The Saccharomyces cerevisiae RAD1 and RAD10 genes are required for the incision step of excision repair, and in addition, they function in mitotic recombination. The RAD1 and RAD10 proteins are associated in a tight complex, and genetic studies have indicated that complex formation is essential for the RAD1/RAD10 controlled biological activities. We have previously identified the RAD10 protein to near homogeneity from yeast and shown that it is a DNA-binding protein with a strong preference for single-stranded DNA. In this study, we purify the RAD1 protein to near homogeneity from yeast and show that it also binds single-stranded DNA preferentially and that the RAD1/RAD10 complex possesses an endonuclease activity. We characterize the RAD1/RAD10 endonuclease activity on both single-stranded and double-stranded DNAs, using agarose gel electrophoresis and trichloroacetic acid precipitation. The RAD1/RAD10 nuclease exhibits a much higher level of activity on single-stranded DNA than double-stranded DNA. The susceptibility of double-stranded DNA to nicking by the RAD1/RAD10 enzyme is markedly dependent on the degree of negative superhelicity, such that a 15-fold increase in nicking rate is observed from superhelical state $c = 0$ to $c = -0.08$. The enzyme produces $3'$-hydroxy and $5'$-phosphate termini on both single- and double-stranded DNAs. We discuss the role of RAD1/RAD10 endonuclease in nucleotide excision repair and in mitotic recombination.

Excision repair of ultraviolet (UV) damaged DNA in eukaryotes is a complex process involving many genes. Eleven genes have thus far been shown to have a role in this process in the yeast Saccharomyces cerevisiae. Mutations in six of these genes, RAD1, RAD2, RAD3, RAD4, RAD10, and RAD14, render cells highly sensitive to UV light and defective in the incision step of excision repair (Wilcox and Prakash, 1981; Reynolds and Friedberg, 1981; Bankmann et al., 1992). In addition, the RAD25 gene is also expected to be indispensable in the incision step (Gulyas and Donahue, 1992; Park et al., 1992). These seven genes likely encode proteins that mediate the recognition and endonucleolytic scission of damaged DNA. Mutations in the remaining four genes, RAD7, RAD16, RAD23, and MMS19 cause a moderate increase in UV sensitivity and a reduced efficiency in excision repair (Wilcox and Prakash, 1981; Miller et al., 1982a and 1982b). Excision repair is less efficient in nucleosomal regions (Smerdon and Thoma, 1990), and some of these genes might affect the accessibility of damaged DNA in chromatin to the incision complex (Bang et al., 1992).

Genetic studies with deletion mutations of yeast excision repair genes have revealed that some of these genes are multifunctional, acting in processes other than excision repair. Specifically, RAD3 and RAD25 are essential for cell viability (Higgins et al., 1982; Naumovski and Friedberg, 1988; Gulyas and Donahue, 1992; Park et al., 1992), and RAD1 and RAD10 function in mitotic recombination (Schiestl and Prakash, 1988, 1990). RAD1 and RAD10 are required for intrachromosomal recombination between direct repeats, and they also affect the homologous integration of linear DNA molecules and circular plasmids into genomic sequences (Schiestl and Prakash, 1988, 1990). The rad1$\Delta$ (Δ, deletion), and rad10A mutations have very similar effects on mitotic recombination, and the rad1$\Delta$ rad10A double mutant resembles the rad1$\Delta$ and rad10A single mutants in the degree of recombination defect, indicating epistasis. In contrast, a synergistic decline in mitotic recombination occurs when the rad1$\Delta$ or rad10A mutation is combined with the rad52Δ mutation. Thus, RAD1 and RAD10 genes function together in a mitotic recombination pathway that is distinct from the RAD52 double strand break recombination pathway (Schiestl and Prakash, 1990). The effects of RAD1 on mitotic recombination have been analyzed genetically in other studies (Klein, 1988, and see “Discussion” for additional references).

An intimate relationship between the RAD1- and RAD10-encoded proteins was indicated from studies demonstrating that RAD1 and RAD10 interact physically (Bailly et al., 1992; Bardwell et al., 1992). Furthermore, a rad1$\Delta$ mutation which inactivates complex formation with RAD10 causes the same high degree of DNA repair and recombination defects as do the rad1$\Delta$ and rad10A mutations (Bailly et al., 1992). These observations strongly suggested that complex formation is essential for the expression of a biochemical activity that mediates the DNA repair and recombination functions of RAD1 and RAD10 proteins.

Previously, we purified the RAD10 protein from yeast and showed it to be a DNA-binding protein with strong preference for single-stranded DNA (Sung et al., 1992). Here, we report the purification of the RAD1 protein from yeast and demonstrate that the combination of RAD1 and RAD10 proteins possesses an endonuclease activity on single-stranded DNA. In addition, we determine the action of the RAD1/RAD10 endonuclease activity on double-stranded DNA as a function of negative superhelicity and UV damage. We also present...
results of DNA binding studies and provide evidence that the enzyme does not act exonucleolytically.

