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Reconstitution of Human Excision Nuclease with Recombinant XPF-ERCC1 Complex*

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The human XPF-ERCC1 protein complex is one of several factors known to be required for general nucleotide excision repair. Genetic data indicate that both proteins of this complex are necessary for the repair of interstrand cross-links, perhaps via recombination. To determine whether XPF-ERCC1 completes a set of six proteins that are sufficient to carry out excision repair, the human *XPF* and *ERCC1* cDNAs were coexpressed in Sf21 insect cells from a baculovirus vector. The purified complex contained the anticipated 5′ junction-specific endonuclease activity that is stimulated through a direct interaction between XPF and replication protein A (RPA). The recombinant complex also complemented extracts of XP-F cells and Chinese hamster ovary mutants assigned to complementation groups 1, 4, and 11. Furthermore, reconstitution of the human excision nuclease was observed with a mixture of five repair factors (XPA, XPC, XPG, TFIIH, and RPA) and the recombinant XPF-ERCC1, thus verifying that no additional protein factors are needed for the specific dual incisions characteristic of human excision repair.

Nucleotide excision repair in humans consists of dual incisions on both sides of the lesion in the damaged strand, which results in excision of 24–32-nucleotide-long oligomers followed by repair synthesis and ligation (1–3). The enzyme system responsible for the dual incisions is referred to as excision nuclease (2). Individuals lacking excision nuclease suffer from xeroderma pigmentosum (XP),[1] a disease that is characterized by photodermatoses and in some cases by neurological abnormalities (4, 5).

Recently, human excision nuclease has been reconstituted from highly purified proteins including those encoded by *XPA* through *XPG* (6–8). These reconstitutions have helped define the minimum essential set of proteins for the dual incision/excision activity. It was found that all proteins encoded by the XP genes with the exception of the XPE gene product were required for excision. Other proteins necessary for this reaction pathway are replication protein A (RPA, consisting of three subunits), transcription factor TFIIH (composed of 5–8 subunits including XPB and XPD), and ERCC1 (which together with XPF forms a distinct complex). These studies were conducted with proteins purified mostly from human cells; therefore, a requirement for additional proteins that were present in the reconstitution fractions as "contaminants" was a realistic possibility. The need for additional unknown proteins was argued using evidence that a factor (IF7) of one or more proteins that purified through several columns with XPF-ERCC1 and finally separated from this complex by a DEAE resin was essential for incision specificity by the reconstituted human excision nuclease (8). However, a later report with proteins purified to apparent homogeneity failed to confirm the need for an additional factor (7). To clarify the discrepancy raised by these studies, we attempted to use proteins expressed and purified from heterologous systems because the presence of trace amounts of IF7 cannot be excluded with the most extensively purified human cellular preparations of XPF-ERCC1. The recent cloning of *XPF* (*ERCC4*) (9) and subsequent isolation of its cDNA (10, 11) provide the opportunity to produce the recombinant XPF-ERCC1 complex in a surrogate host. Toward this goal, we expressed the XPF-ERCC1 complex in abundance using a baculovirus vector, purified the complex to homogeneity, and verified its activity as a junction-specific endonuclease (12) that incises in the 5′ direction of a DNA lesion (7). Recombinant XPF-ERCC1, referred to hereafter as r(XPF-ERCC1), also complemented extracts of XP-F cells and hamster mutants in complementation groups 1, 4, and 11. The complex is fully capable of reconstituting human excision nuclease when combined with TFIIH, XPC, and recombinant XPA, RPA, and XPG proteins. Our data show that only these six factors are needed for damage-specific dual incision by human excision nuclease.

MATERIALS AND METHODS

Antibodies—The *Nco*I-*Bam*HI fragment of the *XPF* cDNA encoding the N-terminal 260 amino acids of XPF was expressed from pET26b (Novagen) in *Escherichia coli* BL21(DE3) and purified as described previously (10). This polypeptide antigen was used to produce mouse polyclonal antibodies. From one of the positive mice, a monoclonal cell line (6D12) was identified and subcloned by standard procedures at the University of California Berkeley Hybridoma Facility.

