

# Human xeroderma pigmentosum group G gene encodes a DNA endonuclease

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## ABSTRACT

**Because of defective nucleotide excision repair of ultraviolet damaged DNA, xeroderma pigmentosum (XP) patients suffer from a high incidence of skin cancers. Cell fusion studies have identified seven XP complementation groups, A to G. Previous studies have implicated the products of these seven XP genes in the recognition of ultraviolet-induced DNA damage and in incision of the damage-containing DNA strand. Here, we express the XPG-encoded protein in Sf9 insect cells and purify it to homogeneity. We demonstrate that XPG is a single-strand specific DNA endonuclease, thus identifying the catalytic role of the protein in nucleotide excision repair. We suggest that XPG nuclease acts on the single-stranded region created as a result of the combined action of the XPB helicase and XPD helicase at the DNA damage site.**

## INTRODUCTION

Nucleotide excision repair (NER) is the major cellular mechanism that removes DNA damage inflicted by ultraviolet light (UV) and other DNA damaging agents that distort the DNA helix. Inactivation of NER results in highly elevated cell death and a hypermutational phenotype upon challenge with UV and other DNA damaging agents, emphasizing the prominent role of this repair system in minimizing the cytotoxic and genotoxic effects of these agents (1).

The human syndrome xeroderma pigmentosum (XP) results from a defect in the nucleotide excision repair of UV damaged DNA. XP patients exhibit extreme sensitivity to sunlight and they suffer from a high incidence of skin cancers. Cell fusion studies have indicated the existence of seven XP complementation groups, XPA through XPG (1, 2), revealing considerable genetic heterogeneity in this syndrome. Existing evidence indicates that the accomplishment of NER requires, in addition to the products of the seven known XP genes, also the ERCCI gene product (1). The structure and function of NER genes have been conserved to a remarkable degree among eukaryotes, from yeast to human (1).

In human cell free extracts, NER of bulky DNA lesions proceeds *via* a dual incision event at the damage site, excising

the lesion in the form of a DNA fragment  $\approx 30$  nucleotides in length (3). Studies carried out with purified yeast and human NER proteins have indicated that the XPA and XPD proteins function in the recognition of DNA damage (4–6). Subsequent to the recognition step, the DNA helicase activity of XPB protein (7, 8) and of XPD protein (9,10) is likely to effect localized unwinding of the double helix, exposing a single-stranded damage containing region for dual incision. We have expressed the XPG-encoded product in insect cells and purified it to homogeneity. Here, we present our biochemical studies demonstrating that XPG is an endonuclease that generates 3' hydroxyl and 5' phosphate ends. This work therefore identifies XPG as one of the endonucleolytic components that mediates the incision of the damaged DNA strand during NER.

## MATERIALS AND METHODS

### Expression of a $\rho$ -XPG antigen and antibody production

An XPG protein segment from amino acid residue 107 to residue 452 was fused to the N-terminal 116 amino acids of *E. coli* transcriptional terminator  $\rho$  in the plasmid p39AS (11) to yield plasmid pXPG6f, in which the  $\rho$ -XPG fusion is under the control of the P<sub>L</sub> promoter of bacteriophage  $\lambda$ . To induce the synthesis of the  $\rho$ -XPG hybrid polypeptide, *E. coli* strain AR120.A6 harboring pXPG6f was treated with nalidixic acid (40  $\mu$ g/ml) in double-strength Laurie broth at 37°C for 5h as described (11). Cells were disrupted by passage through a French Press, and the particulate material was collected by centrifugation. The  $\rho$ -XPG hybrid polypeptide was solubilized from the particulate material by boiling in SDS sample buffer and purified to homogeneity by preparative SDS-PAGE in 3mm thick 10% gels. A yield of 5mg apparently homogeneous  $\rho$ -XPG protein was obtained from 1 L of bacterial culture. The  $\rho$ -XPG antigen was injected into two rabbits for raising polyclonal antibodies, which were purified from anti-sera by affinity chromatography using a Sepharose column containing covalently linked antigen.

