Nucleotide Excision Repair DNA Synthesis by DNA Polymerase ϵ in the Presence of PCNA, RFC, and RPA†

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ABSTRACT: In eukaryotes, nucleotide excision repair of DNA is a complex process that requires many polypeptides to perform dual incision and remove a segment of about 30 nucleotides containing the damage, followed by repair DNA synthesis to replace the excised segment. Nucleotide excision repair DNA synthesis is dependent on proliferating cell nuclear antigen (PCNA). To study gap-filling DNA synthesis during DNA nucleotide excision repair, UV-damaged DNA was first incubated with PCNA-depleted human cell extracts to create repair incisions. Purified DNA polymerase δ or ϵ, with DNA ligase, was then used to form the repair patch. DNA polymerase δ could perform repair synthesis and was strictly dependent on the presence of both PCNA and replication factor C, but gave rise to a very low proportion of complete, ligated circles. The presence of replication protein A (which is also required for nucleotide excision repair) did not alter this result, while addition of DNase IV increased the fraction of ligated products. DNA polymerase ϵ, on the other hand, could fill the repair patch in the absence of PCNA and replication factor C, and most of the products were ligated circles. Addition of replication protein A changed the situation dramatically, and synthesis by polymerase ϵ became dependent on both PCNA and replication factor C. A combination of DNA polymerase ϵ, PCNA, replication factor C, replication protein A, and DNA ligase I appears to be well-suited to the task of creating nucleotide excision repair patches.

Exposure of genomic DNA to physical or chemical mutagens leads to the formation of a range of bulky lesions that are efficiently removed by nucleotide excision repair. This repair process requires the interaction of many gene products with damaged DNA, some of which also participate in DNA replication and transcription (Weeda & Hoeijmakers, 1993; Aboussekhra & Wood, 1994). A coordinated sequence of events begins with the recognition, incision, and excision of the damage from the DNA. Many of the genes and gene products involved in this stage of the nucleotide excision repair process in mammalian cells have been identified by studying the inherited, autosomally recessive disease xeroderma pigmentosum (XP).[1] The defective nucleotide excision repair pathway in XP cells causes an increased sensitivity to UV light, and can lead to a large increase in the incidence of sunlight-induced skin cancer (Cleaver & Kraemer, 1989).

Normally, damage is excised as part of an oligomer about 30 nucleotides long (Svoboda et al., 1993). A repair patch of about 30 nucleotides is then formed *in vivo* (Cleaver et al., 1991) and *in vitro* (Hansson et al., 1989; Shivji et al., 1992). The repair synthesis stage is known to require the DNA polymerase accessory factor PCNA. Fractionation of cell extracts has been used to separate PCNA from the other components of nucleotide excision repair, and such PCNA-depleted extracts can perform incision of damaged DNA *in vitro*, but not repair synthesis (Shivji et al., 1992, 1994; Nichols & Sancar, 1992). There is good evidence that PCNA also participates in nucleotide excision repair *in vivo*. After UV-irradiation of non-S phase or quiescent fibroblasts, a tight association of PCNA with DNA can be detected (Celis & Madsen, 1986; Toschi & Bravo, 1988; Jackson et al., 1994), indicating sites of repair. An accumulation of PCNA in nonreplicating cells can also be observed after irradiation of human skin *in situ* (Hall et al., 1993). The rapid association of PCNA with UV-irradiated DNA is absent in XP group A cells, which are defective in the initial recognition/incision steps of repair (Miura et al., 1992).

The participation of PCNA in nucleotide excision repair indicates that either DNA polymerase δ (pol δ) or DNA polymerase ϵ (pol ϵ) normally carries out nucleotide excision repair synthesis, since these polymerases are stimulated by PCNA under various conditions (Hübscher & Spadari, 1994). Studies using chemical inhibitors also suggest that pol δ or pol ϵ (but not PCNA-independent DNA polymerase α) functions during nucleotide excision repair (Dresler & Frattini, 1986; Hunting et al., 1991; Popanda & Thielmann, 1992; Coverley et al., 1992). These findings strongly suggest that a further DNA polymerase accessory factor, replication factor C (RFC), is also involved in nucleotide excision repair. RFC is a multisubunit protein complex that functions during DNA replication to load PCNA onto a DNA template in an ATP-dependent manner, creating a "sliding clamp" for DNA polymerases δ and ϵ (Tsurimoto & Stillman, 1991; Lee & Hurwitz, 1990; Lee et al., 1991a; Burgers, 1991; Podust et al., 1992).

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[1] Abbreviations: XP, xeroderma pigmentosum; pol, DNA polymerase; SV40, simian virus 40; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; RPA, replication factor A; nt, nucleotide(s).

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Additionally, the single-stranded DNA binding replication protein A (RPA, also known as HSSB or RF-A) is involved in nucleotide excision repair. RPA was first discovered as an essential component of SV40 viral DNA replication *in vitro*, where it assists in the unwinding of DNA at the replication origin by T-antigen, and in primosome assembly (Wold & Kelly, 1988; Fairman & Stillman, 1988; Kenny et al., 1989; Melendy & Stillman, 1993). RPA is required for DNA synthesis by pol δ and pol ϵ on primed single-stranded DNA templates (Tsurimoto & Stillman, 1989; Lee et al., 1991a,b; Podust & Hübscher, 1993), but RPA is not required with a gapped double-stranded DNA template (Podust et al., 1994). RPA functions during the first stage (damage recognition/incision) of nucleotide excision repair (Coverley et al., 1991, 1992; Shivji et al., 1992), and as an abundant protein in cell nuclei, it might also be expected to participate during the second stage (repair synthesis).

The studies presented in this paper were designed to reconstruct the second stage of nucleotide excision repair of UV-irradiated DNA, by using purified pol δ or ϵ, PCNA, RFC, RPA, and DNA ligase.

