Role of the Rad1 and Rad10 Proteins in Nucleotide Excision Repair and Recombination

(Received for publication, August 7, 1995)

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In Saccharomyces cerevisiae, the RAD1 and RAD10 genes are involved in DNA nucleotide excision repair (NER) and in a pathway of mitotic recombination that occurs between direct repeat DNA sequences. In this paper, we show that purified Rad1 and Rad10 interact with a synthetic bubble structure and incise the DNA at the 5’-side of the centrally unpaired region. When Rad1–Rad10 and purified XPG protein (the human homolog of yeast Rad2 protein) were co-incubated with the DNA substrate, we observed incisions at both ends of the bubble. This reaction mimics the dual incision step in nucleotide excision repair in vivo. In addition, the recent suggestion that Rad1 can act to resolve Holliday junctions (Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1994) Nature 371, 531–534), explaining the recombination defect observed in rad1 mutants, has been further investigated. However, using proteins purified in two different laboratories we were unable to show any interaction between Rad1 and synthetic Holliday junctions. The role that Rad1–Rad10 plays in recombination is likely to resemble its activity in NER by acting upon partially unpaired DNA intermediates such as those formed by recombination mechanisms involving single-strand DNA annealing.

The process of nucleotide excision repair plays a major role in the removal of DNA lesions following DNA damage. In bacteria, excision repair is initiated by three proteins, UvrA, UvrB, and UvrC, which promote a dual incision reaction by degrading the 3rd to 5th phosphodiester bond 3’ to the lesion and the 8th bond at the 5’-side of the lesion. The 12–13-nucleotide-long oligomer containing the lesion is then removed from the DNA, and the single-stranded gap is filled and sealed by DNA polymerases and DNA ligase (1, 2). Dual incision is also observed in eukaryotes, and higher organisms excise a fragment of 27–29 nucleotides in length (3).

The isolation of UV-sensitive mutants has helped to define the genetic complexity of nucleotide excision repair in Saccharomyces cerevisiae, and it is now known that many genes are involved in the process. These include RAD1, RAD2, RAD3, RAD4, RAD7, RAD10, RAD14, RAD16, RAD23, TFB1, SSL1, and SSL2 (1, 4). Remarkably, the sequences of excision repair genes in yeast and mammalian cells are highly homologous, and the enzymology of the two systems is very similar.

The RAD1 and RAD10 genes of S. cerevisiae encode polypeptides that interact to form a stable complex (5–7) that exhibits a structure-specific endonuclease activity. The endonuclease cleaves single-stranded DNA tails with 3’-ends at the junction with duplex DNA (8), indicating that Rad1–Rad10 promotes the 5’-incision event in NER.3 Genetic studies show that rad1 and rad10 mutants also exhibit defects in mitotic recombination (9–14). In particular, RAD1 and RAD10 are required for intrachromosomal recombination between direct repeats and affect the integration of linear DNA molecules and circular plasmids into genomic sequences. RAD1 is also required for double-strand break repair between two direct repeats when the double-strand break occurs within a region of heterology (12). Recently it was suggested that Rad1 can resolve Holliday junctions (15), thus explaining the recombination defect observed in rad1 mutants.

In this paper we have further investigated the specific endonuclease activity of Rad1-Rad10 using substrates that mimic intermediates thought to occur during nucleotide excision repair and recombination. We show that the purified Rad1 and Rad10 proteins cut a synthetic bubble structure at the 5’-side of the centrally unpaired region and that Rad1–Rad10 together with purified XPG protein (the human homolog of yeast Rad2 protein) catalyze a dual incision reaction. In contrast to the report by Habraken et al. (15), we did not detect any cleavage of Holliday junctions by Rad1 or Rad1-Rad10.

