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# Communication

# Purification and Characterization of the XPF-ERCC1 Complex of Human DNA Repair Excision Nuclease*

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**A complex, which consists of ERCC1 (38 kDa) and a 112-kDa protein, was purified from HeLa cells to homogeneity. This complex complemented the nucleotide excision repair defects of rodent ERCC-1, ERCC-4, and human XP-F mutant cell-free extracts, indicating that the 112-kDa protein is XPF/ERCC4 and providing direct biochemical evidence that XPF and ERCC4 are identical. The XPF/ERCC4-ERCC1 complex has an endonuclease activity with preference for single-stranded DNA and a single-stranded region of duplex DNA with a "bubble" structure. This complex also nicks supercoiled DNA weakly, and this nicking activity is stimulated by human replication protein A when the DNA contains UV damage.**

Xeroderma pigmentosum (XP)[1] is a human disease characterized by a high incidence of actinic cancers and in some cases neurological abnormalities; it is caused by a defect in excision repair as a result of mutations in one of seven genes, *XPA* through *XPG* (1). In recent years all of the XP genes required for excision, with the exception of *XPF*, have been cloned and sequenced (2, 3). Previously it was found that cell-free extracts (CFEs) from human XP-F and rodent ERCC-4 mutants did not complement each other *in vitro*, raising the possibility that these two complementation groups contained mutations in the human and rodent homologs of the *XPF* gene (4, 5). In addition, it was found that the complementing activity of XP-F and ERCC-4 mutant CFEs was tightly associated with the ERCC1 protein (6–8). Here we report the purification of the XPF/ERCC4 protein in complex with ERCC1 to homogeneity, providing direct evidence that *XPF* and *ERCC4* are identical genes. The tight complex of XPF/ERCC4 and ERCC1 is a single-stranded DNA-specific endonuclease with weak endonucleolytic activity on supercoiled DNA. The nicking of supercoiled DNA is stimulated by human replication protein A (RPA) when the DNA contains UV damage.

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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; bp, base pair(s); SSB, single-stranded DNA binding.

## MATERIALS AND METHODS

*Purification of the Complex of ERCC1 and XPF/ERCC4 from HeLa Cells*—All purification procedures were done at 0–4 °C. HeLa whole cell extracts were prepared from a 400-liter culture containing approximately $2.5 \times 10^{11}$ cells (about 35 g of protein) as described previously (7). ERCC1-containing fractions were detected by immunoblotting using polyclonal antibodies raised against maltose-binding protein-ERCC1 fusion protein expressed in *Escherichia coli* (6). The HeLa cell extracts were applied to a DE52 column (5 × 21 cm, Whatman) as described (7). The ERCC1 protein was in the flow-through, which was applied to an Affi-Gel Blue column (5 × 18 cm, Bio-Rad) pre-equilibrated with buffer A (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM dithiothreitol, 15.5% glycerol). The bound proteins were eluted with a linear 800-ml gradient of 0.1–0.7 M KCl, with 1.2 liters of buffer A plus 0.8 M KCl, and then with 500 ml of buffer A plus 1.5 M NaSCN. Fractions containing ERCC1 protein were pooled, and ammonium sulfate was added to 0.6 g/ml. Precipitated proteins were pelleted by centrifugation at 15,000 × *g* for 1 h, resuspended in 50 ml of buffer B (25 mM HEPES-KOH, pH 7.6, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol), and dialyzed against the same buffer. The dialysate was applied to a 6-ml glutathione *S*-transferase-XPA protein affinity column pre-equilibrated with buffer B, washed with the same buffer, and eluted with buffer B plus 0.9 M KCl. The pool of ERCC1 fraction (48 ml) was concentrated to 1.6 ml using Centriprep 30 (Amicon), dialyzed against buffer A, and applied to a 4-ml DEAE-Sepharose column (Sigma). The flow-through fractions containing ERCC1 protein were pooled and loaded onto a 2-ml heparin-agarose column (Sigma). The bound proteins were eluted with buffer A plus 0.9 M KCl. The final pool of ERCC1 fraction (2.4 μg) was concentrated using Centricon 30 (Amicon), dialyzed against buffer A, and stored at −80 °C.

