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Purification of PCNA as a nucleotide excision repair protein

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ABSTRACT

Human cell free extracts carry out nucleotide excision repair *in vitro*. The extract is readily separated into two fractions by chromatography on a DEAE column. Neither the low salt (0.1 M KCl) nor the high salt (0.8 M KCl) fractions are capable of repair synthesis but the combination of the two restore the repair synthesis activity. Using the repair synthesis assay we purified a protein of 37 kDa from the high salt fraction which upon addition to the low salt fraction restores repair synthesis activity. Amino acid sequence analysis, amino acid composition and immunobloting with PCNA antibodies revealed that the 37 kDa protein is the proliferating cell nuclear antigen (PCNA) known to stimulate DNA Polymerases δ and ϵ. By using an assay which specifically measures the excision of thymine dimers we found that PCNA is not required for the actual excision reaction *per se* but increases the extent of excision by enabling the excision repair enzyme to turn over catalytically.

INTRODUCTION

The human syndrome xeroderma pigmentosum (XP) is characterized phenotypically by UV sensitivity, genotypically by mutations in seven genes (XPA-G), and biochemically by defective removal of pyrimidine dimers and DNA adducts caused by UV-mimetic agents (1). It has been generally assumed that XP genes encode the subunits of a human nuclease system which excises the modified nucleotides. The cloning of genes which complement the defects of XP-A (2), XP-B (3), and of XP-D (4) have revealed DNA sequences that encode for proteins with zinc finger, ATPase, and helicase motifs. These observations have led credence to this notion. Furthermore, Robins *et al.* (5) have recently used an *in vitro* repair synthesis complementation assay to purify the XP-A protein and have shown that the protein binds preferentially to UV-damaged DNA. However, some reports indicate that XP mutants are capable of removing thymine dimers from DNA *in vitro* (6,7) raising the possibility that purification of XP complementing proteins might not be sufficient to reconstitute the human excision nuclease system. Therefore, we decided to use a 'resolution and reconstitution' assay (8) with cell-free extract, rather than *in vitro* complementation, to purify the components of the human nucleotide excision nucleases from a cell free extract.

We used the repair synthesis assay (9–13) to monitor the purification of proteins involved in nucleotide excision repair and to identify a homogeneous protein of 37 kDa. Protein chemistry analyses revealed that this protein is the well-characterized proliferating cell nuclear antigen (PCNA).

MATERIALS AND METHODS

Materials

HeLa S3 cells were from the stock of Lineberger Cancer Research Center (University of North Carolina). Cells were grown in suspension to a titer of $5-8\times10^5$ cells/ml. DEAE Bio-Gel A and hydroxyapatite (Bio-Gel-HT) were purchased from Bio-Rad, phenyl sepharose from Sigma, and Ultrogel AcA34 from IBF Biotechnics, Inc. The following buffers were used in resolution and reconstitution of repair synthesis activity: Buffer A: 25mM HEPES, pH 7.9, 0.4 mM EDTA, 8 mM $MgCl_2$, 1 mM dithiothreitol, and 17% glycerol. Buffer B: 25 mM HEPES, pH 7.9, 100 mM KCl, 0.4 mM EDTA, 8 mM $MgCl_2$, and 1 mM dithiothreitol.

Purification of PCNA as a Repair Protein

All purification steps were carried out at 0–4°C. Activity and purity of the fractions were monitored by reconstitution of repair synthesis in vitro and by analysis on SDS-PAGE followed by silver staining.

HeLa cell-free extract (CFE) was prepared by the method of Manley *et al.* (14) and following the dialysis step CFE containing 180 mg protein was loaded onto a 30 ml DEAE Agarose column preequilibrated with Buffer A + 100 mM KCl. Unbound proteins (Fraction D1) were washed from the column with 60 ml of the same buffer, and bound proteins (Fraction D2) were eluted with 60 ml of Buffer A + 800 mM KCl. Three-ml fractions were

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collected and the proteins were located by analyzing 2 μl of each fraction by SDS-PAGE. The peak fractions of low salt and high salt eluates were combined separately. The low salt eluate (D1) was divided into 100 μl aliquots and stored at −80°C. The high salt eluate (D2, 9 ml) was processed further.