Plasmid Constructs—For the overexpression of the recombinant XPF-ERCC1 complex in insect cells, both cDNAs were inserted into the p2Bac vector (Invitrogen). First, the *Sac*I-*Hin*dIII fragment of the pMal-ERCC1 construct (13) containing the *ERCC1* open reading frame (ORF) was placed behind the PH promoter of p2Bac, resulting in the plasmid p2Bac-E1. The *Apa*I-*Xba*I fragment of pcER4–34 containing the human *XPF* ORF (10) was then inserted into p2Bac-E1, placing *XPF* under the control of the P10 promoter and creating the final expression construct p2Bac-FE1. Recombinant baculovirus was obtained by standard methods (14) using the BaculoGold transfection kit (PharMingen).

For *in vitro* transcription-translation, the *ERCC1* and *XPF* ORFs were inserted into the pIBI24 vector (IBI). In order to create the appropriate restriction sites for subcloning of *XPF*, 974 bp encoding the N-terminal one-third of the ORF was amplified from the 5′-end of pcER4–34 using the following polymerase chain reaction primers: 5′-

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[1] The abbreviations used are: XP, xeroderma pigmentosum; CFE, cell-free extract; ERCC, excision repair cross-complementing; RPA, replication protein A; ORF, open reading frame; bp, base pair(s); r(XPF-ERCC1), recombinant XPF-ERCC1.

CGCGAATTCAAGGATATAGCTCTAGAGATGGAGTCAGGCAGCCG-GCTCGACGGATT and 5′-AAACAGCCAACCTGAATTCTGACCAAA-CGCTTTTTCCGTT. The *Xba*I-*Bam*HI fragment of this polymerase chain reaction product and the *Bam*HI-*Apa*I fragment excised from pcER4–34 carrying the 3′ section of the *XPF* ORF were cloned into pIBI24, placing *XPF* under the control of the T7 promoter and creating pTB-F. The *ERCC1* cDNA was similarly placed behind the T7 promoter by inserting the *Sal*I-*Xba*I fragment of pMal-ERCC1 into pIBI24, creating pTB-E1.

Purification of Recombinant XPF-ERCC1—Sf21 cells (5×10^8) were infected with the baculovirus expression construct p2Bac-FE1 at a multiplicity of infection of 20, and cells were harvested 48 h after infection. Cell-free extract was prepared as described previously (15) and loaded onto a 20-ml SP-Sepharose column, and the column was developed with a 200-ml linear gradient from 0.1 to 1.0 M KCl in Buffer A (25 mM Hepes-KOH, pH 7.9, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, and 15% glycerol). The r(XPF-ERCC1) complex that eluted at about 0.4 M KCl was located with polyclonal anti-XPF antibodies (10) and polyclonal anti-ERCC1 antibodies (16). Fractions containing r(XPF-ERCC1) were combined, dialyzed against Buffer A + 100 mM KCl, and then loaded onto a 5-ml glutathione *S*-transferase-XPA affinity column (17). The affinity column was washed with 200 ml of Buffer B (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 1 mM EDTA, 10 mM β-mercaptoethanol, and 10% glycerol). r(XPF-ERCC1) complex was eluted with 1.0 M KCl in Buffer B as indicated by both silver staining and immunoblotting of SDS-polyacrylamide gels. Fractions containing r(XPF-ERCC1) were collected and dialyzed against Buffer A plus 100 mM KCl. The yield was typically 150 μg of protein of high purity from 20 mg of total CFE.

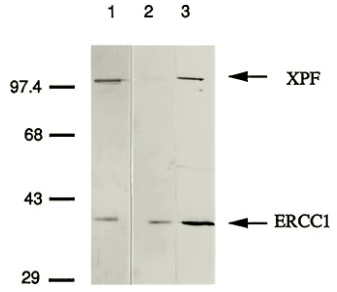

FIG. 1. **Purification of r(XPF-ERCC1).** The r(XPF-ERCC1) complex purified by SP-Sepharose and glutathione *S*-transferase-XPA affinity columns was analyzed on 8.5% SDS-polyacrylamide gel electrophoresis gels followed by silver staining or Western blotting using anti-ERCC1 serum and anti-XPF polyclonal ascites. *Lane 1*, silver-stained gel of purified protein (400 ng); *lane 2*, immunoblot of HeLa CFE (100 μg); *lane 3*, immunoblot of recombinant protein (200 ng). Positions of protein molecular mass standards in kDa are shown.

