In Vitro Reconstitution of the *Schizosaccharomyces pombe* Alternative Excision Repair Pathway†

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ABSTRACT: *Schizosaccharomyces pombe* alternative excision repair has been shown genetically and biochemically to be involved in the repair of a wide variety of DNA lesions. AER is initiated by a damage-specific endonuclease (Uve1p) that recognizes UV-induced photoproducts, base mispairs, abasic sites, and platinum G-G diadducts and cleaves the DNA phosphodiester backbone 5′ to a lesion. Several models exist that employ various mechanisms for damage removal based on the activities of Rad2p, a nuclease thought to be responsible for damage excision in AER. This study represents the first report of the biochemical reconstitution of the AER pathway. A base mispair-containing substrate is repaired in a reaction requiring *S. pombe* Uve1p, Rad2p, DNA polymerase *δ*, replication factor C, proliferating cell nuclear antigen, and T4 DNA ligase. Surprisingly, damage is removed exclusively by the 5′ to 3′ exonuclease activity of Rad2p and not its "flap endonuclease" activity and is absolutely dependent upon the presence of the 5′-phosphoryl moiety at the Uve1p cleavage site.

Modification is constantly incurred in cellular DNA from both endogenous and exogenous sources, and organisms must have efficient pathways to remove such damage in order to survive. If left unrepaired, many lesions can interfere with normal cellular processes and can lead to cell death, mutation, and in some cases neoplastic transformation. DNA repair pathways have evolved in order to allow a cell to maintain its genetic integrity in the face of constant chemical and physical insults to DNA. Ultraviolet radiation is a DNA-damaging agent that induces several types of lesions. Two frequently occurring, biologically relevant damages are the cyclobutane pyrimidine dimer (CPD)[1] and the 6-4 photoproduct (6-4 PP) (*1, 2*). Both of these major UV photoproducts can interfere with DNA replication and transcription (*3*−*5*). Several repair pathways have been previously identified that participate in the removal of CPDs and 6-4 PPs and include DNA photolyase-mediated direct reversal and two excision repair pathways, nucleotide excision repair (NER), and base excision repair (BER) (*6*−*8*). Most organisms possess several UV damage repair pathways, and there is significant variability among different species with respect to the combination of such systems they possess.

The NER pathway has been well characterized and is functionally highly conserved from *Escherichia coli* to humans. The discovery and characterization of the *E. coli* UvrABC proteins helped to define the mechanism of NER (*9, 10*). Lesions are recognized by a multiprotein complex, and excision is initiated by incisions both 5′ and 3′ to the damage, the exact positions varying depending on the organism. Damage is removed as a short oligomer, leaving a gap to be filled in by DNA polymerase and the remaining nick to be sealed by DNA ligase. NER has been reconstituted in vitro and has been shown to recognize a wide variety of damaged substrates (*11, 12*). Although many of these substrates are considered to be bulky, helix-distorting lesions, less distortive single base modifications as well as abasic sites are also recognized by NER (*13*−*15*).

In addition to NER, there is evidence for the existence of an additional excision repair pathway for the removal of both CPDs and 6-4 PPs in *Schizosaccharomyces pombe*. The first component of this pathway is an ATP-independent, damage-dependent endonuclease (UVDE or Uve1p) that makes an incision directly 5′ to either CPDs or 6-4 PPs (*16, 17*). The gene (*uve1*+) encoding Uve1p has been cloned, and mutational analysis indicates that *uve1* mutants in combination with NER mutants are highly UV sensitive compared to NER mutants alone, verifying the importance of Uve1p in the repair of UV photoproducts (*18, 19*). This pathway has been termed alternative excision repair (AER). The species distribution of AER is unknown, although *uve1*+ homo-

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[1] Abbreviations: 6-4 PP, 6-4 photoproduct; AER, alternative excision repair; BER, base excision repair; CPD, cyclobutane pyrimidine dimer; pol *δ*, DNA polymerase *δ*; Exo I, exonuclease I; FEN-1, flap endonuclease/5′ exonuclease; GST, glutathione *S*-transferase; NER, nucleotide excision repair; PCNA, proliferating cell nuclear antigen; RF-C, replication factor C.

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logues have been identified in *Neurospora crassa* and *Bacillus subtilis* (*18, 20*). Recent studies have revealed that the substrate specificity of Uve1p is much broader than originally suspected and includes mispaired bases, abasic sites, platinum G-G diadducts, and other base modifications (*21, 22*). Thus, it appears that AER may be involved in the reversal of a variety of DNA damage in addition to UV photoproducts. That *uve1* mutants display a spontaneous mutator phenotype in a forward mutation assay supports the notion that base mispairs are indeed good substrates for AER in vivo (*22*). Considering all base mispairs are not equally recognized and repaired by conventional mismatch repair, AER may represent a previously described minor repair pathway that is thought to repair all base mispairs (*23, 24*).

