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Reaction Mechanism of Human DNA Repair Excision Nuclease*

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Nucleotide excision repair consists of removal of the damaged nucleotide(s) from DNA by dual incision of the damaged strand on both sides of the lesion, followed by filling of the resulting gap and ligation. In humans, 14–16 polypeptides are required for the dual incision step. We have purified the required proteins to homogeneity and reconstituted the dual incision activity (excision nuclease) in a defined enzyme/substrate system. The system was highly efficient, removing >30% of the thymine dimers under optimal conditions. All of the six fractions that constitute the excision nuclease were required for dual incision of the thymine dimer substrate. However, when a cholesterol-substituted oligonucleotide was used as substrate, excision occurred in the absence of the XPC-HHR23B complex, reminiscent of transcription-coupled repair in the XP-C mutant cell line. Replication protein A is absolutely required for both incisions. The XPG subunit is essential to the formation of the preincision complex, but the repair complex can assemble and produce normal levels of 3′-incision in the absence of XPF-ERCC1. Kinetic experiments revealed that the 3′-incision precedes the 5′-incision. Consistent with the kinetic data, uncoupled 5′-incision was never observed in the reconstituted system. Two forms of TFIIH were used in the reconstitution reaction, one containing the CDK7-cyclin H pair and one lacking it. Both forms were equally active in excision. The excised oligomer dissociated from the gapped DNA in a nucleoprotein complex. In total, these results provide a detailed account of the reactions occurring during damage removal by human excision nuclease.

DNA repair reactions play an important role in preventing cancer development in humans. Individuals with defects in mismatch repair have high incidence of internal cancers (Kolodner and Alani, 1994; Modrich and Lahue, 1996). Similarly, individuals with defective nucleotide excision repair specific for bulky DNA lesions suffer from xeroderma pigmentosum (XP),[1] which manifests itself by extremely high incidence of actinic cancers, increased incidence of cancers of internal organs, and mental and neurological abnormalities (Cleaver and Kraemer, 1989; Friedberg *et al.*, 1995). In humans, mutations in seven genes, *XPA* through *XPG*, cause XP associated with defective excision repair (Cleaver and Kraemer, 1989). In addition, genetic and biochemical studies with rodent cell lines and radiation-sensitive *Saccharomyces cerevisiae* mutants and repair assays with cell-free extracts have implicated several other proteins in the excision step of nucleotide excision repair. In particular, it has been found that the general transcription factor TFIIH (five to eight subunits) (Drapkin *et al.*, 1994; Schaeffer *et al.*, 1994; Wang *et al.*, 1994) and the trimeric replication protein A (RPA or HSSB) are absolutely required for the dual incision step of excision repair (Mu *et al.*, 1995).

Recently, excision repair has been reconstituted from highly purified repair factors in humans (Mu *et al.*, 1995; Aboussekhra *et al.*, 1995) and in the highly analogous *S. cerevisiae* system (Guzder *et al.*, 1995). It has been reported that in both systems the following six fractions are necessary and sufficient for the dual incision activity (Mu *et al.*, 1995; Guzder *et al.*, 1995): XPA (Rad14), RPA, TFIIH (five to eight polypeptides including XPB (Rad25) and XPD (Rad3)), XPC-HHR23B (Rad4-Rad23), XPG (Rad2), and XPF-ERCC1 (Rad1-Rad10). In this study, we have obtained the six fractions of human excision nuclease (excinuclease) free of contaminants and have used synthetic substrates containing either a thymine dimer (T<>T) or a cholesterol molecule at a predetermined site to investigate the reaction mechanism of the excision nuclease. Our results reveal that XPC-HHR23B, in contrast with the other factors that are necessary for all excision reactions, is required for excision of the thymine dimer, but not for excision of a particular cholesterol lesion. In this study, it is also demonstrated that the 3′-incision made by XPG (Harrington and Lieber, 1994; O'Donovan *et al.*, 1994; Matsunaga *et al.*, 1995) precedes the 5′-incision made by XPF-ERCC1 (Matsunaga *et al.*, 1995) and that the CDK-activating kinase constituents (CDK7 and cyclin H) that are present in TFIIH as integral subunits do not interfere with the repair activity of TFIIH and are not required for repair. Following the dual incision, the 27–29-nucleotide-long excised oligomers (Huang *et al.*, 1992) are released in a complex with repair proteins, while the gapped DNA remains complexed with a separate set of repair proteins.

EXPERIMENTAL PROCEDURES

Purification of Human Nucleotide Excision Repair Proteins—Four-hundred liters of HeLa S3 cells were purchased from Cellex Biosciences (Minneapolis, MN), and the cell-free extract was prepared according to Manley *et al.* (1980). The purification scheme in this study was different from our previous design (Mu *et al.*, 1995) in that two additional chromatographic steps, Affi-Gel blue (Bio-Rad) and SP-Sepharose (Pharmacia Biotech Inc.), were used following the first DE52 column (Whatman) chromatagraphy step. The Affi-Gel blue column chromatography step was conducted as described by Park *et al.* (1995b). TFIIH and XPC were found in the gradient elution prior to the 1.5 M NaSCN wash, whereas XPG, RPA, and a minority of XPF-ERCC1 were identified in the fractions eluted with 1.5 M NaSCN by immunoblotting. The majority (>60%) of XPF-ERCC1 came off the Affi-Gel blue column at 0.2–0.4 M KCl and was further purified to near homogeneity as described elsewhere (Park *et al.*, 1995b). The purified complex shown in Fig. 1*A* reconstituted excision nuclease when combined with other factors. However, due to the limited amount of the purest XPF-ERCC1

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[1] The abbreviations used are: XP, xeroderma pigmentosum; RPA, replication protein A; PAGE, polyacrylamide gel electrophoresis; CFE, cell-free extract; ATPγS, adenosine 5′-*O*-(thiotriphosphate); CTD, carboxyl-terminal domain; CDK, cyclin-dependent kinase.

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fraction, some of the experiments were conducted with the XPF-ERCC1 fraction from the penultimate step of purification (Park *et al.*, 1995b).

To further purify XPG, the 1.5 M NaSCN-eluted fractions were loaded onto an SP-Sepharose column (2.5 × 30 cm). A 700-ml gradient of 0.1–0.45 M KCl in storage buffer (25 mM Hepes/KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 17% glycerol) was used to elute this column at 1.3 ml/min. Unlike RPA and XPF-ERCC1, which came in the flow-through fraction, XPG was found to elute at ~0.4 M KCl by immunoblotting. These XPG-containing fractions were subsequently resolved to give homogeneous XPG using procedures modified from Habraken *et al.* (1994).

As to TFIIH and XPC, the fractions from the Affi-Gel blue column containing these factors were applied onto the same SP-Sepharose column and eluted with a 1-liter gradient of 0.1–1.0 M KCl in storage buffer. XPC eluting at ~0.3 M KCl was separated from TFIIH eluting at ~0.16 M KCl. Subsequently, pure XPC as judged by silver-stained SDS-PAGE was obtained using the procedures of Masutani *et al.* (1994). Subsequent TFIIH purification was performed similarly to our previous procedure (Mu *et al.*, 1995), except that Mono S chromatography (HR 5/5, Pharmacia Biotech Inc.) was added as the final step. This 1-ml column was eluted at 0.5 ml/min using a 35-ml gradient of 50–300 mM KCl in buffer C (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 19% glycerol, 10 mM β-mercaptoethanol), and 1-ml fractions were collected. Each fraction was concentrated using an Amicon stirred cell, dialyzed in storage buffer, and stored at −80 °C.

