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Stable and Specific Association between the Yeast Recombination and DNA Repair Proteins RAD1 and RAD10 In Vitro

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The *RAD1* and *RAD10* genes of *Saccharomyces cerevisiae* are two of at least seven genes which are known to be required for damage-specific recognition and/or damage-specific incision of DNA during nucleotide excision repair. *RAD1* and *RAD10* are also involved in a specialized mitotic recombination pathway. We have previously reported the purification of the RAD10 protein to homogeneity (L. Bardwell, H. Burtscher, W. A. Weiss, C. M. Nicolet, and E. C. Friedberg, Biochemistry 29:3119–3126, 1990). In the present studies we show that the RAD1 protein, produced by in vitro transcription and translation of the cloned gene, specifically coimmunoprecipitates with the RAD10 protein translated in vitro or purified from yeast. Conversely, in vitro-translated RAD10 protein specifically coimmunoprecipitates with the RAD1 protein. The sites of this stable and specific interaction have been mapped to the C-terminal regions of both polypeptides. This portion of RAD10 protein is evolutionarily conserved. These results are the first biochemical evidence of a specific association between any eukaryotic proteins genetically identified as belonging to a recombination or DNA repair pathway and suggest that the RAD1 and RAD10 proteins act at the same or consecutive biochemical steps in both nucleotide excision repair and mitotic recombination.

In the budding yeast *Saccharomyces cerevisiae*, the recombination of genetic material is accomplished by multiple, partially overlapping pathways (36). The repair of DNA damage is likewise effected by multiple pathways, some of which intersect with certain recombination pathways (17, 21). Relatively little is known about the complex interrelationships among the members of these pathways, particularly at the biochemical level.

Nucleotide excision repair is a versatile DNA repair pathway which recognizes and removes chemically distinct nucleotide alterations caused by many physical and chemical agents which interact with DNA (16). In the yeast *S. cerevisiae* multiple genes have been identified for this process, at least six of which (*RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, and *RAD14*) are believed to be absolutely required for early steps associated with the recognition and specific incision of damaged DNA (19). The involvement of at least one other gene in these events is suggested by the recent identification of the yeast homolog of the *ERCC3* gene, which is required for nucleotide excision repair in human cells (55). The genetic and, by implication, biochemical complexity of nucleotide excision repair in yeast is also a feature of mammalian cells. Chinese hamster ovary cell mutants defective in nucleotide excision repair fall into 10 distinct genetic complementation groups (43, 49, 50). Similarly, the human cancer-prone hereditary disease xeroderma pigmentosum (XP), which is characterized by defective nucleotide excision repair, includes at least seven genetic complementation groups (12).

In addition to the homology noted above between the human *ERCC3* and yeast *ERCC3* genes, the translated sequences of several yeast genes which were cloned by functional complementation of yeast mutants defective in nucleotide excision repair are homologous with those of human genes cloned by functional complementation of mammalian cell mutants. In particular, the yeast *RAD10*, *RAD3*, and *RAD14* genes share extensive amino acid sequence identity with the human *ERCC1*, *ERCC2*, and *XPAC* genes, respectively (6, 51, 54). The *ERCC2* gene complements the mutant phenotype of cultured cells from XP group D patients (15), and the *ERCC3* gene corrects the mutant phenotype of cells from XP group B (55). Both these forms of XP can be associated with another repair-defective hereditary human disease, Cockayne's syndrome (56). Hence, information about the biochemistry of nucleotide excision repair gleaned from the study of yeast is likely to be generally applicable to other eukaryotes, including humans, and may be relevant to understanding the molecular pathology of XP and Cockayne's syndrome (24, 30, 49).

Little is known about the biochemistry of nucleotide excision repair in eukaryotes. In *S. cerevisiae* only the RAD3 (23, 47) and RAD10 proteins (7, 46) have been extensively purified. The RAD3 protein is a DNA-dependent ATPase with associated processive DNA-DNA helicase (23, 45, 47) and DNA-RNA helicase activities (5, 33). The translocation of the RAD3 protein on DNA is arrested at sites of DNA damage, suggesting a role for this helicase in DNA damage recognition (32). The RAD10 protein binds to both single- and double-stranded DNA at pH 7.5 (7). At pH 6, RAD10 exhibits a preference for single-stranded DNA and can promote the aggregation of complementary single strands (46).

Yeast strains carrying *rad1* (27, 39, 48, 58) or *rad10* (40) loss-of-function alleles exhibit a reduction in certain mitotic recombination events. Mutants defective in the *RAD2*, *RAD4*, or *RAD14* gene do not show this reduction (6, 39, 40, 58). Indeed, they manifest an increased frequency of mitotic recombination, consistent with the channeling of spontaneous DNA damage from the nucleotide excision repair pathway into a recombinational repair pathway (39, 40). Thus, the decreased recombination seen in *rad1* and *rad10* mutants

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is not due to the inactivation of the nucleotide excision repair pathway per se but reflects the direct involvement of the products of these two genes in distinct DNA metabolic pathways.

Mutant *rad1* strains are deficient in recombination between inverted (2) or direct (27, 39, 48, 58) repeats on the same chromosome. Some RAD1-dependent recombination events are repressed by a potential topoisomerase, the product of the *HPR1* gene (1, 3). RAD1-dependent events are stimulated by transcription from either RNA polymerase I (53, 58) or RNA polymerase II (48) promoters. In most of the above systems, the *RAD1* pathway appears to compete for recombinogenic intermediates with the *RAD52* pathway, which is required for most types of meiotic and mitotic recombination. Thus, *rad1 rad52* double mutants exhibit a synergistic decrease in these recombination events (27, 39, 48, 58). *RAD1* is also involved in modulating the pattern of certain interchromosomal recombination events (4).