*In Vitro Excision Assay*—The human excinuclease activity was tested by *in vitro* excision assay using the 140-base pair (bp) substrate containing a cholesterol adduct in the center and a $^{32}P$ label at the sixth phosphodiester bond 5′ to the lesion as described previously (7). The repair reaction mixture (25 μl) contained 35 mM HEPES-KOH, pH 7.9, 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 20 mM NaCl, and 5.6 mM $MgCl_2$, 0.4 mM EDTA, 0.8 mM dithiothreitol, 6.8% glycerol, 2 mM ATP, 20 mM dNTPs, 100 μg/ml bovine serum albumin, ~1 nM substrate, and 50 μg of CFEs. In complementation assays with CFEs, 25 μg of each of two mutant CFEs was included in the reaction mixture. The reaction mixture was incubated for 1 h at 30 °C, and the excision products were analyzed by electrophoresis in 10% denaturing polyacrylamide gels.

*Endonuclease Assay*—Covalently closed single-stranded M13 mp19 DNA (125 ng) or covalently closed supercoiled double-stranded pMLU112 DNA (100 ng) was incubated at 37 °C for the indicated time in 15 μl of nuclease reaction buffer (25 mM HEPES-KOH, pH 7.9, 25 mM KCl, 3 mM $MgCl_2$, 1 mM dithiothreitol, 120 μg/ml bovine serum albumin, and 6.5% glycerol) with the XPF-ERCC1 complex. After incubation, the reaction was terminated with 1% SDS. Samples were analyzed by electrophoresis at 80 V for 1.5 h on 1% agarose gels, followed by staining DNA with ethidium bromide and photography. To test the effect of RPA on XPF-ERCC1 endonuclease activity, non-irradiated or UV-irradiated pMLU112 DNA (100 ng) was incubated with 20 ng of the single-stranded DNA binding proteins from different sources and 165 ng of XPF-ERCC1 fraction from the XPA-affinity column step for 20 min at 30 °C and analyzed as described above.

*Endonuclease Assay for a Linear Duplex DNA Containing a Bubble Structure*—The 5′-labeled bubble substrate was prepared by labeling the oligonucleotide 5′-CCTGCCTAGGATCCAGTAGTCGACTTGGA-CGAACCCGGGATGGAATGGAGTATTCGCCGTGTCCATGGCTGTA-AGTATCCAGGATCCCGG-3′ using T4 polynucleotide kinase and [$\gamma$-$^{32}P$]ATP, followed by annealing to the oligonucleotide 5′-CCGGGATCCTGGATACTTACAGCCATATCAGTTACGCCAGTAT-GCCGATGCTATAAGTTCGTCCAAGTCGACTACTGGATCCTAGGC-AGG-3′. Unpaired regions are underlined. The 90-bp duplex DNA (~1 nM) containing a bubble of 30 nucleotides was incubated for 3 h at 37 °C with the purified XPF-ERCC1 complex as described above. After incubation, 0.1 volume of 0.25 M EDTA and 1 volume of formamide-dye were added to the reaction mixture, and the products were analyzed using a 10% denaturing polyacrylamide gel. S1 nuclease digestion was carried



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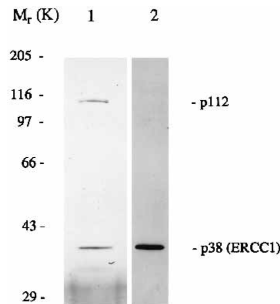


FIG. 1. **Purification of the XPF-ERCC1 complex.** The purified complex was separated on a 10% SDS-polyacrylamide gel and analyzed by silver nitrate staining (*lane 1*, 240 ng) and immunoblotting with anti-ERCC1 antibodies (*lane 2*, 40 ng).

