Characterization of Reaction Intermediates of Human Excision Repair Nuclease*

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Nucleotide excision repair in humans is a complex reaction involving 14 polypeptides in six repair factors for dual incisions on either sides of a DNA lesion. To identify the reaction intermediates that form by the human excision repair nuclease, we adopted three approaches: purification of functional DNA-protein complexes, permanganate footprinting, and the employment as substrate of presumptive DNA reaction intermediates containing unwound sequences 5’ to 3’ to, or encompassing the DNA lesion. The first detectable reaction intermediate was formed by substrate binding of XPA, RPA, XPC-HHR23B plus TFIIH (preincision complex 1, PIC1). In this complex the DNA was unwound on either side of the lesion by no more than 10 bases. Independent of the XPG nuclease function, the XPG protein stabilized this complex, forming a long-lived preincision complex 2 (PIC2). The XPF-ERCC1 complex bound to PIC2, forming PIC3, which led to dual incisions and the release of the excised oligomer. With partially unwound DNAs, thymine-cytosine dimer was excised at a fast rate independent of XPC-HHR23B, indicating that a major function of this protein is to stabilize the unwound DNA or to aid lesion unwinding in preincision complexes.

In humans, nucleotide excision repair is the sole DNA repair activity for removing bulky adducts. The repair reaction involves two basic steps, damage excision by dual incisions and repair synthesis. Mutations that interfere with damage excision give rise to xeroderma pigmentosum (XP) (1). XP is genetically heterogeneous; mutations in seven genes, XPA through XPG, may give rise to the disease (see Ref. 2). Important progress has been made in recent years in understanding the molecular basis of XP and the roles of XP proteins in nucleotide excision repair. These studies have culminated in the molecular basis of XP and the roles of XP proteins.

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In this investigation, we set out to study the events taking place from the damage recognition step to the release of the damage in an oligonucleotide. Specifically, we wished to detect the reaction intermediates of repair protein-DNA complexes and the conformation of DNA within these complexes. Using a biotin-tagged substrate and purified repair factors, we show that the first protein-DNA complex detectable by the streptavidin pull-down assay requires XPA, RPA, XPC-HHR23B, and TFIIH proteins as well as ATP hydrolysis. This complex is termed Preincision Complex 1 (PIC1). PIC1 is relatively unstable and forms a tighter, more stable complex called Preincision Complex 2 (PIC2) in the presence of XPG, even when XPG has lost its 3’-junction nuclease activity because of an active-site mutation. XPF-ERCC1 does not bind to PIC1, but it does bind to PIC2 containing either wild-type or mutant XPG. We call the complex that forms with all six repair factors PIC3. When PIC3 contains the active-site mutant XPG, the human homolog of Rad25; ERCC1 (26–28) and with lower affinity to TFIIH (29). TFIIH binds XPG with high affinity (5, 30) and XPC with lower affinity (16). Finally, RPA binds to XPF-ERCC1 and to XPA (12). These structural and functional properties of the repair factors have led to the proposal of models on assembly and catalysis by excision nuclease. However, no specific intermediates have been detected with either gel retardation or footprinting techniques in support of the reaction mechanism models.

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lesion by 5–10 nucleotides. In PIC2, the unwound region remains the same length as in PIC1 but becomes more stable. PIC3 does not promote further unwinding but leads to dual incisions that expose a 24–32-nucleotide region in the strand complementary to the damaged strand to permanganate modification. Using model substrates containing mispaired DNA 3′, 5′ or 3′ and 5′ to the lesion, it was confirmed that lesion unwinding is on the reaction pathway and that the main function of XPC-HHR23B is to aid in DNA unwinding.

MATERIALS AND METHODS

Repair Factors—Recombinant XPA (10, 32), RPA (33), and XPG (20) were isolated as described. TFIIH and XPC-HHR23B were purified from HeLa cells according to Mu et al. (5, 6). The point mutant of XPG (XPG-D812A) was prepared as described (31).

