Processing of UV Damage in Vitro by FEN-1 Proteins as Part of an Alternative DNA Excision Repair Pathway†

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Received January 19, 1999; Revised Manuscript Received February 17, 1999

ABSTRACT: Ultraviolet (UV) irradiation induces predominantly cyclobutane and (6-4) pyrimidine dimer photoproducts in DNA. Several mechanisms for repairing these mutagenic UV-induced DNA lesions have been identified. Nucleotide excision repair is a major pathway, but mechanisms involving photolyases and DNA glycosylases have also been characterized. Recently, a novel UV damage endonuclease (UVDE) was identified that initiates an excision repair pathway different from previously established repair mechanisms. Homologues of UVDE have been found in eukaryotes as well as in bacteria. In this report, we have used oligonucleotide substrates containing site-specific cyclobutane pyrimidine dimers and (6-4) photoproducts for the characterization of this UV damage repair pathway. After introduction of single-strand breaks at the 5′ sides of the photolesions by UVDE, these intermediates became substrates for cleavage by flap endonucleases (FEN-1 proteins). FEN-1 homologues from humans, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* all cleaved the UVDE-nicked substrates at similar positions 3′ to the photolesions. T4 endonuclease V-incised DNA was processed in the same way. Both nicked and flapped DNA substrates with photolesions (the latter may be intermediates in DNA polymerase-catalyzed strand displacement synthesis) were cleaved by FEN-1. The data suggest that the two enzymatic activities, UVDE and FEN-1, are part of an alternative excision repair pathway for repair of UV photoproducts.

Ultraviolet light produces several types of mutagenic DNA photoproducts (*1*). The two most frequent lesions induced by UVB (290−320 nm) and UVC (190−290 nm) irradiation are the cis-syn cyclobutane pyrimidine dimers (CPDs)[1] and the pyrimidine (6-4) pyrimidone photoproducts [(6-4) photoproducts [(6-4)PPs]].

Several DNA repair pathways for removing these DNA photoproducts exist. A photoreactivation mechanism directly reverts the dimeric photoproducts back to the original bases. This process is carried out by DNA photolyases, which are specific for either CPDs or (6-4)PPs (*2, 3*). Photoreactivating enzymes exist in prokaryotes and lower eukaryotes, and are found in several, but not all, vertebrate species (*4*). The most versatile DNA repair mechanism, nucleotide excision repair (NER), is present in almost all organisms, and is capable of removing both CPDs and (6-4)PPs (*1*). The (6-4) photoproduct is recognized much more efficiently by NER than the CPD (*5*). NER is responsible for the repair of not only UV photoproducts but also a variety of other DNA damage types, including bulky lesions and cross-links (*6*−*8*).

A specialized repair pathway for removal of UV photoproducts involves UV-specific endonucleases. It has long been known that *Micrococcus luteus* and phage T4-infected *Escherichia coli* contain a DNA glycosylase/AP lyase that specifically recognizes CPDs and catalyzes a two-step DNA incision process at the sites of these dimers (*9*−*11*). A similar activity was found in *Saccharomyces cerevisiae* (*12*). These enzymatic activities are considered to be part of a base excision repair pathway. The completion of this pathway has not been extensively studied. Transfection of the T4 endonuclease V gene into repair-deficient rodent or human cells can partially restore UV resistance (*13*, *14*). This suggests that T4 endonuclease V-incised lesions can be processed in mammalian cells, presumably by a pathway that involves removal of the remaining damaged nucleotides as well as gap filling and ligation.

An alternative DNA excision repair pathway for UV damage has been identified more recently. An enzymatic activity, originally found in *Schizosaccharomyces pombe*, recognizes both CPDs and (6-4)PPs and incises the damaged DNA immediately 5′ to the UV photoproducts (*15*−*18*). A similar activity, UV damage endonuclease (UVDE), exists

† This work was supported by grants from the National Institute of Environmental Health Sciences (ES06070 to G.P.P.) and the National Cancer Institute (CA73764 to B.S.).

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[1] Abbreviations: CPD, cis-syn cyclobutane pyrimidine dimer; (6-4)PP, pyrimidine (6-4) pyrimidone photoproduct; UVDE, UV damage endonuclease; FEN-1, flap endonuclease-1; LMPCR, ligation-mediated PCR.

10.1021/bi990105i CCC: $18.00
Published on Web 03/24/1999

in *Neurospora crassa*, and the gene has been cloned from this organism (*17*) as well as from *S. pombe* (*18*). The mechanism that enables further processing of these incised UV-damaged DNA fragments has not yet been elucidated. Genetic evidence indicates that the product of the *S. pombe rad2* gene, a FEN-1 homologue, may be involved in this repair pathway (*19*).

FEN-1 homologues (flap endonucleases) were first purified as exonucleases (*20*) and were later found to operate during DNA replication where they are thought to function during processing of Okazaki fragments (*21*). FEN-1 proteins recognize a 5′-displaced DNA fragment termed a flap structure (*22*−*26*). These enzymes appear to have an additional function in DNA repair. Lindahl (*27*) described the degradation of UV-damaged DNA by the mammalian FEN-1 homologue DNaseIV. Mutants of FEN-1 homologues in *Sa. cerevisiae* (*RAD27*) and *S. pombe* (*rad2*) are sensitive to DNA-damaging agents, and have an increased spontaneous mutation rate (*28*−*30*). FEN-1 proteins possess two types of enzymatic activity: a 5′−3′ double-stranded DNA exonuclease activity which seems to operate preferentially from nicks or gaps and an endonuclease activity that cleaves DNA near the branch point of a 5′ flap structure (*22*−*25*, *31*, *32*).

Various models have been proposed for the processing of UVDE incision intermediates (*4*, *33*, *34*). This repair pathway may involve a 5′−3′ exonuclase or an endonuclease activity that removes the nucleotides containing the UV photolesion. DNA polymerase may extend from the nick and displace the photoproduct-containing DNA to produce a 5′ flap structure. Alternatively, an endonucleolytic activity may act directly on the UVDE-nicked damage-containing DNA to produce an incision 3′ to the damage so that the damage could be removed in the form of a short oligonucleotide. However, biochemical assays have not yet been used to identify such an activity. Here, we show that DNA fragments containing UVDE-incised site-specific CPDs or (6-4)PPs are substrates for direct endonucleolytic processing by flap endonucleases and can also be cleaved as part of a flap structure. The two enzymatic reactions (UVDE and FEN-1) may combine to be part of an alternative excision repair pathway for repair of UV photoproducts.