Fraction D2 (8 ml) was adjusted to 20% saturated ammonium sulfate and applied to a 12-ml phenyl sepharose column preequilibrated with 20% saturated ammonium sulfate in Buffer B. The column was washed with 12 ml of the same buffer and developed with a step gradient (5 steps of 6 ml) of 20% saturated ammonium sulfate to 40% ethylene glycol and a final elution with 24 ml 50% ethylene glycol, all in Buffer B. Three-ml fractions were collected. The active fraction (PS), which eluted with 50% ethylene glycol, was dialyzed against Buffer A + 100 mM KCl and concentrated to 1 ml by ultrafiltration in Centricon 10 (Fraction PS).

Fraction PS was loaded on a 125 ml (1.6 cm×60 cm) AcA34 column which had been calibrated with Sigma SDS-7 molecular weight markers as described by Laue and Roberts (1990) and equilibrated with Buffer A + 100 mM KCl. The column was developed with the same buffer, collecting 2 ml fractions. The activity eluted at fractions corresponding to 80 kDa molecular weight. The active fractions were combined (12 ml) and dialyzed against 20 mM potassium phosphate pH 6.8, 0.4 mM EDTA, 1 mM dithiothreitol, and 17% glycerol (Fraction SC).

Fraction SC (7 ml) was applied to a 5 ml hydroxyapatite column and the column was developed with a 50 ml gradient of 20 mM to 200 mM potassium phosphate, pH 6.8, collecting 2 ml fractions. The active fraction eluted at 75 mM potassium phosphate (Fraction HA1). Chromatography on hydroxyapatite was repeated with a smaller column (2 ml) and a smaller gradient (10 ml) to obtain an active fraction (1.5 ml) with a single protein as determined by silver staining of SDS-PAGE (Fraction HA2).

Protein Chemistry

The amino acid composition of the purified protein and the amino acid sequence of a product from tryptic digestion were determined at the Yale Protein Chemistry Facility by Kathy Stone and Dr. Ken Williams. The sequence obtained from the tryptic peptide was compared with the sequences at the Protein Sequence Database, Protein Identification Resource, National Biomedical Research Foundation.

Substrates

Two substrates were used: pBR322 damaged with cisplatin (11) for repair synthesis, and pUNC1991-4 containing 4 T< >Ts at unique locations (15) for the excision assay. pBR322 and the control plasmid pUC18 were purified through two cesium chloride-ethidium bromide gradients and contained less than 2% nicked DNA. To prepare Pt-adducted DNA, pBR322 was incubated in 1 ml 10 mM Tris HCl, pH 8.0 and 1 mM EDTA containing 59 nM DNA (plasmid) and 1.3 μM cisplatin at room temperature for 24 hrs. Unincorporated drug was removed by dialysis against 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, and 1 mM EDTA. Cisplatin modification was performed in the dark or under yellow light to prevent nicking and as a result the modified DNA contained no more nicks than the control DNA.

The excision assay substrate pUNC1991-4 containing ^{32}P label at the 11th phosphodiester bonds 5′ to the 4 T< >Ts located at unique positions was prepared as described previously (*19*) and contained less than 5% nicked molecules.

Assays

The repair synthesis assay which measures the incorporation of radiolabeled nucleoside into damaged DNA by CFE or a mixture of column fractions was conducted as described by Wood *et al.* (9) and Sibghat-Ullah *et al.* (11) except 4 μCi[$\alpha^{32}P$] dCTP, 61 mM KCl and 6.8% glycerol per reaction were used. 30 μg of the low salt eluate from DEAE-agarose (D1 fraction) was incapable of repair synthesis, but activity could be reconstituted by addition of 30 μg of the high salt eluate (Fraction D2). D2 was subjected to further purification on additional resins, and monitored by restoration of repair synthesis activity to D1.

