Topic Introduction

Analysis of DNA Metabolism in Fission Yeast

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The fission yeast *Schizosaccharomyces pombe* is an excellent model organism to study DNA metabolism, in which the DNA replication and repair mechanisms are evolutionarily conserved. In this introduction we describe a range of methods commonly used to study aspects of DNA metabolism in fission yeast, focusing on approaches used for the analysis of genome stability, DNA replication, and DNA repair. We describe the use of a minichromosome, Ch¹⁶, for monitoring different aspects of genome stability. We introduce two-dimensional gel electrophoresis and immunofluorescent visualization of combed DNA molecules for the analysis of DNA replication. Further, we introduce a pulsed field gel electrophoresis (PFGE) assay to physically monitor chromosome integrity, which can be used in conjunction with a DNA double-strand break (DSB) repair assay to genetically quantitate different DSB repair and misrepair outcomes, including gross chromosomal rearrangements, in fission yeast.

GENOME STABILITY

Genome instability can result from a wide range of events, including metabolic errors, failure to accurately replicate or repair DNA, and missegregation of chromosomes. Genome instability in fission yeast can be assayed from the single gene to the chromosomal level using specific genetic backgrounds. Further, a variety of assays have been developed to monitor genome stability in response to either spontaneous or specific lesion-induced events (Table 1).

A mutator phenotype, in which the mutation rate is significantly increased compared with wild-type cells, can be determined using a number of different approaches: *ade6*[−] or *ade7*[−] mutants grow as red colonies on YES plates, caused by accumulation of a red pigment in cells growing on limiting adenine concentrations [see Introduction: **Growth and the Environment of *Schizosaccharomyces pombe*** (Petersen and Russell 2016)]. Reversion mutations (*Ade*⁺), or mutations in genes upstream of *ade6*⁺ or *ade7*⁺ in the adenine biosynthesis pathway (*Ade*[−]), can thus be detected as white colonies on YES plates. Spontaneous or damage-induced mutation rates in various genetic backgrounds can be determined using this assay, and the mutation rate can be calculated by fluctuation analysis using the Lea–Coulson method of the median (Lea and Coulson 1949; Gutz et al. 1974). Loss of *ura4*⁺ function, detected by the ability of *ura*[−] cells to grow on medium containing 5-fluoroorotic acid (5-FOA) (Boeke et al. 1987), can also be used to determine the mutation rate (Kai et al. 2006). Loss of *ura4*⁺ function has been exploited to monitor different aspects of genome stability in a wide variety of assays (Table 1).

Fission yeast cells carrying a ~530 kb minichromosome, Ch¹⁶ (or variants thereof), an experimentally derived fragment of endogenous ChIII, can be used to determine the rate of gross chromosomal rearrangements or chromosome loss associated with a range of misrepair events. Ch¹⁶ has the

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TABLE 1. Genetic assays and screens developed to monitor various aspects of genome stability in response to spontaneous or environmental events, DNA double-strand breaks, or replication pausing in *S. pombe*

Assay/Screen	Genotype	Phenotype	Select for	Comments and References
Spontaneous or environmentally induced events				
Mutator	ChIII <i>ade6⁻</i>	Red (Ade ⁻)	White (Ade ⁺ or ⁻)	On YEA, <i>ade6</i> or <i>ade7</i> mutants accumulate red pigment due to blocks in purine biosynthesis. Mutator strains show increased white colonies due to reversion or mutation in genes upstream in pathway (Gutz et al. 1974).
Mutator	ChIII <i>ura4⁺</i>	Ura ⁺	Ura ⁻	Mutations leading to loss of <i>ura4</i> function can be selected for using 5-FOA and the rate determined (Kai et al. 2006).
GT repeat instability	ChIII <i>ade6-(GT)₈</i>	Red (Ade ⁻)	White/Pink (Ade ⁺)	Insertion of GT repeats into the <i>ade6</i> gene causes a frame shift and an in frame stop codon, resulting in an Ade ⁻ phenotype. The rate of Ade ⁺ revertants (usually arising from +4, -2, or -8 bp changes) can be determined by fluctuation test. GT stability is maintained by mismatch repair factors (Mansour et al. 2001).
Recombination		Ade ⁻ His ⁺	Ade ⁺ His ⁻ (deletion type) Ade ⁺ His ⁺ (conversion type)	Recombination between direct repeats of <i>ade6</i> mutant alleles results in Ade ⁺ cells through either gene conversion or deletion. Nb <i>ura4⁺</i> can be used instead of <i>his3⁺</i> (Schuchert and Kohli 1988; Osman et al. 1996).
Minichromosome loss and GCRs	ChL	Leu ⁺ Ura ⁺ Ade ⁺	Leu ⁻ Ura ⁻ Ade ⁻ Leu ⁺ Ura ⁻ Ade ⁻	Minichromosome loss and GCRs can be selected for by marker loss (Niwa et al. 1986; Nakamura et al. 2008; Niwa 2016).
Telomere fusion		His ⁻ Leu ⁺	His ⁺ Leu ⁺	Telomere fusion leads to circularization of a <i>his⁺</i> plasmid, splicing out of intronic telomeric repeats and a His ⁺ phenotype (Almeida and Godinho Ferreira 2013).
DNA double-strand break induced events				
End-joining and extracellular DNA capture		Ura ⁻	Ura ⁺	PCR product encoding autonomously replicating <i>ura4⁺</i> is transformed into <i>ura4-D18</i> <i>S. pombe</i> (Decottignies 2005; see also Manolis et al. 2001).
DSB repair, GCRs and chromosome loss		Arg ⁺ G418 ^R Ade ⁺ His ⁺	Arg ⁺ G418 ^S Ade ⁺ His ⁺ (and other combinations)	Repair, misrepair, and failed repair following an HO-induced DSB at <i>MATa</i> can be determined by marker loss profiles (Prudden et al. 2003; Tinline-Purvis et al. 2009; Pai et al. 2016a).
Resection and SSA		His ⁺ Leu ⁻	His ⁻ Leu ⁺	Rapid DSB urg-HO induced DSB induction leads to extensive resection and annealing (SSA) of the <i>LEU2</i> domains to form a functional <i>LEU2⁺</i> gene (Watson et al. 2011).

