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Specific complex formation between proteins encoded by the yeast DNA repair and recombination genes *RAD1* and *RAD10*

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ABSTRACT **The *RAD1* and *RAD10* genes of *Saccharomyces cerevisiae* are required for excision repair of ultraviolet light-damaged DNA, and they also function in a mitotic recombination pathway that is distinct from the double-strand-break recombination pathway controlled by *RAD52*. Here, we show that the RAD1 and RAD10 proteins are complexed with each other *in vivo*. Immunoprecipitation of yeast cell extracts with either anti-RAD1 antibody or anti-RAD10 antibody coprecipitated quantitative amounts of both RAD1 and RAD10 proteins. The level of coprecipitable RAD1 and RAD10 increased when both proteins were overproduced together, but not if only one of the proteins was overproduced. The RAD1/RAD10 complex is highly stable, being refractory to 1 M NaCl and to low concentrations of SDS. By hydroxylamine mutagenesis, we have identified a *rad1* mutant allele whose encoded protein fails to complex with RAD10. The interaction-defective *rad1* mutant resembles the *rad1* or *rad10* null mutant in defective DNA repair and recombination, implying that complex formation is essential for the expression of biological activities controlled by *RAD1* and *RAD10*.**

In humans, the genetic disorder xeroderma pigmentosum (XP) causes extreme sensitivity to sunlight and XP individuals suffer from a high incidence of skin cancers. XP cells exhibit defects in the incision step of excision repair of DNA damaged by ultraviolet (UV) light. Cell fusion studies with skin fibroblasts from XP patients have identified seven XP complementation groups, A–G (1, 2). Eight complementation groups have been identified among UV-sensitive Chinese hamster ovary (CHO) cell lines, and mutants from five of these groups are incision-defective (3). Cross-complementation of UV sensitivity of mutant CHO cell lines by human DNA has led to the isolation of the *ERCC1*, *ERCC2*, and *ERCC3* genes (4–6), and *ERCC3* and *XP-B* represent the same gene (6). Recently, the *XPAC* cDNA was cloned by using as hybridization probe a mouse gene that corrects the excision repair defect of *XP-A* cell lines (7).

The yeast *Saccharomyces cerevisiae* resembles humans in the genetic complexity of excision repair, and at least 10 genes—*RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, *RAD14*, *RAD7*, *RAD16*, *RAD23*, and *MMS19*—are involved in this process. The first 6 of these genes are indispensable for the incision step of repair and they very likely encode proteins that collectively mediate the recognition and endonucleolytic scission of damaged DNA (8, 9). Characterization of genomic deletion mutations has revealed a role of some of these genes in cellular processes unrelated to excision repair. Specifically, *RAD3* is also essential for cell viability (10, 11), and *RAD1* and *RAD10* function in a mitotic recombination pathway that is distinct from the *RAD52* double-strand-break recombination pathway (12).

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The proteins encoded by the human *ERCC1*, *ERCC2*, and *XPAC* genes share remarkable homology with the products of the yeast *RAD10*, *RAD3*, and *RAD14* genes, respectively (4, 5, 9), and a yeast gene homologous to the human *ERCC3* (*XPBC*) gene has also been identified (6). The conservation of the excision repair machinery from yeast to human indicates that information obtained from studies in yeast would be of considerable use in understanding the molecular mechanism of excision repair in humans and in defining the function of *XP* genes.

The requirement in eukaryotes for a large number of genes in excision repair implies a degree of complexity which rivals that of DNA replication, transcription, and cell cycle control, where a hierarchy of protein–protein interactions plays a pivotal role in the modulation of latent biological activities and in the temporal control of key execution points (13–15). Therefore, we have examined whether excision repair proteins associate with each other in a physical complex *in vivo*. The availability of antibodies against four of the proteins, RAD1, RAD2, RAD3, and RAD10, has now allowed us to investigate their possible interaction. In this paper, we demonstrate the existence of a specific and highly stable complex of RAD1 and RAD10 proteins. The biological significance of this physical association is underscored by the failure of a nonfunctional rad1 mutant protein to complex with RAD10. We discuss how these findings could be used in devising strategies for cloning the gene that encodes the human counterpart of *RAD1*.

