Volume 17 Number 12 1989 Nucleic Acids Research

Human nucleotide excision repair *in vitro*: repair of pyrimidine dimers, psoralen and cisplatin adducts by HeLa cell-free extract

Sibghat-Ullah, Intisar Husain, Wendi Carlton and Aziz Sancar

University of North Carolina School of Medicine, Department of Biochemistry, Chapel Hill, NC 27599, USA

Received April 11, 1989; Revised and Accepted May 19, 1989

ABSTRACT

We searched for nucleotide excision repair in human cell-free extracts using two assays: damage-specific incision of DNA (the nicking assay) and damage-stimulated DNA synthesis (the repair synthesis assay). HeLa cell-free extract prepared by the method of Manley *et al.* (1980) has a weak nicking activity on UV irradiated DNA and the nicking is only slightly reduced when pyrimidine dimers are eliminated from the substrate by DNA photolyase. In contrast to the nicking assay, the extract gives a strong signal with UV irradiated substrate in the repair synthesis assay. The repair synthesis activity is ATP dependent and is reduced by about 50% by prior treatment of the substrate with DNA photolyase indicating that this fraction of repair synthesis is due to removal of pyrimidine dimers by nucleotide excision. Psoralen and cisplatin adducts which are known to be removed by nucleotide excision repair also elicited repair synthesis activity 5−10 fold above the background synthesis. When M13RF DNA containing a uniquely placed psoralen adduct was used in the reaction, complete repair was achieved in a fraction of molecules as evidenced by the restoration of psoralen inactivated KpnI restriction site. This activity is absent in xeroderma pigmentosum group A cells. We conclude that our cell-free extract contains the human nucleotide excision repair enzyme activity.

INTRODUCTION

Nucleotide excision repair entails the removal of the damaged nucleotide(s) *en block*, in the form of a short oligonucleotide, from the DNA duplex, filling in the resulting gap by DNA polymerases and sealing the nick by DNA ligase (1). The genetics of nucleotide excision is similar in *E. coli* and man: mutations in any of the 3 genes, *uvrA*, *uvrB*, and *uvrC* in *E. coli* and in any of the 10 genes, XP-A through XP-I and XP-V, in humans make the cells extremely sensitive to UV and UV-mimetic agents such as psoralen, cisplatin, mitomycin-C, benzo[a]pyrene and 4-nitroquinoline oxide (2). At the biochemical level both the *uvr*- and XP mutants fail to incise their DNA following treatment with any of the above-mentioned agents.

The molecular mechanism of the incision step has been elucidated in *E. coli* (3). The UvrA, UvrB, and UvrC proteins constitute an ATP-dependent nuclease (ABC excinuclease) which incises the damaged strand 7 bases 5′ and 4 bases 3′ to the adducted nucleotide. In contrast, studies on human nucleotide excision have been frustrated for lack of a defined *in vitro* system and/or purified proteins. Studies with permeabilized cell systems show that normal human cells but not cells from XP mutants carry out UV-induced incision and repair synthesis in an ATP-dependent manner (4−6). The repair synthesis is accomplished by DNA polymerase δ (7,8) and repair patches of 30−100 nucleotides are produced (9). Recently repair synthesis complementation by microinjection of cell-free

extracts into XP cell lines has been used as an assay to partially purify the XP-A gene product (10).

In vitro studies with cell-free extracts have resulted in some contradictory findings. Mortelmans *et al.* (11) reported that while cell-free extracts prepared by sonication of both normal and XP-A cells excised pyrimidine dimers from exogenously added naked DNA, only extracts from normal cells incised irradiated DNA in chromatin. Kano and Fujiwara (12) confirmed these results and, in addition, reported that cell-free extracts from the XP-C and XP-G groups as well, were deficient in excising thymine dimers from native, but not from partially deproteinized chromatin. Both groups reported that the excision activity was dependent on Mg^{2+}, partially stimulated by dNTPs but not by ATP. These results are in conflict with those obtained by the permeabilized cell systems mentioned above as well as the results to be presented in this report which shows strong ATP dependence of repair synthesis activity. While our studies were in progress Wood *et al* (13) reported that cell-free extracts prepared by the method of Manley *et al.* (14) carried out ATP-dependent repair synthesis with UV irradiated DNA and that this activity was deficient in all XP mutants (XP-A, XP-C, XP-D, and XP-V) tested. The results presented here confirm and extend the observations of Wood *et al.* (13), and furthermore demonstrate that the repair synthesis activity is equally effective on DNA containing pyrimidine dimers, psoralen or cisplatin adducts. We also show that this repair synthesis activity accomplishes repair in a biological sense in that it restores the sensitivity of damaged DNA to a restriction enzyme by excising the psoralen adduct from the restriction site and filling in the gap.

MATERIALS AND METHODS

Materials

The radiosotopes, [α-^{32}P] TTP (3,000 Ci/mmole), and [γ-^{32}P] ATP (7000 Ci/mmole) were obtained from ICN Radiochemicals, Irvine, CA; [α-^{32}P] dCTP (6,000 Ci/mmole) was from Amersham. ATP, dNTPs, phosphoenolpyruvate and pyruvate kinase were purchased from Sigma Chemical Co. (Saint Louis, MO). M13mp19 single-and double-stranded DNAs, restriction enzymes, and polynucleotide kinase were from New England Biolabs (Beverly, MA). HeLa S3 cells were from the stock of Lineberger Cancer Center (University of North Carolina). Normal (GM1989), XP-A (GM2250), and XP-C (GM2498) lymphoid cell lines immortalized with Epstein-Barr virus were purchased from the N.I.G.M.S. Human Genetic Mutant Cell Repository (Camden, NJ).

