Proc. Natl. Acad. Sci. USA
Vol. 76, No. 9, pp. 4586–4588, September 1979
Genetics

Evidence that a single DNA ligase is involved in replication and recombination in yeast

(Okazaki fragments/conditional mutants/gene conversion)

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Contributed by Herschel L. Roman, June 18, 1979

ABSTRACT **The possible existence in yeast of different nuclear DNA ligase enzymes led us to ask whether induced recombination (gene conversion) involves the same ligase as that involved in DNA replication. The conditional *cdc9* mutant is known to be defective, under restrictive conditions, in the rejoining of Okazaki fragments. We show here that under the same conditions, x-ray-induced convertants within the *cdc9* locus are produced with kinetics indicating that most, if not all, of the conversion events require the participation of the *cdc9*-controlled ligase. Thus, the same DNA ligase is involved in DNA replication and in induced gene conversion.**

The continuity of the DNA phosphodiester backbone is ensured by ligase activities, which play, therefore, an essential role in DNA metabolism (1). Conditional ligase mutants have been isolated in different organisms (2–7) and shown to be defective, under restrictive conditions, in the rejoining of the DNA pieces (Okazaki fragments) (8) that are made during replication. Studies of the sensitivity of some of these mutants to the killing effect of radiations and chemicals have suggested that DNA repair is under the control of the same enzyme.

Genetic recombination necessarily involves ligation. It is not known if, *in vivo*, the ligases that are involved in DNA replication and repair mechanisms are also essential for recombination. Recombination may require a different ligase, and in mammalian cells the existence of two different ligases has been demonstrated (9). The experiments reported here were designed to see whether or not, in the yeast *Saccharomyces cerevisiae*, x-ray-induced gene conversion (intragenic recombination) is dependent upon the ligase activity known to be involved in DNA replication and controlled by the *cdc9* locus (7).

MATERIALS AND METHODS

Strains. The strains used are derived from the *cdc9-1* and *cdc9-8* mutants isolated by L. H. Hartwell. The temperature-sensitive character of each of these mutant segregates 2:2 in tetrads of *cdc9/CDC9* diploids. The heteroallelic diploid is of genotype a/α *cdc9-1/cdc9-8*. The homoallelic diploids are a/α *cdc9-1/cdc9-1* and a/α *cdc9-8/cdc9-8*.

Media. The liquid or solid yeast extract/peptone medium with glucose as the carbon source (YEP) is as described by Mortimer and Hawthorne (10).

X-Ray Irradiation. The source was a soft x-ray tube (50 kV, 20 mA). The dose rate was 100 rads sec^{-1} (1 rad = 0.01 gray). Ten milliliters of a cell suspension ($2–8 \times 10^7$/ml, according to the experiment) in saline (0.9% KCl), was irradiated in an open glass petri dish, 10 cm in diameter. In some experiments, cells were first plated and each plate was immediately irradiated.

Experimental Procedure. Stationary-phase cells ($3–4 \times 10^8$/ml) or exponential-phase cells (5×10^6 to 2×10^7/ml) in liquid YEP were harvested, resuspended in saline, sonicated, and counted in a hemocytometer. The stationary-phase cells, which were largely unbudded after sonication, were used in only one experiment reported in this paper, that dealing with the loss of viability in treated and untreated cells kept at 35°C (restrictive temperature) for various lengths of time. At the end of each time period the plates were returned to 22°C (permissive temperature) to allow the surviving cells to grow into colonies. The cells were also counted at the different time points to determine the relationship of the frequency of unbudded cells (in G1) to the frequency of survival. The results are given in Fig. 1A.

All of the experiments dealing with gene conversion were done with exponential-phase cells. The cell concentration was adjusted to $2–8 \times 10^7$ cells per ml. Budding cells were counted as one cell. After irradiation, appropriate dilutions were plated on YEP and incubated at 22°C for the determination of viability; undiluted aliquots were plated on YEP plates prewarmed at 35°C for the detection of recombinants in the *cdc9* gene. No significant lethality was induced by the irradiation. In some experiments, irradiated cells were plated at time 0 at 22°C, and the plates were shifted to a 35°C incubator at different times. The colonies were scored after 4 days of incubation. All platings were done in triplicate, and the experiments were repeated several times.