Pull-down Experiments—We used a 136-base pair duplex with a T6–4T photodimer. The complete nucleotide sequence of the T6–4T substrate is identical to the T6–4T substrate employed previously (34) except that the 5′-terminal guanine of the T6–4T-containing strand was replaced by a cytosine in this study. The oligonucleotide containing a site-specific photoproduct (5′-GTAT6–4TATG-3′) was provided by X. Zhao and was prepared by the method of Smith and Taylor (35). This oligomer was 5′-phosphorylated with [γ-32P]ATP (7,000 Ci/mmol, ICN) using T4 kinase and annealed with other five partially overlapping oligonucleotides. After ligation, the full-length 136-mer with an internal 32P label at the fourth phosphodiester bond 5′ to T6–4T was purified through an 8% denaturing and a 5% nondenaturing polyacrylamide gel. Since the substrate has a protruding guanine and a cytosine at either 3′-end, dGTP and biotinylated dCTP were used to fill in the one-base protruding ends, resulting in the biotinylation of the 3′-terminus of the T6–4T-containing strand. Subsequently, the biotinylated substrate DNA was incubated with streptavidin covalently linked to magnetic beads (Dynabead M-280, Dynal) and became covalently linked to magnetic beads. Following pulling down using a magnet, the substrate was ready for the pull-down experiments.

As illustrated in Fig. 1, the pull-down experiments were initiated by incubating a subset of the six excision nucleases constituents and the magnetic bead-attached substrate (50 fmol) in 25 μl of excision reaction buffer (30 mM Hepes-KOH, pH 7.9, 50 mM KCl, 4 mM MgCl2, 200 μg/ml bovine serum albumin) with or without 2 mM ATP at 30 °C for 60 min. The substrate DNA was then pulled down by holding a magnet beneath the reaction vial and washed twice with 100 μl of excision reaction buffer containing 2 mM ATP to remove loosely bound proteins. The washed DNA pellet was then resuspended in 25 μl of excision reaction buffer with 2 mM ATP containing the repair proteins that were omitted in the first pull-down incubation and incubated at 30 °C for 90 min. To examine if dual incisions had occurred in this second incubation, the reaction mixture was deproteinized, ethanolation-precipitated, and analyzed using an 8% denaturing polyacrylamide gel.

Permanganate Probing for Single-stranded Thymes in the Reaction Intermediates—The protocol of permanganate footprint method was adapted from Refs. 36 and 37. The 5′-terminally 32P-labeled T6–4T substrate was incubated with the indicated amounts of repair proteins at 30 °C for 60 min in excision reaction buffer with 2 mM ATP in a total volume of 25 μl. Subsequently, KMnO4 was added to a final concentration of 5 mM. After 1 min at room temperature, β-mercaptoethanol was supplemented to 1 M to stop the permanganate reaction. Following deproteinization by proteinase K and ethanol precipitation, the DNA was resuspended in 50 μl of 1× T4 DNA ligase buffer and incubated at 30 °C for 90 min. After evaporation under vacuum to remove piperidine, the permanganate-modified thymines were revealed by resolving the DNA products on 8% denaturing polyacrylamide gels.

Bubble Substrates Mimicking Unwound Intermediates—Internally 32P-labeled bubble substrates containing T···G were synthesized as described by Mu and Sancar (25). The 32P label was in the fourth phosphodiester bond 5′ to the photodimer. The sequences of oligonucleotides 1–7 (as indicated in Fig. 1a) used to construct the substrates have been published (25); oligonucleotides 8 and 9 are listed in Table I. The excision reactions of these bubble substrates were performed in the absence of the indicated repair factor under reaction conditions reported by Mu and Sancar (25). All excision reactions were processed as described above and resolved on 8% denaturing polyacrylamide gels. The levels of damage excision were quantified by scanning the dried polyacrylamide gels using PhosphorImager (Molecular Dynamics).