The XPA cDNA tagged with His_6 using the pRSET vector (Invitrogen) was overexpressed in *Escherichia coli* strain DR153 (Park and Sancar, 1993) and purified by a modified procedure of Jones and Wood (1993). The DNA construct bearing the three-subunit human RPA was obtained from Dr. M. Wold (University of Iowa), and the recombinant RPA of apparent homogeneity was prepared from *E. coli* according to Henricksen *et al.* (1994).

Substrate for Excision/Incision Assays—The substrate was a 140-base pair duplex containing either a cholesterol molecule attached at position 70 or a cyclobutane thymine dimer (T<>T) at positions 74 and 75 in the opposite strand (see Fig. 2, *D* and *E*). The complete nucleotide sequence of the cholesterol-containing substrate has been published (Matsunaga *et al.*, 1995). However, the nucleotide sequence surrounding the thymine dimer is different from that of the cholesterol substrate, and the difference is as shown in Fig. 2*D*. Depending on the type of experiment performed, we used 5′-terminally or internally ^{32}P-radiolabeled substrates. Internally radiolabeled duplex substrates were made from six oligomers, whereas end-labeled substrates were assembled from either two oligomers of 140 nucleotides or the same six oligomers used for internally labeled substrate (Matsunaga *et al.*, 1995). Two kinds of cholesterol-containing oligomers were used in this study. Cholesterol-A (see Fig. 2*A*) was from Midland Certified Reagents (Midland, TX), and cholesterol-B was from Operon Biotechnologies, Inc. (Alameda, CA). Cyclobutane thymine dimer-containing oligomer was obtained from Dr. J.-S. Taylor (Washington University, St. Louis, MO) prepared by the method of Smith and Taylor (1993).

Excision/Incision Assays—The excision assays that measure the excised damage-containing oligomer by dual incisions of the human DNA repair excision nuclease were conducted as described previously (Huang *et al.*, 1992; Huang and Sancar, 1994; Mu *et al.*, 1995; Matsunaga *et al.*, 1995). The excision reaction was incubated at 30 °C for 1 h unless indicated otherwise. The amount of each purified repair factor for a typical 25-μl excision reaction is as follows: XPA, 20 ng; TFIIH, 1–2 ng; XPC, 7 ng; XPG, 1–2 ng; XPF-ERCC1, 1–2 ng; and RPA, 300 ng. Quantitation of the excised oligomers of 23–30 nucleotides and the oligomers corresponding to single incision was achieved by scanning the sequencing gel using a PhosphorImager (Molecular Dynamics, Inc.).

Pull-down Assays/Release of Excised Oligomer and Formation of Preincision Complex—The recessed 3′-end of internally radiolabeled duplex substrate was filled with dATP-biotin (Life Technologies, Inc.) using the standard protocol. Incorporation of dATP-biotin was judged to be >90% using a 5% denaturing polyacrylamide gel. Following incubation for 1 h with either reconstituted excision nuclease or cell-free extract (CFE). The streptavidin bead-bound substrate DNA (and therefore the proteins that interacted with the substrate DNA) was collected using a magnetic particle concentrator (Dynal, Inc.) and washed twice with 100 μl of 1 × excision reaction buffer (30 mM Hepes/KOH, pH 7.9, 50 mM KCl, 4 mM $MgCl_2$, 2 mM ATP, 200 μg/ml bovine serum albumin). To determine if the excised oligomers could be pulled down by the beads, both the magnetic bead-bound material and unbound material were deproteinized, ethanol-precipitated, and analyzed using 8% denaturing polyacrylamide gels. Excision reactions to isolate preincision complex were performed with the biotinylated internally radiolabeled substrates using 50 μg of repair-deficient mutant cell extract. To rule out the possibility that repair proteins were pulled down via nonspecific interactions with substrate DNA, 100 ng of pBR322 plasmid were included as competitor DNA for each experiment. After the 1-h 30 °C incubation, the reaction mixture was placed in the magnetic particle concentrator in order to collect streptavidin bead-bound DNA-protein complex. Subsequently, the bead-bound material was subjected to a second excision reaction in the presence of either a repair-deficient mutant cell extract (50 μg) of a different complementation group or the corresponding purified repair factor. The products of this second excision reaction were then examined using 8% denaturing polyacrylamide gels.

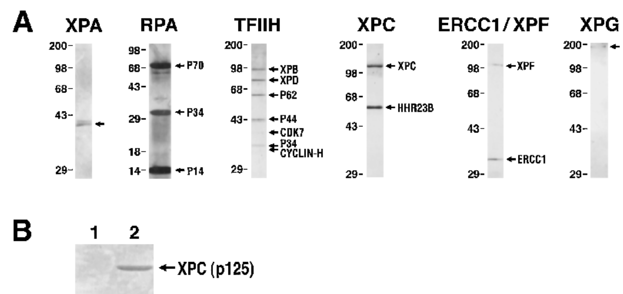

FIG. 1. **The six factors of human excision nuclease.** *A*, silver-stained SDS-PAGE of the six factors. XPA and RPA are recombinant proteins purified from *E. coli*. The other factors were purified from HeLa cells. The amounts of individual factors analyzed by SDS-PAGE were as follows: XPA, 100 ng; TFIIH, 50 ng; XPC, 40 ng; XPG, 40 ng; XPF-ERCC1, 30 ng; and RPA, 800 ng. *B*, lack of XPC in the five fractions that excise cholesterol damage. Ten-fold the amount of the three HeLa cell-derived fractions used in the reconstitution assays was mixed and separated by SDS-PAGE (10%) and subjected to immunoblotting using the ECL detection system according to the manufacturer (Amersham Corp.) (*lane 1*). *Lane 2* contained 1.5 ng of XPC protein as a positive control (<10% of this amount can be detected).

RESULTS

Excision Repair with Purified Proteins—It has been reported that the mixture of highly purified fractions containing XPA, RPA, TFIIH, XPC-HHR23B, XPG, and XPF-ERCC1 was capable of excising DNA lesions in 28-nucleotide-long oligomers from synthetic substrates (Mu *et al.*, 1995) or nicking heavily UV-irradiated plasmid DNA (Aboussekhra *et al.*, 1995). However, some of the fractions used in these reconstitutions contained minor contaminants, and hence, the requirement for additional proteins could not be eliminated (Mu *et al.*, 1995). In fact, it was proposed that an unidentified factor that copurified with XPF-ERCC1 was needed for specific nicking (Aboussekhra *et al.*, 1995). Furthermore, damage-specific nicking was observed in the absence of RPA (Aboussekhra *et al.*, 1995), raising the possibility that RPA was not essential for nicking, but played a stimulatory role. In addition, in both reconstitution systems, only ~1% of the lesions were excised, leading one to suspect that additional factors were needed for optimal activity.

To address the questions raised by these studies, we have purified all six repair factors extensively to obtain proteins of high purity as shown in Fig. 1*A*. The mixture of these fractions excised both thymine dimer and two types of cholesterol lesions (Fig. 2) from DNA in the form of 24–30-nucleotide-long damage-carrying oligomers with high efficiency. Both lesions were excised efficiently and to about the same extent (20–30%) in 5–7 h (Fig. 3). Thus, it appears that the efficiency of the defined excision repair system is comparable to the *in vivo* reaction, suggesting that it is unlikely that additional factors are required for optimal excision. The poor excision efficiency obtained in our previous reconstitution may be attributed to the disproportionate amount of the factors. For instance, we have found that increasing the amount of XPC-HHR23B in the reaction mixture from 15 to 60 ng can severely inhibit excision (data not shown).