Nuclease Assay—A 3′-end-labeled 90-bp duplex and a 90-bp DNA fragment with a 30-nucleotide unpaired sequence ("bubble") in the middle as described previously (12) were used as substrates to test for nonspecific nuclease and junction-specific nuclease activities of r(XPF-ERCC1), respectively. The reaction mixture (7.5 μl) contained 5 fmol of DNA and the indicated amount of r(XPF-ERCC1) in nuclease buffer (25 mM Hepes-KOH, pH 7.9, 10 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 6.5% (v/v) glycerol). The reaction mixture was incubated at 37 °C for 30 min, and the products were analyzed on 8% denaturing polyacrylamide gels followed by autoradiography.

Complementation Assay with Mutant Cell-free Extracts—Reaction mixtures contained 50 μg of mutant CFE, 20 ng of r(XPF-ERCC1), and 25 fmol of the 140-bp cholesterol A substrate (7) in 25 μl of excision buffer (6, 7). The reaction was carried out at 30 °C for 60 min. For cross-complementation, 25 μg of each mutant CFE was mixed in 25 μl of excision buffer with substrate, and the reaction was carried out in an identical manner. Excised fragments were analyzed on 8% denaturing polyacrylamide gels. The mutant CFEs used in this study were XP-A (GM02345B), XP-F (GM08437), ERCC-1 (UV20), ERCC-3 (UV24), ERCC-4 (UV41), ERCC-5 (UV135), and ERCC-11 (UVS1).

In Vitro Transcription-Translation—[^{35}S]Methionine-labeled XPF and ERCC1 proteins were prepared by the TNT Coupled Reticulocyte Lysate System (Promega) using pTB-F and pTB-E1 as templates, either added separately or together in the reaction mixture following the manufacturer's instructions. 10 fmol of ERCC1, 4 fmol of XPF, and 4 fmol of XPF-ERCC1 were used for protein-protein interaction study.

DNA Binding Assay—A 90-bp duplex was terminally labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase as described (12). To obtain UV-damaged DNA, the 90-bp duplex was irradiated with 2500 J/m^2 of 254 nm light from a germicidal lamp at a fluence rate of 250 milliwatts/cm^2. For binding assays, 3 fmol of DNA was incubated with r(XPF-ERCC1) in Buffer A containing 100 mM KCl for 10 min on ice. The reaction mixtures were directly analyzed on 5% native polyacrylamide gels run in 1 × TBE buffer for 90 min at room temperature. The *E. coli* UvrA protein that binds specifically to UV-irradiated DNA (18) was

