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The *RAD7, RAD16*, and *RAD23* Genes of *Saccharomyces cerevisiae*: Requirement for Transcription-Independent Nucleotide Excision Repair In Vitro and Interactions between the Gene Products

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Nucleotide excision repair (NER) is a biochemical process required for the repair of many different types of DNA lesions. In the yeast *Saccharomyces cerevisiae*, the *RAD7, RAD16*, and *RAD23* genes have been specifically implicated in NER of certain transcriptionally repressed loci and in the nontranscribed strand of transcriptionally active genes. We have used a cell-free system to study the roles of the Rad7, Rad16, and Rad23 proteins in NER. Transcription-independent NER of a plasmid substrate was defective in *rad7, rad16*, and *rad23* mutant extracts. Complementation studies with a previously purified NER protein complex (nucleotide excision repairosome) indicate that Rad23 is a component of the repairosome, whereas Rad7 and Rad16 proteins were not found in this complex. Complementation studies with *rad4, rad7, rad16*, and *rad23* mutant extracts suggest physical interactions among these proteins. This conclusion was confirmed by experiments using the yeast two-hybrid assay, which demonstrated the following pairwise interactions: Rad4 with Rad23, Rad4 with Rad7, and Rad7 with Rad16. Additionally, interaction between the Rad7 and Rad16 proteins was demonstrated in vitro. Our results show that Rad7, Rad16, and Rad23 are required for transcription-independent NER in vitro. This process may involve a unique protein complex which is distinct from the repairosome and which contains at least the Rad4, Rad7, and Rad16 proteins.

Nucleotide excision repair (NER) in eukaryotic cells is a complex biochemical process involving multiple gene products (see references 8 and 9 for reviews). By using a cell-free system that faithfully mirrors the genetic requirements for NER in the yeast *Saccharomyces cerevisiae*, it has been directly demonstrated that at least nine proteins are indispensable for this process (25, 30, 31, 34). Disruption or deletion of the *RAD1*, *RAD2*, *RAD4*, and *RAD14* genes confers extreme sensitivity of cells to killing by UV radiation, and cell extracts of such mutants are defective in NER (reviewed in reference 9). Similarly, cells carrying mutations in the essential genes *RAD3*, *SSL1*, *SSL2* (*RAD25*), *TFB1*, and *TFB2*, all of which encode subunits of the RNA polymerase II basal transcription factor IIH (TFIIH), which is required both for transcription and NER (6, 7, 10, 25, 30, 31), are also defective in NER in vitro (6, 30, 31).

Mutational inactivation of the *RAD7*, *RAD16*, or *RAD23* genes confers partial sensitivity to UV radiation (20, 22, 37), and such cells have been reported to retain residual capacity for excision of pyrimidine dimers following exposure to UV light (16–18, 21, 28, 37). Strains carrying mutations in both the *RAD7* and *RAD16* genes are no more sensitive than either single mutant (28), suggesting that the Rad7 and Rad16 proteins operate in the same biochemical pathway. The *RAD7* and *RAD23* genes are located immediately adjacent to the highly conserved cytochrome *c* genes *CYC1* (*RAD7*) and *CYC7* (*RAD23*) on yeast chromosomes X and V, respectively, and it has been suggested that these regions may have arisen by gene duplication (15). There is no homology between the *RAD7* (565 codons) and *RAD23* (398 codons) at the amino acid sequence level, however. Like the *RAD2* gene, which is indispensable for NER, the *RAD7*, *RAD16*, and *RAD23* genes (but not other *RAD* genes required for NER) are transcriptionally up-regulated when cells are exposed to UV radiation (1, 12, 13, 23).

The partial UV radiation-sensitive phenotype of *rad7* and *rad16* mutants may relate to the observation that these genes are uniquely required for NER in certain transcriptionally repressed regions of DNA. Both *rad16* (2) and *rad7* (28, 36) mutants are defective in the excision of pyrimidine dimers from the transcriptionally silent *HML*α and *HMR***a** mating-type locus, yet they support efficient repair of the transcriptionally active *MAT*α locus. Attempts to determine whether this reflects the transcriptional status of these loci or their chromatin conformation suggest a better correlation with the latter parameter (4). Consistent with this correlation, Rad7 protein has been reported to physically interact with Sir3 protein (which is involved in the structural conformation of certain transcriptionally repressed regions of DNA) both in vitro and in the yeast two-hybrid system (19). Additionally, a strain deleted of both the *SIR3* and *RAD7* genes is less sensitive to UV

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radiation than a *rad7* mutant alone (19). There is other evidence in support of a requirement for the Rad7 and Rad16 proteins in NER of transcriptionally silent DNA. NER of the nontranscribed (coding) strand of the transcriptionally active *RPB2* gene is abolished in *rad7* and *rad16* mutants (28). Additionally, unexcised pyrimidine dimers in a *rad7* deletion mutant were identified primarily in the nontranscribed strand of transcriptionally active regions of a minichromosome plasmid substrate carrying precisely positioned nucleosomes and in an ~300-bp nontranscribed region of this plasmid (17). However, in these studies there was no evidence that Rad7 protein is directly involved in the displacement of nucleosomes from damaged DNA during NER (17).

The role of the Rad23 protein in NER in yeast is unclear. A published study failed to identify any correlation between the structural organization and/or transcriptional status of damaged DNA and a requirement for the *RAD23* gene during NER (29). However, a different study reported that a strain deleted of the *RAD23* gene lost strand bias for NER of pyrimidine dimers in transcriptionally active plasmid and genomic DNA (18). Additionally, this study reported a partial defect in overall repair of genomic DNA, suggesting a possible explanation for the partial UV radiation sensitivity of *rad23* mutants (18).

The in vitro NER system that we have previously used (32, 34, 35) has been shown to monitor this DNA repair mode exclusively in transcriptionally silent plasmid DNA (35). In the present study, we have demonstrated an absolute requirement for the *RAD7*, *RAD16*, and *RAD23* genes in this system. We also present evidence that the Rad7, Rad16, and Rad4 proteins are components of a multiprotein complex in yeast. Our observations support the notion that Rad4 protein interacts with Rad23 protein and that like Rad4 protein, Rad23 is a component of a multiprotein supercomplex designated the nucleotide excision repairosome (25). In contrast, the Rad7 and Rad16 proteins do not appear to be subunits of this supercomplex.

