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# Co-correction of the ERCC1, ERCC4 and xeroderma pigmentosum group F DNA repair defects *in vitro*

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**The mammalian *ERCC1*-encoded polypeptide is required for nucleotide excision repair of damaged DNA and is homologous to *Saccharomyces cerevisiae* RAD10, which functions in repair and mitotic intrachromosomal recombination. Rodent cells representing repair complementation group 1 have nonfunctional ERCC1. We report that repair of UV-irradiated DNA can be reconstituted by combining rodent group 1 cell extracts with correcting protein from HeLa cells. Background repair was minimized by employing fractionated rodent cell extracts supplemented with human replication proteins RPA and PCNA. Group 1-correcting activity has a native molecular mass of 100 kDa and contains the 33 kDa ERCC1 polypeptide, as well as complementing activities for extracts from rodent group 4 and xeroderma pigmentosum group F (XP-F) cells. Extracts of group 1, group 4 or XP-F cells do not complement one another *in vitro*, although they complement extracts from other groups. The amount of ERCC1 detectable by immunoblotting is reduced in group 1, group 4 and XP-F extracts. Recombinant ERCC1 from *Escherichia coli* only weakly corrected the group 1 defect. The data suggest that ERCC1 is part of a functional protein complex with group 4 and XP-F correcting activities. The latter two may be equivalent to one another and analogous to *S.cerevisiae* RAD1.**

*Key words:* excision repair/protein complex/RAD mutants/UV light/XP

## Introduction

Genetic and biochemical studies indicate that more than 20 different protein components are needed to carry out nucleotide excision repair of damaged DNA in mammalian cells. During this process, lesions are located, DNA is incised on the damaged strand, an oligonucleotide containing the damage is excised and a repair patch is synthesized (Weeda and Hoeijmakers, 1993). This is the main pathway that cells use to remove damage caused to DNA by the UV component of sunlight. The strategy is versatile and is also used to remove many chemical adducts from DNA.

In humans, a deficiency in nucleotide excision repair is associated with the heritable disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (Weeda and Hoeijmakers, 1993). Individuals with XP are affected with skin lesions (including tumours) at a dramatically high rate. There are seven genetic complementation groups of XP (XP-A to XP-G) and a variant form, representing different components that are thought to work together in a protein complex during DNA repair. Five of the relevant genes (complementing XP-A, B, C, D and G cells) have been isolated. A further source of nucleotide excision repair-defective mutants is a set of UV-sensitive cell lines isolated from established rodent cell cultures. These have been assigned to 11 different complementation groups, and human genes that correct the rodent cell defects are designated *ERCC* (excision repair cross-complementing) genes (Busch *et al.*, 1989; Riboni *et al.*, 1992). Some of the rodent complementation groups have proved to be equivalent to XP or CS complemention groups with group 2 equivalent to XP-D (Weber *et al.*, 1990; Flejter *et al.*, 1992), group 3 to XP-B (Weeda *et al.*, 1990), group 5 to XP-G (O'Donovan and Wood, 1993; Scherly *et al.*, 1993) and group 6 to CS-B (Troelstra *et al.*, 1992). The nucleotide excision repair process is evolutionarily conserved in eukaryotes, and homologues of the *XP* and *ERCC* genes have been identified in many organisms, most notably in *Saccharomyces cerevisiae* (Weeda and Hoeijmakers, 1993).

In order to study the biochemistry of nucleotide excision repair, we have utilized a cell-free system in which damaged plasmid DNA can be repaired during an incubation with extracts from human cells (Wood *et al.*, 1988). This has allowed purification of activities that correct the repair defect in extracts from XP-A (Robins *et al.*, 1991) and XP-G (O'Donovan and Wood, 1993) cells. The DNA binding properties of the XPAC (XP-A complementing) protein indicate that it is involved in the recognition of damage. In addition, studies with the cell-free system have revealed that some factors which participate in semiconservative DNA replication also function during nucleotide excision repair. These include the single-stranded DNA binding protein RPA (Coverley *et al.*, 1991) and PCNA (Nichols and Sancar, 1992; Shivji *et al.*, 1992). *In vitro* analysis is beginning to reveal specific roles for the different components in the reaction mechanism.

One well-characterized human repair gene, *ERCC1*, does not correct any known form of a human DNA repair syndrome (van Duin *et al.*, 1989). Nevertheless, the *ERCC1* gene product plays a critical role in the nucleotide excision repair process. Rodent cells with a defect in ERCC1 are hypersensitive to the toxic effects of agents including UV, acetylaminofluorene, 4-nitroquinoline-1-oxide and mitomycin C (Thompson *et al.*, 1980; Wood and Burki, 1982; Wood *et al.*, 1983; Hoy *et al.*, 1985; Zdzienicka *et al.*, 1987). Extracts from such complementation group 1 mutant cells are deficient in nucleotide excision repair of DNA, and we previously found that this repair defect could be alleviated by mixing group 1 extracts with repair-deficient extracts from rodent group 3 or human XP group B cells (Biggerstaff and Wood, 1992). We now report that repair can be restored to group 1 extracts by an activity from human cells that includes the ERCC1 polypeptide and is associated

with correcting activity for rodent group 4 and human XP-F extracts.

## Results

### *Reconstitution of DNA repair with fractionated cell extracts and purified proteins*

For the studies reported here of complementation of repair-defective rodent cell extracts, we used a scheme in which DNA repair synthesis can be reconstituted with fractionated cell extracts and purified proteins. Whole cell extracts are fractionated by chromatography on phosphocellulose (Fairman and Stillman, 1988). Protein that is adsorbed to the column in buffer with 0.1 M salt and elutes with 1.0 M salt is designated cell fraction II (CFII). Such a fraction prepared from a repair-proficient cell extract contains all of the factors necessary for nucleotide excision repair of DNA except for the single-stranded DNA binding protein RPA and the DNA polymerase accessory factor PCNA (Shivji *et al.*, 1992). Incubation of plasmid DNA with CFII and RPA for 1 h at 30°C leads to stable accumulation of incised repair intermediates in UV-irradiated plasmid DNA (Shivji *et al.*, 1992). Upon addition of PCNA, repair synthesis takes place rapidly and is virtually complete within 10 min. The advantage of this procedure (outlined in Figure 1A) is the extremely low background obtained with CFIIs from repair-defective cells. We have found that by enabling DNA repair synthesis with PCNA for only a brief time at the end of the repair reaction, we can minimize spurious DNA synthesis that takes place during more prolonged incubations and is unrelated to true nucleotide excision repair.