To date, most studies of the AER pathway have focused specifically on Uve1p, and little information exists on the steps after Uve1p-mediated DNA strand scission 5′ to the site of damage. It has been proposed that subsequent events include lesion removal by an endo- or exonucleolytic activity to create a gap that is filled in and ligated by DNA polymerase and ligase, respectively (*25*). Alternatively, strand displacement by DNA polymerase immediately following Uve1p-meidated incision and its subsequent removal by a flap endonuclease (executed by a FEN-1-type protein) have also been proposed as intermediate steps in the AER pathway (*19, 26*). The current model of AER is based on early models of NER, prior to the model detailing dual incisions, which focused primarily on damage excision and resynthesis by the 5′ to 3′ exonuclease and DNA polymerase activities of *E. coli* DNA pol I (*27*).

Genetic studies have implicated *S. pombe rad2*+ in the AER pathway (*19, 28*). *rad2*+ was cloned and identified as a member of the FEN-1 (flap endonuclease/5′ exonuclease) family of proteins which were initially identified as exonucleases essential for the completion of lagging strand DNA replication (*29*). Studies indicate that these proteins possess multiple nuclease activities including a structure-specific endonuclease activity and a 5′ to 3′ double-stranded DNA exonuclease activity (*26, 30*−*33*). More recently, FEN-1 homologues have been identified either genetically or biochemically as key components in several repair pathways including the DNA polymerase δ (pol δ) branch of BER (*34*−*39*). Considering the different nuclease activities that are associated with Rad2p, it is conceivable that it could participate in AER as either an exonuclease or an endonuclease (*26*).

To better understand the mechanism of AER and the specific role of Rad2p, experiments were designed to reconstitute AER in vitro. This system provides an ideal assay to study the biochemical aspects of the repair of a biologically relevant AER substrate, in addition to a novel mechanism for the repair of base mispairs, and may provide insights into the mechanism of repair of other AER substrates. The repair substrate used contained a C/A base mispair that we have previously shown to be efficiently recognized by Uve1p (*22*). The AER repair reaction contained *S. pombe* DNA pol δ and the accessory proteins replication factor C (RF-C) and proliferating cell nuclear antigen (PCNA) in addition to purified Uve1p, Rad2p and T4 DNA ligase. An unexpected finding was that damage is excised by the 5′ to 3′ exonuclease activity of Rad2p as opposed to its flap endonuclease activity and that such exonuclease activity was completely dependent upon the presence of the 5′-phosphoryl terminus created by Uve1p-mediated DNA strand scission near the site of the damage.

EXPERIMENTAL PROCEEDURES

Proteins, Enzymes, and Chemicals. S. pombe Rad2p and a truncated version of Uve1p (Δ228) were overexpressed and purified as described earlier (*26, 40*). Briefly, both proteins were expressed in *Saccharomyces cerevisiae* as N-terminal glutathione *S*-transferase (GST) fusion proteins. cDNAs were cloned into the pYEX-4T1 $CuSO_4$ inducible expression vector and expressed in DY150 cells. Proteins were purified by glutathione affinity chromatography. *S. pombe* pol δ, PCNA, and RF-C were purified as previously described (*41*). T4 DNA ligase, T4 polynucleotide kinase, and terminal transferase were purchased from Promega. [α-^{32}P]ddATP (3000 Ci/mmol), [α-^{32}P]dTTP (3000 Ci/mmol), [α-^{32}P]dATP (6000 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham. All other chemicals were of the highest grade commercially available.

Oligonucleotides. The following oligonucleotides were used in the construction of the various repair substrates and were synthesized by Integrated DNA Technologies, Inc.: oligo A: 5′ TAGGTCAAGCGTTAGCATGCCTGCACGAACTAAGCAATTCGTAATGCATTACAAGTCGCA 3′, oligo B: 5′ TGCGACTTGTAATGCATACGAATTGCTTAATTCGTGCAGGCATGCTAACGCTTGACCTA 3′, oligo C: 5′ TAGGTCAAGCGTTAGCATGCCTGCACGA 3′, oligo D: 5′ ACTAAGCAATTCGTAATGCATTACAAGTCGCA 3′. Oligo E represents oligo D with a modified 5′ end (phosphoryl moiety). Oligonucleotides were constructed such that the final repair substrate contained phosphorothioate linkages (3) terminating both ends of each strand (Figure 1).

Substrates. Oligonucleotides were electrophoresed on a denaturing 20% polyacrylamide gel, visualized by UV shadowing, eluted from the acrylamide in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and ethanol precipitated. Oligo D was 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (*42*). Oligos A, B, D, and E were 3′ end-labeled using terminal transferase and [α-^{32}P]ddATP (*43*). End-labeled or unlabeled oligonucleotides were annealed to the respective complementary strand in TE buffer plus 50 mM $MgCl_2$ by heating to 70 °C followed by slowly cooling to room temperature. Duplex DNA substrates were purified on a 20% nondenaturing polyacrylamide gel, purified as described above, resuspended in TE buffer, and stored at −20 °C. Oligos A and B were annealed to create oligo 60 C/A MM. Oligos B, C, and D were annealed to create oligo 60 C/A MM (Nick-5′ OH). Oligos B, C, and E were annealed to create oligo 60 C/A MM (Nick-5′ P).

