Strand-Specific Binding of RPA and XPA to Damaged Duplex DNA†

Ingrid L. Hermanson-Miller and John J. Turchi*

Department of Biochemistry and Molecular Biology, Wright State University School of Medicine, Dayton, Ohio 45435

Received June 19, 2001; Revised Manuscript Received December 6, 2001

ABSTRACT: The nucleotide excision repair (NER) pathway is a major pathway used to repair bulky adduct DNA damage. Two proteins, xeroderma pigmentosum group A protein (XPA) and replication protein A (RPA), have been implicated in the role of DNA damage recognition in the NER pathway. The particular manner in which these two damage recognition proteins align themselves with respect to a damaged DNA site was assessed using photoreactive base analogues within specific DNA substrates to allow site-specific cross-linking of the damage recognition proteins. Results of these studies demonstrate that both RPA and XPA are in close proximity to the adduct as measured by cross-linking of each protein directly to the platinum moiety. Additional studies demonstrate that XPA contacts both the damaged and undamaged strands of the duplex DNA. Direct evidence is presented demonstrating preferential binding of RPA to the undamaged strand of a duplex damaged DNA molecule.

The major pathway for the repair of bulky adduct DNA damage is catalyzed by nucleotide excision repair (NER)[1] (*1*). A single damaged site must be detected among an expansive background of undamaged DNA. The NER pathway accomplishes this via two distinct mechanisms, global genomic repair (GGR) and transcription coupled repair (TCR) (*2*). Recognition by the GGR pathway relies on specific proteins that bind with higher affinity to damaged DNA than to undamaged DNA. Several proteins have been implicated in the recognition of damaged DNA in mammalian cells including the xeroderma pigmentosum group A protein (XPA), replication protein A (RPA), XPE (DDB), and the XPC−hHR23B complex (*3*). Each of these proteins has been shown to bind preferentially to various types of damaged DNA including cisplatin−DNA adducts, UV-induced damage, and acetylaminofluorene (AAF) adducts. The wide array of chemical structures that are substrates for the NER pathway have also been demonstrated to be repaired at different rates (*4*, *5*). The efficiency of NER-catalyzed repair is correlated with the thermal instability of duplex DNA induced by the specific DNA adducts (*4*).

Interaction of the chemotherapeutic drug *cis*-diamminedichloroplatinum(II) (cisplatin) with DNA induces a variety of DNA adducts including 1,2-d(GpG) or d(ApG) intrastrand adducts, 1,3-d(GpXpG) intrastrand adducts, interstrand cross-links, and monofunctional adducts. Each adduct induces a unique localized distortion of the DNA and results in varying degrees of destabilization of the duplex DNA structure. The intrastrand cisplatin adducts are repaired by the NER pathway, and the degree of distortion induced is reflected in the rates of repair. For example, a 1,2-d(GpG) intrastrand adduct generates a bend angle between 32 and 34 degrees and unwinds the DNA by 23 degrees; however, the local base pairing remains intact around the adduct (*6*, *7*). Repair of the 1,2-d(GpG) adduct occurs at a rate of 20% as compared to repair of the 1,3-d(GpXpG) adduct, which induces a localized denaturation of the duplex 2−4 bp around the lesion (*8*, *9*).

We have developed an assay to assess the strand specificity of damaged DNA binding by the damage recognition proteins, XPA and RPA. Through the use of a photoinduced protein−DNA cross-linking assay, we provide direct evidence that RPA binds preferentially to the strand opposite a single damaged site in duplex damaged DNA, consistent with previously published data (*10*, *11*). XPA does not demonstrate the same preferential binding as RPA but appears to have equal preference for binding both the damaged strand and the strand opposite the damaged strand of the duplex DNA. In addition, evidence is also presented suggesting that both XPA and RPA are in close proximity to the platinum moiety of a duplex DNA damaged with cisplatin.

EXPERIMENTAL PROCEDURES

Materials. DNA oligonucleotides were from Midland Certified Reagents (Midland, TX), Integrated DNA Technologies, Inc. (Coralville, IA), or Operon Technologies, Inc. (Alameda, CA). AciI, T4-polynucleotide kinase, and T7 DNA polymerase were from New England Biolabs (Beverly, MA). Unlabeled nucleotides were from Pharmacia (Piscataway, NJ), and radiolabeled nucleotides were from New England Nuclear (Boston, MA). Cisplatin was from Sigma (St. Louis, MO), and DACH-platinum was kindly provided by S. Chaney (University of North Carolina). The rhRPA expression vector was kindly provided by Marc Wold. All other reagents were purchased from standard suppliers.

† This work was supported by National Institutes of Health awards CA64374 and CA82741 to J.J.T. I.H.-M. was supported by the Wright State University Biomedical Sciences Ph.D. program.

* Corresponding Author: Department of Biochemistry and Molecular Biology, Wright State University, 3640 Colonel Glenn Highway, Dayton, OH 45435. Phone: (937) 775-2853. Fax: (937) 775−3730. E-mail: john.turchi@wright.edu.

[1] Abbreviations: RPA, replication protein A; XPA, xeroderma pigmentosum group A; NER, nucleotide excision repair; DACH, diamminocyclohexane; TCR, transcription coupled repair; GGR, global genomic repair.

