Human Nucleotide Excision Nuclease Incises Synthetic Double-Stranded DNA Containing a Pyrimidine Dimer at the Fourth Phosphodiester Linkage 3′ to the Pyrimidine Dimer†

Satoshi Tateishi,*,‡ Naoko Hori,§ Eiko Ohtsuka,§ and Masaru Yamaizumi*,‡

Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kuhonji 4-24-1, Kumamoto 862, Japan, and Faculty of Pharmaceutical Science, Hokkaido University, Sapporo 060, Japan

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ABSTRACT: Linear 75mer double-stranded DNA containing a single pyrimidine dimer at a unique site was used to investigate pyrimidine dimer-dependent endonuclease activities from human cells. HeLaS3 cell extract incised the target DNA at the fourth phosphodiester linkage 3′ to the pyrimidine dimer. However, incision of the DNA at 5′ side of the pyrimidine dimer was not detected. The incision was also detected in cell extracts prepared from other excision repair-proficient cell lines. Incision was detected only on the DNA strand containing a pyrimidine dimer in the presence of poly(dI-dC)·poly(dI-dC) double strand. The reaction required Mg^{2+} but not ATP. The extract prepared from excision repair-deficient xeroderma pigmentosum (XP) cells belonging to the complementation group A was unable to incise the DNA. Extracts from the complementation groups C, D, and G incised the DNA very weakly at the third phosphodiester linkage 3′ to the pyrimidine dimer, a site different from that incised by normal human cell extract. These results suggest that the observed incision reaction is associated with excision repair in human cells.

The major photochemical DNA damage produced by ultraviolet light (UV)[1] is the formation of cyclobutane pyrimidine dimers. Studies of the repair of these lesions in both prokaryotes and eukaryotes have thus far established two principal processes for their restitution: enzymatic photoreactivation and nucleotide excision repair. Photoreactivation is mediated by photolyase, an enzyme that binds to a site containing dimer and, upon absorption of visible light energy, cleaves the pyrimidine–pyrimidine ring structure, thereby restoring native pyrimidines. The biochemistry of excision repair of UV-irradiated DNA in *Escherichia coli* is now well understood. The multisubunit UvrABC nuclease has been shown to cut a damaged DNA strand on each side of the lesion in an ATP-dependent reaction (Sancar & Rupp, 1983; Yeung et al., 1986), and then a DNA fragment containing the dimer is removed. The resulting gap in the DNA strand is filled by synthesis of new DNA which is finally joined to preexisting DNA.

In human cells, pyrimidine dimers are recognized and removed from the DNA by the process of excision repair, but very little is known about the nature of the proteins involved in pyrimidine dimer recognition and DNA incision. A repair defect has been noted in xeroderma pigmentosum (XP), an inherited desease characterized by extreme photosensitivity and formation of multiple skin cancers. So far, seven complementation groups, A–G, have been identified in xeroderma pigmentosum. Cells from these patients exhibit a reduced level of DNA repair synthesis in response to UV light (Cleaver, 1968) and have been shown to be deficient in excision repair (Zelle & Lohman, 1979; Mitchell et al., 1985). It was reported that cells from most or all XP complementation groups have a defect in the initial incision at damaged sites of DNA (Fornace et al., 1976; Thielmann et al., 1985). Recently, Wood et al. established a cell-free system that can perform repair synthesis on a UV-irradiated plasmid and showed a difference in repair synthesis between normal and XP cells (Wood et al., 1988). Using this system, they found that human single-stranded DNA binding proteins are required for excision repair (Coverley et al., 1991). During preparation of this report, Huang et al. delineated the borders of the repair patch by using a cell-free system containing a plasmid that has four pyrimidine dimers (Huang et al., 1992).

In this study, we prepared synthetic double-stranded DNA (dsDNA) substrate containing a cis–syn pyrimidine dimer at a distinct site to detect a pyrimidine dimer-dependent incision. The DNA is incised at a unique site by normal human cell extracts. In contrast, a cell extract prepared from excision repair-deficient xeroderma pigmentosum complementation group A is unable to incise the DNA, and extracts from complementation groups C, D, and G incise the DNA at a different site than do normal human cell extracts.

MATERIALS AND METHODS

Cell-Free Extract. Extracts were prepared by the method of Manley et al. (Manley et al., 1980) with some modifications. HeLaS3 cells were grown in suspension culture in HEPES-buffered Eagle's MEM supplemented with 10% calf serum, 0.29 g/L glutamine, 0.055 g/L $NaHCO_3$, and 10^5 units/L penicillin. One-liter cultures of cells in late exponential phase ($(5–8) \times 10^5$ cells/mL) were used for the preparation of cell extract. The cell pellet (packed cell volume 2 mL) was rinsed three times in ice-cold phosphate-buffered saline supplemented with 1 mM PMSF, 4 μg/mL leupeptin, and 1 μg/mL pepstatin and then was resuspended in 4 mL of hypotonic buffer (40 mM Tris-HCl [pH 7.9], 1 mM EDTA, 4 mM DTT, 2 mM PMSF, 4 μg/mL leupeptin, and 3 μg/mL pepstatin). The swollen cells were broken by 25–75 strokes in a Dounce homogenizer. Six milliliters of ice-cold solution containing 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, 1

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‡ Kumamoto University School of Medicine.

