Nucleic Acids Research, Vol. 19, No. 17 4623–4629

Removal of psoralen monoadducts and crosslinks by human cell free extracts

Joyce T.Reardon, Peter Spielmann[1], Juch-Chin Huang, Srinivas Sastry[1], Aziz Sancar* and John E.Hearst[1]
Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, NC 27599 and [1]Department of Chemistry and The Division of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720, USA

Received June 11, 1991; Revised and Accepted August 2, 1991

ABSTRACT

Human cell free extracts are capable of carrying out damage-induced DNA synthesis in response to DNA damage by UV, psoralen, and cisplatin. We show that this damage-induced DNA synthesis is associated with removal of psoralen adducts and therefore is 'repair synthesis' and not an aberrant DNA synthesis reaction potentiated by DNA deformed by adducts. By comparing the denaturable fraction of psoralen adducted DNA which becomes labeled in the repair reaction to that of terminally labeled DNA (without repair) we have found that all DNA synthesis induced by psoralen monoadducts is the consequence of removal of these adducts. By the same approach we have obtained preliminary evidence that this *in vitro* system is capable of removing psoralen crosslinks as well.

INTRODUCTION

Nucleotide excision repair is a mechanism by which lesions are removed from DNA in the form of a short oligonucleotide with concomitant filling in of the resulting gap by DNA polymerase and nick closure by ligase (1). Although this repair mechanism has been well-characterized in prokaryotes it remains biochemically ill-defined in mammalian cells. An important step towards characterization of nucleotide excision in humans was the development of a cell free extract system capable of carrying out repair synthesis (2–4). Although this *in vitro* system has been used extensively (5–9) the repair synthesis that can be accomplished in the system under optimal conditions corresponds to removal of only 1–10% of total adducts assuming a 'repair patch' size of 20 nucleotides (3,5). Therefore it has not been possible to directly demonstrate that the *in vitro* system is capable of removing the damaged nucleotides although some indirect evidence for such removal has been presented. Indeed, it could be argued that the so-called 'repair synthesis' is actually damage induced aberrant DNA synthesis which does not result in repair, which is defined as the removal of the base adduct and its replacement with a normal nucleotide.

In this paper we have used DNA containing psoralen adducts to directly address the question of whether all or part of the damage-induced DNA synthesis is associated with adduct removal and therefore is genuine repair synthesis. We found that essentially all DNA synthesis induced by psoralen monoadducts is associated with adduct removal and therefore qualifies as bona fide repair synthesis. We also found that, in this cell free extract system, psoralen crosslinks elicited a higher level of DNA synthesis compared to psoralen monoadducts and that this higher level of synthesis was associated with removal of interstrand crosslinks.

MATERIALS AND METHODS

Materials

HeLa S3 cells were from the stock of Lineberger Cancer Center (University of North Carolina). The radioisotopes, [α-^{32}P]dCTP (6000 Ci/mmole) and [γ-^{32}P]ATP (7000 Ci/mmole) were obtained from New England Nuclear-DuPont (Boston,MA) and ICN Radiochemicals (Irvine, CA), respectively. ATP, dNTPs, pyruvate kinase, and phosphoenolpyruvate were from Sigma Chemical Company (St. Louis, MO). Restriction enzymes, kinase, ligase and DNA polymerase I were from Bethesda Research Laboratories (Gaithersburg, MD) and 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) was purchased from HRI Associates (Emeryville, CA).

Repair Systems

HeLa cell free extract (CFE) capable of damage-induced DNA synthesis was prepared from HeLa S3 cells by the method of Manley *et al.* (10) as described by Sigbhat-Ullah *et al.* (3). Typically, the yield was 7–10 mg total protein per liter of cells; each batch of extract was tested to determine the optimal protein concentration to be used in the repair synthesis assays as the amount of protein which gave optimal signal-to-noise ratio varied somewhat from extract to extract. The CFE was stable for at least 6 months when stored at −80°C and retained its activity after one cycle of thaw-and-refreeze. The subunits of *E. coli* (A)BC excinuclease were purified as described previously (11).

* To whom correspondence should be addressed

Substrates

pBR322 DNA damaged by UV or psoralen (HMT) was our substrate. The plasmid was purified through two CsCl-ethidium bromide density gradients, dissolved in TEN 7.4 (10 mM Tris HCl, pH 7.4, 10 mM NaCl, 1 mM EDTA) and stored at 4°C. For UV damage, the DNA was exposed to 254 nm radiation from a 15 W Westinghouse Germicidal Sterilamp. The DNA was at a concentration of 20 μg/ml and in the form of 2–3 μl droplets during irradiation. We have established that under these conditions 12.5 J/m^2 introduces 1 UV pyrimidine dimer per plasmid. No nicks were introduced into DNA during irradiation with 254 nm.

Psoralen adducted DNA substrates containing either exclusively monoadducts or mostly diadducts (interstrand crosslinks) were prepared by taking advantage of the unique spectral properties of DNA, psoralen, and psoralen-pyrimidine monoadducts: DNA effectively stops absorbing light at $\lambda \geq 310$ nm, the psoralen furan side monoadduct stops absorbing light at $\lambda \geq 390$ nm and psoralen has an absorption extending to at least 410 nm. The irradiation device and light source have been described previously (12), however certain modifications in the filter system were made to suit our purposes. For monoadduct formation we used a 1/4" thick glass-Pyrex filter with about 50% transmission at 365 nm and a 3 mm 350 nm cutoff filter followed by a 389 nm bandpass filter (from Oriel or Baird Atomic). Multiple filters were necessary to remove the strong 365 nm mercury emission line and to protect the bandpass filter from overheating. This filter train resulted in 5 mW/cm^2 of 389 nm (monoadducting) light. For crosslinking, the output of a 2200 W Hg/Xe arc lamp was passed through a 9 cm path length 1.7% $Co(NO_3)_2$/2% NaCl filter which produced 300 mW/cm^2 of 320–380 nm light (12).