10.1021/bi0112863 CCC: $22.00
Published on Web 01/24/2002

Table 1: Synthetic Oligonucleotides

name	sequence (5′−3′)[a]
T	CCCCTATCCTTTCCGCGTCCTTACTTCCCC
TD	CCCCTATCCTTTCCG**D**GTCCTTACTTCCCC
TI	CCCC*X*ATCCTTTCCGCGTCCT*X*ACTTCCCC
TDI	CCCC*X*ATCCTTTCCG**D**GTCCT*X*ACTTCCCC
B	GGGGAAGTAAGGACGCGGAAAGGATAGGGG
BI	GGGGAAG*X*AAGGACGCGGAAAGGA*X*AGGGG

[a] D, cholesterol lesion; *X*, 5′-IdU or BrdU as indicated in figure legends. Site-specific cisplatin of DACH-platinum adducts were positioned at the GCG sequence on oligonucleotides T and TI and were used in Figures 1 and 4.

Human recombinant RPA was purified from *Escherichia coli* as previously described (*11*, *12*), and recombinant human XPA was purified from Sf21 insect cells following infection with a recombinant baculovirus (*13*).

DNA Substrate Preparation. We have designed a series of duplex DNA substrates prepared from single-stranded 30-base oligonucleotides (Table 1). The sequences were designed to allow the site-specific modification of one strand of the duplex, designated the T-strand, with cisplatin to create a 1,3-d(GXG)-Pt lesion (*14*). Alternatively, a cholesterol lesion was incorporated at the same position during the chemical synthesis (*15*). The oligonucleotides were also prepared with halogenated-dUMP substitutions on each strand of the duplex surrounding the site of the DNA adduct. Experiments were performed with both Br-dUMP substitution and I-dCMP substitutions. We found no significant difference, and the specific substrates used are indicated in the figure legends. The duplex substrates are prepared by 5′-end labeling one of the strands using T4 polynucleotide kinase and [γ-^{32}P]ATP. For DNA substrates containing site-specific cisplatin lesions, the T-strand oligonucleotide was modified with either cisplatin or DACH-platinum at a 1:1 drug-to-nucleotide ratio. Platination reactions were incubated overnight in the dark at 37° C in buffer containing 1 mM $NaHPO_4$ (pH 7.5) and 3 mM NaCl. DNA was subsequently purified from unreacted cisplatin or DACH-platinum by ethanol precipitation. The 30-base T-strand oligonucleotides were then annealed to complementary B-strand oligonucleotides, and the duplex substrates were treated with AciI. Undamaged control DNA substrates were not subject to AciI digestion. The full-length 30-bp duplex DNA substrates were then purified by electrophoresis using a 15% native polyacrylamide gel. The DNA substrates were eluted from the gel, treated with mung bean nuclease, and purified from any degraded single-stranded DNA via G-50 spin column chromatography as previously described (*10*). DNA substrates containing the cholesterol lesions were prepared by a similar protocol that involved annealing the appropriate strands, purification via 15% native gel electrophoresis, and elution from the gel as described above. Treatment of the cholesterol-damaged DNA with AciI was omitted as greater than 95% of the TD and TDI oligonucleotides contained the cholesterol lesion (data not shown).

DNA Binding and Photo-Cross-Linking Assay. Binding reactions were performed in 20 mM Hepes (pH 7.2), 1 mM DTT, 70 mM NaCl, 1 mM $MgCl_2$, 50 fmol of [^{32}P]labeled DNA and purified XPA or RPA as indicated in the figure legends. Reactions were allowed to reach equilibrium by incubation at 4° C for 30 min. Individual samples were cross-linked by UV-irradiation using three 15W GE lamps; F15T8 or G15T8 were used providing peak output at 254 or 302 nm. Samples were irradiated on ice for 5 min at a fluence of approximately 20 Jm^{-2} s^{-1}. The reaction products were then separated by 10% SDS−PAGE and visualized by autoradiography. Dried gels were quantified by PhosphorImager analysis using ImageQuant software in volume integration mode. There was no significant difference in the products obtained using the different lamps, and kinetic analysis of the cross-linking reaction demonstrated that a 5-min exposure time was optimal in terms of minimizing nonspecific cross-linking (data not shown). The cross-linking efficiency varied dependent on the specific DNA substrate used but was typically less than 5% of the input DNA containing the halogenated bases.

T4 DNA Polymerase/Exonuclease Assay. DNA binding and photo-cross-linking reactions were performed as described above. After cross-linking, samples were either incubated at room temperature or 95° C for 5 min. T4 DNA polymerase (3 units) was then added once samples were cooled to room temperature and incubated at 37° C for 30 min. Samples were then resolved via 10% SDS−PAGE analysis and visualized by autoradiography.

RESULTS

Photo-Cross-Linking RPA to Damaged DNA. The use of chemical and photo-cross-linking has been used successfully to characterize the interactions between a variety of proteins and their DNA substrates. We have employed the photo-cross-linking technique to determine how the DNA damage recognition proteins, RPA and XPA, interact with damaged DNA. Substrates were designed to contain site-specific damage and also incorporated halogenated base analogues to allow zero distance cross-linking of a protein to the DNA upon exposure to UV light. The duplex DNA substrates were 5′-labeled with [^{32}P] on either the damaged or undamaged strand to assess the strand specificity of the damaged DNA binding. Each 30-bp DNA substrate was either untreated or treated with cisplatin to generate a single 1,3-d(GpXpG) cisplatin adduct. As earlier results demonstrated that RPA binds duplex damaged DNA via denaturation (*10*), it became important to limit RPA-induced denaturation of the duplex DNA to allow accurate determination of the orientation of RPA binding to the duplex DNA structure. Therefore, the RPA-to-DNA ratio was decreased, and higher NaCl concentrations were employed. These conditions resulted in minimal denaturation of the duplex DNA substrates as assessed by high percentage native polyacrylamide gel electrophoresis (data not shown). The photoinduced cross-linking enabled the detection of RPA/DNA complexes that are considerably less stable than the complexes formed under conditions that allow the denaturation of the duplex DNA and are observable by electrophoretic mobility shift assays. The 1,3d(GpXpG) cisplatin−DNA adduct was originally chosen based upon its local bending and unwinding properties and the fact that this adduct is an excellent substrate for NER-catalyzed repair in vitro (*9*, *16*). Initial experiments with RPA suggested that cross-linking to the cisplatin-damaged DNA was independent of the photoreactive base analogues and could occur via direct Pt-protein cross-linking. It has been reported previously that cisplatin can act as an effective photoinduced cross-linking agent (*17*). This group reported