MATERIALS AND METHODS

Oligonucleotides Containing Site-Specific Cyclobutane Pyrimidine Dimers and (6-4) Photoproducts. The synthesis of an oligonucleotide containing a site-specific cis-syn CPD by phosphoramidite chemistry was carried out as described previously (*35*). The sequence was 5′-GCCTTCCCAT^-TGGCTCGCGTCGC, where T^T indicates the position of the dimer. The purified oligomer was phosphorylated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and annealed to an unlabeled oligonucleotide representing the opposite strand. To confirm the presence of a CPD, the duplex DNA was incubated at 37 °C for 30 min with 0.075 μg of T4 endonuclease V in 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 100 μg/mL bovine serum albumin. The cleavage products were separated on 20% denaturing polyacrylamide gels.

The same oligonucleotide containing a (6-4) photoproduct instead of a CPD was synthesized according to the method of Iwai et al. (*36*) with some modifications. The 3′-levulinyl thymidyl (3′−5′) thymidine 2-cyanoethyl phosphotriester was prepared as previously described (*37*). A 1 mM solution (230 mL) of this compound in 15% aqueous acetonitrile was poured into a tray (30 cm × 36 cm) and frozen. The tray was then placed into a UV cross-linker (Stratagene) and irradiated at 1200 J m^{-2} min^{-1} for 20 min. These steps were repeated until the total time of irradiation reached 2 h. After irradiation, the acetonitrile was removed under vacuum and the remaining solution was passed through a HPLC PRP-1 (Hamilton) column (1 cm × 30 cm). The elution of the adsorbed material was performed with a linear gradient of 5 to 35% acetonitrile in water, resulting in separation of the product from the substrate. Fractions were analyzed by reverse-phase HPLC on a C18 column in a 10 to 20% acetonitrile/water gradient. Fractions containing product with a UV absorption maximum at 326 nm were collected and evaporated to dryness. The desired product contained 5−10% of the contaminating material that was impossible to separate by HPLC on either C18 or PRP-1 columns. This material was visualized during HPLC by using two flow-through UV detectors in series each tuned to a different wavelength, the first set at 326 nm (the absorption of the desired product) and the second at 265 nm (the absorption of the contaminating material). The contaminating material was removed by purification on an open column, on silica gel H60 (Fluka) in a gradient of 3 to 10% methanol in dichloromethane. The yield averaged 15%. The rest of the synthesis was carried out as described in the Supporting Information of Iwai et al. (*36*). The identity of the phosphoramidite containing the (6-4)PP and its presence in the oligonucleotide were confirmed initially by its fluorescence emission spectrum with a maximum at 400 nm (*38*). In addition, the sensitivity of the double-stranded oligonucleotide to UVDE and its alkali lability detected by heating in 1 M piperidine followed by electrophoresis through 20% polyacrylamide gels also prove that this photolesion had been introduced.

Oligonucleotides with 5′-displaced flap structures were prepared as follows. The 18-mer control oligonucleotide without photoproduct (5′-TTGGCTCGCGTCGCTCTC) was created by 3′ end labeling on a template strand. This oligonucleotide contained a 5′-phosphate group. The same sequence with a 5′-TT CPD was produced by UVDE cleavage of the annealed CPD oligonucleotide 5′-GCCTT-CCCAT^TGGCTCGCGTCGCTCTC on a complementary strand after 3′ end labeling. These 18-mer UVDE cleavage products were gel purified. After upstream primers UP1 (5′-CCCACCTCGCCTTCCCA) or UP2 (5′-CCCACCTCGC-CTTCCCATTGGC) were annealed to the complementary strand, the 18-mers were annealed subsequently to create the nicked and flapped substrates, respectively. The complementary strand was the 35-mer 5′-GAGAGCGACGC-GAGCCAATGGGAAGGCGAGGTGGG.

UVDE Overexpression and Purification. Recombinant *N. crassa* UVDE protein was purified as follows. *E. coli* cells transformed with pFNcDE (an expression construct with a *tac* promotor in front of the *N. crassa uvde* gene) were grown until the OD_{600} equaled 0.6−0.9 before adding 1 mM IPTG (final concentration) to the cell culture. After incubation for 4−5 h, cells were harvested and a cell lysate was obtained by sonication of the cell pellet in a lysis buffer [20 mM Tris-HCl and 100 mM NaCl (pH 7.5)] containing 1 mM phenylmethanesulfonyl fluoride. Cell extracts were first

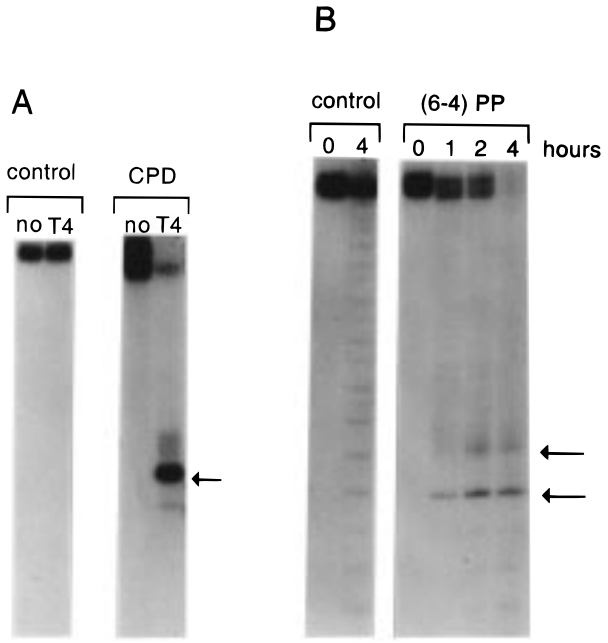

FIGURE 1: Characterization of oligonucleotides containing UV photoproducts. (A) Oligonucleotide with a cis-syn cyclobutane pyrimidine dimer (CPD). Double-stranded control and CPD oligonucleotides were incubated with T4 endonuclease V after 5′ end labeling. The cleavage products were separated on a 20% polyacrylamide gel. (B) Oligonucleotide containing a (6-4) photoproduct. Control and (6-4) photoproduct oligonucleotides were heated in 1 M piperidine at 90 °C for various periods of time after 5′ end labeling. The major piperidine cleavage products (9−11 nucleotides in length) are denoted by arrows.

