**Structure-specific Nuclease Activity in Yeast Nucleotide Excision Repair Protein Rad2**

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Saccharomyces cerevisiae Rad2 protein functions in the incision step of the nucleotide excision repair of DNA damaged by ultraviolet light. Rad2 was previously shown to act endonucleolytically on circular single-stranded M13 DNA and also to have a 5'→3' exonuclease activity (Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1993) Nature 366, 365-368; Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1994) J. Biol. Chem. 269, 31342-31345). Using two different branched DNA structures, pseudo Y and flap, we have determined that Rad2 specifically cleaves the 5'-overhanging single strand in these DNA's. Rad2 nuclease is more active on the flap structure than on the pseudo Y structure. Rad2 also acts on a bubble structure that contains an unpaired region of 14 nucleotides, but with a lower efficiency than on the pseudo Y or flap structure. The incision points occur at and around the single strand-duplex junction in the three classes of DNA structures.

The Rad2 nuclease is related to the E. coli pol I 5'→3' exonuclease in that these enzymes share homology in domains conserved in pol I and related microbial nucleases.

A mammalian 45-kDa 5'→3' exonuclease that is related to E. coli pol I 5'→3' nuclease and that shares homology with yeast Rad2 is required for lagging strand DNA synthesis in reconstituted DNA replication systems (6-10). Following the RNase H1-catalyzed cleavage of primer RNA one nucleotide 5' of the RNA-DNA junction, the 5'→3' exonuclease removes the remaining monoribonucleotide of the RNA primer (9). Like the pol I nuclease, the mammalian enzyme also has a similar structure-specific activity (11). The RTH1 gene encodes the S. cerevisiae counterpart of this mammalian 45-kDa exonuclease (12). Genetic studies with the rth1Δ mutant strain have indicated a role of RTH1 in DNA replication as well as in DNA mismatch repair (12, 13).

The protein encoded by RTH1 and its mammalian counterpart contains ~380 amino acids. Rad2, by contrast, is a much larger protein, containing 1031 residues. The homology between yeast Rad2, RTH1, and their mammalian counterparts is restricted to three regions (for references, see Ref. 12). Moreover, whereas RTH1 and its mammalian counterparts have a role in DNA replication and in mismatch repair, Rad2 is required for NER, but has no apparent involvement in DNA replication and mismatch repair (2, 3, 12).

Thus, Rad2 and RTH1 proteins have diverged functionally. Here, we examine the action of Rad2 nuclease on various DNA structures.

**MATERIALS AND METHODS**

Purification of Rad2 Protein—Rad2 protein was purified from the protease-deficient yeast strain LY2 (MATα, gal1, regi-501, leu2-3, leu2-114, ura3-52, trpl-1, nap3-4, prb1-112) harboring the Rad2-overproducing plasmid pR2.26. In pR2.26, the Rad2 gene is under the control of the synthetic hybrid GAL-PGK promoter inducible by galactose (14). Starting from crude lysate prepared from 200 g of LY2[pR2.26], we used a combination of ammonium sulfate precipitation and chromatographic fractionation on columns of Q-Sepharose, S-Sepharose, Sephacryl S-500, and Mono S to obtain ~100 µg of nearly homogeneous Rad2 protein (14). Purified Rad2 protein was concentrated to 400 µg/ml using a Centricon-30 microconcentrator (Amicon, Inc.) and stored in small portions at ~70 °C. Once thawed, the Rad2 protein solution was kept on ice, where it remains enzymatically active for at least 1 week.

Polyconal Antibodies Specific for Rad2—A β-galactosidase-Rad2 fusion protein was expressed in E. coli carrying the plasmid pKMB (14). The insoluble histidine-polypeptide was purified from inclusion bodies by preparative SDS-polyacrylamide gel electrophoresis and used as antigen for polyclonal antibody production in rabbits. Antibodies were affinity-purified from the rabbit sera by passage through a Sepharose column containing the covalently linked antigen. After dialysis against phosphate-buffered saline (10 mM NaH₂PO₄, pH 7.2, 150 mM NaCl), the antibody solution was concentrated to 2 mg/ml and stored in small portions at ~70 °C. Affinity-purified polyclonal antibodies specific for Rad2 and Rad10 proteins were obtained as described previously (15).