The monoadducted DNA was prepared as follows. Reaction mixtures containing 284 μg/ml of pBR322 and 41 μg/ml [^{3}H]-HMT (150 Ci/mole) in 2.2 ml of 10 mM Tris HCl, pH 7.5, 150 mM NaCl, and 1 mM EDTA were irradiated for two min with 389 nm light. The sample was precipitated three times with ethanol to remove unbound psoralen and then dissolved in TE. To prepare crosslinked DNA one half of the monoadducted sample was irradiated for 10 min with 320–380 nm light. Aliquots of singly and doubly irradiated DNAs were assayed for tritium content from which it was determined that an average of 40 HMT adducts per plasmid was present in both samples. The total yield was 192 μg of monoadducted plasmid and 184 μg of crosslinked plasmid starting with 625 μg of pBR322 for the initial irradiation. To produce HMT modified pBR322 with fewer monoadducts per plasmid the initial irradiation time was reduced to 27 sec. This treatment yielded an average of 8 HMT monoadducts per plasmid.

The types of psoralen adducts produced by this irradiation scheme were determined by hydrolyzing aliquots of DNA to nucleosides and analyzing the products by reverse phase HPLC as described previously (13,14). These analyses revealed the following adduct distributions in the two DNAs. 'Monoadducted-pBR322': 77.2% furan side thymidine adduct, 10.5% furan side cytidine adduct, and 2.2% pyrone side thymidine adduct. No diadduct was detectable in this DNA. 'Crosslinked-pBR322': 58.0% diadduct, 22% furan side thymidine adduct, 9.2% furan side cytidine adduct, and 4.4% pyrone side thymidine adduct. As is apparent from these figures, not all the furan side monoadducts were converted to diadducts under the conditions used in the second irradiation. There are two possible explanations for this failure, either the sample was exposed to an insufficient dose of crosslinking light, or that many of the adducts are in non-crosslinkable sites in the plasmid. The slight decrease in the amount of cytosine adduct seen indicates that some of these adducts are also being driven on to crosslinks. The doubling of the amount of pyrone side adduct in the crosslinked sample relative to the monoadducted sample is due to in-helix photoisomerization of furan side monoadducts in crosslinkable sites (15). The psoralen adducted DNA was about 50% nicked probably due to singlet oxygen formation during irradiation and handling of the plasmid in repeated precipitations. The nicked DNA was removed by an additional centrifugation through a CsCl-ethidium bromide gradient to obtain psoralen adducted DNA with >95% superhelical molecules for use in the repair synthesis assay.

Repair Synthesis Assay

This assay, which measures the incorporation of a radiolabeled nucleotide into damaged DNA, was performed as described (3) except that the concentration of KCl was increased to 75 mM and undamaged M13RFI DNA was not included as an internal control. Rather, unmodified pBR322 was 'repaired' and analyzed in parallel. Repair synthesis with (A)BC excinuclease was conducted essentially as described elsewhere (8). Following repair reaction, the reaction mixture was treated with Proteinase K (100 μg/ml, 30 min at 50°C). DNA was then extracted with phenol and ether, precipitated with ethanol and resuspended in the appropriate buffer for restriction enzyme digestion. DNA was either linearized with EcoRI or digested into multiple fragments with HpaII or a combination of BamHI, BstYI, EcoRI, HincII, PstI and PvuII; the fragments were separated on either 1% agarose gel or 5% non-denaturing polyacrylamide gel. Quantitation of the incorporated radioactivity was accomplished by Cerenkov or liquid scintillation counting of the DNA-containing bands; incorporation into unmodified DNA was subtracted from the incorporation into modified DNA to determine damage-specific incorporation. When indicated, the fragments were excised from the gels, electroeluted and processed further.

Assays to Detect Removal of Psoralen Adducts

The assays measure the fraction of DNA labeled by repair synthesis which no longer contains psoralen. The assay measures the relative amount of psoralen in fragments radiolabeled as a result of damage-induced DNA synthesis compared to the amount of psoralen in fragments labeled by kinasing the total DNA.

For measuring removal of psoralen monoadducts, pBR322 containing 8 HMT monoadducts was subjected to repair synthesis and digested with a mixture of restriction enzymes and then the fragments were separated on a 5% polyacrylamide gel. A total of five fragments ranging in size from 276 bp to 454 bp were excised from the gel, purified by electroelution, precipitated with ethanol and resuspended in TEN 7.4 buffer. Aliquots of these labeled fragments were irradiated with 7.5×10^4 J/m^2 of 366 nm light from two 15 W GE Black Light lamps to convert all crosslinkable psoralen monoadducts (70–80%) into crosslinks. To measure the level of psoralen adducts in total DNA, a sample of pBR322 with 8 monoadducts (as measured by tritium content) was digested with the same mixture of restriction enzymes, kinased, and the same fragments were isolated and subjected to the same irradiation treatment. The DNA fragments labeled by either damage-induced synthesis or terminally labeled by kinasing were then denatured by heating at 95°C for 15 min and allowed to renature at 23°C for 5 min prior to separation on 5–8%

polyacrylamide gels. The gels were autoradiographed and the autoradiograms were scanned by densitometry to estimate the fractions of single-stranded (free of crosslinkable monoadducts) and double-stranded DNA.

