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Ligase-Deficient Yeast Cells Exhibit Defective DNA Rejoining and Enhanced Gamma Ray Sensitivity

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Yeast cells deficient in DNA ligase were also deficient in their capacity to rejoin single-strand scissions in prelabeled nuclear DNA. After high-dose-rate gamma irradiation (10 and 25 krads), *cdc9-9* mutant cells failed to rejoin single-strand scissions at the restrictive temperature of 37°C. In contrast, parental (*CDC9*) cells (incubated with mutant cells both during and after irradiation) exhibited rapid medium-independent DNA rejoining after 10 min of post-irradiation incubation and slower rates of rejoining after longer incubation. Parental cells were also more resistant than mutant cells to killing by gamma irradiation. Approximately 2.5 ± 0.07 and 5.7 ± 0.6 single-strand breaks per 10^8 daltons were detected in DNAs from either *CDC9* or *cdc9-9* cells converted to spheroplasts immediately after 10 and 25 krads of irradiation, respectively. At the permissive temperature of 23°C, the *cdc9-9* cells contained 2 to 3 times the number of DNA single-strand breaks as parental cells after 10 min to 4 h of incubation after 10 krads of irradiation, and two- to eightfold more breaks after 10 min to 2.5 h of incubation after 25 krads of irradiation. Rejoining of single-strand scissions was faster in medium. After only 10 min in buffered growth medium after 10 krads of irradiation, the number of DNA single-strand breaks was reduced to 0.32 ± 0.3 (at 23°C) or 0.21 ± 0.05 (at 37°C) per 10^8 daltons in parental cells, but remained at 2.1 ± 0.06 (at 23°C) or 2.3 ± 0.07 (at 37°C) per 10^8 daltons in mutant cells. After 10 or 25 krads of irradiation plus 1 h of incubation in medium at 37°C, only DNA from *CDC9* cells was rejoined to the size of DNA from unirradiated cells, whereas at 23°C, DNAs in both strains were completely rejoined.

DNA ligase catalyzes the synthesis of phosphodiester bonds between adjacent 3'-hydroxyl and 5'-phosphoryl termini in duplex, base-paired DNA molecules (14, 18). Originally isolated as a temperature-sensitive, cell division cycle mutant of the eucaryote *Saccharomyces cerevisiae* (7–9), *cdc9-1* yeast cells are deficient in DNA ligase (12). At the restrictive temperature of 37°C, the mutant cells synthesize small-sized nascent DNA (12), exhibit enhanced recombination (4, 6), and arrest in medial nuclear division (G2 [2]). The availability of a ligase-deficient mutant has made it possible to test directly for the first time the postulated involvement of DNA ligase in a eucaryotic DNA repair system.

A deficiency in the repair of damaged DNA in *cdc9* cells has not been reported previously. DNA rejoining has been presumed to be defective in *cdc9* mutants because incubation at 37°C after exposure to UV light (12) or 0.3% methyl methanesulfonate (11) renders *cdc9-1* cells more sensitive to killing than parental (*CDC9*) cells. Survival of *CDC9* and *cdc9-1* cells, however, is very similar after 37°C incubation for 1 h after gamma irradiation (11). Ionizing radiation produces direct single-strand scissions of phosphodiester bonds in DNA which can be rejoined directly by DNA ligase. Single-strand breaks are also produced indirectly after the participation of one or more enzymes in the intracellular DNA repair system acting after ionizing irradiation; the final step in the repair system necessitates DNA rejoining. It is reported here that a mutation allelic to *cdc9-1*, *cdc9-9*, confers a deficiency in medium-independent and medium-dependent rejoining of single-strand breaks produced after gamma irradiation. The *cdc9-9* mutation also confers an enhanced sensitivity to killing by gamma rays.

MATERIALS AND METHODS

Yeast strains. Haploid strains are listed in Table 1; they were provided by Louise Prakash, University of Rochester, N.Y.