The excision repair assay measures the release of oligomers containing thymine dimers and was conducted as described by Huang *et al.* (15). Briefly, a semisynthetic plasmid containing four cyclobutane thymine dimers (T< >T) at predetermined sites and ^{32}P label at the 11th phosphodiester bond 5′ to the T< >Ts was incubated with CFE or mixtures of column fractions in 50

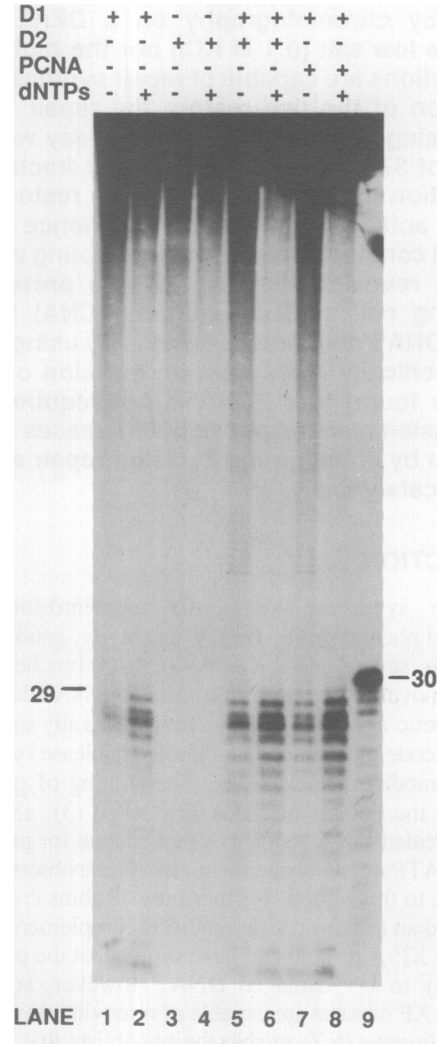

Figure 1. Purification of PCNA by Repair Synthesis Resolution and Reconstitution Assay. On the left are the autoradiographs of repair synthesis assays conducted with control (−) DNA or cisplatin adducted (Pt)DNA. On the right is a schematic of the separation procedure employed to obtain a pure protein essential for repair synthesis activity. Each repair synthesis reaction contained 30 μg D1 and/or 10 μl of the active fraction from the indicated column.

μl repair synthesis buffer (11) with or without dNTPs as indicated. Incubation was at 30°C for 2 hrs. Afterwards the DNA was deproteinized by proteinase K and phenol extraction, precipitated with ethanol, redissolved in formamide-dye and analyzed on 10% polyacrylamide DNA sequencing gel. The excision products are seen as bands of 27−29 nucleotides on an autoradiograph of the gel.

Other Methods

Protein concentration of cell-free extract and DEAE and phenyl Sepharose fractions was determined by Bradford assay (16) using Bio-Rad reagents and bovine serum albumin as standard. Protein concentration of AcA34 and hydroxyapatite fractions was determined by Silver staining and amino acid analysis by the ninhydrin method. SDS-PAGE was done by the method of Laemmli (17) and the gels were silver stained as described by Morrissey *et al.* (18), using Bio-Rad developer. Autoradiographs, photographs of ethidium bromide stained gels, and silver stained SDS-polyacrylamide gels were scanned with a Zeineh Softlaser Scanning Densitometer (Fullerton, CA) for quantifying radiolabel incorporation, DNA, and protein, respectively.

Table 1. Purification of PCNA as a Repair Protein

Fraction	Volume (ml)	Total Protein (mg)[a]	Specific Activity
1. Cell-free Extract	18.5 ml	178.7	8
2. DEAE (D2)	9	20.7	18
3. Phenylsepharose (PS)	3	0.44	307
4. AcA34 (SC)	12	0.19	3106
5. Hydroxyapatite I(HA1)	2	0.02	8400
6. Hydroxyapatite II(HA2)	1.5	0.01	11200

[a]These numbers are based on the assumption that the entirety of each fraction was employed in the next step; in reality 15−40% of each fraction was retained for further analyses.