Figure 1B shows an example of data obtained by this procedure with CFIIs from group 1 and group 3 CHO mutant extracts. When combined with RPA and PCNA as indicated, a CFII from each mutant alone gives extremely low background repair synthesis. Including the two fractionated extracts together in the same reaction mixture reconstitutes the components necessary for nucleotide excision repair, and this can be monitored by measuring DNA repair synthesis.

### *Limited correction with recombinant ERCC1*

We took several approaches in an attempt to correct repair-defective group 1 extracts with protein expressed from human *ERCC1* cDNA. RNA transcribed from the cDNA was used for translation by rabbit reticulocyte lysate. The synthesized protein migrated on SDS−PAGE at ~43 kDa (Figure 2A), although the predicted molecular mass of human ERCC1 is 32.6 kDa (van Duin *et al.*, 1986); this anomalously slow migration of ERCC1 has been noted before (Belt *et al.*, 1991). We were unable to correct the group 1 repair defect with lysate containing *in vitro*-translated ERCC1 (data not shown), and turned instead to production of the protein in *E.coli*. Expression of ERCC1 with an amino-terminal polyhistidine tag was monitored by immunoblotting with an antibody raised against a peptide predicted from the ERCC1 sequence (Figure 2B). Most of the protein was produced as a precipitate that was solubilized and purified by nickel−agarose chromatography and ion exchange FPLC to yield ERCC1 of ~90% purity (Figure 2C). Addition of up to 25 ng of the protein to reactions gave only a small amount of complementation of fractionated group 1 extracts, in comparison with a mixture of group 1 and 3 extract fractions (Figure 2D). In contrast,

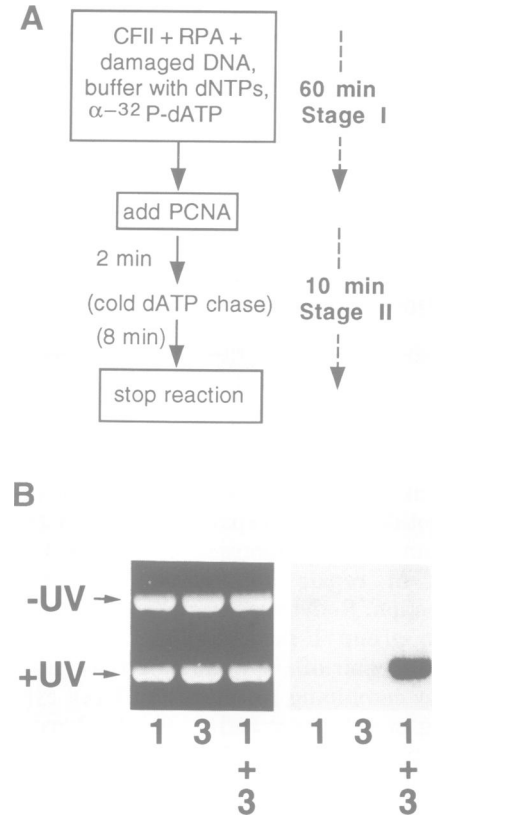


**Fig. 1.** Two-stage reactions to measure DNA repair synthesis. (**A**) Cell fraction II (CFII) is prepared by phosphocellulose chromatography of a whole cell extract as described by Shivji *et al.* (1992), then incubated in reaction mixtures with purified human RPA and PCNA as indicated. The cold dATP chase is optional, but helps reduce background. (**B**) Repair synthesis reactions performed as in panel A included 70 μg CFII protein prepared from a group 1 extract (CHO 43-3B), 1; a group 3 extract (CHO 27-1), 3; or 35 μg protein from each CFII, 1 + 3. Each reaction included 50 ng of RPA in the first stage and 25 ng of PCNA in the second incubation. DNA isolated from the reaction mixtures was linearized with *Bam*HI and separated by agarose gel electrophoresis (left). DNA synthesis was detected by fluorography (right). 60 fmol dAMP was incorporated in the reaction mixture in the final lane.

XP-A extracts can be readily complemented with recombinant XP-A protein produced in a similar way (C.Jones and R.Wood, in preparation).

There are many possible reasons for the low complementing activity of the recombinant ERCC1, but several trivial explanations are unlikely. The histidine tag would not be expected to interfere with correction since the amino-terminus of ERCC1 tolerates many amino acid and length changes; this region is very divergent between human and mouse, and a gene with a deletion of the sequence coding for the 54 amino-terminal amino acids still complements group 1 mutant cells (van Duin *et al.*, 1986, 1988). Critical post-translational modifications of ERCC1 might not take place in *E.coli*, or correct refolding of the recombinant protein might not occur, but we were also unable to obtain correction with the small amount of ERCC1 produced in reticulocyte lysate.

### *Group 1 and 4 fractionated extracts do not complement one another*

Recently it has been shown that the RAD10 protein, the *S.cerevisiae* homologue of ERCC1, forms a tight and specific