MATERIALS AND METHODS

Strains. Yeast strain W303-1B (*MAT***a** *ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1*) and the isogenic strains MGSC104 (*rad7*Δ::*LEU2*), MGSC97 (*rad7*Δ::*URA3*), W303236 (*rad16*Δ::*URA3*), and MGSC101 (*rad23*Δ::*URA3*) were kindly provided by Jaap Brouwer, University of Leiden (28). The yeast two-hybrid strain Y190 was a gift from Stephen Elledge, Baylor College of Medicine (5). Yeast strains LN203-2 (*rad3*), SX46A (*RAD4*::*URA3*) (*rad4*), and BJ2168*rad10*-Δ (*rad10*) have been described previously (33). Strain SX46A Δrad14 (*MAT***a** *rad14*Δ::*HIS ade2 his3-532 trpl-289 ura3-52*) was constructed in this laboratory.

In vitro NER. Yeast whole-cell extracts active for NER and cell extracts containing overexpressed Rad2 protein were prepared as previously described (35). Standard NER assays were carried out in yeast whole-cell extracts as described previously (35). NER was monitored by measuring DNA repair synthesis in plasmid pUC18 DNA containing AAF (*N*-acetyl-2-aminofluorene) adducts. AAF-modified DNA was prepared by treating pUC18 with *N*-acetoxy-2-acetylaminofluorene (the activated form of AAF) and purifying the DNA in a 5 to 20% sucrose gradient as described previously (32). Purified plasmid pGEM-3Zf(+) DNA was included in the reactions as an undamaged internal control. After postreaction treatments and separation of the DNA by 1% agarose gel electrophoresis in the presence of ethidium bromide (32), DNA bands were visualized by UV illumination. DNA repair synthesis was detected by autoradiography of dried agarose gels and quantitated by liquid scintillation counting of excised DNA bands as described previously (32).

Analysis of protein-protein interactions involving full-length and deletion constructs by the yeast two-hybrid system. The *RAD4*, *RAD7*, *RAD16*, and *RAD23* genes were cloned into both pAS1-CYH2 and pACTII fusion vectors (5) carrying the yeast Ga14 DNA-binding and Ga14 transcriptional activation domains, respectively. All two-hybrid assay experiments were carried out with strain Y190 (a Cyh^r derivative of Y153 [5]), and β-galactosidase activity was quantitated as described previously (3). Genes were cloned into the two-hybrid vectors as follows.

The *RAD4* gene was amplified by PCR from yeast DNA of strain rad4/pRAD4, which contains a *RAD4* overexpression plasmid with primers 1 and 2 containing *Nco*I and *Bam*HI sites (Table 1). The PCR product was cloned into the vectors in frame with the *GAL4* gene by using *Nco*I and *Bam*HI sites (Table 1, underlined).

The *RAD7* gene and truncated derivatives were amplified by PCR from yeast strain SX46 genomic DNA by using primers which contain *Nco*I and *Bam*HI sites (Table 1, underlined). The full-length *RAD7* gene was cloned into both of the two hybrid vectors, and truncated *RAD7* fragments were cloned into the pACT II vector at *Nco*I and *Bam*HI sites in frame with the *GAL4* gene.

The full-length *RAD16* gene and truncated *RAD16* fragments were amplified by PCR from plasmid pUB33 (a gift of Jaap Brouwer), using primers which contain *Sfi*I and *Bam*HI sites (Table 1, underlined). The full-length *RAD16* gene was cloned into both two-hybrid vectors, and the truncated fragments were cloned into the vector pAS1-CYH2 at *Sfi*I and *Bam*HI sites in frame with the *GAL4* gene.

The full-length *RAD23* gene and truncated *RAD23* fragments were amplified by PCR from yeast SX46a genomic DNA by using primers which contain *Nco*I and *Bam*HI sites (Table 1, underlined). The full-length *RAD23* gene was cloned into both of the two-hybrid vectors, and truncated *RAD23* fragments were cloned into the vector pACT II at *Nco*I and *Bam*HI sites in frame with the *GAL4* gene.

All junctions of vectors and cloned genes were confirmed by DNA sequencing. The expression of full-length Rad4, Rad7, Rad16, and Rad23 fusion proteins was verified by testing the abilities of the respective plasmids to rescue the UV radiation sensitivity of relevant mutants.

Analysis of Rad7-Rad16 interaction in vitro. The *RAD7* gene was cloned into the *Nco*I/*Sal*I site of plasmid pGEX-KG, generating an in-frame *RAD7-GST* (glutathione *S*-transferase) fusion gene. Following transfection into *Escherichia coli* DH5α, induction of the fusion gene was achieved by addition of 100 μM isopropyl β-D-thiogalactopyranoside (IPTG). Rad7-GST fusion protein was purified from crude bacterial extracts by affinity chromatography on glutathione-agarose as described previously (24). The fusion protein was isolated and immobilized on glutathione-agarose beads. In addition, the GST moiety was independently immobilized on glutathione-agarose beads.

A plasmid containing a hemagglutinin (HA) epitope-tagged *RAD16* gene fused to the Gal4 transcriptional activation domain was transformed into a yeast *rad7* deletion mutant. Whole-cell extracts were prepared from a 100-ml overnight culture resuspended in 500 μl of 20 mM HEPES-KOH (pH 7.6)–10 mM $MgSO_4$–10 mM EDTA–10 mM dithiothreitol, 20% glycerol–protease inhibitors. Whole-cell extracts from wild-type (W303-1B) and *rad16* deletion (W303-236) strains without epitope-tagged genes were similarly prepared.

Rad7-GST fusion protein (500 ng) immobilized on glutathione-agarose beads (50 μl) was incubated overnight at 4°C with 100 μg of whole-cell extract (450 μl) prepared from the *rad7* mutant harboring the HA-tagged *RAD16* plasmid. Whole cell extracts were also incubated with glutathione-agarose beads and with GST-glutathione-agarose beads as controls. Additionally, 100 μg of whole cell extracts prepared from wild-type (RAD^+) and *rad16* (containing no HA epitope-tagged genes) strains were incubated with immobilized Rad7-GST fusion protein. Agarose beads were harvested by centrifugation and were extensively washed with 20 mM HEPES-KOH (pH 7.6)–0.1% Triton X–10 mM $MgSO_4$–10 mM EDTA–10 mM dithiothreitol–20% glycerol–protease inhibitors. Following the final wash, 2× sodium dodecyl sulfate (SDS) loading buffer was added to the beads and samples were electrophoresed through SDS–8% polyacrylamide gels. Proteins were transferred to an Immobilon-P membrane (Millipore) and blotted with a mouse monoclonal anti-HA antibody (12CA5 clone; Boehringer) as instructed by the manufacturer.