Media and culture conditions. Strains were routinely cultured at 23°C with aeration in a medium containing 10 g of yeast extract (Difco Laboratories, Detroit, Mich.), 20 g of peptone (Difco), 20 g of dextrose, and

0.08 g of adenine sulfate per liter (YPAD). Buffered medium (YPADP) contained 0.05 M phosphate. Supplemented synthetic growth medium contained, per liter, 67 g of yeast nitrogen base (Difco) without amino acids or $(NH_4)_2SO_4$, 50 mg of $(NH_4)_2SO_4$, 10 g of succinic acid, 6 g of NaOH, 20 g of dextrose, 60 mg of adenine sulfate, 20 mg of L-tryptophan, 30 mg of L-isoleucine, and 150 mg of L-valine; the pH was adjusted to 5.8. Media were solidified with 2% (wt/vol) agar (Difco) when desired.

For each experiment, precultures of each strain were grown in YPAD the day before labeling to ensure an adequate supply of fresh cells. DNA and RNA were labeled by growing yeast at 23°C with aeration from starting inocula of 5×10^6 cells per ml in supplemented minimal medium (5, 15, 19); [6-H^3]uracil (specific activity, 20 to 30 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was added to 7 μCi/ml or [2-^{14}C]uracil (specific activity, 40 to 60 Ci/mmol; New England Nuclear) was added to 5 μCi/ml. Double-label procedures were used for comparisons of parental and mutant strains; the 6-^{3}H- and 2-^{14}C-labeled radioisotopes were alternated between the two strains in replicate experiments. After approximately 14 h, cells were harvested by centrifugation at 4°C in an RC5B Sorvall SS34 rotor at 3,000 × *g*, washed once in deionized water, chased in supplemented synthetic medium without radiochemical for 60 to 90 min, and then washed twice again with deionized water.

Gamma ray exposures. Washed cells were suspended at 10^7 cells per ml in deionized water and chilled until ice cold. Irradiations were carried out on ice in a cobalt-60 irradiator (J. L. Shepard and Associates, Glendale, Calif.) made available by Christopher Lawrence, University of Rochester. The dose rate was estimated from the decay constant of ^{60}Co after periodic ferrous sulfate dosimetry. Irradiated cells were immediately pelleted by centrifugation at 4°C and either immediately converted to spheroplasts or resuspended for post-irradiation incubation. Both unirradiated and irradiated cells were given post-irradiation incubation periods with aeration.

Cell survival. Cells for survival and DNA studies were grown identically. After irradiation, cells were sedimented immediately at 4°C, and then appropriate serial dilutions were made in cold deionized water before plating in triplicate or quintuplicate on YPAD agar plates.

Spheroplast formation. Washed cells were suspended at approximately 10^8 cells per ml in 0.9 M sorbitol–0.05 M EDTA–0.1 M sodium citrate (pH 5.8; adapted from Forte and Fangman [5]) containing 3 mg of zymolyase 60,000 (Kirin Brewery Co., Ltd., Takasaki, Japan) per ml. After incubation at 34°C, spheroplasts were sedimented in an RC2B SS34 rotor at 2,000 × *g* and gently resuspended in cold 0.1 M sodium citrate and 0.1 M EDTA, and approximately 5×10^6 to 1×10^7 spheroplasts in 100 μl were immediately layered onto the lysis layer atop alkaline sucrose gradients.

Velocity sedimentation and molecular weight determinations. Alkaline sucrose (Ultrapure density gradient grade; Schwartz/Mann, Orangeburg, N.Y.) solutions contained 0.5 M NaCl, 0.01 M EDTA, and 0.2 M NaOH. Gradients were formed in 5-ml polyallomer ultracentrifuge tubes by the addition of 2.5 ml of 5% and 2.4 ml of 20% solutions to a custom-made gradient maker. Gradients were overlaid with 100 μl of a lysis buffer containing 1.3% purified sodium lauroylsarcosinate (97% minimum; Colgate-Palmolive Co., Jersey City, N.J.), 0.6 M NaCl, 0.2 M NaOH, and 0.067 M dissodium EDTA. After gently layering the spheroplasts, 1 h was allowed for lysis of spheroplasts and DNA unwinding. For a sedimentation marker, T4 particles containing [^{14}C]DNA were incubated in 2% Sarkosyl for 10 min at 60°C and layered on gradients. T7 [^{3}H]DNA was used as a low-molecular-weight marker.

