replicative DNA fragment carrying a selectable marker and containing ends homologous to the particular locus in a genome. However, even in *S. cerevisiae*, transformation can result in unwanted (aberrant) integration events, the frequency and spectra of which are quite different for ends-out and ends-in transformation assays. It has been observed that gene replacement (ends-out gene targeting) can result in illegitimate integration, integration of the transforming DNA fragment next to the target sequence and duplication of a targeted chromosome. By contrast, plasmid integration (ends-in gene targeting) is often associated with multiple targeted integration events but illegitimate integration is extremely rare and a targeted chromosome duplication has not been reported. Here we systematically investigated the influence of design of the ends-out assay on the success of targeted genetic modification. We have determined transformation efficiency, fidelity of gene targeting and spectra of all aberrant events in several ends-out gene targeting assays designed to insert, delete or replace a particular sequence in the targeted region of the yeast genome. Furthermore, we have demonstrated for the first time that targeted chromosome duplications occur even during ends-in gene targeting. Most importantly, the whole chromosome duplication is *POL32* dependent pointing to break-induced replication (BIR) as the underlying mechanism. Moreover, the occurrence of duplication of the targeted chromosome was strikingly increased in the *exo1Δ sgs1Δ* double mutant but not in the respective single mutants demonstrating that the Exo1 and Sgs1 proteins independently suppress whole chromosome duplication during gene targeting.

1. Introduction

A linear DNA fragment introduced in the cell of the yeast *Saccharomyces cerevisiae* undergoes recombination with the homologous region in the genome (homologous recombination, HR). In order to perform gene targeting, yeast cells are transformed with a non-replicative DNA fragments carrying a selectable marker and containing ends homologous to the particular locus in the genome. These transforming DNA fragments are either (i) linear fragments used to replace the targeted sequence (gene replacement) [1] or (ii) linearized plasmids that integrate in the targeted locus (plasmid integration) [2,3]. Although both gene replacement and plasmid integration require proteins involved in HR [2–12], these two processes are mechanistically quite different [13]. After homologous pairing of the transforming DNA fragment and targeted sequence, the ends of the transforming DNA fragment point away from each other during gene replacement (ends-out gene targeting, EOGT) whereas during plasmid integration they point toward each other (ends-in gene targeting, EIGT).

In EIGT, the double-strand break (DSB) on the linearized plasmid is almost always repaired by HR resulting in targeted plasmid integration, whereas the frequency of random (non-targeted)

Abbreviations: HR, homologous recombination; EOGT, ends-out gene targeting; EIGT, ends-in gene targeting; DSB, double-strand break; TCD, targeted chromosome duplication; BIR, break-induced replication; bp, base pair; kb, kilobase; nt, nucleotide; PFGE, pulsed-field gel electrophoresis; ss, single stranded.

* Corresponding authors at: Laboratory for Biology and Microbial Genetics, Department of Biochemical Engineering, Faculty of Food Technology and Biotechnology, University of Zagreb, Pierottijeva 6, Zagreb 10000, Croatia.
Tel.: +385 1 483 6013; fax: +385 1 483 6016.
E-mail addresses: astafa@pbf.hr (A. Štafa), iksvetec@pbf.hr (I.-K. Svetec).
[1] Present address: Center for Proteomics, School of Medicine, University of Rijeka, Rijeka 51000, Croatia.

http://dx.doi.org/10.1016/j.dnarep.2014.07.004
1568-7864/

integration due to illegitimate recombination could be estimated to be as low as 0.1% [14]. Moreover, illegitimate integration, including homology-assisted illegitimate recombination [15,16] is the only aberrant event observed so far. Contrary to EIGT, the spectrum of aberrant events during EOGT is wider. Apart from (i) random integration by illegitimate recombination; transformation can result in (ii) integration of the transforming DNA fragment next to the homologous target, generating tandem repeats and (iii) targeted chromosome duplication (TCD) detected in aberrant transformants containing both, allele expected after a successful gene targeting and an untransformed allele (heteroallelic transformants) [14]. Duplication of a targeted chromosome can occur either by extensive DNA synthesis from the 3′-ends of the transforming fragment to the ends of the targeted chromosome by break-induced replication (BIR) [17,18] or by chromosome missegregation [19].

The fidelity of gene targeting and spectra of aberrant events during EOGT can be influenced by different parameters such as the length of the flanking homologies used for targeting [20–22] and GC content of the targeted region [23]. Although as few as 40 bp at each end ensures gene targeting, the success of targeted events increases with the length of flanking homologies [20–23]. Various studies using different eukaryotic model organisms suggest that the fidelity of EOGT is governed by the same rules. Transformation of the protozoa *Trypanosoma brucei* frequently occurs due to HR and fidelity of EOGT depends on the length of flanking homology [24–26]. A high fidelity of gene targeting (percentage of a successful gene targeting) has also been reported for the moss *Physcomitrella patens* [27,28]. Moreover, when flanking homologies are not of the same length, the transforming DNA fragment integrates next to the sequence sharing longer homology, frequently creating tandem repeats as previously reported in the moss [29], mouse cell lines [30] and yeast [14].