Uve1p-Mediated DNA Cleavage Assays. A 0.3 pmol aliquot of oligo 60 C/A MM (either C- or A-containing strand 3′ end-labeled) was incubated with 300 ng of Uve1p in 20 mM Hepes, pH 6.5, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 100 mM NaCl for 30 min at 37 °C (10 μL reaction volume). Reaction mixtures were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. Labeled samples (∼10 000 cpm) were loaded on a 12% denaturing polyacrylamide gel and visualized by phosphorimager analysis (Molecular Dynamics) and autoradiography. Percent

FIGURE 1: In vitro repair substrates. (A) Oligo 60 C/A MM corresponds to the full-length C/A mispair-containing substrate. (B) Oligo 60 C/A MM (Nick-5′ P) represents the Uve1p cleavage product of oligo 60 C/A MM. (C) Oligo 60 C/A MM (Nick-5′ OH) represents a modification of oligo 60 C/A MM (Nick-5′ P) replacing the 5′-phosphoryl with a hydroxyl moiety. The asterisks indicate phosphorothioate linkages in the DNA backbone. The location of the C/A mispair is in boldface.

cleavage was expressed as the ratio of cleavage for each strand relative to total cleavage. Maxam and Gilbert base-specific chemical cleavage reaction products of the labeled strands were run on the gel for size markers.

Complete Repair Assays. A 0.5 pmol sample of unlabeled DNA substrate (oligo 60 C/A MM) was incubated with 300 ng of Uve1p in 20 mM Hepes, pH 6.5, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 100 mM NaCl (10 μL final volume) for 30 min at 37 °C. The reaction (final volume 50 μL) was adjusted to include 40 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, 166 μg/mL BSA, 20 μM dCTP, 20 μM dGTP, 10 μM dATP, 10 μM dTTP, 1 μCi of [α-^{32}P]dTTP, 1 μCi of [α-^{32}P]dATP, and various protein components (Rad2p, 5 μg; pol δ, 0.3 unit; RF-C, 0.26 unit; PCNA, 100 ng; and T4 DNA ligase, 10 units). The mixture was then incubated for an additional 90 min at 37 °C. Reaction mixtures were extracted with phenol/chloroform/isoamyl alcohol (25:24:1), the unincorporated radionucleotides removed using Micro-spin G-25 columns (Pharmacia), and the reaction products ethanol precipitated. Labeled samples were loaded onto a 12% denaturing polyacrylamide gel, and repair products were visualized by autoradiography.

Damage Excision Assays. A 100 fmol aliquot of 3′ end-labeled oligo 60 C/A MM (Nick-5′ OH) or oligo 60 C/A MM (Nick-5′ P) was incubated with various protein components in a 50 μL reaction containing 40 mM Tris-HCl, pH 7.8, 7 mM $MgCl_2$, 2 mM ATP, 0.5 mM DTT, 166 μg/mL BSA, and 20 μM of each dNTP for 90 min at 37 °C. Reaction mixtures were processed as described under Uve1p cleavage reactions and separated on a 20% denaturing polyacrylamide gel. Maxam and Gilbert base-specific chemical cleavage reaction products of a 3′ end-labeled oligo 60 C/A MM were run on the gel for size markers.

RESULTS

Biochemical Reconstitution of the Alternative Excision Repair System. The in vitro AER system was designed to test whether a specific combination of *S. pombe* proteins with known activities was capable of repairing an oligonucleotide substrate containing a Uve1p substrate. The repair substrate used in these assays was a 60mer duplex oligonucleotide (oligo 60 C/A MM) containing a central C/A base mispair that is efficiently cleaved in vitro by Uve1p (Figure 1A). Because Uve1p had been previously shown to cleave all 12 base mispair combinations to various extents, it was important to determine the relative efficiency of cleavage on both strands of oligo 60 C/A MM which was used in subsequent repair assays. Either the C- or the A-containing strand was 3′ end-labeled, annealed to the respective complementary strand, and incubated with Uve1p. Uve1p is capable of cleaving only one strand of a base mispair-containing substrate, producing a population of nicked duplex oligonucleotides with approximately 80% containing the nick on the C strand only and approximately 20% containing the nick on the A strand only [(*22*) and Figure 2A].