RESULTS

To study the assembly of human excision nuclease we used the following general strategy (Fig. 1). Individual repair proteins or combinations of them were mixed with a magnetic bead-linked substrate, and then the DNA and DNA-bound proteins were isolated using a magnet. To the purified DNA-protein complexes, the remaining repair factors were added, and repair was measured by the excision assay. In preliminary experiments we failed to isolate complexes with one to three repair factors and proceeded to conduct experiments with combinations of four and five repair factors. We were able to isolate complexes with four and five repair factors which we named Preincision Complex 1 and 2, respectively. These complexes are most likely on the pathway for formation of excision nuclease complex because they can lead to dual incisions upon encountering the missing components.

![Diagram](image)

**TABLE I**

Oligonucleotides (5′ to 3′) used to assemble bubble-containing model substrates in this study

| Oligonucleotide no. | Nucleotide sequence |
|---------------------|---------------------|
| 8                   | GGGGCAGATTCCAGTCGCCGCGATCCCTGTAAGTGACCTGCTCGACGCCAGTTGCGCTCCATACCTCGCAAAGGGCGAATTCGAGCTCGCCCGGGATCCTCTAGAGTCGACCTGCTGCAGCCCAAGCTTGCGCGAGGTATCCCTCGCAAA |
| 9                   | TGGCCAGCTGCGCGAGATTGCTGCTCGAGGATATCGAATTCCGTACGTGTTCAGGTCC |
Results of pull-down experiments conducted with two factor omissions are shown in Fig. 2. As apparent the only two-factor omission that produced a nucleoprotein complex purifiable by the pull-down assay is the one formed in the absence of XPF and XPF-ERCC1 and XPG proteins (Fig. 2, lane 2). All other pull-down experiments involving pairwise omissions of repair factors failed to produce excision products (data not shown). The formation of this complex (named PIC1) with XPA, RPA, XPC, HHR23B, and TFIIH is ATP-dependent (compare lanes 2 and 3). Most importantly, the addition of the XPG and XPF-ERCC1 nucleases to buffer-washed PIC1 resulted in the dual incision typical of human excision nuclease, indicating that the first four factors formed a structure conducive to assembly of active excision nuclease. Hence it is reasonable to conclude that the complex formed with these four repair factors is on the pathway to dual incision.

Previously, we reported that with the pull-down assay a preincision complex could be isolated from an XPF mutant cell extract, which upon incubation with purified XPF-ERCC1 protein led to excision of damage (6). However, pull-down experiments with a rodent XP-G mutant cell extract failed to yield a productive preincision complex, suggesting a role for XPG in the preincision events (6). This previous result with the rodent XP-G mutant cell extract seemed to be contradictory with the present data shown in Fig. 2 (lane 2), which indicates that a productive preincision complex (i.e., PIC1) can be formed in the absence of both XPG and XPF-ERCC1. Hence, we conducted single factor omission experiments using purified XPF-ERCC1 protein to mimic the presence of XPG and XPF-ERCC1. The results in Fig. 2 (lane 4) show that in the absence of XPF-ERCC1, a productive complex (designated PIC2) must have been pulled down from the mixture of XPA, RPA, TFIIH, XPC-HHR23B, and XPG so that its subsequent encounter with purified XPF-ERCC1 led to damage excision. The excision was not caused by nonspecific association of repair factors with substrate DNA because all other single omission experiments failed to give dual incisions under these conditions (see Fig. 3). As in the case of PIC1, the formation of this second preincision complex, PIC2, was also ATP-dependent (data not shown). In
light of the results of the pull-down experiments using purified proteins, it is very likely that PIC1 may have been pulled down from the rodent XP-G mutant cell extract. Since in that study only the purified XPG protein was added back to the buffer-washed PIC1 (6), dual incisions failed to occur because of the absence of XPF/ERCC1 in the second incubation.