Measurement of UV radiation survival of yeast strains. Quantitative survival of yeast strains after UV irradiation was measured as described previously (6, 30). Cells were grown to mid-log phase (optical density at 600 nm of ~1.0) in synthetic complete dropout medium with galactose and were irradiated with UV light at 254 nm on YP-Gal plates. Plasmids carrying various *RAD7*, *RAD16*, and *RAD23* truncations were cloned into the centromeric plasmid pSW20 at an *Sma*I site. This plasmid was constructed by exchanging the *URA3* selectable marker for a *TRP* marker in plasmid pMW20 containing the *GAL10-1* promoter (a gift from Mark Walberg, University of Texas Southwestern Medical Center, Dallas).

RESULTS

NER in *rad7*, *rad16*, and *rad23* mutant extracts. NER in yeast cell extracts measures DNA repair synthesis in plasmid pUC18 DNA treated with the DNA-damaging agent AAF (32, 34, 35) and proceeds in the absence of detectable transcription (31). Hence, the system exclusively measures transcription-independent NER. To investigate the role of the Rad7, Rad16, and Rad23 proteins in this mode of NER, the cognate genes were disrupted and repair was examined in vitro. Consistent with previous observations (32), NER in extracts of wild-type cells increased linearly with increasing amounts of protein (Fig. 1A, lanes 1 to 3; Fig. 1B). In contrast, extracts of *rad7* (Fig. 1A, lanes 4 to 6; Fig. 1B), *rad16* (Fig. 1A, lanes 7 to 9; Fig. 1B), and *rad23* (Fig. 2) disruption mutants were defective in NER. The

TABLE 1. PCR primers used for protein interaction studies[a]

Gene	Primer no.	Primer sequence
RAD4	1	5′-CATGCCATGGTGAATGAAGACCTGCCCAAGG-3′
	2	5′-CGGGATCCTCAGTCTGATTCCTCTGACATC-3′
RAD7	3	5′-CATGCCATGGAAATGTATCGCAGTAGAAACCGAC-3′
	4	5′-CGGGATCCTTATATACTGTCACTCTGTCTCCC-3′
RAD16	5	5′-CGTACGTAGGCCATGGAGGCCATGCAAGAAGGGGGCTTTATCCG-3′
	6	5′-CACAACGGGATCCGGGTGATGTCACTTAAAAACTCC-3′
RAD23	7	5′-GATGCCATGGTTAGCTTAACCTTTAAAAATTTCAA-3′
	8	5′-CGGGATCCTCAGTCGGCATGATCGCTGAATAG-3′
RAD7(1-553)	3	
	9	5′-CGCGGATCCTAAGGCCTCATTGTGGCCTTTTC-3′
RAD7(1-445)	3	
	10	5′-GGCGGATCCTTACGACAGTGAATCTGTAG-3′
RAD7(1-301)	3	
	11	5′-CGGGATCCTCGTTGATCAGAAATGGTC-3′
RAD7(94-565)	12	5′-CATGCCATGGTTTCCTACGATGCCAGGATG-3′
	4	
RAD7(222-565)	13	5′-CATGCCATGGCCCTGAATGATCATAC-3′
	4	
RAD7(302-565)	14	5′-CATGCCATGGACACATGGGAGAAGTTCTTTG-3′
RAD16(1-778)	5	
	15	5′-CGGGATCCCTATCTGCTAATGGCAGCTTCATCTTG-3′
RAD16(1-669)	5	
	16	5′-CGCGGATCCTTACCCTGAAGCTTCACTGTTTG-3′
RAD16(175-790)	17	5′-GGTTGGCCATGGAGGCCAAGCAGCCGGATGGTATG-3′
	6	
RAD16(202-790)	18	5′-GGTTGGCCATGGAGGCCATTTATGCGGGCGGTGT-3′
	6	
RAD16(267-790)	19	5′-GGTTGGCCATGGAGGCCAGAACCACGGATATCAAAG-3′
	6	
RAD23(1-377)	7	
	20	5′-CGCGGATCCTACACCTGGATAACAAGATCTC-3′
RAD23(1-280)	7	
	21	5′-CGCGGATCCAACAATGGGGCTAAAGC-3′
RAD23(1-163)	7	
	22	5′-CGGGATCCTCTCTTTGGTAGCCC-3′
RAD23(108-398)	23	5′-CATGCCATGGATGCTTCTGCAGCTCCTG-3′
	8	
RAD23(218-398)	24	5′-CATGCCATGGCTGAAGACGACTTATTTGC-3′
	8	
RAD23(281-398)	25	5′-CATGCCATGGAAAACATAAGTGCTAGATATC-3′
	8	

[a] See text for experimental details.

extent of this defect was comparable to that observed with extracts of cells mutated for several other *RAD* genes, such as *RAD10* (Fig. 1A, lanes 10 to 12; Fig. 1B), *RAD4* (Fig. 1A, lanes 13 to 15; Fig. 1B), *RAD14* (Fig. 3, lane 5), and *RAD3* (Fig. 3, lane 6), all of which are known to be indispensable for NER in vitro (25, 31, 34). These results indicate that the Rad7, Rad16, and Rad23 proteins are required for transcription-independent NER in our in vitro system.

Complementation of defective NER in *rad7*, *rad16*, and *rad23* and mutant extracts. Defective NER in cell extracts can typically be complemented by the addition of the relevant purified protein or cell extract from a different NER-deficient mutant (30, 34). Such complementation affords the opportunity of assessing possible physical relationships between two repair proteins. For example, it was previously demonstrated that *tfb1* and *ssl1* mutant extracts do not complement each other for defective NER in vitro, thereby confirming independent evidence that the Tfb1 and Ssl1 proteins are subunits of the core RNA polymerase II basal transcription complex TFIIH (30). To assess possible interactions involving the Rad7, Rad16, and Rad23 proteins, we attempted to complement deficient NER by mixing different mutant extracts. Consistent with the results shown in Fig. 1 and 2, extracts of *rad7*, *rad16*, and *rad23* mutant cells were defective in NER in vitro (Fig. 3, lanes 1 to 3). We failed to observe complementation of this defect when *rad7* and *rad16* mutant extracts were mixed (Fig. 3, lane 12). In contrast, *rad7* and *rad16* extracts were both complemented by extracts of *rad14* (Fig. 3, lanes 13 and 14) or *rad3* (Fig. 3, lanes 17 and 18) cells, suggesting that the Rad7 and Rad16 proteins are complexed in the cell extracts.