MATERIALS AND METHODS

Yeast Strains. Table 1 lists the strains used in this study.

Plasmids for Expression of RAD Proteins in Yeast. pCS41, which is derived from the low-copy yeast vector YCplac33 (16), contains the *RAD1* gene on a 7-kilobase (kb) *Bam*HI fragment (17). pCS108 contains the *rad1* mutant allele obtained by hydroxylamine mutagenesis of pCS41. This *rad1* allele resembles the *rad1* null mutation in defects in DNA repair and mitotic recombination. pRR22, a derivative of the 2μ multicopy yeast plasmid YEp24 (18), contains the *RAD1* gene on an 8.5-kb *Sau*3A1–*Bam*HI fragment. pRR162, pSCW367, and pSUC8 are derivatives of the 2μ *TRP1* plasmid pSCW231 (19) in which the *S. cerevisiae ADC1* promoter controls the expression of the *RAD2*, *RAD3*, and *RAD10* genes, respectively. Plasmids pRR248, pKM8, pSP119, and pPS7 were used for overproducing RAD1, RAD2, RAD3, and RAD10, respectively, in *Escherichia coli* for use as immunogens for antibody production in rabbits.

Determination of Mitotic Recombination Rates. Plasmid pRS6 and the method for the determination of recombination rates have been described (12, 20).

Abbreviation: XP, xeroderma pigmentosum.

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Table 1. Yeast strains

Strain	Genotype
DBY747	*MATa ura3-52 leu2-3,112 trp1-289 his3-Δ1*
DBY747 (*rad1Δ*)	*MATa ura3-52 leu2-3,112 trp1-289 his3-Δ1 rad1Δ::URA3*
DBY747 (*rad10Δ*)	*MATa ura3-52 leu2-3,112 trp1-289 his3-Δ1 rad10Δ::LEU2*
PRY43 (*rad1Δ*)	*MATa ura3-52 leu2-3,112 trp1-289 his3-Δ1 rad1Δ::LEU2* (from DBY747)
RSY6	*MATa ura3-52 leu2-3,112 trp5-27 arg4-3 ade2-40 ilv1-92 HIS3*::pRS6
RSY6 (*rad1Δ*)	*MATa ura3-52 leu2-3,112 trp5-27 arg4-3 ade2-40 ilv1-92 rad1Δ::URA3 HIS3*::pRS6
RSY6 (*rad52Δ*)	*MATa ura3-52 leu2-3,112 trp5-27 arg4-3 ade2-40 ilv1-92 rad52Δ::TRP1 HIS3*::pRS6
RS69-8B (*rad1Δ rad52Δ*)	*MATa arg4 trp5-27 ilv1-92 ura3-52 leu2-3,112 rad1Δ::URA3 rad52Δ::TRP1 HIS3*::pRS6

HIS3::pRS6 indicates the integration of plasmid pRS6 at the chromosomal *HIS3* locus, generating the *his3 Δ3' his3 Δ5'* duplication.

Buffers. Buffer A was 50 mM Tris·HCl (pH 7.5) containing 10% sucrose, 0.6 M KCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, and the following protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstatin A at 20 μg/ml, and phenylmethanesulfonyl fluoride and benzamidine hydrochloride at 0.5 mM. Buffer B was 50 mM Tris·HCl (pH 7.5) containing 50 mM NaCl, 0.2% Triton X-100, and the same set of protease inhibitors as in buffer A. Phosphate-buffered saline (PBS) was 10 mM NaH_2PO_4 (pH 7.2) containing 150 mM NaCl.