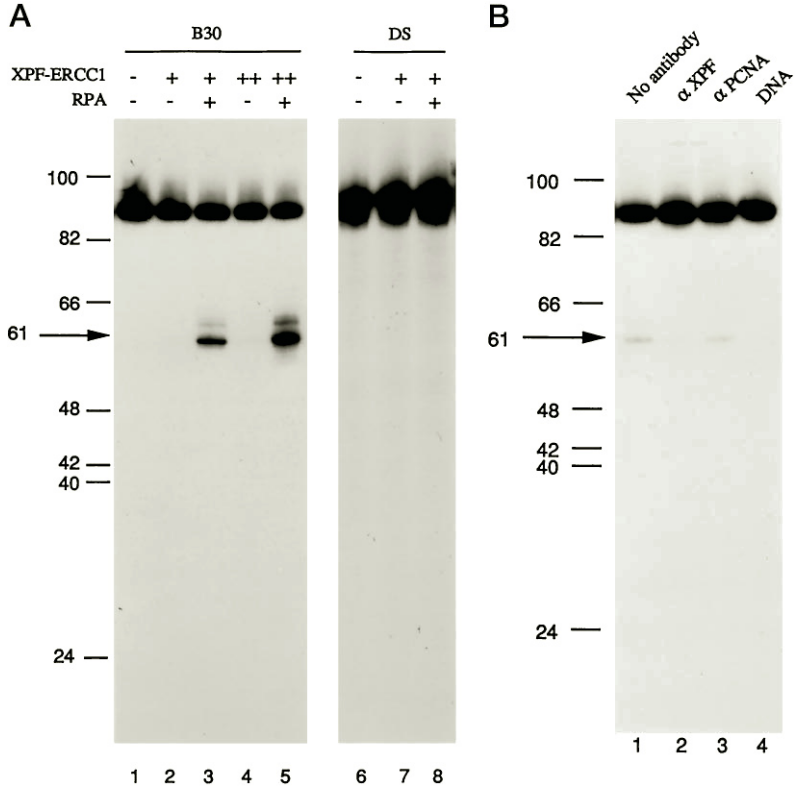

FIG. 2. **Junction-cutting activity of r(XPF-ERCC1).** *A*, bubble-30 and double-stranded DNA substrates. The 3′-end-labeled substrate was incubated with 20 ng (+) or 40 ng (++) of r(XPF-ERCC1) in the absence (−) or presence (+) of RPA (50 ng) as indicated. The 5′ border of the bubble is 57 nucleotides from the label, and the major incision site is 61 nucleotides from the label or 4 nucleotides 5′ to the single-stranded bubble region (12). Double-stranded DNA substrate was incubated with r(XPF-ERCC1) alone (20 ng, *lane 7*) or r(XPF-ERCC1) (20 ng) and RPA (50 ng, *lane 8*) as indicated. In *lanes 2, 3, 4,* and *5*, 0.5, 28, 1.3, and 49% of the substate were incised by r(XPF-ERCC1), respectively. *B*, inhibition of r(XPF-ERCC1) junction cutting by the anti-XPF monoclonal antibody. The bubble substrate was incubated with r(XPF-ERCC1) (10 ng) alone (*lane 2*) or with r(XPF-ERCC1) (10 ng) that had been preincubated for 10 min on ice with 100 ng of either anti-XPF monoclonal antibody (*lane 2*) or an unrelated monoclonal antibody against proliferating cell nuclear antigen (*lane 3*). Then, RPA (50 ng) was added and incubation was continued at 37 °C for 30 min. In *lane 2*, 93% of the junction-cutting activity of r(XPF-ERCC1) was inhibited by anti-XPF antibody. *Lane 4* contained DNA only.

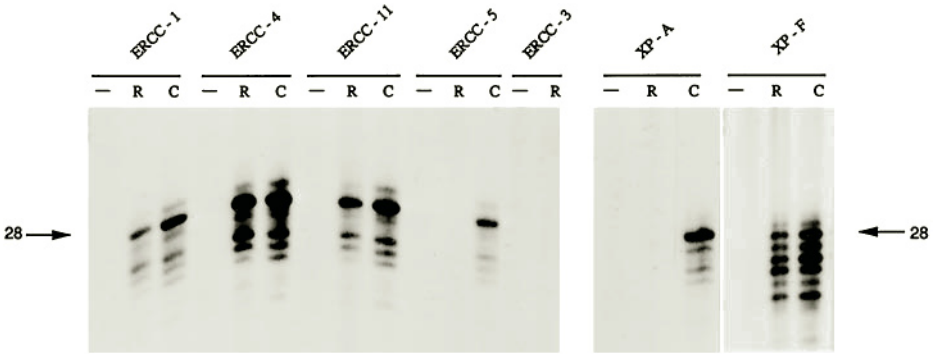

FIG. 3. **Complementation of mutant cell-free extracts.** The 140-bp duplex DNA containing a single adducted cholesterol A (7) was incubated with the indicated mutant CFE to which either r(XPF-ERCC1) (20 ng) was added (*R*), or, for cross-complementation, CFE from Chinese hamster ovary mutant group 3 (ERCC-3) was added as indicated (*C*). The size of the major excision product (28 nucleotides) is indicated by an *arrow*.

used as a positive control. Binding was quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS AND DISCUSSION