### SDS-PAGE and immunoblotting

After electrophoresis in SDS 7% polyacrylamide gels, proteins were transferred onto nitrocellulose (S&S) in a Transblot apparatus (Bio-Rad). Nitrocellulose sheets were probed for 2h

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with 1/1000 dilution of affinity purified anti-XPG antibodies (stock of OD<sub>280</sub>=2) and developed using the indirect peroxidase procedure (12).

#### Immunoprecipitation of XPG from HeLa cell extract

For the immunoprecipitation experiment, 4 OD<sub>280</sub> units of anti-XPG antibodies were cross-linked to 350 $\mu$ l protein A agarose to give anti-XPG immunobeads as described previously (13). To prepare extract, 0.2g HeLa S3 cells were boiled for 5min in 0.5ml 10mM Tris-HCl, pH 7.5, containing 1% SDS and 15mM DTT, diluted with 5ml of buffer I (50mM Tris-HCl, pH 7.5, containing 150mM NaCl, 1% Triton X-100, 5mM EDTA, and protease inhibitors), and clarified by centrifugation (100,000 $\times$ g, 60min). The supernatant was mixed with 50 $\mu$ l of immunobeads at 4°C overnight. After washing three times with 500 $\mu$ l buffer I and once with 600 $\mu$ l 30mM Tris-HCl, pH 7.5, containing 50mM NaCl, 0.2mM EDTA, 0.1% SDS, proteins were eluted from the immunoprecipitate using 50 $\mu$ l 2% SDS for 5min at 37°C and 10 $\mu$ l of the eluate was analysed by immunoblotting.

#### Expression of XPG protein in insect cells

For expression of XPG protein in insect cells, a 3732 bp cDNA fragment containing the complete XPG protein coding frame was inserted into the NotI site of the baculovirus vector pVL1392 (Invitrogen) to yield pXPG7, thus placing XPG under the control of the viral polyhedrin promoter. *Spodoptera frugiperda* (Sf9) cells were co-transfected with linear wild type AcMNPV DNA (Invitrogen) and pXPG7, and recombinants were detected by visual screening for the occlusion negative (polyhedrin negative) phenotype and examined by Southern blotting for the presence of XPG cDNA. Expression of the XPG protein in Sf9 cells harboring XPG-containing recombinant virus was verified by immunoblotting.

#### Purification of XPG protein

For XPG purification, 2 $\times$ 10<sup>8</sup> Sf9 cells were suspended in 16ml recombinant virus solution (10<sup>8</sup> plaque forming units/ml), incubated for 1h at 27°C to allow infection. After being diluted to 150 ml with TNM-FH medium (Gibco/BRL) supplemented with 10% fetal bovine serum, infected cells were incubated for 44h at 27°C in spinner flasks and collected by centrifugation. All the subsequent steps were carried out at 4°C. Cells were suspended in 30ml buffer A (50mM Tris-HCl, pH 7.5, containing 10% sucrose, 400mM KCl, 10mM EDTA, 10mM 2-mercaptoethanol and protease inhibitors — aprotinin, chymostatin, leupeptin, and pepstatin A, each at 10  $\mu$ g/ml, and also 0.5mM each of benzamidine and PMSF) and disrupted in a Dounce glass homogenizer using 20 strokes and pestle A. The crude cell lysate was clarified by centrifugation (100,000 $\times$ g for 60min) and treated with ammonium sulfate (0.23g/ml). The protein precipitate was dissolved in 10ml buffer K (20 mM K<sub>2</sub>HPO<sub>4</sub>, pH 7.4, containing 0.5mM EDTA, 10mM 2-mercaptoethanol, 10% glycerol and protease inhibitors) and dialysed for 3 h against 1 L of the same buffer before being applied onto a Q Sepharose column (1 $\times$ 6 cm), which was eluted with a 100 ml gradient of 0–0.5M KCl in buffer K. Fractions containing the peak of XPG protein, eluting around 300mM KCl, were identified by a combination of Coomassie blue staining of SDS-polyacrylamide gels and immunoblotting. The pool of XPG protein (8 ml) was concentrated to 0.8ml in a Centricon 30 microconcentrator (Amicon) and subjected to molecular sizing in a Sephacryl S300 column (1 $\times$ 40 cm) in buffer K with 30 mM

KCl. The S300 pool was further fractionated in a Mono S column (HR5/5) using a 50 ml gradient of 100–800 mM KCl and XPG protein eluted at  $\approx$ 350 mM. The Mono S pool of XPG protein (100  $\mu$ g total) was nearly homogeneous as revealed by SDS-PAGE and Coomassie staining (Fig. 1B). Purified XPG was concentrated to 300 $\mu$ g/ml and stored in 20 $\mu$ l aliquots at –70°C.

