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Xeroderma pigmentosum variant (XP-V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity

Chikahide Masutani[1], Marito Araki[1,2,3], Ayumi Yamada[1,2], Rika Kusumoto[1,4], Tomokazu Nogimori[1,2], Takafumi Maekawa[1], Shigenori Iwai[5] and Fumio Hanaoka[1,3,6]

[1]Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, [2]The Graduate School of Pharmaceutical Science, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, [3]The Institute of Physical and Chemical Research (RIKEN), Wako-shi, Saitama 351-0198, [4]Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara 630-01 and [5]Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan

[6]Corresponding author
e-mail: fhanaoka@imcb.osaka-u.ac.jp

Xeroderma pigmentosum variant (XP-V) represents one of the most common forms of this cancer-prone DNA repair syndrome. Unlike classical XP cells, XP-V cells are normal in nucleotide excision repair but defective in post-replication repair. The precise molecular defect in XP-V is currently unknown, but it appears to be a protein involved in translesion synthesis. Here we established a sensitive assay system using an SV40 origin-based plasmid to detect XP-V complementation activity. Using this system, we isolated a protein from HeLa cells capable of complementing the defects in XP-V cell extracts. The protein displays novel DNA polymerase activity which replicates cyclobutane pyrimidine dimer-containing DNA templates. The XPV polymerase activity was dependent on $MgCl_2$, sensitive to NEM, moderately sensitive to KCl, resistant to both aphidicolin and ddTTP, and not stimulated by PCNA. In glycerol density gradients, the activity co-sedimented with a 54 kDa polypeptide at 3.5S, indicating that the monomeric form of this polypeptide was responsible for the activity. The protein factor corrected the translesion defects of extracts from three XPV cell strains. Bypass DNA synthesis by the XP-V polymerase occurred only in the presence of dATP, indicating that it can incorporate only dATP to bypass a di-thymine lesion.

Keywords: cyclobutane pyrimidine dimer/DNA polymerase/DNA replication/error-free translesion synthesis/xeroderma pigmentosum variant

Introduction

Xeroderma pigmentosum (XP) is an autosomal recessive human disease characterized by sunlight sensitivity, cutaneous and ocular deterioration, and premature malignant skin neoplasms after exposure to sunlight. XP has been classified into eight complementation groups, XP-A to XP-G and XP-V. Cells from XP-A to XP-G patients have defects in the process of nucleotide excision repair (NER), which eliminates a wide variety of structurally unrelated lesions, including ultraviolet light (UV)-induced cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts, as well as certain chemical adducts. The genes and proteins of XP groups A, B, C, D, F and G have been isolated and found to represent some of the subunits of the core NER machinery. In contrast, cells belonging to the eighth group, XP variant (XP-V), are NER-proficient but display abnormal DNA replication, including reduced ability to elongate nascent DNA strands on UV-irradiated DNA. Thus, the *XP-V* gene product is likely to be involved in the process of DNA replication on damaged DNA known as post-replication repair, but not in NER (Friedberg *et al.*, 1995; Bootsma *et al.*, 1998).

DNA replication in eukaryotic cells is a complex process requiring a variety of proteins to synthesize the leading and lagging strands of DNA in an asymmetric and coordinated manner (Bambara *et al.*, 1997; Waga and Stillman, 1998). UV irradiation of mammalian cells leads to a transient inhibition of DNA synthesis by a variety of mechanisms, including checkpoint mechanisms in G_1–S phase and replication fork block at DNA lesions during the S phase of the cell cycle. The checkpoint responses have been suggested to provide the necessary time to repair damaged DNA before the restart of DNA replication. The inhibition of active replication, however, is not absolute, and some bypass replication eventually takes place before DNA repair. Two subpathways, error-free and error-prone, are thought to be responsible for bypass replication in cells. In yeast, DNA polymerase ζ, the sixth DNA polymerase of eukaryotic cells, which can catalyze *trans*-dimer synthesis, has been identified and shown to be involved in the error-prone pathway (Lawrence and Hinkle, 1996; Nelson *et al.*, 1996a). A RAD6-dependent error-free bypass replication pathway has also been found in yeast cells (Lawrence, 1994). *RAD30* was shown to be involved in this pathway (McDonald *et al.*, 1997; Roush *et al.*, 1998) and to code for a DNA polymerase capable of bypassing di-thymine lesions efficiently (Johnson *et al.*, 1999). However, the molecular mechanisms still remain largely unknown, and very little is known about either pathway in higher eukaryotes. Recent studies using cell-free SV40 origin-dependent replication systems have revealed that extracts from human HeLa cells are capable of bypassing lesions on both leading and lagging strands during replication. The replication was only weakly mutagenic at UV-induced di-thymine sites, suggesting that the error-free bypass pathway had been successfully reconstituted in a human cell-free system (Svoboda and Vos, 1995; Carty *et al.*, 1996; Cordeiro-Stone *et al.*, 1997; Ensch-Simon *et al.*, 1998). More importantly, using a

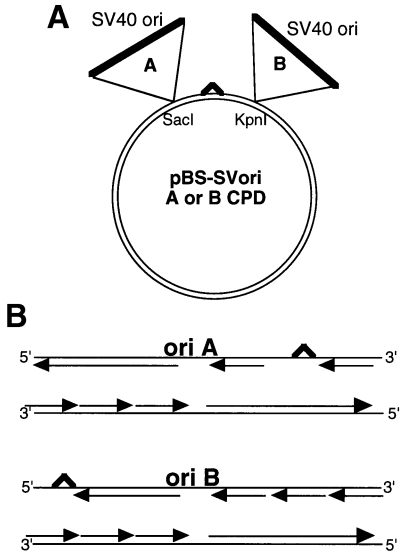

Fig. 1. Schematic representation of the constructs. (**A**) The SV40 origin of DNA replication was cloned at the *Sac*I and *Kpn*I sites of pBS-KS(+) to make pBS-SVoriA and pBS-SVoriB, respectively. A chemically synthesized oligomer containing a *cis–syn* thymine dimer was placed between the *Pst*I and *Hin*dIII sites of pBS-KS(+). (**B**) Replication forks starting from oriA would be expected to encounter the lesion on the lagging strand template whereas those starting from oriB would be expected to encounter the lesion on the leading strand template.

plasmid DNA containing a single CPD on the leading strand, some groups reported that extracts from XP-V cells lacked lesion bypass replication (Cordeiro-Stone *et al.*, 1997; Ensch-Simon *et al.*, 1998; Svoboda *et al.*, 1998; Cordonnier *et al*., 1999).

Here we constructed a sensitive assay system to detect an activity that corrects defects of extracts from XP-V cells, purified the XP-V correcting protein from HeLa cells, and found that the protein has a novel DNA polymerase activity.

Results

Cell-free damage bypass DNA replication

Covalently closed circular templates containing the SV40 origin of DNA replication and a single CPD (Figure 1A) were used to assay damage bypass DNA replication in a cell-free system. Two types of constructs, pBS-SVori-A[CPD or ND (no damage)] and pBS-SVoriB(CPD or ND), were designed to position the SV40 origin in opposite orientations on the DNA template. In this way, replication forks would encounter the lesion on the lagging strand template in the case of pBS-SVoriA and on the leading strand template in the case of pBS-SVoriB (Figure 1B). These constructs were incubated with human cell extracts and newly synthesized products were fractionated on agarose gels containing ethidium bromide. To examine whether the CPD was present on the newly synthesized closed circular DNA molecules (RFI), half of each sample was treated with T4 endonuclease V (T4 endoV), which specifically introduces a nick at the CPD site, and the products were subjected to electrophoresis. This treatment converts RFI molecules to nicked circular molecules (RFII) if damage bypass replication occurs. As shown in Figure 2A, the reactions were almost completely dependent on SV40 large T antigen, indicating that most DNA replication began at the SV40 replication origin and that repair-type syntheses did not occur to any significant extent. This inefficient repair could have several explanations; the assay conditions and cell extracts were optimized for DNA replication but not for repair, and the CPD which is known to be repaired very slowly was used as a lesion. The amount of newly synthesized RFI molecule containing the CPD was ~70% of that of the undamaged RFI molecule, irrespective of whether pBS-SVoriA or pBS-SVoriB DNAs were used as template (Figure 2A, compare lanes 3 with 7 and 11 with 15). On pBS-SVoriB (lesion on the potential leading strand), the incorporation of radioactive material into RFII and RFI molecules was decreased by the presence of CPD. On pBS-SVoriA (lesion on potential lagging strand), however, little difference was observed between undamaged and damaged templates. These results suggest that the lesion on the lagging strand can be overcome by two mechanisms, bypass DNA replication and the synthesis of Okazaki fragments beyond the lesion, resulting in the formation of gapped molecules. In contrast, a lesion on the leading strand can only be overcome by the bypass replication mechanism, as observed by others (Svoboda and Vos, 1995; Cordeiro-Stone *et al.*, 1997). T4 endoV treatment of the products of CPD templates revealed that ~40% of the newly synthesized RFI molecules were sensitive to nicking (Figure 2A, compare lanes 7 with 8 and 15 with 16), in contrast to the cases of undamaged templates in which a much smaller portion was incised by the enzyme (Figure 2A, compare lanes 3 with 4 and 11 with 12). As half of the newly synthesized daughter molecules would be derived from the replication of damage-free parental strands and would therefore be insensitive to nicking by T4 endoV, ~80% of the lesions must have been bypassed by the DNA replication complex.