EXPERIMENTAL PROCEDURES

Proteins—The S. cerevisiae Rad1 and Rad10 proteins were purified to homogeneity as described (16). Two preparations of Rad1 were utilized (prepared independently in the laboratories of E. C. F. and A. E. T.). Rad1 protein was found to be unstable and became inactivated by repeated freeze-thawing, and consequently it was always used from a freshly thawed stock. When complexed with Rad10, Rad1 was considerably more stable. Rad1 and Rad10 were therefore preincubated together on ice for 5 min before addition to the reactions. The two preparations gave very similar results. Escherichia coli RuvC protein (17) and human XPG protein (18) were purified as described elsewhere.

DNA Substrates—The bubble substrate was produced by annealing two 90-mer oligonucleotides. One oligonucleotide contains a central poly(C)30 region, whereas the other contains poly(T)30 to produce a centrally unpaired poly(C):poly(T) region of 30 nucleotides flanked by duplex DNA. The DNA sequences and method of preparation are described elsewhere (19). The poly(T)30-containing strand (oligo 2) was labeled with 32P at the 5’-end prior to annealing. Synthetic Holliday junction X12 was prepared as described previously (20).

Cleavage Assays—Reaction conditions are stated in the figure legends. 32P-Labeled DNA products were analyzed by denaturing 12% polyacrylamide gel electrophoresis using TBE buffer. The gels were dried onto filter paper, exposed to Kodak XAR5 film, and visualized by autoradiography.

RESULTS AND DISCUSSION

A key event in NER in eukaryotic cells is the introduction of dual incisions flanking a damaged site in DNA, leading to the

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*This work was supported by the Imperial Cancer Research Fund (to S. C. W. and R. D. W.), the National Institutes of Health (to E. C. F.), and the Council for Tobacco Research (to A. E. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: NER, nucleotide excision repair; SSA, single-strand annealing; BSA, bovine serum albumin.
excision of a DNA fragment about 30 nucleotides long (3). To investigate the incision step in more detail, we prepared a synthetic bubble structure, which contains a 30-nucleotide-long unpaired region flanked by duplex regions of similar length (Fig. 1). This model substrate is likely to resemble an intermediate in NER, after repair proteins have unwound and separated the DNA strands around the damaged region (19). When purified S. cerevisiae Rad1 and Rad10 proteins were incubated with a synthetic bubble structure in which one strand was 5'-32P-end labeled, we observed cleavage of the labeled strand at the 5'-side of the unpaired region, ~2 bases into the adjacent duplex DNA (Fig. 1, lanes b and c). No products were observed that corresponded to cleavage within the unpaired region or on the 3'-side. The polarity of cleavage is therefore the same as that observed previously with model DNA structures with single-stranded DNA tails with 3'-ends (8). However, the results presented here provide evidence that a free single-stranded DNA end is not required for structure-specific cleavage by Rad1-Rad10, indicating that the duplex 5'-single-strand junction is directly recognized by the endonuclease. Cleavage of the bubble structure required both proteins (Fig. 2, lane d), since neither Rad1 (lane e) nor Rad10 (lane f) alone had any effect on the bubble substrate.

In human cells, the XPG endonuclease makes the 3'-incision during nucleotide excision repair (19). When XPG was compared with Rad1-Rad10, we observed that the bubble substrate was nicked with a polarity opposite to that seen in reactions with the Rad1-Rad10 complex (Fig. 1, lanes e and f). When the bubble substrate was incubated with both XPG and the Rad1-Rad10 protein complex (Fig. 1, lane d), incision products were observed that corresponded to cleavage at the 3'- and 5'-sides of the bubble.

The S. cerevisiae homologue of the human XPG protein is Rad2 (21). Consistent with this, a truncated form of Rad2 has been shown to cleave model DNA structures with the same polarity as XPG (22). The dual incision reaction of NER in yeast is therefore likely to involve Rad2 and the Rad1-Rad10 complex, with the corresponding functions performed in humans by XPG and the ERCC1-ERCC4 protein complex (where ERCC1 is the homologue of Rad10 (23) and ERCC4 is the homologue of Rad12). Although the exact mechanics of damage recognition, DNA unwinding, and incision remain to be elucidated, the results presented here strengthen the view that a partially unwound or open complex around a DNA lesion is an intermediate in the NER process.