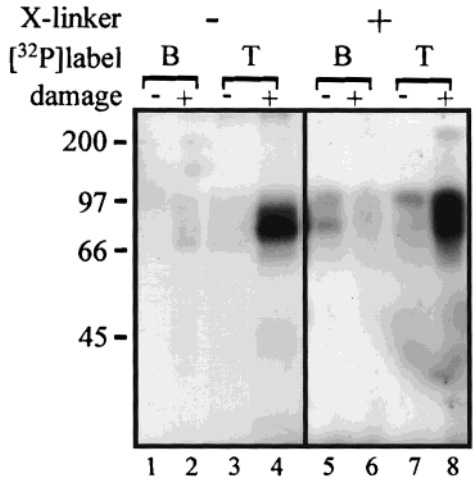

FIGURE 1: Photoactivated cross-linking of RPA to Pt-damaged duplex DNA. The DNA substrates were prepared as described in Experimental Procedures. The T or TI strand was left undamaged (lanes 1, 3, 5, and 7) or damaged with Pt-DACH (lanes 2, 4, 6, and 8). DNA substrates were prepared without photoreactive bases (lanes 1−4) or with the photoreactive BrdUMP bases positioned on the B-strand (lanes 5 and 6) or the T-strand (lanes 7 and 8). The [^{32}P] label was positioned on the B-strand (lanes 1, 2, 5, and 6) or the T-strand (lanes 3, 4, 7, and 8). RPA (9 ng) was incubated with the DNA followed by irradiation at 254 nm. Products were resolved by SDS−PAGE and detected by autoradiography.

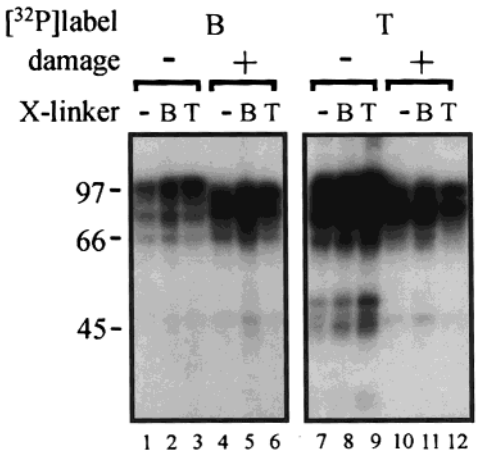

FIGURE 2: Photoactivated cross-linking of RPA to cholesterol-damaged duplex DNA. The DNA substrates were prepared as described in Experimental Procedures. Reactions were performed with undamaged T-strand (lanes 1−3 and 7−9) or cholesterol-damaged T-strand (lanes 4−6 and 10−12). DNA substrates were prepared without photoreactive bases (lanes 1, 4, 7, and 10) or with the photoreactive I-dUMP bases positioned on the B-strand (lanes 2, 5, 8, and 11) or the T-strand (lanes 3, 6, 9, and 12). The [^{32}P] label was positioned on the B-strand (lanes 1−6) or the T-strand (lanes 7−12). RPA (9 ng) was incubated with the DNA followed by irradiation at 302 nm. Products were resolved by SDS−PAGE and detected by autoradiography.

that the cisplatin atom itself formed the point of attachment in the HMG protein−DNA cross-linked complex. Their results also suggested that certain cisplatin analogues would not be as photoreactive as cisplatin since a Pt(en)Cl_2 complex was reported to have undergone photoinduced cross-linking with considerably less efficiency (*17*). A platinum analogue containing a 1,2-diaminocyclohexane modification (Pt-DACH) is very similar to Pt(en)Cl_2 but contains an additional cyclic structure attached to the two ammine groups. We, therefore, investigated UV induced photo-cross-linking of RPA to DNA substrates modified with a single, site-specific (Pt-DACH) lesion. The results obtained using this substrate are shown in Figure 1 and demonstrate that in reactions with DNA substrates damaged with DACH-platinum and are [^{32}P]labeled on the T-strand, cross-linked protein−DNA products were observed in the range of 66−97 kDa. Products in this range suggest cross-linking to the 70-kDa subunit of RPA (*18*, *19*). The presence of the photoreactive bases had minimal effect on the observed level of cross-linking to the RPA protein, while a slight change in product distribution was observed (compare lanes 4 and 8). Reactions performed with undamaged DNA or with the [^{32}P]label on the B-strand resulted in minimal cross-linking. These data were similar to those obtained for cisplatin-damaged DNA and again suggest that the protein−DNA cross-linking is directly via the Pt lesion (data not shown). Together, these results suggest that the 70-kDa subunit of RPA is in contact with the Pt lesion located within the duplex DNA substrate for both 1,3-d(GpXpG)cisplatin−DNA and 1,3-d(GpXpG)DACH Pt adducts.