#### Nuclease assay by agarose gel electrophoresis

Covalently closed single-stranded (ss) M13 mp18 DNA (200ng or 600 pmol nucleotides) was incubated with XPG protein (50ng or 0.37 pmol) for the indicated times at 37°C in 10 $\mu$ l buffer R (50 mM Tris-HCl, pH 8.0, containing 10 mM MgCl<sub>2</sub>, 1 mM DTT and 50  $\mu$ g/ml BSA). The reaction was stopped by adding SDS to a final concentration of 0.5% followed by electrophoresis in 0.9% agarose gels run in 20 mM Tris-acetate, pH 7.5, with 0.5 mM EDTA. DNA was stained with ethidium bromide and photographed through a red filter using Polaroid type 55 film.

#### Determination of the nature of termini in XPG-digested DNA

12  $\mu$ g of covalently closed ssM13 DNA was treated with or without 850 ng of XPG protein for 90min at 37°C in 180  $\mu$ l of buffer R. Following digestion with XPG, one third of the reaction mixture was treated with 20 units of alkaline phosphatase and incubated at 37°C for another 60min. After phenol extraction, DNA was precipitated from the phosphatase treated and untreated samples by adding three volumes ethanol, dried, and redissolved in water to 200ng/ $\mu$ l. For 3'-end-labelling, 2 $\mu$ g of DNA that had not been treated with alkaline phosphatase was incubated with 10 units of calf thymus terminal transferase (Boehringer Mannheim) and 40  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P] dATP (3000 Ci/mmol, Amersham) at 37°C for 30 min. For 5'-end labeling, 2 $\mu$ g of DNA that had been treated with alkaline phosphatase or not was reacted with 5 units of T4 polynucleotide kinase and 25  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P] ATP (6000 Ci/mmol; Amersham) for 30 min at 37°C.

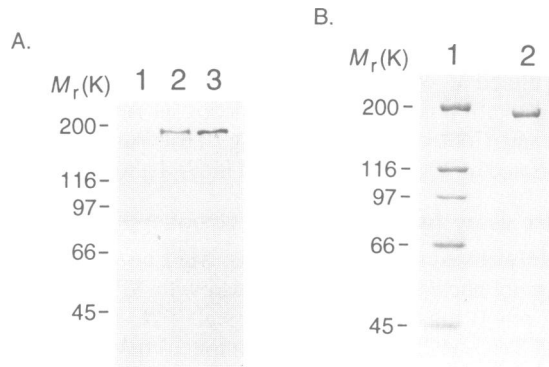
#### Nuclease assay by 5' end-labeling

1.4  $\mu$ g of circular ssM13 DNA was incubated with 250 ng or 500 ng of XPG in 80  $\mu$ l buffer R at 37°C. An 11 $\mu$ l aliquot was taken at 2.5, 5, 10, and 20min, heated for 5 min at 80°C, and then treated at 37°C with 2 units of alkaline phosphatase for 30min. After phenol extraction, the DNA was precipitated with ethanol, redissolved, and 5' end-labeled with 3 units of T4 polynucleotide kinase and 2.5  $\mu$ Ci of [ $\gamma$ -<sup>32</sup>P] ATP (750 Ci/mmol). The labeled DNAs were precipitated with ice-cold 10% trichloroacetic acid (TCA) in the presence of 5 $\mu$ g calf thymus DNA and collected on GF/C filters. After washing the filters with 5ml 10% TCA, they were placed in vials containing 10ml Liquiscint for radioactivity measurement in a Beckman LS 500 TD counter.

