

Molecular Mechanisms of Pyrimidine Dimer Excision in *Saccharomyces cerevisiae*: Excision of Dimers in Cell Extracts

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Cell-free extracts prepared from *rad1-19*, *rad2-2*, *rad3-1*, *rad4-3*, *rad7-1*, *rad10-1*, *rad14-1*, *rad16-1*, and *cyc1-1* (*rad7*) mutants of *Saccharomyces cerevisiae* all catalyze the preferential excision of thymine-containing pyrimidine dimers from ultraviolet-irradiated DNA specifically incised with *M. luteus* ultraviolet deoxy-ribonucleic acid incising activity.

Excision repair in the yeast *Saccharomyces cerevisiae* is a genetically complex process. Mutations at nine different genetic loci (*RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD7*, *RAD10*, *RAD14*, *RAD16*, and *MMS19*) preclude the normal excision of pyrimidine dimers in vivo (13-15, 17, 18, 22, 26, 27). A tenth locus (*UVS12*) that also affects dimer removal from DNA has recently been reported (2) and two additional loci (*CDC8* and *CDC9*) may be involved in pyrimidine dimer excision. *CDC8* is a member of the *RAD3* epistatic group (16), whereas there is suggestive evidence (9) that *CDC9* may be defective in DNA ligase, an activity thought to be necessary for the final strand closure step of excision repair. Although the involvement of most of these loci in the excision repair of UV damage to DNA is well established, neither the nature of their gene products (with the possible exception of *CDC9* [9]) nor their precise role in excision repair is currently known.

We have previously reported the presence of incubation-dependent, single-strand breaks (incisions) in the nuclear DNA of UV-irradiated excision-proficient strains of the yeast *S. cerevisiae* (21, 22). Similar incubation-dependent, single-strand breaks were not observed in the nuclear DNAs of *rad1*, *rad2*, *rad3*, *rad4*, and *rad14* mutants (21, 22). The number of DNA single-strand breaks in the *RAD*⁺ strain S288C was found to be reduced by photoreactivation in vivo (21), indicating that they are related specifically to the excision of pyrimidine dimers. Thus, their absence in the *rad* mutants mentioned above is consistent with the hypothesis that the products of the *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD14* genes are involved in the incision or

preincision steps of excision repair of pyrimidine dimers. Further support for this hypothesis would be provided by the demonstration that the mutants just discussed are not defective in postincisional events required for the excision repair of pyrimidine dimers. We have previously established that lysates prepared from excision-proficient *RAD*⁺ yeasts catalyze the loss of >90% of the thymine-containing pyrimidine dimers from specifically preincised UV-irradiated DNA, whereas less than 10% of the total radioactivity in DNA was converted to an acid-soluble form (20). This dimer-excising activity has a divalent cation requirement that can be satisfied by the addition of Mg²⁺. In the present study, we show that this dimer-excising activity is present at normal levels in extracts prepared from all of the *rad* mutants examined.

The yeast strains used in this study are shown in Table 1. Lysates of wild-type and mutant strains were prepared by the mechanical disruption of cells from mid-log phase cultures as described previously (21). Cells were grown to a density of approximately 6 × 10⁷ cells per ml in 150 ml of YEP medium (1% [wt/vol] yeast extract [Difco Laboratories], 2% [wt/vol] peptone [Difco] and 2% [wt/vol] glucose). Cells were harvested by centrifugation, washed twice with Tris-hydrochloride buffer, pH 7.6, and resuspended in 2.0 ml of 20.0 mM Tris-hydrochloride, pH 7.6, at 0°C. The washed cells were disrupted in 15.0-ml Corex tubes by mixing on a Vortex mixer in the presence of 0.5-mm glass beads by the method of Lang et al. (11). Mixing was applied in 30-s intervals for a total of 4 min. Care was taken to keep the lysates on ice between Vortex cycles. Cellular disruption was generally 95% or greater, as determined by examination of cell refractility by phase-contrast microscopy. Lysates were harvested by aspiration with the

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TABLE 1. Source of *rad* mutants used