Gene targeting is managed by a number of proteins also involved in repair of chromosomal DSB by HR. DSB repair by HR starts by processing the DNA ends by 5′-3′ nuclease activity producing 3′-single stranded DNA tails. DNA resection is performed by several proteins including the MRX/Sae2 complex, Exo1 exonuclease, Sgs1 helicase and Dna2 endonuclease [31–33]. In the absence of Exo1 and Sgs1 only short 3′ single stranded DNA tails form and extensive resection is prevented. Exo1 is also involved in mismatch repair [34], survival of telomerase deficient cells [35] processing of the stalled replication forks [36] and its inactivation increases the frequency of gap repair and crossing over in the transformation assay [9]. Sgs1, a homolog of *Escherichia coli* RecQ helicase [37], functions in extensive resection of DNA ends and dissolves double Holliday junctions into non-crossovers [38,39]. Inactivation of the *SGS1* gene increases the efficiency of transformation in both EOGT and EIGT assay [12,40–42]. Langston and Symington [12] proposed that Sgs1 inhibits ends-out recombination not by heteroduplex rejection, as reported during single strand annealing [43], but by unwinding flanking sequences homologously paired with their genomic target. It has been shown that inactivation of both extensive DNA resection pathways, in the *exo1Δ sgs1Δ* double mutant, results in the increased transformation efficiency, using either the transforming DNA fragments for EOGT or chromosome fragments, presumably due to decreased degradation of the linear DNA [42,44]. Furthermore, the *exo1Δ sgs1Δ* double mutant shows an increased frequency of *de novo* telomere addition [42,44,45]. Therefore, inactivation of *EXO1* and *SGS1* genes could influence not only the efficiency of transformation [41,42] but also the fidelity of EOGT, as observed by Chung et al. [42], and spectrum of aberrant transformation events. Moreover, the proportion of all transformation outcomes, including targeted chromosome duplication, could be influenced by the design of the gene targeting assay.

DNA replication initiated at origins of replication during S-phase, as well as by BIR at DSBs, requires several DNA polymerases [reviewed in 46]. It has been shown that BIR is dependent on Pol32, a non-essential subunit of the Pol δ polymerase complex [47]. Therefore, the *pol32* mutation could be used to distinguish whether chromosome duplications observed during gene targeting occurs by BIR or chromosome missegregation.

In this study we followed transformation efficiency, fidelity of gene targeting and spectra of aberrant events in five EOGT and one EIGT assay. Heteroallelic transformants having both a transformed and an untransformed, targeted allele that arose due to TCD were ubiquitous aberrant events in all EOGT assays but, for the first time, they were also detected in the EIGT assay. We show that the appearance of TCD during EOGT is *POL32* dependent pointing to BIR as the underlying mechanism. Inactivation of *EXO1* and *SGS1* genes synergistically increased the transformation efficiency, during both EOGT and EIGT, as reported previously [41,42]. However, this effect was associated with a striking decrease in the fidelity of gene targeting due to increase of TCD. In addition, frequency of TCD was not increased in respective single mutants demonstrating that Exo1 and Sgs1 proteins independently suppress gene targeting-associated chromosome duplication.

2. Materials and methods

2.1. Plasmids and the transforming DNA fragments

All plasmids used for yeast transformation are constructed in this study. Plasmid pLUH is a derivate of pLS42 [48] carrying *LEU2*, *URA3* and *HIS3* genes and it was used as a circular replicative control in transformation experiments. All other non-replicative (integrative) plasmids used for generation of the DNA fragments for transformation are listed in Table 1. The pGU2 and pAGU2 plasmids differ only by the *RG* region, the central part of the *ARG4* gene (539 bp, EcoRV-Bsp1407I end-filled fragment) inserted in the NaeI site of the plasmid pGU2 backbone creating pAGU2. The plasmid pGU2 is a derivative of the pGEM-7ZF(−) (Promega) plasmid. It is constructed by deleting and end-filling EcoRI-HindIII fragment from the multiple cloning site (MCS) and Kpn2I-MluI region from the backbone. Resulting plasmid was used to create pGU2 by inserting the *URA3* fragment in the end-filled BamHI site. The *URA3* fragment was S1-treated 1638 bp EcoRI–EcoRI fragment from pCR2.1.TOPO-URA3 which was obtained by insertion of 1621 bp *URA3* PCR fragment (primers: ZG1 5′-gggaagacaagcaacgaaac-3′ and ZG4 5′-ttcattggttctggcgaggt-3′) in the plasmid pCR2.1.TOPO.

The plasmid pLGU+4 was constructed by inactivation of the NcoI site in the *URA3* gene by cutting and end-filling. Additionally, NcoI-MroI cut and end-filled *LEU2* PCR fragment (1815 bp, primers: YUPL5 5′-atataCCATGGcattatttttttcctcaacat-3′ and YDOL3 5′-atataTCCGGAgtgtttttattgttgtgtat-3′, containing NcoI and MroI sites, respectively) was inserted in the NaeI site of the plasmid backbone.

The pAUHI and pAULI plasmids were constructed by inserting the BamHI–BamHI end-filled *HIS3* gene (1771 bp) and NcoI-MroI end-filled *LEU2* gene (1815 bp) in the StuI site in the *URA3* gene of the plasmid pAGU2, respectively. The plasmid pADULI was constructed by replacing the StuI-EcoRV fragment from the *URA3* gene (248 bp) of the plasmid pAGU2 with the NcoI-MroI end-filled *LEU2* gene (1815 bp).

Plasmid pAGUS was constructed by replacing PvuII-PvuII fragment (2038 bp) containing 1638 bp *URA3* region of the plasmid pAGU2 with the SmaI–SmaI fragment (1104 bp) carrying shorter *URA3* region from the plasmid pTZGU [14]. Standard media and procedures were used for the cultivation of the *E. coli* strains (DH5α and XL1blue) and DNA manipulations [49].