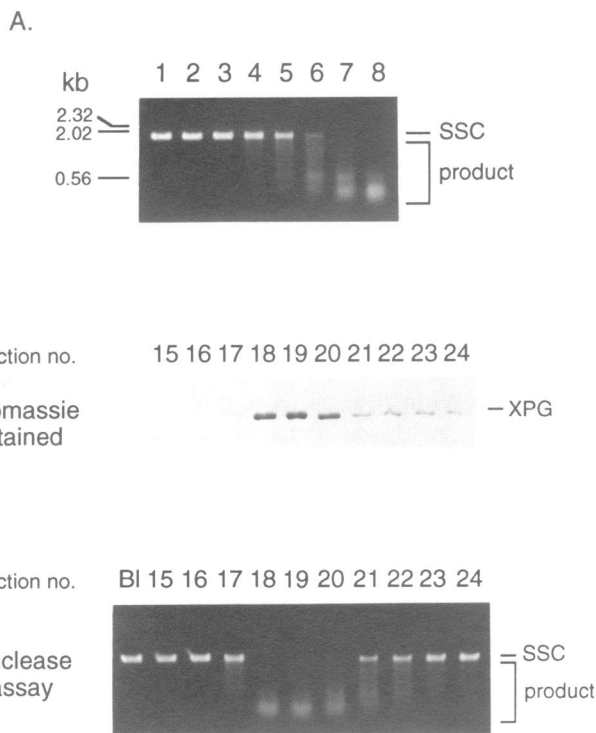
## RESULTS

#### Production of antibody specific for XPG protein

For the purpose of raising polyclonal antibodies against the XPG protein, we fused a portion of the XPG protein encompassing amino acids 107 to 452 with the N-terminal 116 residues of the *E. coli* transcriptional terminator protein  $\rho$ . Synthesis in *E. coli* strain AR120.A6 of the  $\rho$ -XPG hybrid protein is driven by the  $\lambda$  P<sub>L</sub> promoter and induced by treatment of cells with nalidixic acid. The  $\rho$ -XPG polypeptide is found exclusively in the particulate fraction after cell breakage, and was purified to homogeneity by preparative SDS-PAGE (see Materials and



**Figure 1.** Expression of XPG protein in Sf9 insect cells. (A) Immunoblot analysis. The nitrocellulose blot of a 7% denaturing polyacrylamide gel was probed with affinity purified anti-XPG antibodies. Lane 1, extract from Sf9 cells infected with wild type baculovirus; lane 2, extract from Sf9 cells infected with the recombinant baculovirus Bv.XPG1 that contains the XPG gene under the control of the viral polyhedrin promoter; lane 3, 10 ng of purified XPG protein. (B) Purity analysis. A 7% denaturing polyacrylamide gel containing 1 μg of purified XPG protein (lane 2) was stained with Coomassie blue. Molecular weight markers were run in lane 1.



**Figure 2.** XPG protein is a single-stranded DNA endonuclease. (A) Degradation of circular ssDNA by XPG protein. Lane 1, ss M13 viral DNA alone; lane 2 ss M13 DNA incubated with 50 ng of purified XPG in absence of  $Mg^{2+}$  for 30 min.; lane 3, ss M13 DNA incubated in the presence of  $Mg^{2+}$  and 0.5% SDS for 30 min.; lanes 4–8, ssM13 DNA incubated with 50 ng of XPG in the presence of  $Mg^{2+}$  for 2.5, 5, 10, 20, 30 min, respectively. (B) Co-elution of nuclease activity with XPG protein. Fractions 15 to 24 from the Mono S step of XPG purification were concentrated individually and 2 μl were subjected to SDS[EN]PAGE and staining with Coomassie blue to examine their XPG content. The same concentrated Mono S fractions, 0.6 μl each, were tested for ssDNA endonuclease activity. Symbols: SSC, single-stranded circular DNA; product, XPG digested DNA; BI, single-stranded circular DNA incubated without XPG.

Methods). After dialysis against phosphate buffered saline, the  $\rho$ -XPG protein was mixed with Freund's adjuvant and injected into two rabbits for producing polyclonal antibodies. Anti-XPG antibodies were purified from rabbit sera by affinity chromatography and used in immunoblotting and immunoprecipitation.

