Requirement of DNA Polymerase Activity of Yeast Rad30 Protein for Its Biological Function*

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The RAD30 gene of Saccharomyces cerevisiae encodes a DNA polymerase, Polγ. The RAD30 protein shares homology with the yeast Rev1 and the Escherichia coli DinB and UmuC proteins. Although these proteins contain several highly conserved motifs, only RAD30 has been shown to possess a DNA polymerase activity. To determine whether the DNA polymerase activity of RAD30 was essential for its biological function, we made a mutation in the highly conserved SIDE sequence in RAD30, in which the aspartate and glutamate residues were changed to alanine. The resulting mutant protein lacked DNA polymerase activity, and this mutation inactivated the biological function of the protein.

UV-induced DNA damage presents a block to the DNA replication machinery. To maintain the continuity of the DNA during replication, UV lesions encountered by the replication machinery are circumvented by both error-free and error-prone means. In the yeast Saccharomyces cerevisiae, genes in the RAD6 epistasis group function in the replication of DNA-containing lesions generated by UV light and by other DNA-damaging agents. Mutations in the RAD6 and RAD18 genes confer extreme sensitivity to UV light, and these mutants are defective in postreplicative bypass of UV-damaged DNA and in UV-induced mutagenesis (1). Rad6, a ubiquitin-conjugating enzyme, exists in vivo in a complex with Rad18, a DNA-binding protein (2, 3). How Rad6-Rad18 protein-dependent ubiquitination promotes error-free and mutagenic postreplicative bypass is not known.

Of the genes in the RAD6 epistasis group, REV1, REV3, and REV7 are required for mutagenic bypass of UV damage, and yeast lacking any of these genes is nonmutable by UV light (1). The REV3 and REV7 proteins form DNA polymerase ζ, which shows limited ability to bypass a cis-syn thymine-thymine dimer (4). Rev1 is a deoxyxycytidyl transferase that can incorporate a dCMP residue opposite an abasic site (5). The RAD5 gene is required for error-free postreplicative bypass of UV lesions, and it encodes a DNA-dependent ATPase (6, 7). The RAD30 gene affects an alternate pathway of error-free bypass of UV lesions, and the rad5Δ rad30Δ double mutant exhibits a synergistic increase in UV sensitivity over either single mutant (8). Rad30 shares homology with the yeast Rev1 protein and with the E. coli DinB and UmuC proteins (8, 9). We have recently shown that RAD30 encodes a novel eukaryotic DNA polymerase, named Polγ, which has the unique ability to efficiently replicate a cis-syn thymine-thymine dimer-containing template, and it inserts two A residues across from the dimer (10).

The presence of biochemical activity in a protein does not necessarily imply the requirement of that activity in the biological function of the protein. For example, the deoxycytidyl transferase activity of Rev1 seems to have no role in the bypass of UV-damaged DNA templates (5). The S. cerevisiae RAD3 protein and its human counterpart, XPD, both possess DNA helicase activity (11, 12), and they are required for nucleotide excision repair and for RNA polymerase II transcription. Mutational inactivation of the DNA helicase activity of these proteins, however, impairs only the repair function and not the transcription function (13–15). Thus, the DNA helicase activities of Rad3 and XPD are required for DNA repair but not for transcription. Here, we examine whether the DNA polymerase activity of RAD30 is necessary for its role in damage bypass. For this purpose, we altered the aspartate and glutamate residues present in the highly conserved domain of serine, isoleucine, aspartate, and glutamate (SIDE) in RAD30 to alanines. The resulting RAD30 mutant protein lacks DNA polymerase activity, and this mutation inactivates the biological function of RAD30. Thus, the DNA polymerase activity of RAD30 is indispensable for its role in damage bypass.