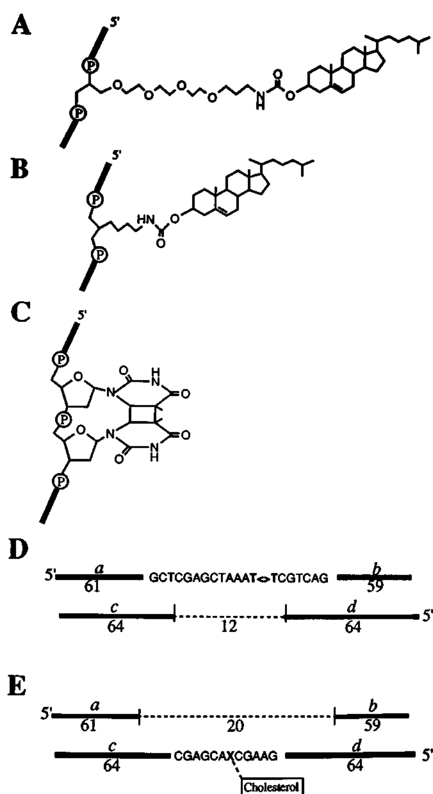

FIG. 2. **Substrates used in this study.** *A*, and *B*, the two types of cholesterol substrates. *C*, thymine dimer. All of these lesions were incorporated into 140-base pair duplexes as described under "Experimental Procedures." For convenience, lesion structures shown in *A* and *B* were termed cholesterol-A and cholesterol-B, respectively. *D* and *E*, schematic drawings of the full-length duplex substrates for the thymine dimer and cholesterol and the oligonucleotides (*a–d*) used for substrate construction.

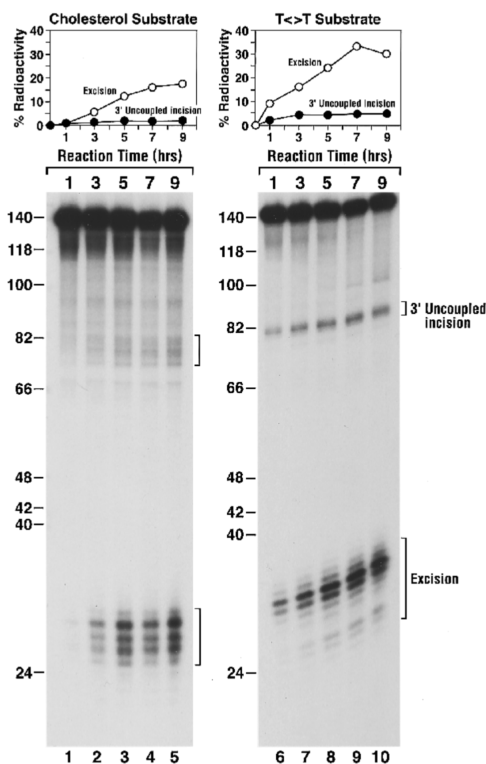

FIG. 3. **Excision of cholesterol-A lesion and thymine dimer by reconstituted human excision nuclease.** *Upper panels*, quantitative analyses of the data. The percent of the input substrate that was either nicked 3′ only or excised is plotted. *Lower panels*, sequencing gels showing the excision of cholesterol-A and the thymine dimer. Cholesterol was excised mainly in the form of 26–28-nucleotide-long oligomers; the thymine dimer was excised in the form of 28–32-nucleotide-long oligomers. The reaction mixtures contained 1 nM DNA substrate and the following concentrations of the repair factors: 20 nM XPA, 0.2 nM TFIIH, 1.6 nM XPC, 0.5 nM XPG, 0.5 nM XPF-ERCC1, and 100 nM RPA.

Requirement for Individual Factors in Dual Incision—Previously, we have shown that all six factors (Fig. 1*A*) are required for excision (Mu *et al.*, 1995). However, that study did not address the question of whether or not single incision occurs when one or more of these six factors are omitted. Particularly, the report of Aboussekhra *et al.* (1995) that a reduced proportion of nicked damaged molecules was observed when RPA was absent suggested that either the 3′- or the 5′-nick can take place without RPA. Hence, we conducted excision/incision assays using the defined excision nuclease system and a 140-base pair duplex with a single thymine dimer under the conditions where one of factors was omitted from the complete system. In this assay, 5′-nick without 3′-incision (uncoupled 5′-incision) is expected to generate fragments in the size range of 88–92 nucleotides, whereas uncoupled 3′-incision is predicted to generate fragments 79–82 nucleotides in length (Huang *et al.*, 1992; Matsunaga *et al.*, 1995). The results of this experiment are shown in Fig. 4*A*. In the complete system, the 27–30-nucleotide-long excision products and, to a lesser extent, the 79–82-nucleotide fragments due to uncoupled 3′-incision appeared (Fig. 4*A*, *lane 3*). Under these experimental conditions, ~20% of the 3′-incisions were not coupled to 5′-incision. Omission of any factor, with the exception of XPF-ERCC1, completely abolished both 5′- and 3′-incisions. Significantly, omission of XPF-ERCC1 gave rise to single incision at the 3′-side of the lesion exclusively (*lane 8*). Moreover, the extent of this uncoupled 3′-incision in the absence of XPF-ERCC1 was equal to the sum of the excision plus uncoupled 3′-incision in the complete system (*lane 3 versus 8*). This suggests that 3′-incision occurs at a normal level in the absence of XPF-ERCC1, the protein responsible for 5′-incision. A second significant point in Fig. 4*A* is that in the absence of RPA (*lane 9*), there was no detectable (<5% of the signals in *lanes 3* and *8*) excision or uncoupled 5′- or 3′-incision, indicating that RPA is required for both incisions.

XPC-HHR23B Is Not Required for Excision of Certain Lesions—Human XP-C mutant cell lines exhibit a unique biochemical phenotype. Even null XP-C mutants repair thymine dimers and (6-4) photoproducts of the template strand of the actively transcribing genes at a normal rate (Venema *et al.*, 1991; van Hoffen *et al.*, 1995). To accommodate these observations, it was hypothesized either that a stalled RNA polymerase substitutes for XPC in the assembly of excision nuclease or that the structure of damaged DNA in the stalled complex obviates the need for the XPC protein. When we tested a cholesterol-containing substrate (cholesterol-B) (Fig. 2*B*) in addition to the thymine dimer substrate in reconstitution experiments, a surprising result was obtained. Fig. 4*B* shows that, in contrast with the thymine dimer, the cholesterol-B lesion was excised in the absence of XPC (*lane 7*). However, the level of excision without XPC was lower than that of the complete system, and omission of XPC caused more degradation of the substrate than the omission of any of the other factors. Similarly, omission of XPC and XPF-ERCC1 led to extensive degradation without excision (*lane 3*), consistent with the notion that in the absence of XPF-ERCC1, 3′-incision was made. These results suggest that XPC is not required for either the 3′- or the 5′-incision with this particular substrate. Without the protection of XPC, the incised DNA as well as the full-length