Complete repair reactions contained *S. pombe* Uve1p, Rad2p, DNA pol δ, PCNA, RF-C, T4 DNA ligase, a damage-containing duplex oligonucleotide (Figure 1A), and [^{32}P]dNTPs. Repair products should contain labeled patches of DNA and were analyzed following denaturing gel electrophoresis and autoradiography. Repair reactions that contained all of the components generated a full-length, labeled 60mer (Figure 2B, lane 5). One interpretation of this result is that a full-length product may be produced by pol δ-mediated strand displacement synthesis (immediately following Uve1p-mediated DNA strand cleavage) as opposed to Rad2p-mediated excision followed by fill-in and ligation of a small gap. When Rad2p is omitted from the reaction (Figure 2B, lane 3), the band representing full-length product observed in lane 5 was not detected. This indicates that the formation of this product is completely dependent on the presence of Rad2p and it is not the result of strand displacement synthesis by pol δ. Production of labeled 60mer product was absolutely dependent on the presence of Uve1p, Rad2p, pol δ, PCNA, RF-C, and DNA ligase. In the absence of either PCNA or RF-C, pol δ did not incorporate nucleotides into a substrate containing a seven-nucleotide gap (data not shown). Reactions containing pol δ and Rad2p produced a series of products ranging in size from 29 to 36 nucleotides (Figure 2B, lanes 4 and 5). These products represented the portion of substrate in which a short DNA segment containing the C/A mispair had been removed and replaced with a pol δ-dependent repair patch approximately 1−7 nucleotides in length. As Uve1p cleaves the C-containing strand of the mispair almost exclusively, the repair intermediates observed in lanes 4 and 5 are indicative of nucleotides incorporated into the repair patch as indicated in boldface (Figure 2B). When T4 DNA ligase was included in the reactions, a product was produced that migrated with full-length 60mer representing a completely repaired duplex oligonucleotide. Repair was dependent upon the presence of the C/A base mispair; similar experiments using a substrate

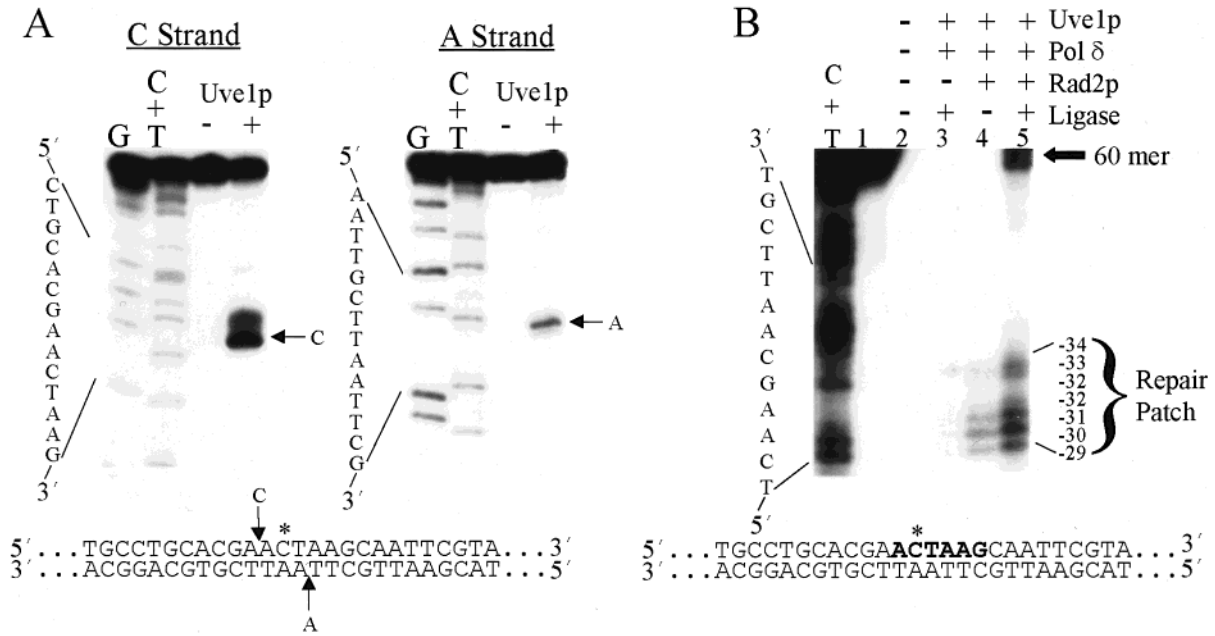

FIGURE 2: (A) Determination of the major Uve1p cleavage site of 3′ end-labeled oligo 60 C/A MM. 0.3 pmol of oligo 60 C/A MM (C-containing strand labeled on the left; A-containing strand on the right) was incubated with 300 ng of Uve1p for 30 min at 37 °C. The Uve1p cleavage sites for each strand are indicated by arrows to the right and below the gel and designated C and A for each respective strand. Uve1p cleavage of the C-containing strand of oligo 60 C/A MM represents approximately 80% of the total cleavage while the A-containing strand is cleaved approximately 20%. (B) In vitro repair of a C/A base mispair-containing substrate. Oligo 60 C/A MM was incubated with the various reaction components indicated above the gel for a total of 120 min at 37 °C. The DNA sequence of the substrate in the vicinity of the repair patch is indicated below the gel. The repair patch intermediates and the corresponding nucleotide position (relative to the 5′ end) are indicated to the right of the gel with the repair patch nucleotide sequence in boldface below the gel. The arrow represents the full-length, ligated repair product (60mer). The left lane contains the C+T base-specific chemical cleavage ladder with the corresponding sequence indicated to the left of the gel. Reaction products were separated on a 12% denaturing polyacrylamide gel and visualized by autoradiography. The asterisk indicates the location of the C/A mispair.

in which a C/G base pair replaced the C/A mispair failed to produce repair products (data not shown). The assay was designed to account for optimal activity conditions for all of the proteins used (*40*, *41*, and data not shown). Although data presented here follow a two-step reaction procedure with the incision step utilizing a buffer with lower pH and higher NaCl concentrations, subsequent experiments have shown that 60 C/A MM is efficiently processed by all proteins used in these assays under the same buffer conditions (data not shown). All subsequent assays utilized an end-labeled oligonucleotide representing a Uve1p-cleaved substrate to allow for a straightforward, direct comparison between individual reaction components (Figure 1B,C).