The involvement of a specialized *RAD1*-dependent pathway for recombination events between repeats may reflect the involvement of regions of homology relatively limited compared with that between homologous chromosomes. In addition, the opposing effects of transcription and topoisomerase function on the *RAD1* pathway may reflect unique topological constraints on certain *RAD1*-mediated recombination events.

The role of the *RAD10* gene in recombination has not been as thoroughly studied. However, in the one system in which both *rad1* and *rad10* mutants were examined either singly or in combination, their effects were indistinguishable and epistatic (40).

An epistatic genetic interaction (e.g., between *RAD1* and *RAD10* in recombination and between *RAD1* or *RAD10* and the other genes required for nucleotide excision repair) formally indicates either that the proteins act at separate steps in a linear pathway or that they act at the same or consecutive steps as part of multiprotein complex. This distinction has important implications as to mechanism, possible intermediates, and interpretation of the complex pathologies of XP (18, 20, 36).

In this study we show that RAD1 and RAD10 proteins form a stable complex in vitro in the absence of DNA. We have also identified regions in both the RAD1 and RAD10 polypeptides which contain binding domains required for this interaction. Although multiple genes have been genetically shown to participate in recombination and/or repair in eukaryotes (21, 24, 36, 49), these results provide the first biochemical evidence for a stable heteromeric complex between the products of such genes.

MATERIALS AND METHODS

Materials. Plasmids pGEM3Zf(+) and pGEM4Z, as well as the SP6, T3, and T7 RNA polymerases; RQ1 DNase and RNasin RNase inhibitor; and the T7 and SP6 promoter primers, were from Promega Biotechnology. Rabbit reticulocyte lysates were obtained from Novagen. In vitro translation-grade [^{35}S]methionine, Amplify fluorographic reagent, and prestained molecular weight markers were from Amersham. Ready Protein scintillation cocktail was from Beckman. Caffeine, anti-rabbit immunoglobulin G-alkaline phosphatase, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indolyl phosphate were from Sigma Chemicals. The m^7(G)ppp(G) cap analog was from Pharmacia/LKB Biotechnology. Type HA nitrocellulose filters were from Millipore. c-*fos* (Ab-2) antibody, c-*jun*-AP-1 (Ab-1) antibody, and protein A-agarose were from Oncogene Science. Restriction enzymes were from Bethesda Research Laboratories and New England Biolabs.

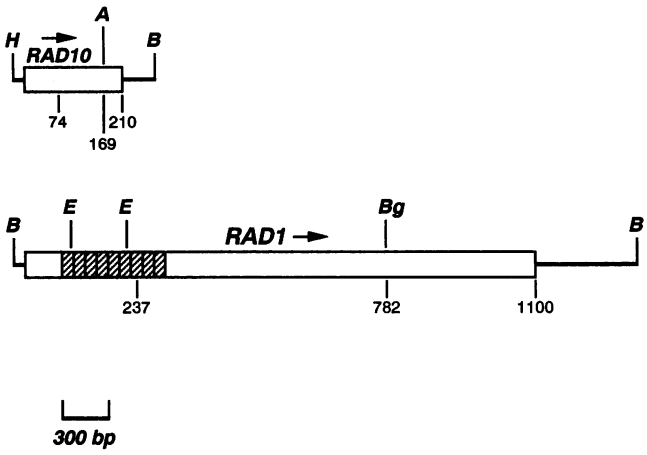

FIG. 1. *RAD10* and *RAD1* DNA fragments used for plasmid constructions. Restriction site abbreviations: *H*, *Hin*dIII; *B*, *Bam*HI; *E*, *Eco*RI; *Bg*, *Bgl*II; *A*, *Acc*I. Boxed regions represent the protein-coding sequences. Hatched boxes within the *RAD1* coding region represent the series of nine leucine-rich motifs (41). Particular amino acids are numbered along the bottom of each gene.

Plasmid constructions. Plasmid constructions employed standard techniques (8, 37). The *RAD1* and *RAD10* inserts used in cloning experiments are shown in Fig. 1. The *RAD1* *Bam*HI fragment contains the *RAD1* gene deleted of its promoter region, so that 19 bp of DNA immediately 5′ to the translational start codon are juxtaposed to 30 bp of DNA located 391 bases upstream (57). This fragment was cloned in the SP6 orientation into pGEM3Zf(+) to generate plasmid pGEM3Zf(+)-SP6*RAD1*. To generate plasmid pGEM4Z-T7*RAD10*, the *RAD10* gene on a *Hin*dIII-*Bam*HI fragment (7) was cloned into the vector pGEM4Z.

Plasmid constructs for the production of N-terminal deletion polypeptides were as follows. For RAD1$_{237-1100}$ (see "Mapping of the RAD1-RAD10 interaction domains"), the *RAD1* *Bam*HI fragment (see above) was digested with *Eco*RI (which cuts internally; Fig. 1) and cloned into pGEM4Z cut with *Bam*HI and *Eco*RI. The first methionine codon in the construct is *RAD1* codon 237. For RAD1$_{782-1100}$, plasmid pGEM3Zf(+)-SP6*RAD1* was linearized with *Xba*I and used as the template in a polymerase chain reaction with the T7 promoter primer and the primer 5′ CGGAATTCACCATG GAAGATCTTTCTCACTACA. This primer is complementary to *RAD1* codons 782 to 787 and substituted an initiation codon for codon 781. Polymerase chain reaction conditions were as described elsewhere (38). The amplified fragment was digested with *Eco*RI and *Bam*HI and cloned into plasmid pGEM4Z digested with *Bam*HI and *Eco*RI. For RAD10$_{74-210}$, plasmid pGEM4Z-T7*RAD10* was linearized with *Hin*dIII and used as the template in a polymerase chain reaction with the SP6 promoter primer and the primer 5′ CGGAATTCACCATGGCTACAGATGACTATAA. This primer is complementary to *RAD10* codons 74 to 79 and substituted an initiation codon for codon 73. The amplified fragment was digested with *Eco*RI and *Bam*HI and cloned into plasmid pGEM4Z digested with *Bam*HI and *Eco*RI.