RESULTS AND DISCUSSION

Rationale of Experimental Procedure. Gene conversion is a recombinational process by which genetic information can be transferred from one DNA molecule to another at least partially homologous in sequence. In a heteroallelic diploid (mutated on each homologous chromosome at different sites in a same gene), a conversion event can lead to the formation of a wild-type gene. This implies necessarily the occurrence of breaks within the gene, whether they are at the initiation sites of the heteroduplex that is postulated as an intermediate in gene conversion or are made during mismatch repair (11, 12). If the ligation of these breaks is not achieved, it is likely that the complete transcription of this gene will not be possible. A conditional mutation in a gene governing this last step of recombination may therefore, under restrictive conditions, prevent the occurrence of successful conversion events.

The ts *cdc9* mutant cells of *S. cerevisiae* are, under restrictive growth conditions (35°C), defective in the rejoining of the Okazaki fragments (7) and arrested before the onset of nuclear division (13). Does the *cdc9* locus also control a ligase that is essential for gene conversion? If it does and the ligase is absent at 35°C, no convertants should be formed at this temperature.

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Abbreviation: YEP, yeast extract/peptone/glucose.

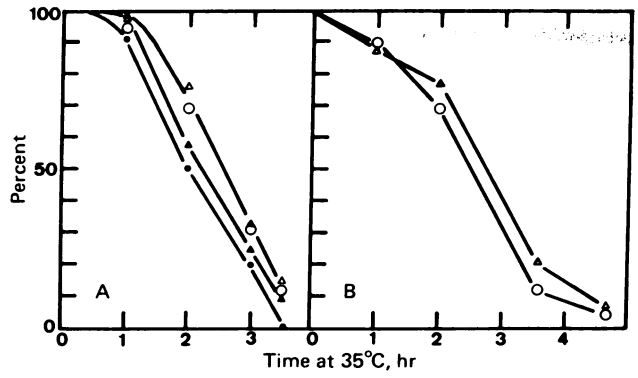

FIG. 1. Kinetics of cell inactivation during incubation at 35°C. (*A*) Stationary-phase cells; percent unbudded cells (closed symbols) and percent survival (open symbols). (*B*) Exponential-phase cells; percent survival. For both figures, the circles correspond to unirradiated cells and the triangles to cells exposed to x-rays (3 krad).

If it does not, we should obtain convertants at 35°C because it is known (14; unpublished results) that gene conversion can be induced in cells that are not replicating their DNA and are abundantly present in exponentially growing cultures. The occurrence of gene conversion in the absence of the *cdc9* ligase could be explained in two ways: (*i*) by the presence of a second ligase, or (*ii*) by the formation of a wild-type *CDC9* gene as a result of nick-translation (15) that starts between the mutant sites and extends beyond the gene during mismatch repair in the heteroduplex.

It is possible to answer this question by studying the induction by x-rays of convertants in the *cdc9* gene itself, using a diploid heteroallelic for this gene. If, following irradiation and plating at 35°C, a transcribable wild-type allele of *cdc9* is formed, the converted cells have the possibility of making a normal gene product and consequently of replicating their DNA normally and forming a colony on the plate. This presupposes that under our experimental conditions the cells are able to recombine and to synthesize the new gene product before being inactivated during incubation at the restrictive temperature.

To find out if there is a specific time or times during the cell division cycle when cell inactivation occurs at 35°C, stationary-phase cells were plated on YEP medium at 35°C and the plates were transferred to 22°C at various times. The cells were largely unbudded after sonication. Treated (3 krad) and untreated cells were handled in the same way. Cells on the plate were examined microscopically to note the onset of budding, a good indicator of the start of DNA synthesis (S) (16). The bud reaches approximately the same size as the mother cell as the terminal phenotype at the restrictive temperature.