To examine the activities of XP-V cell extracts, we used partially and transiently transformed cell mixtures as described in the Materials and methods, because it was hard to make replication-competent cell extracts from nontransformed cells. Using these cell extracts, damage bypass replication activities were examined. As shown in Figure 2B, T antigen-dependent DNA replication was observed with XP7TA cell extracts, indicating that the extracts were as replication proficient as HeLa cell extracts. However, both leading and lagging strand DNA synthesis on CPD templates by XP7TA cell extracts was clearly inhibited, and the amount of newly synthesized RFI molecule with the CPD templates was ~25–30% of those of the undamaged templates (Figure 2B, compare lanes 3 with 7 and 11 with 15). The newly synthesized RFI molecules were relatively resistant to T4 endoV treatment (Figure 2B, compare lanes 7 with 8 and 15 with 16), indicating that XP7TA cell extracts were defective in damage bypass replication. There was a clear difference in the level of newly synthesized RFII molecules between templates containing the lesion on the leading strand template and those containing the lesion on the lagging strand template (compare lanes 7, 8 with 15, 16 in Figure 2A and B). These results suggest that XP7TA cell extracts are deficient in damage bypass replication but proficient in the replication of damage-free parental strands, probably

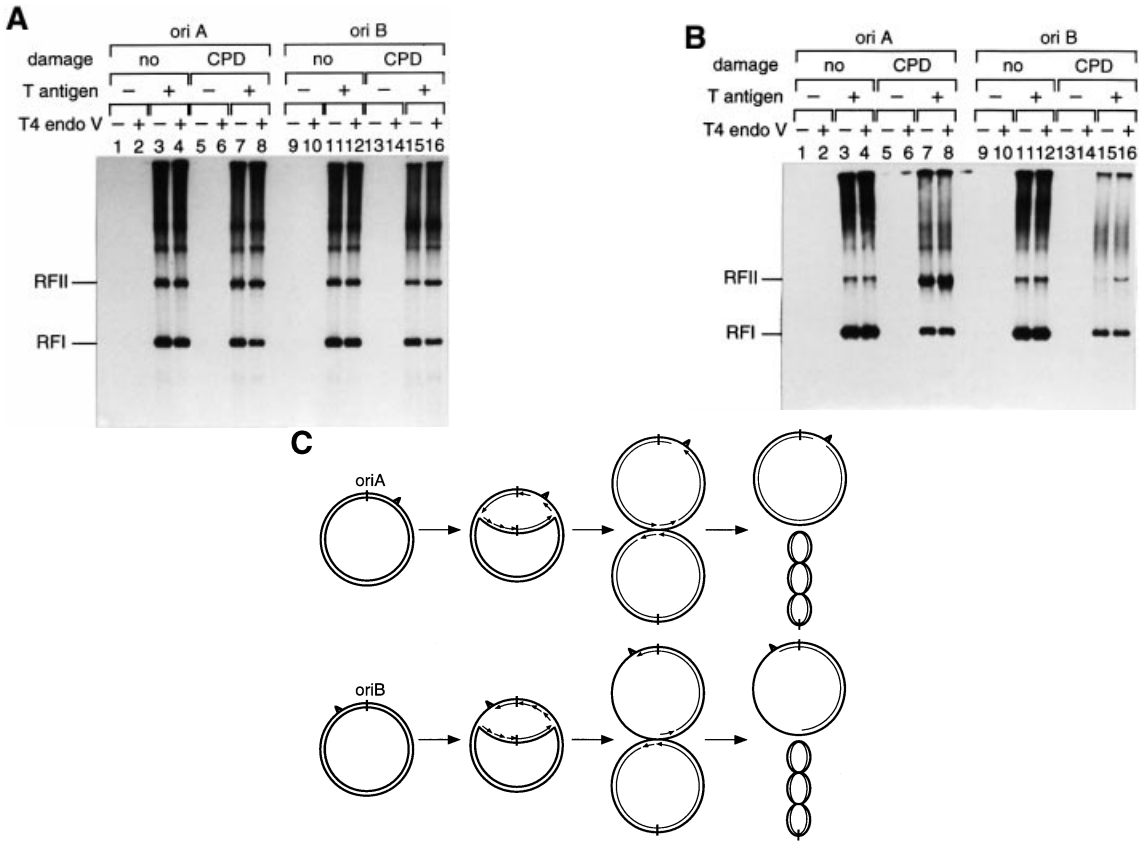

Fig. 2. 'One-step' damage bypass replication assay with human cell extracts. (**A**) HeLa cell extracts were incubated at 37°C with DNA templates in the presence (lanes 3, 4, 7, 8, 11, 12, 15 and 16) or absence (lanes 1, 2, 5, 6, 9, 10, 13 and 14) of SV40 T antigen in the standard reaction mixtures for the one-step assay. Templates used were pBS-SVoriA(ND) (lanes 1–4), pBS-SVoriA(CPD) (lanes 5–8), pBS-SVoriB(ND) (lanes 9–12) and pBS-SVoriB(CPD) (lanes 13–16). After a 60 min incubation the products were purified and incubated in the presence (even numbered lanes) or absence (odd numbered lanes) of T4 endoV, and subjected to an agarose gel electrophoresis in the presence of ethidium bromide. An autoradiogram of the gel is shown. The positions of closed circular (RFI) and nicked circular (RFII) molecules are indicated. (**B**) XP7TA cell extracts were used instead of HeLa cell extracts and analyzed as in (A). (**C**) Potential effects of CPD on *in vitro* DNA synthesis with XP7TA cell extracts are illustrated. Because of the absence of the damage bypass machinery in the XP-V cell extracts, movement of the replication fork from the origin to the right is blocked transiently when the lesion is present on the lagging strand template (oriA). However, leading strand synthesis continues and even lagging strand synthesis would resume after synthesizing new Okazaki fragments beyond the lesion. This would leave a short gap opposite the CPD. This explains why RFII molecules accumulate even without T4 endoV treatment of the products. On the other hand, elongation of the leading strand is blocked almost completely by replication fork movement from the origin (oriB) to the left. This would result in the uncoupling of leading and lagging strand synthesis, resulting in RFI molecules synthesized almost completely by lagging strand synthesis. However, there remain some replication intermediates whose radioactive intensity is not so high because of limited synthesis on the left half side of the replicating molecules and the unstable nature of the replication intermediates at least *in vitro*.

because of the uncoupling of leading and lagging strand synthesis. The capacity of XP7TA cell extracts to bypass the lesion on the lagging strand template by initiating Okazaki fragment synthesis *de novo* on the 5′ side of the lesion was also observed for extracts from SV40-transformed XP4BE cells (Cordeiro-Stone *et al.*, 1997). However, the reduced amount of newly synthesized RFI molecules on CPD templates (25–30% of that on non-damaged templates), which are likely to be the products from undamaged parental strands, indicate that the uncoupling occurred at ~50–60% of the replication forks but the remainder did not. The possible modes of *in vitro* replication on CPD-containing templates by XP-V cell extracts are shown in Figure 2C.