§ Hokkaido University.

[1] Abbreviations: UV, ultraviolet light; XP, xeroderma pigmentosum; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; UDS, unscheduled DNA synthesis.

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mM PMSF, 4 μg/mL leupeptin, and 3 μg/mL pepstatin was added slowly. The solution was transferred to polyallomer ultracentrifuge tubes. Two milliliters of neutralized saturated ammonium sulfate solution was then added drop by drop with gentle mixing. The extremely viscous lysate in a tube was mixed by rotation at 6 rpm for 30 min and centrifuged for 2 h at 80 000 rpm in an RP80 rotor (Hitachi) at 4 °C. The supernatant was withdrawn, leaving the last 0.5 mL behind. Protein was precipitated by addition of 0.4 g/mL ammonium sulfate (neutralized with 1 μL of 1 M NaOH/g of ammonium sulfate). The precipitate was collected by centrifugation, resuspended in 1 mL of dialysis buffer (20 mM Tris-HCl [pH 7.9], 0.2 mM EDTA, 0.5 mM DTT, 0.2 M KCl, 10% glycerol, 0.01% BL-8SY [Nikko Chemicals], 0.01% Nonidet P-40, 0.5 mM PMSF, 4 μg/mL leupeptin, and 1 μg/mL pepstatin), and dialyzed against 500 mL of the dialysis buffer for 12 h at 4 °C. Insoluble materials were removed by centrifugation, and the clarified extract (1 mL/1 L starting culture) was divided into small aliquots. Extracts typically contained 10 mg/mL protein. The extracts were stable for over a year at −80 °C and remained active after one thawing and refreezing.

Other human cell lines, WI38VA13, FL (Fogh & Lund, 1957), XP2OSSV(XP-A), XP4PASV(XP-C), XP6BESV-(XP-D), and XP3BRSV(XP-G) were grown as monolayer cultures in Dulbecco's-modified Eagle's medium supplemented with 10% fetal calf serum, 1 g/L $NaHCO_3$, 10^5 units/L penicillin, and 0.1 g/L streptomycin sulfate at 37 °C under 5% CO_2. These cell lines, except for FL, are established cell lines immortalized with SV-40 virus and are free of mycoplasma. When the monolayers were semiconfluent, cells were detached from the plates by incubating them with 0.13% trypsin and 0.01% EDTA at 37 °C for 5 min and collected by centrifugation. The cell pellet (packed cell volume 0.3 mL from 30 plates) was washed once with medium and twice with phosphate-buffered saline. Cell extracts were prepared by the same method used for HeLaS3 cells.

Preparation of 75mer Double-Stranded DNA. (i) 75mer dsDNA containing a cis–syn cyclobutane pyrimidine dimer was prepared by annealing and ligation of a 14mer oligonucleotide containing a pyrimidine dimer (Inaoka et al., 1989) with a 31mer, a 30mer, and a complementary 75mer (Figure 1). The sequences of the 31mer and the 30mer were GAGCATCCAGATCTAGCACCTCTAGAGTCCG and GCGTCAGTCGACACTAGTGATCAGCTCGTC, respectively. The oligonucleotides were synthesized with an Applied Biosystems Model 381A synthesizer. The 14mer, CGAAG-GTTGGAAGC, was irradiated with UV light (wavelength 280 nm) for 1–2 h using a high-pressure mercury lamp equipped with a UV-25 filter (Toshiba) and a $NiSO_4 \cdot 6H_2O$ (500 g/L) filter. The unique TT in the center of the oligonucleotide was expected to form a cyclobutane pyrimidine dimer after UV irradiation. Oligonucleotide containing a cis–syn cyclobutane dimer was purified by C_{18} reverse-phase HPLC. The modified fraction was collected and repurified by HPLC. The 5′ end of the 31mer sequence was labeled with T4 kinase and [γ-^{32}P]ATP. The 5′ ends of the 14mer sequence, 30mer sequence, and 75mer sequence were phosphorylated with T4 kinase and cold ATP. Four picomoles of each oligonucleotide was mixed and then annealed by heating at 95 °C for 5 min and slow cooling. The annealed oligonucleotides were ligated with T4 ligase, and then the mixture was extracted with phenol/chloroform. DNA was precipitated in the presence of 0.3 M sodium acetate with 2 volumes of ethanol. The ligated product was purified by 8 M urea/6% polyacrylamide gel electrophoresis. The gel slice containing ligated ds75mer oligonucleotide was cut out, and the DNA was eluted from the gel using a C3HV tube (Millipore) and extracted with phenol/chloroform. DNA was precipitated in the presence of 0.3 M sodium acetate with 2 volumes of ethanol, dried, and dissolved in 10 mM Tris-HCl (pH 7.6), 1 mM EDTA.