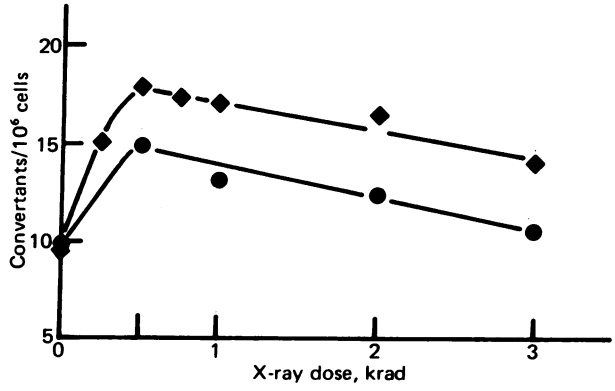

FIG. 2. Induction of convertants under restrictive conditions (two experiments). Cells were in exponential phase when treated.

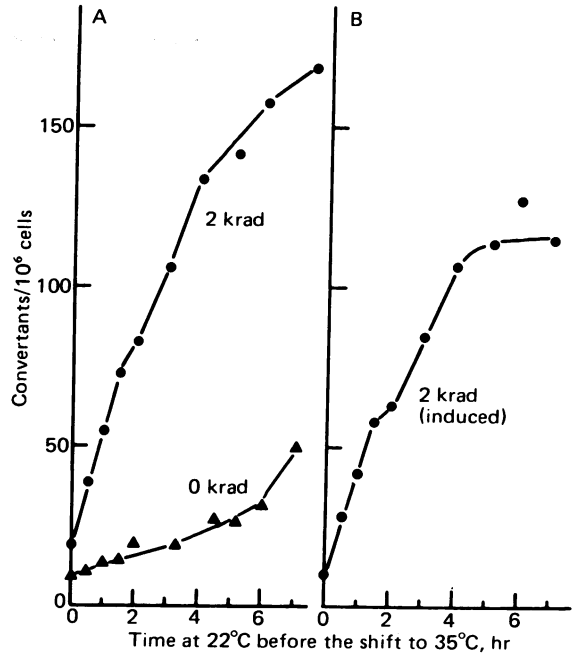

FIG. 3. Induction of convertants in exponential phase cells as a function of time at 22°C after irradiation and before incubation at 35°C. (*A*) Unirradiated (▲) and irradiated (●) cells (2 krad). (*B*) Induction curve after subtraction of the corresponding controls (dose 0).

The inactivation curves of the treated and untreated cells, plotted also in relation to the percent of unbudded (G1) cells, is shown in Fig. 1*A*. It is evident that treated cells are no more sensitive to killing than the untreated control. It is also quite clear that the cells do not die until after entering the S phase. The same experiment was conducted with exponential-phase cells, in which the budding pattern could not be followed as readily as it was in cells that were in stationary phase. The results, which are essentially like those in Fig. 1*A*, are given in Fig. 1*B*.

The evidence that G1 cells are relatively insensitive to killing at 35°C does not by itself answer the question of whether conversion is to be expected at 35°C. Studies on recombination in another cell division cycle mutant, *cdc4*, in which cell division is blocked before S at 35°C, have demonstrated that G1 cells can undergo gene conversion at the restrictive temperature shortly after irradiation (14). The response to γ rays was linearly proportional to the dose, at least up to the maximum of 4 krad used in the experiment. Thus, we conclude that cells heteroallelic for *cdc9* have the capacity for viable conversion events if the *cdc9* ligase is not a requirement.

Conversion under the Restrictive Conditions. Exponentially growing cells, heteroallelic for *cdc9*, were irradiated, plated, and incubated at 35°C for the detection of convertants. According to the stage they are in when plated, the cells progress through different portions of the cycle before reaching the sensitive step. At 22°C, no significant x-ray inactivation was detected in the 0–3 krad dose range used. The frequency of convertants increased up to 0.5 krad, at which 5–10 convertants per 10^6 cells were induced (Fig. 2). At higher doses there was a slow decline in the frequencies of convertant colonies. This amount of induction, although very small, was not due to mutation, as was shown by control experiments in which homoallelic *cdc9-1/cdc9-1* and *cdc9-8/cdc9-8* cells were treated in the same way. The frequency of reversion in the homoallelic diploids increased with dose but was less than $1/10^6$ cells even at 6 krad.