Oligonucleotides Used—The following oligonucleotides were used to construct various DNA substrates. Oligonucleotide A, which contains 50 bases, has the sequence 5'-GGGACTGATCCTGCGCACTTTTGACTTGGCTGCTTTGACTTGGGCG-3'. Oligonucleotide B, a 20-mer, has the sequence 5'-CCCCCAAAAGTCCACAGCC-3'. Oligonucleotide C, a 50-mer, has the sequence 5'-ATGGGTATAGCAGATGACG-3'.

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Rad2 Nuclease

RESULTS

Specific Cleavage of Pseudo Y DNA Structure by Rad2 Protein—To determine whether the Rad2 nuclease activity would mediate the cleavage of a DNA structure that contains single-stranded tails adjacent to a duplex region, we hybridized 32P-labeled oligonucleotide A, C, E, and G were radiolabeled at their 5'-terminus with the bacteriophage T4 polynucleotide kinase and [γ-32P]ATP (Amersham Corp.; 6000 Ci/mmol). DNA substrates were obtained by annealing one of the radiolabeled oligonucleotides to nonlabeled oligonucleotides. Radialabeled oligonucleotide C was hybridized to oligonucleotide A to generate the pseudo Y-1 substrate shown in Fig. 1, and radiolabeled oligonucleotide C was hybridized to oligonucleotides A and B to generate Flap-1, which contains a 5'-overhanging single strand (see Fig. 1). Radialabeled oligonucleotide E was hybridized to oligonucleotide A and radiolabeled oligonucleotide E was hybridized to oligonucleotides A and D to generate the pseudo Y-2 and 3'-overhanging Flap-2 DNA substrates, respectively (see Fig. 1). To obtain the bubble DNA substrate (see Fig. 1), radiolabeled oligonucleotide G and oligonucleotide F were hybridized to each other. Annealing reactions were carried out by mixing 180 pmol of nonradiolabeled oligonucleotides in 50 μl of buffer (50 mM Tris-HCl, pH 8.2, 10 mM MgCl2, 0.2 mM EDTA, 5 mM dithiothreitol, and 0.1 mM spermidine) and incubating at 92°C for 2 min, at 65°C for 10 min, at 37°C for 20 min, and finally at 25°C for 20 min. Annealing mixtures were run at 4°C on 8% polyacrylamide gels in TBE buffer (90 mM Tris borate, pH 8.5, 2 mM EDTA); the region of the gel containing the radiolabeled DNA substrate was excised; and the DNA was eluted from the gel by digestion with T4 DNA polymerase in a reaction mixture containing 2 U/ml T4 DNA polymerase in 10 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 50 mM KCl. The purified DNA substrates were stored at 4°C.

Nuclease Assay—Rad2 protein (2-40 ng or 17-340 fmol) was incubated with 75 fmol of 5'-32P-labeled DNA (7000-20,000 cpm) in reaction buffer (50 mM Tris-HCl, pH 8.0, 5 mM MgCl2, 1 mM dithiothreitol, and 100 μg/ml bovine serum albumin) for 10 min at 30°C in a final volume of 11 μl. The reaction was terminated by adding 11 μl of gel loading buffer (90% deionized formamide in TBE buffer); the mixture was heated for 2 min at 92°C, and a fraction of it (2,500 cpm) was loaded on an 11% polyacrylamide gel containing 7M urea and 16% formamide in TBE buffer. Electrophoresis was carried out for 2 h at 30 V/cm on 0.5-mm-thick polyacrylamide gels that had been prerun at the same constant voltage for 20 min. Gels were soaked in a mixture of 20% methanol and 5% acetic acid for 15 min before being dried. The gel was either subjected to autoradiography to visualize the radiolabeled substrates and products or analyzed in the PhosphorImager 425 (Molecular Dynamics, Inc.) to quantify the various radiolabeled DNA species. The size markers used were purchased from Pharmacia Biotech Inc. and were 5'-end-labeled with [γ-32P]ATP and T4 DNA polymerase kinase.