While the results suggest that RPA is in contact with the damaged strand of the duplex DNA, relative affinities between the two strands cannot be definitively determined because the majority of the cross-linking is via the platinum which is only present on the T-strand. Therefore, we sought to identify a DNA adduct that is recognized by the NER repair machinery that would not be capable of direct cross-linking to proteins. We chose a cholesterol-modified DNA that has been shown to be repaired efficiently by the NER process and is expected to be relatively chemically inert to UV-induced cross-linking. Substrates were again prepared varying the position of the [^{32}P]label and photoreactive bases relative to the cholesterol lesion, and the results are presented in Figure 2. Comparison of these data to those presented in Figure 1 demonstrate a low level of cross-linking for all of the substrates as compared to that observed with the Pt-damaged DNA. The data obtained in Figure 2 required a significantly longer exposure time that resulted in the detection of protein−DNA cross-links in the absence of photoreactive bases. This nonspecific cross-linking was not as apparent in the series of reactions performed with Pt-damaged DNA as the relatively efficient cross-linking by the Pt moiety resulted in shorter exposure times being required to visualize the protein−DNA complexes. For the experiments assessing binding to cholesterol-modified DNA, control reactions using undamaged DNA with the [^{32}P]label on the undamaged B-strand of the duplex resulted in minimal cross-linking of the RPA 70 subunit that was largely independent of the position of the photoreactive base analogues (lanes 1−3). A considerable increase in cross-linking to the B-strand was observed when the cholesterol lesion was incorporated into the T-strand of the duplex and either no photoreactive bases were employed or were positioned on the B-strand (lanes 4 and 5). Relatively little change in cross-linking efficiency was observed when the photoreactive bases were incorporated on the T-strand (lane 6). These results suggest that in the presence of damage, RPA binding is directed to the undamaged strand of the duplex. In reactions performed with substrates containing the [^{32}P]label on the T-strand of the duplex, significant cross-linking was observed in the absence of the cholesterol lesion (lanes 7−9). This is likely a result of the increased affinity

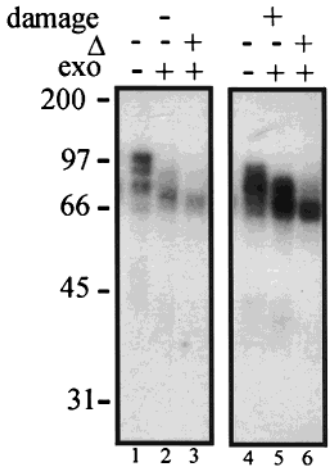

FIGURE 3: Multiple RPA70−DNA complexes. The DNA substrates were prepared as described in Experimental Procedures. Reactions were performed with undamaged T-strand (lanes 1−3) or cholesterol-damaged T-strand (lanes 4−6). DNA substrates were prepared with the [^{32}P]label and photoreactive I-dUMP bases positioned on the B-strand on all DNA substrates. RPA (9 ng) was incubated with the DNA followed by irradiation at 302 nm. Products were resolved by SDS−PAGE and detected by autoradiography directly (lanes 1 and 4), or after digestion with T4 DNA polymerase/exonuclease (lanes 2 and 5), or after heat denaturation followed by T4 polymerase/exonuclease digestion (lanes 3 and 6).

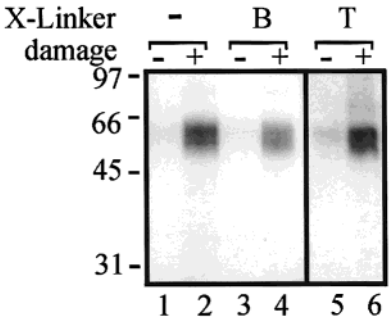

FIGURE 4: Photoactivated cross-linking of XPA Pt-damaged DNA. The DNA substrates were prepared as described in Experimental Procedures. The T or TI strand was left undamaged (lanes 1, 3, and 5) or damaged with cisplatin (lanes 2, 4, and 6). DNA substrates were prepared without photoreactive bases (lanes 1 and 2) or with the photoreactive BrdUMP bases positioned on the B-strand (lanes 3 and 4) or the T-strand (lanes 5 and 6). The [^{32}P] label was positioned on the T-strand for all substrates. XPA (75 ng) was incubated with the DNA followed by irradiation at 254 nm. Products were resolved by SDS−PAGE and detected by autoradiography.

of RPA for polypyrimidine sequences versus polypurine tracts (*20*, *21*). The T-strand of the duplex is pyrimidine rich, whereas the B-strand complement is purine rich. Importantly, even with this relatively high level of nonspecific cross-linking, a dramatic decrease in cross-linking was observed when the cholesterol lesion was positioned on the [^{32}P]labeled strand T of the duplex (lanes 10−12). In the absence of damage, the relative distribution of RPA to the two strands of the duplex is largely dictated by the proportion of pyrimidines in the individual DNA strands. However, in the presence of DNA damage on the T-strand, decreased binding of RPA to the damaged strand is observed and a greater amount of RPA associates with the undamaged strand of the duplex DNA, despite the preference for pyrimidine-rich DNA sequences. Importantly, the signal obtained in these experiments is also dependent on the efficiency of the cross-linking reaction. In addition to the preference of RPA to bind polypyrimidine sequences, pyrimidine bases also are more effective cross-linkers than purine bases. Therefore, the difference in product intensity (lanes 4−6 as compared with lanes 10−12) cannot be attributed to simply differences in bindng affinity but also to the likelihood that a given complex will be cross-linked. Clearly, we would anticipate that the redistribution would be even more dramatic if the DNA substrates did not have the purine/pyrimidine strand bias. Importantly, the data presented in Figure 2 provide direct evidence that the 70-kDa subunit of RPA preferentially interacts with the undamaged strand of a damaged duplex DNA.