Nucleic acids were sedimented in an SW50.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 20°C. The speed and length of the ultracentrifugation were dependent upon the particular experiment. For example, control DNA was routinely sedimented at 8,000 rpm for 20 h ($\omega^2 t = 5 \times 10^{10}$ radians per s). An average of 25 fractions of approximately 0.2 ml was collected from the bottom of each gradient with the aid of a polystaltic pump (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.). Fractions were collected on Whatman no. 17 premarked paper strips by procedures adapted from the method of Carrier and Setlow (1). Paper strips were washed three times in 5% trichloroacetic acid and twice in 95% ethanol. Twelve to twenty-four hours were allowed for each trichloroacetic acid wash to enable RNA hydrolyzed during alkaline sedimentation to diffuse from the paper strips.

Filters were dried, and acid-insoluble radioactivity in each fraction was determined by liquid scintillation counting.

With T4 and T7 DNAs as standards, the gradient system was determined to be isokinetic, and the constant, k, and exponent, α, were experimentally determined for the Studier equation: $S = kM^{\alpha}$. With the aid of a computer program, weight-average molecular weights (M_w) were calculated from distributions of radioactivity in gradients.

The number of single-strand breaks relative to the unirradiated, unincubated control were calculated as follows:

$$10^8 \times \frac{(M_w \text{ unirradiated}/M_w \text{ irradiated}) - 1}{M_w \text{ unirradiated} \div 2}$$

In the interest of simplicity in comparing normal and mutant strains, certain assumptions are inherent in this method of approximating the numbers of single-strand breaks; however, any error associated with the assumptions should be equivalent for both strains. In the experiments reported here, the molecular weight determinations for DNA from unirradiated, unincubated cells and the unirradiated, incubated control cells were usually very similar, except for minor increases in radioactivity in the top 15 to 18% of a few gradients.

RESULTS

Production of single-strand breaks in irradiated *CDC9* and *cdc9* cells. The single-strand breaks produced in parental (prelabeled) DNA were determined by velocity sedimentation of DNA through precalibrated, isokinetic alkaline sucrose gradients. The experimental procedure involved mixing *CDC9* and *cdc9-9* cells before irradiation and carrying out all subsequent experimental procedures on mixed suspensions.

To minimize potential shearing of DNA, spheroplasts were carefully layered onto a lysis layer on density gradients.

Typical sedimentation profiles for quantifying single-strand breaks are shown in Fig. 1. Under the sedimentation conditions typically employed (Fig. 1A; $\omega^2 t = 5 \times 10^{10}$ radians per s), the 80 to 90% of DNA from normal (*CDC9*), unirradiated yeast which is nuclear in origin sediments in a major peak to the bottom (approximately) 70 to 75% of gradients; the mitochondrial DNA of respiratory-proficient strains sediments more slowly than T4 DNA, in the top 25%. The DNAs from unirradiated A364A (*CDC9*) and 288 (*cdc9-9*) cells grown and prelabeled at 23°C yielded comparable profiles (Fig. 1A). Single-stranded nuclear DNA from the unirradiated cells sedimented with an M_w of 1.9×10^8 to 2.2×10^8.

The approximately linear relationship of gamma ray dose to single-strand breaks produced in DNAs from haploid *CDC9* and *cdc9-9* cells is shown in Fig. 2. The same numbers of breaks were usually produced in parental and mutant cells irradiated with the same dose of ionizing radiation.