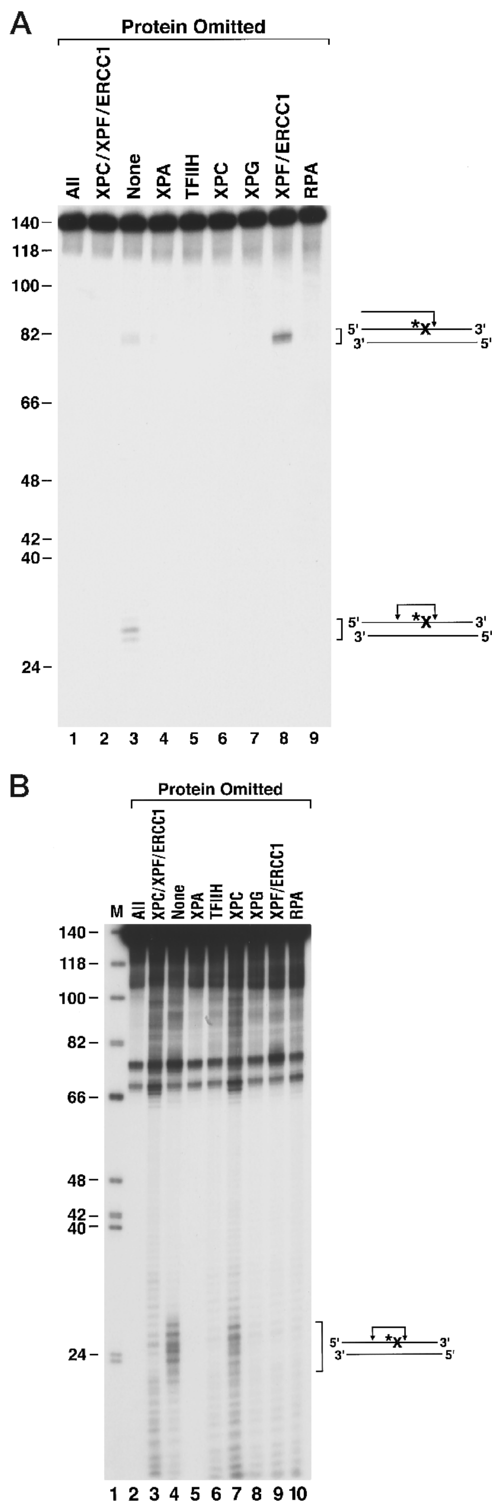

FIG. 4. **Differential requirement for excision of thymine dimer (T<>T) and cholesterol-B lesions.** *A*, excision of T<>T. The positions of the excision product and uncoupled 3′-incision are indicated. Substrate (3.5%) was excised, and 1.5% of the substrate contained uncoupled 3′-incision in the complete system (*lane 3*). In the reaction without XPF-ERCC1 (*lane 8*), 5% of the substrate contained an uncoupled 3′-nick. *B*, excision of cholesterol-B lesion. The positions of the main excision products are shown by a *bracket*. The level of excision in the area indicated by the *bracket* was 3.5% in the complete system (*lane 4*) and 2.7% in the reaction lacking XPC (*lane 7*). *Lane 1*, size markers (*M*) in nucleotides.

substrate were degraded possibly by the nonspecific nuclease activity of XPG or XPF-ERCC1. The phenomenon of XPC-independent excision of cholesterol-B lesion, however, cannot be entirely attributed to the cholesterol molecule because the excision of a second cholesterol-containing substrate, cholesterol-A lesion (Fig. 2*A*), does depend on XPC (data not shown). Without solving the molecular structure of these various substrates, it is impossible to determine why excision of cholesterol-B lesion is XPC-independent. However, the observation that this lesion is excised in the absence of XPC and of transcription may be taken as evidence for the notion that in transcription-coupled repair, the structure of the DNA in the stalled complex rather than RNA polymerase *per se* enables the excision nuclease to assemble and excise damage from the template strand at an essentially normal rate.

Since these results were quite unexpected, we considered the possibility that low level contaminant XPC in our other factors may be responsible for the excision nuclease activity reconstituted without XPC-HHR23B. The mixture of other HeLa cell-derived factors (TFIIH, XPG, and XPF-ERCC1) was tested for XPC contamination by Western analysis. With an assay capable of detecting 10 pg of XPC in our reaction mixture, no XPC was detectable (Fig. 1*B*). This enables us to rule out the presence of contaminating XPC that would support the excision of cholesterol-B lesion by XPC-omitted reconstitution reaction. An implication emerging from these observations is that with certain substrates or with all substrates in special structures as exist during transcription, XPC is not needed for excision nuclease activity.

Assembly of Excision Nuclease—Although the properties of the excision nuclease subunits in isolation have led to plausible models for the assembly of this complex enzyme system (Tanaka and Wood, 1994; Sancar, 1995; Park *et al.*, 1995a), direct evidence for these models was limited. The data presented in Fig. 4*A* (*lane 8*) reveal that the 3′-incision that is made by XPG (Harrington and Lieber, 1994; O'Donovan *et al.*, 1994; Matsunaga *et al.*, 1995) occurred to the normal extent without XPF-ERCC1, demonstrating that the five other fractions can assemble at the lesion site without XPF-ERCC1. Consequently, XPF-ERCC1 may be the last factor to join the assembly.

In contrast, no 5′-incision was observed in the absence of XPG (Fig. 4*A*, *lane 7*). Two explanations were considered for this finding. (i) XPG must be present to form the preincision complex; and (ii) the 5′-incision made by XPF-ERCC1 depends on the presence of the 3′-incision made by XPG, but not on the actual existence of XPG in the preincision complex. Previous studies have shown that inhibition of 3′-incision by anti-XPG antibodies does not interfere with the 5′-incision, indicating that the formation of 5′-incision is not dependent on the production of the 3′-nick (Matsunaga *et al.*, 1995). Hence, the failure to observe 5′-incision in the absence of XPG might be due to the fact that XPG must be present in the preincision complex to enable XPF-ERCC1 to bind and carry out the 5′-incision. To test this model, we conducted the following experiment. CFE from an XP-F or XP-G mutant cell line was mixed with substrate that contained biotinylated nucleotides at the 3′-termini. Following incubation, the substrate and preincision complexes on the substrate were removed from the unbound cellular proteins using streptavidin-attached magnetic beads. An XP-G or XP-F cell-free extract was then added to the immobilized complexes isolated from XP-F or XP-G CFE, respectively. The results reveal that addition of the XP-G extract (which contains XPF-ERCC1) or purified XPF-ERCC1 to the DNA-protein complex isolated from the XP-F extract produced excision (Fig. 5, *lane 2*). In contrast, addition of XP-F CFE to the complexes pulled down from the XP-G extract in the same manner did not result in excision (*lane 4*). From these data, we conclude that XPG enters the preincision complex before XPF

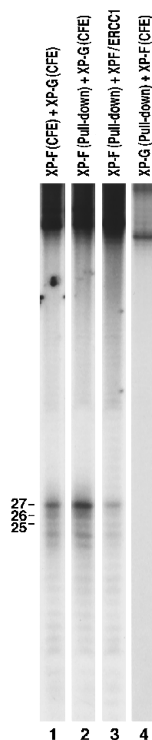

FIG. 5. **Preincision complex formation with extract lacking XPF, but not with extract deficient in XPG.** Cell-free extracts from XP-F (GM08437A) or a Chinese hamster ovary cell line defective in XPG (UV135) were mixed with internally radiolabeled cholesterol-A substrate with a 3′-biotin tag. The preincision DNA-protein complex was isolated by the pull-down method as described under "Experimental Procedures" and complemented with either a second mutant extract or purified repair factor. *Lane 1*, the XP-F plus XP-G cell-free extract was used as a positive control. *Lane 2*, the substrate pulled down in XP-F CFE with streptavidin beads was incubated in XP-G CFE. *Lane 3*, same as *lane 2*, except that purified XPF-ERCC1 (2 ng), instead of the XP-G cell-free extract, was added to the pull-down DNA-protein complex. *Lane 4*, the substrate pulled down following incubation with XP-G CFE by beads was then incubated with XP-F CFE. The positions of the major excision products are marked to the left of the *lane 1*.