### Expression of XPG protein in insect cells and its purification to homogeneity

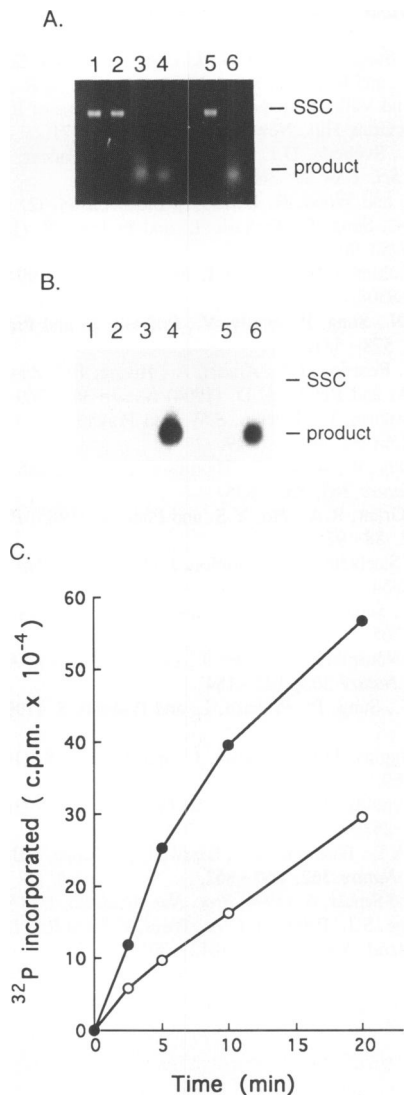
We had initially attempted to produce the complete XPG protein in yeast by use of a variety of yeast promoters including *ADCI*, *PGK*, and the hybrid *GAL-PGK*. However, we did not detect any XPG protein in extracts prepared from yeast strains harboring plasmids that contain XPG cDNA placed distal to these promoters. To determine whether XPG protein could be expressed in insect cells, the XPG cDNA was fused to the baculovirus polyhedrin promoter in the vector pVL1392 (Invitrogen) to give plasmid pXPG7. Linearized wild type baculovirus DNA and pXPG7 were used in co-transfection of Sf9 insect cells to obtain recombinant viruses that contain the XPG gene. One such recombinant Bv.XPG1, among seven that were identified, was picked for further work. Immunoblot analysis with anti-XPG antibodies of extracts from Sf9 cells infected with wild type baculovirus (Fig. 1A, lane 1) and with the recombinant Bv.XPG1 (Fig. 1A, lane 2) indicated the presence of XPG protein in cells carrying the recombinant Bv.XPG1 virus (Fig. 1A, lane 2). XPG protein so expressed has an apparent molecular size of 170 kDa, which is identical to the size of XPG protein immunoprecipitated from extract of Hela cells (data not shown). The predicted size of XPG from nucleotide sequence analysis is 134 kDa (14).

XPG protein was detected in Sf9 cells 24 h after infection with Bv. XPG1 and its level increases at later times. Since the XPG protein becomes increasingly insoluble after 44 h of infection (data not shown), cells were harvested at this time for protein purification. XPG protein was purified from extract of Bv.XPG1 infected Sf9 cells by a combination of ammonium sulfate precipitation and chromatographic fractionation in Q Sepharose, Sephacryl S300, and Mono S as described in Materials and Methods. SDS[EN]PAGE and Coomassie blue staining of XPG protein from the Mono S step showed that the protein is nearly homogeneous (Fig. 1B, lane 2). The identity of purified XPG protein was verified by immunoblotting (Fig. 1A, lane 3). From  $2 \times 10^8$  Sf9 cells infected with Bv.XPG1, we obtained  $\approx 100$ mg XPG protein.