Significantly, comparison of the excision signals in XPG/ERCC1 and XPF/ERCC1 omission experiments shown in Fig. 2 and in repeated experiments conducted under identical conditions indicated that the excision signal obtained with PIC2 is much stronger than that obtained with PIC1. The most likely explanation is that PIC2 is more stable than PIC1 and better survives the repeated washes during pull-down so that upon addition of the omitted factor efficient excision takes place. This interpretation was supported by the experiments that probed the structure of DNA within these complexes as described below.

**Conformation of DNA in PIC1 and PIC2**—The dependence of formation of PIC1 and PIC2 on ATP and the fact that TFIIB has helicase activity (15–17) suggested that the DNA within these complexes might be unwound and that either the degree of unwinding or the protein composition of the unwound complex may be responsible for the differential stability of these complexes. Hence, we probed the DNA for unwinding within these complexes by KMnO₄ treatment, which oxidizes unpaired thymines and renders them cleavable by alkaline treatment.

The substrate was a duplex of 136 base pairs with a centrally located T[6–4]T and a terminal radiolabel in the complementary strand. Fig. 4 indicates the positions of Ts near the lesion in both strands, and Fig. 5a shows the results of permanganate probing of PIC1. In the absence of any repair factor both T(+1) and T(−1) were slightly reactive with permanganate, consistent with previous reports that T[6–4]T photoproducts unwind DNA in the immediate vicinity of the lesion (38, 39). Upon incubation of substrate with XPA, RPA, and XPC-HHR23B, the three factors that have been implicated in damage recognition (3, 4, 24), no additional hypersensitive sites appeared. This indicates that complexes that form with these factors do not stabilize the complex could be caused by KMnO₄ hypersensitivity extending to T(+1) and T(−1) and by the enhanced sensitivity at T(+1) and T(−1) (Fig. 5a, lane 5). This unwinding was not caused by the non-specific helicase action of TFIIB binding to the partially unwound T[6–4]T and enlarging it by the dual (3′ to 5′ to 3′) helicase activities because the unwinding required XPA, RPA, and XPC-HHR23B in addition to TFIIB (Fig. 5a, lanes 6–8). The reaction also required ATP hydrolysis because it was inhibited by ATPγS; hence the unwinding had the characteristics of helicase action (lane 9). As expected, the combination of the four repair factors had no effect on the undamaged DNA control (lanes 11 and 12). Together these data lead us to conclude that XPA, RPA, XPC-HHR23B, and TFIIB make a specific complex with damaged DNA and unwind it around the lesion in both 5′ and 3′ directions more than 5 base pairs but less than 10 base pairs.

**Fig. 4. Numbering scheme for thymines surrounding the lesion.** Thymines 5′ and 3′ to the T[6–4]T are numbered as shown in both strands. The complete sequence of this 136-mer DNA has been published (34).

**Fig. 5. Unwinding of T[6–4]T in PIC1 is dependent on XPA, RPA, XPC-HHR23B, TFIIB, and ATP.** Panel A, mixtures of XPA (20 ng), RPA (250 ng), and XPC-HHR23B (10 ng) were incubated with increasing amounts of TFIIB (lane 2, 0 ng; lane 3, 0.5 ng; lane 4, 3 ng; lane 5, 6 ng), and the T[6–4]T substrate (50 fmol) containing a 32P label at the 5′-terminus of the undamaged strand in 25 μl of excision reaction buffer with 2 mM ATP. After incubation at 30°C for 60 min, unpaired thymines were probed using the permanganate chemical footprinting method. Lanes 5–8 contained 6 ng of TFIIB. In lane 9, the nonhydrolyzable analog of ATP, ATPγS, was added to the reaction mixture to a final concentration of 2 mM before permanganate treatment. A Maxam-Gilbert purine sequencing ladder of the substrate DNA is shown in lane 10. The thymines reactive with permanganate are indicated with open circles and those immediately external to the reactive thymines by closed circles. The permanganate probing experiment was performed with an unmodified substrate DNA of the same nucleotide sequence (lanes 11 and 12) under the indicated conditions. Panel B, diagram showing the “repair bubble” in PIC1.