EXPERIMENTAL PROCEDURES

Extracts and Proteins. Human cell extracts at 20 mg/mL were prepared as described (Wood et al., 1988). Human CFII and CFIA fractions were prepared (Shivji et al., 1992) by loading whole cell extract on a column of phosphocellulose in buffer A (25 mM Hepes−KOH, pH 7.8, 1 mM EDTA, 0.01% NP40, 10% glycerol, and 1 mM dithiothreitol) containing 0.15 M KCl. Flow-through fractions were collected, and bound protein was eluted with buffer A containing 1.0 M KCl. Pooled peak fractions from the flow-through (CFI) and the 1.0 M KCl elution (CFII) were dialyzed against 25 mM Hepes−KOH (pH 7.9), 1 mM EDTA, 17% glycerol, 1 mM dithiothreitol, 12 mM $MgCl_2$, and 0.1 M KCl. CFI was then loaded onto a column of DEAE Biogel (Bio-Rad) equilibrated in buffer A containing 0.15 M KCl. Peak fractions from the flow-through (CFIA) were dialyzed as above. CFIA and CFII contain all proteins essential for nucleotide excision repair except for PCNA.

RPA was purified from HeLa cells (Kenny et al., 1989). Pol δ, pol ϵ, RFC (Mono Q fraction), and PCNA were purified from calf thymus (Podust et al., 1992; Podust & Hübscher, 1993). DNA ligase I was purified from calf thymus as described to give a single band visible after SDS−PAGE (Tomkinson et al., 1990), and was kindly provided by P. Robins and T. Lindahl. T4 DNA ligase was purchased from New England Biolabs. DNase IV was purified as described (Robins et al., 1994) from HeLa cells and was provided by P. Robins and T. Lindahl.

Repair Assay. (A) Damage Excision Stage. UV-irradiated DNA was produced by irradiating plasmid pBluescript KS^+ with 450 J/m^2 UV light, treatment with *Escherichia coli* Nth protein, and repeated purification of closed circular forms as described (Biggerstaff et al., 1991). First-stage reactions to produce DNA substrates produced during repair of UV-irradiated DNA were performed using the HeLa cell fractions and the strategy previously described (Shivji et al., 1992). Reaction mixtures (500 μL) contained 500 μg of CFII, 240 μg of CFIA (containing RPA), 2.5 μg of closed circular UV-damaged plasmid, and 2.5 μg of nondamaged closed circular pHM14 (Rydberg et al., 1990) plasmid DNA. The reaction buffer was as described (Shivji et al., 1992), except that

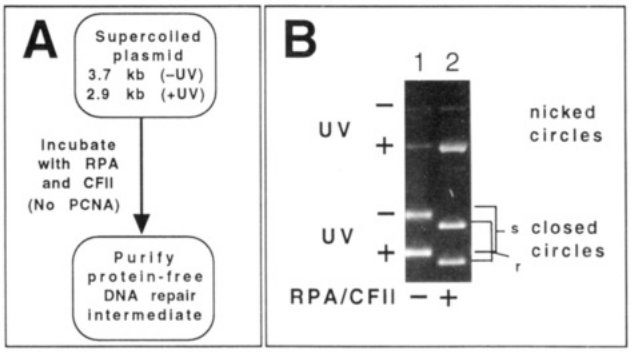

FIGURE 1: Generation of DNA repair intermediates. (A) Procedure used to generate DNA repair intermediates. The closed-circular UV-irradiated plasmid DNA contains pyrimidine dimers and (6-4) photoproducts but is purified free from pyrimidine hydrates. The 2.9 kb UV-irradiated plasmid and a control unirradiated plasmid of 3.7 kb are incubated together with RPA protein and fractionated cell extract (CFII) in the absence of PCNA, where the incision/excision reaction of nucleotide excision repair takes place. (B) A 1% agarose gel, cast and run in buffer containing ethidium bromide, showing the DNA mixture before (lane 1) or after (lane 2) incubation with RPA and CFII. Closed-circular and nicked-circular forms of the irradiated (+) and nonirradiated (−) plasmids are shown. Closed-circular DNA is supercoiled before incubation with the extract, and relaxed after incubation.

[α-^{32}P]dATP was omitted. After a 90 min incubation at 30 °C, the DNA was purified as described (Wood et al., 1988) and dissolved in 50 μL of 10 mM Tris-HCl, 1 mM EDTA.

(B) Synthesis Stage Using Purified DNA and Proteins. For repair synthesis, 25 μL reaction mixtures contained 200−400 ng (5 μL) of the purified intermediate DNA mixture, in repair synthesis buffer containing 45 mM Hepes−KOH (pH 7.8), 70−80 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 20 μM each of dGTP, dCTP, and TTP, 8 μM dATP, 74 kBq of [α-^{32}P]dATP (110 TBq/mmol), 2 mM ATP, 22 mM phosphocreatine (di-Tris salt), 2.5 μg of creatine phosphokinase, 3.4% glycerol, and 18 μg of bovine serum albumin. RPA, PCNA, RFC, pol δ or pol ϵ, and T4 DNA ligase or DNA ligase I were added as indicated, and reactions were incubated at 30 °C for 60−90 min. The DNA was purified by proteinase K digestion, phenol−chloroform extraction, and ethanol precipitation. The products were dissolved in 15 μL of 10 mM Tris-HCl, 1 mM EDTA and separated by electrophoresis on a 1% agarose gel containing 0.25 μg/mL ethidium bromide. DNA recovery was normalized by densitometric scanning of a photographic negative of the agarose gel. Radiolabel in the damaged and nondamaged DNA was quantified by scintillation counting of bands excised from the dried gel and by densitometry of autoradiographs, and corrected for DNA recovery.