Preparation of Immunobeads. Ten OD_{280} units of affinity-purified antibodies or nonimmune rabbit IgG (Sigma) was bound to 1 ml of protein A-Affi-Gel (Bio-Rad) in PBS, then covalently linked in 0.2 M sodium borate, pH 9.05/20 mM dimethyl pimelimidate (crosslinking agent; Sigma) as described (21). Immunobeads were stored at 4°C in 2 volumes of PBS.

Immunoprecipitation. Stationary-phase yeast cultures in YPD medium or synthetic omission media were diluted with YPD to 5×10^6 cells per ml and incubated at 30°C. At titers of $1–2 \times 10^8$ cells per ml, cells were harvested by centrifugation, resuspended in buffer B (3 ml/g of cells), and ruptured by vigorous agitation with 0.5-mm glass beads (1.5 g/g of cells) using eight 30-sec pulses with intermittent chilling on ice. The cell lysate was clarified by centrifugation, and 2 ml of the supernatant was mixed gently on a rocking platform with 15 μl of immunobeads for 2 hr at 4°C. The immunobeads were spun down and washed three times with 300 μl of buffer B. Bound proteins were eluted from immunobeads at room temperature with 300 μl of 1% SDS in 50 mM Tris·HCl (pH 6.8), precipitated overnight with 3 volumes of acetone at −20°C, and dissolved in 50 μl of electrophoresis sample buffer (22), and an aliquot (usually 10 μl) was subjected to SDS/PAGE. The nitrocellulose blots of polyacrylamide gels were probed with 1:1000 dilution of the appropriate anti-RAD antibodies (OD_{280} = 3) and developed by the indirect peroxidase procedure (23).

RESULTS

Coimmunoprecipitation of RAD1 and RAD10. Cell extract from the *RAD*$^+$ yeast strain DBY747 was mixed with immunobeads specific for the RAD1, RAD2, RAD3, or RAD10 proteins and also with beads carrying rabbit IgG. After washing the immunoprecipitates, bound yeast proteins were eluted by treatment with 1% SDS and, after separation by SDS/PAGE, analyzed by immunoblotting with anti-RAD antibodies. Immunobeads specific for RAD1 precipitated a 130-kDa polypeptide that reacted with anti-RAD1 antibodies in immunoblot analysis (Fig. 1*A*, lane 2), and those specific for RAD2, RAD3, and RAD10 precipitated immunoreactive polypeptides of 130 kDa (Fig. 1*B*, lane 3), 89 kDa (Fig. 1*C*, lane 4), and 25 kDa (Fig. 1*D*, lane 5), respectively. The proteins detected (Fig. 1) were encoded by the given *RAD* gene because (*i*) none was precipitated by beads bearing rabbit IgG (Fig. 1, lanes 1) or showed crossreactivity with antibodies against the other RAD proteins in immunoblot analysis (data not shown), (*ii*) the observed molecular weight of the polypeptides was in close agreement with the value deduced from the DNA sequence (8), and (*iii*) the amount of immunoreactive material varied according to the *RAD* gene dosage in all cases (see below, and data not shown).

Interestingly, whereas anti-RAD2 and anti-RAD3 immunobeads precipitated only their cognate antigen (Fig. 1 *B* and *C*), an apparently quantitative amount of RAD1 was coprecipitated by anti-RAD10 immunobeads (Fig. 1*A*, lane 5) and of RAD10 by anti-RAD1 immunobeads (Fig. 1*D*, lane 2). Thus, it appears that the majority of cellular RAD1 and RAD10 molecules are specifically associated in a complex precipitable with antibodies against either protein.