### XPG protein is an endonuclease

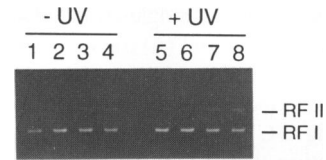
When purified XPG protein was incubated with circular ssM13 DNA, the DNA was converted to forms having increasing electrophoretic mobility in agarose gels as a function of reaction time (Fig. 2A, lanes 4 to 8), revealing that XPG protein has an associated nuclease activity. The nuclease function is endonucleolytic because it acts on covalently closed ssDNA (Fig. 2A) and dsDNA (see later). Expression of the nuclease activity requires  $Mg^{2+}$  (Fig. 2A, compare lane 2 to lane 8), which can not be replaced by  $Ca^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ , or  $Zn^{2+}$  (data not shown). Nuclease action is optimal between pH 8–8.5 and is not affected by KCl up to 100mM, but addition of the protein denaturant SDS to 0.5% abolishes the activity completely (Fig. 2A, compare lane 3 to lane 8).

We have examined the co-elution of endonuclease activity with XPG protein. As shown in Fig. 2B, the ability to degrade circular



**Figure 3.** XPG acts endonucleolytically and generates 3' hydroxyl and 5' phosphate groups. (A) and (B) Nature of termini in XPG digested DNA. ssM13 DNA that had been treated with XPG protein (lanes 3, 4, and 6) and mock-treated ssM13 DNA (lanes 1, 2, and 5) reacted either with calf thymus terminal transferase and [ $\alpha$ - $^{32}$ P] dATP (lanes 5 and 6) or with T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P] ATP (lanes 1 to 4). In lanes 2 and 4, the DNA had been treated with calf intestinal alkaline phosphatase before the incubation with T4 polynucleotide kinase. The samples, each containing 200ng DNA, were analysed in an agarose gel, which was stained with ethidium bromide and photographed (A) before being dried onto a sheet of Whatman 3MM paper and subjected to autoradiography (B). (C) Time dependent generation of 5' phosphate termini by XPG protein. ssM13 DNA was incubated with 3.1  $\mu$ g/ml (○) or 6.2  $\mu$ g/ml XPG protein (●), and at the indicated times, the reaction product was treated with alkaline phosphatase and then labeled with polynucleotide kinase and [ $\gamma$ - $^{32}$ P] ATP as described in Materials and Methods.  $^{32}$ P-labeled XPG digested DNA was precipitated by trichloroacetic acid, collected on Whatman GF/C filters before radioactivity measurement. Symbols used in (A) and (B): SSC, single-stranded circular DNA; product, XPG digested DNA.

ssM13 DNA was found to closely parallel the amount of XPG protein in the Mono S column fractions from the last step of purification. The fact that the endonuclease activity co-elutes with XPG protein (Fig. 2B) coupled with the high degree of purity



**Figure 4.** Nicking of supercoiled DNA by XPG protein. Supercoiled pBR322 DNA (lanes 1 to 4) or UV irradiated supercoiled pBR322 DNA (lanes 5 to 8), 200ng each, was incubated with 30ng (lanes 2 and 6) 60ng (lanes 3 and 7), and 90ng (lanes 4 and 8) of XPG protein at 37°C for 30min as described in Materials and Methods. After electrophoresis in a 0.9% agarose gel, DNA species were revealed by ethidium bromide staining. Symbols: RFI, supercoiled form; RFII, RF with discontinuity in one strand.

of the protein (Fig. 1B) establish that this activity is an intrinsic property of XPG.

### XPG nuclease generates 3' hydroxyl and 5' phosphate termini

To determine the nature of the DNA termini generated by XPG nuclease action, we subjected circular ssM13 DNA that had been treated with XPG protein to  $^{32}$ P-labeling with calf thymus terminal transferase and T4 polynucleotide kinase. The XPG digested DNA has a 3' hydroxyl group as it is labeled efficiently by terminal transferase (Fig. 3A and B, lane 6), and the presence of a 5' phosphate is indicated by the fact that efficient labeling of the DNA by polynucleotide kinase requires prior treatment with alkaline phosphatase (compare lanes 3 and 4 in Fig. 3A and B).

To examine the kinetics of formation of DNA ends, XPG digested DNAs were labeled at the 5' end followed by trichloroacetic acid precipitation of  $^{32}$ P-labeled DNA. Figure 3C shows that more DNA ends are generated by XPG with increasing reaction time and protein concentration, again indicating an endonucleolytic mode of action.