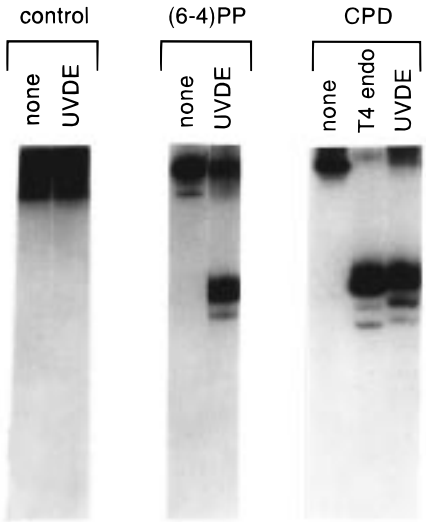

FIGURE 2: Reaction of UVDE with oligonucleotides containing photolesions. Double-stranded control, (6-4) photoproduct, and CPD oligonucleotides were incubated with UVDE or T4 endonuclease V after 3′ end labeling and separated on 20% polyacrylamide gels.

applied to heparin−Sepharose. Substantial activity of UVDE was recovered in 500 mM NaCl from the column. Since the *N. crassa* UVDE contains several consecutive histidine residues, UVDE can be further purified by applying the heparin fraction onto a Ni−NTA agarose column. The eluate was then applied onto Q-Sepharose, yielding >95% pure UVDE.

Flap Endonuclease Overexpression and Purification. Human flap endonuclease-1 was overexpressed and purified

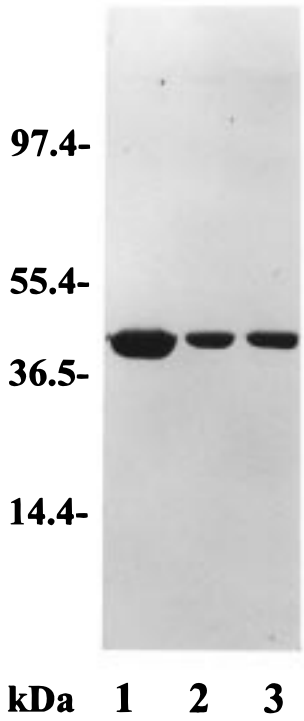

FIGURE 3: SDS−polyacrylamide analysis of purified FEN-1 proteins. Human FEN-1 (lane 1), *S. pombe* Rad2 protein (lane 2), and *Sa. cerevisiae* Rad27 protein (lane 3) were purified from overproducing *E. coli* strains and separated on a Coomassie-stained 10% SDS−polyacrylamide gel. Molecular mass markers are indicated on the left.

following our established procedures (*39*). *S. pombe rad2* nuclease cDNA was amplified by PCR from a *λ*yes *S. pombe* cDNA library (ATCC) using two oligonucleotide primers: R2F-Nco, 5′-ccATGGGAATTAAAGGTATG (mismatched sequence are in lowercase letters and the restriction site is underlined); and R2R-Hind, 5′-CAaagCTTCGAAATCAACGC. The blunt-ended product was inserted into the pCR-Script SK(+) cloning vector (Stratagene), subcloned into the T7 RNA polymerase expression vector pET28b (Invitrogen), and sequenced to verify the coding region of the enzyme according to the previously published sequence (*28*). The resulting plasmid was transformed into the BL21(DE3) *E. coli* host strain. The established expression and purification procedures for human FEN-1 were used to obtain the pure and active recombinant Rad2 enzyme except that fast protein liquid chromatography was used to automate the process. Similarly, the gene encoding *Sa. cerevisiae* Rad27 nuclease was PCR-amplified using a *Sa. cerevisiae* genomic library (the gene has no intron). The library was a gift from A. Bailis (City of Hope). The two primers for the PCR were R27F-Nco (5′-CCGGAAGAAAAAccATGGG) and R27R-Hind (5′-GAAgCttCTCATCAACTTCCC). The recombinant active enzyme was for the first time purified for this study. The purification procedure was identical to that used for the *S. pombe* Rad2 enzyme.

UV Damage Processing Reactions. The 23-mer oligonucleotide containing a CPD or (6-4)PP was 3′ end labeled with Klenow fragment in the presence of dTTP and [^{32}P]-dCTP. After labeling, this oligonucleotide becomes a 27-mer and ends in TCTC-3′. The labeled 27-mer was gel purified and annealed to the complementary strand. Reaction of the oligonucleotides with UVDE was carried out in a