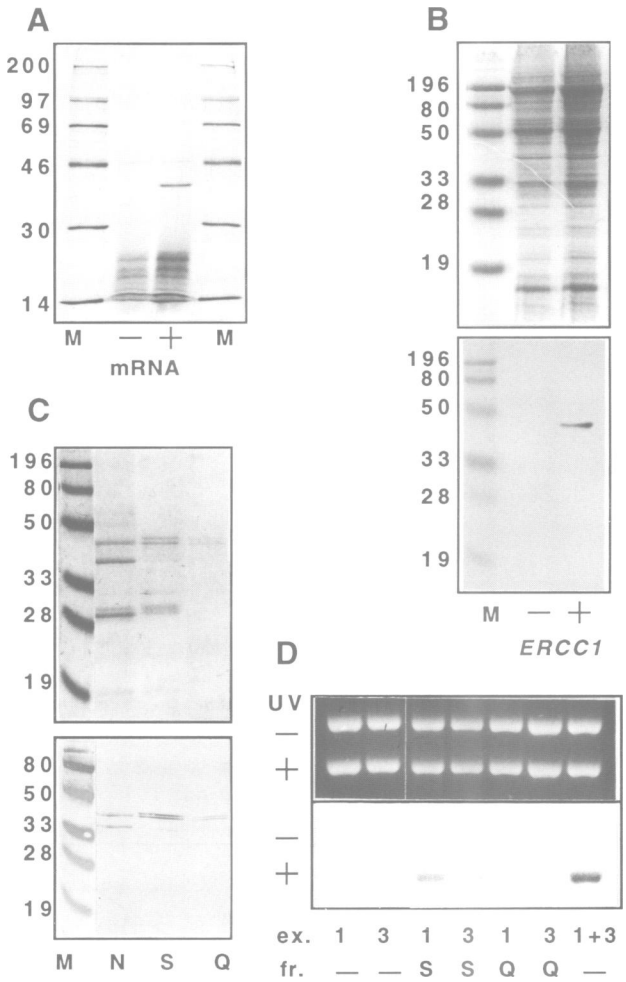


**Fig. 2.** Human ERCC1 protein produced in *E.coli*. (**A**) *In vitro* translation of human ERCC1 by rabbit reticulocyte lysates primed with (+) or without (−) *ERCC1* mRNA. Lanes M contain $^{14}$C-labelled markers. (**B**) Synthesis of polyhistidine-tagged ERCC1 in *E.coli*. M, prestained markers (Bio-Rad); −, pET15b vector only; +, pET15b/*ERCC1*. Top, SDS−polyacrylamide gel of whole cell lysates, stained with Coomassie Blue; bottom, immunoblot with anti-ERCC1 antibody 1. (**C**). Top, SDS−PAGE of ERCC1 sequentially purified from *E.coli* pellet by N, chromatography on a nickel−agarose column; S, an FPLC Mono S column; and Q, an FPLC Mono Q column. M, prestained markers. Bottom, immunoblot with anti-ERCC1 antibody 1. The additional cross-reacting bands in fractions N and S may represent proteolytic fragments of ERCC1 which retain the polyhistidine tag. (**D**) DNA repair synthesis reactions with 70 μg protein from a CFII of group 1 cells or group 3 cells as indicated (ex.) with PCNA and RPA (see Figure 1) and added recombinant ERCC1 (fr.). 125 ng protein was added of fractions purified by FPLC on the Mono S column (S), and 25 ng protein was added of the more purified material from the Mono Q column (Q). Top, agarose gel; bottom, fluorograph showing repair synthesis.

association with the RAD1 protein (Bailly *et al.*, 1992; Bardwell *et al.*, 1992). The two yeast proteins appear to work together to perform a critical function in nucleotide excision repair, and also to participate in a pathway of mitotic recombination. This suggested that by analogy with the yeast system, ERCC1 might be tightly associated with another protein in the cell extracts. This could make it difficult to correct the defect in group 1 mutant cell extracts with recombinant ERCC1 protein, since the latter would have to replace defective protein that was bound firmly in a complex.

We tested the ability of phosphocellulose-fractionated

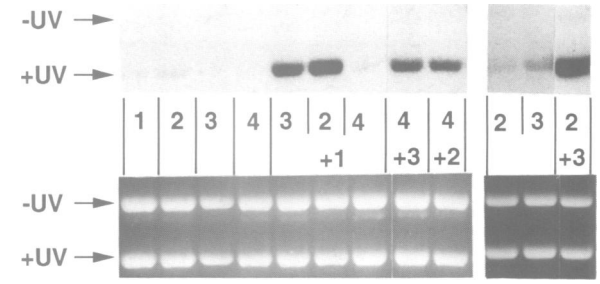


**Fig. 3.** Repair synthesis reactions using CFIIs from cells of different complementation groups. Each two-stage reaction with RPA and PCNA included 90 μg protein from a CFII of the indicated complementation group, or a mixture of 45 μg CFII protein from each of two different complementation groups. Top, fluorograph; bottom, agarose gel of linearized plasmid DNA stained with ethidium bromide.

extracts (CFIIs) from the group 1 mutant to complement such fractionated extracts from other repair groups. Interestingly, a group 1 CFII was unable to complement a CFII from a group 4 mutant, even though CFIIs from groups 1 or 4 could complement those from groups 2 and 3 (Figure 3), or extracts from group 5 (O'Donovan and Wood, 1993). The lack of complementation *in vitro* between groups 1 and 4 is intriguing because cells from groups 1 and 4 share an exceptional sensitivity to DNA interstrand cross-linking agents such as mitomycin C that is not displayed by mutants from the other characterized groups (Hoy *et al.*, 1985). This hypersensitivity to cross-linking agents might reflect a recombination defect in group 1 and 4 mutants in addition to the nucleotide excision repair defect, reminiscent of the shared repair/recombination defect in yeast *rad1* and *rad10* mutants (Weeda and Hoeijmakers, 1993). A tight functional association of ERCC1 and ERCC4 could provide a reason for the poor or absent complementation *in vitro* (Figures 2D and 3). Isolation of the *ERCC4* gene has not been reported, and so analysis of a putative ERCC1−ERCC4 association cannot yet be carried out by the co-immunoprecipitation methods that were used for demonstrating the RAD1−RAD10 association in yeast. Instead, we used a biochemical approach and examined the behaviour of ERCC1 and ERCC4 correcting activities from human cell extracts.

### *Co-purification of group 1 and group 4 correcting activities*

In initial experiments, we observed that group 1 complementing activity from calf thymus tissue elutes during gel filtration with an anomalously high molecular weight (unpublished data). To analyse this further in human cell extracts, a CFII prepared from HeLa cells was concentrated by ammonium sulphate precipitation and fractionated by gel filtration chromatography in a buffer that included 1 M KCl to minimize nonspecific ionic interactions. Fractions were dialysed and tested for their ability to correct CFIIs prepared from group 1 and 4 extracts, after supplementation with RPA and PCNA in two-stage reactions as outlined in Figure 1A. The correcting activities for group 1 (Figure 4A) and group 4 (Figure 4B) coincided exactly (Figure 4C). To ascertain whether the ERCC1 polypeptide was present in the complementing fractions, protein was analysed by immunoblotting with anti-ERCC1 antibody. The presence of ERCC1 in the fractions correlated exactly with correcting activity