Damage Is Excised by the 5′ to 3′ Exonuclease Activity of Rad2p and Not by Its Flap Endonuclease Activity. To further delineate the mechanism of AER, it was necessary to determine the nature of the Rad2p nuclease activity in the repair assays. A 3′ end-labeled substrate was generated [oligo 60 C/A MM (Nick-5′ P)] that resembled the major Uve1p-mediated DNA cleavage product at the site of a C/A mispair and contained 3′-hydroxyl and 5′-phosphoryl termini following DNA strand scission (Figure 1B). This substrate was utilized to distinguish between the 5′ to 3′ exonuclease and flap endonuclease activities of Rad2p in the repair reactions. When oligo 60 C/A MM (Nick-5′ P) was incubated with Rad2p alone (Figure 3A, lane 2), a series of products was observed which ranged from 1 to 6 nucleotides shorter that the fragment length corresponding to the nick immediately 5′ to the base mispair site. This result indicates that Rad2p processes this substrate via a 5′ to 3′ exonuclease and not via a flap endonuclease, which would have resulted in a single cleavage product visualized as one band on the gel. A range of 1−6 nucleotides was removed, which corresponded to the size of the repair patch (Figure 2B). Similar reactions were carried out in the presence of pol *δ*/PCNA/RF-C to determine if this substrate was processed in a similar manner under conditions where DNA polymerase might cause strand displacement and result in subsequent Rad2p-mediated flap endonuclease activity (as evidenced as a single gel band). In the presence of pol *δ*, the Rad2p exonuclease-mediated degradation products observed in lane 2 were still present (Figure 3A, lane 3), suggesting that any pol *δ*-mediated strand displacement that may have occurred did not have any effect on the 5′ to 3′ exonuclease activity of Rad2p. However, these experiments do not rule out the possibility that upon addition of Rad2p to the reaction, its inherent exonuclease activity may interfere with the ability of DNA polymerase to extend the upstream strand and may obscure flap endonuclease cleavage that could be observed in the reaction. To address this possibility, assays were performed in which the order of addition of reaction components was varied. Under conditions where oligo 60 C/A MM (Nick-5′ P) was incubated initially with either Rad2p (Figure 3A, lane 4) or pol *δ*/PCNA/RF-C (Figure 3A, lane 5), the only substrate processing observed was Rad2p-mediated 5′ to 3′ exonuclease degradation, similar to the previous observations (Figure 3A, lane 2). This result indicates that under conditions that favor strand displacement synthesis and subsequent 5′ flap formation, the only activity of Rad2p on the substrate is the 5′ to 3′ exonuclease activity and not flap endonuclease activity. The pattern of Rad2p-mediated substrate degradation, shown here for a Uve1p-