Little if any complementation was observed with mixtures of *rad4* and *rad23* mutant extracts (Fig. 3, lane 9), consistent with previous observations that Rad4 protein purified from yeast extracts is tightly associated with Rad23 protein (11). Extracts of both *rad7* and *rad16* mutants were also only weakly complemented by extracts of *rad4* mutant cells (Fig. 3, lanes 7 and 8, respectively). To confirm that the *rad4* extracts were otherwise competent for NER in vitro, we demonstrated efficient complementation when such extracts were mixed with those from a *rad14* mutant (Fig. 3, lane 16). In contrast to the weak correction of defective NER in *rad7* and *rad16* extracts by addition of *rad4* mutant extracts, the former extracts were efficiently corrected by mixing with extracts of *rad23* cells (Fig. 3, lanes 10 and 11). Hence, the Rad7, Rad16, Rad4, and Rad23

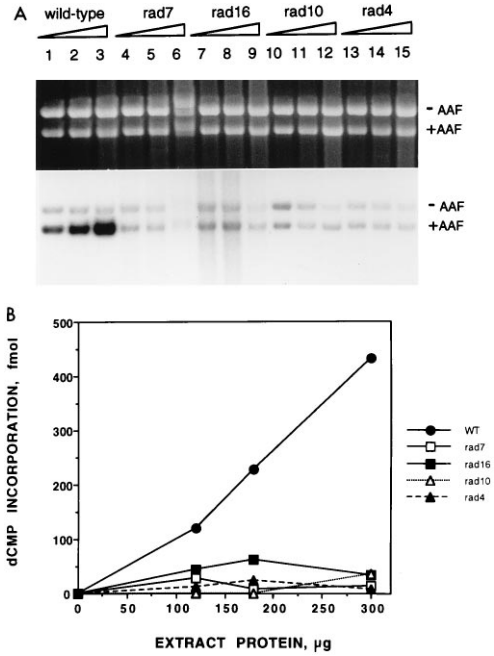

FIG. 1. NER in *rad7* and *rad16* deletion mutant extracts. (A) In vitro NER was performed in various yeast cell extracts as indicated with extract concentrations at 120 μg (lanes 1, 4, 7, 10, and 13), 180 μg (lanes 2, 5, 8, 11, and 14), and 300 μg (lanes 3, 6, 9, 12, and 15). Repair reactions were at 26°C for 2 h, and the repair products were processed as described in Materials and Methods. +AAF, pUC18 DNA containing AAF adducts; −AAF, undamaged pGEM-3Zf(+) DNA. The top panel shows the ethidium bromide-stained gel, and the bottom panel is an autoradiogram showing DNA synthesis. (B) Damage-specific DNA repair synthesis in A was quantitated by scintillation counting of the DNA bands excised from the gel. WT, wild type.

proteins are apparently not in the same protein complex. Conceivably Rad4 protein is physically associated with Rad7 and Rad16 proteins in one complex and independently associated with Rad23 protein in a second complex.

Direct examination of protein interactions. To confirm the suggested interactions between Rad4 and Rad23 proteins and between the Rad4, Rad7, and Rad16 proteins, we directly examined pairwise interactions of these proteins in the yeast two-hybrid in vivo system. We observed interactions between

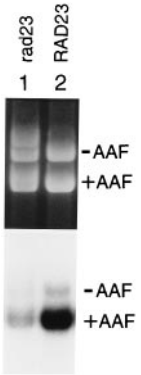

FIG. 2. NER in *rad23* deletion mutant extract. In vitro NER was performed in 300 μg of *rad23* deletion mutant extract (lane 1) or its isogenic wild-type extract (lane 2) at 26°C for 2 h. +AAF, pUC18 DNA containing AAF adducts; −AAF, undamaged pGEM-3Zf(+) DNA. Top, ethidium bromide-stained gel; bottom, autoradiogram.

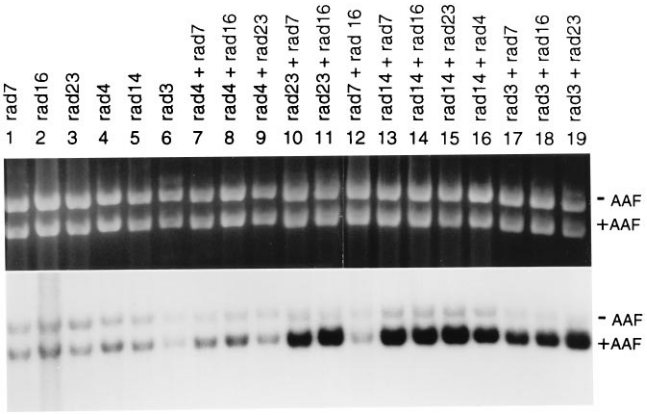

FIG. 3. In vitro complementation for NER among various *rad* mutant extracts. Standard in vitro NER was performed in various *rad* mutant extracts as indicated (lanes 1 to 6). Repair reaction mixtures contained 150 μg of whole-cell extract supplemented with 30 μg of cell extract containing Rad2 protein that was overexpressed in the respective mutant strain. To carry out in vitro complementation between two *rad* mutant extracts (lanes 7 to 19), 150 μg of whole-cell extract from each strain was mixed as indicated in the presence of 50 μg of cell extract containing Rad2 overexpressed in one of the complementing strains. The repair assays were performed as for lanes 1 to 6. +AAF, pUC18 DNA containing AAF adducts; −AAF, undamaged pGEM-3Zf(+) DNA. Top, ethidium bromide-stained gel; bottom, autoradiogram.

the Rad4 and Rad7, Rad4 and Rad23, and Rad7 and Rad16 proteins (Fig. 4 and Table 2) but not between Rad23 and Rad16 or Rad23 and Rad7 in the yeast two-hybrid system (Table 2).