RESULTS

Preparation of Nucleotide Excision Repair Intermediates Suited To Measure DNA Synthesis. In order to reconstitute the synthesis stage of nucleotide excision repair, we used DNA substrates that were created by dual incision during nucleotide excision repair *in vitro*. Figure 1A outlines the procedure for generating first-stage DNA repair intermediates. As described previously, fractionated HeLa cell extract (phosphocellulose fraction CFII) and RPA protein were incubated with a mixture of UV-irradiated and unirradiated DNA for 1 h. In the absence of PCNA, incision and excision of a damaged oligonucleotide can occur, but repair synthesis by the PCNA dependent DNA repair polymerase is prevented

(Shivji et al., 1992; Nichols & Sancar, 1992). Figure 1B shows an agarose gel of a DNA mixture before incubation (lane 1) and after recovery from the incision/excision reaction (lane 2). The repair intermediates appear as nicked circles, specifically formed in damaged DNA. Formation of the incision in damaged DNA is RPA-dependent (Shivji et al., 1992). After incubation in the extract, covalently closed circles are relaxed by topoisomerases, and migrate slightly faster in the gel in the presence of ethidium bromide (Figure 1B, lanes 1 and 2).

An important step in the preparation of the UV-irradiated DNA used in these experiments was prior treatment with *E. coli* Nth protein (endonuclease III) to remove hydrated pyrimidine photoproducts, followed by repurification of closed circular material. The hydrated pyrimidines are good substrates for base excision repair initiated by a DNA glycosylase in human cell extracts, and nicking at such photoproducts would obscure the incisions created by nucleotide excision repair (Wood et al., 1988).

Repair DNA Synthesis by Pol δ and Pol ϵ in the Absence of RPA. The DNA mixture containing the first-stage repair intermediates was purified by phenol−chloroform extraction and ethanol precipitation, and then used as the substrate for gap-filling by pol δ and pol ϵ. In Figure 2, reaction mixtures also included T4 DNA ligase and (where indicated) the polymerase accessory factors PCNA and RFC. The same reaction conditions were used in the gap-filling reactions as were used in the first-stage reactions or with whole-cell extracts. The reaction buffer includes 70−80 mM KCl, 2 mM ATP, and an ATP-regenerating system.

In the absence of RPA, the requirement of the two polymerases for accessory proteins was very different [Figure 2A (top panel), lanes 1−7 for pol δ, and lanes 8−14 for pol ϵ]. Synthesis by pol δ was dependent upon both PCNA and RFC, while pol ϵ did not require either factor to produce closed circular products. The data are quantified in Figure 2B (bottom panel). The amounts of DNA polymerases used (0.9 unit for pol δ and 4.8 units for pol ϵ) were chosen to give similar total amounts of synthesis in the substrate in the presence of PCNA and RFC under these reaction conditions. With pol δ, 15 ng of PCNA per reaction mixture was limiting, and more synthesis could be achieved by including 100 ng of PCNA per reaction (Figure 2, lane 5 vs lanes 6 and 7). Addition of 300 ng of RPA had little effect on the overall level of synthesis by either polymerase, but slightly decreased the proportion of closed-circular products (lanes 7 and 14).

A further striking difference in the behavior of the two polymerases was the different proportions of closed-circular products created. Synthesis by the pol δ/PCNA/RFC holoenzyme gave rise to only a small proportion of closed-circular products in the presence of T4 DNA ligase (Figure 2). In contrast, most of the products produced after synthesis by pol ϵ in the presence of T4 DNA ligase were closed-circular, fully repaired products. Thus, pol ϵ seemed better suited than pol δ for the purpose of nucleotide excision repair synthesis, because it gave rise to a higher fraction of complete repair patches.

Similarity of Products Obtained with T4 Ligase and Mammalian DNA Ligase I. The proportion of ligated products was low when pol δ holoenzyme and T4 DNA ligase were used together, and it seemed possible that mammalian DNA ligase I might be more compatible with the system than the enzyme from bacteriophage. DNA ligase