The same method was employed to measure the removal of psoralen crosslinks except the irradiation step to convert monoadducts to crosslinks was omitted. A total of eight fragments ranging in size from 67 bp to 309 bp were analyzed. Again, fragments labeled either as a result of damage-induced synthesis or kinasing were subjected to denaturation-renaturation and then separated on polyacrylamide gels. The fractions of single-stranded DNA in the two DNAs were compared to determine whether DNA labeled by damage-induced synthesis contained a greater fraction of single-stranded DNA compared to total DNA (labeled by kinasing).

Data Analysis

The rationale of our assay is as follows: since only 1−10% of the DNA is repaired it would be difficult to detect a 1−10% decrease in adducts if total DNA were used in the analysis. However, if one analyzes only those molecules which have undergone damage-induced DNA synthesis then the background signal from unrepaired molecules is eliminated. Psoralen chemistry allows one to estimate the number of psoralen adducts in the radiolabeled subpopulation and therefore determine if DNA synthesis is associated with loss of the adduct. At low doses psoralen molecules adduct Ts at 5'TpA sequences and these monoadducts can be converted into crosslinks with 70−80% efficiency by irradiation with 366 nm. Thus, if one exposes DNA to 366 nm light following repair synthesis and then looks at the fraction of the labeled DNA which becomes single-stranded upon denaturation by heating at 95°C, only the psoralen in radiolabeled (repaired) DNA is measured. By conducting the same type of treatments on terminally labeled DNA (kinased) the level of psoralen in total DNA is similarly determined. From a comparison of these levels it is determined whether DNA labeled by repair synthesis contains a lower frequency of psoralen photoproducts. Since the psoralen damage is more-or-less randomly distributed the data must be analyzed using the Poisson formula. Thus a fragment containing an average (m) of one psoralen adduct per molecule will have a fraction, P(0), of 37% with no adducts at all and a fraction of 18% with two adducts and so on. In applying Poisson statistics to this specific problem it is expected that the P(0) class will not be labeled by damage-specific resynthesis and that the P (≥ 2) class will remain as duplex even if one of the psoralens has been removed by repair. The possibility of removal of two adducts from a restriction fragment in this low efficiency repair system is considered to be negligible. Therefore only the removal of psoralen from the P(1) class is assumed to contribute to an increase in single-stranded DNA, P(0) class. The P(0) class of total DNA is measured by analyzing kinased (unrepaired) fragments and from this value m is calculated knowing that the various classes of psoralen adducts are estimated from $P(k) = e^{-m} m^k/k!$ where k = frequency class. We assumed (1) damage-induced DNA synthesis always results in elimination of one adduct, (2) the probability of repairing more than one adduct in a fragment is zero and (3) the probability of repair synthesis in a fragment is directly proportional to the number of adducts present in that fragment. With these assumptions then, and provided that there is no background incorporation, for a fragment with an average of m psoralen monoadducts, the fraction of single-stranded DNA in total DNA after denaturation is expected to be $P(0) = e^{-m}$ while the fraction that becomes single-stranded in repaired DNA should be $P(1)/(1-P_0)$. This is the ratio of the P(1) class to the total of the classes of adducted fragments as the P(0) class is assumed to not contribute to specific labeling and the labeling into the P(>1) classes does not result in generation of single-stranded DNA. Thus, for any given m in total DNA determined experimentally from analysis of kinased fragments, $P(1)/(1-P_0)$ is calculated. If damage-induced DNA synthesis is not associated with repair, the fraction of single-stranded DNA should be equal to P(0) for both kinased and internally (repair) labeled DNA; however, if *all* damage-induced DNA synthesis is associated with adduct removal then the fraction that becomes single-stranded DNA in the repair synthesis reaction should be $P(1)/(1-P_0)$. By comparing the fraction of single-stranded DNA in the kinased and 'repaired' DNAs we were able to determine whether the 'repaired' DNA was indeed repaired. The analysis for the repair of crosslinked DNA was similar.

RESULTS

Repair Synthesis with DNA-MA and DNA-XL

Previous work (2,3) has shown that psoralen-damaged DNA elicits DNA synthesis by HeLa CFE. There is a 90% loss in repair activity when HeLa CFE is heat-inactivated (45°C, 12 min) prior to repair synthesis. The previous work was conducted with a substrate containing mostly MA and an unknown level of crosslinks. We wished to determine the relative efficiencies of the two forms of adducts as substrates for repair synthesis. We also used UV-damaged DNA as our reference substrate since its dose response is well characterized. In agreement with earlier reports, at modification levels of 8 adducts per molecule UV-damaged or psoralen-adducted DNA give comparable repair signals (Table 1). At higher doses the UV signal appears to measure linearly up to 20 adducts per molecule while the signal with psoralen-adducted DNA starts to level off. However, when the monoadducts are converted to crosslinks the signal more than doubles. This is in contrast with what is observed with the *E. coli* nucleotide excision repair system; in this system, which was reconstituted with purified UvrA, UvrB and UvrC proteins, DNA polymerase I (PolI) and ligase, conversion of monoadducts to crosslinks drastically reduces the repair signal. Even though (A)BC excinuclease incises psoralen monoadducts and crosslinks with near-equal efficiency the crosslink cannot be further processed by the enzyme and the two nicks generated on the furan side of the crosslink do not function as primers for PolI (16−19).