Medium-independent DNA rejoining. We first measured the capacities of gamma-irradiated parental and mutant cells to rejoin single-strand breaks in the absence of medium. It was reasoned that, under these conditions, new ligase molecules should not be synthesized after irradiation, and DNA rejoining should be proportional to the functional activity of ligase molecules present at the time of irradiation. Moreover, the initial step in the rejoining reaction catalyzed by DNA ligase utilizes the energy of a cofactor (14), in whose absence ligation is expected to be limited. In the absence of medium, therefore, rejoining of DNA single-strand breaks would also be expected to be limited by energy reserves in the cell at the time of irradiation.

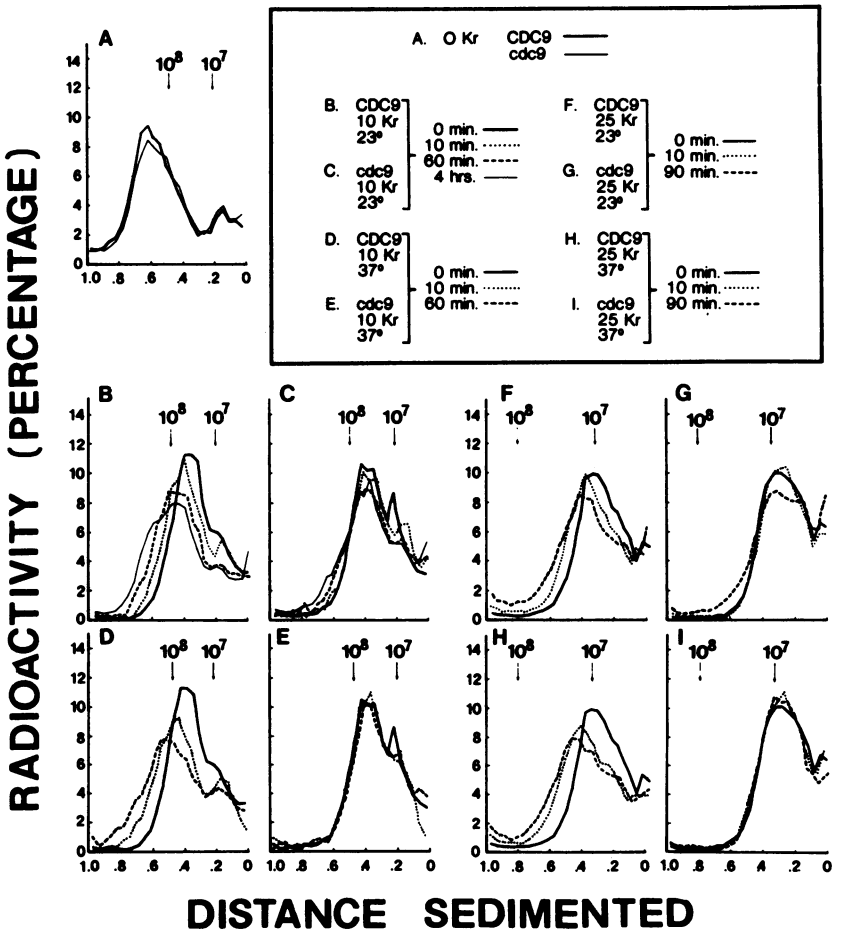

FIG. 1. Typical alkaline sucrose gradient sedimentation profiles illustrating the effects of 10 and 25 krads of gamma irradiation and subsequent incubation in deionized water on the sedimentation rates of DNA from *CDC9* and *cdc9-9* cells.