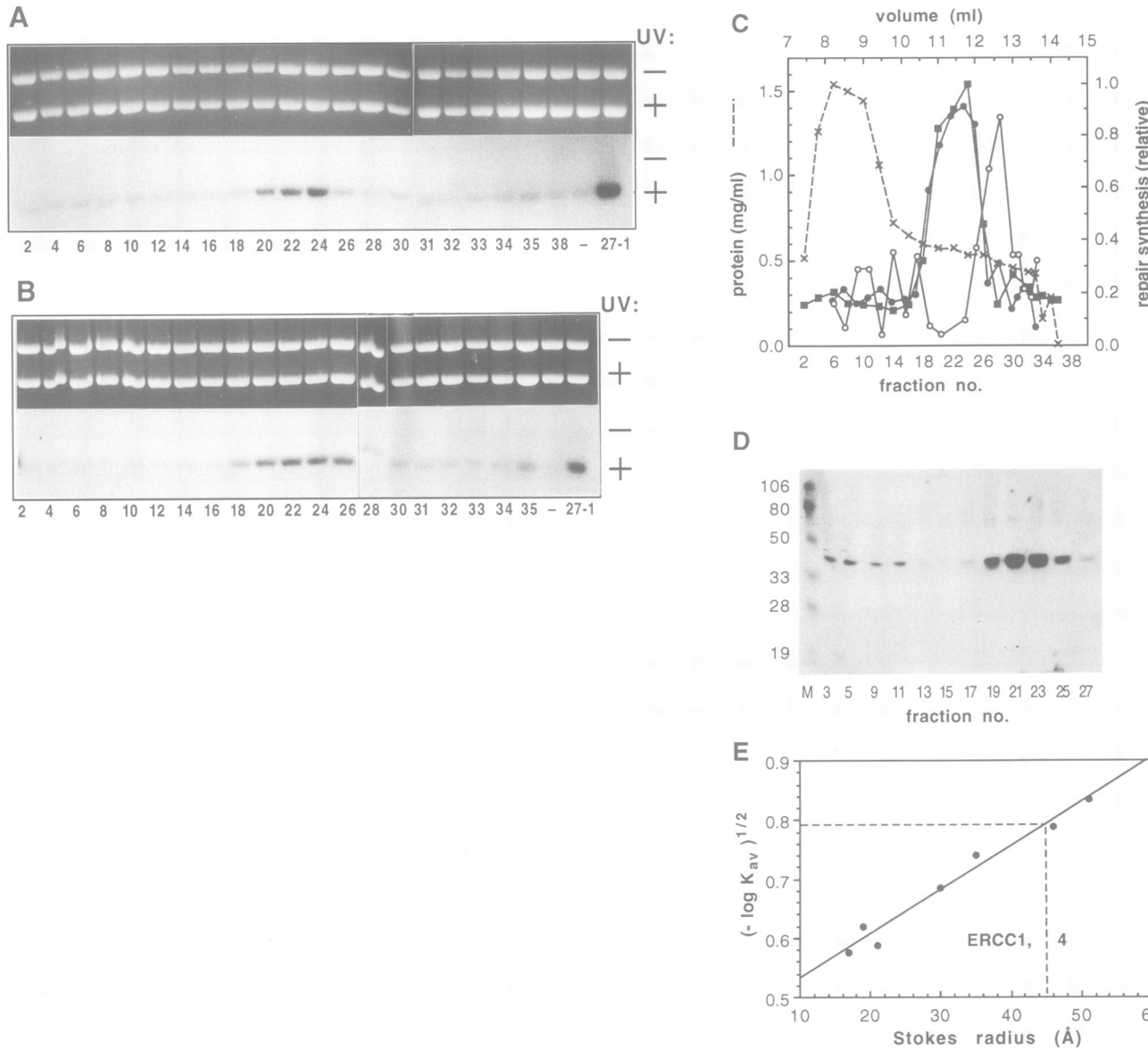


**Fig. 4.** Fractionation of group 1 and 4 correcting activity by size using gel filtration chromatography. (**A** and **B**) A 10 μl aliquot of the indicated fraction from a Superose 12 column was added to 90 μg of CFII from group 1 cells (43-3B, panel A), or group 4 cells (UV41, panel B) and DNA repair synthesis measured using RPA and PCNA as described. No column fraction was added to lanes marked '–'. Lanes marked '27-1' represent reactions with a mixture of 45 μg of CFII from group 3 cells (27-1) and 45 μg of CFII from 43-3B (A), or UV41 (B). (**C**) Plot of data from A (group 1, closed circles) and B (group 4, closed squares), with total protein eluted from the column (dashed line). The open circles show the correcting activity for a CFII from XP-A cells by the same fractions used in A and B. Relative repair synthesis (giving similar synthesis in early fractions) is plotted for clarity; at the respective peaks of complementing activity the fmol dAMP incorporated were 75 (group 1), 40 (group 4) and 25 (XP-A). (**D**) Immunoblot of aliquots of the indicated fractions from the Superose column using anti-ERCC1 antibody 2. M, pre-labelled markers. (**E**) Determination of the Stokes radius of group 1- and group 4-correcting activity from a plot (Siegel and Monty, 1966) of the experimentally determined $(-\log K_{av})^{1/2}$ versus the Stokes radius of the indicated markers (bovine γ-globulin, 51 Å; aldolase, 46 Å; bovine serum albumin, 35 Å; ovalbumin, 30 Å; chymotrypsin, 21 Å; myoglobin, 19 Å; cytochrome C, 17 Å). Blue dextran was used to determine the void volume of the column (7.7 ml).

for group 1 and 4 extracts (Figure 4D). The ERCC1 and ERCC4 correcting activities had a Stokes radius of 45.5 Å (Figure 4E). Correcting activities for XP-A extract and for CFII from a group 3 extract were also assayed; XP-A correcting activity (Robins *et al.*, 1991) emerged at a position corresponding to ~40 kDa as expected (Figure 4C), while group 3 correcting activity eluted earlier than the ERCC1/ERCC4 peak at a position corresponding to ~230 kDa (data not shown).

To obtain a more accurate measurement of the native molecular mass of the group 1 and 4 correcting activities, the sedimentation coefficient was determined after centrifugation on a glycerol gradient. Group 1 and group 4 correcting activities had an estimated sedimentation coefficient of 5.4S (Figure 5). Combining the Stokes radius and S value and assuming a partial specific volume of 0.725 g/cm$^3$ (Siegel and Monty, 1966) yields an estimate of 100 kDa for the native molecular mass of group 1 and group 4 correcting activities, and a frictional coefficient of 1.5.