### XPG nuclease action on supercoiled DNA

Because of its involvement in NER, we examined whether XPG would incise UV damaged DNA preferentially. For this purpose, supercoiled pBR322 DNA that had or had not been irradiated with a high UV dose of 500J/m<sup>2</sup> was incubated with XPG protein, and the reaction mixtures were analysed in agarose gels followed by staining of DNA with ethidium bromide. As shown in Fig. 4, XPG converts a small portion of the supercoiled DNA (RFI) into form II DNA (RFII), and UV irradiation of DNA has no effect on nicking, indicating that XPG by itself does not act preferentially on UV damaged DNA. As discussed below, damage specific incision by XPG will require its interaction with the damage recognition components of the NER machinery. We did not observe any conversion of supercoiled DNA to linear DNA by XPG under our reaction conditions.

### DISCUSSION

By use of baculovirus, we have successfully produced the XPG gene product in Sf9 insect cells and find that the XPG protein so expressed has the same molecular size as cellular XPG immunoprecipitated from Hela extract. A purification procedure was devised to obtain nearly homogeneous XPG protein from extract of insect cells expressing the protein. Results from our biochemical studies indicate that the XPG protein has nuclease activity. The XPG nuclease activity is endonucleolytic because

(i) it acts on covalently closed single-stranded and double-stranded DNAs (Fig. 2 and Fig. 4) and (ii) the number of ends generated by XPG protein in ssDNA as assayed by 5' end labeling and TCA precipitation increases with reaction time and XPG concentration (Fig. 3C). These findings are congruent with our recent demonstration that RAD2 protein, the yeast homolog of XPG, also possesses an endonuclease activity (15). We show that XPG by itself does not act preferentially on UV irradiated supercoiled DNA (Fig. 4), indicating that XPG endonuclease is targeted to the damage site *via* its interaction with other NER components.

Mechanistically, the process of NER can be divided into several steps, namely, recognition of the DNA damage, unwinding of the duplex DNA, incision at the 5' side and at the 3' side of the damage, and finally, turnover of the incision protein complex and the damage containing DNA fragment followed by repair synthesis to fill the excision gap. Biochemical characterization of various purified human and yeast NER proteins has yielded important insights into the probable biological functions of these components and has also been invaluable in conceptual understanding of the repair process. XPA (4) and its yeast homolog RAD14 (5) have an affinity for UV damaged DNA, indicating a role of these proteins in the initial stage of damage recognition. Our recent work on yeast RAD3, which is remarkably homologous to XPD, has indicated that it too binds UV damaged DNA preferentially (6), implicating XPD in damage recognition as well. XPB (8) and XPD (10) and their yeast counterparts RAD25 (7) and RAD3 (9, 16) possess a DNA helicase activity that can be used in effecting an open conformation of the damaged DNA, allowing scissions to be made in the single-stranded region so created. Results obtained in our present study indicate that XPG carries out one of the scissions that occur in the damaged DNA strand. The other scission is expected to be mediated by a complex of the ERCC1 and ERCC4 (XPF) proteins, whose yeast counterparts RAD10 and RAD1 combine to form an endonuclease (17, 18). Because the XPG protein does not act preferentially on UV damaged DNA (this work) and the same is expected to hold for the predicted ERCC1/ERCC4 (XPF) complex (17), interaction of XPG and of the ERCC1/ERCC4 (XPF) complex with the DNA damage recognition components, either directly or through an intermediary, will be necessary to incorporate these endonucleases into the NER machinery. In fact, evidence already exists for the interaction of the ERCC1/ERCC4 (XPF) complex with the damage recognition component XPA (19,20). Our previous work with the rad3-Arg48 mutant protein that does not have helicase activity has indicated a role of this activity in the post-incision phase of repair (16), and it is probable that the RAD25 helicase activity also plays a post-incision role (7). Subsequent to dual incision in the damaged DNA strand, the combined helicase function of XPB/RAD25 and XPD/RAD3 could effect turnover of the incision protein complex and the damage containing DNA fragment to allow for repair synthesis to fill the resulting gap.

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