The EMBO Journal vol.12 no.9 pp.3693-3701, 1993

# Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F

**A.J.van Vuuren, E.Appeldoorn, H.Odijk, A.Yasui[1], N.G.J.Jaspers, D.Bootsma and J.H.J.Hoeijmakers**

Department of Cell Biology and Genetics, Medical Genetic Center, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands and [1]Research Institute for Tuberculosis and Cancer, Tohuku University, Sendai, Japan

Communicated by D.Bootsma

**Nucleotide excision repair (NER), one of the major cellular DNA repair systems, removes a wide range of lesions in a multi-enzyme reaction. In man, a NER defect due to a mutation in one of at least 11 distinct genes, can give rise to the inherited repair disorders xeroderma pigmentosum (XP), Cockayne's syndrome or PIBIDS, a photosensitive form of the brittle hair disease trichothiodystrophy. Laboratory-induced NER-deficient mutants of cultured rodent cells have been classified into 11 complementation groups (CGs). Some of these have been shown to correspond with human disorders. In cell-free extracts prepared from rodent CGs 1-5 and 11, but not in a mutant from CG6, we find an impaired repair of damage induced in plasmids by UV light and *N*-acetoxy-acetylaminofluorene. Complementation analysis *in vitro* of rodent CGs is accomplished by pairwise mixing of mutant extracts. The results show that mutants from groups 2, 3, 5 and XP-A can complement all other CGs tested. However, selective non-complementation *in vitro* was observed in mutual mixtures of groups 1, 4, 11 and XP-F, suggesting that the complementing activities involved somehow affect each other. Depletion of wild-type human extracts from ERCC1 protein using specific anti-ERCC1 antibodies concomitantly removed the correcting activities for groups 4, 11 and XP-F, but not those for the other CGs. Furthermore, we find that 33 kDa ERCC1 protein sediments as a high mol. wt species of ~120 kDa in a native glycerol gradient. These results strongly suggest the presence of a pre-existing enzyme complex in mammalian cell extracts, harbouring at least the products of the *ERCC1* and *ERCC4* genes. This complex also carries complementing activites of XP group F and rodent CG11. We postulate that the complex, like the one in *Saccharomyces cerevisiae* involving the RAD1 and RAD10 proteins (the latter being the homologue of ERCC1), functions in both NER and recombinational repair.**

*Key words:* Chinese hamster mutant/complementation analysis/damaging agents/nucleotide excision repair/repair complex

## Introduction

Nucleotide excision repair (NER) is a DNA repair system that has evolved in all living organisms for the removal of lesions inflicted on DNA by mutagenic radiations (notably UV light) or numerous chemical agents. It is a multi-enzyme process involving specific recognition and dual incision of the damaged strand, followed by lesion removal, gap-refilling and strand ligation (Grossman and Yeung, 1990; Selby and Sancar, 1990; Hoeijmakers, 1991). In man, genetic defects in NER of UV-induced DNA damage give rise to the severe disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) or PIBIDS. Besides hypersensitivity of the skin to sunlight and progressive neurological dysfunction, a dramatically increased risk of skin cancer (XP) or developmental abnormalities (CS) entail the clinical hallmarks of these disorders (Cleaver and Kraemer, 1989; Nance and Berry, 1992). PIBIDS is a photosensitive form of the brittle hair disorder trichothiodystrophy (Lehmann, 1987). Somatic cell hybridization studies have revealed extensive genetic heterogeneity: in XP, at least seven NER-defective complementation groups (named A to G) exist whereas in CS two (CS-A and CS-B) have been identified so far (Lehmann, 1982; Vermeulen *et al.*, 1991) and two complementation groups have been found in PIBIDS patients (Stefanini *et al.*, 1992, 1993).