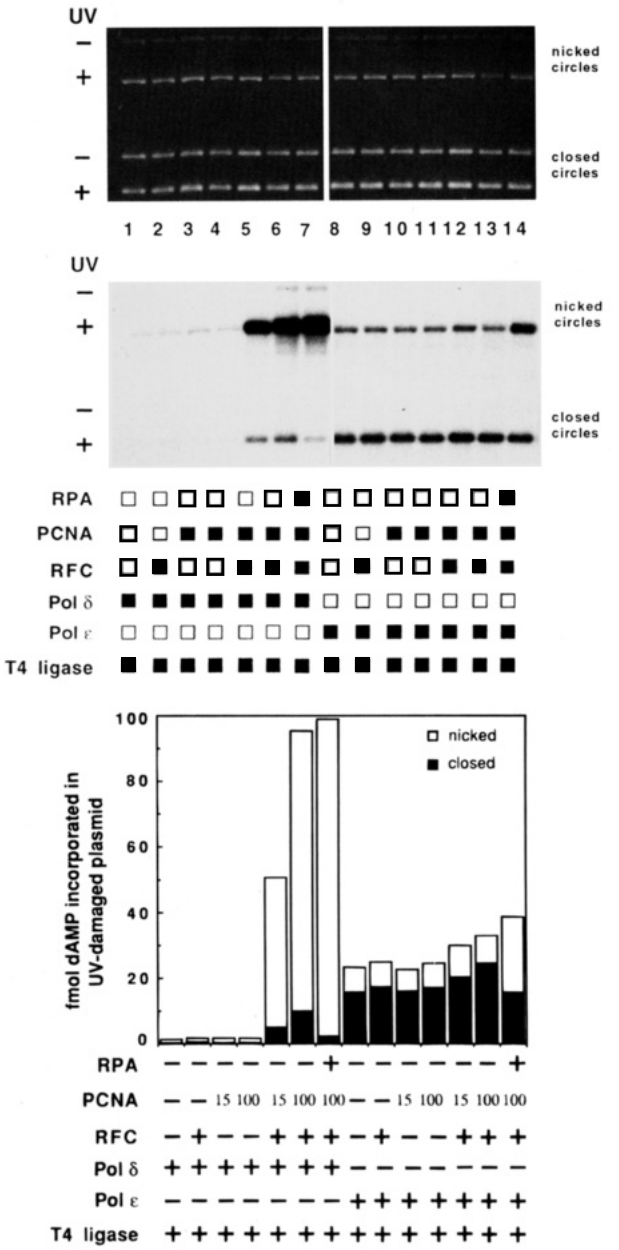

FIGURE 2: Gap-filling repair synthesis by pol δ and ϵ in the absence of RPA. (A, top panel). Purified DNA mixture as in Figure 1B, lane 2, was incubated in reaction buffer containing ATP, dNTPs, and [α-^{32}P]dATP, with either pol δ (0.9 unit, lanes 1−7) or pol ϵ (4.8 units, lanes 8−14) in the presence of T4 DNA ligase (120 units). RFC (90 ng) and PCNA (15 ng, lanes 3, 5, 10, and 12; 100 ng, lanes 4, 6, 7, 11, 13, and 14) were added where indicated by the filled boxes. In lanes 7 and 14, RPA (300 ng) was included. The top part of this panel is an ethidium bromide stained agarose gel showing the nicked and closed-circular forms of irradiated (+) and nonirradiated (−) DNA. The bottom part of the panel is an autoradiograph of the agarose gel showing repair synthesis. (B, bottom) Quantification of data for the UV-irradiated DNA, from part A. Closed bars, synthesis in closed-circular DNA; open boxes, synthesis in nicked circular DNA.

I appears to function both in semiconservative DNA replication and in DNA repair (Lindahl & Barnes, 1992; Waga et al., 1994). Figure 3A (top panel) shows a comparison of the products obtained in reaction mixtures containing either T4 DNA ligase or purified calf thymus DNA ligase I (Tomkinson et al., 1990). Irrespective of which ligase was used, pol ϵ gave a higher proportion of complete (closed-circular) repair products in comparison with pol δ. Inclusion of RPA in the reaction mixtures had little effect on the level of overall repair synthesis by either polymerase, but RPA

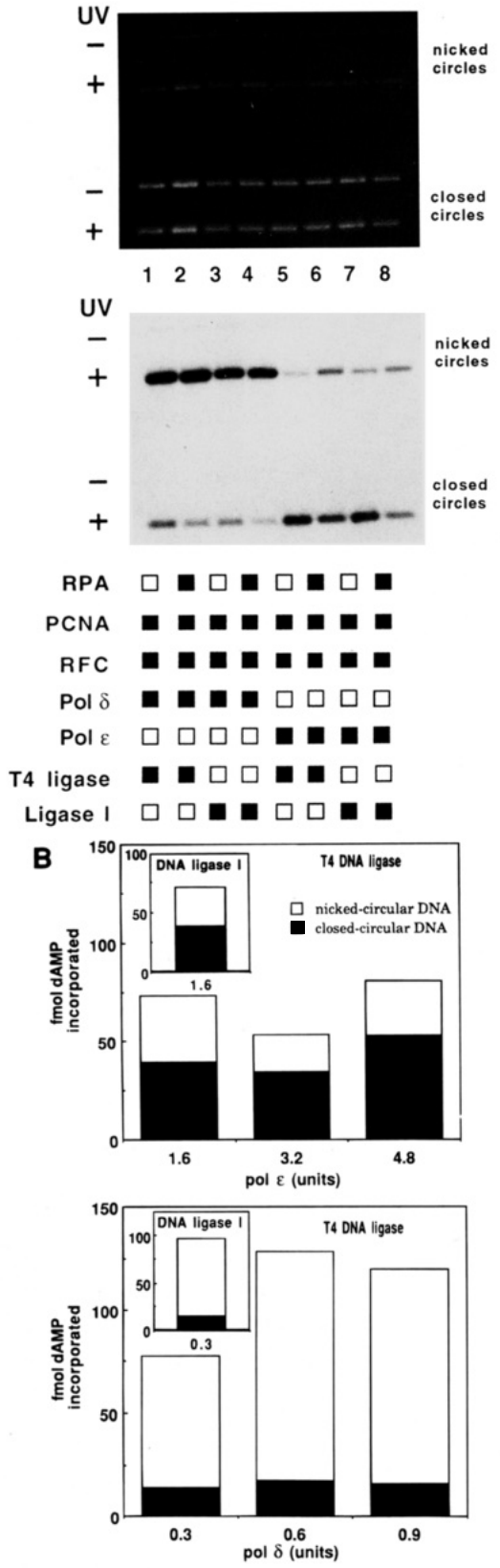

FIGURE 3: (A, top panel) Repair synthesis with T4 DNA ligase or mammalian DNA ligase I. Reactions were performed and analyzed as in Figure 2, with either pol δ (0.9 unit, lanes 1−4) or pol ϵ (4.8 units, lanes 5−8), PCNA (440 ng), RFC (47 ng), and either T4 DNA ligase (80 units) or calf thymus DNA ligase I (200 ng) where indicated by the closed boxes. (B, bottom panel) Repair synthesis with different amounts of pol ϵ (top) or δ (bottom). DNA was incubated with the indicated amounts (units) of pol δ or pol ϵ in the presence of 300 ng of RPA, 440 ng of PCNA, 47 ng of RFC, and 80 units of T4 DNA ligase (main graphs) or 200 ng of DNA ligase I (insets). The graph shows repair synthesis in nicked-circular DNA (open boxes) and closed-circular DNA (closed boxes).

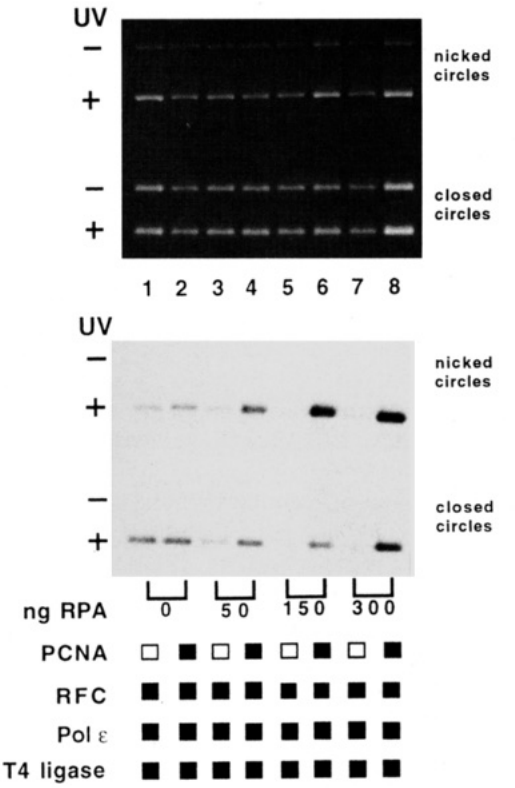

FIGURE 4: PCNA dependence of synthesis by pol ϵ in the presence of RPA. All reaction mixtures contained pol ϵ (4.8 units), RFC (90 ng), T4 DNA ligase (120 units), and either no PCNA (lanes 1, 3, 5, and 7; open boxes) or 100 ng of PCNA (lanes 2, 4, 6, and 8; closed boxes). Reactions in lanes 3−8 also included the indicated amounts of RPA. Reactions were carried out and analyzed as in Figure 2.