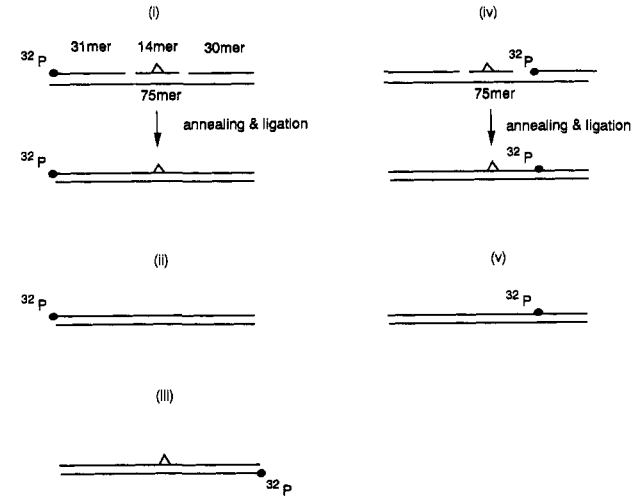

FIGURE 1: Structures of the 75mer dsDNA substrates. (i) 75mer dsDNA containing a pyrimidine dimer prepared by annealing and ligation of a 14mer oligonucleotide containing a pyrimidine dimer, a 5′-labeled 31mer, a 30mer, and a complementary ss75mer; the Δ represents a pyrimidine dimer; (ii) is identical with (i), except for the absence of a pyrimidine dimer; (iii) is 75mer dsDNA (5′ end of the lower strand was labeled with ^{32}P); (iv) is 75mer dsDNA (seventh phosphodiester linkage 3′ to the pyrimidine dimer was labeled with ^{32}P); (v) is identical with (iv) except for the absence of a pyrimidine dimer.

For control experiments, the following DNA substrates shown in Figure 1 were prepared.

(ii) 75mer dsDNA without a pyrimidine dimer was prepared by the same method as (i), except that nonirradiated 14mer was used.

(iii) 75mer dsDNA labeled at the 5′ end of the lower strand of the DNA was prepared by the same method as (i), except that the 75mer oligonucleotide instead of the 31mer was labeled with ^{32}P.

(iv) 75mer dsDNA labeled at the seventh phosphodiester linkage 3′ to the pyrimidine dimer was prepared by the same method as (i), except that the 30mer oligonucleotide instead of the 31mer was labeled with ^{32}P.

(v) The same procedure was followed as for (iv), except that nonirradiated 14mer was used instead of 14mer containing a pyrimidine dimer.

Incision Reaction. Standard 50-μL reaction mixtures contained 2 ng of 75mer dsDNA (5000 cpm), 25 mM HEPES–KOH (pH 7.8), 0.02 μg/mL poly(dI-dC)·poly(dI-dC) double strand (Pharmacia), 70 mM KCl, 1 mM $MgCl_2$, 0.01 mM $ZnCl_2$, 1 mM DTT, 0.5 mM EDTA, 1 mM ATP, 20 mM phosphocreatine, 2.5 μg of creatine kinase (Boehringer Mannheim), 1% glycerol, and 50 μg of extract protein. Reactions were carried out at 25 °C.

Product Analysis by Autoradiography. Reactions were stopped by addition of EDTA to 20 mM, and then the mixtures were extracted with phenol/chloroform. DNA was precipitated in the presence of 0.3 M sodium acetate with 2 volumes of ethanol, washed with 70% ethanol, and then dried. DNA was dissolved in 2 μL of 90% formamide containing dye and loaded onto a 8 M urea/6% polyacrylamide gel and run at 30 V/cm for 2 h. An autoradiograph of the dried gel was obtained using Fuji AIF RX film and an intensifying screen for 12 h

at −80 °C. Band intensities on the autoradiograph were quantified using a Fuji BAS 2000 image analyzer.

RESULTS

Preparation of DNA Bearing a Pyrimidine Dimer. A 75-bp dsDNA substrate containing a cis–syn pyrimidine dimer at a unique site was prepared as described in Materials and Methods (see Figure 1). A pyrimidine dimer was located in the center of the upper strand and the 5′ end of the upper strand was labeled with ^{32}P. Purified DNA substrate contained no impurities as judged by denaturing gel electrophoresis. The DNA substrate was constructed to have six restriction enzyme cleavable sites. Cleavage occurred at the expected sites (data not shown).

T4 endonuclease V is known to incise UV-irradiated DNA at the sites of cyclobutane pyrimidine dimers (Grafstrom et al., 1982). More than 95% of the DNA substrate was cleaved at the expected site by treatment with T4 endonuclease V, indicating that almost all molecules of the DNA substrate had a pyrimidine dimer at the unique site. When an identical DNA substrate without a pyrimidine dimer was incubated with T4 endonuclease V, the cleaved product was not observed (data not shown).

Detection of a Pyrimidine Dimer-Dependent Incision. We used denaturing gel electrophoresis to monitor the fate of DNA substrate after incubation with extracts from normal human cells. Initially, we prepared cell extracts from EB virus-immortalized lymphoid cells and incubated DNA substrate with these cell extracts. Wood et al. showed that human lymphoid cell extracts promote repair synthesis on covalently closed circular DNA containing pyrimidine dimers (Wood et al., 1988). However, human lymphoid cell extracts degraded linear DNA substrates irrespective of the presence of pyrimidine dimer, and this made detection of pyrimidine dimer dependent-endonuclease activities difficult. Such degradation activity was found to be much lower in cell extracts prepared from fibroblastic cells and epithelial cells rather than lymphoid cell lines. Therefore, we used mainly extract prepared from HeLaS3 cells for the following experiments.

