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Genetics

Enhanced mitotic recombination in a ligase-defective mutant of the yeast *Saccharomyces cerevisiae*

(DNA metabolism/cell cycle)

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ABSTRACT **The temperature-sensitive *Saccharomyces cerevisiae* cell cycle mutant *cdc9* is defective in DNA ligase, and the DNA synthesized at the restrictive temperature contains many single-strand breaks. We find that holding a diploid homozygous for *cdc9* at the restrictive temperature and then plating cells at the permissive temperature gives rise to increased intragenic and intergenic recombination. In the latter case, recombinants signaled by the *ade2* locus rise to about 4% of the survivors after 6 hr of incubation at the restrictive temperature. We propose that the single-strand breaks left in DNA synthesized at the restrictive temperature may lead to recombination.**

Many studies on genetic recombination in prokaryotes and eukaryotes have made use of mutants that show alterations in recombination frequency. Early work mainly involved mutants that showed a reduction or abolition of recombination (1). More recently, interest has focused on mutants with increased levels of spontaneous recombination, termed "hyper-rec" mutants. Such mutants have been studied in prokaryotes and in a number of eukaryotes, including *Ustilago maydis* (2), *Aspergillus nidulans* (3–6), and *Saccharomyces cerevisiae* (7–9). In *Escherichia coli*, the gene products of several hyper-rec mutants have been identified (10), but none of the eukaryotic mutants has been associated with a specific enzymatic lesion and the mechanisms leading to increased recombination in eukaryotes are obscure. Here we have studied recombination in a yeast mutant with a known enzymatic lesion in DNA metabolism. The mutant, *cdc9*, is a conditional mutant of the cell cycle (11) and has been found to be defective in an ATP-dependent DNA ligase (12).

We report here that mitotic recombination is strongly enhanced in homozygous *cdc9* diploid cells held at the restrictive temperature before being plated at the permissive temperature.

MATERIALS AND METHODS

Strains. The strains used in this study were constructed in this laboratory from strains already available. The *cdc9-1* mutant was originally provided by L. H. Hartwell (Department of Genetics, University of Washington, Seattle, WA). Strains carrying other genetic markers were provided by R. C. von Borstel or by the Yeast Genetic Stock Center (University of California, Berkeley, CA). The linkage relationships shown below are those determined or cited by Mortimer and Hawthorne (13). Gene symbols are those given by Plischke *et al.* (14).

The homozygous diploid of *cdc9-1* used in these experiments (strain g572A) had the following genotype:

$$\frac{\alpha\ cdc9\text{-}1}{a\ cdc9\text{-}1}\ \frac{CAN1\ ura3}{can1\quad +}\ \circ\ \frac{+\qquad his1\text{-}1}{hom3\text{-}10\ his1\text{-}7}$$

$$\frac{+\quad +}{lys\ tyr1}\ \frac{+}{ura1}\ \frac{+}{ade2}\ \frac{+}{leu2}$$

It was isolated as a mitotic segregant from a strain, g572, of similar genotype but heterozygous for *cdc9*. Cells of g572 were plated on YPD agar, exposed to a low dose of UV irradiation ($\approx$300 ergs/mm^2), and incubated at 25°C. Among the survivors, a clone was identified (g572A) that did not grow at 37°C and whose cells were arrested at this temperature with the cell cycle morphology characteristic of *cdc9* strains ("dumb-bells," see ref. 11). Genetic analysis confirmed that this strain was homozygous for *cdc9*. In addition, four spore-clones from a single meiotic tetrad of g572A were crossed with unrelated wild-type strains. The crosses were analyzed, and all four were found to contain only one temperature-sensitive mutation, confirming that g572A did not contain any other temperature-sensitive mutations unlinked to *cdc9*.

Media and Genetic Procedures. The complex growth medium used was YPD, which consists of 1% Difco yeast extract, 2% Bacto peptone, and 2% glucose (all wt/vol). McClary's medium (15) was used for inducing sporulation. Auxotrophs were scored on Wickerham synthetic minimal medium (16) with appropriate additions. Canavanine medium consisted of Wickerham's medium with appropriate additions containing 30 mg of L-canavanine per liter. COM medium was identical to canavanine medium except that no canavanine was added. Solid media contained 2% (wt/vol) agar.