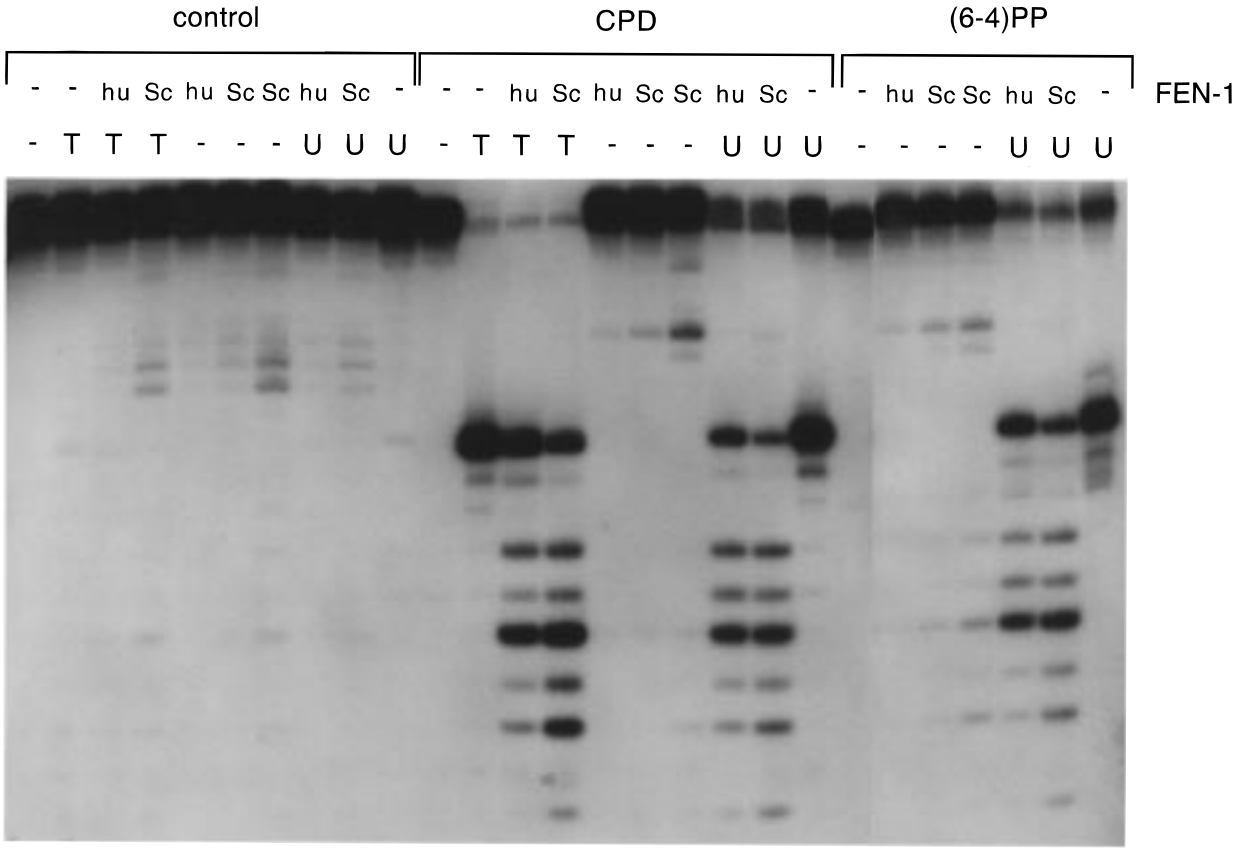

FIGURE 4: Activity of human and *Sa. cerevisiae* FEN-1 on oligonucleotides containing UV photoproducts and on T4 endonuclease V- or UVDE-nicked DNA substrates. The control oligonucleotide, CPD oligonucleotide, or (6-4) photoproduct oligonucleotide was incubated with UVDE (U) or T4 endonuclease V (T), followed by human (hu) FEN-1 or *Sa. cerevisiae* (Sc) FEN-1 proteins, or with the respective enzymes alone. Double-stranded oligonucleotides were 3′ end labeled. The concentration of FEN-1 proteins was 0.3 ng/μL (3 ng per reaction), except for the control Sc lanes which contained 0.1 and 0.3 ng/μL, respectively.

buffer containing 50 mM Tris-HCl (pH 7.9), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 5 ng/μL UVDE at 37 °C for 1 h. T4 endonuclease cleavage was carried out in 50 mM Tris-HCl (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 100 μg/mL bovine serum albumin for 1 h at 37 °C. After UVDE or T4 endonuclease cleavage, DNA was phenol extracted and ethanol precipitated. Standard FEN-1 reactions were carried out in 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, and 50 μg/mL bovine serum albumin for 1 h at 30 °C. The final concentration of FEN-1 proteins was between 0.1 and 0.3 ng/μL.

Ligation-Mediated PCR. Ligation-mediated PCR on UVDE- and FEN-1-processed DNA was performed on genomic DNA from human fibroblasts irradiated with a dose of 2000 J/m^2 of UVC light delivered from a germicidal lamp. The UV-irradiated DNA (10 μg) was incubated with UVDE at a concentration of 20 ng/μL for 2 h at 37 °C and then for 2 h at 30 °C with 10 ng/μL *S. pombe* FEN-1 in reaction buffers as described above. The DNA was extracted with phenol/chloroform and precipitated with ethanol. Ligation-mediated PCR using primers specific for the lower strand of the promoter of the human *PCNA* gene was performed as described previously (*40*).

RESULTS

Oligonucleotide Substrates. Oligonucleotides containing site-specific CPDs and (6-4)PPs as well as a control oligonucleotide containing no photodamage were used as substrates for enzymes in the in vitro UV damage processing reactions. These oligonucleotides were produced using phosphoramidite building block chemistry to introduce either a 5′-TT CPD or a 5′-TT (6-4) photoproduct into an oligonucleotide sequence. T4 endonuclease V digestion was used to confirm the identity of the CPD oligonucleotide (Figure 1A). This enzyme is a DNA glycosylase/AP lyase specific for cis-syn cyclobutane pyrimidine dimers. To confirm the presence of the (6-4)PP, the oligonucleotide was heated in 1 M piperidine at 90 °C for extended periods of time to cleave DNA at the sites of (6-4) photoproducts (Figure 1B). Control oligonucleotides are degraded only nonspecifically under these conditions. The main products of piperidine cleavage of the (6-4)PP oligonucleotide were 9−11 nucleotides in length, which is where the (6-4) product had been introduced into the sequence.

UVDE Reactions on DNA Photoproducts. Incubation of CPD and (6-4)PP oligonucleotides with the UV damage endonuclease from *N. crassa* (Figure 2) resulted in cleavage of both substrates, as previously described (*17*). The cleavage specificity of *N. crassa* UVDE is identical to that of *S. pombe* UVDE (*18*). UVDE cleavage generated one main band, which migrated approximately one-half of a nucleotide position slower than the T4 endonuclease-derived CPD oligonucleotide cleavage product after 3′ end labeling. This is consistent with the reported UVDE cutting mechanism, which results in cleavage just 5′ to the photodamage (*15*, *17*).