### *In vitro complementation of ERCC1 and 4 extracts with XP extracts*

As outlined in the introduction, the UV-sensitive rodent groups 2, 3 and 5 correspond to XP complementation groups

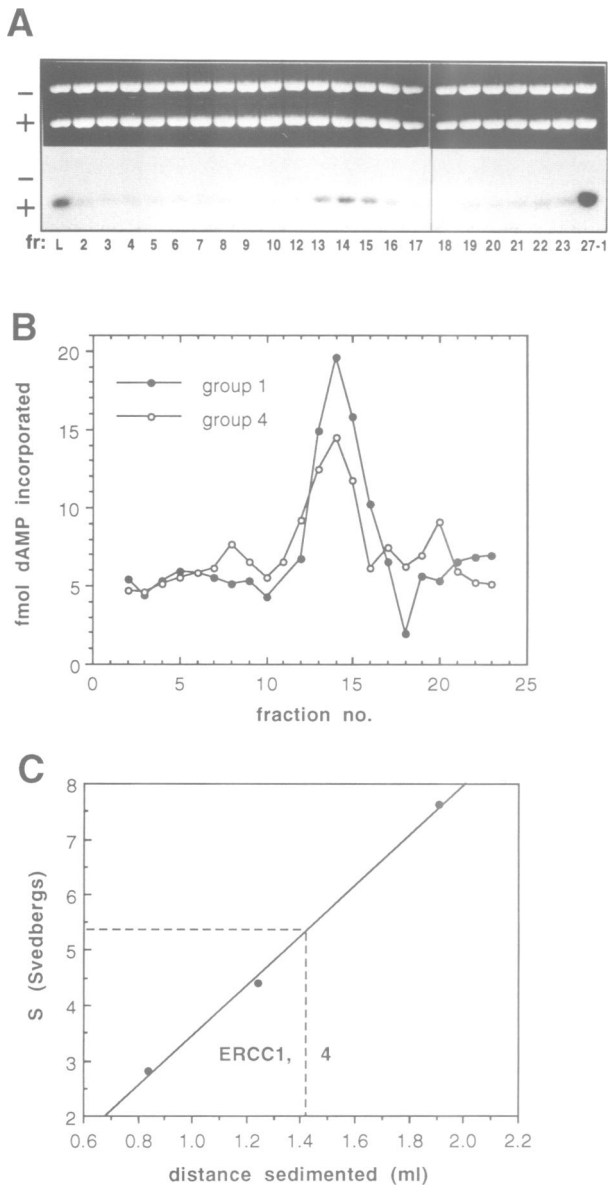


**Fig. 5.** Sedimentation velocity of group 1 and 4 correcting activities. (**A**) Samples of the indicated fractions (fr.) from the gradient were assayed for DNA repair correcting activity with 90 μg CFII from 43-3B group 1 cell extracts (shown in the figure) or from UV41 group 4 extracts. An aliquot of the sample loaded on the gradient was also assayed (L). The lane marked '27-1' represents a reaction with a mixture of 45 μg of CFII from CHO 27-1 group 3 cells and 45 μg of CFII from 43-3B group 1 cells. Top, agarose gel of linearized plasmid DNA (−, unirradiated; +, irradiated) stained with ethidium bromide; bottom, fluorograph of the gel. (**B**) Quantification of correcting activity. The peak of group 1 and 4 correcting activities corresponds to a position 1.425 ml from the top of the gradient. An immunoblot of the protein fractions with anti-ERCC1 antibody (not shown) confirmed that ERCC1 polypeptide was associated with the active fractions. (**C**) Plot of the sedimentation position of markers in a parallel gradient versus known S-values (alcohol dehydrogenase, 7.6S; bovine serum albumin, 4.4S; carbonic anhydrase, 2.8S) showing derivation of the sedimentation coefficient for ERCC1/ERCC4 correcting activity.

D, B and G respectively. The *ERCC1* gene on chromosome 19 cannot, however, correct cells of any XP group (van Duin *et al.*, 1989) and it is of interest to know whether group 4 might correspond to one of the remaining XP groups. The assignment of *ERCC4* to the short arm of human chromosome 16 (Liu *et al.*, 1989) rules out a correspondence with XPAC on chromosome 9 (Tanaka *et al.*, 1990). We also know that group 4 and XP-A correcting activities separate during purification (Figure 4C) as do group 4 and XP-C correcting activities (M.Shijvi and R.Wood, unpublished). The partial repair defect in cells from the XP-E group is associated with a distinct 125 kDa DNA binding protein (Hwang and Chu, 1993). XP group F appeared to be the only remaining possible human equivalent of group 4 and so XP-F extracts were examined for their ability to complement group 1 and 4 extracts in the fractionated system.

When tested, a CFII from XP-F cells was in fact unable to complement a CFII from group 1 or 4, but could complement fractionated extracts from other tested groups, including group 3 (Figure 6A) and XP-A. Further, XP-F correcting activity from HeLa cell extracts eluted during gel filtration in a peak that coincided exactly with the peak of group 1 and group 4 correcting activity (Figure 6B). Together, the data indicate that XP-F correcting activity is associated with group 1 and group 4 correcting activities.

### *ERCC1 polypeptide in repair-defective CHO and human cells*

To check for the presence of ERCC1 in UV-sensitive human and rodent cells, immunoblotting was performed with an antibody raised against a synthetic carboxy-terminal peptide predicted from the human ERCC1 sequence. The 13-residue oligopeptide used for immunization is very conserved between man and mouse, with only a single difference between the two species (Asp in human and Glu in mouse), and the mouse and human proteins differ in overall length by only one amino acid (van Duin *et al.*, 1988). We anticipated that a similar degree of conservation in the hamster protein would allow detection of ERCC1 in CHO extracts. In extracts from CHO cells, the principal band recognized by the antibody migrated at 38 kDa (Figure 7A), slightly faster than the human protein (Figure 4D). Immunoblotting of equal amounts of protein from repair-proficient CHO-9 cells and from rodent groups 1−6 showed that the 38 kDa band was reproducibly reduced in amount in the group 1 and group 4 extracts (Figure 7A) and in corresponding CFIIs (not shown). The cell line CHO 83951A is a transfectant of group 1 43-3B cells with the human *ERCC1* gene. As expected, an immunoblot of cell extract from this line showed the presence of human ERCC1 and a greatly reduced amount of hamster ERCC1. In human cells, ERCC1 was present in similar amounts in normal, XP-A, XP-B, XP-C, XP-D and XP-G extracts (Figure 7B). However, in extracts from the XP-F cell line GM8437, the amount of ERCC1 was reduced relative to all other XP or normal extracts tested (Figure 7B).