Since the excision assay suggests that PIC2 is more stable in PIC1, we wished to learn about the structure of the DNA in this complex. Under optimal conditions for formation of PIC1, we did not detect any effect of XPG (which defines the PIC2) on DNA sensitivity to permanganate. However, upon carrying out the KMnO₄ reaction with limiting amounts of TFIIB, a clear XPG effect was observed. As shown in Fig. 6 both T(+4) and T(−5) became more sensitive to KMnO₄ oxidation with increasing concentrations of XPG. However, even with the highest concentrations of XPG used, the extent of unwinding in PIC2 was indistinguishable from that in PIC1. It thus appears that PIC1 is less stable than PIC2 and that by using high concentrations of TFIIB, the equilibrium is shifted toward fully unwound conformation. The presence of XPG in the complex stabilizes it, possibly through its interaction with TFIIB (5, 30) and RPA (11). However, it is also known that the PIC2 is capable of making the 3′-incision (6). Thus, this apparent stabilization of the complex could be caused by KMnO₄ hypersensitivity induced by a flap structure resulting from the 3′-incision. Hence, we wished to determine whether or not the PIC2 complex required the 3′-incision activity of XPG protein. For this purpose we used the XPG(D812A) mutant. This is an...
active-site mutant, and the D812A substitution completely abolishes the structure-specific endonuclease activity of XPG without affecting its other biochemical properties (31). When PIC2 was reconstituted with XPG(D812A), it exhibited the same properties as the PIC2 formed with the wild-type enzyme (Fig. 6, lane 9). Thus, it is concluded that binding of the XPG polypeptide to other proteins in the preincision complex stabilizes PIC1 and that the XPG nuclease activity is dispensable for this phenomenon.

**PIC3**—The XPG and XPF ERCC1 proteins are the 3'- and 5'-nuclease factors of human excision nuclease. In contrast to XPG, the pull-down experiments failed to reveal the presence of a preincision complex containing XPF-ERCC1. As the pull-down assay is a rather harsh method for isolating DNA-protein complexes, it was conceivable that such complexes did not survive the repeated washes. The unwinding assay provided us another opportunity to test whether such complexes form. Thus, we conducted KMnO₄ probing with all repair factors except XPG using the same T[6–4]T substrate containing a ³²P radiolabel at the 5'-terminus of the damaged strand. Consistent with what was observed with experiments performed with substrate radiolabeled in the undamaged strand (Figs. 5 and 6), with this substrate as well the combination of XPA, RPA, XPC-HHR23B, and TFIIH unwound t(+2), t(+10), and t(+6) (Fig. 7, lane 2). This result enabled us to narrow down the 5'-boundary of the excision bubble to be between t(+2) and t(+10). The addition of increasing amounts of XPF-ERCC1 did not affect the permanganate footprint, nor did it result in 5'-incision (lanes 3–5 and 9–10). These data support the conclusions from pull-down experiments that XPF-ERCC1 does not enter the excision nuclease complex before XPG and that the presence of XPG in the nuclease complex is required for XPF-ERCC1 to make the 5'-incision. This is consistent with the results obtained when the excision nuclease was reconstituted with the active-site mutant XPG protein (31).

In fact, the active-site XPG mutant enabled us to identify a third excision nuclease complex, which we call PIC3. PIC3 forms when all of the repair factors are present. The excision
nuclease assembled in the presence of the five repair factors plus XPG(D812A) does not make the 3' incision (31) and yielded permanganate hypersensitivity pattern essentially identical to that of the PIC2 (Fig. 8, lanes 5 and 6). Thus, the presence of XPF-ERCC1 in the complex does not affect the size of the excision bubble. In contrast, if instead of XPG(D812A) wild-type XPG is included in the reaction mixture, dual incisions take place, and a region extending from T(-18) to T(-5) becomes hypersensitive to permanganate. This result is in agreement with our earlier findings that upon dual incisions the human excision nuclease (in contrast with the bacterial excision nuclease) releases the excised oligomer and hence generates a single-stranded gap equal in size to the excised fragment (6). This explains the hypersensitivity of the T residues from T(+18) to T(-5) in the excision gap (Fig. 8, lane 7).