FIG. 1. DNA substrates used. Asterisks indicate the position of the 5'-32P label, and the oligonucleotides that carry the label are underlined. The conditions used for annealing the oligonucleotides and other experimental details that pertain to the construction of the substrates are described under "Materials and Methods."
Rad2 Nuclease Cleaves Flap Structures More Efficiently than Pseudo Y—The results presented in Figs. 2 and 3 indicate that Rad2 nuclease acts on the 5'-overhanging single-stranded tail in both pseudo Y and flap structures. Since Flap-1 differs from pseudo Y-1 only in possessing oligonucleotide B, which is the exact complement of the 3'-overhanging single strand in the latter, a direct and meaningful comparison of the relative cleavage efficiencies of the two classes of DNA structure was possible. Since 32P-labeled oligonucleotide C was common to pseudo Y-1 and Flap-1, the two DNA structures used in the comparison had the same molar specific radioactivity. As shown in Fig. 4, the Flap-1 substrate was cleaved by Rad2 protein at a significantly higher efficiency than was pseudo Y-1. For instance, whereas ~90% of Flap-1 was cleaved by 10 ng of Rad2 protein, ~30% of pseudo Y-1 was cleaved by the same quantity of Rad2 (Fig. 4). The conclusion regarding the relative activities of Rad2 protein on the pseudo Y and flap structures was validated in at least three other independent experiments (data not shown).

The DNA structure-specific nuclease activity of Rad2, as assayed using Flap-1 DNA as substrate, requires Mg2⁺, which cannot be replaced by Ca2⁺, Co2⁺, Cu2⁺, or Zn2⁺, although Mn2⁺ is partially effective (Table I). The flap cleavage activity is not affected by KCl concentrations up to 50 mM, but higher amounts of the salt result in significant inhibition of the activity (data not shown), and the activity is abolished by 0.1% SDS (Table I).

Cleavage of 5'-Overhanging Single Strand in “Bubble” Structure—To investigate whether Rad2 nuclease would act on a single-stranded region located in a bubble structure, we hybridized 32P-labeled oligonucleotide G to nonlabeled oligonucleotide A, 20 ng (lanes 4 and 6) of Rad2 protein. The radiolabeled cleavage products were revealed by autoradiography after electrophoresis of the reaction mixtures. B, inhibition of flap cleavage by Rad2 antibodies. The Flap-1 substrate (75 fmol; lanes 1–5) was incubated with 20 ng of Rad2 protein for 10 min (lanes 2–5) in the presence of 1 μg of affinity-purified antibodies specific for Rad2 (lane 3), Rad1 (lane 4), or Rad10 (lane 5). The positions in nucleotides of the size markers are shown in lane M.

structure because (i) no cleavage of oligonucleotide A used in the construction of pseudo Y-1 occurred at the same (Fig. 2, lane 2) and higher (data not shown) concentrations of Rad2 protein, and (ii) the 3'-overhanging single strand in a similar DNA structure (pseudo Y-2) that was obtained by hybridizing radiolabeled oligonucleotide E to nonlabeled oligonucleotide A (see Fig. 1 and “Materials and Methods”) was not cleaved by Rad2 (Fig. 2, lane 4).