The results from Figure 2 also revealed a variety of products in the 66−97 kDa that could be either higher order complexes or perhaps the 70-kDa subunit also cross-linked with the 14- or 32-kDa subunits to the DNA substrate. To ascertain the source of the multiple products, we employed nuclease digestion of the DNA, and the results are presented in Figure 3. We used a 3′ to 5′ exonuclease activity such that the 5′-[^{32}P] label would remain undigested, and any accessible DNA with a 3′ terminus would be degraded. The results revealed that the products decreased in size as a result of nuclease digestion (lanes 1 and 2), but multiple products were still present, especially in complexes formed with damaged DNA (lanes 4 and 5). Therefore, we heat-denatured the cross-linked protein−DNA products prior to nuclease digestion to make more of the DNA accessible to nuclease-catalyzed digestion (lanes 3 and 6). The results demonstrate that all of the products can be digested to result in a single product of approximately 66 kDa. This product is consistent with the 70-kDa subunit of RPA being cross-linked to the DNA substrate and was confirmed by cross-linking reactions performed with single-stranded DNA which has been shown to predominantly cross-link to the central region of the 70 kDa subunit of RPA (*19*, *22*). These results suggest that the difference in product size observed with binding duplex DNA substrates can be attributed to varying lengths of DNA cross-linked to the 70-kDa subunit and not to the formation of higher ordered complexes with either the 14- or 32-kDa subunits of RPA.

XPA Photo-Cross-Linking to Damaged DNA. A second essential component of the NER repair pathway is the XPA protein, which is also involved in recognition of DNA damage. Similar to results obtained with RPA binding platinum-damaged DNA, XPA can also be photo-cross-linked to the cisplatin adduct (Figure 4). The DNA substrates used in Figure 4 have the [^{32}P]label positioned on the undamaged strand of the duplex. Cross-linked protein−DNA products are only observed in reactions performed with damaged DNA. Considering the degree of damage specificity and the relative insensitivity of cross-linking to the presence of the photoreactive bases in the duplex, we conclude that XPA is also cross-linked to the DNA via the Pt moiety. XPA, therefore, is also in close proximity to the damaged bases of the duplex DNA substrate, similar to RPA. Similar results were also obtained measuring XPA cross-linking to DNA substrates containing the Pt-DACH adduct (data not shown). To assess binding of XPA to damaged DNA without the caveat of direct cross-linking to the Pt, we employed DNA substrates containing the cholesterol lesion. The results presented in Figure 5 demonstrate relatively inefficient cross-

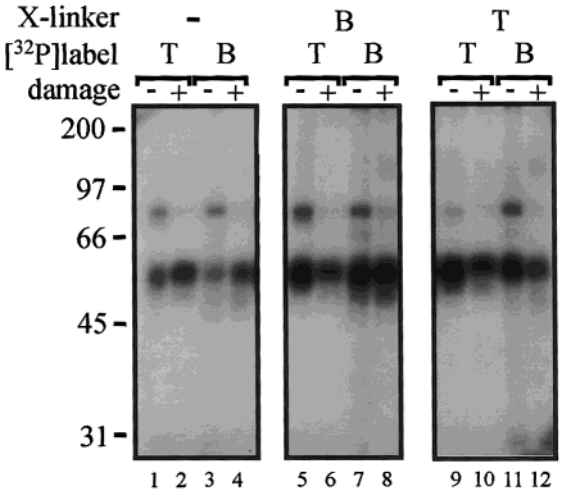

FIGURE 5: Photoactivated cross-linking of XPA cholesterol-damaged DNA. The DNA substrates were prepared as described in Experimental Procedures. Reactions were performed with undamaged T-strand (odd lanes) or cholesterol-damaged T-strand (even lanes). DNA substrates were prepared without photoreactive bases (lanes 1−4) or with the photoreactive I-dUMP bases positioned on the B-strand (lanes 5−8) or the T-strand (lanes 9−12). The [^{32}P] label was positioned on the B-strand (lanes 3, 4, 7, 8, 11, and 12) or the T-strand (lanes 1, 2, 5, 6, 9, and 10). XPA (75 ng) was incubated with the DNA followed by irradiation at 302 nm. Products were resolved by SDS−PAGE and detected by autoradiography.