In addition to these naturally occurring mutants, a large collection of laboratory-induced, UV-sensitive, NER-defective mutants have been obtained from cultured rodent cells. By restoration of UV resistance in cell hybrids, 11 complementation groups (CGs) were discerned among these mutant cell lines (for recent overviews see Thompson *et al.*, 1988; Busch *et al.*, 1989; Stefanini *et al.*, 1991; Zdzienicka *et al.*, 1991; Riboni *et al.*, 1992; Collins, 1993). Cross-sensitivity to chemicals producing bulky DNA lesions is a feature of the UV-sensitive mutants in all groups. However, representatives from groups 1 and 4 are unique with regard to the property of extreme sensitivity of DNA crosslinking agents, such as mitomycin C (Busch *et al.*, 1989).

A number of excision repair cross-complementing (*ERCC*) genes, capable of correcting the NER defects in the rodent CGs have been isolated using gene transfer strategies. Characterization has provided clues to the function of their gene products and their involvement in human disease (for reviews see Hoeijmakers and Bootsma, 1990; Friedberg, 1992; Hoeijmakers, 1993). For instance, based on sequence motifs, both the genes *ERCC2* and *ERCC3* are expected to encode proteins with helicase activity. These genes are now known to be involved in XP-D and XP-B, respectively (Weeda *et al.*, 1990; Flejter *et al.*, 1992; Weber, cited in Lehmann *et al.*, 1992). A third putative helicase, encoded by *ERCC6*, is required for preferential NER of actively transcribed DNA segments and is defective in CS group B (Troelstra *et al.*, 1992). The *ERCC5* gene was recently found to be responsible for XP-G (O'Donovan and Wood, 1993; Scherly *et al.*, 1993). The ERCC1 protein is not associated with the defect in any of the XP, CS or PIBIDS groups (Van Duin *et al.*, 1989). On the other hand, the XPAC protein is affected in XP-A (Tanaka *et al.*, 1989), but in none of

the rodent CGs 1–7 or CG 11 (our unpublished results).

As another outcome of the characterization of NER genes it has become clear that they are highly conserved in evolution. Thus, homologues for most cloned mammalian NER genes could be identified in lower eukaryotes, particularly in yeast. As examples, the *ERCC1, 2, 3* and *5* genes share extensive sequence homology at the protein level with the *RAD10, RAD3, RAD25/SSL2* and *RAD2* genes of *Saccharomyces cerevisiae*, respectively (Van Duin *et al.*, 1986; Weber *et al.*, 1990; Gulyas and Donahue, 1992; Park *et al.*, 1992; Scherly *et al.*, 1993). Therefore, it is likely that the basic features of the NER mechanism are conserved from yeast to man as well and that the homologues have functional equivalence. Both yeast and mammalian studies indicate that some NER genes play a role in other cellular processes. For instance, *RAD1* and *RAD10* are required for mitotic recombination (Schiestl and Prakash, 1988, 1990) and recently a direct involvement of the ERCC3 protein in transcription initiation was found (Schaeffer *et al.*, 1993).

A cell-free *in vitro* assay of mammalian NER is another important tool to elucidate the functions of the NER gene products at the enzymological level. The *in vitro* system measures repair synthesis in UV-irradiated plasmid DNA mediated by isolated human cell extracts (Wood *et al.*, 1988). The assay can mimic *in vitro* complementation analysis of XP extracts (Hansson *et al.*, 1991; Coverley *et al.*, 1992) and can detect repair of bulky lesions induced by chemical agents (Hansson *et al.*, 1989; Sibghat-Ullah *et al.*, 1989; Sibghat-Ullah, 1990). It has served to assess the topology of the early endonucleolytic step of human NER (Huang *et al.*, 1992), the involvement in NER of additional protein factors such as PCNA and SSB/RP-A (Coverley *et al.*, 1991, 1992; Nichols *et al.*, 1992; Shivji *et al.*, 1992) and the purification of XPAC protein from calf thymus (Robins *et al.*, 1991).