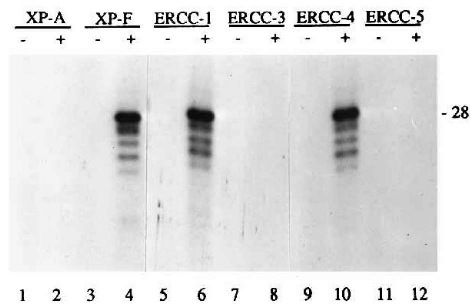


FIG. 2. **Specific complementation of XP-F, ERCC-1, and ERCC-4 mutants by the XPF-ERCC1 complex.** CFEs from different repair complementation groups were incubated in the standard reaction mixture in the absence (−) or presence (+) of the purified XPF-ERCC1 complex (8 ng). *Lanes 1* and *2*, XP-A (GM02345B); *lanes 3* and *4*, XP-F (GM08437A); *lanes 5* and *6*, ERCC-1 (UV20); *lanes 7* and *8*, ERCC-3 (UV24); *lanes 9* and *10*, ERCC-4 (UV41); *lanes 11* and *12*, ERCC-5 (UV135). *28* indicates the position of the main excision product.

out at 20 °C for 20 min in 30 mM sodium acetate (pH 4.6), 1 mM zinc acetate, 50 mM NaCl, and 5% glycerol with 0.1 unit of S1 nuclease (Life Technologies, Inc.).

*Immunodepletion of the XPF-ERCC1 Complex by Anti-ERCC1 Antibody*—Protein A-agarose beads (30 μl, Santa Crutz) were incubated with 60 μl of preimmune serum or immune serum raised against ERCC1 protein for 1 h at 4 °C. The beads were washed extensively with buffer A and incubated with 1.3 μg of XPF-ERCC1 fraction from the XPA-affinity column step for 1 h at 4 °C. After spinning down the beads, the supernatant was carefully removed and used in the endonuclease assay with single-stranded M13 DNA.

*Quantitation of the Endonuclease Activity*—The intensities of DNA bands were measured by scanning the photographs of ethidium bromide-stained agarose gels with a Molecular Dynamics computing densitometer series 300 instrument, and the average numbers of the nicks per DNA molecule were calculated by the P(0) class of Poisson distribution.

## RESULTS AND DISCUSSION

*Purification of XPF/ERCC4 in the Complex with ERCC1 from HeLa Cell-free Extract*—The ERCC1-containing complex was purified from 400 liters of HeLa cell culture using procedures modified from Mu *et al.* (7) in which an XPA-affinity chromatography is the major purification step, since XPA protein binds to ERCC1 protein with high affinity (6, 9). The affinity-purified complex was purified further through two additional columns, DEAE-Sepharose followed by heparin-agarose, and only two polypeptides were detected in the final fraction by silver staining (Fig. 1, *lane 1*). One is at 38 kDa and was identified as ERCC1 based on the calculated molecular weight from the cDNA sequence (10) and Western blotting with anti-ERCC1 antibodies (Fig. 1, *lane 2*). The other protein has an apparent molecular mass of 112 kDa.

It was recently reported that partially purified ERCC1 fractions complemented both XP-F and ERCC-4 CFEs as well as ERCC-1 CFE (7, 8), raising the possibility that the 112-kDa protein, which makes a complex with ERCC1, is XPF and/or ERCC4. The purified complex was tested for complementing CFEs of mutant cell lines for excision activity (Fig. 2). As expected, the complex complemented rodent ERCC-1 mutant extract (*lane 6*). In addition, the complex complemented XP-F and ERCC-4 CFEs as well (*lanes 4* and *10*), while no other mutant extract (XP-A, ERCC-3 (XP-B), and ERCC-5 (XP-G)) was complemented (*lanes 2, 8,* and *12*). Furthermore, the complementation of both XP-F and ERCC-4 CFEs by the complex was inhibited by anti-ERCC1 antibodies (data not shown). This inhibition was overcome by the addition of the excess amount of the purified complex, indicating that both complementations were due to the ERCC1-containing complex but not to a minor contaminant in our preparation. These results lead us to conclude that the 112-kDa protein is XPF, which in turn is identical to ERCC4. Previously, it was found that CFEs from human XP-F and rodent ERCC-4 mutants did not complement each other *in vitro* for either excision (4) or repair synthesis (5, 11) activity, and it was suggested that the *ERCC4* gene might be identical to the *XPF* gene or that XPF-ERCC1 was in a complex with ERCC4 (4, 5, 11). Here we show direct biochemical evidence that XPF and ERCC4 are identical,[2] in spite of earlier evidence based on somatic cell hybrids suggesting that *ERCC4* and *XPF* might be different genes (12).