Purification of RAD10 protein. The RAD10 protein was purified to homogeneity from an overexpressing yeast strain

by using three chromatographic steps as previously described (7).

Antiserum production. RAD10 antibodies were produced and affinity purified as previously described (7). Polyclonal antiserum to full-length RAD1 protein expressed in *Escherichia coli* was produced and affinity purified by a similar procedure (12a).

In vitro transcription templates. Plasmids pBSSK-T3*jun* and pGEM3-T7c-*fos* (28) were used for the generation of in vitro-translated Jun and Fos proteins. The plasmids were linearized with *Cla*I and *Hin*dIII, respectively. For the production of full-length RAD1 protein, plasmid pGEM3Zf (+)-SP6*RAD1* was linearized with *Sma*I. For the production of the $RAD1_{1-784}$ deletion protein, plasmid pGEM3Zf(+)-SP6*RAD1* was linearized internally with *Bgl*II (Fig. 1). For the production of the $RAD10_{1-169}$ deletion polypeptide, plasmid pGEM4Z-T7*RAD10* was linearized internally with *Acc*I (Fig. 1). All other transcription templates were linearized with *Bam*HI. Templates were then purified by phenol-chloroform extraction and ethanol precipitation.

In vitro transcription and translation. Synthesis of capped RNA from plasmid DNA templates (see above) using bacteriophage RNA polymerase was as described elsewhere (29). Rabbit reticulocyte lysate translation reactions were prepared as described by the manufacturer with 0.8 mM Mg^{2+}, 80 mM K^+, 10 μg of RNA per ml, and 0.1 to 0.5 mCi of [^{35}S]methionine per ml (final concentrations). Caffeine was added to a final concentration of 5 mM to enhance the translational yield (14, 42). All experiments were reproduced in the absence of caffeine. Reactions were at 30°C for 2 h. Translation yields were quantitated by trichloroacetic acid precipitation as described previously (31) by using nitrocellulose filters. These results were converted into the absolute protein yield by taking into account the endogenous concentration of methionine in the reticulocyte lysate and the number of methionines per protein molecule.

Ammonium sulfate precipitation. Immediately following the 2-h translation incubation an equal volume of 4 M saturated ammonium sulfate in TE buffer (10 mM Tris, pH 7.5; 1 mM EDTA) was added to the translation reaction mixtures at room temperature. Samples were incubated in ice water for 20 min and then microcentrifuged for 20 min at 14,000 × *g* at 4°C. The supernatant was removed, and the pellets were washed with ice-cold 2 M ammonium sulfate–2 mM dithiothreitol in TE buffer. The tubes were briefly recentrifuged, the last traces of ammonium sulfate were removed, and the pellets were resuspended in immunoprecipitation buffer.

Buffers. Buffer A is as described elsewhere (7). Buffer B contains 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.2; 75 mM KCl, 4 mM $MgCl_2$, 5 mM sodium bisulfite, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% (vol/vol) Tween 20, 12.5% glycerol, 0.5 μg of leupeptin per ml, 0.7 μg of pepstatin per ml, and 0.1 mM phenylmethylsulfonyl fluoride. Buffer C is buffer B containing 15% ethylene glycol.

Immunoprecipitation. Immunoprecipitation reactions were performed in buffer B (for reactions using RAD1 antiserum and for appropriate controls) or buffer C (for reactions using RAD10 antiserum and for appropriate controls). Proteins were mixed in 200 μl of buffer and incubated for 30 min at 30°C. The reaction mixtures were then microcentrifuged for 10 min at 14,000 × *g*, and the supernatant was transferred to a tube containing 15 μl of protein A-agarose and 1 μg of the relevant antibody. The tubes were rocked for 1 h at room temperature and then spun in a microcentrifuge for 30 s at 2,500 rpm. Supernatants were removed, and the pellets were washed twice with 1 ml of ice-cold buffer B or C. The pellets were then resuspended in scintillation cocktail for quantitative analysis or in 30 μl of sample buffer (26) for gel analysis. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were treated twice for 15 min with 50% methanol–12% acetic acid, soaked for 30 min in fluorography reagent, dried under vacuum, and exposed to X-ray film for 4 to 8 h.