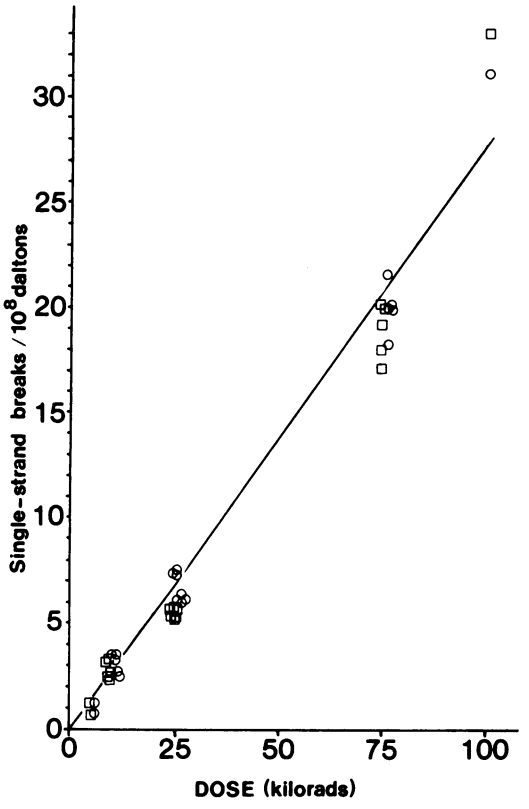

FIG. 2. Production of single-strand breaks in DNA from *CDC9* (□) and *cdc9-9* (○) yeast cells as a function of gamma ray dose. Symbols represent determinations from separate experiments.

Figure 1 shows DNA rejoining in the absence of medium. The single-stranded DNA from cells irradiated with 10 and 25 krads of gamma rays before spheroplast formation sedimented with an M_w of $5.5 \pm 0.7 \times 10^7$ and $3.0 \pm 0.2 \times 10^7$, respectively. A 10-min aerobic incubation of cells in deionized water (or 0.05 M phosphate buffer [data not shown]) after irradiation but before conversion of the cells to spheroplasts resulted in increased sedimentation rates of DNA from *CDC9* cells (Fig. 1B, D, F, and H) but not of DNA from *cdc9* cells (Fig. 1C, E, G, and I). Parental cells exhibited additional time-dependent increases in faster-sedimenting DNA after 1 to 4 h of posttreatment incubation (Fig. 1B and D). In contrast, post-irradiation incubation of irradiated mutant cells at the restrictive temperature of 37°C resulted in no detectable increases in DNA sedimentation rates (Fig. 1E and I), and post-irradiation incubation at 23°C resulted in negligible increases in faster-sedimenting DNA (Fig. 1C and G).

From sedimentation profiles like those shown in Fig. 1, average numbers of single-strand breaks were calculated and plotted as a function of the length of post-irradiation incubation (Fig. 3). Losses in single-strand breaks in parental and mutant cells could then be quantitatively compared to provide an indication of the kinetics of DNA rejoining at each temperature.

At 23°C, *cdc9* cells contained two to three times the number of DNA single-strand breaks as parental cells after incubation for 10 min to 4 h after irradiation with 10 krads (Fig. 3A) and two- to eightfold more breaks after 10 min to 2.5 h of incubation after irradiation with 25 krads (Fig. 3C). Rates of rejoining gradually decreased as the length of the post-irradiation incubation increased. At 37°C, the number of breaks in mutant DNA remained practically constant, whereas DNA was rapidly rejoined in parental cells (Fig. 3B and D). Rates of DNA rejoining in parental cells were rapid during 10 min of incubation but decreased during longer post-irradiation incubation.

Rejoining after incubation in medium. DNA rejoining in *CDC9* cells irradiated with 10 krads of gamma rays was faster during 10 min of post-irradiation incubation in growth medium (Fig. 4A) than during 10 min of incubation in the absence of medium (Fig. 1B). This rapid DNA rejoining in medium represented a reduction from 2.5 ± 0.07 to 0.32 ± 0.3 (23°C) or 0.21 ± 0.05 (37°C) single-strand breaks per 10^8 daltons (Fig. 5). DNA rejoining was slower in the *cdc9-9* mutant (Fig. 4B and 5). After 10 (Fig. 5) or 25 (data not shown) krads of irradiation and subsequent incubation at 23°C in medium for 1 h, DNAs in both parental and mutant cells were rejoined to the size of DNAs from unirradiated cells. At the restrictive temperature, however, mutant cells failed to rejoin single-strand breaks