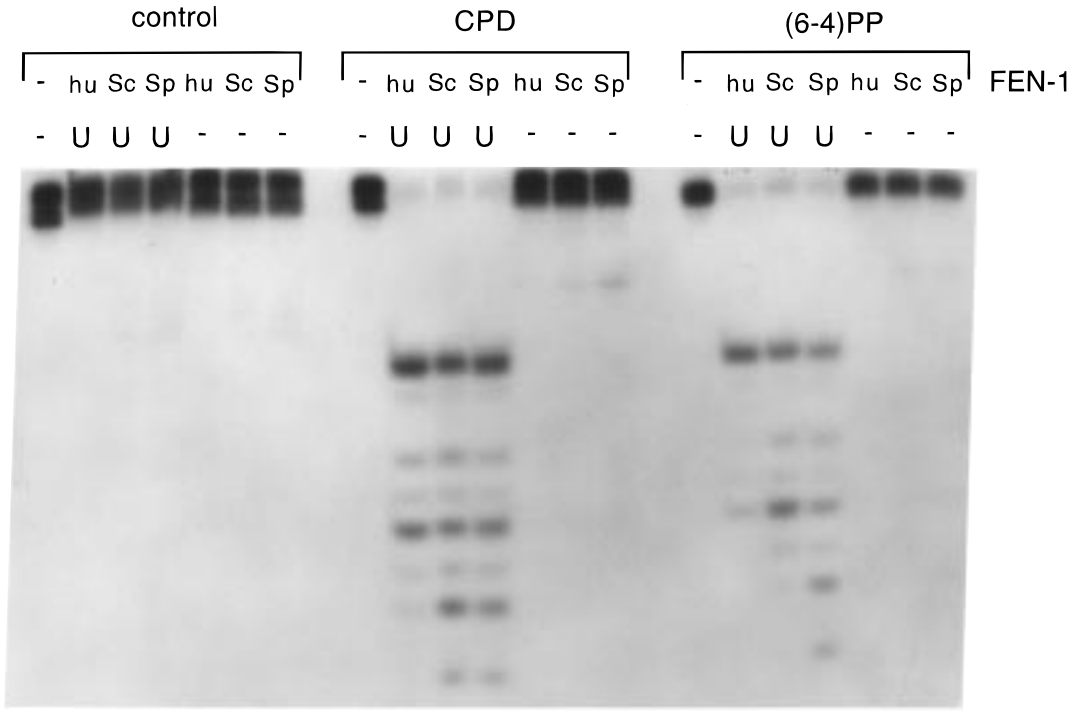

FIGURE 5: Comparison of human, *Sa. cerevisiae*, and *S. pombe* FEN-1 activities on UVDE-nicked DNA. The control oligonucleotide, CPD oligonucleotide, or (6-4)PP oligonucleotide was incubated with or without UVDE (U) followed by human FEN-1 (hu), *Sa. cerevisiae* (Sc), or *S. pombe* (Sp) FEN-1 proteins. Double-stranded oligonucleotides were 3′ end labeled. The concentration of FEN-1 proteins was 0.1 ng/μL.

FEN-1 Reactions on UVDE-Nicked DNA. FEN-1 proteins were purified from overproducing *E. coli* strains. The purity of the proteins is shown in Figure 3. We first characterized the interaction of human FEN-1 and *Sa. cerevisiae* FEN-1 (Rad27 protein) with UVDE-nicked DNA after 3′ end labeling of the double-stranded sequence (Figure 4). FEN-1 displayed low levels of 5′−3′ exonuclease activity on control oligonucleotides that contained no photoproduct and on DNA substrates that contained either a CPD or a (6-4)PP (Figure 4). However, when photoproduct-containing substrates had been incubated with UVDE to introduce nicks 5′ to the photolesions, FEN-1 displayed an entirely different cleavage specificity. A ladder of bands became apparent that had increased mobility relative to the UVDE-nicked product. The strongest bands were found five and seven nucleotide positions 3′ to the major UVDE cleavage site; i.e., cleavage had taken place three and five nucleotide positions 3′ to a photolesion. A similar FEN-1 specificity was observed for sequences containing either a CPD or a (6-4)PP. FEN-1 alone produced some cleavage products with lower mobilities on the (6-4)PP sequence (Figure 4). However, such FEN-1 activity in the absence of UVDE cleavage is minimal with the CPD oligonucleotide or the control oligonucleotide. The low activity of FEN-1 alone on the (6-4)PP oligonucleotide may be explained by a stimulation of FEN activity by the photoproduct itself perhaps mediated by transient melting of the 5′ end of the oligonucleotide or by the instability of this photoproduct creating small amounts of strand breaks during the incubation conditions. Recent data show that a (6-4) photoproduct strongly destabilizes the DNA double helix (*41*). The results presented in Figure 4 suggest that UVDE-nicked DNA containing either CPDs or (6-4)PPs can be processed directly by FEN-1.

We also analyzed whether the FEN-1 reaction is specific for UVDE-nicked DNA or whether a substrate containing a CPD nicked with the CPD-specific DNA glycosylase/AP lyase activity of T4 endonuclease V can also be processed by FEN-1. Figure 4 shows that the latter is the case. The reaction products created by FEN-1 from UVDE-nicked DNA or T4 endonuclease V-nicked DNA were almost identical (Figure 4, middle). In addition, Figure 4 shows that the *Sa. cerevisiae* FEN-1 homologue Rad27 has the same cleavage specificity as the human enzyme.

UVDE-like activities have so far been detected only in three organisms: *N. crassa*, *B. subtilis*, and *S. pombe*. We have then purified and tested the *rad2* gene product of *S. pombe*. This protein, which is highly homologous to both human and *Sa. cerevisiae* FEN-1 (*42*), has recently been characterized biochemically and was shown to possess flap endonuclease-like activities (*34*). Figure 5 shows that *S. pombe* FEN-1 efficiently processed UVDE-nicked substrates in a manner similar to those of the other two FEN-1 homologues.