Here, we report the results of a systematic study of NER in UV-sensitive mutants of various rodent CGs using the *in vitro* assay. In attempting *in vitro* complementation analysis, we encounter selective absence of correction, when cell extracts from representatives of groups 1, 4 and 11 are mixed. Results from antibody depletion experiments and sedimentation analysis indicate that these specific patterns of *in vitro* non-complementation can be explained by the presence of a high mol. wt repair enzyme complex, comprising at least the products of the *ERCC1* and *ERCC4* genes. In addition, the factors able to complement XP group F and CG11 are associated with this complex.

## Results

### *In vitro repair activity in Chinese hamster cell extracts*

Two cell-free extracts, prepared from wild-type Chinese hamster cells (CHO9) and from a NER-deficient strain belonging to CG1 (43-3B), were compared with respect to their ability to mediate repair synthesis *in vitro* using UV-irradiated plasmid DNA (450 J/m$^2$) as a substrate. As shown in Figure 1A, UV-dependent repair synthesis was observed *in vitro*, with only a very slight incorporation of $^{32}$P-labelled dATP in the non-damaged plasmid. With these substrates no significant difference in repair synthesis was detected between a wild type (CHO9) and a CG1 mutant extract (43-3B).

Besides pyrimidine dimers and 6-4 photoproducts UV induces oxidative thymine species as a minor UV lesion, which is efficiently recognized by a NER-independent endonuclease activity present in human extracts. Plasmids can be cleared from such damage by treatment with the *Escherichia coli* Nth protein (Wood *et al.*, 1988). Using a 'cleaned' UV-irradiated substrate [UV(*nth*)] a clear difference between the repair activities of wild-type CHO9 and mutant 43-3B extracts became apparent (Figure 1B). Similar results were detected with plasmids damaged by *N*-acetoxy-2-acetyl-aminofluorene (AAF) (Figure 1C), which causes almost exclusively *N*-(guanin-8-yl) adducts (Landegent *et al.*, 1984). Apparently, the endonuclease-triggered activity is very potent in CHO extracts, necessitating the use of AAF or UV(*nth*) substrates to measure NER activity. The observed levels of repair synthesis of human and Chinese hamster repair-proficient protein extracts, when expressed as fmol $^{32}$P incorporated per ng plasmid, were in the same range and linearly dependent on the amount of extract added (data not shown).

As in CG1, a clear defect in *in vitro* NER is present in the tested rodent CG2, 3, 4, 5 and 11, as well as in XP-A and XP-F (Table I), in agreement with their inability to incise UV damage *in vivo*. In an extract derived from CG6 a normal response was observed, in line with the near normal

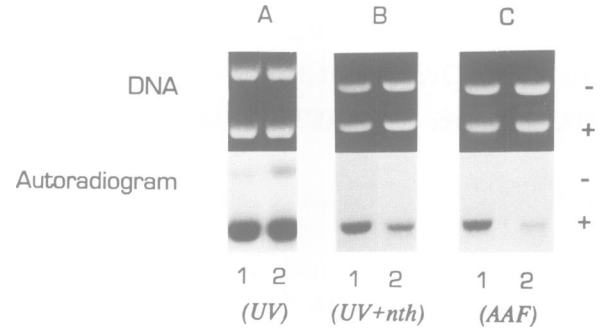


**Fig. 1.** Damage-dependent incorporation of [$^{32}$P]dATP into various DNA substrates. Plasmid DNAs were incubated with 200 μg of wild-type (CHO9, lanes 1) or CG1 (43-3B, lanes 2) extract in the repair assay. After incubation isolated plasmids were linearized and separated on agarose gels. Upper panels show ethidium bromide-stained gels and lower panels are autoradiograms of the dried gels. The positions of non-damaged and damaged substrates are indicated by a minus (−) and a plus (+). (**A**) Plasmids exposed to 450 J/m$^2$ of UV light; (**B**) plasmids exposed to 450 J/m$^2$ UV and further processed using Nth protein; (**C**) plasmids exposed to AAF.