Table 1. Damage-Specific Nucleotide Incorporation (fmol dCMP) During Repair Synthesis by Human and *E. coli* Systems on DNA Damaged by UV or Psoralen.

Repair System	Type Damage/Number of Adducts Per Plasmid				
	UV/8	UV/20	Pso MA/8	Pso MA/40	Pso XL/40
HeLa CFE	97±17	196±32	83±38	128±26	298±58
UvrABC + PolI	n.d.*	382±14	429±109	309±52	95±24

The Pso XL substrate contains both monoadducts (17) and crosslinks (23). The values given (fmol±s.e.m.) are the averages of 3−9 experiments using HeLa CFE (6 extract preparations) or 2 experiments using the *E. coli* system. The incorporation into UM DNA, which averaged 60 fmol, was subtracted from total incorporation in damaged DNA to determine damage-specific incorporation.
*not determined.

One would expect that the *E. coli* system would generate a repair synthesis signal for XL DNA that is at least equivalent to that observed for the MA DNA substrates since the crosslinked substrate contains 17 monoadducts in addition to the crosslinks. We can not unambiguously explain the significant decrease in repair synthesis signal for XL DNA; however, one possible explanation is that the extensive crosslinking in the XL DNA interferes with the tracking of UvrAB and the transient unwinding that occurs during the loading of UvrB to the damage site by the A_2B_1 complex (20). Whether the increased repair synthesis observed with HeLa CFE constitutes true repair synthesis will be addressed later.

Removal of Psoralen MA by HeLa CFE

While the results presented above as well as several previous reports clearly show damage-induced DNA synthesis by HeLa CFE it has not been shown unambiguously that this synthesis is the result of filling-in of the single strand gaps generated by nucleotide excision nuclease(s). To demonstrate that adducts were removed from DNA we took advantage of a unique property of psoralen photochemistry: the psoralen-furan side thymine monoadduct absorbs a second photon and in this excited state makes a pyrone side adduct with an appropriately positioned thymine in the complementary strand. Thus, by a second round of irradiation with 366 nm, psoralen monoadducts can be converted with 70–80% efficiency to interstrand crosslinks. Thus, following repair synthesis the frequency of psoralen adducts remaining in radiolabeled DNA can be determined easily by measuring the fraction of rapidly renaturable radioactive DNA following heat denaturation-rapid renaturation. By comparing this fraction with that obtained with unrepaired DNA (5′-terminally labeled) a quantitative estimate between label incorporation and adduct removal can be made.

The results of such a denaturation-rapid renaturation experiment are shown in Figure 1. As can be seen qualitatively from this figure the fraction of DNA that remains single-stranded in DNA labeled by repair synthesis is higher than the fraction of single-stranded DNA in total DNA and thus the repair-labeled DNA must have less psoralen adducts compared to total DNA. The resolution of the single-stranded DNA into two bands was unique to the 276 bp fragment shown in this figure. However, the appearance of two bands was not unique to 'repaired' DNA as the unrepaired single-stranded DNA (kinased) resolved into two bands in other experiments (data not shown).

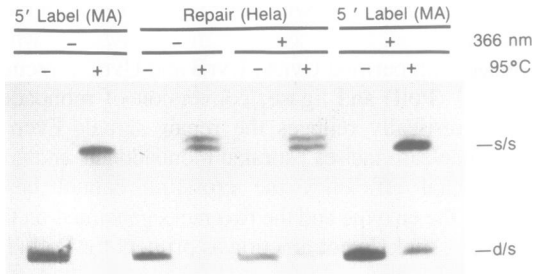

Figure 1. Repair of Psoralen Monoadducted (MA) DNA by HeLa CFE. Plasmid pBR322 containing 8 MA per molecule was incubated as described except each reaction mixture contained 300 ng DNA and 5 μCi [α-^{32}P]dCTP. Following incubation, repaired DNA was restricted and the BamHI-HincII$_{(276bp)}$ fragment was gel purified. One half of the repaired and thus internally labeled DNA, indicated as Repair (HeLa), was irradiated with a black light (+366 nm) and one half was left untreated (−366 nm). Damaged DNA, which was not repaired but which was 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, indicated as 5′ Label (MA), was similarly irradiated or not. Prior to resolution on a 5% polyacrylamide gel, one half of each sample was heat denatured (+95°C) or left in its native state (−95°C). The relative positions of single- (s/s) and double-stranded (d/s) DNA are shown. In some experiments, 5′ labeled UM DNA was electrophoresed in parallel; these fragments comigrated with untreated (−366 nm) MA DNA of the same size.