Standard methods for crosses and other genetic procedures in yeast were used (17). Cells were diluted and washed (where necessary) in saline (0.9%) before they were plated.

Determination of Recombination Frequency. A culture of g572A was grown overnight at 21°C in YPD medium with shaking to a density of approximately 10^7 cells per ml. At t = 0 hr, the culture was shifted to 37°C and shaking was continued. At hourly intervals samples were diluted and plated on YPD agar, and undiluted samples were washed and plated on defined medium without histidine (HIS medium). All plates were incubated at 21°C and subsequently counted to monitor both viability and the number of whole or sectored red colonies (on YPD plates) or histidine prototrophs (on HIS plates). After counting, YPD plates were replica-plated to canavanine me-

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dium and to COM medium to monitor expression of the recessive *can1* gene, which determines resistant to the amino acid analogue L-canavanine (18). In the principal experiment, an average of 1540 colonies per time point was monitored to determine the frequency of red colonies and an average of 1127 colonies per time point was monitored for canavanine resistance. On the HIS plates, an average of 3.9×10^6 live cells was plated per time point to determine the frequency of histidine prototrophs.

RESULTS

The mutant *cdc9* is a conditional cell cycle mutant in which cells at the restrictive temperature are blocked in mitosis (11). Cells transferred to the restrictive temperature before or during DNA synthesis (S phase) will complete that round of synthesis (11), despite the absence of detectable DNA ligase (12). However, the DNA synthesized at 37°C contains many single-strand breaks (12), and we wished to determine the frequency of recombination under these conditions. To do this, g572A, a diploid homozygous for *cdc9-1*, was held at 37°C and at intervals cells were plated at the permissive temperature. Because the strain was heteroallelic at the *his1* locus, the number of histidine prototrophs arising provides a measure of intragenic recombination, although reverse mutation could also give rise to some prototrophic cells. To detect recombination in longer intervals the strain was heterozygous for the recessive *ade2* and *can1* genetic markers. The expression of these alleles results in red colonies on the YPD plates for *ade2* (19) and colonies resistant to L-canavanine for *can1* (18). Such colonies may represent cells homozygous for these recessive markers, arising from mitotic conversion at the locus, or recombination between the gene and its centromere. Alternatively, they may represent cells hemizygous for the mutant allele, arising from chromosomal nondisjunction or deletion of the wild-type gene.

Results for viability of g572A are given in Fig. 1. There was an initial rise in colony-forming units/ml, which can be accounted for by post-S phase cells completing a residual division before arresting at the following nuclear division (11). After this, viable numbers declined exponentially, and this continued throughout the period of the experiment.

Fig. 2 shows the number of histidine prototrophs, the number of whole or sectored red colonies, and the number of whole or sectored canavanine-resistant colonies per surviving colony-forming unit plotted against time at 37°C. There was a small increase in histidine prototrophs, amounting to 1.2 induced events per 10^4 survivors after 6 hr. There was a much greater

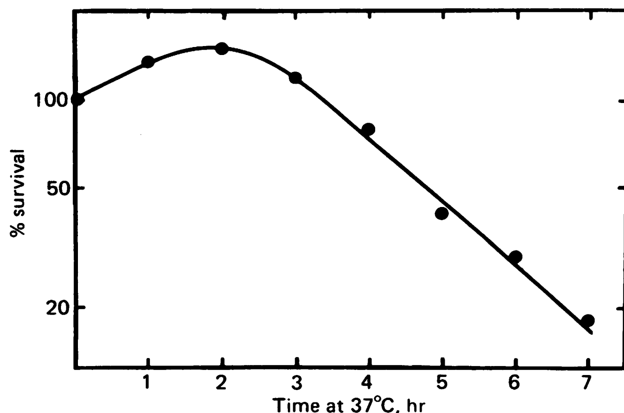

FIG. 1. Survival of g572A at 37°C. A culture of g572A was held at 37°C in YPD medium. At intervals, samples were spread on YPD agar and incubated at 25°C.