Genetic evidence indicates that, in addition to their role in NER, the Rad1 and Rad10 proteins are involved in a pathway of mitotic recombination (1, 4), possibly by playing a role in the resolution of recombination intermediates (such as Holliday junctions). Recent studies reported that the Rad1 protein (1-5 μg/ml) could resolve synthetic Holliday junctions by endonucleolytic cleavage (15). Since the structure of a Holliday intermediate is quite different from intermediates that occur during NER, we compared the activities of Rad1 and Rad1-Rad10 with RuvC protein, the E. coli Holliday junction resolvase (24, 25). Fig. 2 (lane d) shows that Rad1-Rad10 cut the bubble structure efficiently whereas RuvC protein was inactive on this substrate (lane e). We also compared the activities of Rad1-Rad10 and RuvC on a synthetic Holliday junction X12 (20). Resolution by RuvC is known to occur by the introduction of symmetrical related nicks at the site of the crossover (20). When the junction was 5'-32P-labeled in strand 2, we observed the nick in this strand by denaturing polyacrylamide gel electrophoresis (Fig. 3, lane h). However, using preparations of Rad1 produced in two different laboratories, we were unable to detect any evidence of nicking when the junction was incubated with 1.2-12.5 μg/ml purified Rad1 protein (Fig. 3, lanes b-e, and data not shown). Incubation of the junction with the Rad1-Rad10 complex also failed to yield cleavage products (Fig. 3, lane g). Experiments carried out with a different junction (X0 (20)) also provided negative results (data not shown). Furthermore, we could find no evidence that Rad1 or Rad10 could bind to the synthetic Holliday junctions, either individually or as a

2 L. Thompson, personal communication.
complex, as measured by bandshift assays (data not shown).

Taken together, these results show that under conditions in which the Rad1-Rad10 complex is highly active and cleaves 85% of the bubble structure (Fig. 3, lane i), it fails to nick the synthetic Holliday junction in a manner expected of a junction-specific resolvase. Though we cannot account for the ability of others to demonstrate Holliday junction resolution by Rad1 (15), it is known that synthetic Holliday junctions tend to "breathe" during incubation and may be prone to attack by contaminating single-strand endonucleases (26).

If Holliday junctions do not serve as targets for Rad1-Rad10, what role do these proteins play in recombination? Studies of budding yeast indicate that there are two pathways of double-strand break-induced recombination (27). One of these pathways (single-strand annealing or SSA) is utilized during recombination that involves direct repeats (12), which the Rad1-Rad10 complex is highly active and cleaves (12), who observed that Rad1-Rad10 fail to cleave synthetic Holliday junctions. Synthetic Holliday junction X12 (0.5 ng, 5′-32P-labeled on oligo 2) was incubated with Rad1 and Rad10 to show that the proteins were active. Reaction products were analyzed by denaturing 12% polyacrylamide gel electrophoresis and visualized by autoradiography.

The unwound region (modeled in the experiments described here by a synthetic bubble structure) is recognized by the Rad1-Rad10 complex and Rad2, which introduce structure-specific incisions at the 5′- and 3′-sides, respectively. In mammalian cells, similar cuts would be made by ERCC1-ERCC4 complex and XPG, respectively. Following incision and removal of the damage-containing digonucleotide, the gap would be filled by DNA synthesis by SSA, B model for the recombinational repair of double-strand breaks by SSA. Following the initiation of recombination by the introduction of a double-stranded break, the broken DNA ends are resected by the action of a 5′-3′-exonuclease. Complementary over-lapping 3′-ends then anneal, and Rad1-Rad10 trims off the 3′-ended unpaired single strands. The DNA is then repaired by synthesis and ligation, resulting in a deletion product in which sequences between two direct repeats have been lost.

The specific cleavage of DNA nucleotide excision repair intermediates (Fig. 4A).

Acknowledgments—We thank Iain Goldsmith and the Imperial Cancer Research Fund Oligonucleotide Synthesis Unit for making the oligonucleotides and John Nicolson for photography.