Reactions with Model Substrates Mimicking Reaction Intermediates—The data presented so far show the formation of DNA intermediates with melted base pairs not exceeding 10 unpaired base pairs on either side of the lesion during the assembly of the excision repair nuclease. We wished to know whether by using premelted DNA (bubble structure) we could abrogate the requirement for some of the repair factors. We have shown previously that a substrate with a structure mimicking a transcription bubble stalled at a lesion, that is, a thymine dimer followed by a 10-nucleotide bubble, could be repaired in the absence of XPC-HHR23B (25). This finding provides support for the intermediacy of lesion-stalled transcription bubble in transcription-coupled repair and explains the XPC-independence of transcription-coupled repair (40, 41).

To investigate the role of unwinding in excision, we prepared three types of substrates. T<->T(3'-10) and T<->T(5'-10) contained 10 mismatched pairs in a row immediately next to a T<->T either at the 3'- or the 5'-side of the photodimer, respectively. The third substrate contained 20 mispaired bases with a T<->T in the center of the mismatched run (T<->T(20)) (Fig. 9A). In light of the role of TFIIH in unwinding the DNA in the PIC1 and PIC2, we were especially interested in whether or not the requirement for TFIIH could be circumvented by using unwound substrates. Excision assays were conducted with these substrates in the absence of individual factors that are known, or thought, to unwind and stabilize unwound DNA. Photodimers in all three substrates are repaired more efficiently than a T<->T in a nominal duplex DNA [T<->T(0)]. We had reported previously that T<->T(3'-10) is repaired faster than T<->T(0) (25), consistent with the notion that unwinding 3' to the lesion is on the pathway for repair. Here we show that T<->T(5'-10) is also repaired at a more rapid rate than T<->T(0), indicating that unwinding 5' to the lesion is also important for repair (Fig. 9B). Not surprisingly, T<->T(20) was also excised efficiently, further confirming the KMnO4 assay results that both 5' and 3' unwinding are on the pathway for repair.

In addition, we observed that, as in the case of T<->T(3'-10), with T<->T(5'-10) and T<->T(20) substrates excision can occur without XPC-HHR23B. This finding raises the possibility that conditions such as replication, recombination, or other DNA dynamic events creating similar structures may enable cell to repair DNA in the absence of XPC. There is some indirect evidence that indeed such XPC-independent repair occurs in transcriptionally active chromosomal domains (42). Most significantly, however, we find that TFIIH requirement can only very poorly be abrogated in substrate T<->T(20), which is more or less similar to the repair bubble we detected with permanganate (Fig. 9B, lane 22). Thus, it appears that TFIIH plays other roles in excision such as the interactions with XPA (29) and XPG (5, 30) proteins, in addition to its helic unwinding activity.

DISCUSSION

The basic reaction mechanism of human nucleotide excision repair and the proteins required for carrying out this reaction are relatively well understood (3, 4). However, the structures of DNA-protein complexes at various stages of the reaction, damage recognition, dual incision, and release of the excised oligomer are not known. Two general models have been advanced. One model proposes that all of the excision repair factors are assembled in the form of a repairosome capable of carrying out the entire excision reaction in yeast (43) or even the entire excision repair process including excision, repair synthesis, and ligation in humans (44). However, the repairosome proposed for yeast is incapable of carrying out damage excision, perhaps because of lack of RPA in complex with the RAD proteins. Indeed, a systematic study of the question has revealed the ready separation of repair proteins in yeast and provided strong evidence for sequential assembly (7, 8). Similarly, the protein preparation called human repairosome (44) contained many unrelated proteins and only a small fraction of the repair proteins (44). Again, the relative ease with which the repair
proteins could be separated from human cell extracts (5, 6, 45) is evidence that sequential assembly is the modus operandi of excision repair in humans. Indeed, the best efficiency of excision has been accomplished by a reaction carried out with individually purified repair factors (6).