*XPF-ERCC1 Complex Is a Single-stranded DNA Endonuclease*—Since it is known that the presumed yeast homolog of XPF-ERCC1, the RAD1-RAD10 complex, is an endonuclease with a specificity for single-stranded DNA and supercoiled double-stranded DNA (13, 14), we tested the XPF-ERCC1 complex for nuclease activity. Fig. 3, *A* and *B*, shows that the complex degraded single-stranded M13 DNA extensively and nicked supercoiled DNA rather inefficiently with no preferential activity on UV-damaged DNA. These data are consistent with XPF-ERCC1 being a single strand specific endonuclease. The nuclease activity is dependent on $Mg^{2+}$, which can be replaced by $Mn^{2+}$ but not by $Ca^{2+}$, $Co^{2+}$, $Cu^{2+}$, and $Zn^{2+}$ (data not shown). To confirm that the nuclease activity was intrinsic to XPF-ERCC1, the fractions from the last purification column were tested for XPF-ERCC1 by Western blotting with anti-ERCC1 antibodies and for nuclease activity using M13 DNA as a substrate. Fig. 3*C* shows co-elution of ERCC1 (and hence the complex) and of nuclease activity. When fraction 24 containing the nuclease activity was analyzed by silver staining, it contained only two proteins, ERCC1 and XPF (Fig. 1, *lane 1*). Furthermore, the nuclease activity was partially suppressed by immunodepletion of the XPF-ERCC1 complex with anti-ERCC1 antibodies (Fig. 3*D*), consistent with the notion that the endonuclease activity is intrinsic to the XPF-ERCC1 complex.

It has been reported that RAD1-RAD10 specifically cleaves the single strand to double strand junction of Y-shaped DNA only of the strand with the 3′ single-stranded terminus, and hence the preferential nuclease activity on single-stranded M13 DNA by the RAD1-RAD10 complex was attributed to cleavage at the junction of stem-loop structures known to exist in M13 DNA (15). To test for such an activity, we designed a 90-bp partial duplex DNA with a 30-nucleotide "bubble" near the center because of non-complementary sequences. As shown in Fig. 4*A*, the homogeneous fraction containing only the XPF and ERCC1 proteins (Fig. 1) did not show such a preference; rather a ladder in the region corresponding to the "bubble" was observed, even though the fractions from the penultimate purification step had a preference for junction (data not shown).

[2] XPF/ERCC4: in this paper we show that these two proteins are identical, and hence we will use XPF for the rest of the paper.