Improvement of the assay system for the detection of XP-V complementation activity

Cell-free systems have been widely used for the biochemical identification and purification of factors involved in DNA replication. Initially, we tried to use the cell-free damage bypass DNA replication system to purify directly a protein capable of complementing the defects of XP-V cell extracts. Although we were able to detect this activity in HeLa nuclear extracts, it was difficult to detect weak activities in non-concentrated preparations, because DNA synthesis on damage-free parental strands occurs in the assay system with XP-V cell extracts; therefore, the difference of the incorporation between bypass-proficient cell extracts and XP-V cell extracts is logically only 50% at most. To improve the assay, we developed a two-step assay in which the CPD template was first incubated with XP-V cell extracts in the presence of all components except [α-^{32}P]dCTP and the complementing fraction, and then portions of the assay were incubated for a further brief period in the presence of [α-^{32}P]dCTP and the complementing fraction. During the first incubation period, DNA replication machinery will stall at the lesion on the damaged strand but continue on the damage-free strand without incorporating radioactivity. During the short period of the second incubation in the

presence of [α-^{32}P]dCTP and the complementing fraction, radioactivity will be incorporated preferentially into the products of damage bypass replication. As shown in Figure 3, XP7TA cell extracts showed poor DNA synthesis on both pBS-SVoriA(CPD) and pBS-SVoriB(CPD) templates during the second incubation. However, newly synthesized RFI molecules could be observed clearly if the assays were complemented with HeLa nuclear extracts. The RFI molecules were quite sensitive to nicking by T4 endoV, indicating that they were the products of damage bypass replication. We employed this two-step assay system with pBS-SVoriB(CPD) DNA and XP7TA cell extracts for the purification of XP-V complementation activity.

Purification of XP-V correcting protein

XP-V complementation activity was detected in nuclear extracts from HeLa cells and purified as summarized in Table I. Recovery of the activity from the phosphocellulose column chromatography was only 18.5% but was 30.7% after the next hydroxyapatite column chromatography step, suggesting the presence of inhibitors in crude cell

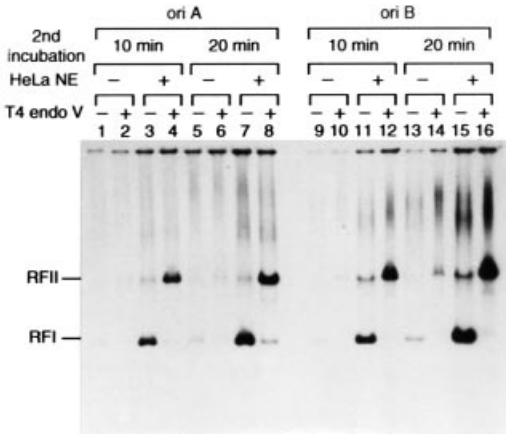

Fig. 3. 'Two-step' damage bypass replication assay with XP-V cell extracts. XP7TA cell extracts were incubated with pBS-SVoriA(CPD) (lanes 1–8) or pBS-SVoriB(CPD) (lanes 9–16) in the standard reaction mixtures for the two-step assay, which does not contain [α-^{32}P]dCTP in the first step. After 60 min incubation [α-^{32}P]dCTP was added to the mixture with (lanes 3, 4, 7, 8, 11, 12, 15 and 16) or without (lanes 1, 2, 5, 6, 9, 10, 13 and 14) nuclear extracts from HeLa cells (2 μg protein). The samples were incubated for another 10 min (lanes 1–4 and 9–12) or 20 min (lanes 5–8 and 13–16). The DNA products were purified and incubated in the presence (lanes of even numbers) or absence (lanes of odd numbers) of T4 endoV, and subjected to agarose gel electrophoresis in the presence of ethidium bromide. An autoradiogram of the gel is shown. The positions of closed circular (RFI) and nicked circular (RFII) molecules are indicated.

fractions. During the final MonoS chromatography step, the activity was found to co-elute from the column with a 54 kDa polypeptide (Figure 4). At this step the specific activity increased 744-fold over that of the starting material (Table I). A portion of the peak from the MonoS column was subjected to glycerol density gradient centrifugation (Figure 5A–C). The 54 kDa polypeptide co-sedimented with the activity at 3.5S, suggesting that this polypeptide comprises the activity and functions as a monomer.

To confirm that the purified MonoS fraction contained XP-V complementation activity, aliquots of the fraction were tested in the one-step (Figure 6A and C) and two-step (Figure 6B and D) assays. In both assay systems (Figures 6A and B), the fraction stimulated the DNA synthesis of XP7TA cell extracts on CPD templates but not on non-damaged templates (compare lanes 1–4 with 5–8 and 9–12 with 13–16). Stimulation occurred irrespective of whether the lesion was located on the lagging (compare lanes 5, 6 with 7, 8) or leading strand template (compare lanes 13, 14 with 15, 16). To examine whether the activity could complement cell extracts other than XP7TA, we prepared two more XP-V cell extracts from XP1RO and XP2SA cells. Although cell-free DNA replication was different depending on the extract, perhaps due to variability in cell transformation efficiencies between experiments or differences in cell growth, both extracts from XP1RO and XP2SA cells showed T antigen-dependent DNA syntheses (data not shown). Addition of the purified factor to extracts from these two XP-V cell extracts significantly enhanced DNA synthesis on damaged templates. As with XP7TA complemented extracts, the replicated DNA was sensitive to nicking by T4 endoV in both the one-step (Figure 6C) and two-step (Figure 6D) assays. On the other hand, the factor did not stimulate the DNA synthesis of HeLa cell extracts. From these results, we conclude that the purified fraction contains a factor required specifically for damage bypass replication in at least three XP-V cell extracts.

The XPV protein fraction has DNA polymerase activity

The XPV fraction was tested for enzymatic activity and found to have DNA polymerase activity. The DNA polymerase activity seems to be intrinsic to the putative XP-V protein because XP-V complementation activity and DNA polymerase activity co-sedimented with the 54 kDa protein during glycerol density gradient centrifugation (Figure 5D versus A). The DNA polymerase activity in the MonoS fraction was characterized by

Table I. Purification of XP-V correcting protein from HeLa cells

	Protein (mg)	Activity (U)	Recovery (%)	Specific activity (U/mg)	Purification (fold)
Nuclear extract	1486	10 047	100	6.76	1
Phosphocellulose	512	1861	18.5	3.63	0.54
Hydroxyapatite	267	3083	30.7	11.55	1.71
AS ppt	105	3033	30.2	28.72	4.25
Hitrap phenyl	22.3	1240	12.3	55.61	8.23
TSK phenyl	7.28	303	3.0	41.62	6.16
TSK HA	0.615	163	1.6	265	39.2
MonoQ	0.240	149	1.5	622.9	92.1
MonoS	0.016	80.5	0.8	5031	744

measuring the incorporation of [α-^{32}P]dCTP into single primed phagemid templates. As shown in Figure 7A, DNA polymerase activity was completely dependent on $MgCl_2$, sensitive to *N*-ethylmaleimide (NEM), resistant to 100 mM but sensitive to 200 mM KCl, resistant to 200 μM aphidicolin (inhibitor of DNA polymerases α, δ and ε) and ddTTP (inhibitor of DNA polymerases β and γ), and not stimulated by recombinant human proliferating cell nuclear antigen (PCNA) (stimulator of DNA polymerases δ and ε). These characteristics suggest that it is distinct from DNA polymerases α, β, γ, δ and ε. In the yeast *Saccharomyces cerevisiae*, two other DNA polymerases, ζ and η, have been identified (Nelson *et al.*, 1996a; Johnson *et al.*, 1999). Both polymerases were reported to catalyze translesion DNA syntheses and have not yet been identified in mammalian cells.