did seem to partially inhibit completion of the repair patch with both T4 DNA ligase and DNA ligase I (Figure 3A).

Since RPA is an abundant protein in cell nuclei, and is required for nucleotide excision repair, further studies included RPA in the reaction mixtures. Reducing the amount of DNA polymerase in the reaction mixtures did not alter the basic difference in product formation between the two polymerases. Different amounts of either pol δ or pol ϵ were tested with either T4 DNA ligase (Figure 3B, bottom panel) or DNA ligase I (Figure 3B, insets). In all cases, synthesis with DNA polymerase ϵ gave rise to mostly closed-circular products, whereas most products with polymerase δ remained in the nicked-circular form.

PCNA Dependence of Pol ϵ in the Presence of RPA. The above results presented something of a paradox. PCNA is known to be required for nucleotide excision repair *in vitro* and *in vivo*, yet the polymerase best-suited to filling these repair gaps appeared to be DNA polymerase ϵ, which could perform synthesis in the absence of PCNA. However, although pol ϵ was PCNA-independent in the absence of RPA (Figure 2), we found that the situation changed dramatically when RPA was present.

Figure 4 shows reactions with pol ϵ in the presence of increasing amounts of RPA. In the absence of RPA, repair synthesis by pol ϵ did not depend upon PCNA (Figure 4, compare lanes 1 and 2). When increasing amounts of RPA were added, repair synthesis in the absence of PCNA was progressively abolished (lanes 3, 5, and 7). However, in the presence of PCNA, synthesis could take place (lanes 4, 6, and 8). With 150−300 ng of RPA per reaction, repair synthesis by pol ϵ was almost fully dependent on the presence of PCNA.

RFC Dependence of Pol δ and ϵ for Gap-Filling Repair in the Presence of RPA. RFC functions during DNA replication and gap-filling synthesis to facilitate the loading of PCNA onto DNA templates (Podust et al., 1994). For this reason, it is expected to participate in nucleotide excision repair, although a direct involvement has not been demonstrated. In the series of experiments reported here, we found that whenever PCNA was required, RFC was also needed. A titration of RFC in repair synthesis by pol δ and ϵ is shown in Figure 5. Based on the results from Figures 3 and 4, this experiment used 150 ng of RPA (to give full PCNA dependent synthesis by pol ϵ), 2.4 units of pol ϵ, and 0.6 units for pol δ. As in Figure 2, no synthesis by pol δ was observed in the absence of RFC [Figure 5A (top panel), lane 1], but synthesis occurred in the presence of increasing amounts of RFC (lanes 2−5). Similarly, synthesis by pol ϵ under these conditions was also stimulated by RFC, although a small amount of synthesis could take place in its absence (Figure 5A, lanes 6−10). The conditions used gave nearly identical amounts of total repair synthesis for the two polymerases (Figure 5B), but as previously, synthesis with pol ϵ led to a higher fraction of products in closed-circular DNA than did synthesis with pol δ (Figure 5B, inset).

Effect of DNase IV on Repair Synthesis by Pol δ and Pol ϵ. The results above indicated that pol ϵ was more effective in producing ligatable products in the repair substrate than was pol δ. A distinct possibility for this difference was a limited strand displacement produced during synthesis by pol δ, resulting in the inefficient ligation. Such strand displacement synthesis by pol δ has been observed with other DNA substrates, for example, on circular single-stranded DNA containing two annealed oligonucleotides forming a gap of 230 nt (Podust & Hübscher, 1993). On a double-stranded DNA circle containing a defined 45 nt gap, pol δ in the presence of RFC and PCNA could fill the gap and synthesize up to 150 nt into the double-stranded region (V. N. Podust, L. Podust, and U. Hübscher, unpublished results). Replication of SV40 DNA *in vitro* with purified components (including pol δ and DNA ligase I) could yield closed-circular products only upon addition of a 5′−3′ exonuclease (Waga et al., 1994) This exonuclease, first designated DNase IV (Lindahl et al., 1969), has been given a variety of names including MF1, FEN-1, factor pL, and the human homolog of *Schizosaccharomyces pombe* rad2 (Robins et al., 1994). It has also been found to facilitate the production of ligated products in reactions including pol δ and DNA ligase I (Turchi & Bambara, 1993). DNase IV also improves the proportion of ligated products obtained with pol δ using the repair substrate described here (Figure 6 and data not shown). Quantification of Figure 6 showed that in the absence of DNase IV, only 0.5% of the products were closed-circular. Addition of 15 or 30 ng of DNase IV gave 21% and 27% closed-circular products, respectively. However, we still did not find conditions that yielded a proportion of closed-circular products as great as that achieved with pol ϵ. Synthesis carried out by pol ϵ gave 40% closed-circular products without DNase IV, while addition of 15 or 30 ng of DNase IV resulted in 43% and 55% closed-circular products, respectively.