Other methods. SDS-polyacrylamide gel electrophoresis (PAGE) of proteins was as described previously (22). Western blotting (immunoblotting) was carried out as previously described (34). After reaction with primary antibody, Western blots were incubated with anti-rabbit immunoglobulin G conjugated to alkaline phosphatase and developed with the color substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

In vitro translation of RAD1 and RAD10. The involvement of RAD1 and RAD10 in two distinct DNA metabolic pathways suggests a close biochemical relationship between these two proteins. To explore potential protein-protein interactions between them, radiolabelled RAD1 and RAD10 proteins were synthesized by in vitro transcription and translation. The cloned *RAD1* and *RAD10* genes (Fig. 1) were tailored into plasmids containing bacteriophage RNA polymerase promoters. Capped transcripts were synthesized in vitro, purified, and used to program translation supported by rabbit reticulocyte lysates in the presence of ^{35}S-methionine. This resulted in the synthesis of radiopure protein representing ~1/20,000 (by weight) of the total protein in the translation reactions (44). We observed that the use of a simple ammonium sulfate fractionation step (see Materials and Methods) resulted in considerable purification of in vitro-translated proteins. Specifically, >90% of in vitro-translated RAD1, RAD10, Jun, or Fos protein precipitated under conditions of 50% ammonium sulfate saturation, whereas <4% of the total lysate protein precipitated under these conditions (data not shown). This represents a >20-fold purification of these in vitro-translated proteins. This procedure also facilitated the complete and rapid exchange of the lysate buffer for buffers required in subsequent experiments.

Figure 2 shows the specificity of the RAD1 and RAD10 antibodies for in vitro-translated proteins as determined by Western blot analysis of translation lysates. Affinity-purified antibodies raised against RAD1 protein expressed in *E. coli* (12a) reacted only with rabbit reticulocyte lysates programmed with *RAD1* RNA (see Fig. 4, lane 2) and not with lysates programmed with *RAD10* RNA (see Fig. 4, lane 3), or lysates which were not programmed with RNA (see Fig. 4, lane 1). Similarly, antibodies to the RAD10 protein reacted only with lysates programmed with *RAD10* RNA (see Fig. 4, lane 4) and not with lysates programmed with *RAD1* RNA (see Fig. 4, lane 5) or lysates not programmed with RNA (see Fig. 4, lane 6).

In vitro-translated RAD10 protein was previously shown to have an electrophoretic mobility indistinguishable from that of the protein purified to homogeneity following overexpression of the cloned *RAD10* gene in yeast cells (7). The RAD1 protein translated in vitro (Fig. 2) or expressed in *E. coli* or yeast cells (not shown) migrates in SDS-PAGE gels with a relative electrophoretic mobility of ~150 kDa. This is considerably larger than the value of ~126 kDa calculated

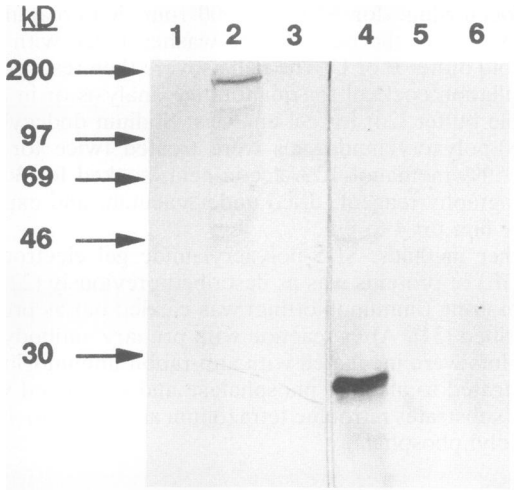

FIG. 2. Specificity of RAD1 and RAD10 antisera; in vitro-translated proteins from rabbit reticulocyte lysates programmed with no RNA (lanes 1 and 6), *RAD1* mRNA (lanes 2 and 5), or *RAD10* mRNA (lanes 3 and 4). The Western blot was reacted with RAD1 antibodies (lanes 1 to 3) or RAD10 antibodies (lanes 4 to 6).

from the size of the *RAD1* open reading frame. The anomalous electrophoretic mobility of the RAD1 protein may be a consequence of its high content of acidic amino acids, as has been observed with several other acidic proteins (9, 25, 35). Electrophoretic analysis of radiolabelled in vitro-translated RAD1 protein revealed the presence of several polypeptides of lower molecular weight (Fig. 3, lane 3). These presumably result from premature translation termination, a result frequently observed when large transcripts are translated in vitro (11, 44).

Coimmunoprecipitation of RAD1 and RAD10. The technique of coimmunoprecipitation has been successfully used by others to study protein-protein interactions with in vitro-translated proteins (28). We investigated whether the RAD1 protein would specifically coprecipitate with the RAD10 protein in the presence of RAD10 antiserum and protein A-agarose carrier beads. A typical result of such an experiment is shown in Fig. 3. In the absence of the RAD10 protein minimal amounts of in vitro-translated RAD1 protein were nonspecifically precipitated by the antibody-bead complex (lane 4). However, in the presence of in vitro-translated RAD10 protein, the RAD1 protein was efficiently coprecipitated (lane 5).

The specificity of this interaction was demonstrated in several ways. Firstly, it should be noted that the interaction occurred in the presence of a 1,000-fold excess (by weight) of unlabelled lysate proteins. Such a vast excess of irrelevant protein would be expected to block any "sticky" sites for nonspecific protein-protein interactions on RAD1 and RAD10 proteins. In fact, we have observed coprecipitation of RAD1 and RAD10 proteins in lysates which were not fractionated by ammonium sulfate precipitation, indicating that the binding reaction in vitro can take place in the presence of a 20,000-fold excess of lysate proteins (data not shown). Secondly, the coprecipitation was strictly dependent on the presence of RAD10 (or RAD1; see below) antiserum (Fig. 3, lane 9). Finally, when selected functionally unrelated proteins were translated in vitro and precipitated with the appropriate antibodies, nonspecific associations with the RAD1 or RAD10 protein were not observed. Thus, for example, in vitro-translated Fos protein did not coprecipitate with the RAD10 protein (Fig. 3, lane 7), nor did the RAD1 protein coprecipitate with the Fos protein (lane 10). Similarly, in vitro-translated Jun protein did not coprecipitate with the RAD10 protein (data not shown), nor did the RAD1 protein coprecipitate with the Jun protein (data not shown).