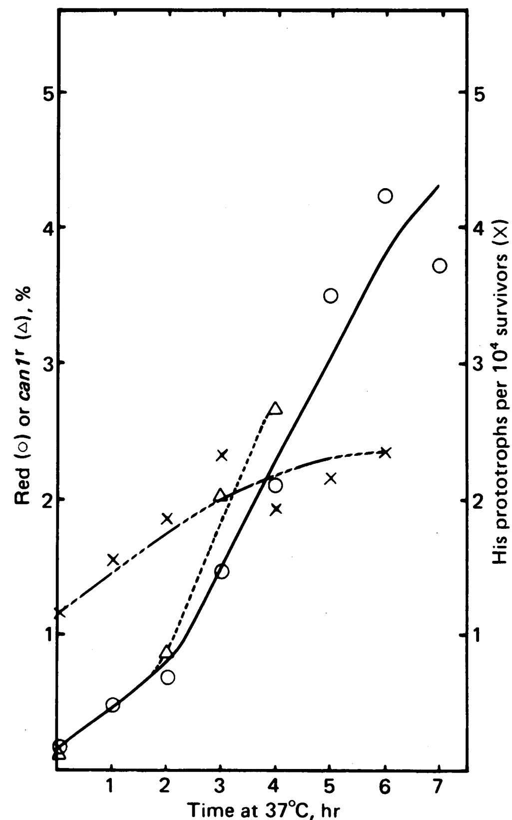

FIG. 2. Mitotic recombination in strain g572A held at 37°C. A culture of g572A was held at 37°C in YPD; at intervals, samples were plated at 25°C to determine frequency of recombinants. Recombination signaled by the *ade2* locus was measured by counting the number of whole or sectored red colonies on YPD agar; recombination signaled by the *can1* locus was measured by determining the number of canavanine-resistant colonies. Intragenic recombination was measured at the *his1* locus by establishing the number of histidine prototrophs. The survival at 37°C was also determined (Fig. 1), and numbers of recombinants obtained are expressed as a proportion of the surviving cells. ○, Percentage of red colonies per survivor; △, percentage of canavanine-resistant colonies per survivor; ×, histidine prototrophs per 10^4 survivors.

relative increase in the frequency of red and canavanine-resistant colonies, amounting in the red colonies to about 4% of the survivors at 7 hr and in the *can1* colonies to 2.5% after 4 hr.

A sharp increase in the frequency of red colonies was also seen in two additional experiments. One was a repeat of the above experiment with strain g572A. In the other we constructed a second homozygous *cdc9-1* diploid strain, by crossing two haploid *cdc9-1* spores, and performed a similar experiment. In contrast, no increase in the frequency of red colonies was seen when the *cdc9-1/+* heterozygous strain, g572, was incubated at 37°C and sampled over a period of 4.5 hr. Hence, the increased recombination is not simply a general characteristic of growth at 37°C, but appears to be a reproducible feature of strains homozygous for *cdc9*.

When putative recombinant clones were examined for survival at 37°C, their viability was found to decline in a fashion similar to that of g572A. Thus, their apparent increase cannot be explained by selective survival of pre-existing cells. This point is emphasized by the occurrence of an absolute increase in putative recombinants in the population. For example, at 3 hr there has been only a 20% decline in viability (Fig. 1), yet there is a 15-fold increase in whole or sectored *can1* colonies

and a 7-fold increase in red colonies (Fig. 2). The histidine prototrophs are likely to have resulted from intragenic recombination since they arise at a frequency (1.2 induced events per 10^4 survivors) far higher than that attributable to spontaneous mutation and independent evidence (unpublished data) indicates that *cdc9* under temperature stress has at most only a minor influence on reversion rates at the *his1* locus. The observed frequency of histidine prototrophs may represent an underestimate of recombination frequency because recombination events involving coconversion of both *his1* alleles will not give rise to prototrophs.

The *ade2* and *can1* colonies could have arisen in several ways (see above), and further characterization was needed to determine the nature of the induced events. Many of the *ade2* and *can1* clones were sectored, indicating that genetic segregation occurred when the cells divided after plating. The proportion of red colonies that were sectored (rather than entirely red) was highest at early time points, probably indicating loss of reciprocal sectors due to lethal sectoring among survivors from later times. To determine the mechanism responsible for the ade^- and *can1* clones, we determined the genotype of each half of 14 sectored red colonies and each half of 18 colonies sectored for canavanine resistance. These sectored colonies were chosen from time points ranging from 1 hr at 37°C to 7 hr at 37°C.