Although the preponderance of both experimental evidence and theoretical consideration are in favor of sequential assembly, the individual steps are not well understood. In particular, the damage recognition step remains rather ill defined (51). It seems that the optimal approach to understand the damage recognition step is to identify distinct DNA protein complexes that were detected in this study is PIC1 whose formation is absolutely dependent on XPA, RPA, XPC, and TFIIH. It is thought that in yeast the Rad14 (Rad1-Rad10) complex, which is the structural homolog of XPA (XPF-ERCC1), can be isolated as a very stable complex. It is possible that within PIC1 the XPF-ERCC1 binding site of XPA is no longer accessible to these proteins. Whatever the reason for lack of binding of XPF-ERCC1, the practical consequence is that we cannot constitute a complex capable of making the 5’-incision in the presence of XPG. It is perhaps of relevance to note that in yeast the Rad14 (Rad1-Rad10) complex, which is the structural homolog of XPA (XPF-ERCC1), can be isolated as a very stable complex. It is thought that in yeast the Rad14 (Rad1-Rad10) complex is essential for any damage-dependent nicking to occur, and hence an uncoupled 3’-incision is not observed in the absence of Rad1-Rad10 in the yeast excision nuclease system (7, 8).

Another noteworthy finding of our studies is that the XPC-HHR23B complex is dispensable for excision of T(3'-10) but not only from the T(3'-10) and T(20) but also from the T(20) and T(10). It is still not clear whether the XPC-HHR23B complex is actually involved in the repair process or if it is merely a stabilizing factor for the PIC1 complex. Further studies are needed to clarify this issue.

A surprising finding in this study was the failure to detect a strong interaction between XPF-ERCC1 and PIC1 which forms in the presence of the XPA protein. It has been shown that XPA binds quite tightly to the XPF-ERCC1 complex (26, 28). In fact, this interaction is so strong and specific that an XPA affinity column is the main purification step for the XPF-ERCC1 complex (46). Furthermore, the XPF-ERCC1 complex is the subunit of the excision repair nuclease that carries out the 5’-incision (6, 20, 47). It is possible that within PIC1 the XPF-ERCC1 binding site of XPA is no longer accessible to these proteins. Whatever the reason for lack of binding of XPF-ERCC1, the practical consequence is that we cannot constitute a complex capable of making the 5’-incision in the absence of XPG.

**FIG. 9.** Model substrates mimicking reaction intermediates of excision nuclease. Panel A, substrate constructs. Oligonucleotides used to construct these substrates are indicated by the # sign, and the position of a 32P radiolabel in each substrate is shown. The sequences of oligomers 1–7 have been published (25). Oligomers 8 and 9 are listed Table I. Panel B, internally radiolabeled substrates as shown in panel A were incubated either with the entire set of excision nuclease components or with partial mixtures with the indicated omissions. The reaction products generated by dual incisions of the excision nuclease are indicated by a bracket. Reactions similar to those in lanes 1–5 and 11–15 have been published previously (25). To reveal the weak signal in the absence of TFIIH, a longer exposure to an x-ray film of the area covering the excision products is shown in lanes 17–20. The percents of substrate excised were: lane 2, 1.5%; lanes 7 and 9, 4.9%; lanes 12 and 14, 5.4%; lanes 17 and 19, 2.9%.
lies between $T(11)$ and $t(6)$, whereas the $3'$-boundary of the observed bubble lies between $T(+10)$ and $t(+6)$, whereas the $3'$-boundary is between $T(-5)$ and $T(-11)$. Thus the unpaired region as drawn reflects an approximation to dual incision sites. The dual incision sites of this substrate have been determined in a previous study (34).