**Table I.** Residual activity of the NER mutants *in vitro*

| CG | Cell strain | Residual activity (% of wild type)[a] | |
|---|---|---|---|
| 1 | 43-3B | 27 ± 4 | (*n* = 13) |
| | UV85 | 22 ± 4 | (*n* = 10) |
| 2 | UV5 | 21 ± 3 | (*n* = 11) |
| 3 | 27-1 | 23 ± 3 | (*n* = 20) |
| 4 | UV41 | 28 ± 7 | (*n* = 5) |
| | UV47 | 17 ± 3 | (*n* = 6) |
| 5 | UV135 | 24 ± 4 | (*n* = 9) |
| 6 | 14-60-23B | 116 ± 19 | (*n* = 3) |
| 11 | UVS1 | 24 ± 4 | (*n* = 6) |
| XP-A | XP12RO-SV | 15 ± 5 | (*n* = 5) |
| XP-F | XP2YO | 16 ± 9 | (*n* = 3) |

[a]Mean ± SEM (*n* = number of experiments).

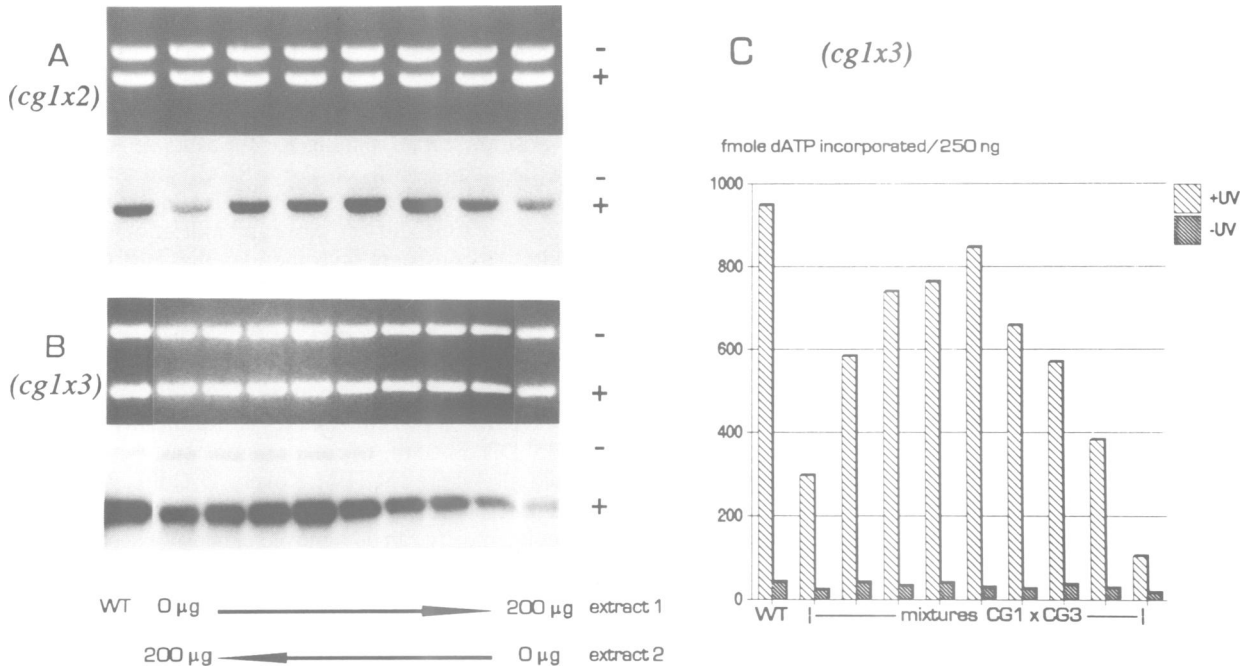


**Fig. 2.** Complementation of repair activity in mixtures of CG1 versus CG2 (**A**) and CG1 versus CG3 (**B** and **C**). In both mixtures UV(*nth*) damaged plasmids were used. Extracts were mixed in different ratios totalling 200 µg of protein. Increments were 25 and 30 µg in (A) and (B/C), respectively. Cell extracts used are in (A) 43-3B (CG1) and UV5 (CG2) and in (B/C) 43-3B (CG1) and 27-1 (CG3), extracts 1 and 2, respectively. First lanes in each panel contain 200 µg of CHO9 extract (WT). Because the amounts of recovered DNA in (B) are not completely equal in all lanes, a bar graph of this experiment is shown in (C), representing normalized data obtained after liquid scintillation counting of the $^{32}P$ incorporation and scanning of the DNA fluorograms.