Physical Complex of RAD1 and RAD10. To establish that coprecipitation of RAD1 and RAD10 was due to complex formation between the proteins rather than to fortuitous immunological crossreactivity, we repeated the immunoprecipitation experiments with extract from the *rad1Δ* and *rad10Δ* mutant strains isogenic to the *RAD*$^+$ strain DBY747 used in prior experiments. Although the *rad1Δ* or *rad10Δ* mutation had no significant effect on the cellular level of RAD10 or RAD1 protein, respectively, no RAD10 protein was precipitated with anti-RAD1 immunobeads in the extract from the *rad1Δ* strain (Fig. 2*A Lower*, lane 2), and conversely, no RAD1 protein was precipitated with anti-RAD10 immunobeads from the *rad10Δ* extract (Fig. 2*B Upper*, lane

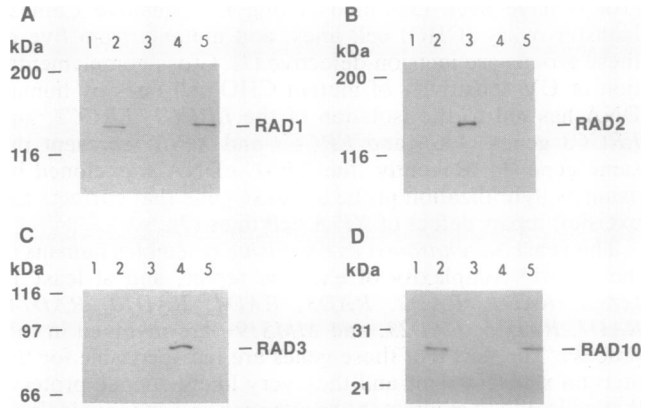

FIG. 1. Immunoprecipitation of RAD proteins. Extract from yeast strain DBY747 (*RAD*$^+$) was incubated with protein A-Affi-Gel beads carrying nonimmune rabbit IgG or affinity-purified IgG specific for a RAD protein. Bound yeast proteins were eluted from immunobeads by treatment with 1% SDS and analyzed by immunoblotting. In each panel, samples in lanes 1–5 originated from immunoprecipitation with control beads containing nonimmune rabbit IgG and with immunobeads specific for RAD1, RAD2, RAD3, and RAD10, respectively. Nitrocellulose blots were probed with anti-RAD1 (*A*), anti-RAD2 (*B*), anti-RAD3 (*C*), and anti-RAD10 (*D*) antibodies. No other protein bands except the ones shown were detected in the immunoblots.

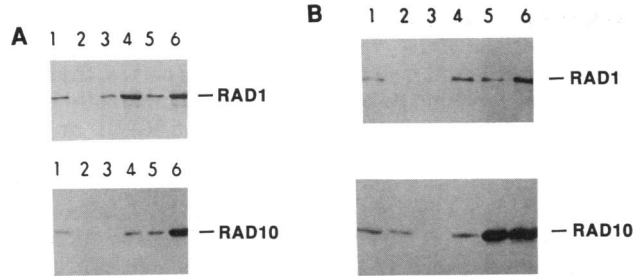

FIG. 2. RAD1 and RAD10 are physically associated *in vivo*. Extracts from DBY747 (*RAD*$^+$), its isogenic *rad1*Δ and *rad10*Δ derivatives, DBY747 harboring pRR22 (2μ, *RAD1*), DBY747 harboring pSUC8 (2μ, *ADC1–RAD10*), and DBY747 harboring both pRR22 and pSUC8 were subjected to immunoprecipitation. The SDS eluates from anti-RAD1 (*A*) and anti-RAD10 (*B*) immunobeads were analyzed by immunoblotting with anti-RAD1 (*Upper*) and anti-RAD10 (*Lower*) antibodies. In each panel, lanes 1–6 contain, respectively, samples from *RAD*$^+$, *rad1*Δ, *rad10*Δ, *RAD*$^+$(pRR22) overproducing RAD1, *RAD*$^+$(pSUC8) overproducing RAD10, and *RAD*$^+$(pRR22, pSUC8) overproducing both RAD1 and RAD10.