In preliminary experiments, we could not detect any pyrimidine dimer-dependent reaction product. We reasoned that the binding of a putative pyrimidine dimer-specific nuclease to the target DNA was interfered by a large amount of nonspecific DNA binding proteins in the cell extract. The addition of a competitor DNA to the cell extract would reduce the concentration of the nonspecific DNA binding proteins. Therefore, we carried out the reactions with various concentrations of poly(dI-dC)·poly(dI-dC) double strand.

Figure 2A shows that when 75mer dsDNA containing a pyrimidine dimer [Figure 1 (i)] was incubated with HeLaS3 cell extract at 25 °C for 10 h, a distinct product appeared. The reaction required an appropriate concentration of poly(dI-dC)·poly(dI-dC) double strand. In the absence of poly(dI-dC)·poly(dI-dC) double strand, no specific product was observed. In the presence of 0.02 μg/mL poly(dI-dC)·poly(dI-dC) double strand, incision occurred at a site several bases 3′ to the pyrimidine dimer, as judged by comparison with the product of incision by T4 endonuclease V. The amount of incision product decreased with increasing concentration of poly(dI-dC)·poly(dI-dC) double strand. The product was not seen when 0.5 μg/mL poly(dI-dC)·poly(dI-dC) double strand was present.

When an identical DNA substrate without a pyrimidine dimer was incubated with HeLaS3 cell extract, the product was not observed (Figure 2B). These results indicate that the reaction is dependent on pyrimidine dimer.

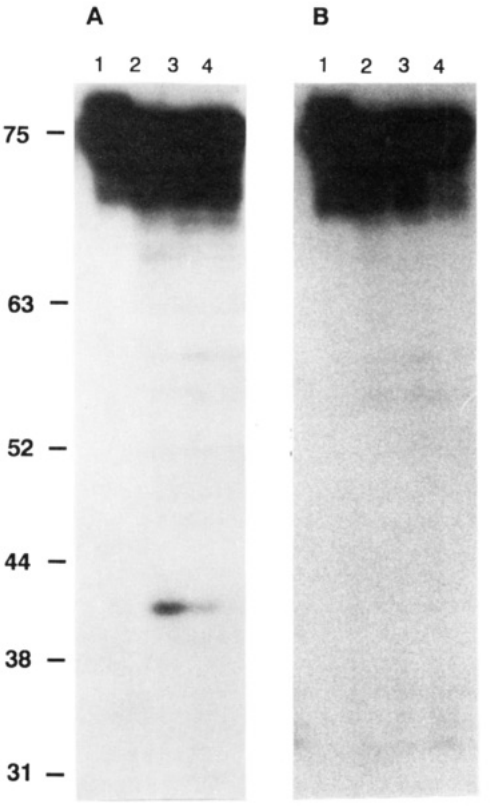

FIGURE 2: Pyrimidine dimer dependent-incision by HeLaS3 cell extract. (A) 75mer dsDNA containing a pyrimidine dimer (Figure 1 (i)) was incubated with HeLaS3 cell extract at 25 °C for 10 h in the presence of various concentrations of poly(dI-dC)·poly(dI-dC) double strand. Lane 1, 0 μg/mL; lane 2, 0.02 μg/mL; lane 3, 0.1 μg/mL; lane 4, 0.5 μg/mL poly(dI-dC)·poly(dI-dC) double strand. After the incubation, DNA was separated on a denaturing gel and visualized by autoradiography as described in Materials and Methods. (B) Reactions were carried out as in (A) except that 75mer dsDNA without a pyrimidine dimer (Figure 1 (ii)) was used as a substrate. The positions of the DNA markers (mer) are indicated by bars; 63-, 52-, 44-, 38-, and 31mer DNA were prepared by digesting 75-bp DNA with *Bcl*I, *Sal*I, *Hin*PI, T4 endonuclease V, and *Bst*UI, respectively.

The activity was detected not only in HeLaS3 cell extract but also in extracts from other excision repair-proficient cell lines such as FL and WI38VA13 (data not shown). To confirm that the reaction was promoted by pyrimidine dimer-dependent endonuclease, we carried out the following experiments.

First, DNA substrate labeled with ^{32}P at the 5′ end of the lower strand of the DNA [Figure 1 (iii)] was incubated with HeLaS3 extract. No product from the lower strand was observed, indicating that the reaction occurred only on the upper strand containing a pyrimidine dimer (Figure 3, lane 3).