(continued)

TABLE 1. Continued

Assay/Screen	Genotype	Phenotype	Select for	Comments and References
Replication fork arrest-induced events				
Recombination and GCRs		Ura ⁺	Ura ⁻	RTF-induced replication fork stalling at the RTS sites results in GCRs (Lambert et al. 2005)
Recombination and GCRs		Ade ⁻ His ⁺	Ade ⁺ His ⁻ (deletion type) Ade ⁺ His ⁺ (conversion type)	Replication fork blockage using RTF promotes direct repeat recombination (Ahn et al. 2005)

DSB, double-strand break; GCR, gross chromosomal rearrangement; RTF, replication termination factor.

advantages that it is mitotically and meiotically stable, it is nonessential, and *S. pombe* can tolerate ChIII disomy (Niwa et al. 1986; Matsumoto et al. 1987). Ch¹⁶ variants have been used to characterize aspects of centromere and telomere biology, DNA replication, DNA repair, and to determine the rates of spontaneous and break-induced chromosome loss in particular mutant backgrounds. Ch¹⁶ and its many variants continue to be a very useful tool for monitoring different aspects of genome stability (Matsumoto et al. 1987; Niwa et al. 1989; Takahashi et al. 1994; Prudden et al. 2003; Nakamura et al. 2008). For detailed protocols to determine spontaneous and break-induced minichromosome loss and genome stability, see Protocol: **Determination of the Frequency of Minichromosome Loss to Assess Chromosomal Stability in Fission Yeast** (Niwa 2016) and Protocol: **DNA Double-Strand Break Repair Assay** (Pai et al. 2016a).

DNA REPLICATION

DNA replication must be temporally regulated in coordination with other events of the cell cycle to guarantee that complete chromosome duplication takes place only once every cell cycle. Unscheduled or aberrant replication could result in genome instability and potential aneuploidy of the daughter cells.

Eukaryotic replication initiates at multiple discrete loci along the chromosomes called replication origins (ORI), which share two important features: the first is the presence of many more ORIs than the minimum number required to replicate the genome. This excess means that not every ORI is used in every cell cycle. In *S. pombe*, direct measurement of ORI efficiency (Daigaku et al. 2015) identified ~1000 ORIs that divided into two broad categories: a few hundred efficient ORIs (>60% efficiency) and a large number of inefficient ORIs. The average probability of firing a given ORI during S-phase is thus <30%, an estimation consistent with previous studies (Cotobal et al. 2010). The second property is that eukaryotic ORIs have very degenerate sequences, making their identification difficult on the basis of sequence alone. In *S. cerevisiae*, an 11–17-bp long element present in all ORIs is necessary but not sufficient for ORI activity (Theis and Newlon 1997). No consensus elements have been identified in the ORIs of *S. pombe*. However, somewhat paradoxically, it is possible to predict the localization of ORIs in the *S. pombe* genome because most colocalize with intergenic regions that contain 250–500-bp long tracts with an A+T content significantly higher than the genome average (Segurado et al. 2003). This elevated A+T composition seems to be the only requirement for the specification of *S. pombe* ORIs as shown by the fact that exogenous A+T-rich sequences without homology with the *S. pombe* genome can initiate replication as efficiently as endogenous ORIs (Cotobal et al. 2010). Analysis of *S. pombe* DNA replication at the DNA level is described in Protocol: **Two-Dimensional Gel Electrophoresis of DNA Replication Intermediates in *Schizosaccharomyces pombe*** (Sánchez and Antequera 2016), in which replication intermediates can be identified.