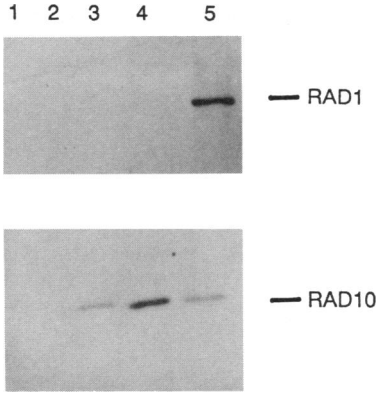

FIG. 3. Strength of RAD1/RAD10 association. The RAD1/RAD10 complex from 5 ml of DBY747 extract was immobilized on 50 μl of anti-RAD1 immunobeads, washed with buffer B, and then eluted sequentially with 10 volumes of 0.5 M NaCl (lane 1), 1 M NaCl (lane 2), 0.05% SDS (lane 3), 0.1% SDS (lane 4), and 1% SDS (lane 5). The content of RAD1 (*Upper*) and RAD10 (*Lower*) in the various eluates was examined by immunoblotting.

3). These results argue strongly that RAD1 and RAD10 form a physical complex.

We then investigated how the level of immunoprecipitable RAD1/RAD10 complex might be influenced by increased expression of either one or both proteins. For this purpose, the multicopy plasmids pRR22 (2μ, *RAD1*) and pSUC8 (2μ, *ADC1–RAD10*) were introduced singly or in combination into the *RAD*$^+$ strain DBY747, and the overproduction of RAD1 and RAD10 proteins in the plasmid-bearing strains was confirmed by immunoprecipitation (Fig. 2). Evidently, overproduction of RAD10 or RAD1 alone has no observable effect on the cellular level of the other protein (Fig. 2*A Upper*, lane 5; Fig. 2*B Lower*, lane 4), nor does it alter appreciably the amount of the other protein coprecipitating (Fig. 2*A*, lanes 4; Fig. 2*B*, lanes 5). Taken together, our results demonstrate that complex formation between RAD1 and RAD10 is highly efficient and that neither of the two proteins is apparently limiting in wild-type cells with regard to this process. Consistent with these observations, when both RAD1 and RAD10 proteins were overproduced in the same cell, the amount of coprecipitating RAD1 and RAD10 proteins increased (Fig. 2, lanes 6). Even when we overproduced the RAD2 and RAD3 proteins, we did not observe any evidence for their involvement in complex formation with each other or with the RAD1 and RAD10 proteins (data not shown).

To test whether RAD1 interacts with RAD10 in the absence of other proteins, we employed a modified immunoblotting technique (13). Briefly, proteins in extract from RAD1-overproducing cells were resolved by SDS/PAGE and transferred onto nitrocellulose. After a renaturation step, the blot was incubated with purified RAD10 (24) and then probed with antibodies. In this analysis, nitrocellulose-bound RAD1 protein formed a complex with RAD10 as detected by reactivity of the RAD1 band to anti-RAD10 antibodies (data not shown).

RAD1/RAD10 Complex Is Highly Stable. To test the strength of RAD1/RAD10 interaction, the complex of RAD1 and RAD10 from wild-type cells was immobilized on anti-RAD1 immunobeads and subjected to successive elution steps with increasing concentration of NaCl (0.5 M and 1 M) followed by the protein denaturant SDS (0.05%, 0.1%, and 1%), and eluates were examined by immunoblot analysis (Fig. 3). Since RAD1 was bound to the immunobeads directly by the anti-RAD1 antibodies, its dissociation from the immunocomplex was evident only at the highest concentration of SDS (1%) used (Fig. 3 *Upper*, lane 5). The experimental design was therefore such that examination of the RAD10 content in the various eluates would yield information regarding the affinity of the RAD1/RAD10 complex. As much as 1 M NaCl did not effect the dissociation of RAD10 from the immunocomplex, and, as determined by densitometry, 25%, 58%, and 17% of RAD10 molecules were eluted by 0.05%, 0.1% and 1% SDS, respectively (Fig. 3 *Lower*, lanes 3–5). Thus, the complex between RAD1 and RAD10 is very stable, being refractory to 1 M NaCl and partially stable to low concentrations of SDS. We verified this conclusion by using anti-RAD10 immunobeads to isolate the RAD1/RAD10 complex (data not shown).