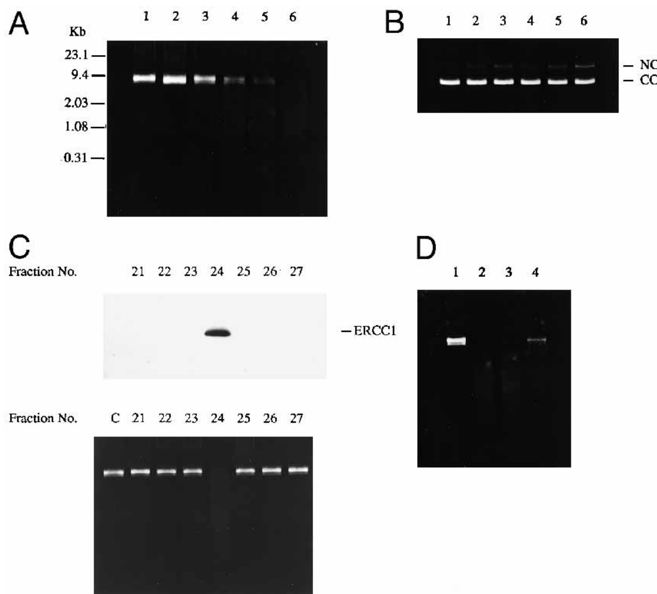


FIG. 3. **The XPF-ERCC1 complex is an endonuclease.** *A,* degradation of covalently closed single-stranded DNA by the XPF-ERCC1 complex. *Lane 1*, DNA alone; *lane 2*, DNA with 16 ng of the XPF-ERCC1 complex and 0.5% SDS; *lanes 3–6*, DNA with 4, 8, 12, and 16 ng of the XPF-ERCC1 complex, respectively, and the average numbers of nicks per DNA molecule introduced by the XPF-ERCC1 complex were 0.2, 1.2, 2.2 and 4.0, respectively. *kb*, kilobase pairs. *B*, nicking of covalently closed supercoiled double-stranded DNA by the XPF-ERCC1 complex. *Lanes 1–3*, non-irradiated DNA; *lanes 4–6*, UV-irradiated (1 kJ/m$^2$) DNA. *Lanes 1* and *4*, DNA alone; *lanes 2* and *5*, DNA with 8 ng of the XPF-ERCC1 complex; *lanes 3* and *6*, DNA with 16 ng of the XPF-ERCC1 complex. The average number of nicks per DNA molecule introduced by 16 ng of the XPF-ERCC1 complex was 0.12. *NC*, nicked circular DNA; *CC*, covalently closed supercoiled DNA. *C*, co-elution of nuclease activity with the XPF-ERCC1 complex. Fractions 21–27 (1 ml each) from the last purification step on heparin-agarose were concentrated to 350 $\mu$l individually. *Top panel*, 20 $\mu$l of the concentrated fractions was analyzed for the XPF-ERCC1 complex by immunoblotting with anti-ERCC1 antibodies; *bottom panel*, 4 $\mu$l of the same concentrated fractions were assayed for nuclease activity with single-stranded M13 DNA. On the average 5.0 nicks/DNA molecule were introduced by the XPF-ERCC1 complex in fraction 24. *C*, control DNA with no addition of fraction. *D*, immunodepletion of nuclease activity by anti-ERCC1 antibodies. The XPA-affinity column-purified fraction was used for this experiment. *Lane 1*, DNA alone; *lanes 2–4*, DNA plus XPF-ERCC1 fractions treated with protein A alone (*lane 2*), protein A plus preimmune serum (*lane 3*), and protein A plus immune serum (*lane 4*), respectively. The average numbers of nicks/DNA molecule in *lanes 2–4* as calculated by the P(0) class of Poisson distribution were >6, 3.6, and 1.0, respectively.