Detection of FEN-1 Incisions by Ligation-Mediated PCR. To demonstrate that FEN-1 incision takes place also on long DNA fragments, we have used genomic DNA irradiated with germicidal UV light at 2000 J/m^2. This DNA was incubated with UVDE or T4 endonuclease V and FEN-1 (*rad2* gene product of *S. pombe*) to produce incisions. Ligation-mediated PCR (LMPCR; *43*) was then used to map these incision breaks in the promoter of the human *PCNA* gene which contains several hot spots for UV damage (Figure 6). UV photoproducts had been previously mapped along the same sequences by LMPCR (*40*). UVDE incisions alone give a very low signal in LMPCR (Figure 6), similar to T4 endonuclease V breaks (*44*), presumably because these

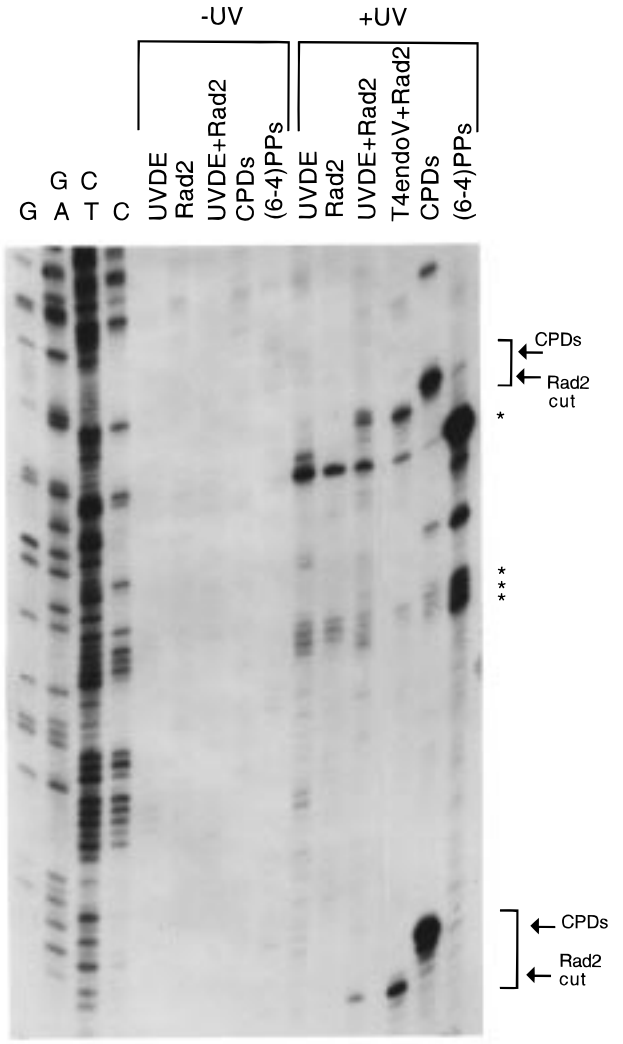

FIGURE 6: Analysis of FEN-1 cleavage products by ligation-mediated PCR. Genomic DNA from fibroblasts was irradiated at 2000 J/m^2 with a germicidal UV lamp. This DNA was incubated with UVDE, T4 endonuclease V, *S. pombe* FEN-1 (Rad2 protein), or a combination of two enzymes. The promoter of the human *PCNA* gene was analyzed by ligation-mediated PCR using primers specific for the lower strand between nucleotides −150 and −50. The sequence-dependent frequency of UV-induced CPDs and (6-4)PPs was mapped by T4 endonuclease/photolyase and piperidine cleavage, respectively. Chemical DNA sequencing reactions are shown on the left. The asterisks denote nonspecific degradation products derived from (6-4)PPs under these incubation conditions. FEN-1 cleavage products occurring 3′ to CPDs are denoted with brackets. These bands migrate faster in the LMPCR assay.

breaks are not compatible with the ligation step of ligation-mediated PCR. However, the lanes containing UVDE or T4 endonuclease V in combination with FEN-1 protein did contain specific signals near sites of strong CPD formation. The fragments resulting from FEN-1 cleavage migrated approximately three nucleotide positions faster than the respective fragments derived from mapping of UV lesions by T4 endonuclease/photolyase cleavage. This indicates that FEN-1 can process UVDE-incised DNA directly on long DNA fragments and that these breaks can be detected by ligation-mediated PCR. Under these conditions, only weak signals were obtained from (6-4)PPs, presumably because there was too much nonenzymatic degradation of (6-4)PPs.

FEN-1 Reactions on Substrates That Contain CPDs in a Flap Configuration. DNA polymerases may catalyze extension reactions on nicked substrates produced by UVDE cleavage, resulting in displacement of the damaged strand. To test whether these intermediates are substrates for FEN-1, we have constructed oligonucleotides with a 5′ flapped DNA structure that contains a CPD at the 5′ end. As controls, oligonucleotides with a nick in the same sequence context were used together with identical substrates containing no photoproduct. All substrates containing nicks or flaps, with or without CPD, were cleaved by *S. pombe* FEN-1 (Figure 7). Incubation in 1 mM $MgCl_2$ increased the extent of cleavage of the nicked substrates relative to incubation in 10 mM $MgCl_2$. The higher salt concentration presumably reduces "breathing" at the 5′ terminus of the fully annealed oligonucleotides, thereby interfering with the ability of FEN-1 to move beyond the damaged site. With the flapped substrates, cleavage occurred predominantly at the junction where the displaced strand meets the double strand while cleavage of the nicked substrate took place at several positions. The oligonucleotides containing the CPD were cleaved somewhat more efficiently than the controls without lesion. Very efficient cleavage occurred with the flapped substrate that had a 5′-displaced CPD (Figure 7).