level of unscheduled DNA synthesis and incision in cultured cells (Thompson *et al.*, 1988). Some variation of residual activity in separate extracts of these NER-deficient mutants was found, ranging from 15 to 30% of the wild-type response. We conclude that NER can be detected readily *in vitro* using rodent cell-free extracts and that this repair requires the active presence of the products of the *ERCC1, 2, 3, 4, 5* or *11* genes, in addition to those of *XPAC* and *XPFC*.

### *In vitro complementation analysis*

Since *in vitro* complementation with human extracts can be achieved by mixing representatives of different CGs of XP (Wood *et al.*, 1988), it is expected that mutant extracts from various rodent CGs should complement each other as well. To determine the complementation patterns of the various Chinese hamster CGs in more detail, CG1, 2, 3, 4, 5 and 11 were tested, together with human CGs XP-A and XP-F. In view of the normal levels of repair activity *in vitro*, CG6 could not be used for analysis.

First, extracts from CG1 and 2 or CG1 and 3 were combined in various proportions and *in vitro* NER was measured. A substantial, ratio-dependent increase of repair synthesis was found with maximal repair activity around a mixing ratio of 1:1 (Figure 2). Restoration of repair synthesis was observed in the damaged plasmids only. Some variation in different experiments was experienced, but the presence of correction was unequivocal.

Then, proportional mixing experiments were conducted with other CGs. The results from a number of typical tests are shown in Figure 3 and the complete data set is summarized in Table II. Clear complementation was observed in any mutual combination of CG2, 3, 5 and XP-A and in combination with extracts from CG1, CG4 and CG11. Unexpectedly, pairwise mixing of representatives of CG1, CG4 or CG11 did not yield levels of repair synthesis significantly different from those in the deficient extracts alone (Figure 3). Similarly, XP-F extracts failed to complement CG1, 4 and 11, whereas clear correction was observed with CG2 and CG3. This pattern of non-complementation was consistently observed using independent isolates and with different representatives of CG1 and CG4 (Table II). These results indicate absence of correction in all combined extracts of CG1, CG4, CG11 or XP-F.

### *Apparent molecular weight of ERCC1 protein on glycerol gradients*

The consistent pattern of non-complementation *in vitro* between groups 1, 4, 11 and XP-F suggests that the corresponding proteins somehow affect each other; the presence of a defect in one has an effect on the activities of the others. Such a situation may occur, for instance, when these gene products form a protein complex *in vivo*, of which the subunits cannot exchange under the conditions of the *in vitro* assay. To investigate this possibility, a NER-proficient HeLa extract was size-fractionated on a 10–30% glycerol gradient. To minimize effects of buffer and salt the gradient was run in the conditions of the repair assay together with internal mol. wt markers. The presence of ERCC1 protein in gradient fractions was monitored by SDS–PAGE and immunostaining with a specific anti-ERCC1 antiserum (for characterization of the antiserum see below). ERCC1 runs on SDS gels with an apparent mol. wt of 39 kDa, which is larger than the calculated mol. wt of 33 kDa. This is probably due to the proline-rich N-terminus, since truncated ERCC1 missing the N-terminal segment migrates at the