**EXPERIMENTAL PROCEDURES**

Buffers—Cell breakage buffer (CBB) was 50 mM Tris-Cl, pH 7.5, containing 10% sucrose, 600 mM KCl, 10 mM EDTA, 10 mM 2-mercaptoethanol, and the following protease inhibitors: phenylmethylsulfonyl fluoride and benzamidine hydrochloride at 0.3 mM, and aprotinin, chymostatin, leupeptin, and pepstatin at 10 μg/ml. The lysozyme-containing chromatography buffer (L) was 200 mM KH2PO4, pH 7.4, containing 10% glycerol, 1 mM DTT, and 0.5 mM EDTA; the aforementioned protease inhibitors were included in this buffer when needed. The reaction buffer (buffer R) used in the RAD1/RAD10 nuclease assays was 20 mM Tris-HCl, pH 7.5, containing 50 mM KCl, 5 mM MgCl2, 1 mM DTT, and 60 μg/ml BSA. Other buffers were: (i) 50 mM Tris-HCl, pH 7.5, containing 50 μg/ml BSA, 5 mM MgCl2, 1 mM ATP, and 1 mM DTT (buffer L, for T4 DNA ligase), (ii) 50 mM Tris-HCl, pH 7.5, containing 50 μg/ml BSA, 50 mM KCl, 10 mM MgCl2, and 1 mM DTT (buffer T, for calf thymus topoisomerase I), and (iii) 10 mM Tris-HCl, pH 7.0, with 0.1 mM EDTA (TE, for dissolving DNAs).

**Gel Electrophoresis and Immunoblotting—**SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (1970). Gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 25% methanol, 12.5% acetic acid in water, and destained using the same mixture lacking acetic acid. Nitrocellulose gels (Schematic S) and SDS-polyacrylamide gels (7% for RAD1 and 11% for RAD10) were probed with affinitry purified antibodies (OD,y, 10,000 counts/min/pg and 57,000 counts/min/pg, respectively. Nitrocellulose filters were washed with 1% followed by 10 μl(100 pg) of sonicated calf thymus DNA and 125 ng of RAD10 were used. After incubation at 30 °C, SDS was added to 1% followed by 10 μl(100 pg) of sonicated calf thymus DNA and the pool of which (fraction IV, total 3 ml) was diluted with 700 μl of 7% glycerol before being applied onto a Mono Q (HR5/5, Pharmacia LKB Biotechnology Inc.), equilibrated with buffer A containing 100 mM KCl, and washed with 5 ml of the same buffer after sample application. Chromatographic development of Mono Q was with a 30-ml gradient of 100-500 mM KCl in buffer A. Fractions (0.5 ml each) containing RAD1, eluting at ≥350 mM KCl, were pooled (fraction V, total 2 ml) and concentrated to 300 μl using a Centricron-30 device. The yield of nearly homogeneous RAD1 protein was 300 μg with a recovery of 30%. RAD1 protein is stable for at least 1 week at 0 °C and for at least 1 year at -70 °C.

**Purification of RAD1 Protein from Yeast—**Yeast strain CMY135 harboring the RAD10 overproducing plasmid pSUC8 (2 μm, ADC1::RAD10) was cultured at 30 °C in complete synthetic medium lacking tryptophan to a cell density of 4×10^10 cells/ml. RAD10 protein was precipitated from clarified cell extract prepared from 100 g of yeast cells by treatment with ammonium sulfate (0.31 g/ml), and was further enriched by sequential ion-exchange steps on DEAE-Sepharose, Bio-Rex-70, and Mono S, as described previously (Sung et al., 1992). The Mono S pool (fraction III, 4 ml) was concentrated to 0.8 ml using a Centricron-10 device (Amicon) and fractionated by size exclusion chromatography on a column of S100HR (1 × 45 cm, total volume 35 ml) equilibrated and developed in buffer A containing 150 mM KCl. The S100HR pool (3 ml, fraction IV) was concentrated to 250 μl; the yield was 400 μg of homogeneous RAD10 with a recovery of 20%. RAD10 protein is stable for at least 1 week at 0 °C and for at least 2 years at -70 °C.

**Nitrocellulose Filter Binding—**The 3H-labeled viral (ss) and replicating form (ds) M13mp18 DNAs had specific radioactivities of 42,000 counts/min/μg and 57,000 counts/min/μg, respectively. Nitrocellulose filters (HAWP 25 from Millipore) were soaked in 0.3 M NaOH for 12 min at 25 °C (Noranelli and Lieberman, 1980). The filters were rinsed thoroughly with water and soaked at 25 °C for 3 h in 20 mM Tris-HCl, pH 7.0. Assay mixtures (10 μl) were assembled in buffer R, and contained 50-300 ng of RAD1 and 100 ng of DNA. After a 15-min incubation at 30 °C, the assay mixtures were diluted with 90 μl of ice-cold buffer R lacking BSA and sucked dropwise through filters. After washing with 5-μl aliquots of buffer R, filters were soaked in 1% followed by 100 μl of 10% trichloroacetic acid. Samples were incubated on ice for 10 min and centrifuged at 4 °C for 5 min in a microfuge. An aliquot (60-90 μl) of the clear supernatant was mixed with 10 μl of Liquiscint before radioactivity measurement. To determine radioactivity that remained acid precipitable, the quantity of 10% trichloroacetic acid-lacking buffer R was added and washed with 100 μl of 10% trichloroacetic acid. The filters were washed with 10 ml of each ice-cold 10% trichloroacetic acid.
acid and ethanol, mixed with 10 ml of Liquiscint, and assayed for associated radioactivity.