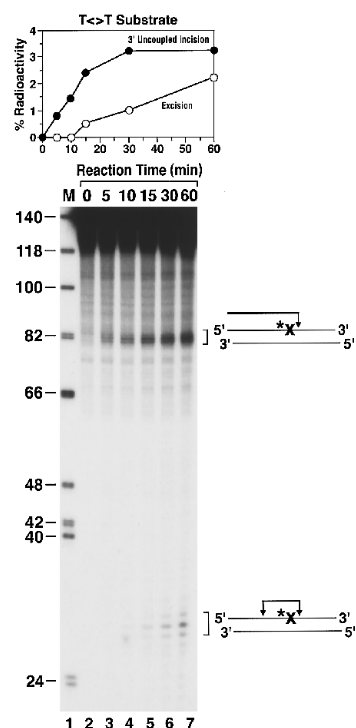

FIG. 6. **Order of incision.** *Upper panel*, shown are quantitative analyses of the data in the *lower panel*. *Lower panel*, internally radiolabeled thymine dimer substrate was incubated with the reconstituted excision nuclease. Aliquots were taken at the indicated time points, quenched with 20 μg of proteinase K, and analyzed on an 8% sequencing gel. The uncoupled 3′-incision appeared before the excision product, which required both 3′- and 5′-incisions. Uncoupled 5′-incision (which is not observed) would have produced fragments of 88–92 nucleotides. *Lane 1*, size markers (*M*) in nucleotides.

and that XPG is required for positioning of the XPF-ERCC1 nuclease in nicking at the 5′-side of damage.

Order of Incision—The finding that XPG, along with other factors except XPF-ERCC1, can make the 3′-incision raised the possibility that the 3′-incision may precede the 5′-incision. However, in a previous study, it was found that anti-XPG antibodies inhibited 3′-incision while having only a modest effect on 5′-incision, whereas anti-ERCC1 antibodies inhibited both 3′- and 5′-incisions to the same extent (Matsunaga *et al.*, 1995). These observations are consistent with 5′-incision preceding and being necessary for 3′-incision. To investigate the order of incision directly, a kinetic experiment was performed to examine the appearance of the two incisions. The *lower panel* of Fig. 6 shows the results of this experiment, and the *upper panel* shows the quantitations of uncoupled 3′-nicks and excision that represents the 5′-nicks coupled to 3′-nicks on internally radiolabeled substrate. At early time points (*lanes 3* and *4*), 3′-nicks started to build up, while no 5′-incision (excision) was detectable, suggesting that 3′-incision happens first. While the uncoupled 3′-nicks seemed to level off after 30 min, dual incision (excision) continued to accumulate. The results of kinetic experiments over a much longer period of time using the same T<>T substrate were as shown in Fig. 3: the level of uncoupled 3′-nicks remained approximately constant at <5%, whereas the dual incision went up to 30%. Although the lag time between the two 3′- and 5′-incisions seemed to vary somewhat (see the level of T<>T excision at the 60-min time point in Figs. 3 and 6), the appearance of 3′-incision ahead of 5′-incision has been reproducible. Thus, analogous to the bacterial excision nuclease (Lin and Sancar, 1992), we conclude that in human excision nuclease, the 3′-incision precedes the 5′-incision.

Role of TFIIH and CDK-activating Kinase in Excision—The general transcription factor TFIIH is an essential general excision repair factor (Drapkin *et al.*, 1994; Schaeffer *et al.*, 1994; Wang *et al.*, 1994). Its polypeptide composition ranges from 5 to 10 depending on the purification procedure (Gerard *et al.*, 1991; Conaway and Conaway, 1993; Zawel and Reinberg, 1995). The precise function of TFIIH in excision nuclease is not known; however, two of its subunits, XPB and XPD, have helicase activities that are thought to be important for locally unwinding the duplex and thus targeting the nuclease subunits to their proper sites. Indeed, it has been shown that excision is absolutely dependent on ATP hydrolysis (Svoboda *et al.*, 1993). However, it was not clear whether both 3′- and 5′-incisions required ATP hydrolysis. To answer this question, we performed an incision assay with 5′-terminally labeled DNA in a standard reaction mixture with an increasing amount of ATPγS. Fig. 7 shows that the poorly hydrolyzable ATPγS competed with ATP and inhibited both 5′- and 3′-incisions, ruling out the possibility that uncoupled single incision may occur without ATP hydrolysis. This lends credence to the notion that TFIIH helicase-directed unwinding of DNA (not just ATP binding-induced conformational change) is required for targeting the nuclease subunits and enabling them to attack the appropriate phosphodiester bonds.

Recently, the CDK7-cyclin H pair was found to be a constituent of TFIIH and to be responsible for its kinase activity (Roy

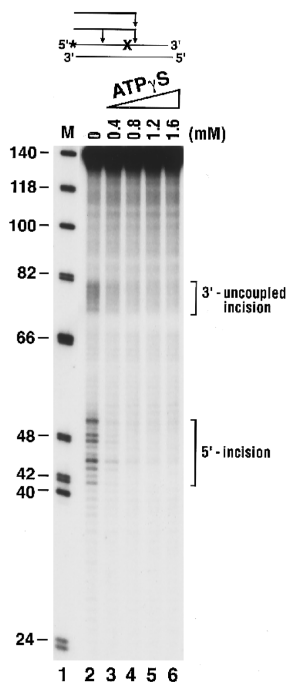

FIG. 7. **ATP hydrolysis is required for both 3′- and 5′-incisions.** To the excision assay containing 2 mM ATP were added the indicated amounts of ATPγS (Pharmacia Biotech Inc.), and the reaction was carried out for 60 min at 30 °C with 5′-terminally radiolabeled cholesterol-A substrate. *Lane 1*, size markers (*M*) in nucleotides.

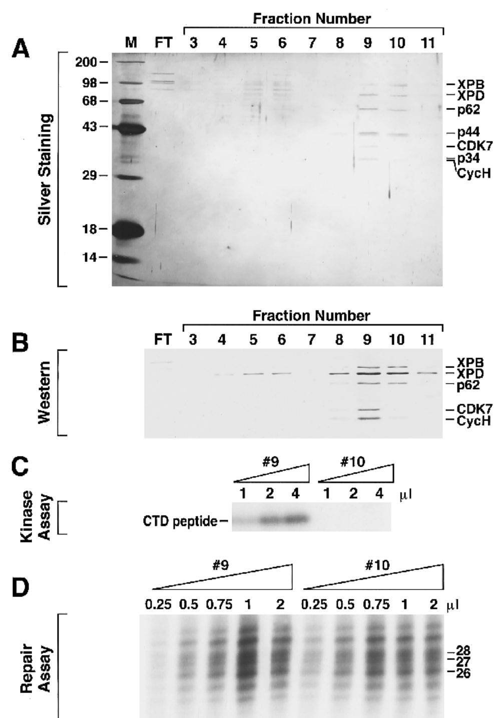

FIG. 8. **Roles of TFIIH subunits in excision.** *A*, column profile of the final Mono S column chromatography step visualized by silver-stained SDS-PAGE (10%). *B*, analysis of the same fractions by immunoblotting using a mixture of anti-XPB, anti-XPD, anti-p62, anti-CDK7, and anti-cyclin H (*CycH*) antibodies. *C*, CTD peptide kinase assay with holo-TFIIH and core TFIIH using increasing amounts of fractions 9 and 10. Each microliter of TFIIH fractions 9 and 10 contained ~1 ng of protein as determined by the dye binding protein assay (Bio-Rad). Kinase assay was conducted as described by Aprelikova *et al.* (1995). *D*, excision assays with holo-TFIIH and core TFIIH. The positions of the major excision products are indicated. *M*, size markers in nucleotides; *FT*, flow-through fractions.