Like other fungi, *S. pombe* lacks a thymidine transporter, which would allow uptake of external thymidine, and a thymidine kinase, which would phosphorylate thymidine to thymidine monophosphate and allow it to enter the pool of nucleotides that will be incorporated into DNA. Thus, *S. pombe*

cannot incorporate the thymidine analog bromodeoxyuridine (BrdU), which is widely used to study replication dynamics in mammalian cells. To circumvent this problem, *S. pombe* strains expressing the genes for the human equilibrative nucleoside transporter 1 (hENT1) and the herpes simplex virus thymidine kinase (tk), which efficiently incorporates BrdU and another 5-halogenated analogs of thymine, such as CldU and IdU, have been generated (Hodson et al. 2003; Sivakumar et al. 2004). These strains make it possible to follow replication dynamics by pulse-labeling with BrdU and other analogs. The analogs can be detected by immunoprecipitation of replicated regions with anti-BrdU antibodies followed by high-throughput sequencing (Hayano et al. 2012; Tazumi et al. 2012). Replicated DNA can also be identified by immunofluorescent visualization on combed DNA molecules; see Protocol: **Analysis of DNA Replication in Fission Yeast by Combing** (Lebofsky et al. 2006; Patel et al. 2006; Czajkowsky et al. 2008; Iyer et al. 2016; Gallo et al. 2016). If the identification of specific genomic regions in the DNA is needed, the combed DNA molecules can be hybridized in situ with fluorescent labeled probes against the genomic regions of interest (Lebofsky et al. 2006; Patel et al. 2008).

DNA REPAIR

DNA can incur multiple lesions, either through scheduled metabolic events, such as mating-type switching and meiotic double-strand breaks, or through endogenous metabolic errors, or exposure to genotoxic agents, such as ionizing and ultraviolet radiation. These events can give rise to different classes of DNA damage, including base damage, nucleotide damage, DNA interstrand or intrastrand crosslinking and the formation of single-strand or double-strand breaks (DSBs). Each of these error types has the capacity to cause mutations that can lead to cell death. In addition, misrepair of such lesions can lead to genome instability, for example, through joining of noncontiguous DSB ends, which can lead to dicentric and acentric chromosomes (and to oncogene activation in higher eukaryotes). Cells have therefore evolved a DNA damage response (DDR) pathway and a number of specific DNA repair pathways including base excision repair, nucleotide excision repair, mismatch repair, and double-strand break repair. Together these are responsible for maintaining genome stability in response to such lesions (Friedberg et al. 2006). An overview of assays that monitor DNA repair and genome stability is provided in Table 1.

Here we focus on the repair of DSBs, which are commonly studied because they are the most lethal of these lesions if not repaired. DSBs are repaired in eukaryotes by either of two evolutionarily conserved repair pathways: homologous recombination (HR) and nonhomologous end joining (NHEJ). HR is initiated by resection of the 5' end of the DSB to generate a 3' single-stranded DNA (ssDNA) overhang. This resected 3' end then invades the sister chromatid or homologous chromosome, which is used as a template for DNA replication and accurate repair (Heyer et al. 2010). This process requires a number of evolutionarily conserved factors including replication protein A (RPA), Rad52, and Rad51 (Raji and Hartsuiker 2006). During NHEJ, the broken ends are rapidly bound and protected by the Ku70/80 heterodimer, which in mammalian cells facilitates recruitment of the DNA-dependent protein kinase (DNA-PKcs; absent in yeast). Following processing, DNA ends are ligated by the DNA ligase 4 (Lig4)/XLF/XRCC4 complex. Alternative Ku-independent NHEJ pathways have also been identified (Lieber 2010). In logarithmically growing fission yeast, DSB repair is performed predominantly by HR, which is thought to reflect the fact that ~70% of cells are in haploid G2 phase and thus benefit from a sister chromatid as a potential repair template. In contrast, DSB repair occurs through NHEJ in G1. Both classical and alternative NHEJ pathways have been described in *S. pombe* (Manolis et al. 2001; Decottignies 2005; Li et al. 2012).

Molecular approaches have been developed to monitor the efficiency of DSB resection and HR repair in *S. pombe* and to detect the appearance of Rad11 (an RPA subunit), Rad52, or Rad51 foci in response to DSB induction, either using immunohistochemistry or live cell imaging in conjunction with fluorescent protein-tagged repair proteins (Carneiro et al. 2010). A protocol for pulsed field gel electrophoresis (PFGE), which can be used to physically monitor DSB repair and to detect

chromosomal rearrangements arising from misrepair, is presented in Protocol: **Using Pulsed-Field Gel Electrophoresis to Analyze *Schizosaccharomyces pombe* Chromosomes and Chromosomal Elements** (Pai et al. 2016b). A range of genetic assays have also been developed to determine the efficacy of DSB repair and homologous recombination in fission yeast (Osman et al. 1996; Virgin et al. 2001; Prudden et al. 2003; Du et al. 2006; Watson et al. 2011). Such a DSB assay is described in Protocol: **DNA Double-Strand Break Repair Assay** (Pai et al. 2016a), which can be used to quantify the repair and misrepair outcomes of a DSB generated within the context of a nonessential minichromosome (Cullen et al. 2007; Tinline-Purvis et al. 2009; Moss et al. 2010).

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