Further confirmation of these interactions was provided by the results of deletion analysis using the two-hybrid system with accompanying complementation of the UV radiation-sensitive phenotype of relevant mutant strains. As shown in Table 3, deletion of the N-terminal 107 or 217 amino acids of Rad23 protein (398 amino acids) did not abolish its ability to interact with Rad4 protein. However, the loss of just the C-terminal 21 amino acids did. Consistent with these results, a plasmid encoding Rad23 protein deleted of the N-terminal 107 amino acids partially corrected the UV radiation sensitivity of a *rad23* deletion mutant (Fig. 5A). Phenotypic correction was not observed with a plasmid carrying a deletion of the C-terminal 118 amino acids of Rad23 protein (Fig. 5A).

In a similar vein, we observed that deletion of the C-terminal 12 or N-terminal 93 amino acids of Rad7 protein (565 amino acids) weakened but did not abolish its interaction with both Rad4 and Rad16 proteins (Table 3). More extensive deletion involving the loss of the C-terminal 120 amino acids of Rad7 protein abolished its interaction with Rad16 but not Rad4 protein (Table 3), and deletion of the N-terminal 221 amino acids of Rad7 abolished its interaction with Rad4 but not Rad16 protein (Table 3). Rad7 polypeptides missing the N-terminal 301 or C-terminal 264 amino acids failed to interact with either Rad4 or Rad16 protein (Table 3). The plasmid encoding Rad7 protein deleted of the N-terminal 93 amino acids (which, as just stated, still interacts with both Rad4 and Rad16 proteins), partially corrected the sensitivity of a *rad7* mutant to UV radiation (Fig. 5B). However, none of the other Rad7 truncations shown in Table 3 had this effect (Fig. 5B).

The effect of truncating the Rad16 protein was also examined. Deletion of the N-terminal 174 amino acids of Rad16 (790 amino acids) did not abolish the ability of the protein to interact with Rad7 protein (Table 3). Furthermore, this truncated protein could partially rescue the UV sensitivity of a

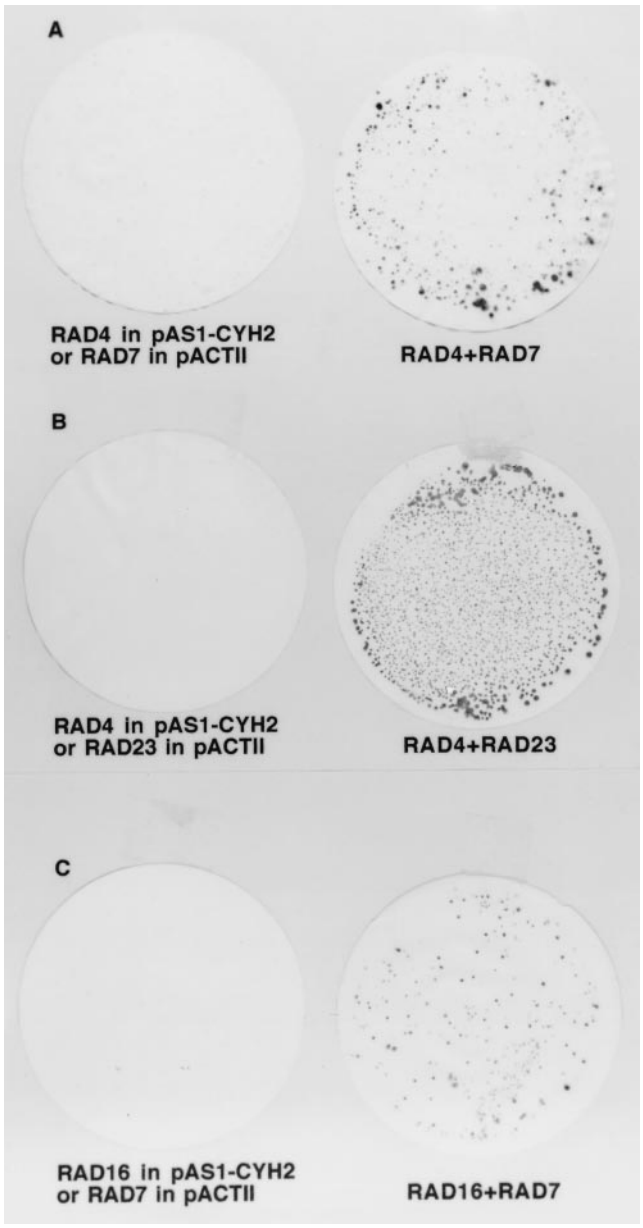

FIG. 4. Protein interactions between Rad4 and Rad7, Rad4 and Rad23, and Rad7 and Rad16 determined by the yeast two-hybrid system. Yeast Y190 cells were transformed with one or two yeast two-hybrid plasmids containing the *RAD4*, *RAD7*, *RAD16*, or *RAD23* gene fused in frame with the Gal4 DNA-binding domain or the Gal4 transcriptional activation domain. Transformants were transferred to nitrocellulose filters. The filters were immersed in liquid nitrogen and then incubated in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) for qualitative β-galactosidase assays. A positive interaction between a pair of fusion proteins resulted in blue colonies on the filter (right panel), whereas control colonies containing only one fusion protein remain white (left panel).

rad16 deletion mutant (Fig. 5C). Several other truncated Rad16 polypeptides constructed, including one missing just the C-terminal 12 amino acids, failed to interact with Rad7 or detectably rescue its UV-sensitive phenotype (Table 3 and Fig. 5C).

Collectively, these results not only confirm interactions between the various proteins studied but additionally define specific regions of some of them that are required for these interactions. The C-terminal half of Rad23 protein is required for its interaction with Rad4 protein. The N-terminal region of Rad7 protein is required for its interaction with Rad4 protein, and the C-terminal region of Rad7 protein is required for its interaction with Rad16 protein. Finally, both the N- and C-

TABLE 2. Detection of Pairwise interactions among Rad4, Rad7, Rad16, and Rad23 fusion proteins, using the yeast two-hybrid system