To generate single DNA strands from duplex DNA, the latter was boiled for 3 min in H2O and chilled immediately in ice-water mixture for 5 min.

Determination of Nature of Termini in RAD1/RAD10-digested DNA—Three µg of ds- (90% supercoiled and 10% nicked) M13 DNA was incubated at 30 °C for 60 min in buffer R (final volume 50 µl) without or with RAD1 and RAD10 at 600 and 250 ng, respectively. After phenol extraction of reaction mixtures, DNA was isolated by ethanol precipitation and dissolved in 15 µl of TE. Ligation mixtures contained 1 µl of RAD1/RAD10-nicked DNA or DNA from the minus enzyme control, with or without 1.5 units of T4 DNA ligase (Boehringer Mannheim) in 10 µl of buffer L. The reaction proceeded at 15 °C for 15 h, and the ligase was inactivated by heating at 70 °C for 10 min before electrophoresis in a 0.7% agarose gel containing 4.5 µM chloroquine to effect the separation of ligated DNA (relaxed circular form) from the nicked circular form.

Superoiling of DNA—DNA preparations with varying degrees of negative superhelical density were obtained using the procedure of Keller (1975). M13mp18 RF DNA (6.5 µg of DNA, 90% supercoiled) was incubated for 10 min at 37 °C in 100 µl of buffer T with 0–16 µM ethidium bromide. Following the addition of 12 units of calf thymus topoisomerase 1 (Bethesda Research Laboratories), relaxation of DNA proceeded at 37 °C for 150 min. The reaction mixtures were extracted once each with phenol, phenol-CHCl3, and CHCl3. The DNA was precipitated by the addition of 2.5 volumes of ethanol, washed with 70% ethanol, dried, and dissolved in TE to 150 µg/ml.

RESULTS

Purification of RAD1 and RAD10 Proteins—Overexpression of RAD1 and RAD10 in yeast was achieved by fusing their coding frames to the promoter of the highly expressed alcohol dehydrogenase I (ADCI) gene. Yeast strain CMY135 was used as host for the plasmids pRR168 (ADCI-RAD1) and pSUC8 (ADCI-RAD10). Immunoblot analyses of cell extracts using affinity purified antibodies (Bailly et al., 1992; Sung et al., 1992) revealed that RAD1 and RAD10 proteins are overproduced in CMY135 to an extent where each constitutes approximately 0.05–0.1% of total cellular protein (Fig. 1, A and B, lane 2). The amount of RAD1 and RAD10 in CMY135 harboring the ADCI vector pSCW231 lacking the RAD1 or RAD10 gene is too low to detect under the conditions employed (Fig. 1, A and B, lane 1). Detection of wild type level of RAD1 and RAD10 requires their prior enrichment from cell extract by immunoprecipitation; RAD1 and RAD10 proteins immunoprecipitated from extract of CMY135 and other yeast strains migrate in denaturing polyacrylamide gels in the same position as the overproduced proteins (Bailly et al., 1992). The observed molecular mass of RAD1 is 130 kDa and that of RAD10 is 25 kDa, in good agreement with the predicted molecular masses of 126.4 and 24.3 kDa for the two proteins (Reynolds et al., 1985, 1987).

To purify RAD1 protein, clarified extract from 100 g of CMY135 (pRR168) was treated with 0.21 g/ml of ammonium sulfate to precipitate RAD1 quantitatively and approximately 15% of total protein. The protein precipitate was dissolved and, after dialysis to remove ammonium sulfate, sequentially fractionated in columns of Bio-Rex 70, Q Sepharose, hydroxyapatite, and Mono Q (see “Experimental Procedures”). The RAD1 pool from the Mono Q step (fraction V) was analyzed by SDS-PAGE and staining with Coomassie Blue, and judged to be nearly homogeneous (Fig. 1A, lane 4). Immunoblot analysis established the authenticity of the purified protein and revealed that immunoreactive polypeptides migrating below RAD1 protein in cell extract (Fig. 1A, lane 2), which most likely corresponded to proteolytic fragments of RAD1, had been removed during purification (Fig. 1A, lane 3). Thus, RAD1 protein was purified 2000-fold from cell extract, with a yield of 300 µg of apparently homogeneous protein and a recovery of 30%.