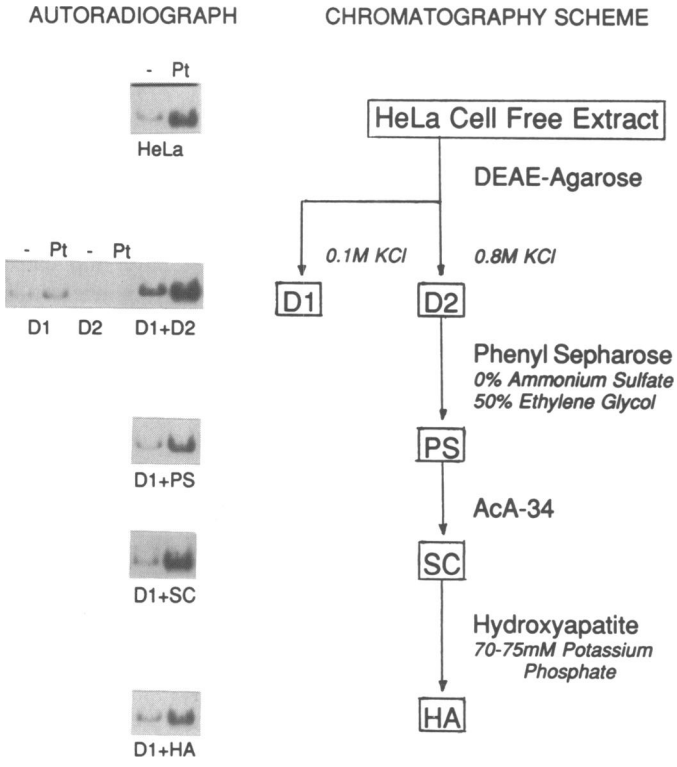

Figure 2. Hydroxyapatite Column Activity Profile. Fractions of 0.5 ml were collected and tested for repair synthesis complementing activity upon addition to the D1 fraction. The top panel shows an autoradiogram of the repair synthesis assays and in the bottom panel the elution profile of the 37 kDa protein is compared to the activity profile. Substrate(S) DNA is 300 ng pBR322, 25 cisplatin adducts/plasmid and control (C) is 300 ng pUC18, unmodified. Protein concentrations were determined by silver staining of SDS-polyacrylamide gels and amino acid analysis by the ninhydrin method.

RESULTS

Resolution and Reconstitution of Repair Synthesis Activity

To isolate the subunits of the human excision nuclease(s) we decided to separate HeLa CFE into two fractions unable to carry out repair synthesis and then purify the protein(s) necessary for activity from each of these fractions. Fig. 1 shows that passing the CFE extract through a DEAE resin equilibrated with 0.1 M KCl is sufficient for the initial separation. About 80% of the proteins pass through the column (D1) while the 20% which are retained are eluted with 0.8 M KCl (D2). Although the low-salt fraction contains some residual repair synthesis activity, the high salt fraction is completely devoid of activity. Yet upon addition of D2 to the low-salt fraction the repair synthesis signal is enhanced by a factor of 5−10. Therefore we decided that D2 contained one or more proteins necessary for carrying out nucleotide excision repair synthesis and proceeded to purify these proteins. The DEAE high salt fraction was further purified through successive chromatography on phenyl sepharose, AcA34 (gel permeation), and hydroxyapatite resins. The activity always eluted as a single peak as evidenced by the fact that mixing with fractions from outside of the peak did not improve the repair

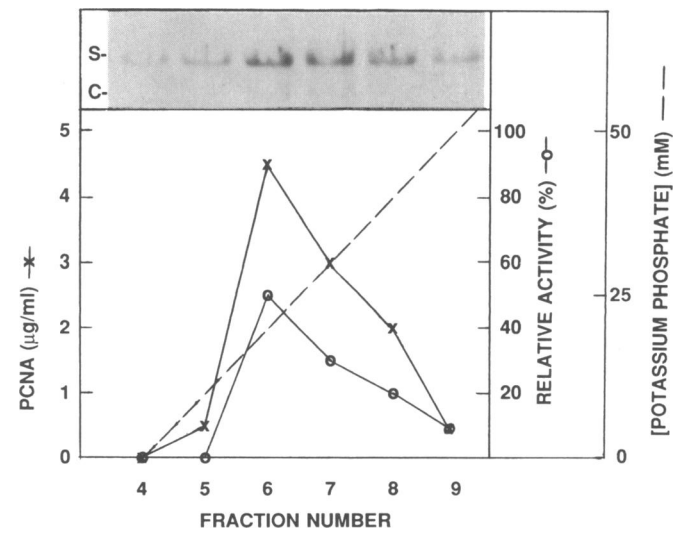

Figure 3. SDS-PAGE Analysis of the Purification Fractions. The following amounts of protein were loaded into the lanes; cell free extract (CFE) 10 μg; High Salt DEAE (DE), 4 μg; Phenyl Sepharose (PS), 2 μg; AcA34 (SC), 1 μg; Hydroxylapatite I (HA1), 0.2 μg; Hydroxylapatite II (HA2), 0.1 μg. The gel was silver stained. The mass of the size markers are indicated on the side.

synthesis signal achieved with D1 plus peak column fractions (data not shown).