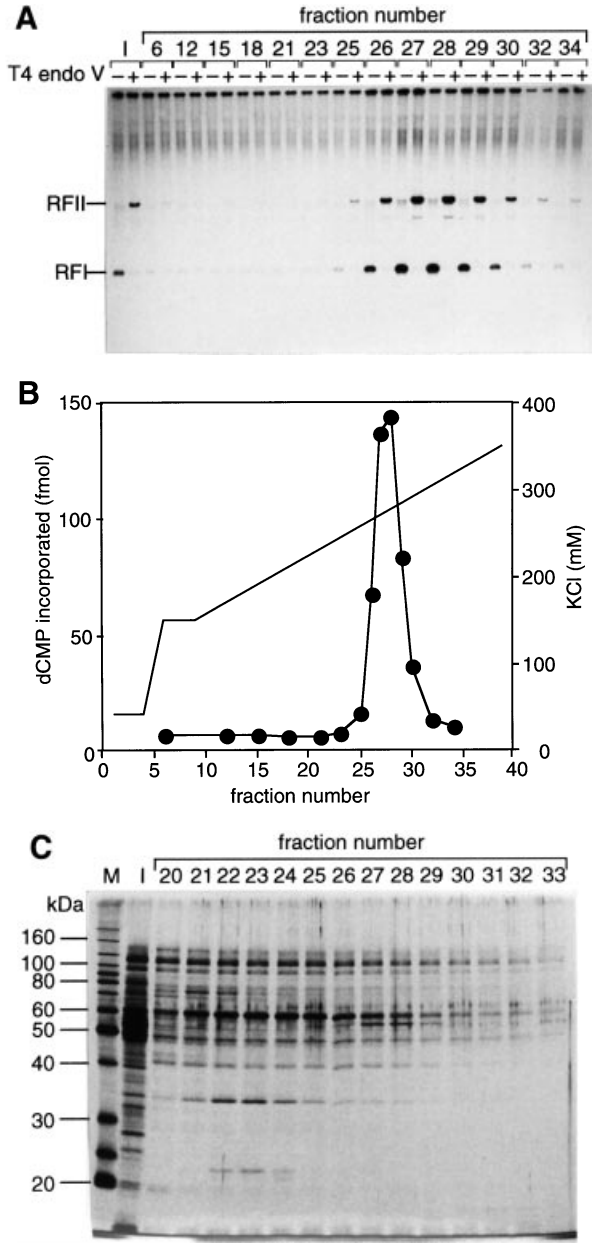

Fig. 4. Purification of XP-V complementation activity.
(A) and (B) Fractions from the MonoS column were examined for XP-V complementation activity in the 'two-step' damage bypass replication assay containing XP7TA cell extracts and pBS-SVoriB(CPD) DNA template. (**A**) An autoradiogram of the gel is shown. (**B**) Incorporation of radioactive material into nicked circular (RFII) molecules after T4 endoV treatment in (A) was measured using a BAS2500 Imaging Analyzer (Fujix). (**C**) Fractions were subjected to SDS–PAGE (12%) and the gels were stained with silver.

To examine the translesion activity of the DNA polymerase in the XPV fraction, a 30mer DNA containing a lesion was synthesized and annealed to a 5′ ^{32}P-labeled 13mer or 16mer DNA, and used as a template (Figure 7B). Like DNA polymerase α, the activity was completely dependent on the template strand and DNA of up to 30mer in length was synthesized on the non-damaged template (Figure 7C, lanes 5–7). On the CPD template, DNA polymerase α stopped DNA synthesis one nucleotide before the CPD whereas the DNA polymerase activity in the XPV fraction was still able to synthesize 30mer long DNA (Figure 7C, lanes 9–11), indicating that this DNA polymerase can bypass CPD on the template. In contrast, when the DNA template contained a (6-4) photoproduct, the polymerase activity associated with the 54 kDa protein was inhibited and could not bypass the lesion (Figure 7C, lanes 13–15), suggesting that this DNA polymerase is specific for bypassing CPDs. However, unlike DNA polymerase α which again terminated DNA synthesis one nucleotide before the (6-4) photoproduct, the polymerase activity in the XP-V complementation fraction incorporated one more nucleotide (Figure 7C, lanes 13–15). The simultaneous presence of DNA polymerase α and the XPV fraction did not alter the efficiency of translesion synthesis.

To examine which nucleotide was incorporated opposite the CPD, polymerization was performed in the presence of only one kind of deoxyribonucleotide in reactions containing a 16mer primed 30mer template with a CPD (Figure 7B). With this template, first and second nucleotides are incorporated opposite a di-thymine site. As shown in Figure 7D, bypass synthesis occurred only in the presence of dATP, indicating that the DNA polymerase can incorporate only dATP to bypass a di-thymine lesion. However, the reaction stopped mainly after incorporating one more dATP opposite dC and, to a lesser extent, after incorporating another dATP opposite dT. In the cases of dCTP, dGTP or dTTP, the DNA polymerase stopped the reaction after incorporating one nucleotide opposite the first nucleotide of the CPD. These findings suggest that the DNA polymerase in the XPV fraction lacks proofreading exonuclease activity and functions like a terminal deoxynucleotide transferase at the end of elongated DNA chains under these conditions.

Discussion

The replication defect of XP-V cell extracts and purification of XP-V complementation activity

XP is a UV-sensitive syndrome which can be divided into eight complementation groups designated A, B, C, D, E, F, G and V. Each of groups A–G has been shown to be defective in some aspect of NER which eliminates UV-induced lesions on DNA. The UV sensitivity of XP-V cells has been difficult to explain because cells from XP-V patients show normal repair activities after UV-irradiation. Recent studies have revealed that extracts from XP-V cells are deficient in DNA replication on

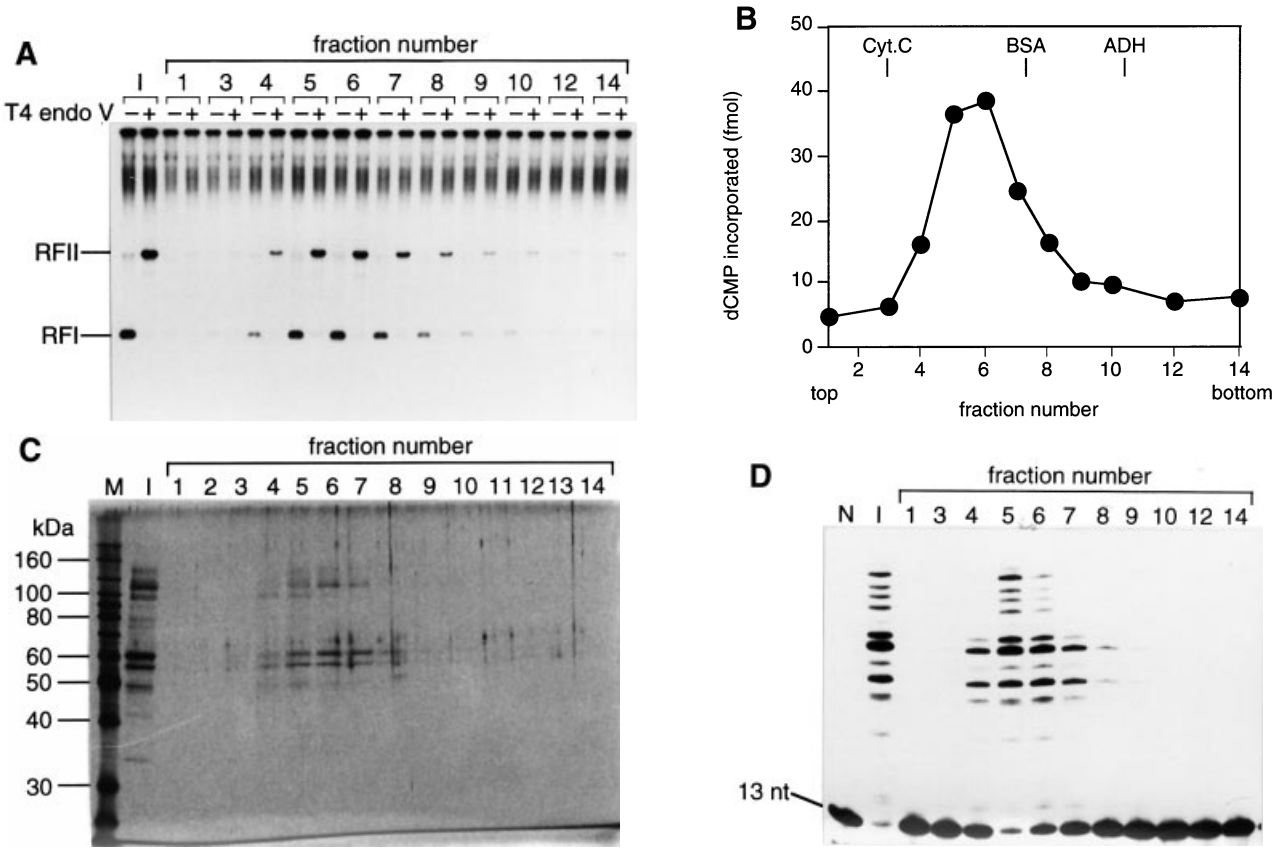

Fig. 5. Glycerol density gradient centrifugation of XPV protein. The MonoS fraction 27 was subjected to glycerol density gradient centrifugation. (A) and (B) Fractions from the glycerol density gradient were examined for XP-V complementation activity in the two-step damage bypass replication assay containing XP7TA cell extract and pBS-SVoriB(CPD) DNA template. (**A**) An autoradiogram of the gel is shown. (**B**) Incorporation of radioactive material into nicked circular (RFII) molecules after T4 endoV treatments in (A) was measured using a BAS2500 Imaging Analyzer (Fujix). The positions of marker proteins in a parallel gradient are indicated. (**C**) Fractions were subjected to SDS–PAGE (10.5%) and the gel was stained with silver. (**D**) Fractions were examined for DNA polymerase activity on a template composed of a [^{32}P]13mer annealed to a 30mer DNA containing the CPD as depicted in Figure 7B.

templates containing lesions (Cordeiro-Stone *et al.*, 1997; Ensch-Simon *et al.*, 1998; Svoboda *et al.*, 1998). More recently, extracts from XP-V cells were found to be defective in translesion DNA synthesis (Cordonnier *et al.*, 1999).