DISCUSSION

UV Damage Repair Systems. Presumably, the most dominant force for the development of DNA repair systems has been the exposure of organisms to solar UV irradiation. It is therefore not surprising that several different UV repair mechanisms have evolved. The most versatile and widespread mechanism is nucleotide excision repair, which is found in almost every organism. It requires the complex interplay of various protein factors, as is evident from analysis of the UVR system in bacteria (*6*, *45*, *46*) or the even more complex machinery that operates in eukaryotic cells (*47*, *48*). Other, seemingly much simpler repair pathways exist for the repair of UV damage in a variety of species (*4*) but, for unknown reasons, have not always been conserved throughout evolution. One group of repair enzymes are photolyases, which can efficiently repair CPDs or (6-4)PPs (*2*, *3*). CPD DNA glycosylases, which introduce a nick at one of the two glycosylic bonds in a CPD and generate an abasic site and a strand break, have been characterized (*9*−*11*). The resulting intermediates are processed further, but the precise mechanism has not been characterized. The FEN-1-like activity of *E. coli* DNA polymerase I may be involved in processing of these intermediates in bacteria. CPD DNA glycosylases have been identified in T4-infected *E. coli*, in the eubacterium *M. luteus*, and in a *Chlorella* virus (*49*) which may be derived from bacterial origin. UVDE repair mechanisms have so far been found in only three organisms, two eukaryotic and one prokaryotic (*4*). However, it is possible that this mechanism is more widespread than is currently thought.

FEN-1 in Base Excision Repair. The involvement of FEN-1 nucleases in base excision repair pathways has recently been demonstrated (*50*−*53*). It was shown that FEN-1 is essential for repair of a reduced AP site or an AP site analogue, both of which cannot be processed through β-elimination by DNA polymerase β. This pathway, which has been termed long patch base excision repair with gap filling of several nucleotides, is initiated by an AP endonuclease incision at the 5′ side of an AP site. In one pathway,

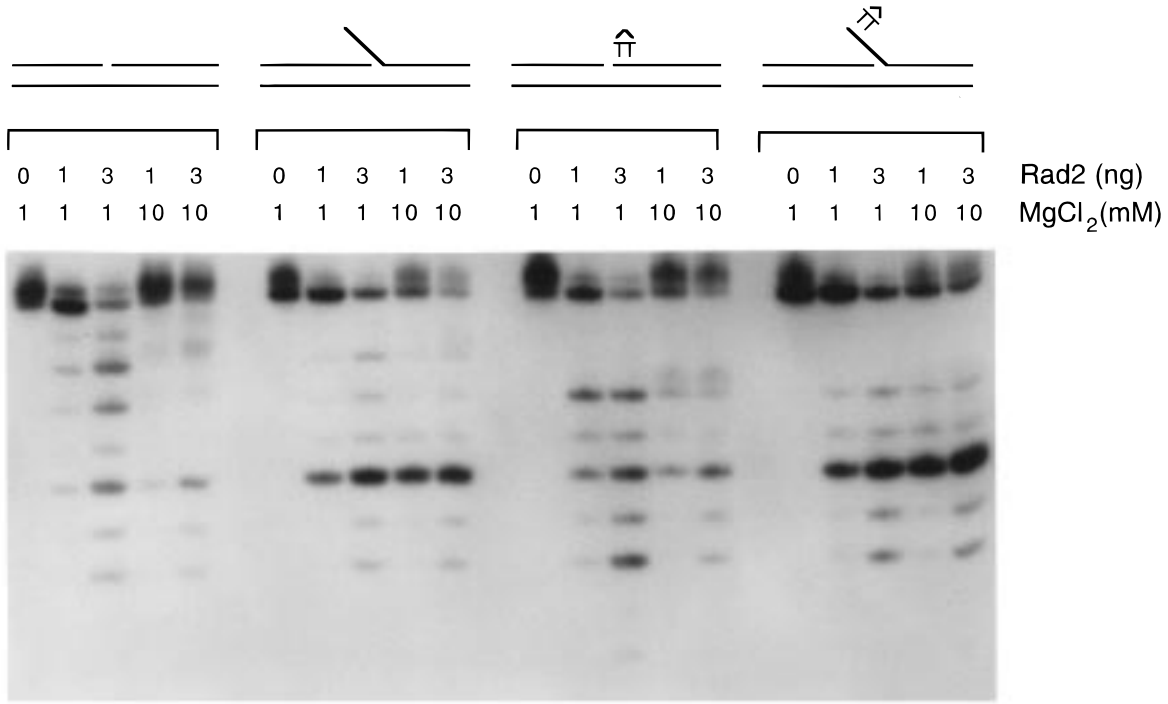

FIGURE 7: Activity of *S. pombe* FEN-1 on substrates containing a nick or flap structure. Control or CPD-containing strands with 5′-phosphate groups were annealed to form nicked or flapped double-stranded structures as indicated. These substrates were incubated with either no enzyme or *S. pombe* FEN-1 using 1 or 3 ng of protein per reaction in 10 μL of buffers containing either 1 or 10 mM $MgCl_2$.

polymerase β cooperated with FEN-1 for incision (*52*, *53*), but another pathway was shown to take place in the absence of DNA synthesis (*53*). It is presently unclear whether long patch base excision repair in vivo is entirely dependent on DNA polymerase δ/PCNA or whether DNA polymerase β is also involved (*52*−*59*). Cell extracts from a DNA polymerase β knockout mouse repaired an AP site analogue as efficiently as a polymerase β-proficient isogenic cell extract, but this PCNA-dependent pathway was shown to be dependent on circular DNA substrates (*59*).

FEN-1 in UV Damage Repair. The mechanistic details of the UVDE repair pathway have not yet been determined. Here we have shown that intermediates produced by UVDE incision can be processed by homologues of the FEN-1 family of nucleases.

PCNA participates in DNA synthesis by DNA polymerase δ and has been shown to stimulate FEN-1 activity (*60*). We could detect only a 1.5−2-fold stimulation of FEN-1 activity by PCNA with any of the substrates used, even with a >300-fold molar excess of PCNA trimers over FEN-1 (data not shown). Our results with UVDE incision products show that FEN-1 proteins can incise these intermediates directly in the absence of any accessory factors such as PCNA and also in the absence of a primer extension reaction that would create a flap structure. In addition, we show that flap structures containing a CPD at the 5′ end of the flap are processed by FEN-1 with high efficiency.