In the purification of RAD10 protein, extract from 100 g of CMY135 (pSUC8) was treated with ammonium sulfate, followed by chromatography on DEAE-Sephacel, Bio-Rex-70, and Mono S, as described previously (Sung et al., 1992). The Mono S pool was subjected to molecular exclusion chromatography using Sephacryl-100, which gives superior separation of RAD10 from the trace amounts of undesired protein contaminants than Sephadex G-75 employed in our original protocol (Sung et al., 1992). The RAD10 protein so purified...
was nearly homogeneous as determined by Coomassie Blue staining of a denaturing polyacrylamide gel (Fig. 1B, lane 4), and did not contain the proteolytic product migrating below RAD10 observed on immunoblot of cell extract (Fig. 1B, lanes 2 and 3). RAD10 protein was purified 1000-fold, and the final yield was 400 μg with a recovery of 20%.

DNA Binding Properties of RAD1 and RAD10—The interaction of purified RAD1 protein with 3H-labeled viral single stranded (ss) and replicating double-stranded form (ds) M13 DNAs was examined by nitrocellulose filter binding. The assay measures the ability of DNA-binding proteins to effect the retention of DNA on alkali-treated nitrocellulose filters (see “Experimental Procedures”). RAD1 protein (50–300 ng or 0.4–2.4 pmol) was incubated with 100 ng of DNA (≈300 pmol of nucleotides) for 15 min. Reaction mixtures were diluted with buffer, sucked slowly through nitrocellulose filters, followed by liquid scintillation spectrophotometry to determine the amount of radioactivity associated with filters. The percentage of input ss- and ds-DNAs retained on nitrocellulose filters was proportional to the amount of RAD1 protein (Fig. 2). Maximal retention (≈85%) of ss-DNA was observed at 250 ng of RAD1, or at 150 nucleotides/RAD1 monomer (Fig. 2). The binding of ds-DNA was considerably less efficient than that of ss-DNA. Specifically, at 200 ng RAD1 where 75% of input ss-DNA was bound, only 20% of ds-DNA was retained on the filter, and binding of ds-DNA reached only 30% at 300 ng of RAD1 (125 nucleotides/RAD1 monomer). Irradiation of DNA with a high UV dose of 500J/m² did not alter the percentage of ss- or ds-DNA retained on filters (Fig. 2), indicating that RAD1 does not recognize UV-induced DNA damage. Binding of ss- and ds-DNAs does not require Mg²⁺, nor is it influenced by 1 mM ATP (data not shown). We showed previously that RAD10 possesses a DNA binding activity that is highly specific for ss-DNA but shows no preference for UV damaged ss- or ds-DNA, and does not require Mg²⁺ or ATP (Sung et al., 1992).

RAD1 and RAD10 Together Exhibit a Nuclease Activity—Purified RAD1 and RAD10 proteins were examined for the ability to hydrolyze various ribonucleoside triphosphates in the absence or presence of ss- and ds-DNAs, and, because of their involvement in genetic recombination, for DNA strand exchange activity with or without ATP. We found that RAD1 and RAD10, alone or when mixed, possess neither NTPase activity nor the ability to promote joint molecule formation between linear ds and ss viral M13 DNAs or between linear ds and ss viral φX174 DNAs (data not shown).

Interestingly, in strand exchange assays, we observed the degradation of the covalently closed ss-M13 viral DNA but not the linear ds form. Fig. 3 shows a time course experiment in which 120 ng of RAD1 (0.95 pmol) and 50 ng of RAD10 (2 pmol) were incubated at 30 °C for 5–30 min with 300 ng of covalently closed ss viral M13 DNA (~900 pmol of nucleotides). Progressing with time, the DNA was converted to forms with increasing electrophoretic mobilities (Fig. 3, lanes 6–10) and at 30 min, the size of DNA products was coincident with the marker DNA in the range of 70–310 base pairs (Fig. 3, lane 10). The ability to degrade DNA was contingent upon the presence of Mg²⁺ (Fig. 3, lane 4), which could not be substituted for by Ca²⁺, Mn²⁺, Cu²⁺, or Zn²⁺ (data not shown). The nuclease activity was abolished by the addition of 0.5% SDS to the reaction mix at the beginning (Fig. 3, lane 5). The inclusion of 1 mM ATP did not have any effect on the nuclease activity. Degradation of DNA absolutely requires both RAD1 and RAD10, as neither of the two proteins alone, even in an amount four times that used in the combination (Fig. 3, lanes 2 and 3), showed any nuclease activity. The RAD1/RAD10 activity also degrades covalently closed ss viral φX174 DNA in a similar manner.

The Nuclease Activity Is Intrinsic to RAD1 and RAD10—To confirm that the nuclease activity is intrinsic to the complex of RAD1/RAD10, we investigated if the nuclease activity increases with overproduction of RAD1 and RAD10, and if the activity coelutes chromatographically with both proteins. For RAD1, extract from RAD1 overproducing strain CMY 135 (pRR168) was processed through ammonium sulfate precipitation and Bio-Rex 70 batch absorption, followed by fractionation of the Bio-Rex 70 pool on a column of Q Sepharose using a KCl gradient (see “Experimental Procedures”). RAD1 appeared in fractions 14–19 of the gradient, as identified by immunoblotting (Fig. 4A, panel 1). The peak and flanking fractions (fractions 4–26) were assayed for ss-nuclease activity.