Materials and Methods

Yeast Strains and Plasmids—Genetic studies were done using yeast strain EMY74.7 (MATα his3α-100, leu2-3, 112, trp1Δ, ura3-52) and its derivatives. Genomic deletions of RAD genes were generated by the gene replacement method. To generate the rad30Δ mutation, 1.4- and 1.1-kilobase PCR products corresponding to the 5′- and 3′-flanking regions of the RAD30 gene, respectively, were directionally cloned into pUC19. The URA3 "geneblaster fragment," which contains the yeast URA3 gene flanked by the duplicated Salmonella typhimurium hisG gene (16), was then inserted in between these PCR products. The rad30Δ deletion generating plasmid, pR30.2, when digested with the restriction enzyme EcoRI, releases a 6.4-kilobase fragment, which when introduced into yeast deletes nucleotides from position +42 to position +1800 of the 1896-nucleotide RAD30 open reading frame. To generate the rad5Δ mutation, yeast strains were transformed with the rad5Δ generating plasmid pBJ22 digested with XbaI as described (6). Deletions were confirmed by PCR analysis of genomic DNA. Loss of the URA3 gene was selected for by plating strains on medium containing 5-flouro-orotic acid.

Mutation of the RAD30 Gene and Plasmid Constructions—To generate the rad30Δ Asp155→Ala, Glu156→Ala mutation, we first isolated the wild type RAD30 gene from the yeast genome by gap repair. The wild type gene was then cloned into pUC19, generating plasmid pBJ579. To create the DE to AA mutation, the MOPH site-specific mutagenesis kit (5 Prime → 3 Prime, Inc., Boulder, CO) was employed using the mutagenic oligonucleotide 5′-GTGCGAAGGCGACTATTGCTGCAG-TATTCTTGTATTGCG-3′, which contains the codons for amino acids AA instead of DE at positions 155 and 156, respectively, in RAD30. To increase the yield of mutant-containing plasmid, pBJ579 was grown in dut ung E. coli in medium containing uracil and was used as template for DNA synthesis from the mutagenic primer. The resulting DNA was ligated and introduced into mutS+ E. coli strain BMH 71-18, which cannot repair mismatches and degrades the uracil-containing template.

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† The abbreviations used are: PCR, polymerase chain reaction; GST, glutathione S-transferase.
Plasmid DNA containing the rad30 Δ− gene was isolated, and the presence of the mutation was confirmed by sequencing. The DNA fragment containing the rad30 mutation was then used to replace the wild type fragment in the pUC19-derived plasmid pHJ639, generating plasmid pHJ643. To overexpress the mutant protein, the rad30 Ala155-Ala156 mutant gene was fused in-frame with the glutathione S-transferase gene under control of the galactose-inducible phosphoglycerate kinase promoter, generating plasmid pHJ643. Plasmids pHJ640 and pHJ646 contain the wild type and rad30 Ala155-Ala156 mutant genes, respectively, in the low copy CEN plasmidYCplac111. These constructs contain approximately 800 nucleotides of 5′-flanking RAD30 sequence. 

**Purification of Rad30 Ala155-Ala156 Mutant Protein—Rad30 Ala155-Ala156 mutant protein was purified from the protease-deficient yeast strain BJ5464 as described previously for the wild type Rad30 protein (10).**

**DNA Polymerase Activity—**DNA polymerase activity was assayed as described previously (10). Reactions (10 μl) containing 25 mM KPO₄ (pH 7.0), 5 mM MgCl₂, 5 mM dithiothreitol, 100 μM each of the four dNTPs, and 10 nM of 5′-32P-labeled oligonucleotide primer annealed to an oligonucleotide template were incubated for 5 min at 30 °C with either 2.5 nM of wild type GST-Rad30 protein or 10 nM of GST-Rad30 Ala155-Ala156 mutant protein. Substrates S-1, S-2, S-3, and S-4 have been described previously (10).