It thus appeared that the high-salt DEAE fraction contained a single protein which was required to restore the repair synthesis activity of D1. The activity eluted as a protein of 80 kDa molecular weight on the sizing column and coincided with a polypeptide of M_r=37,000 at the final purification step on a hydroxyapatite column (Fig. 2). Thus, we concluded that the repair protein was a dimer of a 40 kDa polypeptide. Table 1 summarizes the purification steps and in Fig. 3 the fractions are analyzed by SDS-PAGE and silver staining. Specific activity is expressed as pmole of dCMP incorporated/mg protein. In a standard repair synthesis reaction mixture, containing 50 μg HeLa cell-free extract and 0.3 μg of Pt-adducted pBR322, 400 fmol dCMP is incorporated in 2 hours at 30°C.

Table 2. Amino acid Compositions of the 'Repair Protein' and PCNA

Amino acid	Repair Protein[a]	PCNA[b]
Cys	ND	6
Asx	32	30
Thr	11	12
Ser	21	25
Glx	37	31
Pro	7	7
Gly	ND	14
Ala	21	19
Val	21	21
Met	6	10
Ile	13	14
Leu	27	29
Tyr	8	7
Phe	8	8
His	5	3
Lys	15	16
Arg	ND	8
Trp	ND	1

[a]Based on 232 amino acids not including cys, gly, arg and trp which were not determined (ND).
[b]Composition based on published sequence (19).

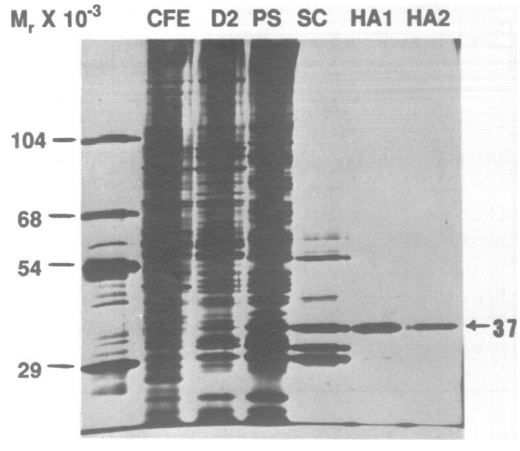

Figure 4. Excision of T< >T in the Absence of PCNA. DNA containing internal label located at the 11th phosphodiester bond 5' to T< >Ts was incubated with D1 with or without D2 (75 nM PCNA) or purified PCNA (25 nM) in the absence or presence of dNTPs as indicated. The reaction products were then analyzed on a 10% sequencing gel. Equal amounts of radioactivity were loaded into all lanes. The positions of the primary excision products are marked by arrows.

PCNA is a Repair Protein

It is apparent from Fig. 3 that after the final purification step restoration of repair synthesis activity to D1 is associated with a single protein. To gain a better understanding of the role of this protein in repair we obtained the amino acid composition and compared it to other proteins of similar molecular weight which are known to be involved in DNA repair, namely the XP-A protein and DNA polymerase β. The amino acid composition shown in Table 2 is entirely different from those of the latter two proteins. To further characterize our protein we cleaved the protein with trypsin and cyanogen bromide, isolated fragments and sequenced one which was in reasonable yield and high purity. The 17- amino acid sequence obtained -SQTSNVDKEEEAVTIE- was in complete agreement with the residues 182−198 of human proliferating cell nuclear antigen, PCNA). Comparisons of the two proteins (Table 2) determined that their compositions were identical within experimental error. Dot immunoblotting with PCNA monoclonal antibody positively identified the purified protein as PCNA (data not shown). As further proof, human PCNA was overexpressed in *E. coli* BL 21 (DE3) on pT7hPCNA plasmid (K. Fien and B. Stillman, personal communication) as described for *S. cerevisiae* PCNA (20). The overexpressed PCNA was purified as described in Materials and Methods for HeLa extract. Chromatographic behavior was identical for PCNA from both sources, and the overexpressed protein substituted for equivalent amounts of HeLa extract PCNA in the repair synthesis assay (data not shown). Thus, we conclude that the repair protein we have purified is PCNA.