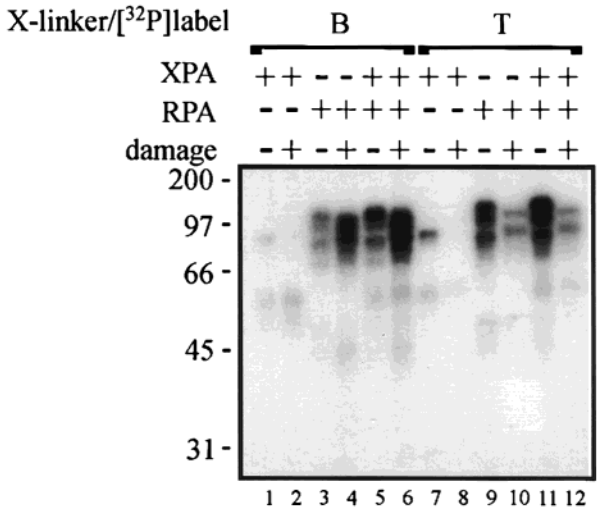

FIGURE 6: Photo-cross-linking of XPA/RPA to cholesterol-damaged DNA. The DNA substrates were prepared as described in Experimental Procedures. Reactions were performed with undamaged T-strand (odd lanes) or cholesterol-damaged T-strand (even lanes). DNA substrates were prepared with the [^{32}P]label and photoreactive I-dUMP bases positioned on the B-strand (lanes 1−6) or the T-strand (lanes 7−12). Reactions were performed with XPA (15 ng) and RPA (3 ng) as denoted in the figure followed by irradiation at 302 nm. Products were resolved by SDS−PAGE and detected by autoradiography.

linking as judged by the exposure times required for the DNA substrates prepared without the photoreactive bases (lanes 1−4). A slight increase in protein−DNA cross-linking was observed in reactions performed with the photoactivatible bases on the B-strand (lanes 5−8) or on the T-strand (lanes 9−12). The most dramatic difference observed in these reactions is the presence of two distinct complexes in reactions performed with undamaged DNA (odd lanes). The faster complex likely represents a single XPA−DNA complex, while the apparent molecular weight of the slower complex is consistent with two XPA molecules bound and cross-linked to the same DNA. This larger complex was not observed in control reactions that were performed in the experiments with cisplatin or Pt-DACH damaged DNA as the exposure times were considerably shorter as a result of the efficient cross-linking of XPA to the Pt moiety. Interestingly, only a single XPA−DNA complex is observed in reactions performed with cholesterol-damaged DNA (even lanes). These results are consistent with a model where the presence of DNA damage alters the positioning of XPA on the duplex DNA substrate.

As XPA and RPA have been demonstrated to directly interact in the absence of DNA (*23*), we sought to assess the interaction of the RPA/XPA complex with damaged DNA via the photo-cross-linking. For these reactions, the proteins were premixed to allow complex formation, and then DNA was added to the reactions. The results presented in Figure 6 demonstrate that in reactions containing both RPA and XPA the addition of XPA resulted in an increase in RPA cross-linking to the B strand of the duplex DNA (compare lanes 3 to 5 and 4 to 6). The presence of damage on the T-strand again resulted in an increased binding of RPA to the undamaged B strand, and this preference was retained in the presence of XPA. RPA binding to the T-strand was also stimulated by XPA but was only apparent in the absence of damage (lanes 7−12). In reactions with damaged DNA, RPA cross-linking to the damaged strand was not altered by the addition of XPA (compare lanes 4 to 6 and 10 to 12). We were unable to observe a complex that would be indicative of an RPA/XPA−DNA complex. While identification of this complex has been reported, stability is increased by the XPC/hHR23B complex and TFIIH and dependent on DNA length (*24*, *25*). Importantly, in the presence of XPA, RPA still retains preferential binding to the undamaged strand of the duplex damaged DNA.

DISCUSSION

The exploitation and refinement of a common photo-cross-linking procedure has enabled the investigation of the spatial orientation of the DNA damage recognition proteins XPA and RPA on damaged DNA. Our data provide direct evidence that RPA binds preferentially to the undamaged strand of a duplex damaged DNA substrate (Figure 2). These data are corroborated by both equilibrium and pre-steady-state analysis of the interaction of RPA with damaged DNA (*10*, *11*, *20*). In addition, RPA has been shown to bind single-stranded DNA with a defined polarity such that placement of RPA on the undamaged DNA strand allows for the two structure specific nucleases to be positioned 5′ and 3′ of that damaged site (*18*, *26*). The binding of RPA is thought to proceed via the denaturation of the DNA substrate and subsequent high-affinity binding to the single-stranded DNA generated (*10*). Previous results from our lab demonstrate that the RPA−duplex DNA complex is not stable under conditions of high RPA to DNA ratios. These conditions result in denaturation of the duplex substrate and subsequent binding of RPA to the single-stranded DNA. Therefore, we suspect that the RPA−duplex DNA complex is not observed by traditional EMSA analysis (*10*). The photoinduced cross-linking experiments, however, favor the retention of the duplex, in effect capturing the intermediate not seen in previous experiments.