Preparation of Cell-free Extracts

We prepared our cell-free extracts by the method of Manley *et al.* (14). Briefly, cells were grown in RPMI-1640 or Joklik modified MEM media to about 10^6 cells/ml, harvested, washed, and swollen by exposure to a hypotonic buffer and then broken open by a Dounce homogenizer. The lysate was centrifuged at 100,000g for 180 min and the pellet was discarded. To the supernatant ammonium sulfate was added to 45% saturation. The precipitate was collected by centrifugation and dissolved in a minimum volume of buffer containing 20 mM Hepes, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 17% glycerol. The solution was then dialyzed at 4°C against 500 ml of the same buffer for 8–12 hrs and the small amount of precipitate formed during dialysis was removed by centrifugation and discarded. The clear supernatant is referred to as cell-free extract (Manley extract) and contains protein at 10–20 mg/ml. The extract was frozen in aliquots in dry ice-ethanol and stored at −80°C.

Preparation of Substrates

We used either randomly modified DNA (by UV, cisplatin, or psoralen) or DNA containing a single psoralen adduct at a defined position. Randomly damaged DNA was prepared by treating pBR322 with UV, psoaralen plus near UV, or cisplatin. The plasmid DNA was purified through two sequential CsCl-ethidium bromide density gradients. UV damaged pBR322 DNA was obtained by irradiating DNA at a concentration of 20 μg/ml in droplets of 2−3 mm in thickness. Under this irradiation condition 12.5 Jm^{-2} of 254 nm from a germicidal lamp produces 1 inactivating adduct (85−90% of which are pyrimidine dimers) per molecule as determined by the transformation assay (15). Psoralen adducted DNA was prepared as follows. pBR322 DNA (50 μg/ml) was mixed with 1−4 μCi/ml of ^{3}H-HMT (specific activity 10 Ci/mole, HRI Associates, Emeryville, CA) and incubated at room temperature in the dark for 30 min, the reaction mixture (500 μl) was then put into a Petri dish, placed on ice and irradiated with 40−60 Jm^{-2} sec^{-1} of 365-nm light from a Blacklight source (Black Light Lamp, Model B-100, Spectronic Inc., Westbury, NY) for 15 min (16). The unincorporated HMT was removed by exhaustive dialysis against 10 mM Tris·HCl/1 mM EDTA. The number of adducts (mainly thymine-furan side monoadducts) per plasmid was determined from the radioactivity that remains associated with DNA. Platinum adducted DNA was prepared by incubating pBR322 (50 μg/ml) with 8 nM [^{3}H]-$PtCl_2$(dach) (230 Ci/mole) in 1 mM Tris-HCl, pH 7.5/10 mM NaCl/1 mM EDTA. The incubation was at room temperature for 2 hrs. The unincorporated drug was removed by dialysis. The number of adducts per plasmid was estimated by measuring the radioactive label incorporated into DNA. DNA containing UV 6−4 photoproducts and other non-pyrimidine dimer photoproducts was obtained by photoreactivating UV irradiated pBR322 DNA. The plasmid (50 μg/ml) was irradiated with 250 Jm^{-2} of 254 nm and then 0.2 μg of DNA was mixed with 2 μg of *E. coli* DNA photolyase (17) in 50 μl reaction buffer containing 50 mM Tris·Cl, pH 7.5/50 mM NaCl/1 mM EDTA/lmM β-Mercaptoethanol/bovine serum albumin, 100 μg per ml. The mixture was incubated at room temperature for 30 min and then placed into a Plastic Petri dish on ice and photoreactivated with Black Light at a fluence rate of 60 $Jm^{-2}s^{-1}$ for 15 min. The photoreactivated sample as well as the control non-photoreactivated DNA were then extracted with phenol and ether and precipitated with ethanol before use in the incision or repair synthesis assays. M13mp19RF DNA containing a single psoralen adduct at the polylinker region was prepared by the method of Kodadek and Gamper (18). The T4 replication proteins used in preparing this substrate were a kind gift of Dr. William Konigsberg, Yale University.

The Incision Assay

This assay measures the conversion of covalently closed DNA to open circles by nicking of the phosphodiester backbone. The reaction mixture contained in 120 μl 40 mM MOPS, pH 7.5/50 mM KCl/15 mM $MgSO_4$/1 mM EDTA/1 mM dithiothreitol/2 mM ATP/[^{3}H]-pBR322 DNA (6.5×10^4 cpm/μg), 90 ng, and 1−5 μl extract (10−50 μg protein). The mixture was incubated at 37°C for 15−30 min and the reaction stopped by addition of EDTA to 45 mM. The mixture was then layered on top of a 4 ml sucrose gradient containing 5−20% sucrose/0.5 M NaCl/0.01 M EDTA/0.2 M NaOH. The gradient was centrifuged at 0°C for 3 hrs at 50,000 rpm in a Beckman SW 60 rotor. Fractions of 0.25 ml were collected, 0.25 ml H_2O was added, then mixed with 5 ml of Scintiverse I (Fischer Scientific) and the radioactivity in each sample was quantitated by