Purification of Recombinant XPF-ERCC1—To obtain a highly purified sample of XPF-ERCC1, we constructed a baculovirus expression vector (p2Bac-FE1) containing the cDNAs from both *ERCC1* and the recently cloned *XPF* (from pcER4–34; Ref. 10). Infection of Sf21 insect cells resulted in overexpression of both proteins, which purified as a complex through SP-Sepharose followed by XPA-affinity columns (16, 17). The use of the XPA-affinity step enabled us to isolate 150 μg of r(XPF-ERCC1) from 20 mg of cellular protein with greater than 95% homogeneity. This compares favorably with the yield of $<$1.0 μg of protein purified from 22 g of HeLa CFE (from a 250-liter cell culture; Ref. 17). After separating the proteins by SDS-gel electrophoresis, analysis by silver staining verified that the preparation was free of protein contaminants (Fig. 1). Antibodies to each of the two subunits detected proteins from the purified preparation that migrated exactly as the 38-kDa (ERCC1) and 112-kDa (XPF) proteins extracted from HeLa cells. This agreement indicates that neither one of the overexpressed proteins undergoes post-translational modification in Sf21 cells, and that the insect cells are capable of carrying out the same post-translational modification as human cells.

Functional Properties of r(XPF-ERCC1)—Two related enzymatic activities have been attributed to XPF-ERCC1 that help to explain its function in excision repair. The complex incises the 5′ junction of DNA bubble and loop structures four nucleotides 5′ to the junction (12) and makes the 5′ incision of the dual incision human excision nuclease (7, 19). Purified r(XPF-ERCC1) was tested for these properties. When a DNA bubble substrate was used, r(XPF-ERCC1) made the specific cleavage expected, and this activity was stimulated 40–50-fold by the addition of RPA (Fig. 2*A*). The complex did not nick double-stranded DNA (Fig. 2*A*, lanes 7 and 8). Incision activity was severely inhibited by the anti-XPF monoclonal antibody, demonstrating the dependence of the reaction on the XPF protein (Fig. 2*B*).

We also tested r(XPF-ERCC1) for complementation of excision nuclease (20), an activity that we consider to be the most specific for the native complex (17). r(XPF-ERCC1) was found to complement extracts from XP-F cells and the Chinese hamster ovary mutants of complementation groups 4 (*ERCC4/XPF*) and 1 (*ERCC1*), but not extracts from XP-A cells and groups 3 (*ERCC3/XPB*) and 5 (*ERCC5/XPG*) (Fig. 3). The recombinant protein also complemented the Chinese hamster ovary complementation group 11 mutant (21), which is now known to be a member of group 4 (10, 11).

Protein and DNA Interactions of XPF and ERCC1—As we have observed previously, XPF-ERCC1 complex binds tightly to XPA, *e.g.* during its purification on the XPA-affinity column. Moreover, XPF-ERCC1 appears to interact with the RPA protein, since the junction endonuclease activity is greatly stimulated by RPA (12). To study the contribution of the two members of the complex in these binding interactions, the proteins were expressed either singly or in combination in an *in vitro* transcription-translation system and tested for binding to XPA or RPA (Fig. 4). In agreement with earlier reports (16, 22, 23), we found that ERCC1 (*lane 1*) and XPF-ERCC1 (*lane 3*) bound to XPA. XPF by itself weakly adsorbed to the XPA-affinity resin (*lane 2*), suggesting that the association of the complex with XPA is mediated mostly by ERCC1. In contrast, ERCC1 did not bind to RPA (*lane 5*), as XPF did (*lane 6*), and therefore XPF must mediate the binding of the complex to RPA (*lane 7*). These results demonstrate that the binding observed previously (in which XPF-ERCC1 bound to RPA with particularly high affinity in the presence of the DNA bubble substrate (12)), occurs through a direct interaction between XPF and RPA.