![Fig. 2. RAD1 protein binds-DNA.](image)

**Fig. 2.** RAD1 protein binds-DNA. 3H-labeled ss viral M13 DNA without (●) or with (○) UV treatment and 3H-labeled M13 ds replicating form DNA (70% supercoiled and 30% nicked circular) without (▲) or with (△) UV treatment were incubated with RAD1 protein. Nucleoprotein complexes were collected on nitrocellulose filters as described under “Experimental Procedures.”

![Fig. 3. Degradation of covalently closed ss viral M13 DNA by the mixture of purified RAD1 and RAD10 proteins.](image)

**Fig. 3.** Degradation of covalently closed ss viral M13 DNA by the mixture of purified RAD1 and RAD10 proteins. Lane 1, 300 ng of DNA; lane 2, DNA incubated with 500 ng of RAD1 protein for 30 min in the presence of Mg²⁺; lane 3, DNA incubated with 200 ng of RAD10 protein for 30 min in the presence of Mg²⁺; lane 4, DNA incubated with 50 ng of RAD10 protein for 30 min in the presence of Mg²⁺; lane 5, DNA was incubated for 30 min with 120 ng of RAD1 and 50 ng of RAD10 without Mg²⁺ (lane 4), or with Mg²⁺ and 0.5% SDS (lane 5). Lanes 6–10, DNA was incubated in the presence of Mg²⁺ with 120 ng of RAD1 and 50 ng of RAD10 together for 5, 10, 15, 20, and 30 min, respectively. The low percentage (0.7%) agarose gel used in this experiment did not separate well the low molecular weight products formed at the later time points. These gel conditions were used to obtain a compact pattern of the low molecular weight products to facilitate their visualization by ethidium bromide staining.
FIG. 4. RAD1 and RAD10 are subunits of the nuclease. A, nuclease activity coelutes with RAD1. Fractions 4–26 from the Q Sepharose step in RAD1 purification from extract of RAD1 overproducing strain CMY135 (pRR168) were examined by immunoblotting with anti-RAD1 antibodies (panel I) or for nuclease activity in the presence of 50 ng of purified RAD10 (panel II). No digestion of DNA was detected with the equivalent Q fractions from the strain CMY135 harboring the vector pSCW231 which lacks the RAD1 gene (panel III). The level of RAD1 protein in these control column fractions was too low to be detected by immunoblot analysis (data not shown). B, nuclease activity coelutes with RAD10. Bio-Rex 70 fractions 30–76 in RAD10 purification from extract of RAD10 overproducing strain CMY135 (pSUC8) were analyzed by immunoblotting with anti-RAD10 antibodies (panel I) or assayed for nuclease activity in the presence of 120 ng of purified RAD1 (panel II). Nuclease activity was missing in the equivalent fractions from the strain CMY135 (pSCW231) carrying the RAD10 gene (panel III), where the level of RAD10 was too low to detect.

In gel electrophoresis (data not shown) and in assays employing trichloroacetic acid precipitation (see later), the nuclease activity was markedly inhibited by antibodies specific for RAD1 or RAD10 while remaining refractory to antibodies against the unrelated RAD6 protein. Taken together, our results strongly indicate that the nuclease activity is an inherent property of the RAD1-RAD10 protein complex.

The RAD1/RAD10 Activity Is Endonucleolytic—The ability of RAD1/RAD10 nuclease to act on covalently closed ss-DNA

The peak and flanking fractions (fractions 30–76) were assayed for the ability to degrade ss-DNA in the presence of purified RAD1. The nuclease activity coeluted with the RAD10 protein (Fig. 4B, panels I and II) and was observed only upon the addition of purified RAD1 (data not shown). The activity was absent in equivalent fractions that originated from the strain CMY135 (pSCW231) carrying the RAD10 gene (Fig. 4B, panel III), where the level of RAD10 was too low to detect.

In gel electrophoresis (data not shown) and in assays employing trichloroacetic acid precipitation (see later), the nuclease activity was markedly inhibited by antibodies specific for RAD1 or RAD10 while remaining refractory to antibodies against the unrelated RAD6 protein. Taken together, our results strongly indicate that the nuclease activity is an inherent property of the RAD1-RAD10 protein complex.

The RAD1/RAD10 Activity Is Endonucleolytic—The ability of RAD1/RAD10 nuclease to act on covalently closed ss-DNA
To examine whether RAD1/RAD10 enzyme has an exonuclease activity, we measured the release of trichloroacetic acid-soluble radioactivity from DNA substrates labeled either uniformly or labeled at the 3' or the 5' end. An exonuclease would produce a rapid release of acid-soluble radioactivity with the end-labeled DNAs but not with the uniformly labeled DNA whereas an endonuclease would cause a slow release of acid-soluble radioactivity from the end-labeled as well as the uniformly labeled DNAs.