Taken together, these data strongly support the positioning of RPA on the undamaged DNA strand. This conclusion contradicts a recently published report demonstrating that RPA apparently binds preferentially to the damaged strand of duplex DNA (*27*). A similar photoinduced cross-linking methodology was employed using oligonucleotides that contained 5-iodo-2′-deoxyuridine (5-IdU) analogues at various positions within the duplex DNA substrates and a single site-specific 1,3-d(GTG) cisplatin adduct (*27*). Using this series of substrates, it was concluded that the location of the primary binding site of the p70 subunit of RPA was to the damaged DNA strand. While control reactions using unmodified DNA substrates were not presented and the placement of the [^{32}P]label was only included on the damaged strand of DNA substrates, cross-linking of RPA to DNA substrates in the absence of any cross-linker was found to be at background levels (G. Krauss, personal communication). Therefore, while it is possible that the direct cross-linking of RPA to the Pt moiety could account for the observed results, differences in UV fluence rates and times of cross-linking could also account for differences in platinum reactivity. The data presented in this paper along with HMG cross-linking to cisplatin modified DNA (*17*) provide strong evidence that cisplatin and its related analogues act with exceptional efficiency as photoinduced cross-linking agents. The ability of the platinum analogue to cross-link RPA to the damaged DNA greatly overshadowed the cross-linking efficiency of the specific halogenated nucleotides (See Figures 1 and 2). While the data obtained with platinum-damaged DNA also are consistent with RPA contacting the damaged DNA strand, data obtained with cholesterol-damaged DNA suggest that the high affinity interaction of the OB-folds of the RPA 70-kDa subunit is with the undamaged DNA stand. In addition, the molar ratio of RPA to DNA employed in our experiments was kept very low to decrease RPA-induced denaturation of the duplex DNA. However, a large excess of RPA that would increase the potential for denaturation of the duplex DNA structure was employed in reactions where an interaction with the damaged strand was reported (*27*). Considering the pyrimidine-rich nature of the damaged DNA strand and the affinity of RPA for pyrimidine-rich DNA, detection of RPA cross-linking to this strand is not unexpected.

Another report also suggested that RPA can bind the damaged single-strand of a UV-damaged duplex DNA (*28*). It was concluded that RPA has a greater affinity for single-stranded DNA containing a pyrimidine (6-4) pyrimidine photoproduct than for undamaged single-stranded DNA (*28*). Clearly, it is possible that RPA displays a preference for a 6-4 photoproduct damaged single-stranded DNA but not cisplatin or cholesterol-damaged single-stranded DNA, and this damage specific effect could account for the different models. These data were derived from the quantification of protein/DNA complexes analysis and the subsequent calculation of binding isotherms and apparent binding constants from EMSA. The data presented, however, revealed inconsistencies in the values presented and in the RPA/DNA complex patterns observed in the EMSA analyses. An apparent loss of radiolabeled DNA is observed as increasing amounts of RPA are titrated into the reaction. Also, in reactions containng 2 fmol of DNA and 1 fmol of RPA, 100% of the DNA substrate was shown to be bound by RPA

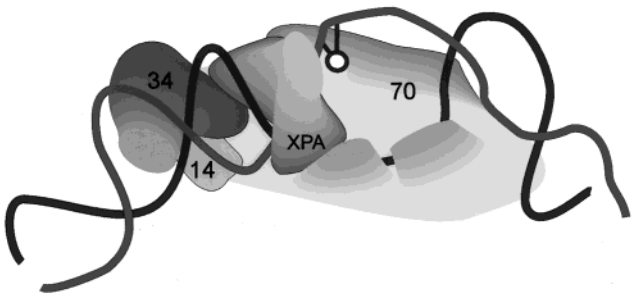

FIGURE 7: Model for RPA−XPA interacting with damaged duplex DNA. The damaged duplex DNA is shown as being partially unwound around the lesion and the 70-kDa subunit of RPA interacting with the undamaged strand of the duplex. The 32-kDa subunit of RPA is poised to interact with the 3′ OH of the damaged strand following incision. XPA is shown to interact with the duplex DNA at the ss−ds DNA junction. In addition, both the 70-kDa subunit of RPA and XPA are positioned in close proximity to the damaged bases of the DNA.

(*28*). Furthermore, at these specific RPA and DNA concentrations, analysis of the products revealed two independent protein−DNA complexes. The second, higher ordered RPA−DNA complex suggests that more than one RPA is bound to a single DNA substrate. These results are difficult to reconcile given the reported excess of DNA in the reactions. The authors also note the presence of contaminating free radiolabel in one DNA substrate preparation that could result in an inaccurate concentration for that substrate. While we cannot rule out a specific 6-4 photoproduct−RPA complex, the positioning of RPA upon the undamaged strand of the DNA duplex is considerably more consistent and compelling in light of existing data and the data presented here (Figure 7).

The photoinduced cross-linking assay was also applied to help elucidate the binding preference and orientation of the damage recognition protein, XPA. It was demonstrated that XPA has little discrimination between binding either the damaged or undamaged strand of the DNA duplex as XPA was cross-linked to both strands with relatively equal efficiencies (Figure 5). Nevertheless, an interesting pattern of XPA binding was observed. For all of the undamaged DNA substrates, not only was a single protein/DNA complex formed but an additional supershifted complex was also apparent. This supershifted complex likely represents an additional XPA molecule bound to the XPA protein/DNA complex forming a complex that contains two XPA molecules bound to a single undamaged DNA substrate. The disappearance of the supershifted complex when the DNA substrate contains damage indicates that the damage effects the ability of XPA to bind the substrate and possibly alters the conformation of XPA binding. Data also suggest that both RPA and XPA are in close enough proximity to the damage to contact the adduct. We suggest that this interaction is not via the central two OB-folds of the RPA 70-kDa subunit, which facilitates the high affinity interaction with single-stranded DNA but with another portion of the RPA 70-kDa subunit.