Mutation	Strain	Background	Obtained from	Originally isolated by	Reference
<i>RAD</i> ⁺	S288C	S288C	D. Gottlieb	R. Snow	24
<i>rad1-19</i>	S967-5b (<i>uvs19</i>)	S288C	D. Gottlieb	R. Snow	6
<i>rad2-2</i>	S226-7c (<i>uvs8</i>)	S288C	D. Gottlieb	R. Snow	24
<i>rad3-1</i>	S222-16a (<i>uvs4</i>)	S288C	J. Game	R. Snow	24
<i>rad4-3</i>	S960-1a (<i>uvs12</i>)	S288C	D. Gottlieb	R. Snow	6
<i>rad7-1</i>	(<i>uvs7 ade2</i>)	197/2d	J. Game	B. Cox and J. Parry	4
<i>cyc1-1 (rad7)</i>	DW18-2B (<i>adel ural cyc1-1</i>)	A364A	L. Prakash	M. Ogur	23
<i>rad10-1</i>	S962-3c (<i>uvs14</i>)	S288C	D. Gottlieb	R. Snow	6
<i>rad14-1</i>	S229-11b (<i>uvs11</i>)	S288C	D. Gottlieb	R. Snow	24
<i>rad16-1</i>	LP1709-6B (<i>adel,2 lys2 ural rad16</i>)	A364A	L. Prakash	B. Cox and J. Parry	4

aid of a Pasteur pipette, after which the glass beads were washed three to four times with 1.0 ml of 20.0 mM Tris-hydrochloride, pH 7.6. The washes were pooled with the original lysate. The lysate was clarified by centrifugation at 10,000 × *g* for 30 min and made 10% (vol/vol) with respect to glycerol in a final volume of 6.0 ml.

Substrate for the assay of dimer-excising activity was prepared from purified [*methyl*-³H]-thymidine-labeled *Escherichia coli* DNA. The purified DNA was irradiated with a low-pressure mercury germicidal lamp. Exposures were adjusted to convert 1.3 to 1.6% of the labeled thymine bases into thymine-containing pyrimidine dimers. The irradiated DNA was then incubated with a partially purified preparation of *Micrococcus luteus* containing pyrimidine dimer-specific DNA-incising activity that introduces single-strand scissions 5' to the sites of dimers (8). In this way, strand breaks were introduced at 80 to 90% of the thymine-containing pyrimidine dimers in the *E. coli* DNA. After reprecipitation and dialysis against 1.0 mM EDTA-20.0 mM Tris-hydrochloride, pH 7.6, the specifically preincised DNA was incubated with extracts of *S. cerevisiae* as previously described (21). Standard reactions contained 12 to 18 μg of preincised DNA (1×10^5 to 1.5×10^5 cpm), 10% glycerol, 10 mM MgCl₂, 20 mM Tris-hydrochloride, pH 7.6, and 50 μl of lysate. All lysates were prepared from the same number of cell equivalents (2×10^9 cells per ml), and all had protein concentrations of 7 to 8 mg/ml after clarification by centrifugation. Reactions were at 30°C for the times indicated in Fig. 1. Reactions were terminated by the addition of EDTA (20 mM final concentration) and by freezing the samples at -20°C. The thymine dimer content of the labeled DNAs was determined by thin-layer chromatography of acid-precipitable DNA as described by Reynolds et al. (20).

Lysates prepared from the *RAD*⁺ strain S288C contained enzyme activity that catalyzed

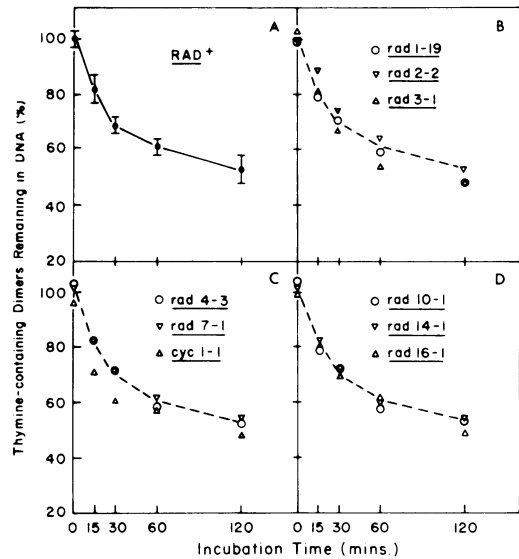


FIG. 1. Removal of thymine-containing pyrimidine dimers from preincised UV-irradiated DNA. 100% = 1.3 to 1.6% of the radioactivity present in the DNA in the form of thymine-containing pyrimidine dimers. Error bars in (A) denote standard deviations for six separate determinations with four independently prepared lysates of S288C (*RAD*⁺).