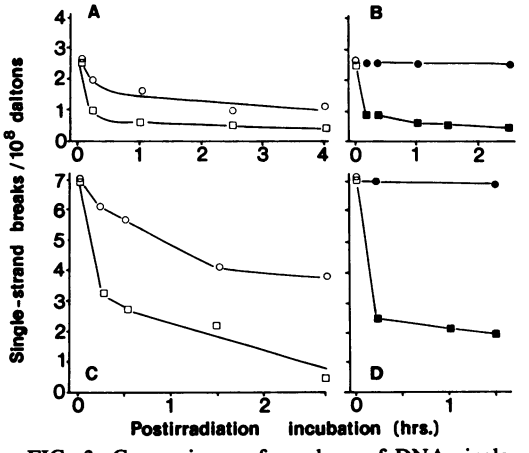

FIG. 3. Comparisons of numbers of DNA single-strand breaks after 10 (A and B) or 25 (C and D) krads of irradiation of *CDC9* (□, ■) and *cdc9-9* (○, ●) cells and subsequent incubation in deionized water at 23 (○, □) or 37°C (●,■).

TABLE 1. Isogenic yeast strains (a *ade1 ade2 ura1 his7 lys2 tyr1 gal1*)

Strain	Allele	Defect	Source (reference)
A364A	*CDC9* (wild type)	(Normal)	Rochelle Esposito[a] (7)
244	*cdc9-1*	Ligase defective	Leland Hartwell[b] (7–9)
288	*cdc9-9*	Ligase defective	Leland Hartwell

[a] University of Chicago, Chicago, Ill.
[b] University of Washington, Seattle.

after 1 h of incubation in medium (Fig. 5). In contrast to post-irradiation incubation in the absence of medium (Fig. 1 and 3), small reductions in the numbers of breaks were detected in DNA from mutant cells after post-irradiation incubation at 37°C in medium (Fig. 4 and 5). In both strains, breaks are undoubtedly very rapidly rejoined during and immediately after gamma irradiation, and this very rapid component of DNA rejoining appears to be enhanced after incubation in medium.

Cell survival. Typical survival curves for *CDC9* and *cdc9-9* strains are shown in Fig. 6. Mutant cells were significantly more sensitive than parental cells to killing by ionizing radiation. Routinely, parental cells were made slightly more resistant to killing after 90 min of preirradiation incubation at 37°C (in the absence of medium), whereas preincubated mutant cells were made somewhat more sensitive (Fig. 6).

While our experiments were in progress, the survival of *CDC9* cells was reported to be similar to that of *cdc9-1* cells after gamma irradiation (11). Numerous quantitative comparisons of the growth of the *cdc9-1* and *cdc9-9* mutants at elevated temperatures, however, had suggested to us that the *cdc9-1* mutation was less severe and thus could be leaky. Table 2 contrasts, for example, the capacities of *CDC9*, *cdc9-1*, and *cdc9-9* strains to grow at elevated temperatures. The growth of *cdc9-1* cells was reduced by only approximately 10 and 25% at 27 and 30°C, respectively, but was markedly curtailed at both 34 and 38°C. In contrast, *cdc9-9* cells grew very slowly and formed abnormally small and fewer colonies at 30°C, even after prolonged incubation. Growth of *cdc9-9* cells was effectively stopped at 38°C and was more inhibited than *cdc9-1* cells at 34°C. Because the *cdc9-1* mutation appeared less severe, the *cdc9-9* allele was employed for the DNA-rejoining studies.

DISCUSSION

The results presented in this paper indicate that ligase-deficient yeast cells are defective in their capacity to rejoin single-strand breaks at the permissive and restrictive temperatures. After equivalent DNA damage by high-dose-rate gamma irradiation was produced in normal and ligase-deficient cells, both rapid and slower DNA rejoining were found to be defective in mutant cells. The *cdc9-9* cells were also considerably more sensitive than parental (*CDC9*) cells to killing by gamma rays (Fig. 6).