The specificity of the interaction between the RAD1 and RAD10 proteins was further confirmed by using antisera to the RAD1 protein. The RAD10 protein coprecipitated with the RAD1 protein in the presence of RAD1 antiserum (Fig. 4, compare lanes 4 and 5), whereas the Fos (lane 7) and Jun (not shown) proteins did not coprecipitate with RAD1 under

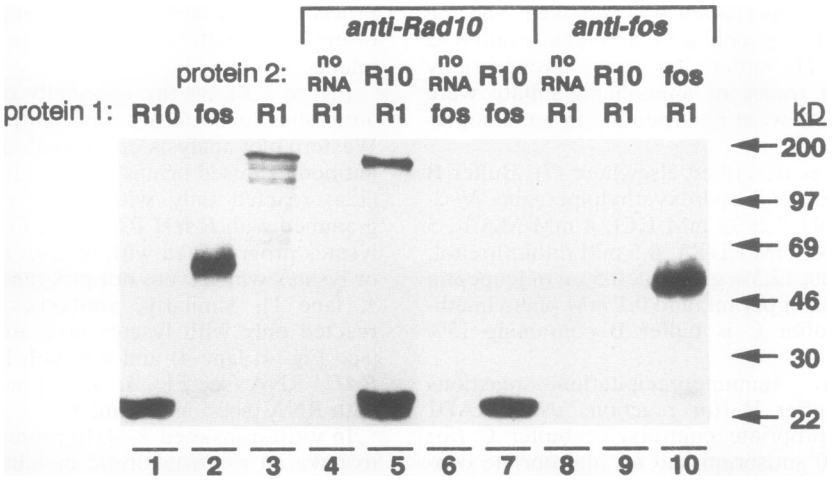

FIG. 3. Coimmunoprecipitation of in vitro-translated RAD1 protein. RAD10, Fos, and RAD1 proteins were translated in vitro, ammonium sulfate precipitated, and loaded directly onto an SDS–10% PAGE gel (lanes 1 to 3) or immunoprecipitated with anti-RAD10 (lanes 4 to 7) or anti-Fos (lanes 8 to 10) antibodies, as described in Materials and Methods, prior to gel analysis. Immunoprecipitation reaction mixtures contained 33 ng of RAD1 (lanes 4, 5, 8, 9, and 10), 26 ng of RAD10 (lanes 5, 7, and 9), and/or 33 ng of Fos (lanes 6, 7, and 10). Reticulocyte lysate not programmed with exogenous RNA was added to lanes 4, 6, and 8 so that all immunoprecipitation reaction mixtures (lanes 4 to 10) contained 30 μg of total protein. The positions of molecular weight markers are indicated on the right.

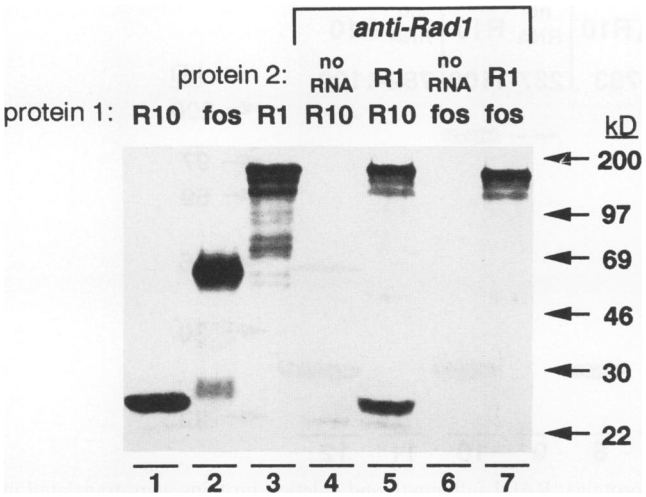

FIG. 4. Coimmunoprecipitation of in vitro-translated RAD10 protein. RAD10, Fos, and RAD1 proteins were translated in vitro, ammonium sulfate precipitated, and loaded directly onto an SDS–10% PAGE gel (lanes 1 to 3) or immunoprecipitated with anti-RAD1 antibodies (lanes 4 to 7), as described in Materials and Methods, prior to gel analysis. Immunoprecipitation reaction mixtures contained 33 ng of RAD1 (lanes 5 and 7), 26 ng of RAD10 (lanes 4 and 5), and/or 33 ng of Fos (lanes 6 and 7). Reticulocyte lysate not programmed with exogenous RNA was added to lanes 4 and 6 so that all immunoprecipitation reactions (lanes 4 to 7) contained 30 μg of total protein. The positions of molecular weight markers are indicated on the right.