The reduced amount of ERCC1 in the group 1 extract suggests that the mutant protein synthesized in these cells may be poorly expressed or unstable. Moreover, the lower amount of ERCC1 in group 4 and XP-F extracts suggests that ERCC1 may be unstable in the absence of normal ERCC4 protein partner.

## Discussion

### *Association of ERCC1 and ERCC4 activities*

The results presented here indicate that the ERCC1 and ERCC4 polypeptides are associated in a protein complex. This suggestion is based on several observations. First, *in*

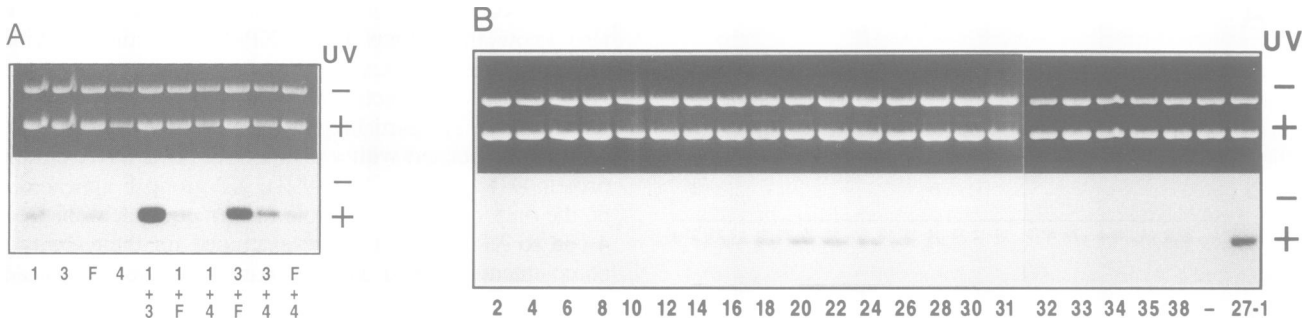


**Fig. 6.** XP-F correcting activity. (**A**) Inability of CFIIs from XP-F cells to complement CHO group 1 and 4 fractionated extracts. Each reaction included 90 μg protein from a CFII of the indicated CHO complementation group (1, 3 or 4) or from XP-F cells (F), or 45 μg protein each in mixtures of CIIs from two different complementation groups, in two-stage reactions with RPA and PCNA. Top, agarose gel of linearized plasmid DNA stained with ethidium bromide; bottom, fluorograph. (**B**) A 10 μl aliquot of the indicated Superose 12 fraction (same as used in Figure 4) was added to 90 μg of CFII from XP-F cells and DNA repair synthesis measured using RPA and PCNA as described. No column fraction was added to the lane marked '–'. In the reactions with the fractions of highest activity, ~25 fmol dAMP were incorporated. The last lane represents a reaction with a mixture of 45 μg of CFII protein from XP-F cells with 45 μg of CFII protein from CHO 27-1 (group 3) cells.

*vitro* complementation does not occur between protein fractions from group 1 and group 4 mutants, even though the *ERCC1* and *ERCC4* genes are clearly distinct and mutant cells from the two groups are able to complement one another *in vivo* upon cell fusion (Busch *et al.*, 1989). Second, the correcting activities for group 1 and 4 mutants co-purify during phosphocellulose chromatography, gel filtration chromatography and glycerol gradient sedimentation as an activity with a native molecular mass of 100 kDa that contains the 32.6 kDa ERCC1 polypeptide. Based on the latter result alone one could hypothesize that ERCC1 exists in cell extracts as a homotrimer and that ERCC4 exists independently in a 100 kDa form. This would not, however, explain the lack of *in vitro* complementation between fractionated group 1 and 4 extracts. It is much more likely that the ERCC1 and ERCC4 proteins are associated with one another, by analogy with the 24 kDa RAD10 and 126 kDa RAD1 proteins of *S.cerevisiae*. An obvious prediction is that ERCC4 will show sequence similarity to a part of the *S. cerevisiae* RAD1 protein. We have used the carboxy-terminal anti-ERCC1 antibody 2 in an attempt to co-immunoprecipitate a polypeptide associated with ERCC1 from [$^{35}$S]methionine-labelled protein of HeLa cells. Examination of the immunoprecipitate after SDS–PAGE showed major bands of 43 kDa (the migration position of ERCC1), 71 kDa and 78 kDa as well as minor or contaminating bands of 45, 51 and 60 kDa (not shown). The 71 kDa or 78 kDa band may correspond to ERCC4, but a definitive identification and further analysis must await purification of the ERCC4 polypeptide and generation of antibodies against it. In an accompanying paper van Vuuren *et al.* (1993) show that an anti-ERCC1 antibody can simultaneously deplete group 1 and group 4 correcting activities from a HeLa cell extract, providing independent and further evidence that ERCC1 and ERCC4 are present together in a protein complex.

In addition to a defect in nucleotide excision repair, *rad1* and *rad10* mutants of *S.cerevisiae* have defects in a pathway of mitotic recombination (Schiestl and Prakash, 1988; Aguilera and Klein, 1989; Thomas and Rothstein, 1989; Schiestl and Prakash, 1990). To explain this recombination defect in *rad1* mutants, Fishman-Lobell and Haber (1992) proposed that RAD1 protein is an endonuclease that processes single-stranded DNA ends. Their data equally fit