In this paper, we constructed assay systems to detect bypass DNA replication on CPD templates in human cell extracts. DNA synthesis by XP-V cell extracts was clearly inhibited on CPD templates, especially when the lesion was located on the leading strand template, as was observed by others (Cordeiro-Stone *et al.*, 1997; Svoboda *et al.*, 1998). When the lesion was located on the lagging strand template, an increase in the level of open circular products was observed. The open circular products are likely to result from the synthesis *de novo* of Okazaki fragments on the 5′ side of the lesion as described previously (Svoboda and Vos, 1995; Cordeiro-Stone *et al.*, 1997). These results indicate that extracts from XP-V cells are deficient in translesion synthesis on both lagging and leading template strands.

In a novel two-step assay developed in our laboratory, we succeeded in observing a clearer difference between XP-V and HeLa cell extracts for translesion DNA synthesis. This was particularly important for the purification of XP-V complementation activity using nuclear extracts from HeLa cells. As shown in Figure 3, the addition of nuclear extracts to XP-V extracts gave efficient incorporation of radioactive material into closed circular molecules whose increased sensitivity to nicking by T4 endoV suggested that XP-V complementation activity present in the cell extracts had allowed bypass of the DNA lesion.

The two-step assay system allowed us to detect XP-V complementation activity in HeLa nuclear extracts irrespective of whether the XP-V cells were derived from three independent patients living in different countries. The assay system was not artificial as the purified factor worked not only in the two-step assay but also in the one-step assay. It must be mentioned that the factor did not stimulate DNA replication on ND templates, indicating that the factor has a specific role to play in translesion synthesis. One of the basal replication proteins, RPA, was reported to be able to correct the defects of XP-V cell extracts (Ensch-Simon *et al.*, 1998). In the present purification procedure RPA was separated from XP-V correcting activity after the first column chromatography step on phosphocellulose. Also, the fraction which contained RPA did not show any detectable XP-V correcting activity (data not shown).

DNA polymerase activity of the XPV protein fraction

We found that the XPV protein fraction had DNA polymerase activity. In mammals four nuclear DNA

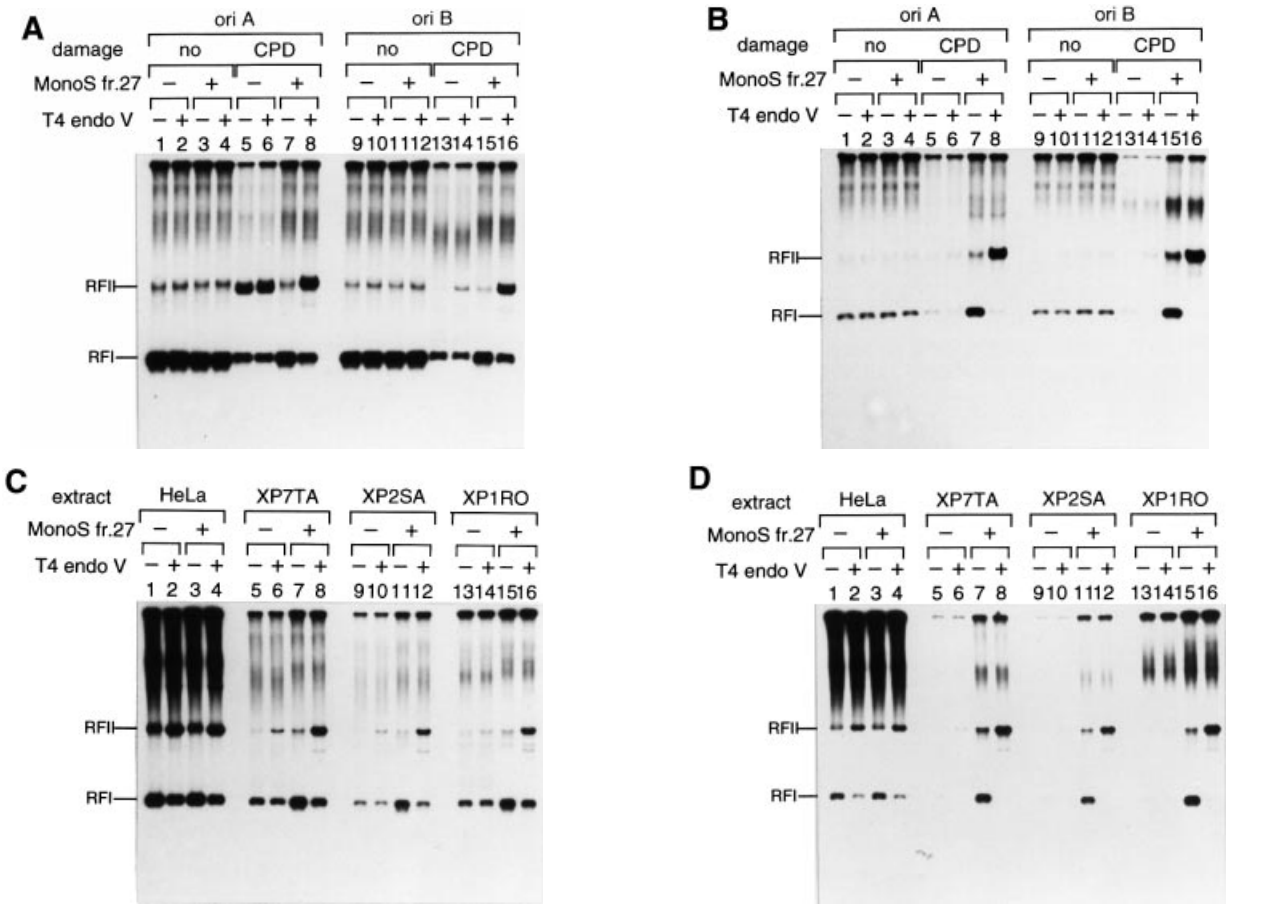

Fig. 6. XP-V complementation activity of the MonoS fraction. (A) and (B) Complementation of damage bypass replication defects of XP-V cell extracts on both leading and lagging strands by the MonoS fraction. (**A**) One-step assay. XP7TA cell extracts were incubated with DNA templates in the presence (lanes 3, 4, 7, 8, 11, 12, 15 and 16) or absence (lanes 1, 2, 5, 6, 9, 10, 13 and 14) of MonoS fraction 27 (10 ng) in the standard reaction mixture for the one-step assay. Templates used were pBS-SVoriA(ND) (lanes 1–4), pBS-SVoriA(CPD) (lanes 5–8), pBS-SVoriB(ND) (lanes 9–12) and pBS-SVoriB(CPD) (lanes 13–16). The DNA products were purified and incubated in the presence (even numbered lanes) or absence (odd numbered lanes) of T4 endoV, and subjected to agarose gel electrophoresis in the presence of ethidium bromide. An autoradiogram of the gel is shown. (**B**) Two-step assay. An experiment comparable with (A) was done by the two-step assay. (C) and (D) Complementation of the three XP-V cell extracts by the MonoS fraction. (**C**) Cell extracts were incubated with pBS-SVoriB(CPD) in the presence (lanes 3, 4, 7, 8, 11, 12, 15 and 16) or absence (lanes 1, 2, 5, 6, 9, 10, 13 and 14) of MonoS fraction 27 in the standard reaction mixture for the one-step assay. Cell extracts used were prepared from HeLa (lanes 1–4), XP7TA (lanes 5–8), XP2SA (lanes 9–12) and XP1RO cells (lanes 13–16). The products were analyzed as described in (A). (**D**) Two-step assay. An experiment comparable with that of (C) was done by the two-step assay.