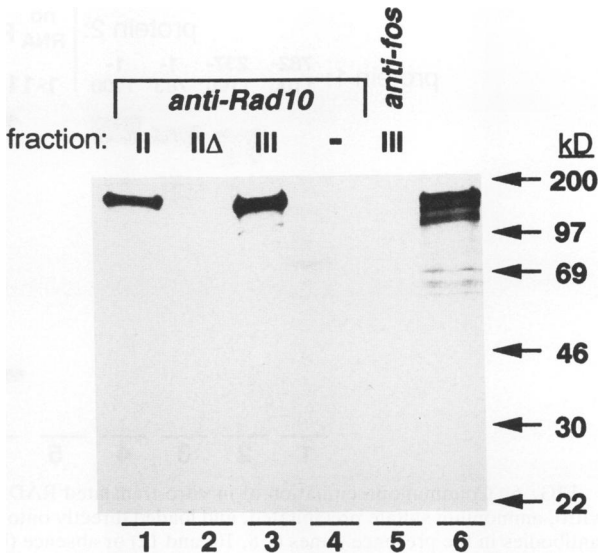

FIG. 5. Coimmunoprecipitation of in vitro-translated RAD1 protein with purified RAD10 protein. RAD1 protein was translated in vitro, ammonium sulfate precipitated, and loaded directly onto an SDS–10% PAGE gel (lane 6) or immunoprecipitated with anti-RAD10 (lanes 1 to 4) or anti-Fos (lane 5) antibodies in the presence of purified RAD10 protein fractions prior to gel analysis. Immunoprecipitation reaction mixtures contained 33 ng of RAD1 in 20 μg of total lysate protein and one of the following: 0.5 μl of RAD10 fraction II, purified from a yeast strain carrying the overexpression vector pG12*RAD10*, in buffer A (~30 ng of RAD10 protein) (lane 1); 0.5 μl of fraction II from a *RAD10* deletion strain carrying the vector pG12 (lane 2); 30 ng of RAD10 fraction III in 20 μl of buffer A (lanes 3 and 5); or 20 μl of buffer A (lane 4). The positions of molecular weight markers are indicated on the right.

these conditions. These results were strictly dependent on the presence of RAD1 antiserum. Additionally, the RAD10 protein was not coprecipitated in the presence of either the Fos or Jun protein and Fos or Jun antiserum (data not shown).

Coimmunoprecipitation of RAD1 with purified RAD10 protein. We previously described a three-step procedure for the purification of the RAD10 protein, consisting of successive hydroxyapatite, phosphocellulose, and blue Sepharose chromatography steps (7). Fraction II, obtained after the first two steps, is highly enriched for the RAD10 protein (~80% pure). Fraction III consists of essentially homogeneous RAD10 protein. Fraction II, generated by a precisely parallel purification of extracts from a yeast strain in which the chromosomal *RAD10* gene is deleted and which carries just the cloning vector, yields a preparation which is totally devoid of the RAD10 protein (7). When these fractions were used in the coimmunoprecipitation assay, in vitro-translated RAD1 protein was coimmunoprecipitated with RAD10 protein fractions II (Fig. 5, lane 1) and III (lane 3) but not with fraction II from the *RAD10* deletion strain (lane 2) or with a control containing just buffer (lane 4). The observed coprecipitation was thus strictly dependent on the presence of the RAD10 protein. Additionally, coprecipitation was strictly dependent on the presence of RAD10 antiserum (lane 5). In vitro-translated Fos and Jun proteins were not coprecipitated with purified RAD10 protein (data not shown).

Quantitation of coimmunoprecipitation. A modification of the coimmunoprecipitation protocol permitted a quantitative evaluation of the interaction between the RAD1 and RAD10 proteins. One protein species was translated in vitro in the absence of radiolabel and was mixed with a second ^{35}S-labelled protein in the standard coimmunoprecipitation reaction mixture. The precipitated complex was resuspended in scintillation cocktail, and radioactivity was quantitated. The results of such experiments are shown in Table 1. The RAD10 protein was coprecipitated with RAD1 at a level ~18-fold above the background, and RAD1 was coprecipitated with RAD10 at a level ~5-fold over the background

TABLE 1. Quantitative analysis of coimmunoprecipitation

Labeled protein	Unlabeled protein[a]	Antiserum	% Precipitation[b]	SE[c]	No. of independent expts
RAD10	No RNA	Anti-RAD10	0.25	0.1	9
RAD10	RAD1	Anti-RAD1	4.6	0.4	9
RAD10	Fos	Anti-RAD1	0.2	0.1	4
Fos	No RNA	Anti-RAD1	0.4	0.1	4
Fos	RAD1	Anti-RAD1	0.4	0.1	4
RAD1	RAD10	Anti-RAD1	29.6	2.2	6
RAD1	No RNA	Anti-RAD10	0.65	0.1	13
RAD1	RAD10	Anti-RAD10	3.2	0.5	13
RAD1	Fos	Anti-RAD10	0.8	0.2	4
Fos	No RNA	Anti-RAD10	0.8	0.2	5
Fos	RAD10	Anti-RAD10	0.9	0.2	5
RAD10	RAD1	Anti-RAD10	41.2	3.6	4

[a] No RNA, reticulocyte lysate not programmed with exogenous RNA.
[b] Percentage of incorporated counts precipitated.
[c] Standard error between independent experiments.