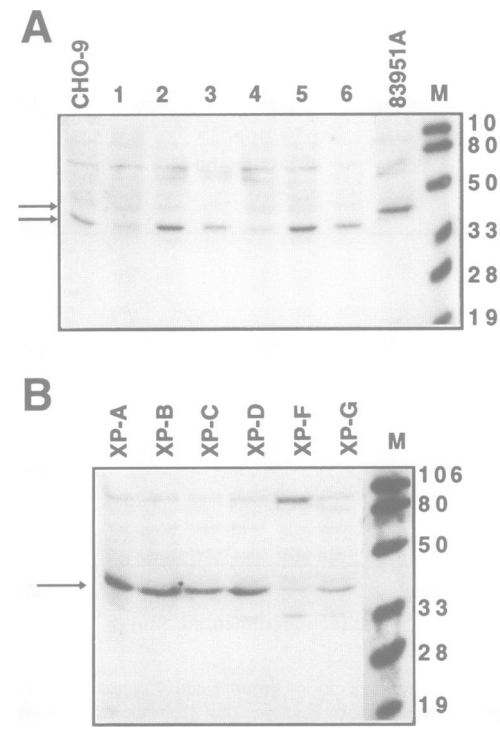


**Fig. 7.** Immunoblots of extracts from CHO and human cells. (**A**) Anti-ERCC1 antibody 2 (1/20 dilution) was used to blot protein (40 μg per lane) from whole cell extracts of CHO-9 cells and cells of complementation groups 1 (43-3B), 2 (UV5), 3 (27-1), 4 (UV41), 5 (UV135) and 6 (UV61), and from CHO83951A, a transformant of 43-3B expressing human ERCC1. The migration positions of human ERCC1 (upper arrow) and hamster ERCC1 (lower arrow) are indicated. Lane M shows prelabelled markers. (**B**) Immunoblot (using a 1/20 dilution of antibody 2) of whole cell extract protein (100 μg per lane) from human XP cells of groups A (GM2345), B (GM2252), C (GM2246), D (GM2253), F (GM8437) and G (XPG83). The arrow indicates the migration position of human ERCC1.

a model in which the RAD1–RAD10 complex forms a nuclease. Recently, Tomkinson *et al.* (1993) demonstrated that RAD1 and RAD10 indeed associate to form a single-stranded DNA endonuclease activity, and that either protein alone lacks such activity. At least one analogous endonuclease activity is needed in human nucleotide excision repair since dual incisions are introduced flanking a lesion

(Huang *et al.*, 1992). Formation of a functional endonuclease by the association of ERCC1 with ERCC4 (or the XP-F protein) would be somewhat reminiscent of the nuclease formed by the association of *E.coli* UvrB and UvrC proteins (Van Houten and Snowden, 1993). A similarity between a portion of the carboxy-terminus of ERCC1 and UvrC has been noted, and it has been suggested that the two proteins might be responsible for a similar function (Doolittle *et al.*, 1986; van Duin *et al.*, 1988). However, residues known to be critical for the nuclease function of UvrC lie outside the region of similarity with ERCC1 (Lin and Sancar, 1992).

The lower amount of ERCC1 polypeptide in group 4 and XP-F extracts (Figure 7) may indicate that ERCC1 is unstable in the absence of a normal protein partner. This could explain why ERCC1 over-production is only modest even when its mRNA is highly expressed (Belt *et al.*, 1991). Further, this would provide one reason for the lack of complementation between group 1 and 4 extracts *in vitro*, even though fusion of cells from the two groups leads to complementation *in vivo*. In a hybrid cell, both ERCC1 and ERCC4 would be synthesized and could associate with one another to form a stable complex. *In vitro*, neither a group 1 nor a group 4 extract would be able to contribute sufficient ERCC1 to a complex.

### *Relationship of ERCC4 and XPFC*

Is the *ERCC4* gene equivalent to the XP-F complementing gene *XPFC*? Since neither *ERCC4* nor *XPFC* nor the corresponding complementing proteins have been isolated we cannot provide a definitive answer, but the findings reported here indicate that such an equivalence is a distinct possibility. XP-F is generally a mild form of XP, with patients showing sun-sensitivity but few tumours. Skin fibroblasts from such individuals have been reported to repair pyrimidine dimers at a slow but detectable rate (Hayakawa *et al.*, 1981). If ERCC4 defects give rise to both faulty nucleotide excision repair and altered intrachromosomal recombination (as anticipated by the hypersensitivity to DNA cross-linking agents and the analogy with yeast RAD1), this might be expected to have a more extreme effect in man. Perhaps most viable XP-F mutations have 'leaky' point mutations in the *XPFC* gene. Recently an unusual, severely affected XP-F patient with neurological symptoms has been reported (Moriwaki *et al.*, 1993) and it will be of interest to explore the extent of the repair and recombination defect in this patient.

Chromosome analysis in human–hamster cell hybrids indicates that *ERCC4* is on the short arm of human chromosome 16 (Liu *et al.*, 1989). On the other hand, Saxon *et al.* (1989) noted partial alleviation of the UV-sensitivity of XP-F fibroblasts when a fragment of chromosome 15 was introduced by microcell-mediated transfer. However, the possibility could not be eliminated that a different chromosome might have been capable of more extensive phenotypic complementation, and so the mapping results do not preclude an identity of *ERCC4* and *XPFC*. In a pioneering approach to examine the relationship between XP-F and rodent repair complementation groups, Thompson *et al.* (1985) made proliferating hybrids between rodent group 1, group 4 and XP-F cells. Although CHO UV41 group 4 cells clearly formed repair-proficient hybrids with XP-A, XP-C and XP-D cells, efficient complementation was not observed in a cross of UV41 and XP-F cells, and in fact was the lowest of any cross investigated. In retrospect this is consistent with an equivalence of ERCC4 and XPFC.

The work reported here brings together the ERCC1 correcting activity for group 1 mutants with correcting activities for group 4, the only remaining rodent group of the six initially described (Thompson *et al.*, 1981; Busch *et al.*, 1989) for which a complementing gene has not been identified, and for XP-F, the only XP group which has not heretofore been associated with a gene or protein defect. The data indicate that these activities are associated in a functional protein complex.

## Materials and methods

### *Cell lines*

CHO cell lines 43-3B (group 1) and 27-1 (group 3) were isolated after treatment of CHO-9 cells with the point mutagen ethylnitrosourea as described (Wood and Burki, 1982). CHO cell lines UV41 (group 4, isolated after treatment of CHO AA8 cells with the frameshift mutagen ICR-170) and other lines with the UV prefix were provided by Dr D.Busch. CHO 83951A was provided by J.Hoeijmakers (Westerveld *et al.*, 1984). Normal and XP cell lines were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). GM8437 is the only transformed XP-F cell line available from the repository.