M13 RF DNA linearized with BglII was labeled with $^{32}$P at the 3' end using the Klenow fragment of *Escherichia coli* DNA polymerase I, or at the 5' end by treatment with T4 polynucleotide kinase following enzymatic removal of the preexisting 5'-phosphate. The end-labeled DNAs and linear ds-M13 DNA uniformly labeled with $^{3}$H were heat-denatured to generate single strands, and incubated with RAD1/RAD10 nuclease. The RAD1/RAD10 enzyme was found to be inefficient in the release of acid-soluble radioactivity. To improve the signal in this assay, we used 2.5 times more RAD1 and RAD10 than the protein amounts used in the gel assays (Fig. 3). A slow release of radioactivity was observed with all three labeled DNAs; for instance, after 10 min of incubation, where all of the covalently closed ss viral M13 DNA had been converted to a much smaller form as indicated from the gel assay (Fig. 3, lane 7), we observed the release of only ~2% acid-soluble radioactivity from the three labeled DNAs, even with the 2.5-fold increase in the RAD1/RAD10 amounts. The slow release of acid-soluble radioactivity and the similar rate of degradation of the end labeled and uniformly labeled DNAs suggest that RAD1/RAD10 enzyme does not possess any significant exonuclease activity.

The RAD1/RAD10 nuclease activity was further examined using M13 ss- and ds-DNAs labeled uniformly with $^{3}$H. Nuclease action on ss-DNA requires the simultaneous presence of RAD1 and RAD10, and of Mg$^{2+}$, and is not affected by the addition of 1 mM ATP (Table I), all in agreement with results obtained using the gel electrophoretic assay. Little release of acid-soluble radioactivity was observed with the ds-DNA (Table I). UV irradiation (500 J/m²) of ss- and ds-DNAs did not influence the nuclease activity (Table I).

### Table I

**RAD1/RAD10 nuclease is specific for single-stranded DNA**

<table>
<thead>
<tr>
<th>Proteins and alterations to reaction conditions</th>
<th>Trichloroacetic acid-soluble radioactivity</th>
<th>% relative activity</th>
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<tr>
<td>RAD10</td>
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</table>

UV irradiation (500 J/m²) of ss- and ds-DNAs did not influence the nuclease activity (Table I).

**Antibody Inhibition of RAD1/RAD10 Nuclease**—Release of acid-soluble radioactivity from uniformly $^{3}$H-labeled ss-DNA is strongly inhibited by affinity purified anti-RAD1 and anti-RAD10 antibodies (Baillie et al., 1992; Sung et al., 1992). For instance, at 4 μg of anti-RAD1 and anti-RAD10, the nuclease activity was inhibited by 85 and 65%, respectively (Fig. 5). In contrast, the addition of an excess of antibodies specific for the unrelated RAD6 protein (Sung et al., 1988) had no effect on DNA digestion (Fig. 5). A strong and specific inhibition of the nuclease activity by anti-RAD1 and anti-RAD10 antibodies was also observed in the gel assay (data not shown). These observations further confirm that the nuclease activity resides in the RAD1-RAD10 complex.

**Nicking of Double-stranded DNA Is Strongly Dependent on Negative Superhelical Density**—To characterize RAD1/RAD10 nuclease action on double-stranded DNA, we used preparations of covalently closed M13 RF DNA differing in their degree of negative superhelicity (designated by the symbol $\sigma$). Such DNA preparations were obtained by treating supercoiled M13 DNA isolated from *E. coli* with increasing amounts of the DNA helix intercalating agent ethidium bromide, followed by relaxation of DNA with calf thymus topoisomerase I, and extraction with organic solvents to remove ethidium (Keller, 1975, see “Experimental Procedures”). Since ethidium reduces the average rotation angle in the DNA double helix, upon its removal, the hitherto relaxed DNA acquires negative superhelical turns whose number being determined by the concentration of the intercalating agent present during incubation with topoisomerase (Keller, 1975). Using this method, we prepared M13 DNAs with $\sigma$ values ranging from 0 (relaxed) to −0.08, in increments of −0.02. To display the topological differences of these DNA preparations (Fig. 6A, lanes 1–5), they were analyzed in a 0.7% agarose gel containing 18 μM chloroquine together with negatively supercoiled DNA.
Fig. 6. Effect of negative superhelicity on nicking of ds-DNA. A, DNA preparations with increasing negative superhelicity were analysed in a 0.7% agarose gel containing 15 μM chloroquine to display their topological differences. Lanes 1-5 contain DNAs with σ of 0, -0.02, -0.04, -0.06, and -0.08, respectively, prepared by treatment of naturally supercoiled DNA with 15 μM chloroquine and calf thymus topoisomerase I as described under "Experimental Procedures." In lane 3, the supercoiled and nicked forms are not separated. In all other lanes, the slower migrating band is the nicked form. B and C, nicking of DNA by the RAD1/RAD10 nuclease is strongly stimulated by negative superhelicity but unaffected by UV irradiation of DNA. The series of increasingly supercoiled DNAs (σ of 0 to -0.08) and naturally supercoiled DNA (σ of -0.06) were incubated with RAD1/RAD10 nuclease for 15 min with or without prior UV treatment (500 J/m²). Reaction mixtures were electrophoresed in agarose gels with 15 μM chloroquine (for DNA with σ of 0, lanes 1 and 2) or without it (for DNAs with σ ranging from -0.02 to -0.08, lanes 3-10). The gels were stained with etidium bromide, photographed, and the photographic negatives scanned by densitometry to generate data points for C. Since UV irradiation has no effect on RAD1/RAD10 nicking of DNAs as indicated in C, only the gels containing DNA samples that had not received UV treatment are shown in B, where DNAs with σ of 0, -0.02, -0.04, -0.06, -0.08 (lanes 1, 3, 5, 7, and 9, respectively), were treated with the RAD1/RAD10 nuclease. 