Rad2 Nuclease Cleaves Flap DNA Structure—The murine FEN-1 nuclease, a structural homolog of Rad2 protein, does not cleave pseudo Y DNA structures, but acts efficiently on “flap” DNA structures that contain a 5'-overhanging single strand (11), cleaving the 5'-overhanging single-stranded tail in the flap structures at and around the single-strand-duplex junction. However, FEN-1 does not act on flap structures that contain a 3'-overhanging single-stranded tail (11). The results presented above indicate that, unlike FEN-1, Rad2 nuclease cleaves pseudo Y DNA efficiently, showing specificity for the 5'-overhanging single strand in pseudo Y (Fig. 2). To investigate whether Rad2 also cleaves flap DNA, we hybridized 5'-32P-labeled oligonucleotide C to nonradiolabeled oligonucleotides A and B to form the Flap-1 substrate, which contains a 20-nucleotide 5'-overhanging single strand (Fig. 1), and we also hybridized 5'-radiolabeled oligonucleotide E to nonlabeled oligonucleotides A and D to form the Flap-2 structure, which contains a 3'-overhanging single strand (Fig. 1). The two flap substrates were then incubated with Rad2 protein under reaction conditions employed for the cleavage of pseudo Y-1. As shown in Fig. 3A, Rad2 protein cleaved Flap-1 highly efficiently, yielding a major product of 21 nucleotides (~74%), a lesser product of 20 nucleotides (~21%), and a trace of a product of 19 nucleotides (~5%). From the sizes of the cleavage products, it could be deduced that the major site of cleavage is located at 1 base inside the duplex region, and the minor sites are at the single strand-duplex junction and 1 base into the single-stranded region (see Fig. 6). In contrast, no cleavage of the 3'-overhanging single strand in Flap-2 was detected at the highest amount of Rad2 protein under the same reaction conditions (Fig. 3A, lane 6). Thus, the Rad2 flap cleaving activity closely resembles that of the FEN-1 protein in showing specificity for a 5'-overhanging single strand only.

The Flap-1 and pseudo Y-1 cleaving activities are intrinsic to Rad2 protein because (i) the Rad2 protein used in this study is essentially homogeneous, and (ii) the cleavage of Flap-1 (Fig. 3B) and pseudo Y-1 (data not shown) was strongly inhibited by affinity-purified antibodies raised against Rad2 protein (compare lanes 3 and 2) expressed in and purified from E. coli (14), but it was unaffected by antibodies specific for Rad1 and Rad10 proteins (lanes 4 and 5).

Rad2 also acts on 5'-overhanging single-stranded tail in flap DNA. A, cleavage of the flap structure. Flap-1 (lanes 1-4) and Flap-2 (lanes 5 and 6) DNAs (75 fmol each) were incubated for 10 min at 30 °C without (lanes 1 and 5) and with 10 ng (lane 2), 15 ng (lane 3), and 20 ng (lanes 4 and 6) of Rad2 protein. The radiolabeled cleavage products were revealed by autoradiography after electrophoresis of the reaction mixtures. B, inhibition of flap cleavage by Rad2 antibodies. The Flap-1 substrate (75 fmol; lanes 1-5) was incubated with 20 ng of Rad2 protein for 10 min (lanes 2-5) in the presence of 1 μg of affinity-purified antibodies specific for Rad2 (lane 3), Rad1 (lane 4), or Rad10 (lane 5). The positions in nucleotides of the size markers are shown in lane M.
TABLE I
Effect of divalent metal ions on Rad2-mediated flap cleavage

| Reaction components | Activity (%) |
|---------------------|-------------|
| Complete reaction   | 100         |
| Complete reaction + 0.1% SDS | <0.5 |
| -Mg²⁺        | <0.5      |
| -Mg²⁺, +Mn²⁺    | 24        |
| -Mg²⁺, +Ca²⁺    | <0.5      |
| -Mg²⁺, +C₀²⁺    | <0.5      |
| -Mg²⁺, +Zn²⁺    | <0.5      |

* Complete reaction 1.Mg²⁺ was the same as that of Mg₂⁺.

DISCUSSION

Our work indicates that Rad2 cleaves flap and pseudo Y structures and that it is more active in cleaving flap structures than pseudo Y. In this regard, Rad2 resembles the mammalian FEN-1 and S. cerevisiae RTH1 nuclease, which are also more efficient at cleaving flap structures than pseudo Y (11). A variety of experiments have indicated that the E. coli polI 5′ → 3′ exonuclease gains access to the cleavage site by moving from the free 5′-end of single-stranded DNA to the site of cleavage at the junction with duplex DNA (5). Biochemical studies of FEN-1 and calf thymus 5′ → 3′ exonuclease have indicated a similar requirement for a free 5′-end for strand cleavage to occur. In agreement with these observations, we find no cleavage of bubble structure by the S. cerevisiae RTH1 protein that we have purified to near homogeneity (data not shown). Unlike RTH1 and FEN-1 nuclease, Rad2 cleaves the bubble structure, albeit with a lower efficiency than the flap or pseudo Y structure. The differential ability of Rad2 and RTH1 proteins to cleave bubble DNA may reflect the affinity of these proteins for binding bubble DNA. Rad2 may possess a domain that confers the ability to bind bubble DNA, and the inability of RTH1 to cleave bubble DNA may arise from the absence of this domain.