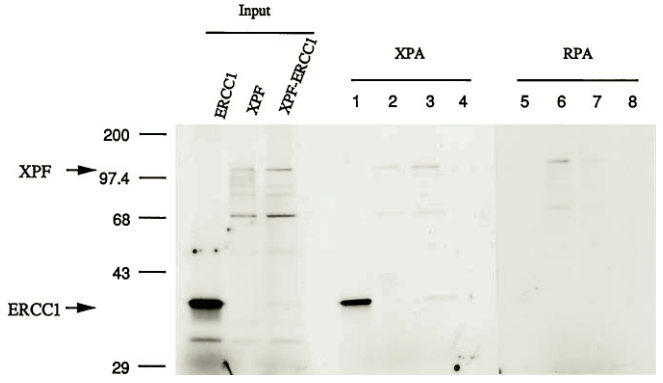

FIG. 4. **XPF protein binds to XPA and RPA.** ^{35}S-Labeled proteins were made by an *in vitro* transcription-translation system, and products were separated by SDS-gel electrophoresis (*Input*, 70% ERCC1, 40% XPF, and 20% XPF-ERCC1 used for the assay were loaded directly). Note that the expression of XPF results in a 70-kDa protein in addition to the full-length product. This is a proteolytic fragment of XPF that is also seen in aged HeLa CFE or after XPF expression in *E. coli*, but not after XPF expression in insect cells. For binding to XPA the transcription-translation product was mixed with maltose-binding protein-XPA attached to amylose beads and incubated at 4 °C for 1 h. After extensive washing with Buffer A + 0.5% Nonidet P-40, the resin was placed in SDS-loading buffer, heated at 95 °C for 5 min, and analyzed on an 8.5% SDS-gel. The input proteins were as follows: *lane 1*, ERCC1; *lane 2*, XPF; *lane 3*, XPF-ERCC1; *lane 4*, UvrB-maltose-binding protein bound to amylose and mixed with XPF-ERCC1 as a negative control. In *lanes 1*, *2*, and *3*, 36%, 8%, and 7% of the input proteins were pulled down, respectively. For binding to RPA, the radiolabeled proteins were mixed with recombinant RPA (31), then anti-p34 monoclonal antibody (Oncogene Science) and protein A-agarose were added, and the mixture was incubated at 4 °C for 1 h. Bound proteins were analyzed as described above. The lanes contained the following: *lane 5*, ERCC1; *lane 6*, XPF; *lane 7*, XPF-ERCC1. The sample run in *lane 8* was identical to that in *lane 7*, but did not include the anti-p34 antibody. In *lanes 6* and *7*, 7% and 5% of the input proteins were co-immunoprecipitated, respectively.

Although XPA (24), RPA (25, 26), and XPC (27) have been

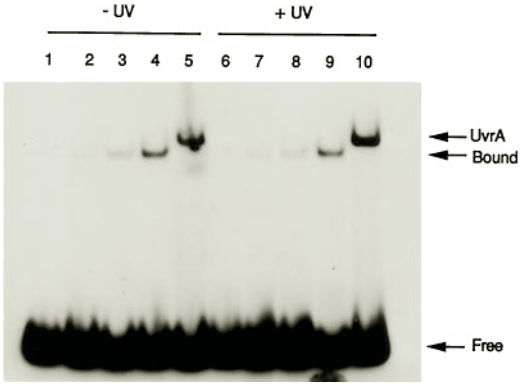

FIG. 5. **DNA binding activity of r(XPF-ERCC1).** A 90-bp duplex without (−UV) or with 2500 J/m^2 irradiation (+UV) was used in a gel mobility shift assay on a 5% native polyacrylamide gel. All lanes contained 3 fmol of DNA and the following amounts of r(XPF-ERCC1): *lanes 1* and *6*, none; *lanes 2* and *7*, 16 ng; *lanes 3* and *8*, 32 ng; and *lanes 4* and *9*, 64 ng. In *lanes 5* and *10*, 7 ng of UvrA was used as a positive control. In *lanes 4* and *9*, 8.3% and 11.3% of the input DNA bound to r(XPF-ERCC1), respectively. In *lane 5* and *10*, 12% and 30% DNA of the input DNA bound to UvrA protein, respectively.