A rad1 Mutant Protein Defective in Interaction with RAD10 Lacks Biological Activity. To address the biological significance of RAD1/RAD10 complex formation, we screened for a *rad1* mutation that impairs this interaction. The *RAD1* gene in the low-copy plasmid pCS41 was mutagenized by treatment with hydroxylamine, and five *rad1* mutant plasmids defective in both excision repair and mitotic recombination were identified. Extract from the *rad1*Δ strain PRY43 harboring these mutant plasmids was subjected to immunoprecipitation with anti-RAD1 immunobeads. No rad1 protein was detected in extracts from *rad1*Δ cells carrying four of these plasmids (data not shown), whereas pCS108 encoded a rad1 protein identical in size and in cellular level to RAD1 (see below). Our genetic and biochemical studies with the mutant *rad1* allele in pCS108 are described below.

The pCS108-borne *rad1* mutation conferred a dramatic increase in UV sensitivity, similar to that seen in the *rad1*Δ strain (Fig. 4). To examine the effect of this *rad1* mutation on mitotic recombination, we introduced the plasmids pCS41 (*RAD1*) and pCS108 (*rad1*), and also the vector YCplac33, into the *rad1*Δ and *rad1*Δ *rad52*Δ strains carrying the chromosomal *his3* Δ*3′ his3* Δ*5′* duplication and determined the rate of formation of *HIS3*$^+$ recombinants (12, 20). The *rad1*Δ or *rad52*Δ mutation lowered the rate of formation of *HIS3*$^+$ recombinants about 8-fold, and this rate declined a further ≈30-fold in the *rad1*Δ *rad52*Δ double mutant (Table 2). The rate of formation of *HIS3*$^+$ recombinants in the *rad1*Δ and *rad1*Δ *rad52*Δ mutant strains was not affected by the introduction of plasmid pCS108. Thus, the *rad1* gene in pCS108 is almost identical to the *rad1*Δ mutation in defective DNA repair and recombination.

To determine whether the rad1 mutant protein interacts with RAD10, we treated extract of *rad1*Δ cells harboring pCS41 or pCS108 with anti-RAD1 immunobeads and examined coprecipitation of RAD10. The cellular levels of RAD1 and the mutant rad1 proteins are apparently the same, and the