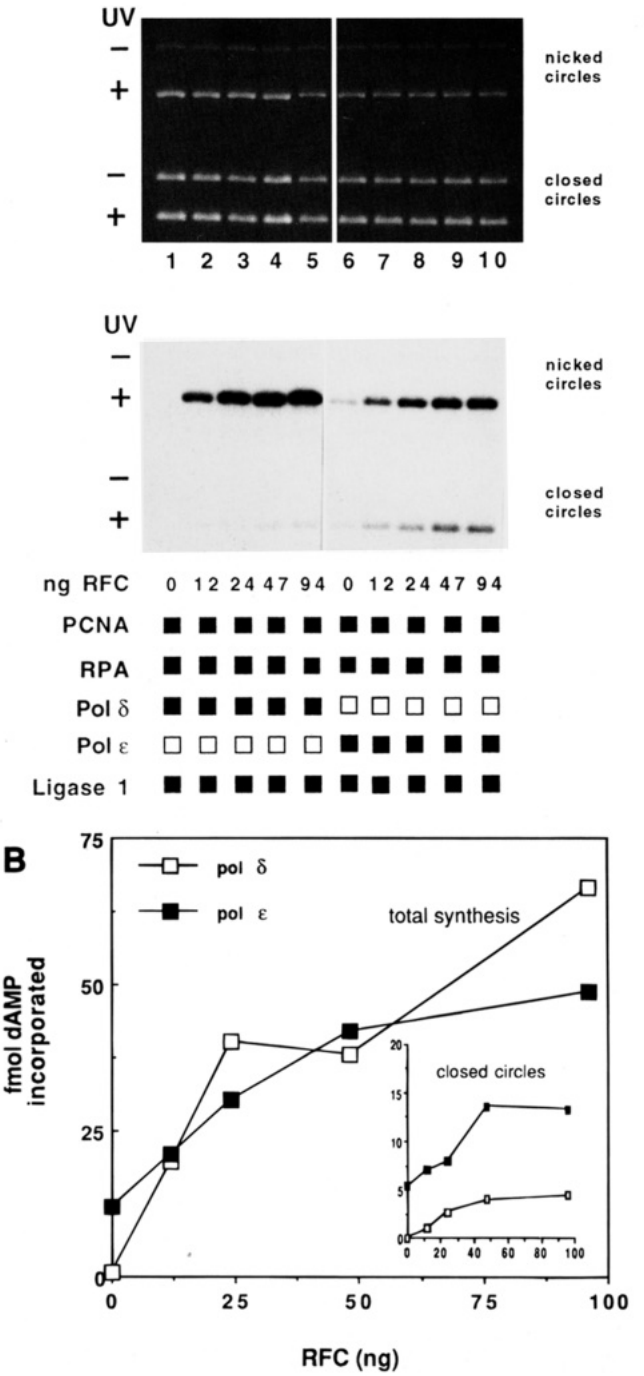

FIGURE 5: Dependence of repair synthesis by pol δ and ϵ on RFC. A, top panel) Reactions were performed and analyzed as in Figure 2. As indicated by the closed boxes, reaction mixtures included either pol δ (0.6 unit) or pol ϵ (2.4 units), RPA (150 ng), PCNA (100 ng), DNA ligase I (120 ng), and the indicated amounts of RFC. (B, bottom panel) Quantification of the data from part A. The main graph shows the sum of repair synthesis in nicked and closed UV-irradiated DNA, and the inset shows synthesis in the closed circular forms only, for pol δ (open symbols) and pol ϵ (closed symbols).

DISCUSSION

Dependence of Gap-Filling Synthesis by Pol ϵ on PCNA and RFC in the Presence of RPA. The principal findings of this study are that pol ϵ is well-suited to filling DNA single-strand gaps produced as reaction intermediates by nucleotide excision repair and that when the single-stranded DNA binding protein RPA is present this synthesis strongly depends on the accessory proteins PCNA and RFC. The substrate used contained incisions created during nucleotide excision repair by human cell extracts *in vitro*, which releases oligomers 27−32 nt long from UV-irradiated DNA (Svoboda et al., 1993), even in PCNA-depleted extracts (Nichols & Sancar, 1992). Filling in of the repair gap after oligomer release gives a patch size *in vitro* of about 30 nt (Shivji et

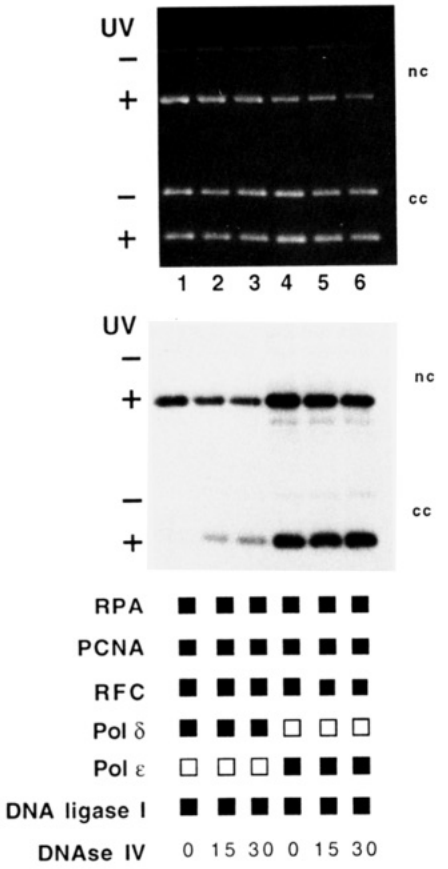

FIGURE 6: Effect of DNase IV on repair synthesis by pol δ and ϵ. Reactions were performed and analyzed as in Figure 2. Reaction mixtures included either pol δ (0.3 unit) or pol ϵ (3.2 units), RPA (150 ng), PCNA (100 ng), RFC (47 ng), DNA ligase I (80 ng), and the indicated amounts (ng) of DNase IV.

al., 1992). Inclusion of PCNA in the reaction mixtures increases total oligomer release, probably because repair synthesis assists turnover of the incision protein complex (Nichols & Sancar, 1992). For the synthesis reactions used here, DNA was purified by phenol−chloroform extraction, obviating this turnover function.