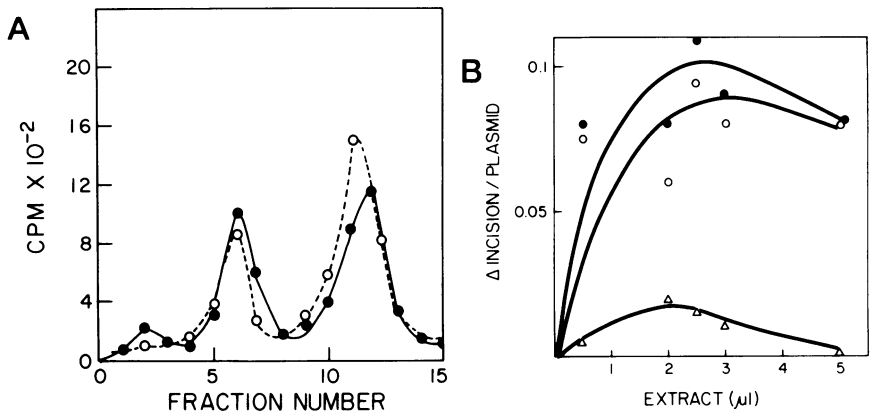

Figure 1. Incision of UV-[^{3}H]pBR322 by HeLa Cell-free Extract.A. Alkaline sucrose gradient profile. Unirradiated DNA or DNA containing 3.3 pyrimidine dimers per plasmid (90 ng) was incubated with cell extract (36 μg protein) and centrifuged through the gradient. Fractions of 0.25 ml were collected and the radioactivity in each was quantitated. The order of the fractions (from left to right) is from bottom to top of the gradient. ●, unirradiated DNA; ○ irradiated DNA. The DNAs were 85% superhelical in the absence of extract. B. Effect of photoreactivation on nicking of DNA. UV irradiated (●, 3.5 dimers/molecule), and irradiated-photoreactivated DNA (○) were incubated with the indicated amounts of extract (15 μg/ml protein) and the number of nicks per plasmid was determined by alkaline sucrose gradients. Δ, dimer-specific incision.

scintillation counting. The average number of nicks per plasmid was estimated from the fraction of DNA that remains superhelical, assuming Poisson distribution of nicks. The total amount of DNA recovered in all fractions was greater than 75% of input DNA.

The Repair Synthesis Assay

This assay measures the incorporation of radioactive nucleotides into damaged DNA. The repair synthesis was quantitated either by TCA precipitation or by separating the DNA on agarose gels followed by autoradiography. When the quantitation was done by TCA precipitation the assay was conducted as follows. The reaction mixture (50 μl) contained 40 mM Tris. HCl, pH 7.5/50 mM KCl/8 mM $MgCl_2$/5 mM ATP/4 nM dNTPs each/1 μCi [α-^{32}P]TTP/0.16 M sucrose/2 mM dithiothreitol/0.67 mM EDTA/0.2 μg pBR322 and Manley extract, 0−50 μg. The mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 50 μl of cold 10% TCA. The pellet was collected by centrifuging in a microfuge for 10 min and the pellet was washed with 5% cold TCA. The pellet was dissolved in 1M perchloric acid, neutralized with 50 μl 1N NaOH and the acid precipitable counts were measured by scintillation counting. This assay is rapid and quantitative; however it yields greater background and shows more variability than the autoradiographic assay described below.

When the repair synthesis product was analyzed by auto-radiography we adopted the procedure of Wood *et al* (13) for our purposes. The reaction mixture (50 μl) contained 40 mM Hepes, pH 7.9/50 mM KCl/8 mM $MgCl_2$/1 mM dithiothreitol/0.4 mM EDTA/200 μg/ml bovine serum albumin/4% glycerol/2 mM ATP/50 μg phosphoenolpyruvate/5 units pyruvate kinase/dATP, dGTP an TTP 20 μM each/8 μM dCTP and 2 μCi [α-^{32}P]dCTP/200 ng pBR322/200 ng M13RFI (undamaged, internal control)/Manley extract, 1−6 μl (10−90 μg). The mixture was incubated at 30°C for

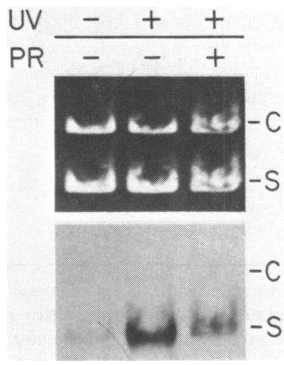

Figure 2. Repair of pyrimidine dimers by cell-free extract. Non-irradiated, UV-irradiated (UV) and irradiated-photoreactivated (PR) pBR322 (200 ng) were mixed with M13RFI DNA and incubated with 45 μg of cell-free extract for 3 hrs in repair synthesis buffer. The reaction products were analyzed by agarose gel electrophoresis followed by autoradiography. *Top*, photograph of gel stained with ethidium bromide. *Bottom*, autoradiograph of the gel. S, substrate (pBR322); C, (internal control, undamaged M13mp19).

3 hrs unless otherwise stated. The DNA was then extracted with phenol and ether and precipitated with ethanol, resuspended in 25 μl of EcoRI buffer, digested with EcoRI and separated on 1% agarose gel. The gel was dried and autoradiographed at −80°C for 12−20 hrs. To quantitate the radioactivity incorporated into DNA, the bands were cut out, placed into scintillation vials and the radioactivity measured by Cerenkov counting. In this assay to correct for variable recovery between samples, undamaged M13mp19FRI is included as internal standard. Both plasmids are cut only once by EcoRI and therefore the autoradiographs of the repair synthesis products reveal two bands of ca. 7kbp (M13RF) and 4 kbp (pBR322), incorporation into the former being a measure of nonspecific synthesis and into the latter a measure of specific synthesis. Digestion of DNA with a restriction enzyme is essential for this analysis because the substrate becomes heavily catenated by topoisomerases present in the extract and doesn't enter the gel without digestion to unit length by a restriction enzyme (13). In addition in this assay, repair synthesis is strongly stimulated by the ATP regeneration systems included in the reaction mixture. Presumably this is due to hydrolysis of ATP by the many ATPases present in the extract during the long incubation period (3 hrs) compared to the 30 min reaction time in the TCA precipitation assay.