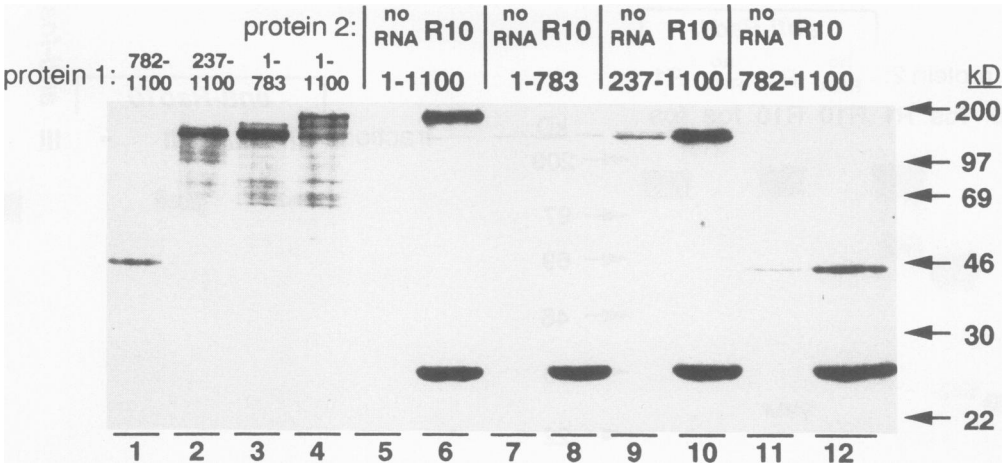

FIG. 6. Coimmunoprecipitation of in vitro-translated RAD1 deletion proteins. RAD1 full-length and deletion proteins were translated in vitro, ammonium sulfate precipitated, and loaded directly onto an SDS–10% PAGE gel (lanes 1 to 4) or immunoprecipitated with anti-RAD10 antibodies in the presence (lanes 6, 8, 10, and 12) or absence (lanes 5, 7, 9, and 11) of 26 ng of in vitro-translated RAD10 protein prior to gel analysis. Immunoprecipitation reactions contained 260 fmol of RAD1 full-length or deletion protein (lanes 5 to 12). Reticulocyte lysate not programmed with exogenous RNA was added to lanes 5, 7, 9, and 11 so that all immunoprecipitation reaction mixtures (lanes 5 to 12) contained 30 μg of total protein. The positions of molecular weight markers are indicated on the right.

(Table 1). About 30% of the total RAD1 protein present in the immunoprecipitation reactions was precipitated by the RAD1 antiserum (Table 1). This level was not significantly different in the presence or absence of RAD10 protein (data not shown), indicating that the RAD10 protein did not inhibit the binding of RAD1 antiserum to epitopes on the RAD1 protein. Similarly, the RAD1 protein did not inhibit the binding of RAD10 antiserum to epitopes on the RAD10 protein (data not shown).

The data in Table 1 were used to estimate the binding affinity of the RAD1-RAD10 interaction and yielded an equilibrium dissociation constant for the RAD1-RAD10 interaction of $\approx 3 \times 10^{-9}$ M, assuming that RAD1 and RAD10 associate as a heterodimer. This value likely represents a minimum estimate of the dissociation constant under these conditions, since some of the antibody species in the polyclonal antisera may block the RAD1-RAD10 interaction. Additionally, some of the RAD1-RAD10 complexes may dissociate during the nonequilibrium conditions which apply during the washing steps.

Mapping of the RAD1-RAD10 interaction domains. To localize the domains of RAD1 and RAD10 proteins involved in heteromer formation, incomplete polypeptides were tested in the coimmunoprecipitation assay. Proteins truncated in the C-terminal region were synthesized by linearizing the transcription templates at internal restriction sites. Plasmid constructs for the production of N-terminal deletions were engineered as described in Materials and Methods. The results obtained with three RAD1 deletion polypeptides are shown in Fig. 6. $RAD1_{1-783}$, a mutant protein in which amino acids 784 to 1100 are deleted, did not coprecipitate with the RAD10 protein (lanes 7 and 8). However, the polypeptide $RAD1_{237-1100}$, in which amino acids 1 to 236 are deleted, retained the ability to coprecipitate with RAD10 (lanes 9 and 10). Indeed, a mutant consisting of only amino acids 782 to 1100 coimmunoprecipitated with the RAD10 protein at levels clearly above the background (lanes 11 and 12). These data indicate that the major domain for interaction with RAD10 lies within the C-terminal 29% of the RAD1 polypeptide. These results are consistent with the observation that the RAD1 species which are coprecipitated with the RAD10 protein do not include the lower-molecular-weight forms which are believed to reflect prematurely terminated polypeptides and hence are expected to be missing C-terminal domains (Fig. 3, compare lanes 3 and 5).

Two RAD10 deletion polypeptides were tested for their ability to coprecipitate with the RAD1 protein, and the results of these experiments are shown in Fig. 7. A RAD10 deletion polypeptide consisting of only the first 169 amino acids no longer coprecipitated with RAD1 (lanes 6 and 7). However, the polypeptide $RAD10_{74-210}$, which is missing the N-terminal 73 amino acids, retained the ability to coprecipitate with RAD1 (lanes 8 and 9). Hence, we conclude that the C-terminal 65% of RAD10 is sufficient for binding to the RAD1 protein and that the C-terminal 20% of RAD10 is necessary for this interaction.

DISCUSSION

RAD1 and *RAD10* are two of at least seven genes which are believed to be absolutely required for damage-specific recognition and/or incision of DNA during nucleotide excision repair in yeast cells. In addition to their role in nucleotide excision repair, the *RAD1* and *RAD10* gene products (but not other *RAD* gene products required for nucleotide excision repair) participate in certain mitotic recombination events in yeast cells (40). The present study demonstrates that the RAD1 and RAD10 proteins form a specific and stable heteromeric complex in vitro in the absence of damaged DNA. The RAD1-RAD10 interaction occurs in the presence of a 20,000-fold excess (by weight) of heterologous protein, a situation not unlike that anticipated in vivo. The specificity of this protein-protein interaction was established by showing that neither RAD1 nor RAD10 protein coimmunoprecipitated with functionally unrelated in vitro-translated proteins such as Fos and Jun, nor did Fos or Jun coimmunoprecipitate with RAD1 or RAD10. Additionally, the results were in all cases dependent on the presence of the appropriate antiserum and antigen. Specificity was further established by the use of purified RAD10 protein and by the