polymerases, α, β, δ and ε, have been characterized biochemically (Wood and Shivji, 1997; Burgers, 1998). The polymerase activity of the XPV fraction was distinct from the activities of other DNA polymerases with respect to sensitivity to inhibitors and stimulators as shown in Figure 7A. Importantly, the polymerase activity of the XPV fraction catalyzed bypass DNA synthesis opposite the CPD by itself. There have been some reports of lesion bypass DNA synthesis by mammalian DNA polymerases. DNA polymerase δ has been reported to be able to bypass CPDs and abasic sites in the presence of PCNA (O'Day *et al.*, 1992; Mozzherin *et al.*, 1997). DNA polymerase β has also been reported to bypass cisplatin adducts and abasic sites efficiently (Hoffmann *et al.*, 1995, 1996; Efrati *et al.*, 1997). However, translesion synthesis by these polymerases was reported to be a highly mutagenic process. In contrast, the DNA polymerase in the fraction complementing the XP-V extracts was able to utilize only dATP, strongly suggesting that translesion synthesis by this DNA polymerase was not so mutagenic as to bypass the di-thymine site which is the major type of UV-induced products. As for the minor types of CPDs including cytosine, we could not yet examine whether the polymerase can bypass the lesion mainly due to the rapid deamination of C in these CPDs.

In the yeast *S.cerevisiae* two DNA polymerases, ζ and η, have been shown to be involved in translesion synthesis (Nelson *et al.*, 1996a; Johnson *et al.*, 1999). DNA polymerase ζ consists of two gene products, Rev3 and Rev7, and has been reported to be able to bypass CPDs (Nelson *et al.*, 1996a). Another gene product, Rev1, which has deoxycytidyl transferase activity, was shown to be involved in the pathway (Nelson *et al.*, 1996b). Although we do not know whether the polymerase is mutagenic *in vitro*, DNA polymerase ζ is likely to be a mutagenic polymerase because mutations in *REV3* suppress mutagenesis in yeast cells (Quah *et al.*, 1980). Recently *RAD30* was shown to encode the novel DNA polymerase η (Johnson *et al.*, 1999). DNA polymerase η can bypass CPDs by incorporating dATP like the polymerase in putative XPV protein described here. In addition to these similar biochemical characteristics, XP-V cells show hypermutability *in vivo*

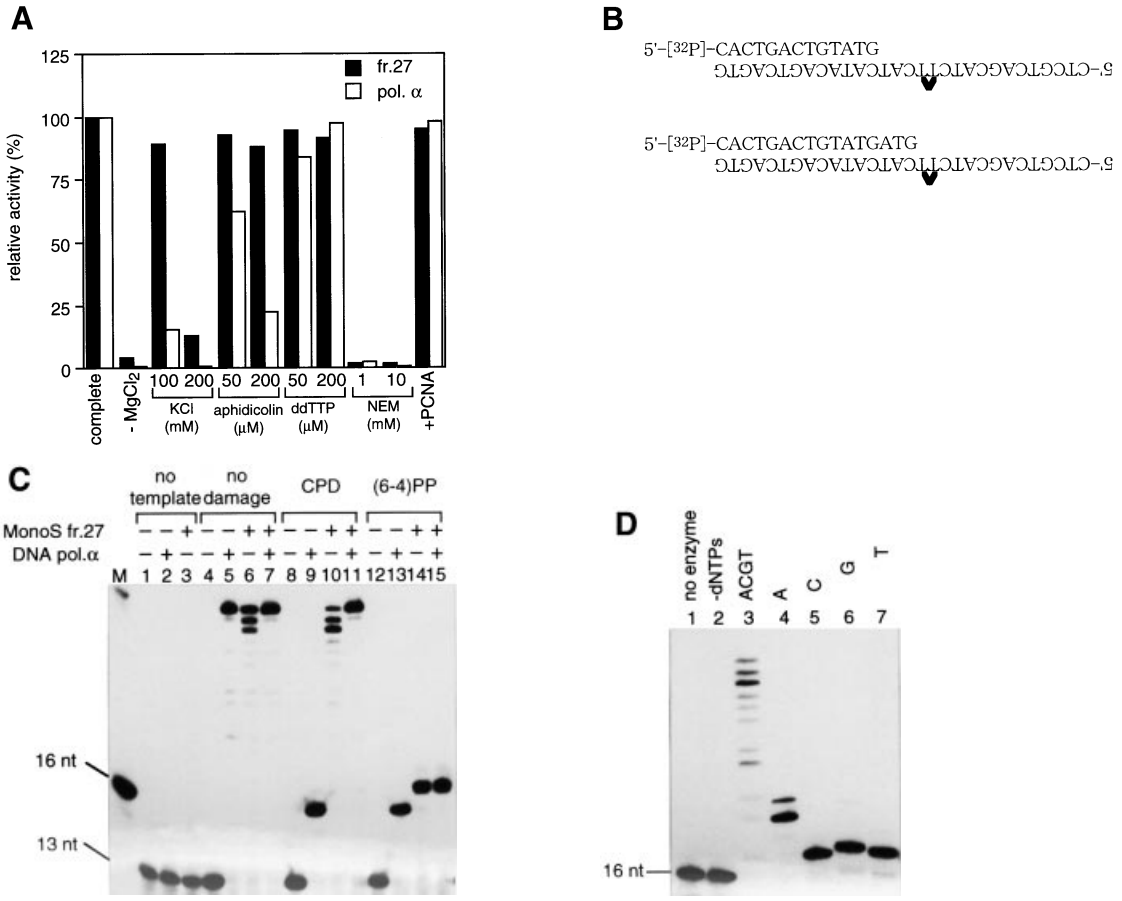

Fig. 7. DNA polymerase activity of the MonoS fraction. (**A**) DNA polymerase activities were examined in reaction mixtures containing single primed DNA templates [T7 primer annealed with single-stranded pBS-KS(+)] and [α-^{32}P]dCTP. The concentrations of dCTP and dTTP were lowered to examine replication sensitivity to aphidicolin and dTTP. DTT was omitted from the reactions when NEM was tested. As for PCNA, 20 ng of recombinant protein was added. MonoS fraction 27 (10 ng) (solid bars) or DNA polymerase α (0.5 ng) (open bars) was incubated on ice for 10 min with or without the components indicated and further incubated at 37°C (1 h for fraction 27 and 30 min for pol α). The incorporation of radioactive material into acid insoluble fractions was determined. The relative replication activity for each condition was calculated as the ratio of the replication activity of the complete reaction and plotted. Net incorporation of dCMP at 100% was 0.27 and 1.46 pmol for MonoS fraction 27 and DNA polymerase α, respectively. (**B**) DNA templates for the lesion bypass DNA polymerase assay. Oligomers (13mer and 16mer) were labeled with [^{32}P] at their 5′-ends and annealed with the 30mer DNA. The 30mer DNA contained a CPD, (6-4) photoproducts, or no damage at the bridged TT site. (**C**) CPD bypass DNA polymerase activity. MonoS fraction 27 (1 ng) and/or DNA polymerase α (0.25 ng) were incubated at 37°C for 30 min with the 5′ ^{32}P-labeled 13mer primer annealed to the 30mer DNA as indicated. After incubation, samples were heat denatured in the presence of 50% formamide and subjected to polyacrylamide gel electrophoresis under denaturing conditions. The products were detected by autoradiography. M: 5′ ^{32}P-labeled oligomers (30mer and 16mer) were used for markers. (**D**) The MonoS fraction 27 (1 ng) was incubated with 5′ ^{32}P-labeled 16mer primer annealed to the 30mer DNA containing the CPD in the presence (lane 3) or absence (lane 2) of the four dNTPs. In lanes 4–7 only one kind of dNTP indicated was included in the reaction mixtures.

and *in vitro* after UV irradiation like RAD30 cells (Wang *et al.*, 1991, 1993; Misra and Vos, 1993; McGregor *et al.*, 1999). These observations suggest that the DNA polymerase in the XP-V complementing fraction is the human counterpart of yeast DNA polymerase η. This conclusion is also supported by the sequence of the cDNA clone coding for the 54 kDa protein purified in this paper (C.Masutani, R.Kusumoto, A.Yamada, N.Dohmae, M.Yokoi, M.Yuasa, M.Araki, S.Iwai, K.Takio and F.Hanaoka, manuscript submitted).