Second, DNA substrate labeled at the seventh phosphodiester linkage 3′ to the pyrimidine dimer [Figure 1 (iv)] was incubated with HeLaS3 extract (Figure 3, lane 4). A pyrimidine dimer-dependent product was also observed. The size of the product was several bases shorter than that produced from 5′-labeled DNA (compare lanes 1 and 4 in Figure 3). These two products seemed to correspond to the two fragments produced by an incision of the upper strand near the pyrimidine dimer. When DNA labeled at the 3′ end of the upper strand was used as a substrate, the same product was observed (data not shown). In this case, several additional products were observed on the gel. However, these products were also

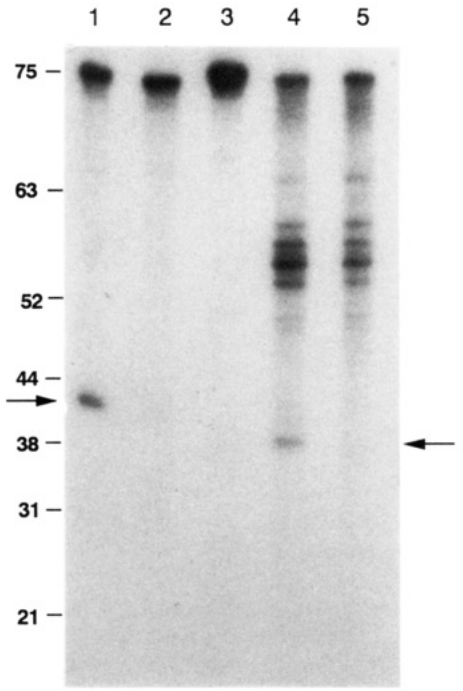

FIGURE 3: Incision reactions of various DNA substrates promoted by HeLaS3 cell extract. DNA substrates were incubated with HeLaS3 cell extract at 25 °C for 10 h in the presence of 0.02 μg/mL poly(dI-dC)·poly(dI-dC) double strand. The DNA substrate used in each reaction was the following: lane 1, 75mer dsDNA containing a pyrimidine dimer [see Figure 1 (i)]; lane 2, 75mer dsDNA identical with (i) except for the absence of a pyrimidine dimer [Figure 1 (ii)]; lane 3, 75mer dsDNA labeled with ^{32}P at the 5′ end of the lower strand of the DNA [Figure 1 (iii)]; lane 4, 75mer dsDNA labeled with ^{32}P at the seventh phosphodiester linkage 3′ to the pyrimidine dimer [Figure 1 (iv)]; lane 5, identical with (iv) except for the absence of pyrimidine dimer [Figure 1 (v)]. Arrows indicate positions of the incision products. The positions of the DNA markers (mer) are indicated by bars; 63-, 52-, 44-, 38-, 31-, and 21mer DNA were prepared by digesting 75-bp DNA with *Bcl*I, *Sal*I, *Hin*PI, T4 endonuclease V, *Bst*UI, and *Xba*I, respectively.

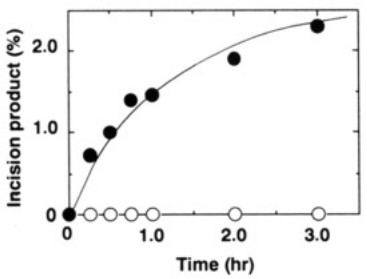

FIGURE 4: Kinetics of the pyrimidine dimer-dependent incision. DNA substrate with (●) or without a pyrimidine dimer (○) was incubated with 1 mg/mL HeLaS3 cell extract at 25 °C in the presence of 0.02 μg/mL poly(dI-dC)·poly(dI-dC) double strand as described in Materials and Methods. At the indicated times, aliquots of reaction mixtures were taken for analysis.

observed when DNA substrate without a pyrimidine dimer was used (Figure 3, lane 5). These products were possibly produced by 5′–3′ exonuclease present in the cell extract.

Kinetics of the Incision Reaction. To determine the kinetics of the pyrimidine dimer-dependent incision reaction, DNA substrate containing a pyrimidine dimer was incubated at 25 °C, and aliquots of the reaction mixture were taken at various times. Figure 4 shows that the incision product could be detected at 15 min, and the amount of the incision product increased for 3 h and then remained constant for 20 h. We estimate that about 2.5% of the DNA substrate was incised in 3 h. In contrast, no incision product was detected even at 3 h when the DNA substrate without a pyrimidine dimer was

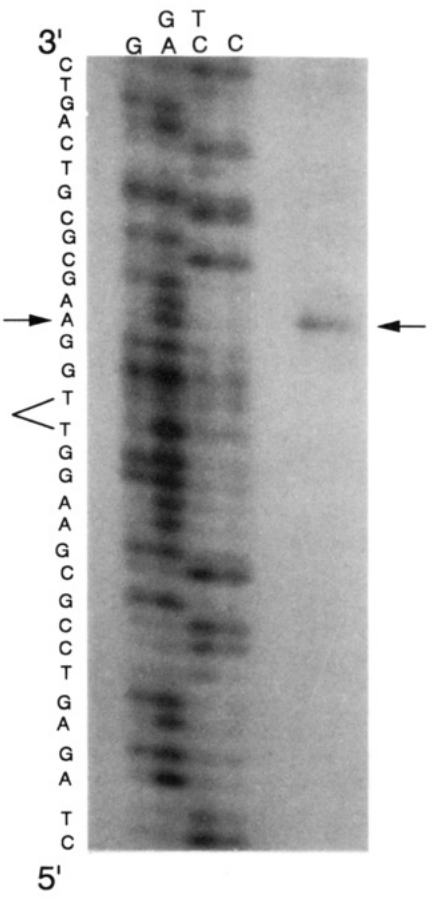

FIGURE 5: Location of the incision site. 75mer dsDNA containing a pyrimidine dimer was incubated with HeLaS3 cell extract at 25 °C for 3 h in the presence of 0.02 μg/mL poly(dI-dC)·poly(dI-dC) double strand. The length of the incision product was compared with 75mer dsDNA chemically cleaved by the method of Maxam and Gilbert (Maxam & Gilbert, 1980). The letters of the left lane represent the positions of nucleotides of the DNA. Arrows indicate the position of the incision product.