Gal4 DNA-binding domain fusion	Gal4 activation domain fusion	Mean β-galactosidase activity ± SD (Miller units)	Relative interaction
Vector	Vector	1.1 ± 0.2	1.0
Vector	Rad4	0.7 ± 0.08	0.6
Rad4	Vector	0.3 ± 0.03	0.3
Vector	Rad7	0.4 ± 0.04	0.4
Rad7	Vector	0.8 ± 0.2	0.7
Rad7	Rad4	0.8 ± 0.3	0.7
Rad4	Rad7	24.2 ± 4.9	22.0
Vector	Rad23	0.7 ± 0.1	0.6
Rad23	Vector	0.4 ± 0.08	0.4
Rad4	Rad23	186.6 ± 27.3	170.0
Rad23	Rad4	2.1 ± 0.3	1.9
Vector	Rad16	3.5 ± 0.2	3.2
Rad16	Vector	0.2 ± 0.04	0.2
Rad4	Rad16	3.2 ± 0.3	2.9
Rad16	Rad4	0.4 ± 0.08	0.4
Rad16	Rad7	5.9 ± 0.5	5.4
Rad7	Rad16	9.2 ± 1.5	8.4
Rad23	Rad16	1.3 ± 0.4	1.2
Rad16	Rad23	0.2 ± 0.03	0.2
Rad7	Rad23	0.3 ± 0.06	0.3
Rad23	Rad7	0.2 ± 0.06	0.2

[a] Comparison of various test plasmids against appropriate vector controls. All interactions, including vector controls, were normalized to a basal value of 1.0, determined when only vectors were used.

TABLE 3. Mapping of interaction domains of the Rad7, Rad16, and Rad23 proteins

Protein with deletion	Interacting protein		
	Rad4	Rad7	Rad16
Rad23(1-398) (full length)	+++		
Rad23(108-398)	++		
Rad23(218-398)	+		
Rad23(281-398)	−		
Rad23(1-377)	−		
Rad23(1-280)	−		
Rad23(1-163)	−		
Rad7(1-565) (full length)	+++		++
Rad7(1-553)	+		±
Rad7(1-445)	+		−
Rad7(1-301)	−		−
Rad7(94-565)	+		+
Rad7(222-565)	−		+
Rad7(302-565)	−		−
Rad16(1-790) (full length)		++	
Rad16(1-778)		−	
Rad16(1-669)		−	
Rad16(175-790)		+	
Rad16(202-790)		−	
Rad16(267-790)		−	

[a] Interactions were measured by the β-galactosidase filter assay (see Materials and Methods). The strengths of the interactions were judged by the relative intensity of the color of colonies on filters.

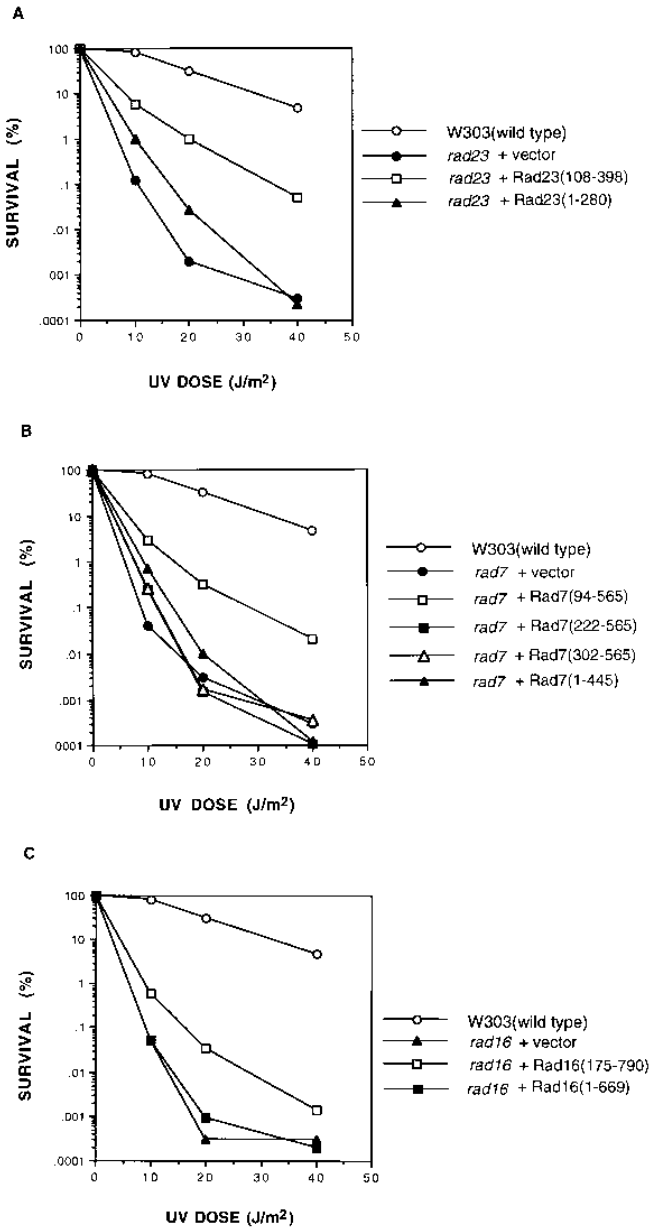

FIG. 5. Rescue of the UV radiation sensitivity of *rad23*, *rad7*, and *rad16* deletion mutants by various plasmid-borne deletion constructs of the *RAD23*, *RAD7*, and *RAD16* genes. (A) A plasmid that expresses Rad23 protein deleted of the N-terminal 107 amino acids (open squares) partially rescues the UV sensitivity of a *rad23* mutant transformed with the vector alone (solid circles), whereas a plasmid that expresses Rad23 protein deleted of the C-terminal 118 amino acids (solid triangles) does not. (B) A plasmid expressing Rad7 protein deleted of the N-terminal 93 amino acids (open squares) partially rescues the UV sensitivity of a *rad7* mutant transformed with the vector alone (solid circles). Plasmids expressing more extensive N-terminal truncations of Rad7 protein (solid squares and open triangles) and a plasmid expressing Rad7 protein deleted of the C-terminal 120 amino acids (solid triangles) do not rescue the UV sensitivity of the *rad7* mutant. (C) A plasmid that expresses Rad16 protein deleted of the N-terminal 174 amino acids (open squares) partially complements the UV sensitivity of a *rad16* mutant transformed with the vector alone (solid triangles). A plasmid that expresses Rad16 protein deleted of the C-terminal 121 amino acids (solid squares) does not.

terminal regions of Rad16 protein are required for its interaction with Rad7 protein.