Role of PCNA in Nucleotide Excision Repair

The identification of PCNA as a repair protein was not surprising because in humans PCNA is known to stimulate DNA polymerase δ (21) and polymerase ε (22). Recent studies have implicated polymerase ε and δ in repair synthesis following nucleotide excision (23−25). We also considered the possibility that PCNA might actually participate in nucleotide excision repair at a preincision/excision step.

To find out whether PCNA participates in the excision or resynthesis steps or both we conducted the excision assay with various fractions and combinations in the absence or presence of dNTPs. The excision assay which utilizes T< >T containing

Table 3. Effect of dNTP and PCNA on Excision and Repair Synthesis Activities.

	Excision Activity[a]		Repair Synthesis Activity (fmole dCMP)	
	−dNTP	+ dNTP[b]	− dNTP[c]	+ dNTP[d]
D1	100	264 ± 11	6	109
D2(PCNA)	0	0	0	0
D1+D2(PCNA)	245 ± 20	463 ± 55	5	748

a. Activity relative to D1-dNTP (100 arbitrary units). Average of 3−4 experiments (± SEM).
b. 20 μM each of dATP, dCTP, dGTP and dTTP added to each 50 μl reaction.
c. 14 nM (4 μCi) [α^{32}P]dCTP (specific activity 6000 Ci/mmole) added to each 50 μl reaction.
d. 20 μM each of dATP, dGTP and dTTP, 8 μM dCTP and 4 μ Ci[α^{32}P] dCTP added to each 50 μl reaction.

DNA with radiolabel in the vicinity of T< >T has been used successfully by Huang *et al.* (15) to demonstrate that human cells excise T< >T in 27−29 nucleotide-long oligomers by incising at precise positions with regard to T< >T. Fig. 4 shows the results of these experiments conducted with D1 and with D1 plus PCNA. As is apparent from the figure, D1 (which has less than 3% of the amount of PCNA found in D2, as determined by dot immunoblotting with monoclonal PCNA antibody, data not shown) carries out excision in the absence of dNTPs (lane 1) indicating that PCNA is not involved in the excision reaction *per se*. In lane 2 it is seen that dNTPs stimulated the level of excision even without PCNA. Addition of PCNA to D1 in the absence of dNTPs also stimulated excision (lane 5 and 7). Maximum stimulation was achieved in the presence of both dNTPs and PCNA (lanes 6 and 8). These results (summarized in Table 3) would imply that PCNA is involved in the resynthesis step by stimulating the repair DNA polymerase.