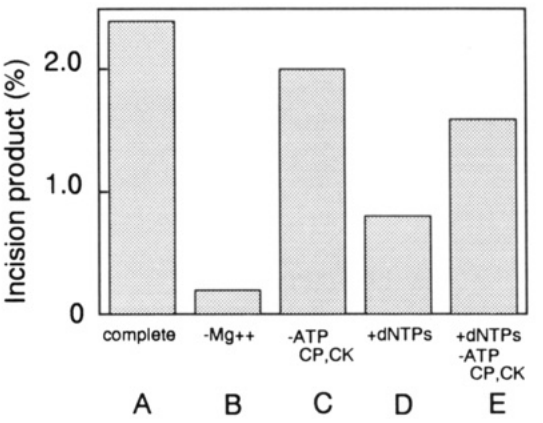

FIGURE 6: Effects of Mg^{2+}, ATP, and deoxynucleotides on the incision reaction promoted by HeLaS3 cell extract. (A) Complete reaction conditions containing Mg^{2+}, ATP, and the ATP-regeneration system. (B) Mg^{2+} was omitted. (C) ATP and the ATP-regeneration system were omitted. (D) A deoxynucleotide mixture (dATP, dGTP, dCTP, and TTP) was added to the reaction mixture (final concentration 1 mM). (E) Same as (D) except ATP and the ATP-regeneration system were omitted.

used. These data are consistent with previous reports that the removal of pyrimidine dimers from UV-irradiated DNA is slow as measured directly by chromatography (Regan, 1968; Meyn et al., 1974) or indirectly by detecting residual photoadducts (Zelle & Lohman, 1979; Williams & Cleaver, 1978; Mitchell et al., 1985).