KpnI Site Regeneration Assay

M13mp19(Fu) contains a furan-side monoadducted T in the KpnI site of the phage RFI DNA and as a result it is resistant to KpnI digestion (17). This assay measures the degree of restoration of susceptibility of the DNA to KpnI digestion as a result of repair synthesis. Covalently closed—but not superhelical—form (RF IV) of M13mp19(Fu) is incubated with Manley extract under the repair synthesis condition, extracted with phenol, precipitated with ethanol and then digested with PvuII restriction endonuclease. This enzyme cuts on both sides of the polylinker region generating a fragment of 322 bp with the KpnI site 105 bp from one terminus and 217 bp from the other. The PvuII fragment is terminally labeled with [γ-^{32}P]ATP and polynucleotide kinase and then subjected to cleavage by KpnI

Table 1. Repair Synthesis on UV-pBR322 Before and After Photoreactivation with DNA Photolyase

Treatment	No. of Adducts Per Plasmid	DNA Synthesis (cpm)	Mole dCMP/Mole Adduct
−UV	0	75	−
+UV	5.0	670	0.18
+UV+PR	1.2	355	0.37

pBR322 was incubated for 3 hrs in 50 μl repair synthesis buffer containing 200 ng DNA (69 pmole plasmid), 2 μCi[α-^{32}P]dCTP(5 Ci/mole) and 45 μg of extract. Following repair synthesis, the DNA was linearized with EcoRI, separated on 1% agarose gel, located by autoradiography and then cut out of the gel for quantitating DNA synthesis by Cerenkov counting. The average number of 'lethal adducts' per plasmid before and after photo-reactivation (PR) were determined by the transformation assay. The corresponding gel is shown in Figure 2.

restriction endonuclease. The reaction products are separated by electrophoresis on a 5% polyacrylamide gel which is then autoradiographed.

Other Methods

The *E. coli* ABC excinuclease which was used as a positive control in some of the experiments was purified as described previously (3). For the KpnI site regeneration assay with this enzyme we also included DNA polymerase I and T4 DNA ligase in the reaction mixture. Thereafter, the reaction products were processed as in repair synthesis with cell-free extract.

To evaluate the effect of superhelicity on repair synthesis by Manley extract we relaxed psoralen-damaged pBR322 DNA with *E. coli* topoisomerase I as follows. pBR322 containing 3 psoralen or cisplatin adducts per molecule was incubated in 50 mM Tris HCl, pH 7.5/50 mM KCl/10 mM $MgCl_2$/0.5 mM dithiothreitol/0.1 mM EDTA/30 μg/ml/DNA, 200 ng bovine serum albumin, and topiosomerase I (Bethesda Research Laboratories), 2 units. The mixture was incubated at 37°C for 30 min and then the DNA was extracted with phenol and ether and precipitated with ethanol for use in the repair synthesis assay.

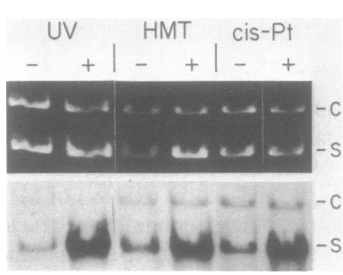

Figure 3. Repair of DNA damaged by UV, psoralen (HMT), and cisplatin. The damaged DNAs (200 ng) contained an average of 5.5, 3, and 3 adducts per pBR322 molecule, respectively, and undamaged M13RFI DNA (200 ng) as internal control. The cell-free extract was 45 μg in all assays. The top and bottom panels are photograph and autoradiographs of the agarose gels.

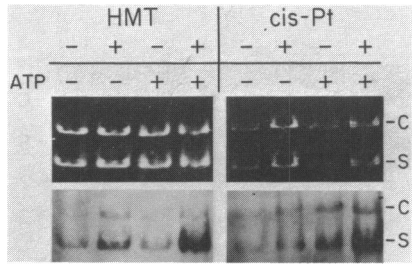

Figure 4. ATP requirement for repair synthesis. DNAs (200 ng) modified with posralen or cisplatin were incubated with 45 μg of extract under repair synthesis condition in the presence or absence of ATP as indicated. The top and bottom panels are photographs and autoradiographs of the corresponding agarose gels.

Table 2. Requirements for Repair Synthesis in Manley Extract

Reaction Condition	^{32}P-TMP Incorporated (cpm) pBR322	UV-pBR322	Δcpm	% Activity
Complete	1680	2440	760	100
−ATP	540	630	90	10
$-MgCl_2$	540	110	90	0
$-ATP-MgCl_2$	700	610	–	0
−dGTP	740	900	160	20
−DNA	–	–	80	10

The 50 μl reaction mixture contained 300 ng pBR322 (irradiated with 200 J/m^2 of 254 nm where indicated) and 30 μg of extract. After 30 min incubation at 37°C the DNA synthesis was measured by TCA precipitation followed by scintillation counting. The high background synthesis was due the fact that the incubation was only for 30 min and the DNA used in the control experiments was moderately nicked.