Direct cross-linking of both XPA and RPA to the Pt-moiety of the damaged duplex suggests that both proteins are in close proximity to the lesion. However, this interpretation is tenuous as the specific amino acid identified in the HMG-1 box protein shown to be specifically cross-linked

was separated by a considerable distance as measured in a HMG-1/Pt-DNA cocrystal (*17*). In addition, the decreased efficiency of Pt(en)Cl_2 to cross-link HMG domain proteins may be unique to the binding properties of HMG domain proteins and not a universal phenomenon where the Pt(en)-Cl_2 complex is less photoreactive than its cisplatin counterpart. Therefore, Pt-DACH platinum would not necessarily be a less effective cross-linking agent in the context of different DNA binding proteins such as XPA and RPA. The increased binding observed when using Pt-DACH as the DNA damaging agent could also be attributed to an increased ability of the repair proteins to recognize this lesion in comparison to the cisplatin−DNA adduct. If Pt-DACH alters the DNA structure in a slightly different manner than cisplatin, it is plausible that XPA and RPA would bind to the Pt-DACH modified DNA more efficiently, thereby, resulting in the greater amount of photoinduced cross-linked protein/DNA complexes. The model presented (Figure 7) positions the 70-kDa subunit of RPA binding the undamaged strand of the duplex, while remaining within proximity to the DNA lesion. XPA is positioned in contact with both strands of the duplex DNA at the ss−ds DNA junction. XPA is also positioned in proximity to the lesion and contacting the 70- and 34-kDa subunits of RPA.

ACKNOWLEDGMENT

We thank Marc Wold for the RPA expression vector, Steve Chaney for the DACH-Pt, and Steve Patrick for purified RPA and oligonucleotide designs.

REFERENCES

1. Lindahl, T., Karran, P., and Wood, R. D. (1997) *Curr. Opin. Genet. Dev. 7*, 158−169.
2. Hanawalt, P. C. (1998) *DNA Damage and Repair Vol. 2: DNA Repair in Higher Eukaryotes* (Nickoloff, J. A., Hoekstra, M. F., Eds.) pp 1−8, Humana Press Totowa, NJ.
3. Batty, D. P. and Wood, R. D. (2000) *Gene 241*, 193−204.
4. Gunz, D., Hess, M., and Naegeli, H. (1996) *J. Biol. Chem. 271*, 25089−25098.
5. Hess, M., Gunz, D., and Naegeli, H. (1996) *Nucleic Acids Res. 24*, 824−828.
6. Kasparkova, J., Mellish, K. J., Qu, Y., Brabec, V., and Farrell, N. (1996) *Biochemistry 35*, 16705−16713.
7. Takahara, P. M., Frederick, C. A., and Lippard, S. J. (1996) *J. Am. Chem. Soc. 118*, 12309−12321.
8. Moggs, J. G., Szymkowski, D. E., Yamada, M., Karran, P., and Wood, R. D. (1997) *Nucleic Acids Res. 25*, 480−491.
9. Zamble, D. B., Mu, D., Reardon, J. T., Sancar, A., and Lippard, S. J. (1996) *Biochemistry 35*, 10004−10013.
10. Patrick, S. M., and Turchi, J. J. (1999) *J. Biol. Chem. 274*, 14972−14978.
11. Patrick, S. M., and Turchi, J. J. (1998) *Biochemistry 37*, 8808−8815.
12. Henricksen, L. A., Umbricht, C. B., and Wold, M. S. (1994) *J. Biol. Chem. 269*, 11121−11132.
13. Hermanson, I. L., and Turchi, J. J. (2000) *Protein Express. Purif. 19*, 1−11.
14. Turchi, J. J., Patrick, S. M., and Henkels, K. M. (1997) *Biochemistry 36*, 7586−7593.
15. Mu, D., Hsu, D. S., and Sancar, A. (1996) *J. Biol. Chem. 271*, 8285−8294.
16. Wakasugi, M., Reardon, J. T., and Sancar, A. (1997) *J. Biol. Chem. 272*, 16030−16034.
17. Kane, S. A., and Lippard, S. J. (1996) *Biochemistry 35*, 2180−2188.
18. Kolpashchikov, D. M., Khodyreva, S. N., Khlimankov, D. Y., Wold, M. S., Favre, A., and Lavrik, O. I. (2001) *Nucleic Acids. Res. 29*, 373−379.
19. Lavrik, O. I., Nasheuer, H. P., Weisshart, K., Wold, M. S., Prasad, R., Beard, W. A., Wilson, S. H., and Favre, A. (1998) *Nucleic Acids Res. 26*, 602−607.
20. Patrick, S. M., and Turchi, J. J. (2001) *J. Biol. Chem. 276*, 22630−22637.
21. Kim, C., Snyder, R. O., and Wold, M. S. (1992) *Mol. Cell Biol. 12*, 3050−3059.
22. Kolpashchikov, D. M., Pestryakov, P. E., Wlassoff, W. A., Khodyreva, S. N., and Lavrik, O. I. (2000) *Biochemistry-Moscow 65*, 160−163.
23. Matsuda, T., Saijo, M., Kuraoka, I., Kobayashi, T., Nakatsu, Y., Nagai, A., Enjoji, T., Masutani, C., Sugasawa, K., Hanaoka, F., Yasui, A., and Tanaka, K. (1995) *J. Biol. Chem. 270*, 4152−4157.
24. Wakasugi, M., and Sancar, A. (1999) *J. Biol. Chem. 274*, 18759−18768.
25. Wakasugi, M., and Sancar, A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 6669−6674.
26. deLaat, W. L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N. G. J., and Hoeijmakers, J. H. J. (1998) *Gene Dev. 12*, 2598−2609.
27. Schweizer, U., Hey, T., Lipps, G., and Krauss, G. (1999) *Nucleic Acids Res. 27*, 3183−3189.
28. Lao, Y., Gomes, X., Ren, Y., Taylor, J., and Wold, M. (2000) *Biochemistry 39*, 850−859.

BI0112863