Expression of β-galactosidase resulting from the interaction between the Rad16 and Rad7 fusion proteins in both of the two-hybrid vector orientations tested was weaker than that observed with the Rad4-Rad7 and Rad4-Rad23 interactions (Table 2). To confirm the interaction between Rad7 and Rad16 proteins in vitro, we expressed the *RAD7* gene in frame with the *GST* gene. Rad7-GST fusion protein was purified by affinity chromatography on glutathione-agarose and was immobilized on glutathione-agarose beads. The beads were incubated overnight at 4°C with a whole-cell extract derived from a *rad7* deletion mutant harboring a plasmid that expresses HA epitope-tagged Rad16 protein fused to the transcriptional activation domain of yeast Ga14 protein. Following incubation, the agarose beads were collected by centrifugation. After extensive washing, samples were run on SDS-polyacrylamide gels and blotted with a monoclonal anti-HA antibody. We detected a single band corresponding to the expected size of the HA-tagged Rad16-Ga14 fusion protein (~106 kDa) (Fig. 6). To confirm the specificity of this interaction, we incubated whole-cell extracts containing HA-tagged Rad16 fusion protein with glutathione-agarose beads alone and with glutathione-agarose beads bound to just the GST moiety. No HA-tagged Rad16 fusion protein was detected in either of these experiments (Fig. 6). We also confirmed the specificity of the anti-HA antibody for the HA epitope with extracts from untagged wild-type and *rad16* mutant strains. When these extracts were incubated with immobilized Rad7-GST fusion protein, the HA antibody did not detectably cross-react with any yeast proteins (Fig. 6).

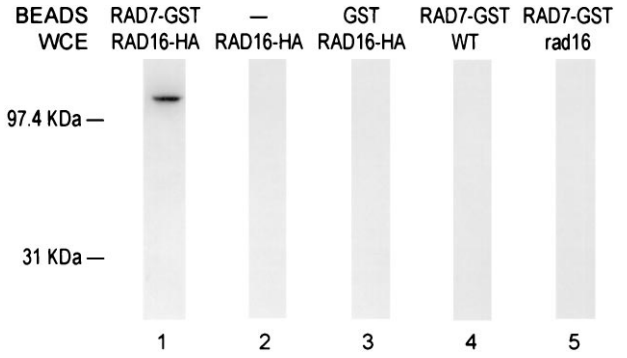

FIG. 6. Interaction between the Rad7 and Rad16 proteins in vitro. Purified Rad7-GST fusion protein immobilized on glutathione-agarose beads binds HA epitope-tagged Rad16 protein fused to the yeast Ga14 transcriptional activation domain (lane 1) (see text for details). Epitope-tagged Rad16-Ga14 protein does not bind to agarose beads complexed to just glutathione (lane 2) or glutathione with GST (lane 3). Similarly, no Rad16 protein signal was detected with the anti-HA antibody after incubation of the Rad7-GST fusion protein immobilized on glutathione-agarose beads with whole-cell extracts (WCE) of untagged wild-type (WT) (lane 4) or *rad16* mutant (lane 5) cells.

Relationship between the NER repairosome and Rad7, Rad16, and Rad23 proteins. A large multiprotein complex designated the nucleotide excision repairosome has been purified from yeast extracts (25). This supercomplex comprises at least the core TFIIH subcomplex in which seven polypeptides have been identified (6), the Rad1-Rad10 endonuclease complex, and the Rad2, Rad4, and Rad14 proteins (25). To determine if the repairosome also contains the Rad7, Rad16, and Rad23 proteins, we attempted to correct defective NER in vitro by using previously characterized repairosome preparations (25). As shown in Fig. 7A, we observed significant correction of defective repair synthesis in extracts of a *rad23* mutant, suggesting that Rad23 protein is present in the repairosome complex. The level of phenotypic complementation was not as complete as that observed when the repairosome fractions were added to extracts of other *rad* mutants, such as *rad3* or *rad10* mutants (data not shown). Furthermore, ~3-

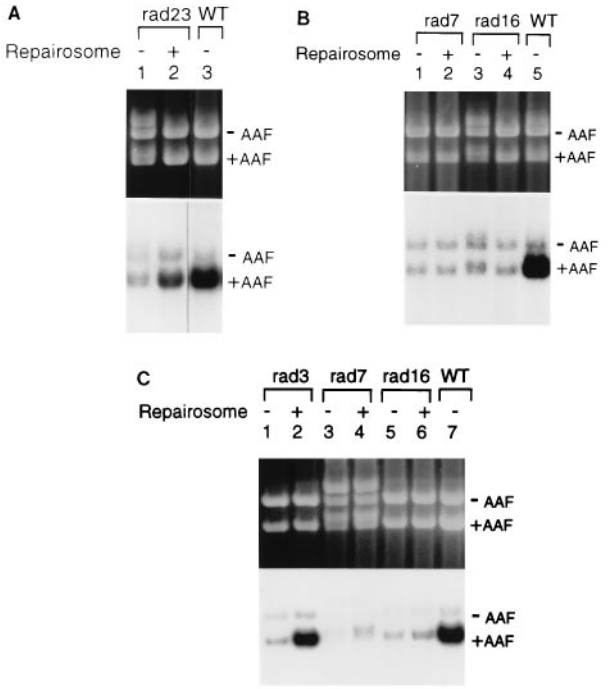

FIG. 7. Complementation of defective NER in *rad7*, *rad16*, and *rad23* mutant extracts by purified repairosome preparations. (A) In vitro NER was performed with 300 μg of *rad23* mutant or isogenic wild-type (WT) extracts with (+) or without (−) repairosome (6 μg). The repairosome was fraction 29 following Sepharose CL-2B chromatography as described previously (25). (B) In vitro complementation with the repairosome was similarly carried out in 300 μg of *rad7* and *rad16* mutant extracts. (C) In vitro NER in *rad3*, *rad7*, and *rad16* mutant extracts (300 μg) was performed in the presence (+) or absence (−) of 8 μg of repairosome (fraction 24 from the Ni-agarose column as described previously [25]). +AAF, pUC18 DNA containing AAF adducts; −AAF, undamaged pGEM-3Zf(+) DNA. Top, ethidium bromide-stained gel; bottom, autoradiogram.

fold-higher levels of the repairosome fraction were required to observe complementation of *rad23* extracts than of *rad3* or *rad10* extracts (data not shown). These results suggest that Rad23 protein may have been inactivated or lost in some repairosome complexes during their storage. However, we cannot formally eliminate the possibility that the repairosome fraction comprises more than one complex, only one of which contains Rad23 protein.