To establish whether all repair synthesis was associated with adduct removal, five fragments ranging in size from 276 bp to 454 bp were isolated from pBR322/MA8 which had been subjected to repair synthesis. If 'repair synthesis' was not the result of adduct removal then the P(0) class (single-stranded fraction) of kinased DNA would be the same as the DNA labeled by 'repair synthesis'. Because the damage-specific incorporation for this substrate was low, we allowed for 30% background synthesis. Because this non-specific labeling would contribute equally to all classes, the equation used to determine the expected fraction single-stranded DNA following repair synthesis was:

$$P_0' = \frac{[P(1)] + [0.3(P_0 + P_1)]}{[\Sigma\ p\ (k)\cdot k] + [0.3]}$$

The results obtained with these five fragments are summarized in Table 2. Several conclusions can be made from this table. First, the P(0) class of the individual fragments does not decrease uniformly with fragment size as would have been expected if psoralen adducts were uniformly distributed in pBR322. As a result, in calculating the P_0', the experimentally determined m values for individual fragments must be used rather an m value obtained based on fragments size and the total number of adducts in pBR322. Secondly, the calculated P_0' is significantly different from P(0) indicating the importance of this type of analysis rather than simply comparing P(0) values. Finally, the experimentally determined single-stranded DNA fraction, with the exception of the 295 bp fragment, is in good agreement with the calculated P_0' which was calculated assuming that all damage-specific synthesis is correlated with adduct removal. Therefore, we conclude that damage-induced DNA synthesis in our system is true repair synthesis resulting from the filling in of gaps generated by damage removal.

Table 2. Fraction of Fragments that Migrate as Single-Stranded DNA Following Repair of Psoralen Monoadducted DNA by HeLa Cell Free Extract.

Fragment Size (bp)	Observed Fraction of 5′ Labeled Total DNA (P_0)	Fraction of Repair Labeled DNA	
		Observed (P_0)	Expected[1]
454	0.34 ± 0.01	0.61 ± 0.07	0.43
400	0.60 ± 0.06	0.76 ± 0.07	0.71
377	0.50 ± 0.02	0.66 ± 0.04	0.61
295	0.79 ± 0.04	0.77 ± 0.04	0.89
276	0.69 ± 0.02	0.84 ± 0.02	0.81

[1]The equation used to determine expected fraction is:

$$\frac{[P(1)] + [0.3(P_0 + P_1)]}{[\Sigma\ p(k)\cdot k] + [0.3]}$$

Plasmid pBR322 containing 8 MA per molecule was treated as described in the legend to Figure 1. The relative amounts of single-stranded DNA in each lane were determined by scanning the resulting autoradiographs with a Zeineh Soft Laser Scanning Densitometer. The values reported for observed fractions single-stranded DNA are the averages of 2–5 experiments. The values for expected fraction single-stranded DNA following repair synthesis were determined from the average values observed for damaged DNA, as described in the text.

Removal of Psoralen Crosslinks

In a manner analogous to that for psoralen monoadducts we reasoned that analysis of radiolabeled DNA following repair synthesis with crosslinked substrate would reveal whether 'repair synthesis' resulted in crosslink repair. The rationale and method of analysis were the same except there was no post-repair irradiation to convert monoadducts to crosslinks. Furthermore, in calculating the crosslink-induced radiolabel incorporation, the signal from MAs present in crosslinked DNA was taken into account as well as non-specific incorporation (an average of 12%), so that in the crosslinked DNA we only looked at the average signal from crosslink repair. We estimate that MAs comprise 27% of the total lesions if crosslinks are considered as two lesions. The equation used to determine the expected fraction single-stranded DNA following repair synthesis was:

$$P_0' = \frac{[P(1)] + [0.12 + 0.27]}{[\Sigma\ p(k)\cdot k] + [0.12 + 0.27]}$$

With these corrections made, then, the fraction of single-stranded DNA in repair-labeled and total (terminally labeled) DNA of eight fragments can be compared to determine whether there was crosslink removal. A representative gel for such an analysis is shown in Figure 2 and the quantitative analysis for 8 fragments is summarized in Table 3. In all fragments analyzed the fraction of repair-labeled DNA that becomes single-stranded upon denaturation-rapid renaturation was higher than for terminally labeled DNA and in most cases the fraction that became single-stranded approximated the fraction that was calculated assuming all labeling results from the elimination of crosslinks.

In *E. coli* crosslinks are incised efficiently by the excision repair enzyme (A)BC excinuclease but the two incisions made on the furan-side adducted strand do not constitute an efficient primer site for PolI in the absence of the recombination protein RecA. As a consequence a stronger 'repair synthesis' signal is obtained with monoadducted DNA relative to crosslinked substrate in a defined *E. coli* system consisting of (A)BC excinuclease, PolI, ligase and the necessary substrates and cofactors. It was surprising then that HeLa CFE yielded a stronger repair synthesis signal with crosslinked DNA. Therefore, we considered the possibility that the 2−3-fold increase in repair synthesis associated with crosslinked DNA may actually result from aberrant synthesis initiated at a nick either on the 5′or 3′ side of the crosslink which is either ligated to the fragment without crosslink removal or continues in the form of strand displacement, or nick translation. Neither of these cases is expected to yield uniform size single-stranded labeled fragments free of crosslinks upon denaturation. Therefore, we analyzed the fragments of pBR322 which were labeled by repair synthesis on DNA sequencing gels. A representative example is shown in Figure 3. As seen in the figure the radioactivity incorporated into the crosslinked DNA (lanes 6 and 12) is in fact associated with single-stranded fragments with defined sizes of about 200 bp or less, with larger fragment smearing due to label incorporation into the P (> 1) class; note the reduced intensity of distinct bands larger than this size. This smearing corresponds to the double-stranded DNA following denaturation-rapid renaturation of repaired XL DNA (see Figure 2); when gel purified repaired DNA fragments were analyzed on sequencing gels, distinct bands in the higher molecular weight regions were evident (data not shown). The same appears to be true also for repair with the defined *E. coli* system. However, most of the repair signal in this system is due to incorporation of label into monoadducted DNA (see Table 1) and as a consequence the labeled fragments are denaturable.