Two pathways of translesion synthesis and repair

In yeast cells at least two pathways of translesion synthesis are known. One is the error-prone pathway which involves Rev1, Rev3 and Rev7, and the other is the error-free pathway which involves Rad30 as described above. In human cells comparable pathways are expected to exist, because a human homologue of *REV3* has been cloned (Gibbs *et al.*, 1998; Xiao *et al.*, 1998) and a mutant with defective translesion synthesis and abnormal hypermutability after UV irradiation, an XP-V, has been described. As described above, the polymerase activity of the XP-V complementing fraction was not error-prone at least during bypass of the CPD; therefore, the absence of the polymerase activity is likely to account for the hypermutable phenotype of XP-V cells, in which only the error-prone pathway is working. Interestingly, the XPV DNA polymerase did not bypass a (6-4) photoproduct, which is another major UV-induced product, suggesting that the error-free pathway involving XPV DNA polymerase does not work to bypass (6-4) photoproducts. This explains the results of a previous report which indicated that (6-4) photoproducts are more difficult to bypass than CPDs in human cell extracts (Carty *et al.*, 1996). The results also suggest that the XPV polymerase identified here contributes the most to bypass activity in human cell

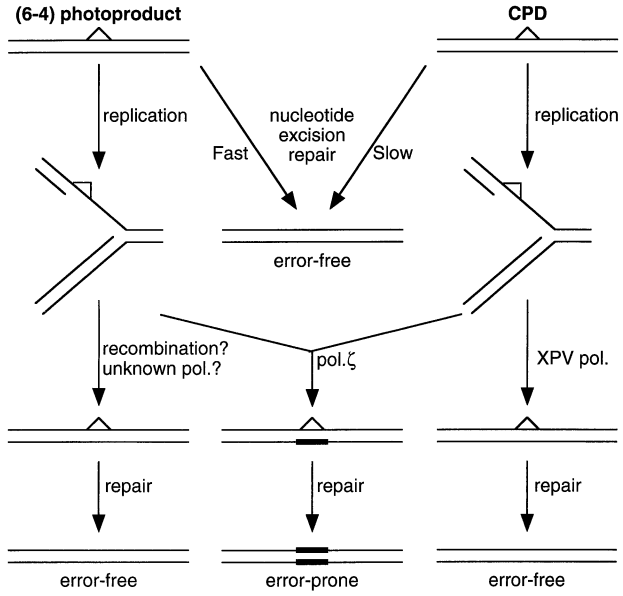

Fig. 8. Possible pathways for error-free and error-prone repair of CPD and (6-4) photoproduct in human cells.

extracts. It is possible that error-prone DNA polymerases can bypass (6-4) photoproducts because previous studies revealed that (6-4) photoproducts were much more mutable than CPDs. However, (6-4) photoproducts have been reported to be repaired much faster by the NER pathway than CPDs (Friedberg *et al.*, 1995). These observations suggest that cells prevent UV-induced mutations by removing (6-4) photoproducts through the rapid NER pathway before the onset of DNA replication and/or S phase checkpoints but allow the bypass of CPDs by the error-free pathway involving the XPV DNA polymerase because the repair of CPDs is too slow (Figure 8).

Materials and methods

Cells and cell-free extracts

Three non-transformed XP-V fibroblasts, XP7TA, XP2SA and XP1RO, were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. HeLa cells were grown in spinner flasks at 37°C with 5% CO_2 in DMEM supplemented with 5% calf serum.

XP-V cells for cell-free DNA replication extracts were cultivated as follows. XP-V cells were inoculated into a dish (ID 100 mm) at a density of 20% confluence. The cells were cultured at 37°C with 5% CO_2 for 15 h and then the pMT-IOD plasmid (kindly provided by Dr K.Sugasawa), which carries the SV40 origin and the large T antigen sequence with a small deletion at the core origin, was transfected into the cells using the LipofectAMINE PLUS reagent (Gibco-BRL) as described by the manufacturer. The cells were cultured with two medium changes per week. After 1 month of culture, many focuses appeared on the dish, and the cells were harvested and inoculated into four dishes (ID 150 mm). Cell culture was continued with 10–30 roller bottles (850 cm^2) followed by gradual scale up.

Cell extracts for cell-free DNA replication assays were prepared according to published protocols (Eki *et al.*, 1991).

For protein purification, HeLa cells were harvested at a density of 10^6 cells/ml, washed with phosphate-buffered saline and then with hypotonic buffer [20 mM HEPES–KOH pH 7.4, 1.5 mM $MgCl_2$, 5 mM KCl, 2 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 μg/ml aprotinin, 0.2 μg/ml leupeptin, 0.1 μg/ml antipain and 50 μM EGTA] and stored at –80°C.

Plasmid DNA substrates

A 0.23 kbp DNA fragment from SV40 DNA (5171–160) was cloned into the *Sac*I (A) or *Kpn*I (B) site of pBS-KS(+) by blunt-end ligation to generate pBS-SVoriA or pBS-SVoriB plasmid, respectively. Two synthetic ND 30mer and 38mer oligonucleotides were annealed to each other and cloned between the *Pst*I and *Hin*dIII sites of pBS-SVoriA and pBS-SVoriB to generate pBS-SVoriA30 and pBS-SVoriB30, respectively. The constructs were verified by DNA sequencing. The sequences of the oligomers were 5′-CTCGTCAGCATCTTCATCATACAGTCAGTG-3′ for the 30mer and 5′-AGCTCACTGACTGTATGATGAAGATGCTG-ACGAGTGCA-3′ for the 38mer. The 30mer oligonucleotide containing a CPD or a (6-4) photoproduct at the underlined site was chemically synthesized as described previously (Murata *et al.*, 1990; Iwai *et al.*, 1996). The *Hin*dIII–*Pst*I large fragments from pBS-SVoriA and pBS-SVoriB were partially digested by *Escherichia coli* exonuclease III, and mixed with single-stranded circular DNA derived from pBS-SVoriA30 and pBS-SVoriB30, respectively, as well as the 5′-phosphorylated CPD30mer. The mixtures were heat-denatured, reannealed and then treated with T4 DNA ligase. Double-stranded closed circular plasmids containing a single CPD [pBS-SVoriA(CPD) and pBS-SVoriB(CPD)] were purified by cesium chloride–ethidium bromide density gradient centrifugation. DNA substrates lacking the lesion [pBS-SVoriA(ND) and pBS-SVoriB(ND)] were prepared in the same way except that the ND30mer oligonucleotide was used instead of the CPD30mer.

Damage bypass DNA replication assay (one-step assay)

Standard reactions of 10 μl contained 40 mM creatine phosphate–Tris pH 7.7, 7 mM $MgCl_2$, 4 mM ATP, 200 μM each of the other three rNTPs, 20 μM [α-^{32}P]dCTP (37 kBq), 100 μM each of the other three dNTPs, 10 mM DTT, 250 μg/ml bovine serum albumin (BSA), 100 μg/ml creatine phosphokinase, 2.5 μg/ml plasmid DNA, 40 μg/ml SV40 large T antigen, 5 mg/ml of human cell extract, and 0.5 μl of sample. After incubation at 37°C for 1 h, the reactions were terminated by the addition of Na_3EDTA at a final concentration of 25 mM. The mixtures were treated with 25 μg/ml of bovine pancreatic ribonuclease A for 5 min at 37°C and then with 200 μg/ml of proteinase K for 1 h at 37°C in the presence of 0.5% SDS. DNA was purified by phenol–chloroform extraction and ethanol precipitation and then dissolved in 30 μl of buffer V (25 mM Tris–HCl pH 8.0, 2.5 mM Na_3EDTA, 15 μg/ml BSA). The samples were divided into two tubes and incubated for 0.5 h at 37°C in the presence or absence of 33 μg/ml of T4 endoV. After the addition of one-fifth of the final volume of loading dye (50% glycerol, 0.25% bromophenol blue), the samples were subjected to 0.9% agarose gel electrophoresis with Tris–borate–EDTA buffer containing 0.2 μg/ml ethidium bromide. Autoradiography was performed at –80°C with Fuji New RX X-ray film. The incorporation of radioactive materials was quantified with a Fuji BAS2500 bioimaging analyzer.