### *Fractionation of extracts*

Whole cell extracts were prepared from $10^9$ CHO cells as described previously (Biggerstaff and Wood, 1992). 80 mg of extract protein was loaded on to a 5 ml column of phosphocellulose (Whatman P11) as described (Shivji *et al.*, 1992). Protein that was adsorbed by the column in buffer containing 0.1 M KCl but eluted in buffer containing 1.0 M KCl was collected and designated cell fraction II (CFII). The CFII was concentrated in a 10 ml Amicon pressure ultrafiltration device to a protein concentration of ~5 mg/ml, dialysed against Buffer A [25 mM HEPES–KOH (pH 7.9), 1 mM EDTA, 17% glycerol, 1 mM dithiothreitol (DTT), 12 mM $MgCl_2$ and 0.1 M KCl] and stored in aliquots at −80°C.

### *Gel filtration and glycerol gradient sedimentation*

A CFII protein fraction from HeLa cells (12 ml, 60 mg) was concentrated by precipitation with 0.44 g/ml ammonium sulphate and centrifugation. The pellet was resuspended and dialysed for 1 h versus Buffer B [25 mM HEPES–KOH (pH 7.8), 1.0 M KCl, 2 mM DTT, 1 mM EDTA and 0.01% (v/v) NP-40]. 0.2 ml (7 mg) protein was loaded on to a 1 cm × 30 cm Superose 12 column (Pharmacia) equilibrated in Buffer B and run at 0.4 ml/min. Fractions (0.2 ml) were collected and dialysed for 3 h against Buffer A before use in assays for complementation of DNA repair synthesis.

Fractions 21 and 23 from the Superose 12 column were pooled and 200 μl was layered on a 3.5 ml 15–35% (v/v) glycerol gradient in Buffer B and centrifuged in a Beckman SW60Ti rotor at 47 000 r.p.m. (227 000 $g_{av}$) for 29 h at 4°C. Fractions (150 μl) were collected from the bottom of the gradient and dialysed for 3 h against Buffer A before use. An identical gradient containing markers was run at the same time and fractions were collected. Sedimentation positions of the markers were determined by SDS–polyacrylamide gel electrophoresis, using densitometry to quantify the intensity of staining with Coomassie Blue.

### *In vitro DNA repair reactions*

Plasmid pBluescript KS+ (Stratagene) was UV-irradiated (450 J/m$^2$) and treated with *E.coli* Nth protein, and closed-circular forms were isolated as described by Wood *et al.* (1988) and Biggerstaff *et al.* (1991) to obtain DNA free of pyrimidine hydrates. Reaction mixtures contained 250 ng each of UV-irradiated pBluescript KS+ and nondamaged control plasmid pHM14, 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dGTP, dCTP and TTP, 8 μM dATP, 74 kBq of [α-$^{32}$P]dATP (111 TBq/mmol), 40 mM phosphocreatine (di-Tris salt), 2.5 μg creatine phosphokinase (type I, Sigma), 3.4% glycerol and 18 μg bovine serum albumin. 50–90 μg CFII protein from mutant cell lines was included in assays of column fractions or 70–90 μg of total CFII protein in complementation assays with ~150 ng of purified RPA protein or 18 μg of CFIA (DEAE Biogel flow-through fraction) protein (Shivji *et al.*, 1992). Reactions were incubated for 1 h at 30°C. Purified PCNA (25 ng) was then added and reactions were incubated for a further 10 min at 30°C. Plasmid DNA was purified from the reaction mixtures,

linearized with *Bam*HI and separated by electrophoresis on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide. Results were quantified by scintillation counting of excised DNA bands and by using a Molecular Dynamics densitometer with ImageQuant software.

### *ERCC1 expression*

The human ERCC1 coding sequence was isolated from a human fibroblast cDNA library (Keyse and Emslie, 1992) by PCR using the primers GATCGGAATTCCCATATGGACCCTGGGAAGGACAAAGA (5′ end of gene) and GATCGGTCGACTCAGGGTACTTTCAAGAAGGGCTCGTG (3′ end of gene). This facilitated its cloning into expression vectors via the *Nde*I and *Sal*I restriction sites. Upon full sequencing of the insert, three base changes caused by *Taq* polymerase were found, two at nucleotides 141 and 369, which did not change the amino acid, and one at nucleotide 337. The latter change was corrected by *in vitro* mutagenesis (Kunkel, 1985) after subcloning the gene into the *Hin*dIII and *Xba*I sites of pBluescript KS−. This construct was used to produce mRNA from the T7 promoter for *in vitro* translation using rabbit reticulocyte lysate and a translation kit (Boehringer Mannheim) including CAP analogue and [$^{35}$S]methionine. For expression in *E.coli*, the gene was cloned into the *Xho*I and *Nde*I sites of pET15b (InVitrogen) to yield a construct with an amino-terminal polyhistidine tag. BL21 (DE3) pLysS cells were grown at 37°C to an $OD_{600}$ of 0.6, IPTG (1 mM) was added and incubation continued for 3 h. The cells were disrupted by sonication and the lysate centrifuged (10 min, 11 000 *g*). The pellet was resuspended in buffer C (0.1 M sodium phosphate, 10 mM Tris−HCl) containing 8 M urea (pH 8.0), loaded on to a nickel−agarose column (Qiagen), eluted with buffer C containing 6 M guanidine−HCl (pH 5.8) and dialysed against buffer A to give fraction N. This fraction (80 μg protein) was loaded on to a 1 ml FPLC Mono S column (Pharmacia) in buffer B containing 0.1 M KCl and 0.1 mM PMSF and the flow-through (50 μg protein, designated fraction S) was loaded on to an FPLC Mono Q column (Pharmacia) in the same buffer. This flow-through (fraction Q), and fraction S were dialysed against buffer A before use in repair assays.

### *Antibodies*

Peptide 1 ($NH_2$-GVPNPSGPPAR-COOH) corresponds to amino acids 9−19 of the deduced human ERCC1 polypeptide (van Duin *et al.*, 1986). Peptide 2 ($NH_2$-ARRLFDVLHEPFL-COOH) corresponds to amino acids 282−294 near the carboxy-terminus of the protein. Rabbit polyclonal antibodies were raised against peptides coupled to bovine thyroglobulin (T1001, Sigma) with carbodiimide (Peptide 1) or glutaraldehyde (Peptide 2). Antisera were affinity-purified on columns containing peptide coupled to bovine serum albumin (Sigma RIA grade) linked to activated Sepharose, and then dialysed against phosphate-buffered saline A before storage at −80°C. Immunoblotting was as described by Robins *et al.* (1991).

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