In contrast to the substantial complementation observed by addition of the repairosome fraction to extracts of *rad23* mutants, we failed to observe any correction of defective repair synthesis in *rad7* or *rad16* mutant extracts (Fig. 7B). To investigate the possibility that Rad7 and Rad16 proteins are normally present in the repairosome but were lost during purification, we also tested a less pure fraction of the repairosome (Ni-agarose fraction [25]). Once again no phenotypic correction of defective NER was observed in the *rad7* and *rad16* extracts, though this repairosome fraction was fully active in complementing defective NER in a *rad3* mutant extract (Fig. 7C). These results suggest that the repairosome(s) purified according to the procedure described by Svejstrup et al. (25) does not include the Rad7 and Rad16 proteins.

DISCUSSION

NER in the yeast *S. cerevisiae* is a complex biochemical process that requires a large number of gene products (reviewed in reference 9). Previous studies have demonstrated the copurification by several distinct fractionation procedures of multiple proteins which are indispensable for early events in NER, prompting the notion of a preformed multiprotein complex (repairosome) in yeast cells (25). This repairosome includes at least the core basal transcription factor TFIIH, the Rad1-Rad10 endonuclease complex, and the Rad2, Rad4, and Rad14 proteins. Mutations in genes that encode known subunits of the complex eliminate all detectable NER in vivo (8, 9) and confer extreme levels of sensitivity to DNA-damaging agents (8, 9). Hence, it seems reasonable to conclude that the repairosome is required for NER in both transcriptionally silent and transcriptionally active regions of the genome.

The specific biochemical requirements for NER in transcriptionally active regions of the genome are not known, and to date there are no reports of a cell-free system that uniquely monitors transcriptionally dependent NER. In contrast, both nuclear and whole-cell extracts can be prepared from yeast which support transcriptionally independent NER (31, 32, 34, 35). Evidence for this conclusion stems from (i) the use of bacterial plasmid substrates that are devoid of yeast RNA polymerase II promoters, (ii) the failure to detect any evidence of RNA transcripts by either the incorporation of radiolabeled RNA precursors into acid-insoluble products or by electrophoretic analyses following incubations under standard NER conditions, and (iii) the absence of ribonucleotide precursors in the reaction mixtures due to extensive dialysis of the extracts. Previous in vivo studies have established a specific requirement for the yeast *RAD7* and *RAD16* genes for NER of transcriptionally silent DNA (2, 28, 36). In light of the evidence summarized above that our experimental conditions monitor NER exclusively in transcriptionally silent DNA, we sought to exploit this system for investigating the role of the *RAD7* and *RAD16* genes in NER in vitro. Our results confirm a requirement for functional *RAD7* and *RAD16* genes for NER in transcriptionally silent DNA in vitro. The additional observation that the products of these genes interact with one another in vivo and in vitro, and that Rad7 protein also interacts with Rad4 protein, suggests that the products of the *RAD4*, *RAD7*, and *RAD16* genes operate in a protein complex comprising at least these three proteins. This putative complex is apparently distinct from the repairosome since the latter complex can correct defective NER in various other *rad* mutant extracts but failed to complement defective NER in extracts of either *rad7* or *rad16* mutants. However, it remains to be firmly established that the Rad7 and Rad16 proteins are not preferentially lost and/or inactivated during purification of the repairosome.

Our observation that Rad4 protein also interacts with Rad23 protein correlates with studies indicating that XPC protein, the human structural homolog of yeast Rad4 protein (reviewed in reference 9), interacts with human HHRAD23B protein (14), one of two known human homologs of yeast Rad23 protein. While direct evidence for a preformed nucleotide excision repairosome in human cells is lacking, it is interesting that our finding that Rad23 protein is apparently present in the yeast repairosome is supported by the observation that fractionation of HeLa whole-cell extracts by Sephacryl S500 chromatography identified both XPC protein and HHRAD23B protein in fractions greater than 669 kDa in size (26). Additionally, both of these proteins cofractionated with human TFIIH during chromatography of crude extracts on heparin-Ultragel columns (26).

The apparent physical association of Rad4 protein with Rad7 and Rad16 proteins is at first glance contradictory to the findings that mutants which are completely deleted of the *RAD4* gene are distinctly more sensitive to UV radiation than are *rad7* or *rad16* deletion mutants and that Rad4 protein is a

component of the repairosome. We are therefore led to the suggestion that Rad4 protein may play two roles in NER in yeast. One of these relates to a specific requirement for a catalytically active NER complex (repairosome). The other may be linked to the requirement for the Rad7 and Rad16 proteins during NER of transcriptionally silent DNA. The latter role of Rad4 protein in yeast correlates with the observation that mutations in the human *XPC* gene result in defective NER in transcriptionally silent DNA (27). Consistent with this model, we have recently identified a mutant form of Rad4 protein that interacts with Rad23 protein but not with Rad7 protein in the two-hybrid system (37a).

A question of considerable interest is whether a requirement for the *RAD7* and *RAD16* gene products uniquely in the repair of transcriptionally silent DNA specifically reflects the transcriptional quiescence of the substrate DNA or a requirement for these gene products to process a chromosomal conformation that precludes access of the repair machinery to sites of base damage in DNA. Despite the fact that the input substrate for NER consists of purified plasmid DNA devoid of preformed nucleosomes, this DNA may be organized into higher-ordered chromatin-like structures during incubation with our cell extracts. Our working hypothesis that Rad7 and Rad16 operate in a complex that is specifically required for the processing of chromosomal structures is consistent with the observation that neither of these proteins is apparently required for NER in a reconstituted in vitro system (11) and with the observation that Rad7 protein interacts with Sir3 protein, which has been implicated in the packaging of certain regions of DNA into transcriptionally repressed chromatin (19).

We have no explanation for the observation that in contrast to the highly UV radiation-sensitive phenotype conferred by deletion of all other nonessential genes which encode subunits of the repairosome, deletion of the *RAD23* gene (which in our hands results in defective NER in vitro) confers only a partial sensitivity to UV light. In this regard, it might be relevant that the human genome contains two genes which are structurally homologous with *RAD23*, designated *HHR23A* and *HHR23B* (14). Conceivably yeast contains a second, as yet unidentified functional (if not structural) homolog of the *RAD23* gene.

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The first two authors contributed equally to this study.

ADDENDUM IN PROOF

Immunoblotting studies with antibodies to purified Rad23 protein have identified this protein as a component of the nucleotide excision repairosome (K. Rodriguez, S. H. Reed, A. E. Tomkinson, and E. C. Friedberg, unpublished observations).

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