Analysis of *ade2* Colonies. Each half of the 14 red colonies was placed on sporulation medium and, where sporulation occurred, meiotic tetrads were analyzed. In 10 of the colonies, the red half gave 4 red:0 white spore clones per tetrad, whereas the white half gave the reverse. These are therefore likely to have arisen by reciprocal mitotic crossing-over. A less likely explanation, that they arose as a consequence of segregation of homologous centromeres resembling that normally seen in meiosis, is not excluded by these data but cannot account for findings relating to the canavanine-resistant colonies (see below).‡ Two of the remaining four colonies failed to sporulate and could not be analyzed, but the white half-colonies in the other two cases were heterozygous for *ade2*; hence, the red sectors of these probably arose by conversion or deletion. One of these red sectors failed to sporulate and the other gave no tetrads with more than two live spores. Lack of sporulation and spore inviability in colonies expressing *ade2* or *can1* events were most frequent among colonies from late time points and probably reflect genetic damage induced elsewhere in the genome. In summary, out of 12 sectored red colonies analyzed, 10 arose by reciprocal recombination whereas 2 arose by another mechanism.

Analysis of *can1* Colonies. Additional information was obtained concerning the mechanism responsible for generating the canavanine-resistant sectors. The *CAN1* gene is located on the left arm of chromosome V (13), and our strain also carried the *URA3* gene in heterozygous condition on the same chromosome arm. In addition, it was heterozygous at *hom3* and heteroallelic at *his1*, both on the right arm of chromosome V (13). We first determined whether both alleles of *his1* were still present in cultures from each of the 36 half-colonies involved in the 18 *can1* events studied. In 17 colonies both halves were clearly heteroallelic at *his1*, as judged by the frequency with which spontaneous and UV-induced histidine prototrophs arose. The presence of both copies of the *his1* locus indicates that they were not monosomic for chromosome V and suggests that normal mitotic centromere segregation has occurred. The canavanine-resistant sectors therefore most likely arose as a result of events involving only the left arm of that chromosome.

‡ We would like to thank B. S. Cox for pointing this out to us.

In the 18th colony, the canavanine-sensitive half was still heteroallelic for *his1* but the resistant half was not. This half also differed in that it expressed the *hom3-10* allele. Thus, the canavanine-resistant sector may have arisen from chromosome loss or nondisjunction, leading to monosomy, or from separate events on the left and right arms. Neither half of this colony could be analyzed meiotically owing to poor sporulation and spore viability, although the canavanine-sensitive half was heterozygous at *CAN1* on the basis of mitotic tests (see below).

The canavanine-sensitive halves of the 17 colonies were further tested to determine the status of the *URA3* and *CAN1* loci. Diploid colonies that are heterozygous at *CAN1* can be distinguished from those that are homozygous for the dominant canavanine-sensitive allele by plating cells on canavanine medium. The heterozygotes give rise to many reistant outgrowths (resulting from mitotic recombination), whereas diploids homozygous or hemizygous for sensitivity give few, if any, such outgrowths. When the 17 canavanine-sensitive half-colonies were tested in this way, 2 gave an ambiguous response, 5 were found to contain the resistant heterozygous allele, and 10 were homozygous (or hemizygous) for sensitivity.

The five colonies with one half heterozygous could have arisen in several ways, including mitotic conversion or deletion of the *can1* allele. In three cases, we sporulated both halves of these colonies; the recovery of three or four live spores from some tetrads in each case makes deletion of the chromosome arm unlikely. Hence, these five colonies most likely represent conversional events. Surprisingly, in two of the three cases analyzed, a coincident conversion at the *URA3* locus was seen.

The 10 colonies in which the canavanine-sensitive sector contained no resistant allele most likely represent classical mitotic crossing-over events between the centromere and the *CAN1* locus. Surprisingly, in 6 out of 10 of these cases the canavanine-sensitive half-colony expressed a *ura3* phenotype, suggesting an event in the centromere-*URA3* interval, and in only 4 cases was the event in the interval between *URA3* and *CAN1* although the latter interval is much longer on the meiotic map (13). Data from meiotic tetrads from two colonies expressing a *ura3 CAN1* half confirmed that each had resulted from a reciprocal recombination event between *ura3* and the centromere.