et al., 1994; Serizawa *et al.*, 1995; Shiekhattar *et al.*, 1995). Furthermore, it was reported that microinjection of antibodies to CDK7 inhibited excision repair *in vivo* (Roy *et al.*, 1994), leading to the conclusion that CDK7 was essential for excision repair. Our purest TFIIH fraction (fraction 9 of the last Mono S column chromatography step) contained stoichiometric CDK7 and cyclin H subunits. However, in the last purification step, a second form of TFIIH devoid of CDK7 and cyclin H was also obtained (fraction 10) (Fig. 8, *A* and *B*), as evidenced by immunoblottings using anti-CDK7 and anti-cyclin H antibodies and a kinase assay to detect the phosphorylation of a tetraheptapeptide repeat (YSPTSPS) of the carboxyl-terminal domain (CTD) of RNA polymerase II. Fig. 8*C* shows that the CDK-activating kinase-lacking TFIIH fraction 10 has <2% of the CTD peptide kinase activity of the TFIIH holoenzyme (faction 9), consistent with the notion that CDK7-cyclin H is responsible for the kinase activity of TFIIH. To ensure that CDK7-cyclin H was not in our other HeLa cell-purified basal repair factors (XPC, XPG, and XPF-ERCC1), we performed the same kinase assay on both individual factors and a combination of the entire set and found no indication of CTD peptide kinase activity (data not shown). When the two forms were tested for reconstitution of excision nuclease activity, no difference was detected between the form containing CDK7-cyclin H and the fraction without it (Fig. 8*D*). Thus, we conclude that the CDK7-cyclin H pair neither is necessary for nor interferes with the repair function of TFIIH.

Fates of Excised Oligomer and Gapped DNA—Following the dual incision in bacterial excision repair, two of the repair proteins, the excised oligomer, and the gapped DNA remain associated in a complex. This complex is later disrupted by repair synthesis proteins, which ultimately fill the repair gap and restore the duplex (Orren *et al.*, 1992). Given the bacterial precedent, we wished to know if in humans the excision nuclease (or a subassembly of it), the excised oligomer, and the gapped DNA remain in a postexcision complex prior to the disruption by repair synthesis proteins. Internally radiolabeled substrate with a terminal biotin tag was incubated with either *E. coli* (A)BC excinuclease or human excision nuclease, and then the unrepaired substrate and gapped DNA were removed from the reaction mixture by using streptavidin-attached magnetic beads. Both the streptavidin-bound fraction and the unbound material were analyzed on a sequencing gel. Fig. 9 shows that in this experimental system, >90% of the DNA was biotinylated and removed from solution by the streptavidin beads (*lanes 2* and *3*). When the *E. coli* excinuclease products were analyzed in this manner, it was found that all of the excised oligomer remained associated with the gapped duplex (*lane 4 versus 5*). This is in agreement with previous studies that showed that after incision the UvrB and UvrC subunits and the excised oligomer remain in the same complex with the gapped DNA (Caron *et al.*, 1985; Orren *et al.*, 1992). To our surprise, with the human excision nuclease system, ~90% of the excised oligomer was left in the streptavidin-unbound fraction, showing that the excised oligomer is off the gapped duplex DNA (*lane 6 versus 7*). Thus, unlike the bacterial system, in humans, the excised oligomer is released without the aid of repair synthesis proteins.

To investigate whether or not the excised oligomer and the gapped DNA were also released from the excision nuclease or its subunits or subassemblies, we conducted an excision assay using a mixture of internally and 5′-terminally labeled substrates. The reaction products were analyzed on nondenaturing polyacrylamide gels. As shown in Fig. 10*A*, three major bands (bands a–c) were observed in addition to free substrate (*lane 2*). The DNA within these bands was analyzed on sequencing gels.

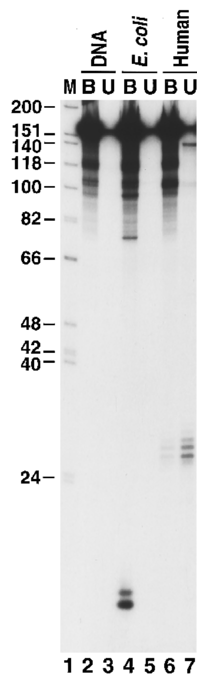

FIG. 9. **Human excision nuclease releases excised oligomers from gapped duplex.** The 3′-terminally biotinylated, internally radiolabeled cholesterol-A substrate 156 nucleotides in length (Huang and Sancar, 1994; Shi *et al.*, 1987) was incubated with *E. coli* or human excision nuclease. The DNA was then pulled down with streptavidin beads, and the streptavidin bead-bound (*B*) and -unbound (*U*) fractions were analyzed on a sequencing gel. *Lane 1*, size markers (*M*) in nucleotides; *lanes 2* and *3*, substrate DNA incubated only in reaction buffer and then separated by streptavidin beads; *lanes 4* and *5*, reaction with the bacterial enzyme; *lanes 6* and *7*, reaction with the human excision nuclease. The locations of bacterial (12–13 nucleotides) and human (26–28 nucleotides) excision products are indicated. The excision reaction with the *E. coli* UvrA, UvrB, and UvrC proteins was carried out as described by Thomas *et al.* (1985).

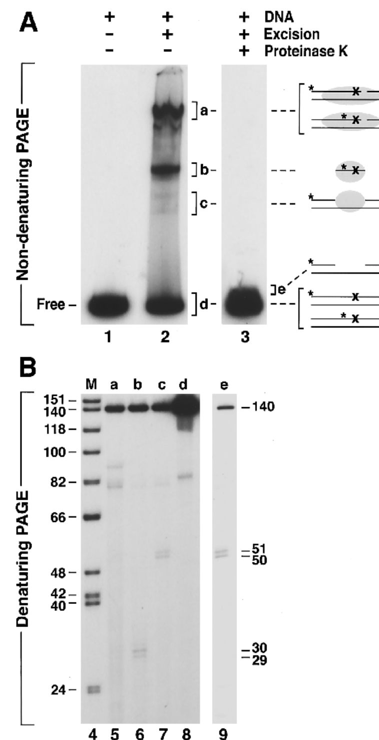

FIG. 10. **Analysis of postexcision complexes.** *A*, analysis of reaction products using nondenaturing PAGE. Excision reaction with a mixture of internally and terminally labeled T<>T substrates was performed, and the products were separated on a 4% nondenaturing polyacrylamide gel. *Lane 1*, DNA alone; *lane 2*, total excision reaction mixture; *lane 3*, total excision reaction mixture treated with proteinase K. Nondenaturing gel electrophoresis conditions were as described by Mu *et al.* (1995). *B*, analysis of the DNA in the DNA-protein complexes on sequencing gels. *Lane 4*, size markers (*M*) in nucleotides; *lanes 5–9*, bands a–e, respectively, from the gels shown in *A*. The positions of the excision product at 29–30 nucleotides and of the 5′-incision at 50–51 nucleotides are indicated. Note that due to the large amount of unrepaired DNA, all retarded bands contained some full-length substrate as a contaminant.

Fig. 10*B* (*lane 5*) shows that the slowest migrating band (band a) contained unrepaired DNA and DNA with an uncoupled 3′-nick. The middle band (band b) contained the excised oligomer, but not gapped DNA, as evidenced by the lack of 50–51-nucleotide-long fragments generated from the 5′-labeled DNA by 5′-incision (*lane 6 versus 7*). Finally, the fastest migrating DNA-protein complex (band c) contained the gapped DNA and no excised oligomer (*lane 7*). To ensure that band c was not protein-free gapped DNA with anomalous migration due to its unusual structure, the same reaction mixture was treated with proteinase K prior to analysis by native gels. As seen in Fig. 10*A* (*lane 3*), under these conditions, only two closely migrating bands were observed: the unrepaired DNA and a second, fainter band (band e). Analysis of band e on a sequencing gel revealed that it contained the gapped DNA, as evidenced by the presence of 50–51-nucleotide-long labeled oligomers (Fig. 10*B*, *lane 9*). Therefore, the retarded band c in *lane 2* represents a protein-bound form of gapped DNA.