The single-stranded binding DNA protein RPA is needed during nucleotide excision repair (Coverley et al., 1991), in a function at an early stage prior to gap-filling repair synthesis (Coverley et al., 1992; Shivji et al., 1992). The RPA may assist in recognition of DNA damage (Clugston et al., 1992). It could also stabilize the gapped intermediate structure, since the binding site size of human RPA homotrimer on single-stranded DNA is 20−30 nucleotides (Kim et al., 1992; Seroussi & Lavi, 1993; Blackwell & Borowiec, 1994), which corresponds well to the size of the incision/excision gap formed in UV-irradiated DNA (Huang et al., 1992). It is therefore appropriate to consider and investigate repair gap-filling by DNA polymerases in the presence of RPA, since this is likely to reflect the most physiological situation.

Numerous studies have shown that pol δ is stimulated by PCNA to synthesize longer chains of DNA. Such stimulation takes place on a variety of templates such as poly(dA)-oligo(dT), primed single-stranded DNA, and SV40 DNA, and at both high and low ionic strengths (Tan et al., 1986; Tsurimoto & Stillman, 1989; Lee et al., 1991a; Podust et al., 1992; Eki et al., 1992; Waga et al., 1994). In contrast, pol ϵ is not affected by PCNA in many assays, and indeed it was first isolated as a "PCNA-independent" polymerase (Nishida et al., 1988; Focher et al., 1989). These studies were performed at low ionic strength (30 mM or less), where both polymerases are most active.

Significantly, however, pol ϵ is stimulated by PCNA under other types of assay conditions. Lee et al. (1991b) found that DNA synthesis by pol ϵ on poly(dA)-oligo(dT) is highly dependent on PCNA and its loading factor RFC at higher ionic strengths (130 mM NaCl or 175−225 mM potassium glutamate). In the absence of salt, synthesis by pol ϵ on a poly(dA)-oligo(dT) substrate does not require PCNA and RFC. At high salt, synthesis by pol ϵ alone is greatly inhibited on this substrate, and addition of PCNA and RFC restored DNA synthesis (Lee et al., 1991b).

The PCNA dependence of pol ϵ is also more marked with some DNA substrates than with others. DNA synthesis by human and bovine pol ϵ on singly-primed M13 DNA is highly dependent on both PCNA and RFC, even at low ionic strength (Lee et al., 1991b; Podust et al., 1992). A detailed enzymatic analysis indicated that PCNA both facilitates primer recognition by pol ϵ and enhances the elongation rate of pol ϵ (Maga & Hübscher, 1995).

The present study demonstrates a further condition under which synthesis by pol ϵ depends on PCNA and RFC: the filling of nucleotide excision repair gaps in the presence of RPA (Figures 4 and 5). Presumably, the accessory factors greatly facilitate loading of pol ϵ onto a template containing a single-stranded gap of ~30 nt which might be covered by RPA. Since the repair patch is short, PCNA is not required for processivity in the sense of promoting synthesis of long products. Rather, it appears that PCNA (and RFC) functions to direct and anchor the pol ϵ onto the repair primer/template junction (and perhaps displace RPA), so that gap-filling synthesis can occur in a controlled manner. It is not yet clear how critical the gap size is in dictating the PCNA dependence of pol ϵ in the presence of RPA. In a previous study, a closed-circular DNA template containing a defined 45 nt gap was used, and gap-filling by pol ϵ in the presence of RPA or *E. coli* single-stranded DNA binding protein was also found to be dependent on PCNA and RFC (Podust et al., 1994).

Relationship to Other in Vitro and in Vivo Studies. The PCNA dependence of nucleotide excision repair *in vitro* and *in vivo* indicates that either pol δ or pol ϵ is involved, and the data presented here suggest that pol ϵ is best-suited for the gap-filling step *in vitro*. DNase IV can, however, improve the yield of ligatable products in reactions with pol δ. Zeng et al. (1994) recently found that antibodies against human pol δ reduced synthesis mediated by nuclear extracts in UV-irradiated DNA. However, it is unclear whether the damage-dependent DNA synthesis observed in that study represented true nucleotide excision repair. It would be useful to reinvestigate the activity of the antibody in a system where a dependence on XP gene products can be demonstrated (Biggerstaff et al., 1993; O'Donovan et al., 1994).

It is difficult to determine which polymerase normally participates in nucleotide excision repair *in vivo*. Organisms often have several backup systems to perform the same function, and perhaps either pol ϵ or pol δ can participate in nucleotide excision repair synthesis in the cell. In an assay using permeabilized nuclei from UV-treated cells, Nishida et al. (1988) identified a human DNA polymerase that was needed for repair synthesis in UV-irradiated DNA. The repair synthesis was dependent on expression of XP genes, and hence was due to nucleotide excision repair. This

polymerase was subsequently identified as pol ϵ (Syväoja et al., 1990). Addition of exogenous PCNA had no effect on the repair activity of the permeabilized nuclei (Nishida et al., 1988). However, since rapid loading of PCNA onto UV-irradiated DNA occurs during repair, the repair synthesis in this assay could still have been PCNA dependent, because sufficient PCNA would already have been present in the nuclei. In conclusion, most available data suggest that pol ϵ is the best candidate for the nucleotide excision repair polymerase.