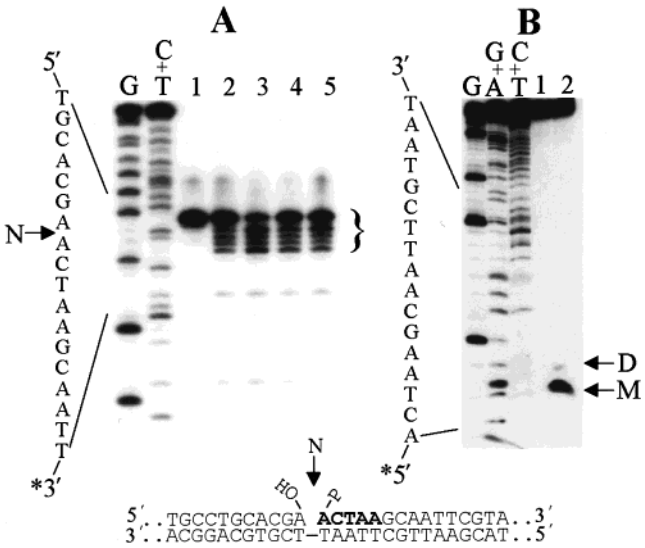

FIGURE 3: Damage excision by the 5′ to 3′ exonuclease activity of Rad2p. (A) 100 fmol of 3′ end-labeled oligo 60 C/A MM (Nick-5′ P) was incubated with either Rad2p alone or Rad2p in combination with pol *δ*/PCNA/RF-C for 90 min at 37 °C. The left lanes indicate Maxam and Gilbert sequencing reaction products of 3′ end-labeled oligo 60 C/A MM with the corresponding sequence listed to the left of the gel. Lane 1, no treatment; lane 2, Rad2p; lane 3, Rad2p/pol *δ*/PCNA/RF-C; lane 4, Rad2p for 45 min followed by the addition of pol *δ*/PCNA/RF-C for the remaining 45 min; lane 5, pol *δ*/PCNA/RF-C for 45 min followed by Rad2p for the remaining 45 min. The sequence of the full-length 60mer is indicated to the left of the gel. Rad2p-mediated cleavage products (~6 nucleotides) are indicated in boldface in the partial substrate sequence below the gel. The arrows to the left and bottom of the gel (N) indicate the position of the nick. (B) Oligo 60 C/A MM (Nick-5′ P) was constructed with a ^{32}P-labeled phosphoryl moiety at the site of the nick to confirm Rad2p-mediated exonucleolytic degradation. Lane 1, no treatment; lane 2, Rad2p. The positions of the mononucleotide (M) and dinucleotide (D) are indicated to the right of the gel. The sequence of the labeled strand is indicated to the left of the gel. Reaction products were separated on a 20% denaturing polyacrylamide gel and visualized by electrophoresis.

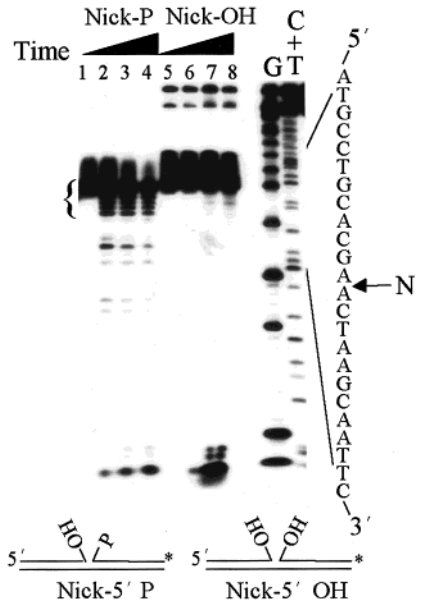

FIGURE 4: Efficient degradation by Rad2p is dependent upon a 5′-phosphoryl moiety at the Uve1p cleavage site. 100 fmol of 3′ end-labeled oligo 60 C/A MM (Nick-5′ P) (lanes 1−4) or oligo 60 C/A MM (Nick-5′ OH) (lanes 5−8) was incubated with Rad2p for 0, 30, 60, or 90 min. The arrow to the right of the gel (N) indicates the position of the nick. The sequence of the full-length 60mer is indicated to the right of the gel. Reaction products were processed and visualized as described in Figure 3.

incised base mispair-containing substrate, is similar to recently reported data for Rad2p using a Uve1p-incised CPD-containing substrate (*44*).

It is possible that although the Rad2p-mediated processing of oligo 60 C/A MM (Nick-5′ P) observed in Figure 3A was characteristic of a processive exonuclease activity, the pattern of degradation could represent a nested set of endonucleolytic cleavage products of varying sizes. To more conclusively show whether Rad2p was acting as an exonuclease or endonuclease, a substrate with a ^{32}P-labeled 5′ terminus (the 5′ terminus of the nick) was utilized. For this substrate, exonuclease activity would be revealed as a single band one nucleotide in length whereas endonuclease activity could be distinguished by a series of bands similar to that observed in Figure 3A. Rad2p treatment of this substrate resulted in one major cleavage product that was one nucleotide in length (Figure 3B, lane 2). In addition, there was a minor band corresponding to a dinucleotide. This is consistent with previous reports that the 5′ to 3′exonuclease activity of Rad2p homologues (FEN-1 proteins) has been shown to remove longer segments of DNA in addition to mononucleotides (*45*). We conclude that the Rad2p-mediated degradation of oligo 60 C/A MM (Nick-5′ P) is in fact due to its 5′ to 3′ exonuclease and not its flap endonuclease activity.

5′ to 3′ Exonuclease Degradation by Rad2p Is Dependent upon a 5′-Phosphoryl Moiety at the Uve1p Cleavage Site. The possibility that the nature of the 5′ terminus may have a significant effect on the DNA damage processing activities of FEN-1 proteins such as Rad2p was addressed. The 5′ terminus of oligo 60 C/A MM (Nick-5′ P) contains a phosphoryl moiety that is replaced with a hydroxyl in oligo 60 C/A MM (Nick-5′ OH). With increasing time (from 0 to 90 min), treatment of the phosphoryl-containing oligo 60 C/A MM (Nick-5′ P) substrate resulted in a pattern of exonucleolytic degradation products similar to those previously observed for Rad2p (Figure 4, lanes 1−4). Replacing the 5′-phosphoryl terminus with a hydroxyl group significantly altered Rad2p processing of the substrate (Figure 4, lanes 5−8). Although there was evidence for 5′ to 3′ Rad2p-mediated exonucleolytic degradation of oligo 60 C/A MM (Nick-5′ OH), as demonstrated by the presence of fast migrating reaction products, the overall efficiency of cleavage was greatly decreased. The pattern of degradation was altered, and the removal of the first 1−6 nucleotides was no longer observed. This result demonstrates the importance of the phosphoryl moiety for Rad2p-mediated exonucleolytic degradation. It is not clear, however, how the 5′-terminal phosphoryl moiety alters Rad2p activity. The finding that the most efficient substrate tested for the 5′ to 3′ exonuclease activity of Rad2p corresponded to a Uve1p-mediated DNA cleavage product provides direct biochemical support for the notion that Rad2p is involved in Uve1p-mediated excision repair.