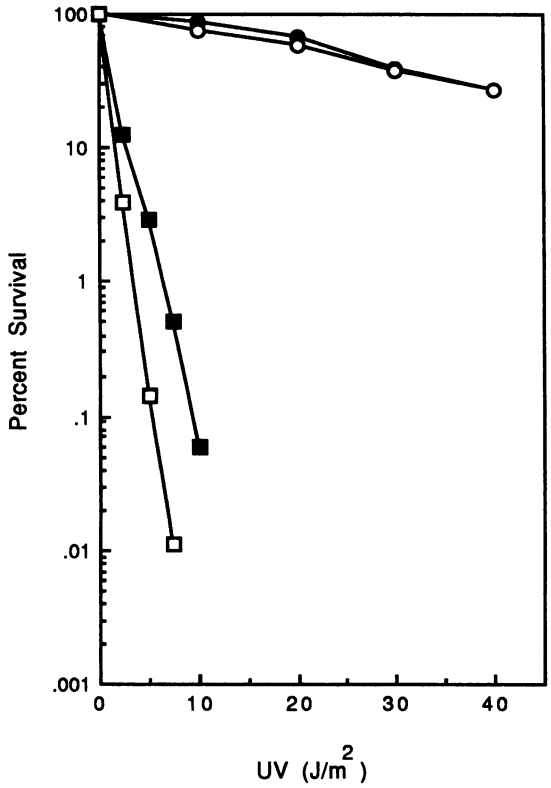

FIG. 4. Survival after UV irradiation of *RAD*$^+$ strain and *radl*Δ strain carrying the *RAD1* or *radl* mutant plasmid. Cells were grown in synthetic complete medium or in synthetic complete medium lacking uracil for maintenance of plasmids. ●, RSY6 (*RAD*$^+$); ○, RSY6 (*radl*Δ) carrying the *RAD1* plasmid pCS41; ■, RSY6 (*radl*Δ) carrying the *radl* mutant gene in plasmid pCS108; □, RSY6 (*radl*Δ) carrying the vector YCplac33.

two proteins have identical electrophoretic mobilities (Fig. 5 *Upper*, lanes 1 and 2). In contrast to the coprecipitation of RAD10 with anti-RAD1 immunobeads in extract containing RAD1 protein (lane 1), we did not observe any coprecipitation of RAD10 with the mutant rad1 protein (lane 2). Conversely, immunoprecipitation of RAD10 coprecipitated the RAD1 protein (lane 3) but not the mutant rad1 protein (lane 4). These observations indicate that the protein encoded by the *radl* mutant gene in pCS108 is defective in interaction with RAD10.

DISCUSSION

In this study, we have examined by immunoprecipitation four yeast excision repair proteins—RAD1, RAD2, RAD3, and RAD10—for physical association. Our results provide strong evidence that the majority of RAD1 and RAD10 proteins exist in a complex *in vivo*. The evidence for association between the RAD1 and RAD10 proteins comes from the repeated observation of coprecipitation of apparently quantitative amounts of both proteins by anti-RAD1 or anti-RAD10 antibodies. A common epitope on the two proteins recognizable by either antibody preparation cannot account for coprecipitation, since no RAD1 protein was precipitated from extracts of the *radl0*Δ strain by anti-RAD10 antibodies, and conversely, anti-RAD1 antibodies did not precipitate RAD10 from extracts of the *radl*Δ strain. The complex between RAD1 and RAD10 proteins is very stable; it withstands 1 M NaCl and is partially resistant to low concentrations of SDS.

Our studies with the *radl* mutant protein defective in complex formation with RAD10 provide important clues regarding the functional significance of this interaction. The finding that the interaction-defective *radl* mutant resembles the *radl*Δ or the *radl0*Δ mutant in DNA repair and mitotic recombination defects implies that complex formation between RAD1 and RAD10 is necessary for the expression of biochemical activities indispensable for DNA repair and recombination. RAD10 protein has been purified to homogeneity in our laboratory; it shows single-stranded-DNA-binding activity and renatures complementary DNA strands (24). We have previously speculated that during excision repair, binding of single-stranded DNA by RAD10 could be utilized subsequent to the recognition and unwinding of the damage site by components of the incision complex (24). The RAD1/RAD10 complex might act at such a later step in excision repair. In genetic recombination, the RAD1/RAD10 complex, with or without other protein components, may catalyze the strand exchange reaction (24). Alternatively, the RAD1/RAD10 complex may have a function common for both repair and recombination. For instance, the complex may possess a latent nuclease activity, whose activation requires interaction with other excision repair proteins at the damage site, that mediates the specific incision of damaged DNA. In combination with other proteins, such an activity might also effect the initiation of recombination. Rigorous evaluation of these and other possibilities will be feasible with the purification of the RAD1 protein, and for this purpose, our findings suggest the possibility of using RAD10-Sepharose as an affinity step for purifying RAD1 from a yeast strain overproducing RAD1.