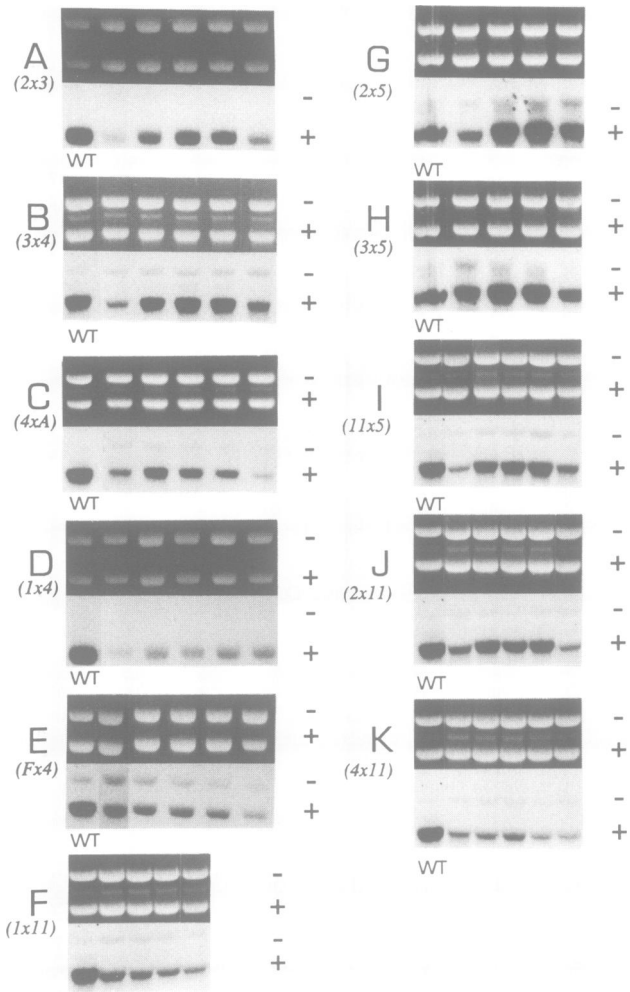


**Fig. 3.** *In vitro* complementation analysis. Substrate plasmids are damaged (+) with AAF (**B**, **C** and **F**–**K**) or with UV(*nth*) (**A**, **D** and **E**). Panels consist of an ethidium bromide fluorogram (top) and an autoradiogram (bottom). Each first lane (left) represents wild-type extract, either HeLa (**E**) or CHO9 (other panels), taken as an internal control. Second and last lanes are mutant extracts alone (200 μg). The lanes in between are mixtures of extracts in different ratios of extract 1 (left) and extract 2 (right), with a total of 200 μg of protein. Extracts 1 versus 2 are: (A) UV5 (CG2) versus 27-1 (CG3); (B) 27-1 (CG3) versus UV47 (CG4); (C) UV41 (CG4) versus XP12RO (XP-A); (D) UV85 (CG1) versus UV41 (CG4); (E) XP2YO (XP-F) versus UV47 (CG4); (F) UV85 (CG1) versus UVS1 (CG11); (G) UV5 (CG2) versus UV135 (CG5); (H) 27-1 (CG3) versus UV135 (CG5); (I) UVS1 (CG11) versus UV135 (CG5); (J) UV5 (CG2) versus UVS1 (CG11); (K) UV41 (CG4) versus UVS1 (CG11).

predicted position (data not shown). The profile of the glycerol gradient depicted in Figure 4 showed that, under native conditions, the protein sediments as a uniform band between the 67 and 149 kDa mol. wt markers, peaking on the position corresponding to ~120 kDa. Although non-specific aggregation of the ERCC1 protein cannot be completely excluded, the overall protein patterns analysed on SDS–PAGE did not show any indication of this. These results are consistent with the idea that ERCC1 resides in a complex.

### *ERCC1 protein produced by E.coli fails to correct CG1 extracts in vitro*

Human ERCC1, overproduced in *E.coli* as a ubiquitin–ERCC1 fusion protein, was tested for *in vitro* complementation. Details of the overexpression constructs will be published elsewhere (M.H.M.Koken, H.Odijk, M.Van Duin, M.Fornerod and J.H.J.Hoeijmakers, in preparation). The ubiquitin moiety can be removed either by a specific ubiquitin lyase (UBP1 protein) from *S.cerevisiae* (Tobias and Varshavsky, 1991) or by ubiquitin-specific lyase activity present in Manley-type extracts incubated under the conditions of the repair assay (Figure 5A, lanes 1–6). The free full-length ERCC1 protein migrated at 39 kDa in SDS–PAGE (Figure 5A) at the same position as ERCC1 protein synthesized in a reticulocyte lysate *in vitro* (Figure 5C, lane 6).