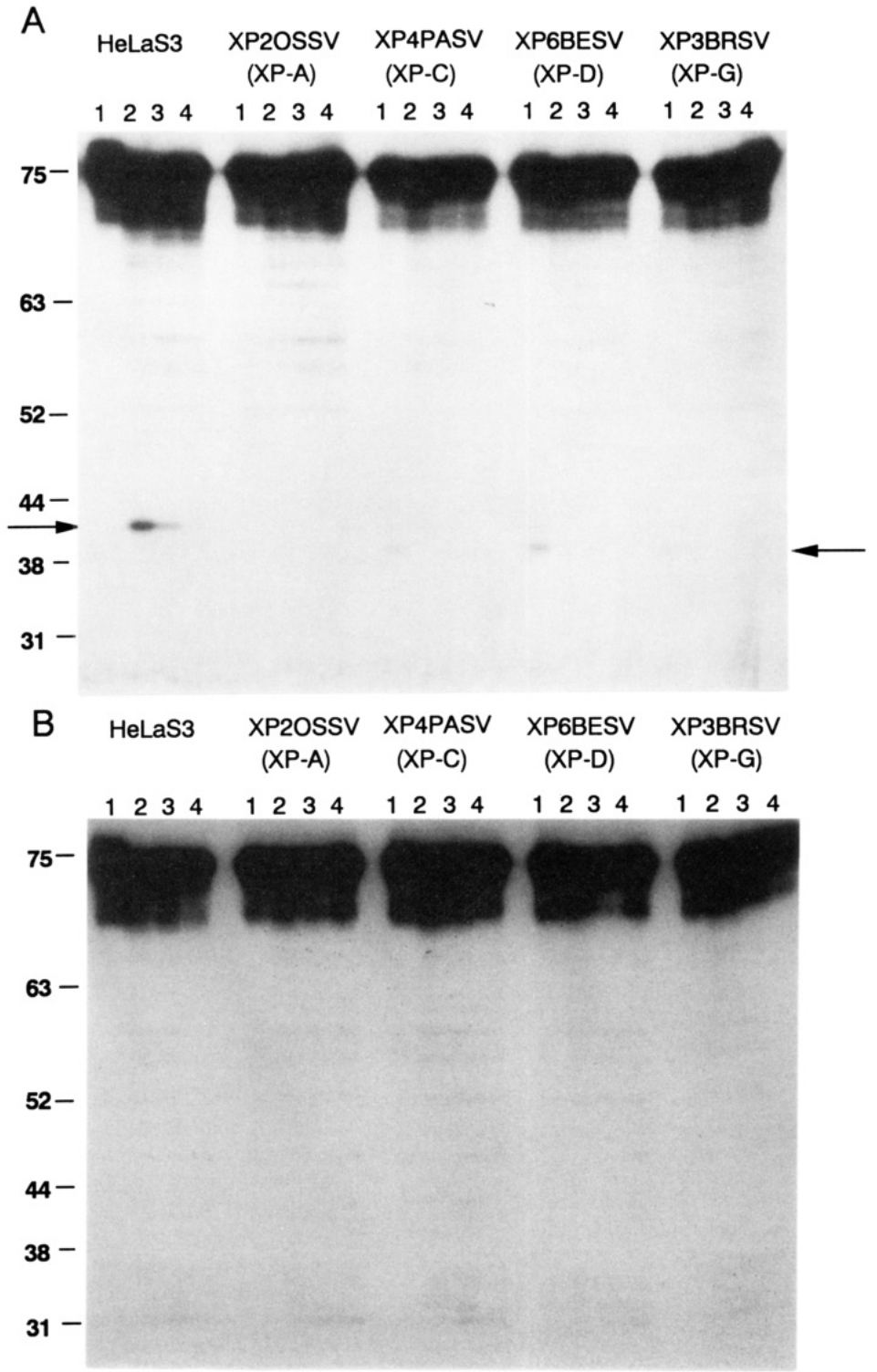

FIGURE 7: Incision reaction promoted by cell extracts prepared from XP cells. (A) 75mer dsDNA containing a pyrimidine dimer [Figure 1 (i)] was incubated with cell extracts from HeLaS3 (normal), XP2OSSV (XP-A), XP4PASV (XP-C), XP6BESV (XP-D), or XP3BRSV (XP-G) cells at 25 °C for 10 h in the presence of various concentrations of poly(dI-dC)·poly(dI-dC) double strand. The concentration of poly(dI-dC)·poly(dI-dC) double strand in the reaction mixture was 0 μg/mL (lane 1), 0.02 μg/mL (lane 2), 0.1 μg/mL (lane 3), or 0.5 μg/mL (lane 4). (B) Reactions were carried out as in (A) except that 75mer dsDNA without a pyrimidine dimer [Figure 1 (ii)] was used. Arrows indicate the position of the incision products. The position of the DNA markers (mer) is indicated by bars; 63-, 52-, 44-, 38-, and 31mer DNA were prepared by digestion of 75bp DNA with *Bcl*I, *Sal*I, *Hin*PI, T4 endonuclease V, and *Bst*UI, respectively.

Determination of the Incision Site of DNA. To determine the location of the incision site, the length of the incision product was compared with the 75mer dsDNA chemically cleaved by the Maxam and Gilbert method. Figure 5 shows that the incision site was located between the third adenosine and the fourth adenosine 3′ to the pyrimidine dimer. Thus, the DNA was incised at the fourth phosphodiester linkage 3′ to the pyrimidine dimer.

Effects of ATP, Mg^{2+}, and Deoxynucleotides. In excision repair, ATP and Mg^{2+} are thought to be required for the incision step and/or oligonucleotide displacement step, rather than repair replication. The cell-free system for excision repair promoted by lymphoid cell extract requires ATP and an ATP regenerating system (Wood et al., 1988). However, the pyrimidine dimer-dependent incision reaction we observed was dependent on Mg^{2+} but independent of ATP (Figure 6B,C).

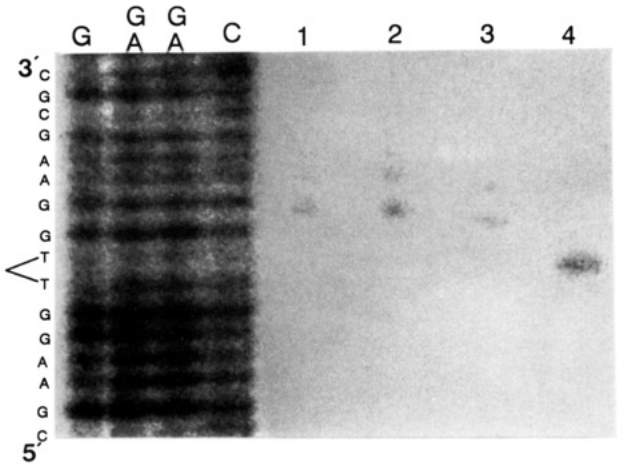

FIGURE 8: Location of the incision site. 75mer dsDNA containing a pyrimidine dimer was incubated with 1 mg/mL XP4PASV (XP-C) cell extract (lane 1), 1 mg/mL XP6BESV (XP-D) cell extract (lane 2), 1 mg/mL XP3BRSV (XP-G) cell extract (lane 3), or 0.1 μg/mL T4 endonuclease V (lane 4) at 25 °C for 10 h. The length of the incision products was compared with 75mer dsDNA chemically cleaved by the method of Maxam and Gilbert (Maxam & Gilbert, 1980).

Repair replication events require deoxynucleotides as precursors for DNA replication. Under our standard conditions described above, deoxynucleotides were not added to the reaction mixture. When all four deoxynucleotides were added to the reaction mixture, the amount of incision product decreased (Figure 6D). The effect of deoxynucleotides on the reaction was reduced when ATP and the ATP-regenerating system were omitted from the reaction mixture (Figure 6E).