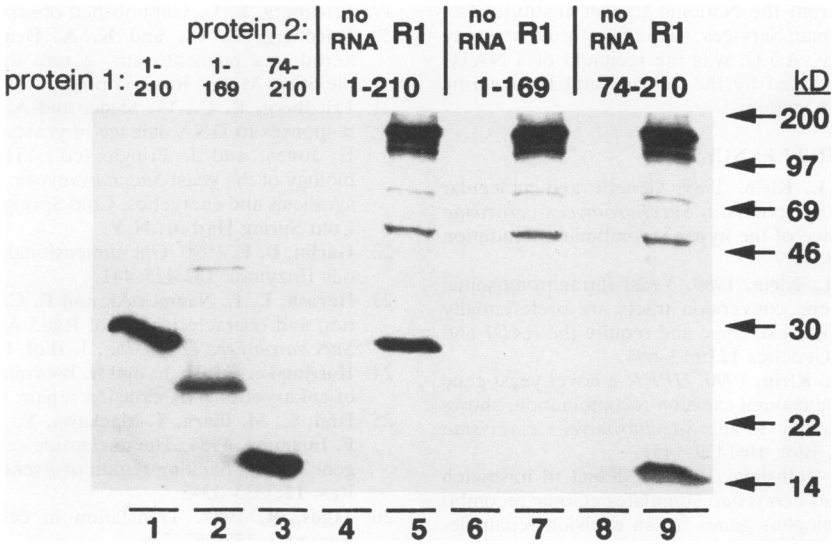

FIG. 7. Coimmunoprecipitation of in vitro-translated RAD10 deletion proteins. RAD10 full-length and deletion proteins were translated in vitro, ammonium sulfate precipitated, and loaded directly onto an SDS–12% PAGE gel (lanes 1 to 3) or immunoprecipitated with anti-RAD1 antibodies in the presence (lanes 5, 7, and 9) or absence (lanes 4, 6, and 8) of 33 ng of in vitro-translated RAD1 protein prior to gel analysis. Immunoprecipitation reactions contained 1 pmol of RAD10 full-length or deletion protein (lanes 4 to 9). Reticulocyte lysate not programmed with exogenous RNA was added to lanes 4, 6, and 8 so that all immunoprecipitation reaction mixtures (lanes 4 to 9) contained 30 μg of total protein. The positions of molecular weight markers are indicated on the right.

observation that the deletion of critical domains from each protein abolished the observed interaction.

Both the *RAD1* and *RAD10* genes are very weakly expressed in yeast cells. We have calculated that there are ~100 molecules of RAD10 protein per cell. If we assume that the RAD1 protein is present at about the same level, the dissociation constant we have estimated ($K_d \approx 3 \times 10^{-9}$) indicates that in vivo >80% of the RAD1 and RAD10 molecules exist as a heteromeric complex at any instant in time, assuming that conditions in vivo roughly approximate those of our in vitro experiments. Thus, at least some of the multiple proteins required for nucleotide excision repair may associate prior to their interaction with damaged DNA. In this regard, it is interesting to note that so-called bipartite nuclear targeting signals have been identified in the amino acid sequences of the *RAD1*, *RAD2*, *RAD4*, and *RAD14* genes but not those of the *RAD3* and *RAD10* genes, which are also required for the specific incision of DNA (13, 19a). Transportation of the polypeptides encoded by the latter genes to the nucleus may be dependent on their stable association with the RAD1, RAD2, RAD4, or RAD14 protein prior to nuclear entry.

In the case of the RAD10 protein the N-terminal 35% of the protein was found to be dispensable for stable interaction with the RAD1 protein. This region is poorly conserved between the *RAD10* and the homologous human (51) and mouse (52) *ERCC1* genes and is known to be dispensable for function in the human gene (51). The C-terminal 65% of the RAD10 protein is sufficient and the C-terminal 20% of the polypeptide is necessary for interaction with the RAD1 protein. The C-terminal two-thirds of *RAD10* is evolutionarily conserved among yeast, mice, and humans (51, 52). These results suggest that the RAD1 interaction domain of *RAD10* may be conserved in eukaryotes.

Recently, a specialized computer search algorithm was used to identify a series of nine tandem leucine-rich motifs in the N-terminal third of the putative RAD1 polypeptide (41). These repeats were proposed to function in a tight and specific interaction with an unknown protein (41). It is clear from our data that these repeats are not involved in the RAD1-RAD10 interaction. In the polypeptide $RAD1_{237-1100}$ seven of the nine leucine-rich motifs are deleted, and $RAD1_{782-1100}$ is completely devoid of these repeats (see Fig. 1), yet both of these incomplete polypeptides retained the ability to coprecipitate with the RAD10 protein. If these repeats do mediate a protein-protein interaction, a heterotrimeric complex involving RAD1, RAD10, and a third protein may exist. The C-terminal 29% of RAD1 protein is necessary and largely sufficient for interaction with RAD10. No obvious sequence motifs have been identified in this region, and it is not possible to assess the evolutionary conservation of this region, since the sequences of RAD1 homologs from other species have not yet been published.

Electron microscopic evidence suggestive of the existence of a large multiprotein recombination complex has been reported (10). Similarly, it has been suggested that the involvement of multiple gene products in the initial steps of nucleotide excision repair reflects the existence of a multiprotein damage recognition and incision complex (17, 18). This study provides the first biochemical evidence of a heteromeric association between any eukaryotic nucleotide excision repair proteins presumed to be involved in these early steps. In addition, these results suggest that the biochemically relevant species of the RAD1 and RAD10 proteins during nucleotide excision repair and/or mitotic recombination may be a RAD1-RAD10 heteromer.

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