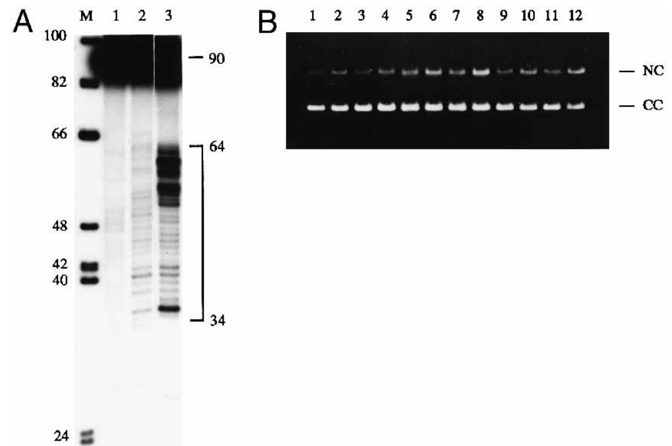


FIG. 4. **Characterization of the endonuclease activity of the XPF-ERCC1 complex.** *A,* cleavage of a 90-bp duplex DNA with a 30-nucleotide bubble by the XPF-ERCC1 complex. *M*, size markers. *Lane 1*, DNA alone; *lane 2*, DNA plus the XPF-ERCC1 complex (8 ng); *lane 3*, DNA plus S1 nuclease (0.1 unit). *B*, stimulation of the nicking activity of the XPF-ERCC1 complex on UV-irradiated double-stranded DNA by human RPA. *Lanes 1–4* contain non-irradiated DNA and *lanes 5–12* contain UV-irradiated DNA (4 kJ/m$^2$). *Lane 1*, DNA alone; *lane 2*, DNA plus XPF-ERCC1; *lane 3*, DNA plus human RPA; *lane 4*, DNA plus XPF-ERCC1 and human RPA; *lane 5*, UV DNA alone; *lane 6*, UV DNA plus XPF-ERCC1; *lane 7*, UV DNA plus human RPA; *lane 8*, UV DNA plus XPF-ERCC1 and human RPA; *lane 9*, UV DNA plus yeast RPA; *lane 10*, UV DNA plus XPF-ERCC1 and yeast RPA; *lane 11*, UV DNA plus *E. coli* SSB; *lane 12*, UV DNA plus XPF-ERCC1 and *E. coli* SSB. *NC*, nicked circular DNA; *CC*, covalently closed supercoiled DNA. All *lanes* contained 100 ng of DNA, whereas 165 ng of the XPA-affinity-purified XPF-ERCC1 and 20 ng of the single-stranded DNA binding proteins were used as indicated. The average numbers of nicks/DNA molecule in four experiments including the one shown here were 0.08 for UV-DNA plus XPF-ERCC1 (*lane 6*) and 0.15 for UV DNA plus XPF-ERCC1 and human RPA (*lane 8*). The yeast RPA and *E. coli* SSB had no significant effect on the level of nicking.

The single-stranded DNA-specific S1 nuclease gave a similar pattern of incision, although the two enzymes differed with regard to the sequence effect on cleavage within the single-stranded region. Nevertheless, these results strongly support the idea that XPF-ERCC1 cleaves M13 DNA and bubble structures as a single-stranded DNA-specific endonuclease but not because of its specific attack on stem-loop structures in M13 DNA.

*Stimulation of Endonuclease Activity of XPF-ERCC1 Complex by Human RPA*—Recent data using anti-ERCC1 antibodies suggested that XPF-ERCC1 makes the 5′ incision (16) in the dual incisions of human excinuclease (17). Having found that XPF-ERCC1 is in fact a single-stranded DNA-specific endonuclease, the question arises as to what factors make it specific for incising damaged DNA only at the 5′ site 20–24 nucleotides away from the lesion. Two human excision repair proteins, XPA and RPA, are known to bind preferentially to damaged DNA (18, 19). Therefore, we considered the possibility that either or both of these proteins might target XPF-ERCC1 to damage. When human RPA was added to the nuclease assay with supercoiled double-stranded DNA, the weak XPF-ERCC1 nicking activity on UV-damaged DNA was stimulated by ~90% but not on undamaged DNA (Fig. 4*B*, *lane 8*). This stimulation is specifically caused by human RPA because neither yeast RPA (*lane 10*) nor *E. coli* single-stranded DNA binding (SSB) protein (*lane 12*) can induce the same effect. On the other hand, we did not detect any effect of XPA on the nicking activity (data not shown). The mechanism of stimulation of the XPF-ERCC1 nuclease activity by human RPA is not known at present. Nevertheless, our data provide some clues as to how the non-specific nuclease activity of XPF-ERCC1 is made to act specifically with the aid of other excision repair proteins known to be essential for precise incisions by human excision nuclease.

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