The ability of FEN-1/RTH1 to cleave 5′-end single-stranded DNA at its junction with duplex DNA has led to the suggestion that Rad2 cleaves the damaged DNA strand on the 3′-side of the damage during NER (11). While the manner of cleavage of model DNA substrates by Rad2 reported in our present work

and by Rad1-Rad10 reported by Bardwell et al. (16) is congruent with the proposal that these proteins incise the damaged DNA strand on the 3′- and 5′-sides of the damage, respectively, direct evidence demonstrating this cleavage pattern in NER is as yet unavailable. Since neither the Rad2 nor Rad1-Rad10 nuclease has any affinity for damaged DNA (14, 17, 18), they must be targeted to the damage site via interaction with the damage recognition factors. The interaction of human XPA with the ERCC1 protein (19, 20) would suggest that the Rad1-Rad10 nuclease is targeted to the damage site via interaction with the damage recognition protein Rad14. Interaction with the other components of the NER machinery may target Rad2 to the damage site. It remains to be determined whether the site of placement of the Rad1-Rad10 and Rad2 nucleases on the damaged DNA strand is coincident with the cleavage pattern of these enzymes on model DNA substrates. The recent reconsti-

3 R. Bambara, personal communication.
tion of the incision step of NER with purified components in yeast (3) should make it feasible to ascertain the manner of assembly and the site of cleavage by these nucleases.

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REFERENCES
1. Huang, J. C., Svoboda, D. L., Reardon, J. T., and Sancar, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668
2. Prakash, S., Sung, P., and Prakash, L. (1993) Annu. Rev. Genet. 27, 33–70
3. Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995) J. Biol. Chem. 270, 12973–12976
4. Konrad, E. B., and Lehman, I. R. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 2048–2051
5. Lyamichev, V., Brow, M. A. D., and Dahlberg, J. E. (1993) Science 260, 778–783
6. Goulian, M., Richards, S. H., Heard, C. J., and Biggsby, B. M. (1990) J. Biol. Chem. 265, 18461–18471
7. Ishimi, Y., Claude, A., Bullock, P., and Hurwitz, J. (1988) J. Biol. Chem. 263, 19723–19733
8. Turchi, J. J., and Bambara, R. A. (1993) J. Biol. Chem. 268, 15136–15141
9. Turchi, J. J., Huang, L., Murante, R. S., Kim, Y., and Bambara, R. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9803–9807
10. Waga, S., Bauer, G., and Stillman, B. (1994) J. Biol. Chem. 269, 10923–10934
11. Harrington, J. J., and Lieber, M. R. (1994) Genes & Dev. 8, 1344–1355
12. Sommers, C. H., Miller, E. J., Dujon, B., Prakash, S., and Prakash, L. (1995) J. Biol. Chem. 270, 4193–4196
13. Johnson, R. E., Kovvali, G. K., Prakash, L., and Prakash, S. (1995) Science 269, 238–240
14. Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1993) Nature 366, 365–368
15. Bailly, V., Sommers, C. H., Sung, P., Prakash, L., and Prakash, S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8273–8277
16. Bardwell, A. J., Bardwell, L., Tomkinson, A. E., and Friedberg, E. C. (1994) Science 265, 2082–2085
17. Sung, P., Prakash, L., and Prakash, S. (1992) Nature 355, 743–745
18. Sung, P., Reynolds, P., Prakash, L., and Prakash, S. (1993) J. Biol. Chem. 268, 26391–26399
19. Li, L., Elledge, S. J., Peterson, C. A., Bales, E. S., and Legerski, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5012–5016
20. Park, C.-H., and Sancar, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5017–5021