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REFERENCES

Aboussekhra, A., & Wood, R. D. (1994) *Curr. Opin. Genet. Dev. 4*, 212.
Biggerstaff, M., Robins, P., Coverley, D., & Wood, R. D. (1991) *Mutat. Res. 254*, 217.
Biggerstaff, M., Szymkowski, D. E., & Wood, R. D. (1993) *EMBO J. 12*, 3685.
Blackwell, L. J., & Borowiec, J. A. (1994) *Mol. Cell. Biol. 14*, 3993.
Burgers, P. M. J. (1991) *J. Biol. Chem. 266*, 22698.
Celis, J. E., & Madsen, P. (1986) *FEBS Lett. 209*, 277.
Cleaver, J. E., & Kraemer, K. H. (1989) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., & Valle, D., Eds.) 6th ed., p 2949, McGraw-Hill, New York.
Cleaver, J. E., Jen, J., Charles, W. C., & Mitchell, D. L. (1991) *Photochem. Photobiol. 54*, 393.
Clugston, C. K., McLaughlin, K., Kenny, M. K., & Brown, R. (1992) *Cancer Res. 52*, 6375.
Coverley, D., Kenny, M. K., Munn, M., Rupp, W. D., Lane, D. P., & Wood, R. D. (1991) *Nature 349*, 538.
Coverley, D., Kenny, M. K., Lane, D. P., & Wood, R. D. (1992) *Nucleic Acids Res. 20*, 3873.
Dresler, S. L., & Frattini, M. K. (1986) *Nucleic Acids Res. 14*, 7093.
Eki, T., Matsumoto, T., Murakami, Y., & Hurwitz, J. (1992) *J. Biol. Chem. 267*, 7284.
Fairman, M. P., & Stillman, B. (1988) *EMBO J. 7*, 1211.
Focher, F., Gassmann, M., Hafkemeyer, P., Ferrari, E., Spadari, S., & Hübscher, U. (1989) *Nucleic Acids Res. 17*, 1805.
Hall, P. A., McKee, P. H., Menage, H., Dover, R., & Lane, D. P. (1993) *Oncogene 8*, 203.
Hansson, J., Munn, M., Rupp, W. D., Kahn, R., & Wood, R. D. (1989) *J. Biol. Chem. 264*, 21788.
Huang, J. C., Svoboda, D. L., Reardon, J. T., & Sancar, A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3664.
Hübscher, U., & Spadari, S. (1994) *Physiol. Rev. 74*, 259.
Hunting, D. J., Gowans, B. J., & Dresler, S. L. (1991) *Biochem. Cell Biol. 69*, 303.
Jackson, D. A., Hassan, A. B., Errington, R. J., & Cook, P. R. (1994) *J. Cell Sci. 107*, 1753.
Kenny, M. K., Lee, S.-H., & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9757.
Kim, C., Snyder, R. O., & Wold, M. S. (1992) *Mol. Cell. Biol. 12*, 3050.
Lee, S. H., & Hurwitz, J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5672.
Lee, S.-H., Kwong, A. D., Pan, Z.-Q., & Hurwitz, J. (1991a) *J. Biol. Chem. 266*, 594.
Lee, S.-H., Pan, Z.-Q., Kwong, A. D., Burgers, P. M. J., & Hurwitz, J. (1991b) *J. Biol. Chem. 266*, 22707.
Lindahl, T., & Barnes, D. E. (1992) *Annu. Rev. Biochem. 61*, 251.
Lindahl, T., Gally, J. A., & Edelman, G. M. (1969) *Proc. Natl. Acad. Sci. U.S.A. 62*, 597.
Maga, G., & Hübscher, U. (1995) *Biochemistry 34*, 891.
Melendy, T., & Stillman, B. (1993) *J. Biol. Chem. 268*, 3389.
Miura, M., Domon, M., Sasaki, T., & Takasaki, Y. (1992) *J. Cell. Physiol. 150*, 370.
Nichols, A. F., & Sancar, A. (1992) *Nucleic Acids. Res. 20*, 3559.
Nishida, C., Reinhard, P., & Linn, S. (1988) *J. Biol. Chem. 263*, 501.
O'Donovan, A., Davies, A. A., Moggs, J. G., West, S. C., & Wood, R. D. (1994) *Nature 371*, 432.
Podust, L. M., Podust, V. N., Floth, C., & Hübscher, U. (1994) *Nucleic Acids Res. 22*, 2970.
Podust, V. N., & Hübscher, U. (1993) *Nucleic Acids Res. 21*, 841.
Podust, V. N., Georgaki, A., Strack, B., & Hübscher, U. (1992) *Nucleic Acids Res. 20*, 4159.
Popanda, O., & Thielmann, H. W. (1992) *Biochim. Biophys. Acta 1129*, 155.
Robins, P., Pappin, D. J. C., Wood, R. D., & Lindahl, T. (1994) *J. Biol. Chem. 269*, 28535.
Rydberg, B., Spurr, N., & Karran, P. (1990) *J. Biol. Chem. 265*, 9563.
Seroussi, E., & Lavi, S. (1993) *J. Biol. Chem. 268*, 7147.
Shivji, M. K. K., Kenny, M. K., & Wood, R. D. (1992) *Cell 69*, 367.
Shivji, M. K. K., Grey, S. J., Strausfeld, U. P., Wood, R. D., & Blow, J. J. (1994) *Curr. Biol. 4*, 1062.
Svoboda, D. L., Taylor, J. S., Hearst, J. E., & Sancar, A. (1993) *J. Biol. Chem. 268*, 1931.
Syväoja, J., Suomensaari, S., Nishida, C., Goldsmith, J. S., Chui, G. S. J., Jain, S., & Linn, S. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 6664.
Tan, C., Castillo, C., So, A., & Downey, K. (1986) *J. Biol. Chem. 261*, 12310.
Tomkinson, A. E., Lasko, D. D., Daly, G., & Lindahl, T. (1990) *J. Biol. Chem. 265*, 12611.
Toschi, L., & Bravo, R. (1988) *J. Cell Biol. 107*, 1623.
Tsurimoto, T., & Stillman, B. (1989) *EMBO J. 8*, 3883.
Tsurimoto, T., & Stillman, B. (1991) *J. Biol. Chem. 266*, 1950.
Turchi, J. J., & Bambara, R. A. (1993) *J. Biol. Chem. 268*, 15136.
Waga, S., Bauer, G., & Stillman, B. (1994) *J. Biol. Chem. 269*, 10923.
Weeda, G., & Hoeijmakers, J. H. J. (1993) *Semin. Cancer Biol. 4*, 105.
Wold, M. S., & Kelly, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2523.
Wood, R. D., Robins, P., & Lindahl, T. (1988) *Cell 53*, 97.
Zeng, X. R., Jiang, Y. Q., Zhang, S. J., Hao, H. L., & Lee, M. Y. W. T. (1994) *J. Biol. Chem. 269*, 13748.

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