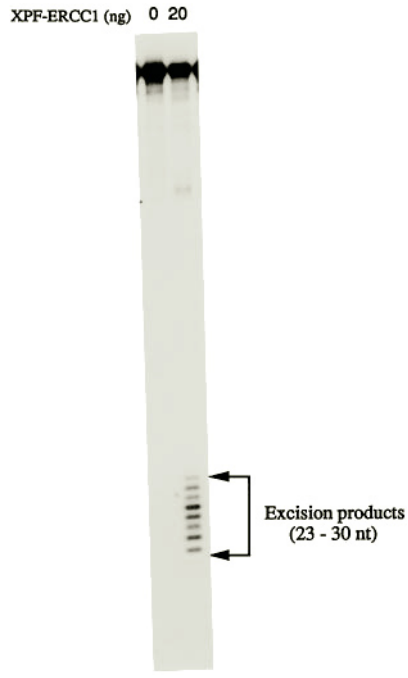

FIG. 6. **Reconstitution of human excision nuclease with r(XPF-ERCC1).** A 140-bp DNA substrate (5 fmol) with internally labeled thymine cyclobutane dimer (7) was incubated in 25 μl of excision buffer containing 300 ng of rRPA, 40 ng of rXPA, 7 ng of XPC, 2 ng of TFIIH, 7.5 ng of rXPG, and 0 or 20 ng of r(XPF-ERCC1) as indicated. The reaction was carried out at 30 °C for 90 min, and the products were analyzed on an 8% denaturing polyacrylamide gel. The range of the major excision products is indicated by the *bracket*.

found to bind somewhat specifically to damaged DNA, the issue of damage recognition remains one of the unsolved questions of the human excision nuclease reaction mechanism. We tested for binding of r(XPF-ERCC1) to UV-damaged DNA to find out if it contributed to damage recognition. However, unlike the UvrA subunit of *E. coli* excision nuclease that discriminates between damaged and undamaged DNA, r(XPF-ERCC1) bound nearly as well to both substrates (Fig. 5). Thus, we conclude that even though XPF-ERCC1 has an intrinsic affinity to DNA, it does not contribute to locating lesions and in this respect is comparable to the homologous Rad1-Rad10 protein complex of *Saccharomyces cerevisiae* (28).

Reconstitution of Human Excision Nuclease with Purified Proteins and r(XPF-ERCC1)—Although significant progress has been made recently in identifying and assigning functions to all of the proteins involved in human excision repair (6–8) and the highly homologous *S. cerevisiae* system (29, 30), the two reports on human excision nuclease differed on an important point. Mu *et al*. (6, 7) found that XPA, RPA, TFIIH, XPC, XPG, and XPF-ERCC1 were necessary and sufficient for reconstituting human excision nuclease, whereas Aboussekhra *et al*. (8) reported that, in addition to these six proteins, an incision factor (IF7) was required. In the latter report, this factor appeared to be tightly bound to XPF-ERCC1 and was separated from it at the last step of a 7-column purification scheme. Therefore, the possibility remained that the nominally pure XPF-ERCC1 complex used in other studies might contain IF7, which would explain the discrepancy between the two reports. Hence, we reasoned that r(XPF-ERCC1) purified from a heterologous source would be useful for solving this problem. We combined r(XPF-ERCC1) with the other known factors: XPA, RPA, and XPG, overexpressed and purified from *E. coli* or insect cells, and XPC and TFIIH, purified to near-homogeneity from HeLa cells (7). This set of highly purified proteins was able to carry out precise excision of an adducted oligonucleotide, thus giving firm evidence that XPF-ERCC1 protein is both necessary and sufficient to reconstitute human excision nuclease when added to the other five excision repair factors (Fig. 6). Indeed, studies with the highly homologous *S. cerevisiae* system have arrived at the same conclusion; that is, the yeast homologs of the six factors are necessary and sufficient for specific incision/excision of UV-damaged DNA (29, 30).

CONCLUSION

Human excision nuclease has been reconstituted from purified repair factors and characterized in considerable detail (2, 3). However, the repair factors are of low cellular abundance, precluding detailed mechanistic studies with proteins purified from natural sources. Of the six repair factors, XPA, RPA, XPG, and XPC are currently available in recombinant forms that can be purified in abundant quantities. As reported here, the availability of the *XPF* cDNA (10) has enabled us to demonstrate direct interaction of XPF with RPA, a finding that may help explain how RPA stimulates the nuclease function of XPF-ERCC1 and how it may target it to its proper site of action in the excision nuclease complex. The use of recombinant protein in the reconstituted system has also confirmed that no additional factors are needed for reconstituting human excision nuclease. When the recombinant form of TFIIH becomes available, it will be possible to perform mechanistic experiments with human excision nuclease such as those that have been conducted with prokaryotic excision nuclease for many years.

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