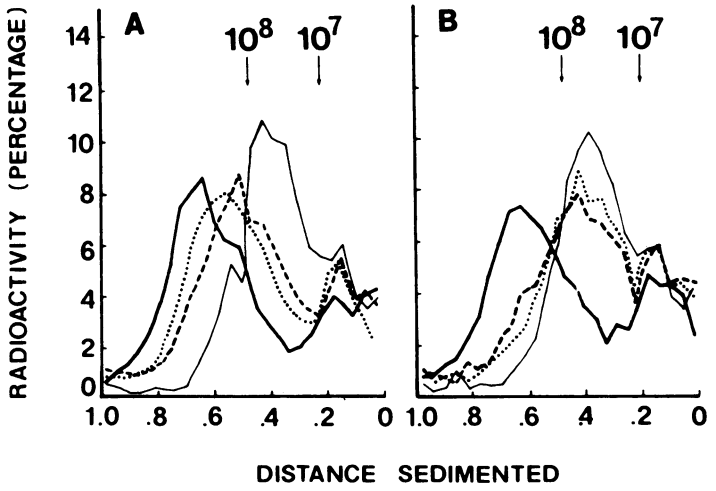

FIG. 4. Typical DNA sedimentation profiles showing the effects of 10 krads of gamma irradiation on late-logarithmic-phase haploid cells and subsequent incubation in buffered medium (YPADP). (A) *CDC9*; (B) *cdc9-9*. (——), 0 krads; (——), 10 krads, (-----), 10-min post-irradiation incubation at 23°C; (·····), 10-min postirradiation incubation at 37°C.

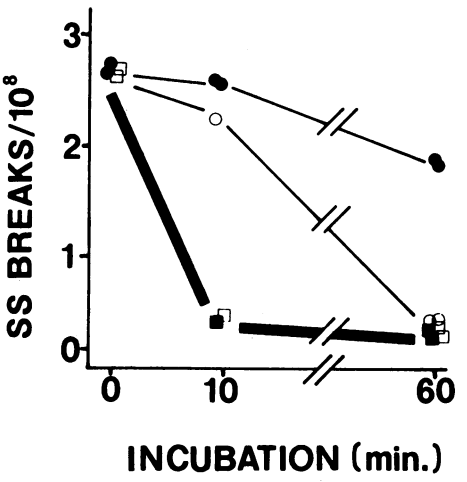

FIG. 5. Comparisons of single-strand breaks after 10 krads of irradiation of *CDC9* (□, ■) and *cdc9-9* (○, ●) cells and subsequent incubation for 0, 10, or 60 min in buffered growth medium at 23 (□, ○) or 37°C (■, ●).

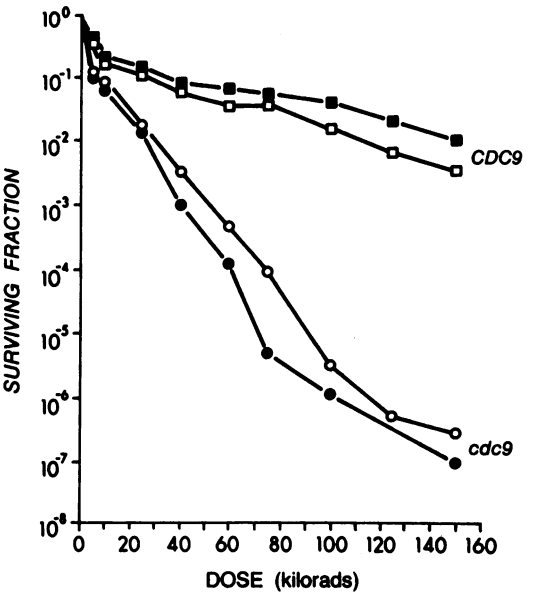

Fig. 6. Survival of irradiated *CDC9* (□, ■) and *cdc9-9* (○, ●) cells which had been incubated with aeration for 90 min in deionized water at 23 (□, ○) or 37°C (■, ●) before irradiation.