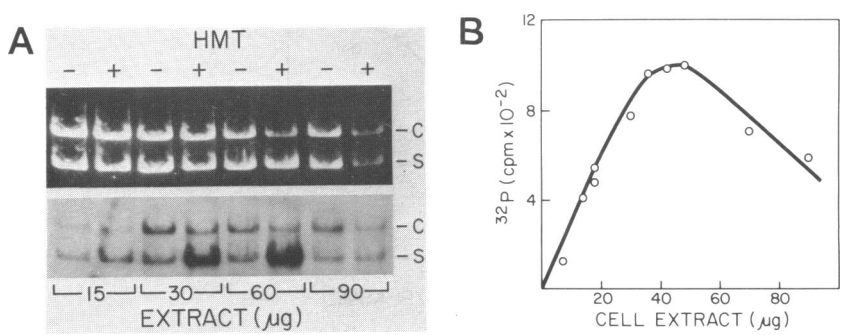

Figure 5. Effect of extract (protein) concentration on repair synthesis. A. Psoralen damaged and control DNAs (200 ng) were incubated with the indicated amounts of extract for 3 hrs and repair synthesis was visualized by autoradiography. B. UV damaged (200 J/m^2)DNA was incubated with varying amounts of extract for 30 min and repair synthesis was quantitated by TCA precipitation. The background incorporation (which ranged from 50 to 70% of the signal) has been subtracted from the numbers plotted.

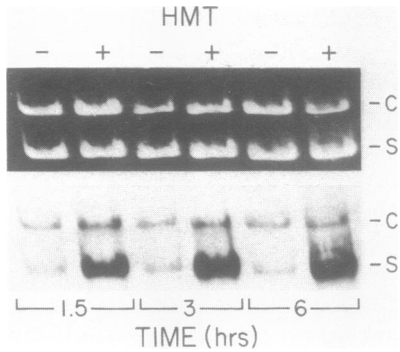

Figure 6. Time course of repair synthesis. Undamaged or psoralen (HMT)-damaged DNA (200 ng) were incubated with 45 μg of extract for the indicated time periods and repair synthesis was measured by agarose gel electrophoresis (top) followed by autoradiography (bottom).

RESULTS

Incision of UV-irradiated DNA

We find that the incision assay, which was instrumental in the initial characterization of nucleotide excision in *E. coli* (19), is only marginally successful with human cell-free extracts. We used UV-irradiated pBR322 to detect damage specific incision activity in Manley extract. The DNA was incubated with various amounts of extract and then analyzed on alkaline sucrose gradients. The profile of a representative gradient is shown in Figure 1A. The cell-free extract produced 0.32 and 0.4 nicks per plasmid in non-irradiated and irradiated DNA respectively. In other experiments with different preparations of cell-free extract UV-specific nicks were obtained in the range of 0.05−0.10 nicks per plasmid. In order to show that the UV-specific nicks were made by the excision nuclease and not the ubiquitous redoxyendonuclease (glycosylase-AP endonuclease, 20) which acts on saturated pyrimidines that are also generated by UV, the incision assay was performed on UV-DNA before and after photoreactivation with DNA photolyase. Photolyase is specific

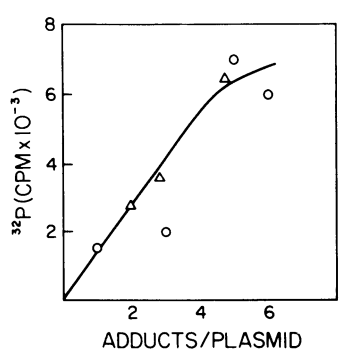

Figure 7. Dose dependence of repair synthesis. pBR322 DNA (300 ng) containing the indicated numbers of psoralen (○), or cisplatin (Δ) adducts was incubated with 20 μg of extract for 30 min in repair synthesis buffer and the repair synthesis was measured by TCA precipitation. The background incorporation into undamaged DNA (which ranged from 70% of the signal at 1.1 adducts per plasmid to 51% at 6 adducts per plasmid) has been subtracted from the data points.

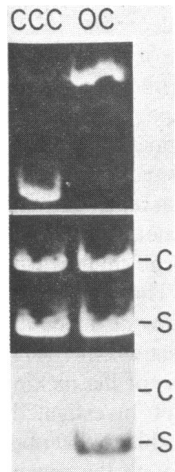

Figure 8. Effect of nicks on background synthesis. pBR322 DNA that had accumulated 'spontaneous' nicks upon storage was separated into superhelical and nicked circles by CsCl-ethidium bromide density gradient and these two forms were used separately in the repair synthesis assay in the presence of superhelical M13RFI DNA as internal control. *Top*, the superhelical (CCC) and open circular forms (OC) that were used in the repair synthesis assays. *Middle*, the same DNAs plus M13RFI after repair synthesis and digestion with EcoRI. *Bottom*, autoradiograph of the gel shown in the middle panel.

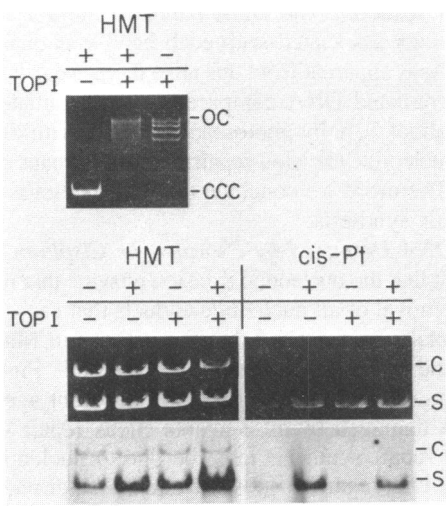

Figure 9. Effect of superhelicity on repair synthesis. Undamaged, psoralen (HMT), or cisplatin (cis-Pt)-adducted pBR322 DNAs (200 ng) were incubated in repair synthesis reaction buffer with 45 μg of extract for 3 hrs either before or after treatment with *E. coli* topoisomerase I (Top I). *Top*, photograph of agarose gel of DNA before and after Topoisomerase I treatment. *Bottom*, the repair synthesis assays with the superhelical and relaxed DNAs. Note the cisplatin adducted DNA did not contain the M13 internal standard during repair synthesis.