With the recent cloning and characterization of human excision repair genes, it is becoming abundantly clear that the excision repair machinery in eukaryotes is evolutionarily conserved. The protein encoded by human *ERCC1* shows homology to yeast RAD10, but the gene encoding the human counterpart of yeast *RAD1* has not yet been cloned. In view of the conservation of the excision repair machinery, it is highly probable that ERCC1 is also tightly associated with the

Table 2. Effect of the *radl* mutation in plasmid pCS108 on mitotic recombination of the *his3* Δ3′ *his3* Δ5′ duplication

Strain	Genotype	Plasmid	No. of *His3*$^+$ recombinants per 10^4 viable cells			Recombination rate × 10^4 (mean ± SD)	% *RAD*$^+$ recombination	% *rad52*Δ recombination
			Culture 1	Culture 2	Culture 3			
RSY6	*RAD*$^+$	None	5.29	2.83	5.04	4.39 ± 2.12		
RSY6	*radl*Δ	pCS41	3.50	4.45	4.93	4.29 ± 1.12	100	
		pCS108	0.75	0.55	0.63	0.64 ± 0.15	14.9	
		YCplac33	0.56	0.61	0.41	0.52 ± 0.17	12.1	
RSY6	*rad52*Δ	None	0.5	0.38	0.5	0.46 ± 0.12		
RS69-8B	*radl*Δ *rad52*Δ	pCS41	0.289	0.429	0.369	0.362 ± 0.010		100
		pCS108	0.029	0.035	0.024	0.029 ± 0.009		8.0
		YCplac33	0.012	0.012	0.025	0.016 ± 0.001		4.4

The *RAD*$^+$, *radl*Δ, and *rad52*Δ strains are isogenic. Plasmids pCS41 and pCS108 carry the *RAD1* and mutant *radl* genes, respectively. YCplac33 is the vector.

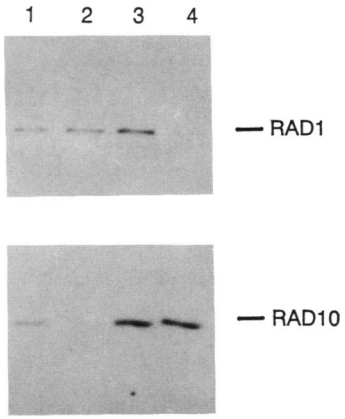

FIG. 5. Failure of a rad1 mutant protein to interact with RAD10, shown by immunoblot analysis of SDS eluate of immunoprecipitates from extracts of *rad1Δ* strain harboring pCS41 (*RAD1*) plasmid (lanes 1 and 3) and of *rad1Δ* strain harboring pCS108 (*rad1*) plasmid (lanes 2 and 4). In lanes 1 and 2, immunoprecipitation was carried out with anti-RAD1 immunobeads, and in lanes 3 and 4, with anti-RAD10 immunobeads. Nitrocellulose blots were probed with anti-RAD1 (*Upper*) and anti-RAD10 (*Lower*) antibodies.

human RAD1 homolog, and this might permit cloning of this gene. Using extracts from wild-type and excision-defective CHO and human XP cell lines metabolically labeled with [^{35}S]methionine, one could identify the RAD1 homolog as a protein coprecipitating with anti-ERCC1 antibody and determine whether this protein was altered or absent in any of the mutants. Should the identification of the RAD1 homolog be successful, amino acid sequence information could then be generated from the protein for cloning the gene. Alternatively, the two-hybrid system developed in yeast to identify genes encoding interacting proteins could make direct cloning of the human gene feasible (25).

The absence of any evidence of complex formation of RAD2 and RAD3 with each other or with RAD1 and RAD10 suggests that RAD2 and RAD3 might be assembled at the damage site in a stepwise manner. RAD3 is a single-stranded-DNA-dependent ATPase and it possesses DNA and DNA·RNA helicase activities (19, 26, 27). RAD3 could function in the early stage of damage recognition, and subsequent ordered assembly of RAD2 and other proteins could be a means of increasing the specificity of the complex for the damage site. Such a mechanism of stepwise assembly of protein components has been proposed to increase the specificity of *E. coli* UvrABC proteins for incision at damage sites in DNA (28).

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