In conclusion, these experiments reveal that even though the excised oligomer is released from the gapped DNA, both the excised oligomer and the gapped DNA are complexed with proteins. Currently, we do not know the identities of the proteins in these complexes. However, it is important that the excision gap is protected by repair proteins from nonspecific nucleases until repair synthesis takes place.

DISCUSSION

Using the excision repair factors purified to homogeneity, we have reconstituted human excision repair nuclease and investigated the roles of the individual components and the sequence of events in the excision reaction. We have used synthetic substrates with either a natural (thymine dimer) or an artificial (cholesterol) lesion at a unique site for these studies. Thus, the combination of repair factors of high purity and of uniform substrate has enabled us to probe the reaction mechanism in considerable detail. In previous reconstitution studies of both human (Mu *et al.*, 1995; Aboussekhra *et al.*, 1995) and yeast (Guzder *et al.*, 1995) excision nucleases, the efficiency of the reconstituted systems was very low. Typically, $<1\%$ of the substrate was repaired. Such low efficiency raised the possibility that the reconstituted systems lacked some additional factors required for optimal activity. The present study shows that, in fact, no additional factors are required for the basal excision reaction. By having sufficient quantities of all of the repair factors, we were able to optimize the concentration of each in the reaction mixture so that the excision rate and extent were comparable to the *in vivo* values. Thus, we consider the results obtained with this system to be applicable to excision repair *in vivo*. In the following text, we attempt to address the roles of the various components in light of results presented in this paper and previous studies and present a reaction scheme (Fig. 11) consistent with existing data on human nucleotide excision repair.

Role of RPA—Using anti-RPA antibodies and cell-free extract, it was found that RPA was required for the repair synthesis step of excision repair (Coverley *et al.*, 1991). More recently, it was reported that RPA was absolutely required for

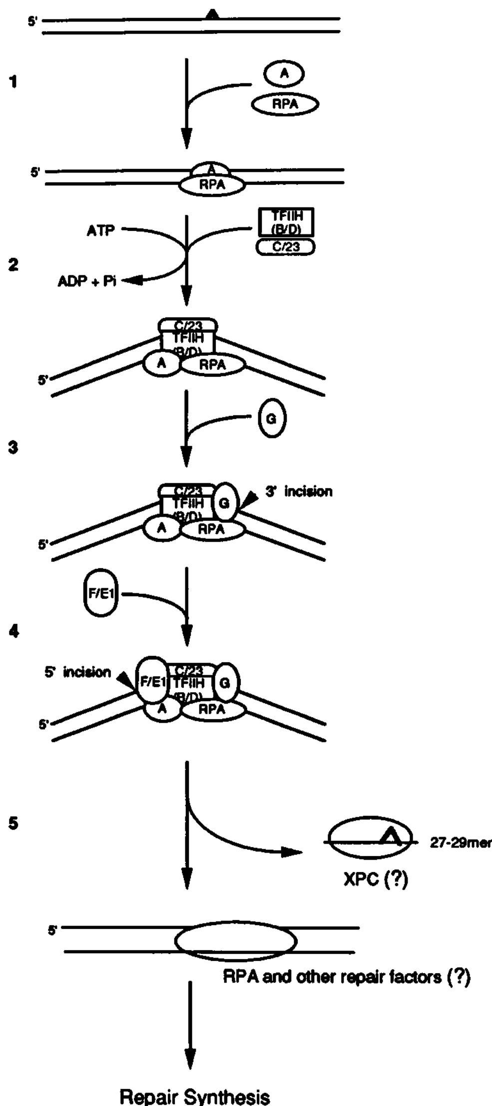

FIG. 11. **Model for mechanism of human excision nuclease.** Damage recognition is achieved by the combination of RPA-XPA (*step 1*), followed by the recruitment of TFIIH and XPC-HHR23B to locally perturb the DNA structure around the lesion in an ATP-dependent manner (*step 2*). The arrival of XPG through its interaction with TFIIH and RPA stabilizes the preincision complex and results in the 3′-incision (*step 3*). Subsequent recruitment of XPF-ERCC1 by XPA to the incision complex produces the 5′-incision (*step 4*). Upon the cutting by XPF-ERCC1 at the 5′-side, the excised lesion-containing oligomer is dissociated from the gapped duplex DNA and bound by protein (possibly XPC), leaving behind the gapped DNA protected by RPA from nucleases (*step 5*).

excision of DNA damage (Mu *et al.*, 1995). However, another study reported that even in the absence of RPA, damage-dependent nicking of substrate plasmid occurred, albeit at a reduced proportion relative to the complete system (Aboussekhra *et al.*, 1995), suggesting that either one of the two nicks could occur without RPA or that both nicks could occur with the other components alone, but that RPA stimulated the reaction. The results presented here clearly show that RPA is absolutely required for both the 5′- and 3′-incisions. In line with this finding, it has been found that RPA dramatically stimulates the 5′-junction incision activity of XPF-ERCC1 and the 3′-junction cutting activity of XPG.[2] Given the recent reports that RPA tightly binds to XPA (He *et al.*, 1995; Matsuda *et al.*, 1995; Li *et al.*, 1995) and our finding that both 3′- and 5′-incisions are absolutely dependent on RPA, we propose that RPA, in combination with XPA, acts as a damage recognition complex and directs the nuclease subunits to the proper incision sites by its specific interactions. The results obtained with yeast reconstituted excision nuclease are in agreement with this model. The highly purified yeast excision nuclease system was found to be absolutely dependent on RPA for nicking of UV-irradiated plasmid (Guzder *et al.*, 1995).

Role of XPC—In humans, the template strand of transcribed genes is repaired preferentially (Mellon *et al.*, 1987). Most XP mutants are defective in genome overall repair as well as in strand-specific repair (Hanawalt, 1994). However, XP-C mutants are exceptional. These mutants repair the template strand of actively transcribing genes at a normal rate, while totally lacking in repair of nontranscribed sequences (van Hoffen *et al.*, 1995). It was therefore proposed that XPC can be substituted by either another protein (*e.g.* RNA polymerase) or a special DNA structure present in a RNA polymerase-stalled complex. The results presented in this paper show that with certain substrates, excision can occur without XPC even in the absence of transcription. In light of these results, we propose that the unique conformation of DNA of the stalled transcription complex rather than RNA polymerase itself plays the crucial role in XPC-independent repair. Interestingly, of all of the yeast homologs of human excision repair proteins, the XPC homolog Rad4 shows the least sequence homology (Legerski and Peterson, 1992; Matsutani *et al.*, 1994), and in contrast to XP-C mutants, yeast Rad4 mutants are totally defective in repair (Prakash *et al.*, 1993; Friedberg *et al.*, 1995). The precise role of XPC is not known. However, this protein binds to DNA nonspecifically with high affinity and avidity (Matsutani *et al.*, 1994)[3] and interacts with TFIIH with moderate affinity (Drapkin *et al.*, 1994). Furthermore, in our reconstitution experiments, omission of XPC led to degradation of the damaged strand, but not the undamaged strand (data not shown), presumably by the exonuclease function of XPG (Habraken *et al.*, 1994). Thus, a plausible function for XPC is that it binds to the damaged strand in the preincision complex and stabilizes the unwound state generated by TFIIH. Following the dual incision, XPC may protect DNA from the nuclease subunits of excision nuclease as well as other nonspecific cellular nucleases.