This indicates that the presence of a single-stranded region of sufficient length around the dimer, regardless of its position relative to the dimer, makes XPC-HHR23B unnecessary for excision. This finding, combined with the high affinity of XPC to single-stranded DNA (23, 24), supports earlier suggestions that the function of XPC is to assist in DNA unwinding and stabilize the unwound structure (24). Clearly, the findings in this report constitute a useful lead on the XPC function in excision repair, which deserves further investigation.

During the course of this study, Evans et al. (48) reported on the unwinding of cisplatin 1,2-d(GpTpG) diadduct containing DNA in cell extracts of normal and excision repair-defective cell lines. The basic conclusion of that study, that excision repair in humans involves the formation of an “excision bubble,” is in agreement with our findings. However, the two studies differ in several aspects. First, as summarized in Fig. 10 the size of the unwound region is less than 20 nucleotides in our study, but it was reported to be ~25 nucleotides by Evans et al. (48). The discrepancy could be due to the extensive unwinding caused by the cisplatin 1,3-d(GpTpG) lesion alone and the fact that the sequences around the lesion in the two studies are different. Second, Evans et al. (48) found unwinding around the lesion even in XPA mutant cell extracts whereas we find that unwinding in our defined system is totally dependent on XPA. Conceivably, DNA-binding proteins or helicases in the XPA cell extract not related to excision repair caused the unwinding of the DNA already unwound due to the cisplatin 1,3-d(GpTpG) lesion. Finally, Evans et al. (48) did not detect any effect of XPG on the unwinding reaction. However, the cell extract they used most likely contained a mutant XPG protein of full length (49), which could assemble with the other repair factors but was unable to carry out the incision reaction. Our studies with wild-type and active-site mutant XPG protein demonstrate the plausibility of this explanation and point to the advantage of studying the assembly reaction with purified proteins rather than cell extracts.

Regarding the assembly of human excision nuclease our results allow us to propose the following minimal scheme (Fig. 11) involving at least three intermediates prior to the, usually concerted, dual incisions. It must be pointed out, however, that although we know the precise repair factors required for making the various preincision complexes, we do not know whether all of the proteins required for making a specific complex are present in that complex. It is conceivable that some of the proteins required for formation of PIC1 actually dissociate from the DNA-protein complex before PIC2 or PIC3 can form. In the *Escherichia coli* excision nuclease system, the (UvrA)_2(UvrB) complex binds to DNA, forming an intermediate that is analogous to “PIC1” detected for the human counterpart in this study. Subsequently UvrA dissociates, leaving behind a stable UvrB-DNA complex (PIC2), which in turn binds to UvrC (PIC3) to initiate the dual incision (for review, see Ref. 3). The function of UvrA is to promote the formation of a productive UvrB-DNA complex. This activity has been termed “molecular matchmaker” (50). It is not unreasonable to expect that one or more of the human excision nuclease constituents may act as molecular matchmakers. Further work on identifying the protein compositions of PIC1 to PIC3 is required to address this possibility.

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![Fig. 10. Location and extent of the repair bubble and its relation to dual incision sites](image1)

FIG. 10. Location and extent of the repair bubble and its relation to dual incision sites. The 5'-boundary of the observed bubble lies between $T(+10)$ and $t(+6)$, whereas the 3'-boundary is between $T(-5)$ and $T(-11)$. Thus the unpaired region as drawn reflects an approximation to dual incision sites. The dual incision sites of this substrate have been determined in a previous study (34).

![Fig. 11. Reaction intermediates of human excision repair nuclease. Panel A, overview of nucleotide excision repair. The triangle stands for a nucleotide damage. Panel B, model of the excision nuclease mechanism highlighting the intermediates detected in this study](image2)

FIG. 11. Reaction intermediates of human excision repair nuclease. Panel A, overview of nucleotide excision repair. The triangle stands for a nucleotide damage. Panel B, model of the excision nuclease mechanism highlighting the intermediates detected in this study.