Considering the quantitative analysis of the level of repair synthesis in Table 1 together with this qualitative analysis of the status of radiolabeled fragments we conclude that the increased repair synthesis observed with crosslinked DNA is associated with disappearance of the crosslink. The most likely explanation of these results for HeLa CFE is that crosslinked DNA induces repair synthesis which results in the removal of the crosslink from at least one strand and closure of the gap following repair synthesis. Whether the crosslink is totally eliminated from DNA or one of the strands remains attached to the crosslinked adduct while the other is filled in by translesion synthesis cannot be ascertained from our data. An interesting (and reproducible) feature of this gel is the shift of the HpaII 190 bp fragment to

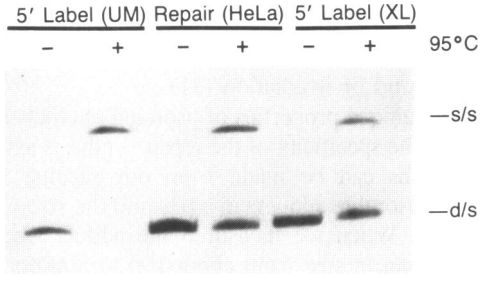

Figure 2. Repair of Psoralen Crosslinked DNA (XL) DNA by HeLa CFE. Plasmid pBR322 containing 23 XL per molecule (+ 17 MA) was incubated as described except each reaction mixture contained 300 ng DNA and 5 μCi [α-^{32}P]dCTP. Following incubation, repaired DNA was restricted and the HpaII-HpaII$_{(309bp)}$ fragment was gel purified. The repaired samples, indicated as Repair (HeLa), contain an internal label introduced during incubation with CFE. Undamaged or damaged DNA which was not repaired, indicated as 5′Label (UM) or 5′Label (XL), were 5′ end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. Prior to resolution on a 5% polyacrylamide gel, one half of each sample was heat denatured (+95°C) or left in its native state (−95°C). The relative positions of single- (s/s) and double-stranded (d/s) DNA are shown.

Table 3. Fraction of Fragments that Migrate as Single-Stranded DNA Following Repair of Psoralen Crosslinked DNA by HeLa Cell Free Extract.

Fragment Size (bp)	Observed Fraction of 5′ Labeled Total DNA (P_0)	Fraction of Repair Labeled DNA	
		Observed (P_0)	Expected[1]
309	0.29±0.03	0.38±0.01	0.47
217	0.20±0.01	0.24±0.01	0.37
160	0.60±0.03	0.74±0.03	0.78
147	0.47±0.02	0.63±0.02	0.67
122	0.53±0.05	0.75±0.03	0.72
110	0.50±0.05	0.72±0.04	0.69
76	0.56±0.02	0.72±0.06	0.75
67	0.73±0.02	0.79±0.04	0.88

[1]The equation used to determine expected fraction is:

$$\frac{[P(1)] + [0.12 + 0.27]}{[\Sigma\ p(k)\cdot k] + [0.12 + 0.27]}$$

Plasmid pBR322 containing 23 XL (+ 17 MA) per molecule was treated as described in the legend to Figure 2. The relative amounts of single-stranded DNA in each lane were determined by scanning the resulting autoradiographs with a Zeineh Soft Laser Scanning Densitometer. The values reported for observed fraction single-stranded DNA are the averages of 3−8 experiments. The values for expected fraction single-stranded DNA following repair synthesis were determined from the average values observed for damaged DNA, as described in the text.

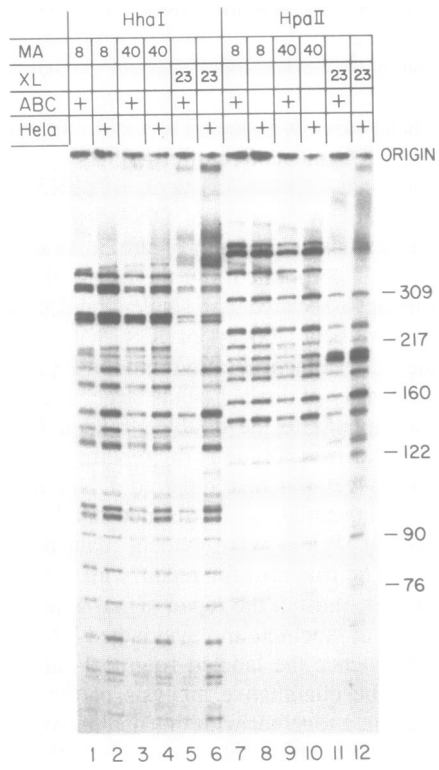

Figure 3. Repair of Psoralen Damaged DNA by HeLa CFE and *E. coli* ABC system. Plasmid pBR322 containing the indicated type and number of adducts was incubated as described in the text; DNA samples in lanes 5–6 and 11–12 also contained 17 MA per plasmid. Following repair synthesis, DNA was restricted with HhaI (lanes 1–6) or HpaII (lanes 7–12) and resolved on an 8% sequencing gel. The amounts of DNA loaded in each lane were different as we aimed to obtain bands of approximaely equal intensity and, therefore, do not reflect the true level of repair synthesis for different numbers and classes of psoralen adducts. Size markers (in bp) are shown to the right.

an apparent size of 200 bp (lanes 11 and 12); the corresponding fragments from MA DNA (lanes 7–10) migrate at the expected position as do the HhaI 190 bp fragments (lanes 1–6).