the preferential release of a significant fraction of the thymine-containing pyrimidine dimers from acid-precipitable preincised DNA (Fig. 1A). Typically, about 50% of the thymine in thymine-containing pyrimidine dimers was released during a 2-h incubation at 30°C, with less than 5% of the total labeled thymine in the DNA being converted to an acid-soluble form. When lysates were prepared from *rad1-19*, *rad2-2*, *rad3-1*, *rad4-3*, *rad7-1*, *cyc1-1 (rad7)*, *rad10-1*, *rad14-1*, and *rad16-1* mutants, the kinetics and extent of removal of dimers from acid-precipitable DNA were indistinguishable from those observed with lysates prepared from the exci-

sion-proficient *RAD*⁺ strain S288C (Fig. 1B to D).

The mutants used in this study are all single *rad* gene mutants that are defective in pyrimidine dimer excision *in vivo* and are also markedly sensitive to UV radiation (R. H. Haynes and B. A. Kunz in J. Strathern, J. Broach, and E. W. Jones, ed., *The Molecular Biology of the Yeast Saccharomyces*, in press). Thus, if any of them were defective in postincisional dimer-excising nuclease activity, it is highly likely that in wild-type cells only a single such activity would be operative and that a defect in this activity would be reflected in cell-free extracts. Such a predicted result is observed in extracts of *E. coli*. Genetic and biochemical studies in this organism indicate that DNA polymerase I is the principal activity responsible for excision of pyrimidine dimers (7) and indeed extracts of *E. coli polA*⁺ cells readily catalyze the preferential loss of thymine-containing pyrimidine dimers from specifically preincised UV-irradiated DNA *in vitro*, whereas extracts of *polA* cells do not (5).

The activity that we have observed in extracts of both wild-type and mutant strains could conceivably reflect the spurious action of enzymes that are not involved in pyrimidine dimer excision *in vivo*, thereby completely masking a true excision nuclease defect. This possibility cannot be fully eliminated until mutants of *S. cerevisiae* known to be defective in dimer-excising nuclease activity are available for study. However, in this regard it is worth noting that such a phenomenon is not observed in extracts of *polA* strains of *E. coli*, despite the fact that such extracts contain other enzyme activities that in purified form can catalyze the excision of thymine-containing pyrimidine dimers from preincised DNA (3, 12, 25). With this reservation in mind, the demonstration in the present experiments that none of the *rad* mutants examined are defective in thymine dimer-excising activity *in vitro* strongly suggests that this is also true *in vivo* and supports our previous conclusion (21, 22) that mutants defective in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD14* loci code for functions directly involved in DNA incision or preincision events during pyrimidine dimer excision in *S. cerevisiae*. Our results are not in agreement with those recently reported by Bekker et al. (1). These authors claim to have demonstrated incision of UV-irradiated DNA with cell-free preparations of wild-type *S. cerevisiae* and of mutants defective in the *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD16* loci and thus suggested that some of these *rad* mutants may be defective in pyrimidine dimer-excising activity.

In light of our observation that the *rad7*,

rad10, and *rad16* mutants contain normal levels of thymine dimer-excising activity *in vitro*, it is possible that in wild-type yeasts these loci also code for functions required for the incision of UV-irradiated DNA *in vivo*. This conclusion can be substantiated by direct examination of the capacity of mutants at the *RAD7*, *RAD10*, and *RAD16* loci to catalyze the formation of incubation-dependent, single-strand breaks in nuclear DNA. Such studies are in progress. Work is also in progress to purify and characterize the dimer-excising activity that we have identified in extracts of *S. cerevisiae*.

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