REFERENCES
1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, American Society for Microbiology, Washington, D. C.
2. Sancar, A., and Sancar, G. B. (1988) Annu. Rev. Biochem. 57, 29–68
3. Huang, J. C., Svedoba, D. L., Reardon, J. T., and Sancar, A. (1993) Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668
4. Kraka, S., Sung, P., and Kraka, R. (1992) Annu. Rev. Genet. 25, 33–70
5. Bailly, V., Sommers, C. H., Sung, P., Kraka, L., and Kraka, S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8273–8277
6. Bardwell, J. A., Cooper, A. J., and Friedberg, E. C. (1992) Mol. Cell. Biol. 12, 3041–3049
7. Bardwell, A. J., Bardwell, L. J., Johnson, D. K., and Friedberg, E. C. (1993) Mol. Microbiol. 8, 1117–1188
8. Bardwell, A. J., Bardwell, L., Tomkinson, A. E., and Friedberg, E. C. (1994) Science 265, 2082–2085
9. Schiestl, R. H., and Prakash, S. (1988) Mol. Cell. Biol. 8, 3619–3626
10. Schiestl, R. H., and Prakash, S. (1990) Mol. Cell. Biol. 10, 2485–2491
11. Bailis, A. M., Arthur, L., and Rothstein, R. (1992) Mol. Cell. Biol. 12, 4898–4903
12. Fishman-Lobell, J., and Haber, J. E. (1992) Science 258, 480–484
13. Zehfs, B. R., McWilliams, A. D., Lin, Y.-H., Hoekstra, M. F., and Keil, R. L. (1992) Genetics 126, 41–52
14. Thomas, B. J., and Rothstein, R. (1989) Genetics 123, 725–738
15. Habraken, Y., Sung, P., Kraka, L., and Kraka, S. (1994) Nature 371, 531–534
16. Tomkinson, A. E., Bardwell, A. J., Tappe, N., Ramos, W., and Friedberg, E. C. (1994) Biochemistry 33, 3505–3511
17. Dunderdale, H. J., Sharples, G. J., Lloyd, R. G., and West, S. C. (1994) J. Biol. Chem. 269, 5187–5194
18. O’Donovan, A., Scherly, D., Clarkson, S. G., and Wood, R. D. (1994) J. Biol.
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19. O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C., and Wood, R. D. (1994) Nature 371, 432–435
20. Bennett, R. J., Dunderdale, H. J., and West, S. C. (1993) Cell 74, 1021–1031
21. Scherly, D., Nouspikel, T., Corlet, J., Uda, C., Barroch, A., and Clarkson, S. G. (1993) Nature 363, 182–185
22. Harrington, J. J., and Lieber, M. R. (1994) Genes & Dev. 8, 1344–1355
23. van Duin, M., de Wit, J., Oeljik, H., Westerveld, A., Yasui, A., Koken, M. H. M., Hoeijmakers, J. H. J., and Bootsma, D. (1986) Cell 44, 913–923
24. Dunderdale, H. J., Benson, F. E., Parsons, C. A., Sharples, G. J., Lloyd, R. G., and West, S. C. (1991) Nature 354, 506–510
25. Iwasaki, H., Takahagi, M., Shiba, T., Nakata, A., and Shinagawa, H. (1991) EMBO J. 10, 4381–4389
26. West, S. C. (1993) Nature 363, 27–28
27. Fishman-Lobell, J., Rudin, N., and Haber, J. E. (1992) Mol. Cell. Biol. 12, 1292–1303
28. Klein, H. L. (1988) Genetics 120, 367–377
29. Ivanov, E. L., and Haber, J. E. (1993) Mol. Cell. Biol. 15, 2245–2251
30. Saffran, W. A., Greenberg, R. B., Thalerscheer, M. S., and Jones, M. M. (1994) Nucleic Acids Res. 22, 2823–2829
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*J. Biol. Chem.* 1995, 270:24638-24641.
doi: 10.1074/jbc.270.42.24638

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