DISCUSSION

Nucleotide excision repair involves several formal steps: incision, excision, resynthesis, and ligation. In theory any of the first three could be used to quantify DNA repair. In the incision assay, either conversion of superhelical DNA to open circular form is measured by agarose gel electrophoresis or the average molecular weight of linear DNA of non-uniform size is determined by alkaline sucrose gradient following treatment with the nicking enzyme. In the excision assay the removal of a radiolabeled adduct is measured in the form of acid soluble oligonucleotides. Although these assays could be quite sensitive when conducted properly, in general they are insensitive to levels of incision/excision below 0.05 events per kbp. In contrast, a repair synthesis assay under appropriate conditions can be quite sensitive. Thus, assuming that an adduct forming-chemical, such as psoralen, and a NTP (nucleoside triphosphate) of the same specific activity are available and assuming that the repair patch is about 20 nucleotides in length the repair synthesis assay would be 20:4=5 times more sensitive than the excision assay. In practice dNTPs of much higher specific activities are available compared to psoralen, 4-nitroquinoline-oxide, acetylaminofluorene or other chemicals which make base adducts excised by nucleotide excision nucleases and the sensitivity of the resynthesis assay is routinely 50–100-fold higher than the incision or excision assays (3).

Because of its high sensitivity, the resynthesis assay was successfully applied to a human cell free system to demonstrate nucleotide excision repair (2–4) under conditions where neither the incision nor the excision assays could provide any convincing signal (3,8). The lack of repair synthesis signal in extracts from XP cell lines known to be defective in nucleotide excision repair provided strong evidence that this was indeed repair synthesis (2).

Despite this advantage in sensitivity, the repair synthesis assay suffers an important disadvantage in specificity. It could be argued, for example, that the increased DNA synthesis observed with damaged DNA merely reflects the increased susceptibility to nicking of damaged DNA by non-specific nucleases to generate primers for DNA polymerases which may incorporate radiolabel 5′ or 3′ to the adducted nucleotide without ever removing the adduct. There have been three other reports indicating that repair synthesis is associated with adduct removal. Sibghat-Ullah *et al.* (3), using a plasmid substrate uniquely adducted with psoralen at a Kpn I site, found that following repair synthesis the Kpn I site (free of psoralen) was restored in a small fraction of the molecules. Using a similar type of substrate, Hansson *et al.* (6) found that label was preferentially incorporated into a 248 bp fragment surrounding the adduct. It could be argued that the small level of adduct removal or specific incorporation were due to nick translation into the small fraction of nicked molecules which invariably exists in these substrates. Heiger-Bernays *et al.* (7) found that HeLa CFE alleviated the replication blocking effect of adducts in an SV40 replication system. It could also be argued that the DNA synthesis observed in this system following treatment with the extract represented nick translation in damaged substrate initiated by non-specific nucleases and not bona fide replication.

The unique photochemistry of psoralen enabled us to address the question of specificity in the *in vitro* repair synthesis assay. Psoralen intercalates into DNA with high preference at TA sequences. Irradiation with 366 nm causes covalent addition of the furan ring to the 5,6 double bond of thymine; the monoadduct then can absorb a second photon and become adducted via the pyrone ring to the thymine in the other strand to create an interstrand crosslink. By controlling the wavelength and dose of irradiation one can obtain DNA containing only monoadducts which can later be converted into crosslinks with high efficiency by a second round of irradiation (21).

Utilizing the unique properties of psoralen photochemistry we have increased the specificity of the repair synthesis assay. Three main conclusions can be made from our results. First, the distribution of psoralen adducts in a plasmid the size of pBR322 is non-random. When we measured the adduct frequency in fragments ranging in size from about 100 to 500 bp we found some fragments contained either higher or lower numbers of adducts than determined from the numbers predicted based on the total number of adducts present in the plasmid. This is not surprising as psoralen has high affinity to 5′TpA sites and still higher affinity to such sites in A-T rich stretches. However, inspection of the sequences of the 'hot spot' and 'cold spot' fragments failed to reveal any unusual distribution of such sequences with one notable exception. Perhaps more subtle sequence modifiers are responsible for the observed non-random distribution of psoralen adducts. The one exception is the HpaII

190 bp fragment which has three times the number of 5'TpA sites as does the HhaI 190 bp fragment; this HpaII fragment has a greater number of psoralen target sites (5'TpA)/unit length than other HpaII fragments. This may somehow be correlated with the observed band shift (Figure 3), although we can not at this time explain why the shift is not also seen in MA DNA.

The most significant conclusion to be made from this work, however, is that 'damage-induced DNA synthesis' observed with psoralen adducted DNA in cell free extracts is 'repair synthesis' in that it is associated with removal of psoralen monoadducts. In general, all P_1 class repair-labeled DNA is no longer crosslinkable upon a second round of UV-irradiation. As the adducts are distributed according to a Poisson distribution within the fragments, some of the repair labeled DNA is converted into crosslinks by UV irradiation. However, the fraction which is convertible to crosslinks is in good agreement with the fraction of fragments expected to have more than one adduct. There are two exceptions to this observation. In one case (454 bp) a higher fraction of repair labeled DNA is converted than would be predicted from a random distribution of adducts. In the second case (295 bp) a lower fraction than expected became single-stranded upon denaturation-renaturation. We expect that these are due to non-Poisson adduct distribution in the two cases, having a single hot spot in the former and two or more cold spots in the latter case.