DISCUSSION

This study represents the first report of a complete biochemical reconstitution of the *Schizosaccharomyces pombe* AER pathway. Based on both genetic and biochemical

analyses of the repair-initiating protein Uve1p, AER is a versatile mechanism by which cells can remove a variety of DNA lesions to include UV photoproducts, abasic sites, and base−base mispairs. Based on the known properties of proteins thought to be involved in this pathway, assays were designed to reconstitute repair of a C/A base mispair-containing substrate. Under the assay conditions used here, repair was dependent upon the presence of a damaged substrate, Uve1p, Rad2p, pol δ, PCNA, RF-C, and T4 DNA ligase with a resulting repair patch of approximately 1−7 nucleotides in length (Figure 2B and data not shown). In addition to conventional mismatch repair, *S. pombe* is thought to possess an additional, less efficient repair system responsible for correcting all combinations of base mispairs with a resulting repair patch of approximately 10 bases (*23*, *24*). Data presented here are consistent with the notion that this system and base mispair removal by AER may represent the same pathway or may represent a third, yet uncharacterized, pathway for the removal of base mispairs.

The gene for *S. pombe* DNA ligase (*cdc17*+) has been cloned and sequenced (*46*), but the corresponding protein has yet to be expressed and characterized. In our studies, T4 DNA ligase was substituted for *S. pombe* DNA ligase, and its utility has been demonstrated as a ligase component of other in vitro reconstituted excision repair systems (*47*, *48*). A major objective of this investigation was to study the mechanism of Rad2p-mediated damage excision during AER as opposed to the ligation step, although the absolute effect of employing T4 DNA ligase on this system is currently unknown. It should be noted that a recent study reports that human DNA ligase I may influence the size of the repair patch produced during BER (*49*).

Although genetic analysis has provided some insight into the components required for AER (*19*, *50*), previous studies have not addressed the biochemistry of the repair components. The current model for AER is shown in Figure 5. Following Uve1p cleavage 5′ to the damage site, there are two possibilities for damage excision. The nick could be processed first by DNA polymerase, extending the 3′ end of the upstream strand, leaving a displaced strand containing the original damage. Rad2p could subsequently cleave this 5′ flap structure at the branch point, releasing a damage-containing oligomer. Alternatively, the damage could be excised directly by Rad2p as a 5′ to 3′ exonuclease, leaving a gap to be resynthesized by DNA polymerase and the DNA backbone sealed by DNA ligase.

Several results presented in this study help to elucidate the mode of damage excision employed by AER. By using a 3′ end-labeled substrate identical to a Uve1p-mediated DNA cleavage product, subsequent processing steps could be analyzed. The only Rad2p-mediated substrate processing observed was 5′ to 3′ exonucleolytic degradation at the Uve1p cleavage site, an activity that was completely dependent on the presence of a 5′-terminal phosphoryl group. Rad2p removed a short segment of DNA from the substrate, leaving a gap that corresponded precisely in size to that of the repair patch for the completely reconstituted system (Figure 2B). When reactions proceeded under conditions that favored the formation of a 5′ flap-containing intermediate prior to Rad2p processing, there was no evidence for flap endonuclease cleavage (Figure 3A, lanes 4 and 5). We have previously demonstrated that the recombinant version of