**UV Sensitivity and UV Mutagenesis—**Yeast strains were grown to mid-exponential phase in selective medium, washed, sonicated to disrupt cell clumps when necessary, and resuspended in sterile distilled water to a density of 2 × 10⁸ cells/ml. Cell suspensions were diluted, spread onto the appropriate medium, and irradiated at a dose rate of 1 J/m²/s. Plates were incubated in the dark, and colonies were counted after 3–5 days. For UV-induced mutagenesis, cells were processed as described above, and appropriate dilutions were plated on synthetic complete medium for viability determinations and on synthetic medium lacking arginine but supplemented with canavanine for determination of can ¹ mutation frequencies.

**RESULTS AND DISCUSSION**

**Conserved Domains in Rad30 and Related Proteins—**Rad30 shares significant homology with the *S. cerevisiae* Rev1 protein and the *E. coli* UmuC and DinB proteins (8, 9). Previous studies have indicated that Rev1 is a deoxyctydyl transferase that transfers a dCMP residue to the 3′-end of a DNA primer in a template-dependent reaction (5) and that Rad30 is a DNA polymerase that can efficiently replicate DNA containing a thymine-thymine dimer (10). The *E. coli* UmuC and UmuD’ proteins promote damage bypass by DNA polymerase III, but the mechanism of their action remains to be elucidated (17, 18). DinB is required for the untargeted mutagenesis of unirradiated α phage grown in pre-UV irradiated *E. coli* cells (19).

Alignment of the amino acid sequences of Rad30 and its related proteins indicates the presence of five conserved motifs, I–V (Fig. 1). Of particular interest are motif I, which contains a conserved aspartate residue flanked by conserved hydrophobic residues on both sides, and motif III, which contains the highly conserved sequence SIDE. Even though the Rad30 family of proteins shares no sequence homology with any other known prokaryotic or eukaryotic DNA polymerases, motif III resembles motif C, which is common to the entire polymerase family and which also contains an invariant aspartate residue and another highly conserved acidic residue (20). Motif I of the Rad30 family may be analogous to motif A, which also is common to the entire polymerase family and which contains an invariant aspartate residue and another highly conserved acidic residue. Motif I of the Rad30 family is unique in that it contains an invariant aspartate residue (20). Motif I of the Rad30 family is unique in that it contains an invariant aspartate residue (20).
The final fractionation step was separated on a 9% denaturing polyacrylamide gel and stained with Coomassie Blue. Lane 1, molecular mass standards; lane 2, Rad30 Ala155-Ala156 mutant protein (400 ng); lane 3, Rad30 wild type protein (300 ng). B, lack of DNA polymerase activity in the Rad30 Ala155-Ala156 mutant protein. Wild type GST-Rad30 (2.5 nM) was incubated with the DNA substrates (10 nM) S-1, S-2, S-3, and S-4 (lanes 1–4, respectively). The GST-Rad30 Ala155-Ala156 mutant protein (10 nM) was incubated with the DNA substrates (10 nM) S-1, S-2, S-3, and S-4 (lanes 5–8, respectively) for 5 min at 30 °C.

The rise in the frequency of mutations was carried out in the presence of all four dNTPs (100 μM each). nt, nucleotides.

The Ala155-Ala156 mutation on UV mutagenesis, we examined the sensitivity of the rad30 strain, but at 10 J/m², the rad5Δ rad30Δ strain shows a sharp rise in the frequency of canI mutations. Introduction of the rad30 Ala155-Ala156 mutant gene in the rad30Δ strain or in the rad5Δ rad30Δ strain had no effect on the incidence of UV-induced canI mutations. As expected, the wild type RAD30 gene lowered the frequency of UV-induced mutations in the rad30Δ strain to the wild type level and in the rad5Δ rad30Δ strain to the rad5Δ level (data not shown).

In summary, inactivation of the DNA polymerase activity of Rad30 causes complete loss of the biological function of this protein, thus indicating the requirement of this DNA polymerase activity in damage bypass. Our studies also suggest the possibility that the aspartate 155 and glutamate 156 residues present in the highly conserved sequence SIDE in Rad30 play an important role in catalysis. It is possible that these residues are part of the active site of the enzyme and that they coordinate the binding of divalent metal ions.

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