Finally, and unexpectedly, we found that psoralen crosslinks induced a higher level of repair synthesis compared to monoadducts. A trivial explanation of this phenomenon would be that upon conversion to a crosslink a psoralen monoadduct becomes a diadduct and in a sense the adduct level in DNA doubles and therefore it is to be expected that the 'repair synthesis' signal should go up. However, at the level of DNA and psoralen adducts (40 MA per pBR322) used in these experiments, the adduct level is near saturating and a two-fold increase in monoadducts would not result in a two-fold increase in signal. Furthermore, the increase in repair synthesis upon conversion of monoadducts to crosslinks is more than two-fold. It is thus clear that one crosslink induces more repair synthesis than do two monoadducts. Whether this is because a crosslink is a better substrate than a monoadduct or the 'repair patch' induced by crosslink is larger remains to be determined. It has been reported that *in vivo* HMT crosslinks are removed more rapidly than monoadducts in an actively transcribed human gene (22). However, our data provide strong evidence that the repair synthesis induced by crosslinks is associated with the disappearance of the crosslink and might legitimately be considered as crosslink repair. There are at least two possible ways in which a crosslink may disappear from DNA with a repair synthesis patch. In one mechanism the crosslink is totally removed by first incising one strand, filling in the gap and then removing the 'dangling-crosslinked oligomer' (16) from the other strand. A second mechanism involves bypass DNA synthesis following two incisions made on both sides of the adduct in one strand. Which mechanism is operative in our system remains to be established. However, the apparent disappearance of psoralen crosslinks in this system opens new possibilities for studying crosslink repair in mammalian cells.

ACKNOWLEDGEMENTS

We thank J.J.Lin for critical reading of this manuscript. This work has been supported by the NIH grant GM32833, the Department of Energy grant DE-Ac03-76SF-00098 and in part by the grant CTR1872A from the Council for Tobacco Research–USA, Inc.; JTR was supported by fellowship ES05513-02 from the NIH.

REFERENCES

1. Sancar, A., and Sancar, G.B. (1988) *Annu. Rev. Biochem.* **57**, 29–67.
2. Wood, R.D., Robins, P. and Lindahl, T. (1988) *Cell* **53**, 97–106.
3. Sibghat-Ullah, Husain, I., Carlton, W. and Sancar, A. (1989) *Nucleic Acids Res.* **17**, 4471–4484.
4. Wood, R.D. (1989) *Biochemistry* **28**, 8287–8292.
5. Hansson, J., and Wood, R.D. (1989) *Nucleic Acids Res.* **17**, 8073–8091.
6. Hansson, J., Munn, M., Rupp, W.D., Kahn, R., and Wood, R.D. (1989) *J. Biol. Chem.* **264**, 21788–21792.
7. Heiger-Bernays, W.J., Essigmann, J.M. and Lippard, S.J. (1990) *Biochemistry* **29**, 8461–8466.
8. Sibghat-Ullah, and Sancar, A. (1990) *Biochemistry* **29**, 5711–5718.
9. Coverley, D., Kenny, M.K., Munn, M., Rupp, W.D., Lane, D.P., and Wood, R.D. (1991) *Nature* **349**, 538–541.
10. Manley, J.L., Fire, A., Cano, A., Sharp, P.A., and Gefter, M.L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3855–3859.
11. Thomas, D.C., Levy, M. and Sancar, A. (1985) *J. Biol. Chem.* **260**, 9875–9883.
12. Cimino, G.D., Shi, Y.B., and Hearst, J.E. (1986) *Biochemistry* **25**, 3013–3020.
13. Kanne, D., Straub, K., Rapoport, H., and Hearst, J.E. (1982) *Biochemistry* **21**, 861–871.
14. Straub, K., Kanne, D., Hearst, J.E., and Rapoport, H. (1981) *J. Am. Chem. Soc.* **103**, 2347–2355.
15. Tessman, J.W., Isaacs, S.T., and Hearst, J.E. (1985) *Biochemistry* **24**, 1669–1676.
16. Van Houten, B., Gamper, H.S., Holbrook, R., Hearst, J.E. and Sancar, A. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 8077–8081.
17. Cheng, S., Van Houten, B., Gamper, H.B., Sancar, A. and Hearst, J.E. (1988) *J. Biol. Chem.* **263**, 15110–15117.
18. Sladek, F.M., Munn, M.M., Rupp, W.D. and Howard-Flanders, P. (1989) *J. Biol. Chem.* **264**, 6755–6765.
19. Cheng, S., Sancar, A., and Hearst, J.E. (1991) *Nucleic Acids Res.* **19**, 657–663.
20. Koo, H.-S., Claassen, L., Grossman, L., and Liu, L.F. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 1212–1216.
21. Chatterjee, D.K., and Cantor, C.R. (1978) *Nucleic Acids Res.* **5**, 3614–3633.
22. Vos, J.-M.H., and Hanawalt, P.C. (1987) *Cell* **50**, 789–799.