Fig. 7. Conversion of RAD1/RAD10 nicked ds-M13 RF DNA to the covalently closed relaxed form by T4 DNA ligase indicates the presence of 3'-hydroxyl and 5'-phosphate groups. In the etidium bromide-stained gel, lanes 1 and 2 contain mock-treated DNA incubated without or with DNA ligase, respectively, lanes 3 and 4 contain RAD1/RAD10-nicked ds-DNA incubated without and with T4 DNA ligase, respectively, and lane 5 contains covalently closed relaxed M13 DNA made by treatment of the supercoiled form with calf thymus DNA topoisomerase I. To effect the separation of covalently closed relaxed DNA from the nicked circular form in lanes 4 and 5, 4 μM chloroquine was included in the agarose gel during electrophoresis. Details of the experiments presented in this figure are given under "Experimental Procedures." NC, nicked circular; RC, relaxed circular; SC, supercoiled.

Because of their involvement in nucleotide excision repair, RAD1/RAD10 nuclease was also examined for the ability to incise UV-damaged DNA. The series of increasingly superhelical M13 DNAs were UV-irradiated (500 J/m²) and incubated with RAD1/RAD10 nuclease. As shown in Fig. 6C, damaged DNA was nicked at the same rate as the non-damaged counterpart. Similar results were obtained when the experiments described in this section were repeated with PM2 DNA (data not shown).

Nuclease Activity Generates 3'-Hydroxyl and 5'-Phosphate Termini—RAD1/RAD10 digested ss viral M13 DNA was efficiently labeled by treatment with calf thymus terminal transferase in the presence of [α-32P]dCTP, indicating that RAD1/RAD10 nuclease generates a 3'-hydroxyl group (data not shown). To establish the nature of the 5' terminus in RAD1/RAD10-digested DNA, it was reacted with T4 poly-nucleotide kinase and [γ-32P]ATP, with or without prior treatment of DNA with calf intestinal alkaline phosphatase. Phosphatase treatment of RAD1/RAD10-digested ss-DNA was found to be essential for significant incorporation of 32P label during reaction with poly-nucleotide kinase, indicating a phosphate group at the 5' end (data not shown).

To verify that the RAD1/RAD10 nuclease generates 3'-hydroxyl and 5'-phosphate termini in negatively supercoiled
DNA, we used T4 DNA ligase. As shown in Fig. 7, the RAD1/
RAD10 incision nick can be closed by T4 DNA ligase, indic-
ating the presence of 3′-hydroxyl and 5′-phosphate groups
at the nick. Thus, the RAD1/RAD10 nuclease generates 3′-
hydroxyl and 5′-phosphate end groups on both ss- and ds-
DNAs.

**DISCUSSION**

Among the seven genes required for the incision step of
nucleotide excision repair in *S. cerevisiae*, only RAD1 and
RAD10 also function in mitotic recombination, and they do
so in an epistatic manner (Schiestl and Prakash, 1990). The
protein products of RAD1 and RAD10 are tightly associated
(Bailly et al., 1992; Bardwell et al., 1992) and genetic studies
implicate the requirement of complex formation in the DNA
repair and recombination functions of the two proteins (Bailly
et al., 1992).

We have purified RAD1 and RAD10 proteins to near ho-
mogeneity (≥98% purity) and examined their biochemical
activities singly and in combination. RAD1 and RAD10 pro-
teins bind ss-DNA with considerably higher affinity than ds-
DNA, and they exhibit no preference for UV damaged ss- or
ds-DNA (this work and Sung et al., 1992). Importantly, the
mixture of RAD1 and RAD10 proteins exhibits a nuclease
activity. The RAD1/RAD10 activity is endonucleolytic since it
acts on covalently closed ss-DNA, as has also been recently
observed by Tomkinson et al. (1993), who used a cruder
preparation of RAD1 (≥85% purity). Here, we provide evi-
dence that the endonuclease activity is intrinsic to the com-
plex of RAD1 and RAD10 by showing that (i) the amount of this
activity that can be purified from cell extract increases
substantially with overproduction of the two proteins, (ii) it
coelutes with both proteins during purification, and (iii) it is
strongly and specifically inhibited by anti-RAD1 and anti-
RAD10 antibodies. The RAD1-RAD10 complex does not pos-
sess detectable exonucleolytic activity.