The action of FEN-1 in the long patch base excision repair pathway can be independent of DNA synthesis (*53*). FEN-1 has been shown to act through its 5′−3′ exonuclease or through its endonuclease activity on gaps and nicks in DNA (*32*). It is possible that a similar activity operates on UVDE-incised DNA in the absence of a flap. The extent of base pairing is diminished at pyrimidines, which are part of a CPD, and the double-helical structure is even more distorted at the site of a (6-4) photoproduct (*41*, *61*−*66*). This loosening of DNA duplex stability may create a substrate that is more amenable to FEN-1 processing because of the presence of a partially denatured "pseudo flap" structure. It has been reported that FEN-1 has a 2−4-fold higher affinity for a flap structure than for a nicked site (*31*). Lieber (*32*) has suggested that the 5′−3′ exonuclease activity usually ascribed to FEN-1 is in fact indistinguishable from its endonuclease activity acting on transient flap-like structures generated by breathing of DNA at the very 5′ end of a nicked substrate. The bovine FEN-1 homologue, calf 5′−3′ exo/endonuclease, has been shown to cleave 5′ flap structures that contain a biotin adduct (*25*) or a cisplatinum adduct (*51*). These results, together with our data, suggest that DNA damage present within the 5′ flap structure does not inhibit FEN-1 cleavage, but instead is efficiently removed as part of an oligonucleotide through the endonucleolytic activity of FEN-1.

It is clear that our observations are from in vitro data and that the proof of in vivo validity is still needed. In particular, it will be important to determine whether FEN-1 is the major nuclease in the cell involved in this pathway. Genetic data support the role of FEN-1 in UV damage processing, at least in organisms that possess UVDE activity. Introduction of the *uvde* gene into repair-deficient recipient cells (including *uvrA*, XP-A, and *Sa. cerevisiae rad2* mutant strains) makes the recipient cells more UV resistant (*17*). This suggests that UVDE-induced breaks can be processed by NER-independent pathways, perhaps involving FEN-1-like activities. In *S. pombe*, a *rad2* single mutant shows significant UV sensitivity (*19*, *28*, *67*), but a *rad2/uvde* double mutant is less sensitive than the *rad2* single mutant (*19*). This suggests that UVDE-nicked DNA requires *rad2* for processing. However, in an NER-deficient background, cells that lack UVDE are more sensitive than cells that lack the *rad2* gene

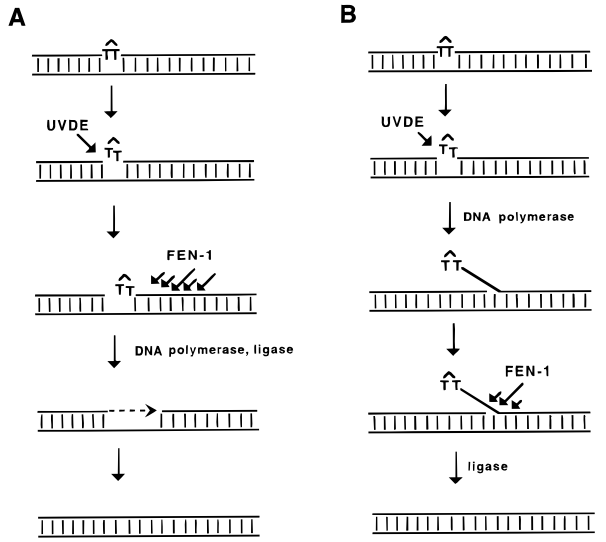

FIGURE 8: Hypothetical models illustrating the repair of a UV photoproduct by the combined action of UVDE, FEN-1, DNA polymerase, and DNA ligase. In pathway A, FEN-1 cleaves the UVDE-nicked DNA directly. In pathway B, a DNA polymerase first creates a flap structure from UVDE-nicked DNA, which is then cleaved by FEN-1.

product, indicating that some of the UVDE nicks can be processed by *rad2*-independent pathways (*19*). Unlike *rad2* mutants, deletion mutants of its *Sa. cerevisiae* homologue *RAD27/RTH1* are not significantly UV sensitive (*29*, *30*). This is consistent with the lack of UVDE activity in *Sa. cerevisiae*.

The dual incision of photoproduct-containing DNA by UVDE and FEN-1 creates a gap in the DNA after release of a short oligonucleotide 4−10 bases in length. The gap left behind by FEN-1 on UV-damaged DNA can be filled by DNA polymerase and ligase to complete the repair reaction. Alternatively, a DNA polymerase may extend from the nick first, followed by FEN-1 cleavage at the junction of the displaced strand and direct ligation (Figure 8A,B). Our results show that both of these FEN-1 cleavage pathways can exist. A comparably sized (two to seven nucleotides) repair patch has been obtained from experiments in which alternative base excision repair pathways of AP sites or AP site analogues were studied (*52*, *56*). FEN-1 is unable to catalyze the release of 5′ terminal deoxyribose phosphates as free sugar phosphates but can liberate these residues as short oligonucleotides (*68*). However, regular AP sites are predominantly repaired by single nucleotide gap filling mechanisms presumably involving the phosphodiesterase activity of DNA polymerase β (*52*, *69*). Besides its involvement in the UVDE repair pathway, the DNA repair activity of FEN-1 may be limited to processing of irregular AP sites or other not directly ligatable strand breaks which may contain damaged bases or sugars at the 5′ ends of the breaks. Such events may play a role in the processing of ionizing radiation damage or oxidatively damaged DNA (*52*). FEN-1 may be involved in other less well characterized repair pathways. For example, Hang et al. (*70*) have recently reported that the major human AP endonuclease cleaves at a position 5′ to a *p*-benzoquinone deoxycytidine adduct. These nicked intermediates may be substrates for FEN-1.

It has been pointed out that the occurrence of gapped or flapped DNA in the UVDE repair pathway (Figure 8) could be a recombinogenic event, or could lead to further nucleolytic DNA degradation, which may have prevented this mechanism from becoming more widespread in evolution (*4*). In nucleotide excision repair, the gaps are protected by a larger protein complex, which may suppress recombination events.

ACKNOWLEDGMENT

We are grateful to A. Sancar for providing *E. coli* photolyase, R. S. Lloyd for T4 endonuclease V, and D. Lee of the Hamilton Corp. (Reno, NV) for the generous donation of the PRP-1 resin.

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BI990105I