for pyrimidine dimers and any decrease in UV-specific nicking as a result of photoreactivation would be indicative of pyrimidine dimer-specific nicking by the extract. The results of a series of such experiments are shown in Figure 1B. It is apparent from this figure that the majority of UV-specific nicks detected by this assay are not made by an enzyme acting on pyrimidine dimers. However, it also appears that at all extract concentrations tested there is, albeit small, dimer-specific incision, indicating that our cell free extract contains nucleotide excision repair activity. These nicking assays were conducted in the presence of ATP. Waldstein *et al* (22) reported an ATP-independent, Mg^{2+}-stimulated endonuclease from calf thymus which was specific for pyrimidine dimers and was inactivated by freeze-thaw. The relation of that activity to ours is unknown. The low signal-to-noise ratio in our experiments made it difficult to study the ATP requirement and therefore we do not know at present whether ATP is essential for dimer-specific nicking. For the same reason the effectiveness of the nicking activity on DNA damaged by other agents (psoralen, cisplatin) was not investigated in detail even though preliminary experiments indicated signals comparable to that obtained with pyrimidine dimers. Instead, we conducted the rest of our study with the repair synthesis assay which was found to be more sensitive than the nicking assay (13).

Repair Synthesis Assay with UV-DNA

This assay measures the pyrimidine dimer-specific incorporation of radioactive nucleotides into UV-irradiated pBR322 DNA. The incorporation of radiolabel is measured either by gel electrophoresis followed by autoradiography or by TCA precipitation. Figure 2 shows the result of a repair synthesis synthesis assay analyzed by autoradiography. Radiolabel is incorporated specifically into UV-irradiated DNA and this repair synthesis is significantly reduced by treating the irradiated DNA with photolyase plus photoreactivating light prior to conducting the repair reaction. The DNA bands of the gel shown in Figure 2 were cut out and the radioactivity associated with each band was quantitated. The results are summarized in Table 1. As is apparent from this table there is ca. 10-fold more incorporation of radiolabel into UV irradiated DNA compared to non-irradiated DNA and this repair synthesis is reduced by about 50% by photoreactivation. Pyrimidine dimer specific repair is a strong indicator of nucleotide excision repair because humans do not have a pyrimidine dimer glyosylase (21). Therefore we conclude that our extract is capable of carrying out nucleotide excision repair synthesis.

Repair Synthesis with DNA Damaged by Psoralen or Cisplatin

Since genetic data suggest that the nucleotide excision enzyme that repairs pyrimidine dimers is also responsible for removal of all nucleotide adducts that cause major helical backbone deformity ('bulky adducts') we reasoned that the activity in Manley extracts should be effective on cisplatin- and psoralen-damaged DNA as well. Figure 3 shows the results of repair synthesis assays on DNAs damaged by 3 different agents. UV, psoralen, and cisplatin. Clearly, DNA damaged by all 3 agents elicits repair synthesis to comparable levels and therefore the repair synthesis must be due to nucleotide excision repair. We characterized this activity with regard to substrate and co-factor requirements by conducting the experiments described below.

Properties of Repair Synthesis Activity

The repair synthesis is ATP dependent (Figure 4) and requires Mg^{2+} and all 4 dNTPs (Table 2). The activity is optimal at a relatively high concentration of about 1 mg/ml of protein (Figure 5A and B). At higher protein concentrations repair synthesis declines due to DNA degradation by the extract. The repair synthesis is relatively slow under our reaction

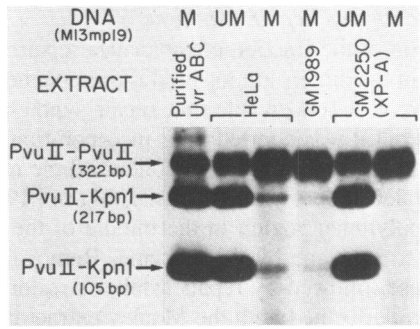

Figure 10. KpnI site regeneration assay. M13mp19 unmodified DNA (UM) or M13mp19(Fu) (M) were incubated with ABC excinuclease or Manley extracts from the indicated cell lines under repair synthesis conditions. Following repair, the $(PvuII\text{-}PvuII)_{322}$ fragment was purified and terminally labeled using polynucleotide kinase. The fragment was then incubated with KpnI, the reaction products separated on a 5% polyacrylamide gel and located by autoradiography. Repair by either the reconstituted *E. coli* nucleotide excision repair system or human cell-free extract restores the sensitivity of DNA to KpnI. GM1989 and GM2250 are immortalized lymphoid cell-lines from normal and XP-A subjects, respectively.

conditions, continuing at a near linear rate for over 3 hrs (Figure 6) in agreement with Wood *et al.* (13). The dose dependence of repair synthesis was also investigated using psoralen and cisplatin damaged substrates. The results shown in Figure 7 indicate that adducts of these two drugs are repaired with equal efficiency and that there is a linear correlation between number of adducts and the level of repair synthesis over a range of 0.25−1.5 adduct/kbp.