In summary, the canavanine-resistant sectored colonies resembled the red-sectored colonies in that most (at least 10 out of 18) resulted from reciprocal mitotic recombination. In most of the remaining colonies, the resistant sector probably arose from a recombination event that was nonreciprocal. Thus we can conclude that the results shown in Fig. 2 reflect a genuine increase in recombination in *cdc9* held at the restrictive temperature.

DISCUSSION

We have shown that holding *cdc9* cells at a restrictive temperature leads to enhanced levels of mitotic recombination in the survivors. Previous work has shown that *cdc9* is defective in DNA ligase and that the DNA synthesized at 37°C contains many single-strand breaks (12). The enhanced recombination that we observe could therefore be a direct consequence of these lesions in the DNA. In *E. coli*, the conditional lethal ligase mutant *lig7* (20) has been found to exhibit enhanced levels of recombination by Konrad (10), who also found additional ligase-defective mutants among a random collection isolated on the basis of a hyperrecombination phenotype. In the same study, Konrad found at least three other classes of hyperrecombination mutants in *E. coli* that resembled the *lig* mutants in showing an excessive amount of DNA present as small

fragments after replication. He attributed the hyper-rec phenotype to this common property of the mutants, suggesting that single-strand nicks or gaps could serve to initiate the recombination process. It seems likely the single-strand breaks observed in *cdc9* (12) could provide the basis of the enhanced recombination, either by initiating the recombination process directly or possibly through induction of a recombinational repair system. The latter possibility is made plausible by the fact that several complex repair systems operate in yeast cells (21, 22).

Alternatively, the enhanced recombination in *cdc9* could be a secondary consequence of the ligase defect. For example, holding cells for several hours in G2 might allow more time for spontaneous recombinational processes to operate. This explanation is made less probable by observations suggesting that most spontaneous mitotic recombination in yeast occurs during the G1 phase (23). However, the possibility of some recombination in G2 cannot be excluded, especially when this phase is prolonged. Studies with other *cdc* mutants that are blocked in G2 but are not directly altered in DNA metabolism may determine whether a prolonged G2 is in itself recombinogenic. Our results do not bear directly on the question of whether the induced recombination events occur in G1, S, or G2. If they arise as a consequence of DNA strand breaks, then they presumably occur after the onset of DNA synthesis at the restrictive temperature or possibly in the next G1 period after the cells are plated at the permissive temperature. If the latter is the case, cell division would occur on plates prior to recombination, and lethality of one or more sister cells must be postulated to account for reciprocally recombined sectored colonies and whole recombinant clones.

A surprising aspect of the induced reciprocal recombination in chromosome V is that 6 out of 10 analyzed events occurred in the interval between the centromere and the *URA3* gene and only 4 between the *URA3* and the *CAN1* locus, despite the fact that the latter interval is 8 times longer on the meiotic map. However, instances of apparent expansion of intervals (relative to meiotic distances) in regions close to centromeres have also been reported in studies of mitotic recombination in other organisms, including *Drosophila* (24) and *Aspergillus* (25), as well as yeast (Robert E. Malone, John E. Golin, and Michael S. Esposito, unpublished data). Hence the distribution of events found in the results with *cdc9* may not be unusual for mitotic cells.

In summary, the observation that temperature stress increases recombination in *cdc9* is consistent with observations on ligase deprivation in *E. coli*. DNA single-strand breaks accumulate in *cdc9* cells, and the increased recombination seen provides some support for the involvement of such breaks in the recombination process. The finding of increased recombination is easily accommodated by most models of recombination because such models usually involve DNA single-strand breaks at an early stage (for a review see ref. 26). Subsequent ligation is probably required to complete recombination, but this could occur in *cdc9* after cells are shifted back to the permissive temperature. There have been many mutants with elevated levels of recombination isolated in several organisms, but *cdc9* in yeast represents a eukaryote in which this phenotype has been associated with a specific enzymatic lesion. Further work may determine whether the ligase coded by the *CDC9* gene itself functions in a late step of recombination and may clarify relationships between DNA replication, recombination, and repair.

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