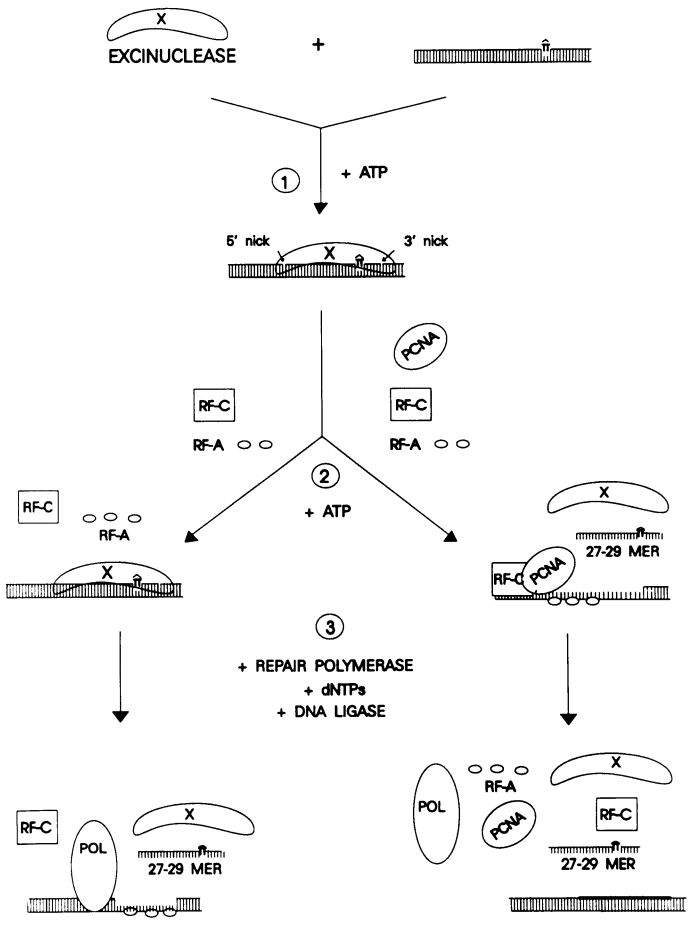

Figure 5. Proposed model for the role of PCNA in human nucleotide excision repair synthesis. Step 1: Excision nuclease (Excinuclease, X) binds to DNA at damage site and makes dual incisions 3′ and 5′ to the damage (19). Step 2: A 'primer recognition complex' forms (17) in the presence of PCNA (right side). The excision nuclease is displaced stimulating enzymatic turnover. In the absence of PCNA (left side), the post-incision complex remains intact. Step 3: Addition of dNTPs results in gap filling by the repair polymerase, followed by ligation. In the absence of PCNA (left side), the excinuclease is displaced by the repair polymerase directly with concomitant gap filling. This mechanism is less efficient overall with regard to enzymatic turnover than that involving PCNA in a 'primer recognition complex' followed by gap filling. Abbreviations: Replication Factor A, RF-A; Replication Factor C, RF-C; DNA Repair Polymerase, POL.

DISCUSSION

The results presented in this study raise two questions: which DNA polymerase is involved in repair synthesis following nucleotide excision repair and how does PCNA stimulate both excision and repair synthesis.

Although all four nuclear DNA polymerases have been implicated in nucleotide excision repair at one time or another, the most recent evidence has implicated DNA polymerase ϵ (26; see 24). Syvaoja *et al.* (26) isolated DNA polymerases δ and ϵ from HeLa cells and, using a permeabilized cell system, found that addition of polymerase ϵ but not polymerase δ resulted in UV-induced repair synthesis. Lee *et al.* (22) have reported that PCNA can stimulate *in vitro* DNA synthesis by both DNA polymerase δ and polymerase ϵ. Our results do not rule out either polymerase as being the repair polymerase but provide strong evidence that whichever polymerase fills-in the excision gap, it carries out this particular reaction in a PCNA-dependent manner. A previous study which reported increased PCNA immunostaining in non S-phase human amnion cells following UV irradiation also implicated PCNA in nucleotide excision repair (27). Thus the *in vivo* results are in agreement with the CFE system and the permeabilized cells.

To investigate whether PCNA directly participates in excision, we conducted thymine dimer excision in the presence and 'absence' (less than 2.5 nM) of PCNA, and in the presence and absence of dNTPs. We found that in the absence of PCNA and dNTPs excision did occur, which led us to conclude that PCNA is not a part of the 'excision nuclease' complex. A stimulatory effect of PCNA on excision was observed in the absence of dNTPs. The excision reaction was stimulated by dNTPs in the absence or presence of PCNA with the most dramatic effect achieved when both PCNA and dNTPs were added to the receptor fraction which apparently contains all other repair proteins. The most likely explanation for these findings is that the basal level of 'nucleotide excision nuclease' is very low and that the enzyme does not act catalytically in the absence of repair synthesis proteins as is the case in *E. coli* (28−30). PCNA along with the repair polymerase and other-yet unidentified proteins-release the excision nuclease and enable it to turnover enzymatically. This model (Fig. 5) might also explain the strong dependence of repair synthesis on the human single-stranded DNA binding protein, RF-A (31). In fact, in an *E. coli* cell-free repair synthesis system containing both the excision nuclease and repair polymerase, no repair synthesis at all was observed unless helicase II was included in the reaction mixture (32) giving the impression that helicase II might be involved in incision, whereas extensive studies have shown that helicase II does not participate in repair prior to the incision/excision reaction. Thus, it appears that the replication complex which includes Pol δ or Pol ϵ, PCNA, RF-A, RF-C and possibly other proteins (helicases) known to be required for human DNA replication (33,34) carry our repair synthesis in human nucleotide excision repair and in doing so enable the excision nuclease to act catalytically.

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