Ligase activity has been detected in crude extracts of *CDC9* cells grown at 23°C, but no ligase activity has been detected in crude extracts of *cdc9-9* cells grown at 23°C (11; Louise Prakash, unpublished results). The deficient rejoining in *cdc9-9* mutant cells is predicted by the ligase deficiency and no doubt accounts for the decreased survival of *cdc9-9* cells. Moreover, the increased rapid strand rejoining in parental cells incubated at 37°C (versus 23°C [Fig. 1, 3, and 4]) correlated with the slightly increased resistance to radiation killing exhibited by the parental cells after incubation at 37°C (Fig. 6). Likewise, the decreased or complete absence of rejoining in mutant cells incubated at 37°C (versus 23°C [Fig. 1, 3, 4, and 5]) correlated with the decreased resistance exhibited by mutant cells after incubation at 37°C (Fig. 6). In this regard, *Escherichia coli* parental and *lig-7*(Ts) cells exhibited analogous temperature dependencies of UV sensitivity (22) but not X-ray sensitivity (3). Particularly in the case of parental yeast cells, the effect of temperature on DNA rejoining and survival is probably due to a generalized temperature dependence of cellular metabolism and recovery mechanisms rather than to a direct effect on DNA ligase. In fact, although it is generally believed that *CDC9* is the structural gene for DNA ligase, this has not been proven. Although regulatory mutants are usually dominant, the possibility still exists that the *cdc9* mutant could be defective in a regulatory function which controls not only DNA ligase but also one or more additional DNA repair enzymes.

In contrast to the ligase-deficient mutants of *S. cerevisiae*, *cdc17* mutants of the fission yeast *Schizosaccharomyces pombe* exhibit a thermosensitive DNA ligase deficiency (16, 17).

TABLE 2. Growth of *CDC9* and *cdc9* at several temperatures[a]

Strain	Survival at following temp (°C)[b]				
	23	27	30	34	38
CDC9	[1.0]	1.0 ± 0.05[c]	1.0 ± 0.08	1.0 ± 0.06	1.0 ± 0.07
cdc9-1	[1.0]	0.89 ± 0.07	0.75 ± 0.09	$1.1 \times 10^{-3} \pm 4 \times 10^{-3}$	$5.6 \times 10^{-5} \pm 7.9 \times 10^{-5}$
cdc9-9	[1.0]	0.92 ± 0.09	0.027 ± 0.01[c]	$2.1 \times 10^{-4} \pm 0.4 \times 10^{-4}$[c]	$<1 \times 10^{-7}$[c]

[a] All three strains were pregrown with aeration in YPAD at 23°C for 60 h. All cells were washed twice with distilled water, appropriately diluted, and then plated on YPAD made with deionized water. Plates were prewarmed to 27, 30, 34, or 38°C before plating cells.

[b] Colony-forming abilities are expressed as the ratio: (mean frequency of colonies at 27, 30, 34, or 38°C)/(mean frequency of colonies at 23°C). The means and standard errors of three separate experiments are given.

[c] Colonies were not visible after the usual incubation period of 3 days. After 6 days of incubation, none of these colonies grew to normal size.

The thermolability of the ligase partially purified from two mutants strongly suggests that the *cdc17* mutation lies in the structural gene for DNA ligase (17). Although DNA repair has not been directly examined in the temperature-sensitive lethal mutants of *Schizosaccharomyces pombe*, other properties of the *cdc17* mutants (16, 17) are very similar to those of both the *cdc9* mutants of *Saccharomyces cerevisiae* and *lig* mutants of *E. coli* (10, 13). The enhanced UV sensitivity of *cdc17* cells at the restrictive temperature suggests that a repair deficiency is correlated with the thermosensitive DNA ligase.

The same DNA ligase appears to be involved in DNA replication (12), recombination (4, 6), and repair (this report; 11, 12) in yeast cells as in *E. coli*. Yet this role for a single DNA ligase is not ubiquitous for eucaryotic cells, since mammalian cells have two serologically unrelated DNA ligases (20, 21). Since one activity increases in proliferating mammalian cells while the other remains at the same level in growing and nongrowing mammalian cells, the two DNA ligases probably play distinct roles in DNA replication and repair (21).

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