Effect of Nicks and Superhelicity on Repair Synthesis

In the course of our experiments we noticed that even with the same batch of cell-free extract and the same batch of DNA there was a gradually increasing level of background synthesis (e.g., compare Tables 1 and 2) and decreasing level of signal with time (weeks to months) even though the extract had been stored at −80°C and the DNA at 4°C during this period. It occurred to us that nicks that accumulate in DNA upon storage might be the cause of high background synthesis and that non-superhelical damaged DNA may not be a substrate for the repair enzyme and that the combination of the two factors was responsible for the disappearance of the signal. To find out if there was a correlation between the non-specific nicks in DNA and the background synthesis, the repair synthesis assay was conducted using non-damaged pBR322 DNAs that were mostly superhelical or mostly nicked. The result is shown in Figure 8. Nicks result in a dramatic increase in background synthesis and therefore the DNA to be used in repair synthesis must be as nick-free as possible. However, superhelical DNA is not required for repair synthesis. With covalently closed but not superhelical substrate (Form IV) that was either synthesized *in vitro* or generated by treating superhelical DNA with topoisomerase I of *E. Coli* we obtain repair synthesis signals comparable to that obtained with superhelical DNA. Figure 9 shows the results obtained with DNA that was relaxed with topoisomerase I. As is apparent from this figure neither the repair synthesis nor the non-specific synthesis is significantly altered by a decrease in superhelicity (the moderate decrease in signal of cisplatin damaged DNA upon relaxation is due to the variability that we typically observe in this assay and it doesn't reflect a true decrease in the signal (data not shown). It thus appears that the apparent lack of repair signal with nicked DNA is due the high background synthesis caused by non-specific nicks.

Restoration of DNA Function by Repair Synthesis

An approximate calculation of the fraction of molecules repaired using the data in Table 1 indicate that only 0.3% of the dimers are repaired if one assumes a repair patch of 30–50 nucleotides. Alternatively, it is conceivable that repair synthesis took place on a larger fraction of the molecules but it was aborted after incorporation of a few nucleotides. To differentiate between these two possibilities we conducted the repair synthesis assay with DNA that contains a posralen adduct at a unique site. M13mp19(Fu) has a psoralen furan side monoadduct at the polylinker region in the middle of the KpnI restriction site and as a result it is resistant to cleavage by this enzyme. Removal of the adduct by the *E. coli* ABC excision nuclease followed by repair synthesis restores the sensitivity to KpnI (23). When the assay was performed with the Manley extract the result shown in Figure 10 was obtained. Extracts made from HeLa cells or lymphoid cells of normal subjects (GM1989) partially (ca. 1–5%) restore the KpnI site while an extract made from lymphoid cells of an XP-A patient (GM2250) cannot. It appears, therefore, that the activity described here carries out repair synthesis to completion and restores the biological function of DNA and thus it is a true repair activity. Furthermore, the absence of this activity in XP-A cells corroborates all our other findings that our cell-free extract contains the human 'excision nuclease' or 'excision nucleases'.

DISCUSSION

In this paper we show that human cell-free extract prepared by the method of Manley *et al.* (14) repairs pyrimidine dimers, cisplatin and psoralen-DNA adducts. This activity is absent in an XP-A cell line. Our results confirm and extend the results of Wood *et al.* (13). We show that at least 50% of the repair synthesis on UV irradiated DNA is due to pyrimidine dimers as prior photoreactivation with *E. coli* DNA photolyase reduces the repair synthesis by 50%. Wood *et al* (13) concluded that the repair synthesis with their UV irradiated substrate was due to pyrimidine dimers because they eliminated molecules with saturated pyrimidines from their substate by prior treatment with *E. coli* endonuclease III. This enzyme incises DNA at sites of pyrimidine hydrates by a glycosylcose-AP endonuclease mechanism (see ref. 1), but does not act on pyrimidine dimers or 6–4 photoproducts. Thus, it could be argued that the repair synthesis observed by Wood *et al* (13) was largely or entirely due to 6–4 photoproducts. However, the results presented in this paper provide unambiguous evidence that pyrimidine dimers are responsible for about half of the signal. The repair synthesis observed on photoreactivated DNA is partly due to dimers remaining after photoreactivation, but mostly to 6–4 photoproducts and to thymine hydrates and other minor photoproducts. We believe the first two are the main cause of residual repair synthesis because we have observed that certain pyrimidine dimers are very resistant to photoreactivation and that in UV-irradiated DNA the 6–4 photoproducts are the preferred substrate for the *E. coli* ABC excinuclease (24), the prototype of excision nucleases. Assuming that most of the repair observed after photoreactivation is due to 6–4 photoproducts, one may conclude that the 6–4 photoproducts are 4–5 fold better substrate than pyrimidine dimers. Whether this extrapolation is justified will only be known when the human excision nuclease is purified and characterized.

Pyrimidine dimers, psoralen and cisplatin adducts are repaired by a single enzyme in *E. coli*, the ABC excinuclease (3). Genetic evidence indicates that the same may be true in humans as well, because XP cells which are defective in repairing pyrimidine dimers are also deficient in repairing psoralen and cisplatin adducts (2). Therefore, we conclude

that the repair synthesis activity elicited by these three agents in our assay must be due to the same enzyme. Indeed, the two XP cell lines we have tested, the XP-A and XP-C (data not shown) are defective in carrying out repair synthesis on DNA damaged by any of these three agents. Thus, we tentatively conclude that humans may have a nucleotide excision nuclease (XP excinuclease) with a substrate spectrum similar to that of *E. coli* ABC excinuclease.