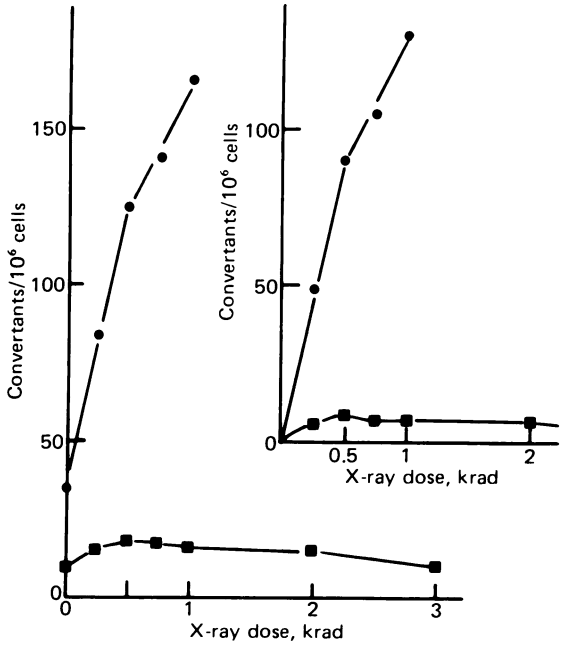

FIG. 4. Induction of convertants in exponential-phase cells as a function of the x-ray dose. ■, Cells incubated immediately at 35°C; ●, cells incubated for 6 hr at 22°C after irradiation. (*Inset*) Curves obtained after subtraction of the backgrounds (dose 0).

Conversion under the Permissive Conditions. The kinetics of induction and the small number of x-ray-induced convertants detected under the restrictive conditions suggest that the *cdc9* temperature-sensitive mutation affects gene conversion also. If so, a temporary incubation of the irradiated cells at the permissive temperature (22°C) after irradiation should allow the conversion events to occur at a higher rate. Fig. 3 shows that this is indeed the case. Unirradiated and irradiated cells (2 krad) were plated at time 0 and incubated for different times at 22°C before the shift to 35°C. The increase in the frequency of convertants is much higher for the irradiated than for the unirradiated cells. Fig. 3*B* shows the induction curve after subtraction of the corresponding controls. A maximum is reached after 4–5 hr at 22°C.

The induction under permissive conditions was studied, as a function of dose, by plating cells on YEP medium, irradiating them with different doses, and holding the plates at 22°C for 6 hr before incubating them at 35°C. Fig. 4 shows that the induction is linear up to 0.5 krad and about 10 times higher than that found for cells immediately incubated at 35°C. At higher doses, where no further induction is found if cells are incubated at 35°C, the rate of induction decreases, but this phenomenon has been observed for other genes, and is not specific for the *cdc9* locus.

The *cdc9* ligase plays, therefore, a major role in x-ray-induced conversion. It is not known whether the ligase itself is thermolabile. If the enzyme is not thermolabile or not completely inactivated at 35°C, the initial rise followed by the decline in the induction curve (Fig. 2) may be interpreted to mean that there is a pool of active ligase molecules in the cells that have been shifted from 22°C to 35°C. If the limiting factor is not the number of lesions in the DNA but the number of active ligase molecules, the increase in the number of lesions with increasing dose of x-rays will result in a decrease in the number of molecules that are available to repair breaks in the *cdc9* locus to complete the conversion event. It is unlikely that the convertants occurring at 35°C are formed by nick-translation during mismatch repair or are due to the transcription of nicked DNA. In either case, the frequency of gene conversion should increase with dose, in contrast with the data presented in Fig. 2. We cannot exclude, however, the possibility that the induced events seen at 35°C are mediated by another ligase not controlled by the *cdc9* locus. The kinetics of induction would indicate that the level of this other enzyme, if it does in fact exist, is also limiting.

In conclusion, these results may be interpreted to mean that a single gene, *CDC9*, known to control the rejoining steps during DNA replication, controls also x-ray-induced gene conversion. Although it is not yet proven, it is likely that *CDC9* is the structural gene of the ligase. The *CDC9* gene product is involved in all the DNA metabolic processes that have been studied with the *cdc9* mutants, including repair after UV (7) and x-rays (unpublished data). It is therefore likely that these cells possess only one nuclear DNA ligase enzyme.

We thank Elizabeth Leney for her expert technical assistance. This research was supported by National Institutes of Health Grant AI-00328 and by Euratom Contract 155761 B10F.

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