SV40 large T antigen was immuno-affinity purified as described (Eki *et al.*, 1991). Bacteriophage T4 endoV gene product was kindly provided by Dr E.Ohtsuka.

Damage bypass DNA replication assay (two-step assay)

Standard reactions of 10 μl contained 40 mM creatine phosphate–Tris pH 7.7, 7 mM $MgCl_2$, 4 mM ATP, 200 μM each of other three rNTPs, 20 μM dCTP, 100 μM each of other three dNTPs, 10 mM DTT, 250 μg/ml BSA, 100 μg/ml creatine phosphokinase, 2.5 μg/ml plasmid DNA, 40 μg/ml SV40 large T antigen, and 5 mg/ml of human cell extract. After incubation at 37°C for 1 h, [α-^{32}P]dCTP (74 kBq) and 0.5 μl of crude or purified XP-V protein were added to the mixtures and incubation was continued for 10 min at 37°C. The reactions were terminated by the addition of Na_3EDTA at a final concentration of 25 mM. Subsequent procedures were exactly the same as for the one-step assay.

DNA polymerase assay

Two types of templates, single primed phagemid DNA and a 13mer or a 16mer oligomer annealed to a 30mer DNA, were used to detect DNA polymerase activity.

The single primed phagemid DNA template was prepared by mixing T7 primer and single-stranded pBS-KS(+) DNA, which was prepared from *E.coli* XL1-blue infected with the helperphage M13KO7, at a molar ratio of 2:1. Standard reactions of 10 μl contained 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 100 μM each of dATP and dGTP, 20 μM dTTP, 10 μM [α-^{32}P]dCTP, 10 mM DTT, 250 μg/ml BSA, 60 mM KCl, 2.5% glycerol, 5% dimethyl sulfoxide and 10 nM primer-template DNA. After incubation at 37°C for 60 min, reactions were terminated by chilling on ice, and acid-insoluble materials were measured as described previously (Masutani *et al.*, 1990).

The 5′-[^{32}P] primer-template DNA was prepared by mixing at a molar ratio of 1:1 the 13mer or 16mer primer, previously labeled at its 5′ end

using T4 polynucleotide kinase and [γ-^{32}P]ATP, with the 30mer DNA containing the lesion. Standard reactions of 10 µl contained 40 mM Tris–HCl pH 8.0, 5 mM $MgCl_2$, 100 µM each of four dNTPs, 10 mM DTT, 250 µg/ml BSA, 60 mM KCl, 2.5 % glycerol and 4 nM 5′-[^{32}P] primer-template DNA. After incubation at 37°C for 30 min, reactions were terminated by the addition of 10 µl of formamide followed by boiling. The products were subjected to 20% polyacrylamide/7 M urea gel electrophoresis followed by autoradiography.

Purification of XP-V correcting activity from HeLa cells

All procedures were carried out at 0–4°C. A frozen stock of HeLa cells (236 ml of packed cell volume) was thawed, suspended in 940 ml of hypotonic buffer and homogenized in an all-glass Dounce homogenizer. The nuclei were obtained by low speed centrifugation followed by high speed centrifugation for 30 min at 25 000 *g* and suspended in 580 ml of buffer 1 (20 mM potassium phosphate pH 7.5, 1 mM Na_3EDTA, 2 mM DTT, 0.5 mM PMSF, 0.4 µg/ml aprotinin, 0.4 µg/ml leupeptin, 0.2 µg/ml antipain and 0.1 mM EGTA). The suspension was made in 0.3 M KCl by the addition of 0.1 volume of buffer 1 containing 3.3 M KCl. An extract was obtained by gentle stirring for 30 min followed by centrifugation for 1 h at 100 000 *g*. The supernatant was dialyzed against buffer 2 (20 mM potassium phosphate pH 7.5, 1 mM Na_3EDTA, 10% glycerol, 1 mM DTT, 0.25 mM PMSF, 0.2 µg/ml aprotinin, 0.2 µg/ml leupeptin, 0.1 µg/ml antipain and 50 µM EGTA) containing 0.1 M KCl and centrifuged for 30 min at 100 000 *g*. The supernatant (nuclear extract) was loaded onto a phosphocellulose column (Whatman P11; 90 ml). The column was washed with the same buffer and the adsorbed proteins were eluted with buffer 2 containing 0.42 M KCl. The eluate was made in 0.42 M KCl by the addition of buffer 2 containing 1.0 M KCl and then loaded onto a hydroxyapatite column (Bio-Rad Bio-Gel HTP; 35 ml). After the column was washed with the same buffer, flow-through fractions were collected and brought to 40% saturation of ammonium sulfate (0.243 g of solid/ml). The precipitates were collected by centrifugation for 30 min at 20 000 *g*, dissolved in 10 ml of buffer 2, and then dialyzed against buffer 2 containing 0.8 M ammonium sulfate. After the removal of insoluble materials by centrifugation, the dialysate was loaded onto a Hitrap Phenyl Sepharose HP (Amersham Pharmacia) with an FPLC system (Amersham Pharmacia). The column was washed with the same buffer and the bound materials were eluted with 30 ml of a decreasing (from 0.8 to 0 M) ammonium sulfate gradient. Active fractions (0.4 M ammonium sulfate) were pooled, dialyzed against buffer 2 containing 2.0 M KCl, and loaded onto a TSKgel Phenyl 5PW column (Tosoh) using the SMART system (Amersham Pharmacia). All the following steps were performed with this system. After the columns were washed with the same buffer, the bound proteins were eluted with 19 ml of a decreasing (from 2.0 to 0 M) KCl gradient. The activity was eluted at ~0.8 M KCl. The active fractions were collected, dialyzed against buffer 2 containing 50 mM KCl and loaded to a TSKgel HA1000 (Tosoh). The column was washed with the same buffer and the proteins were eluted with 19 ml of a linear gradient of potassium phosphate from 20 to 250 mM in solution A (1 mM Na_3EDTA, 10% glycerol, 1 mM DTT, 0.01% Triton X-100, 0.25 mM PMSF, 0.2 µg/ml aprotinin, 0.2 µg/ml leupeptin, 0.1 µg/ml antipain, 50 µM EGTA). Active fractions (~130 mM potassium phosphate) were pooled, dialyzed against buffer 3 [buffer 2 plus 0.01% Triton X-100] containing 40 mM KCl, and loaded onto a MonoQ PC 1.6/5 column (Amersham Pharmacia). After the column was washed with the same buffer, flow-through fractions were collected and loaded onto a MonoS PC 1.6/5 column (Amersham Pharmacia). The column was washed with buffer 3 containing 40 mM and 0.15 M KCl successively, and the bound proteins were eluted with 3 ml of a linear gradient from 0.15 to 0.35 M KCl. Active fractions (100 µl each) were stored at –80°C.

XP-V correcting activity was assayed with XP7TA cell extracts in the two-step assay system. One unit of the activity was defined as the amount of protein required to incorporate 10 fmol of dCMP into T4 endoV sensitive-closed circular forms of pBS-SVoriB(CPD) DNA in XP7TA cell extracts.

Glycerol density gradient centrifugation

A portion (20 µl) of the MonoS fr.27 was layered onto 1.18 ml of a 15–35% (v/v) glycerol gradient in buffer 4 (20 mM potassium phosphate pH 7.5, 1 mM Na_3EDTA, 0.15 M KCl, 1 mM DTT, 0.01% Triton X-100) and centrifuged for 17.5 h at 230 000 *g* at 2°C. Fractions (85 µl each) were collected from the top of the gradient. An identical gradient containing marker proteins was run at the same time. The markers were detected by SDS–PAGE followed by staining with Coomassie Brilliant Blue.

Other methods

DNA polymerase α was immuno-affinity purified from FM3A cells using SJK287 antibodies as described previously (Masutani *et al.*, 1990). PCNA was purified from *E.coli* BL21 (DE3) harboring the pT7-PCNA plasmid (a generous gift from Dr Tsurimoto, NAIST) as described previously (Fien and Stillman, 1992). Protein concentration was measured according to the method of Bradford (1976) with BSA as a standard. SDS–PAGE was performed as described by Laemmli (1970).

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