Role of TFIIH—TFIIH is recruited to the damage site by XPA (Park *et al.*, 1995a). Analogous to its function in transcription, it possibly unwinds DNA surrounding the lesion. Inhibition of both 3′- and 5′-incisions by ATPγS indicates that the helicase function of this factor (Schaeffer *et al.*, 1993) is important for both incisions. The unwinding activity is intrinsic to the XPB and XPD subunits (Weeda *et al.*, 1990; Sung *et al.*, 1993; Weber *et al.*, 1990). The p44 and p34 subunits that have zinc fingers (Humbert *et al.*, 1994) may play a role in DNA binding. An important question concerning TFIIH function is whether or not its kinase component, CDK7-cyclin H, is required for its repair activity. Roy *et al.* (1994) reported that microinjection of anti-CDK7 antibodies inhibited excision repair *in vivo* and concluded that CDK7 (also known as MO15) was needed for repair. In an apparent contradiction, it was reported that in yeast two forms of TFIIH existed, one without Kin28 and CCL1 and one with these proteins and kinase activity (Feaver *et al.*, 1994; Svejstrup *et al.*, 1995). Evidence was presented that the core TFIIH without the kinase component restored repair activity to cell-free extracts of repair-deficient mutants (Svejstrup *et al.*, 1995). In humans, we found that TFIIH with

[2] T. Matsunaga and A. Sancar, unpublished results.

[3] J. T. Reardon and A. Sancar, unpublished results.

or without the CDK-activating kinase subunit was equally active in excision repair using a repair system of highly purified proteins. The fact that the CDK-activating kinase subunits copurify with the core subunits through a seven-column purification scheme leads us to conclude that the interaction of CDK-activating kinase with the core subunits is rather strong. Therefore, with holo-TFIIH, we do not think that the two parts dissociate during the excision reaction. As to the *in vivo* inhibition of excision repair by anti-CDK7 antibodies, it may be taken as evidence that most of the TFIIH is in the holo-TFIIH form *in vivo* and that binding of the antibodies with holo-TFIIH simply prevents its entry into the repair complex due to steric hindrance, rather than that CDK-activating kinase or its activity is required for excision repair.

Roles of XPG and XPF-ERCC1—These are the nuclease subunits of the excision nuclease system. XPG is a single strand-specific endonuclease with preference for single- to double-stranded DNA junctions for the strand making the transition in the 5′ to 3′ direction (O'Donovan *et al.*, 1994). The protein also has a weak 5′ to 3′ exonuclease activity (Habraken *et al.*, 1994). Antibody inhibition experiments suggested that XPG is the nuclease that makes the 3′-incision (Matsunaga *et al.*, 1995), consistent with the specificity of junction cutting activity. XPF-ERCC1 is also a single strand-specific endonuclease (Park *et al.*, 1995b) with special affinity (stimulated by RPA) for junctions where the single-stranded region makes the transition to double-stranded DNA in the 3′ to 5′ direction,[2] consistent with the previous finding that XPF-ERCC1 makes the 5′-incision (Matsunaga *et al.*, 1995). The biotin-streptavidin pull-down experiments described in this paper demonstrated that XPG is required for the formation of a stable preincision complex. Thus, in addition to its nuclease function, XPG has a structural role in maintaining a stable preincision complex, which then recruits XPF-ERCC1. From an XP-F mutant cell-free extract, a DNA-protein complex can be isolated that, upon supplementing with wild-type XPF from a different mutant cell extract or purified XPF protein, leads to excision. The extent of the 3′-incision observed in the defined system without XPF-ERCC1 is equal to that of uncoupled 3′-nicks plus excision by the complete system (Fig. 4*A*, *lanes 3* and *8*), indicating that the complex formed in the absence of XPF-ERCC1 is functionally similar to the one formed in the presence of XPF-ERCC1. In this regard, the human excision nuclease system differs from the yeast excinuclease system despite numerous similarities (including number and sequence of subunits) between the two systems (Prakash *et al.*, 1993; Friedberg *et al.*, 1995): in yeast, uncoupled 3′-incision does not occur in the absence of the Rad1-Rad10 complex, which is the structural and functional homolog of XPF-ERCC1 (Guzder *et al.*, 1995).

In light of the documented interactions between XPA and XPF-ERCC1 (Li *et al.*, 1994; Park and Sancar, 1994) and data presented herein, it is tempting to speculate that XPA not only participates in damage recognition, but also acts as an anchor to recruit XPF-ERCC1 to the proper 5′-incision sites. In other words, this model predicts that XPA must be an integral part of the excision nuclease-DNA complex from the first step of damage recognition to the step prior to 5′-incision.

Human XP-F mutants exhibit a unique phenotype: shortly after UV irradiation, these cells repair damage at 10–15% of the normal level, but excision increases up to 60% with further incubation. Unlike most other XP mutants (with the exception of XP-E), XP-F cells are only moderately UV-sensitive (Cleaver and Kraemer, 1989). Surprisingly, however, CFEs from these mutants are totally defective in excision nuclease activity as determined by an assay capable of detecting 0.1% of normal excision activity (Reardon *et al.*, 1993). In view of the results in this paper that XPF-ERCC1 is not required for 3′-incision, an explanation can be offered for this abnormal phenotype of XP-F cells: the other five factors of the excision nuclease assemble and make the 3′-incision, which ultimately leads to damage removal by nonspecific 3′ to 5′ acting exonuclease(s). Hence, the reaction occurs slowly compared with the normal excision. Such an explanation, in turn, raises an important question: is the uncoupled 3′-incision a normal event during the course of action of human excinuclease, and does it persist for a significant length of time until the XPF-ERCC1 complex diffuses to the lesion site and makes the 5′-incision? When excision reaction was carried out in HeLa CFE, futile 3′-nicking was a rare event, implicating the presence of "a mechanism" in CFE to maintain efficient coupling of the two nicks. This mechanism of keeping tightly coupled dual incision may be accomplished by several means. (i) The entire set of excision repair proteins may exist in a large complex termed "repairosome," as has been suggested for yeast (Svejstrup *et al.*, 1995). (ii) XPF-ERCC1 is actually recruited to the damage site early on, despite the failure to obtain supporting evidence in this study. (iii) An as yet unidentified protein can suppress 3′-incision by XPG. Upon displacement of this protein by XPF-ERCC1, both 3′- and 5′-incisions occur in a concerted but nonsynchronous manner. More experiments are needed to differentiate these models.

Postincision Reactions—In the bacterial excinuclease system, the "excised" oligomer, the two nuclease subunits, and the gapped DNA remain in a complex that is subsequently disrupted by repair synthesis proteins. Surprisingly, in human excinuclease, following the dual incision, the excised oligomer is set free from the gapped DNA. However, both the excised oligomer and the gapped DNA are bound to specific repair proteins, as evidenced by the presence of unique DNA-protein complexes following dual incision. This is especially important for protection of gapped DNA from attack by nonspecific nucleases, which would otherwise produce double strand breaks. At present, we do not know the identity of the proteins associated with either the excised oligomer or the gapped DNA. However, of special note is the intriguing observation that the excised 29-mer complex has slower mobility than the DNA-protein complex of the gapped 140-mer product. Given the 30-nucleotide binding pocket of human RPA (Kim *et al.*, 1994), it is quite conceivable that the gapped DNA is associated with RPA alone and thus primed for gap filling by PCNA-dependent DNA polymerase δ or ϵ (Shivji *et al.*, 1992; Nichols and Sancar, 1992). The excised oligomer might be complexed with XPC since it undergoes degradation in the reactions where excision takes place without XPC. Future work is needed to understand the postincision events and the precise composition of excision nuclease at various stages.

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