Incision Reaction Promoted by Cell Extracts Prepared from XP Cells. XP cells belonging to most complementation groups have a defect in the initial incision step on damaged DNA (Fornace et al., 1976; Thielmann et al., 1985). We compared incision activities of cell extracts from SV-40-transformed XP-A, XP-C, XP-D, and XP-G fibroblasts with that from HeLaS3 cells. The DNA substrate containing a pyrimidine dimer was incubated with these cell extracts at 25 °C in the presence of various concentrations of poly(dI-dC)·poly(dI-dC) double strand. Extract prepared from XP-A cells had no incision activity. When poly(dI-dC)·poly(dI-dC) double strand was absent, extracts prepared from XP-C, XP-D, and XP-G cells incised the DNA weakly at a site different from the site incised by HeLaS3 cell extract (Figure 7A, lane 1). When an identical DNA substrate without a pyrimidine dimer was used, no incision product was observed (Figure 7B). Therefore, the reaction promoted by these XP cell extracts was also dependent on pyrimidine dimer. From comparison to the DNA chemically cleaved by the Maxam and Gilbert method, the major incision site was located at the third phosphodiester linkage 3′ to the pyrimidine dimer (Figure 8). In addition, two other minor sites were observed at the fourth and the fifth phosphodiester linkage 3′ to the pyrimidine dimer.

DISCUSSION

The results reported here show that a DNA substrate containing a pyrimidine dimer is incised by normal human cell extracts at the fourth phosphodiester linkage 3′ to the pyrimidine dimer. The reaction requires an appropriate amount of poly(dI-dC)·poly(dI-dC) double strand. Poly(dI-dC)·poly(dI-dC) double strand would reduce the concentrations of nonspecific DNA binding proteins, resulting in the enhancement of binding of putative pyrimidine dimer-specific endonuclease to DNA.

We conclude that the reaction was performed by a pyrimidine dimer-dependent endonuclease from the following reasons: (i) DNA substrate without a pyrimidine dimer was not incised, (ii) the strand complementary to the strand containing a pyrimidine dimer was not incised, and (iii) the incision product derived from 3′-labeled DNA substrate was detected as well as the product derived from 5′-labeled DNA substrate.

In studies with permeabilized human cells, ATP is required for the damage-specific incision of DNA after exposure to UV light (Dresler & Lieberman, 1983; Kaufmann & Briley, 1987). Wood et al. showed that UV-irradiated plasmid DNA is repaired by normal human cell extracts and that the reaction requires ATP. They speculated that the requirement for ATP is associated with the incision step and/or oligonucleotide displacement step (Wood et al., 1988). The incision reaction we observed was dependent on Mg^{2+} but was independent of ATP. The amount of incision product was decreased by addition of deoxynucleotides to the reaction mixture. This effect was suppressed to some extent when ATP and the ATP-regenerating system were omitted. Therefore, it is possible that the incision of DNA is independent of ATP whereas the displacement of oligonucleotide is dependent on ATP in our cell-free system.

The incision activity was detected not only in HeLaS3 cell extact but also in extracts prepared from other excision repair-proficient cell lines such as FL and WI38VA13 (data not shown). In contrast, DNA substrate was not incised by cell extract prepared from excision repair-deficient XP-A cells. The same substrate was incised weakly with cell extracts prepared from excision repair-deficient cell lines, XP-C, XP-D, and XP-G cells at the third phosphodiester linkage 3′ to the pyrimidine dimer, a site different from that incised by normal human cell extracts. These results suggest that the incision events we observed are associated with excision repair in human cells.

The unusual incision with XP-C, XP-D, and XP-G cell extracts may reflect the defects in excision repair in these cells. It is possible that the nucleotide excision nuclease derived from XP-C, XP-D, or XP-G cell line is not located at an appropriate place in the phosphodiester backbone of the DNA. This would cause the one phosphodiester bond difference of the incision site and the decrease of the activity.

During the preparation of this report, Huang et al. reported that they constructed a cell-free system for nucleotide excision repair, using a human cell extract and a plasmid containing four pyrimidine dimers at unique sites (Huang et al., 1992). In this system, repair synthesis was performed in the presence of deoxynucleoside 5′-[α-thio]triphosphates; therefore repair patches contain phosphorothioate linkages. On the basis of the resistance of these linkages to digestion by exonuclease III, they delineated the 3′ border of the repair patch, as mainly the fourth phosphodiester linkage 3′ to the pyrimidine dimer. Furthermore, on the basis of the sensitivity of the linkages to cleavage by I_2, they determined that the repair patch extends 21–24 phosphodiester linkages 5′ to the pyrimidine dimer. They also detected an excised DNA fragment corresponding to the size of the patch. Thus, they concluded that human nucleotide excision nuclease removes pyrimidine dimer from DNA by incising the damaged strand on both sides of the adduct in a precise manner. In our cell-free system, we detected an incision site located on the 3′ side of the pyrimidine dimer. The incision site corresponds to the 3′ border of the repair patch shown by Huang et al. However, we could not detect incision on the 5′ side of the pyrimidine dimer. It is

most likely that the putative human nucleotide excision nuclease recognizes the site 3′ to the adduct in our DNA substrate but not the site 5′ to the adduct because of the limitation of the length of the DNA.

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