The *E. coli* ABC excinuclease is an ATP dependent enzyme. While our repair synthesis activity was also ATP dependent we cannot conclude from our data that the human excinuclease also requires ATP for activity because even the background DNA synthesis activity was stimulated by ATP. Wood *et al.* (13) reported that DNA synthesis on gapped DNA was ATP independent and concluded that the ATP requiring step must be the incision step. Furthermore, the DNA polymerase δ which is known to be the enzyme involved in repair synthesis does not require ATP (7). Thus, we are inclined to agree that ATP is required for incision even though it appears to stimulate synthesis on undamaged DNA in our assay by some unknown mechanism. It is also important to note that the repair synthesis we observe in our assay is repair in a biological sense in that an adduct is removed and replaced by intact DNA (the KpnI regeneration assay) and thus yields a different end product than nick initiated repair.

Finally, our demonstration of an *in vitro* system capable of repairing cisplatin damaged DNA should open up new approaches to study the mechanism of drug resistance to cisplatin. This drug has been used with great success in treating some cancers; however, its usefulness in treating other cancers has been limited because of the development of drug resistance. It has been reported that at least in some cases this resistance is due to increased DNA repair activity (25,26). The development of an *in vitro* system to study cisplatin repair should make it possible to critically evaluate the contribution of DNA repair to development of drug resistance.

ACKNOWLEDGEMENT

We are grateful to Dr. William Konigsberg for giving us the T4 replication proteins and to Dr. John Hearst for his gift of the psoralen adducted primer. We thank Drs. S. Chaney and C. Selby and A. Nichols for critically reading the manuscript. This work was supported by the NSF grant PCM-8351212 and Grant No. 1878 from the Council for Tobacco Research.

ABBREVIATIONS: HMT, 4′-hydroxymethyl-4,5′,8-trimethylpsoralen; *cis*-Pt or cisplatin, *cis*-$PtCl_2$(dach), *cis*-(1,2-diaminocyclohexane) dichloroplatinum (II); M13mp19(Fu), M13mp19 duplex DNA containing a furan side HMT adduct; XP, xeroderma pigmentosum; TCA, trichloroacetic acid; UV-DNA, UV irradiated DNA; kbp, kilobase pair; bp, base pair.

REFERENCES

1. Sancar, A. and Sancar G.B. (1988) *Annu. Rev. Biochem 57*, 29–67.
2. Friedberg, E.C. (1985) DNA Repair. W. H. Freeman, New York, NY.
3. Sancar, A. and Rupp, W.D. (1983) *Cell 33*, 249–260.
4. Ciarrocchi, G. and Linn, S. (1978) *Proc. Natl. Acad. Sci. USA. 75*, 1887–1891.
5. Dresler, S.L. and Lieberman, M.W. (1983) *J. Biol. Chem. 258*, 12269–12273.
6. Kaufman, W.K., and Briley, L.P. (1987) *Mutation Res. 184*, 237–243.
7. Nishida, C., Reinhard, P. and Linn, S. (1988) *J. Biol. Chem. 263*, 501–510.
8. Dresler, S.L., Gowans, B.J., Robinson-Hill, R.M. and Hanting, D.C. (1988) *Biochemistry. 27*, 6379–6383.
9. Regan, J.D. and Setlow, R.B. (1974) *Cancer Res. 34*, 3318-

10. Yamaizumi, M., Sugano, T., Asahina, H., Okada, Y. and Uchida, T. (1986) *Proc. Natl. Acad. Sci. USA 77*, 5933–5937.
11. Mortelmans, K., Friedberg, E.C., Slor, H., Thomas, G. and Cleaver, J.E. (1976) *Proc. Natl. Acad. Sci. USA 73*, 2757–2761.
12. Kano, Y. and Fujiwara, Y. (1983) *Carcinogenesis 4*, 1419–1424.
13. Wood, R.D., Robins, P. and Lindahl, T. (1988) *Cell 53*, 97–106.
14. Manley, J.L., Fire, A., Cano, A., Sharp, P.A. and Gefter, M.L. (1980) *Proc. Natl. Acad. Sci. USA 77*, 3855–3859.
15. Sancar A. and Rupert, C.S. (1978) *Nature 272*, 471–472.
16. Tessman, J.W., Isaacs, S.T. and Hearst, J.E. (1985) *Biochemistry 24*, 1669–1676.
17. Sancar, A. and Sancar, G.B. (1984) *J. Molec. Biol. 172*, 223–227.
18. Kodadek, T. and Gamper, H. (1988) *Biochemistry 27*, 3210–3215.
19. Seeberg. E., Niseen-Meyer, J. and Strike, P. (1976) *Nature 263*, 524–526.
20. Doetsch, P.W., Helland, D.E. and Haseltine, W.A. (1986) *Biochemistry. 25*, 2212–2220.
21. LaBelle, M. and Linn, S. (1982) *Photochem. Photobiol. 36*, 319–324.
22. Waldstein, E.A., Peller, S. and Setlow, R.B. (1979) *Proc. Natl. Acad. Sci. USA 76*, 3746–3750.
23. Van Houten, B., Gamper, H., Hearst, J. and Sancar, A. (1988) *J. Biol. Chem. 263*, 16533–16560.
24. Myles, G., Van Houten, B. and Sancar, A. (1987) *Nucleic Acids Res. 15*, 1227–1263.
25. Masuda, H., Ozols, R.F., Lai, G-M., Tojo, A., Rothenberg, M. and Hamilton, T.C. (1988) *Cancer Research 48*, 5713–5716.
26. Eastman, A. and Schulte, N. (1988) *Biochemistry 27*, 4730–4734.

This article, submitted on disc, has been automatically converted into this typeset format by the publisher.