In our work, the RAD1/RAD10 nuclease action on ds-DNA
was also characterized. We show that the nicking of ds-DNA
is strongly dependent on the superhelical state of DNA, such
that an increase in negative superhelicity from ρ of 0 to −0.08,
results in ∼15-fold increase in nicking rate. We suggest that
the preferential nicking of increasingly negatively superhelical
DNAs is due to the exposure of ss regions in such DNAs. No
preferential nicking of UV-damaged DNA by the RAD1/
RAD10 nuclease on the damaged strand mediated via its
interaction with proteins already bound there would target
the strand for endonucleolytic scission. The 3′-hydroxyl end
generated by the action of RAD1/RAD10 nuclease could be
subsequently utilized for repair synthesis.

Several lines of genetic evidence suggest that the RAD1/
RAD10 nuclease acts in different stages of recombination,
in initiation, and in the processing of single-stranded tails
generated during some recombination events. HOT1 is a cis-
acting recombination stimulating sequence present in *S. cere-
visiae* ribosomal DNA (rDNA). When HOT1 is inserted at
novel locations in the yeast genome, it stimulates mitotic
recombination in adjacent regions, and this recombination
stimulation is related to transcription by RNA polymerase I
(Stewart and Roeder, 1989). The radΔ mutation lowers the
rate of HOT1 stimulated mitotic recombination (Zehfus et al.,
1990). Transcription by RNA polymerase II also stimulates
mitotic recombination in yeast, and the radΔ mutation
causes a decrease in the level of this recombination too
(Thomas and Rothstein, 1989). The effects of RAD1 on
recombination stimulated by RNA polymerase I or RNA
polymerase II transcription could reflect a role of RAD1/
RAD10 nuclease in the initiation phase. The movement of
the transcriptional machinery through the DNA duplex has
been proposed to produce positive supercoils ahead of the
machinery and negative supercoils behind it. In fact, Ljung-
man and Hanawalt (1992) have recently presented direct
experimental evidence for the existence of negative super-
coiled domains in the 5′ end of transcriptionally active genes
in the human genome. Since RAD1/RAD10 nuclease acts
preferentially on negatively supercoiled DNA, such domains
produced in regions of the genome undergoing active tran-
scription might render these regions susceptible to cleavage
by the RAD1/RAD10 nuclease.

*S. cerevisiae* DNA topoisomerase III encoded by the TOP3
gene relaxes negatively but not positively supercoiled DNA,
as it is specific for single-stranded regions in DNA (Kim and
Wang, 1992). The *top3* null mutant displays an elevated rate
of ectopic recombination between two unlinked, homologous
genes, SAM1 and SAM2 (Bailly et al., 1992). However, *top3*
has no effect when recombination between the gene pair is
initiated by a double strand cut introduced by the HO endo-
nuclease. These observations strongly suggest that elevated
recombination in the *top3* mutant occurs as a result of in-
creased initiation (Bailly et al., 1992). The higher rate of
ectopic recombination between the SAM loci is reduced in the
*top3 rad1* double mutant compared to the *top3* single mutant,
indicating a role for RAD1 in *top3* stimulated recombination.

We suggest that in the absence of TOP3-encoded DNA relax-
ing activity, the negative supercoils that accumulate might be
a substrate for nicking by the RAD1/RAD10 endonuclease.

A role of RAD1 in recombination subsequent to the initia-
tion phase is indicated from experiments in which recombi-
nation is initiated by the introduction of a double strand
break by the HO endonuclease. These studies have been
conducted with yeast plasmids that carry a direct repeat of a
homologous sequence, and in which one of the repeats is
interrupted by the insertion of an HO endonuclease cut site.
Recombination between direct repeats in this system occurs
via two distinct, competing pathways: by a gap repair pathway
that yields gene conversion products, or by a single strand
annealing (SSA) pathway which produces deletions of the
DNA intervening between the two homologous sequences
(Fishman-Lobell et al., 1992, Fishman-Lobell and Haber,
1992), SSA represents a major pathway by which linearized DNA molecules injected into Xenopus oocytes nuclei or transformed into mammalian cells recombine (Maryon and Carroll, 1991; Lin et al., 1990). In the SSA mode of recombination, the ends of a double strand break undergo extensive bidirectional 5' → 3' exonuclease digestion, until complementary regions in direct repeats are exposed and can reanneal. Further nuclease action would degrade the 3'-ended single strand tails. The rad1 mutant is unable to complete recombination between direct repeats when the ends of the double strand break contain non-homology resulting from the insertion of the HO cutting site in one of the repeats. The formation of single-stranded DNA by the 5' → 3' exonuclease activity is apparently not affected by the rad1 mutation, therefore, the defect in recombination is likely due to a defect in the processing of 3' single strand tails (Fishman-Lobell and Haber, 1992). These observations are consistent with a role of the RAD1/RAD10 enzyme in the endonucleolytic removal of the 3' single strand tails during SSA.

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REFERENCES