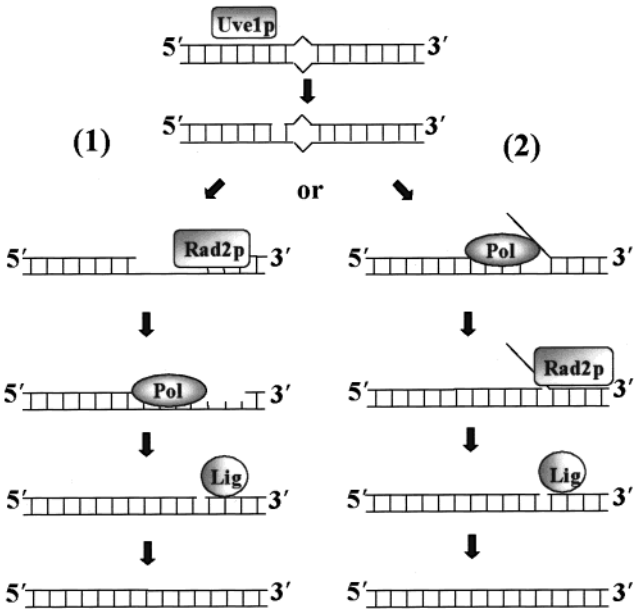

FIGURE 5: Model of alternative excision repair. Repair is initiated by cleavage 5′ to a damage (indicated here as a mispair but could also be any Uve1p-containing substrate such as a CPD) by Uve1p. Pathway 1 depicts the direct removal of the lesion by the 5′ to 3′ exonuclease activity of Rad2p, resulting in a gap. This gap is filled in by pol δ with accessory factors PCNA and RF-C and the DNA backbone sealed by DNA ligase. Alternatively, DNA polymerase could extend the free 3′ terminus at the Uve1p cleavage site initially, allowing for the formation of a displaced 5′ flap to be cleaved by Rad2p as a flap endonuclease, followed by ligation (pathway 2). Under the repair conditions used in this study, the mechanism of repair of a base mispair-containing substrate is similar to that of pathway 1.

Rad2p is able to cleave a 5′ flap substrate (*26*). It should also be emphasized that there is no detectable strand displacement synthesis by pol δ under our conditions in the reconstituted AER system as evidenced by the lack of incorporation of radiolabeled nucleotides in a Uve1p-cleaved substrate in the absence of Rad2p (Figure 2B, lane 3) nor is there extension of a 5′ end-labeled nicked substrate (data not shown). The lack of strand displacement synthesis may be a reflection of the low molar ratio of pol δ to DNA template. The release of mononucleotides almost exclusively by Rad2p (Figure 3B) confirms that the removal of damage is via the 5′ to 3′ exonuclease activity of Rad2p and not a flap endonuclease activity. It has been reported that Rad2p alone is sufficient to process a Uve1p-cleaved CPD-containing substrate (*44*). The different substrates used in our study compared to that of Yoon et al. (varying the location of the ^{32}P label) may account for the different interpretations with respect to the nature of Rad2p nuclease activity. Under all reaction conditions used in this study, Rad2p processes a Uve1p-mediated DNA cleavage product by 5′ to 3′ exonucleolytic degradation of 1−6 nucleotides, demonstrating that the "second" nuclease activity of FEN-1 proteins is the critical function for damage excision during repair in vitro. This exonucleolytic degradation specifically requires a 5′-phosphoryl moiety, and this is the first time this has been reported for a yeast FEN-1 protein and may be relevant for FEN-1 activity during other DNA repair or nucleic acid processing events. Similarly, lambda and calf 5′ to 3′ exonucleases have also been shown to require a 5′-phosphoryl moiety for its 5′ to 3′ exonuclease activity (*51*, *52*).

The substrates utilized to examine the mechanism of AER in this study represent biologically relevant DNA intermediates that would be encountered by DNA repair enzymes in vivo. The Rad2p 5′ to 3′ exonuclease demonstrated here in vitro is thus likely to be an accurate reflection of damage excision during AER in vivo.

There are similarities between the proposed models for AER and long-patch BER with respect to possible mechanisms of damage removal. It has been proposed that Rad2p-like proteins function as flap endonucleases in long-patch BER (*35*). However, our results indicate that damage excision during AER is clearly through the 5′ to 3′ exonuclease activity of Rad2p. The type of nuclease activity utilized during repair may be dictated by the nature of the DNA structural intermediates formed during various stages of the different repair pathways. AP endonuclease cleavage of an abasic site during BER results in the generation of a 5′-terminal sugar moiety while Uve1p cleavage of an abasic site results in a 5′-phosphoryl moiety (*16*, *22*, *53*, *54*). These subtle differences in DNA termini may greatly impact the nuclease activity of Rad2-like proteins; however, it is currently unknown whether Rad2p will directly process an abasic site that has been cleaved by either Uve1p or an AP endonuclease.

There is evidence for Rad2p-independent repair of Uve1p-mediated cleavage products (*19*). Other proteins may function as a 5′ to 3′ exonuclease in AER replacing the requirement for Rad2p. Exonuclease I (Exo I) is a 5′ to 3′ exonuclease that shares homology with FEN-1 proteins and may be one possible candidate for such a nuclease. Genetic evidence suggests that Exo I may participate in excision repair and recombination, although it is currently unknown whether it will process a Uve1p-cleaved substrate (*55*, *56*). In addition, *rad18*+ and *rhp51*+, both of which are thought to be involved in recombination, have been identified as putative components of AER and may be recruited under certain conditions where Rad2p may not be available (*50*).

Although Uve1p homologues have not yet been identified in a large number of organisms, the other required components of AER identified in this study are functionally conserved from *E. coli* to humans. It has been shown that the introduction of Uve1p in *E. coli* (which has no Uve1p sequence homologue) is sufficient to complement the UV sensitivity of a NER mutant, suggesting that the presence of Uve1p will initiate AER and thus provide an organism with additional repair capabilities (*18*). Complementation of UV sensitivity by Uve1p has also been demonstrated for XP cell lines (*18*). Considering the broad substrate specificity of Uve1p, it is reasonable to consider the therapeutic potential of restoring repair capacities of otherwise repair-deficient cells by inducing the multi-lesion repair capabilities of AER.

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