Purified cleaved ERCC1 protein or fusion protein (either crude or partially purified) was added in excess to CG1 extracts. In none of these cases was significant correction observed. The presence of inhibiting factors in the ERCC1 protein preparation was ruled out by adding these proteins also to repair-competent extracts. It appears that in these *in vitro* conditions ERCC1 protein on its own cannot participate in the repair reaction.

### *Characterization of anti-ERCC1 antibodies*

To study the possibility that ERCC1 is involved in a complex, we needed to do experiments at the enzymological level. Therefore a polyclonal antiserum was raised in rabbits against the ubiquitin–ERCC1 protein and affinity-purified on a column carrying immobilized fusion protein. The antisera were characterized in the following ways. First, Western blot analysis visualized a protein band with the exact mol. wt of ERCC1 on SDS–PAGE in crude lysates and Manley-type extracts of HeLa cells (Figure 5D, lanes 4, 5, 7 and 8). This band was not recognized by the preimmune serum (Figure 5D, lanes 1 and 2) and the reaction could be competed by partially purified ERCC1 fusion protein.

**Table II.** Summary of *in vitro* complementation data[a]

| CG→ ↓ | Strain | XP-F XP2YO | XP-A XP12RO | 11 UVS1 | 5 UV135 | 4 UV41 | 4 UV47 | 3 27-1 | 2 UV5 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 43-3B | – | + | – | + | – | – | + | + |
| | UV85 | – | + | – | + | – | – | + | + |
| 2 | UV5 | + | nd | + | + | + | + | + | |
| 3 | 27-1 | + | + | + | + | + | + | | |
| 4 | UV47 | – | nd | – | + | | | | |
| | UV41 | nd | + | – | nd | | | | |
| 5 | UV135 | nd | + | + | | | | | |
| 11 | UVS1 | – | nd | | | | | | |
| XPA | XP12RO | + | | | | | | | |

[a]+, complementation; –, non-complementation; nd, not determined.

No cross-reaction was observed with ubiquitin or with the ERCC1 protein from bovine or Chinese hamster origin (Figure 5D, lanes 6 and 9). Secondly, the antiserum was able to immunoprecipitate $^{35}$S-labelled ERCC1 protein, synthesized *in vitro* by reticulocyte lysate (Figure 5C, lanes 2–5). Finally a complete abolition of UV-induced unscheduled DNA synthesis was observed after micro-injection of the antibodies into repair-proficient human fibroblasts, whereas the preimmune serum did not have any effect (data not shown). These results indicate that the antibodies are able to react specifically with ERCC1 *in vivo* as well as *in vitro*.

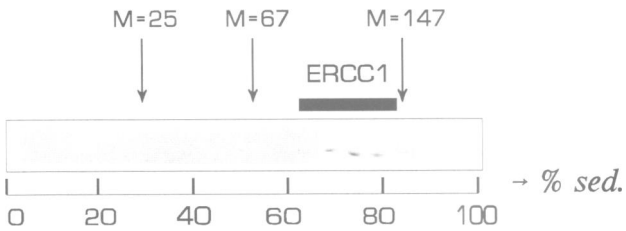


**Fig. 4.** Mol. wt determination of ERCC1 by sedimentation analysis. Active HeLa extract was layered, together with mol. wt standards, on a non-denaturing glycerol gradient and sedimented. The markers are chymotrypsinogen A (25 kDa), albumin (67 kDa) and aldolase (147 kDa). Gradient fractions were analysed on SDS–PAGE to determine the marker positions and immunoblotted to assess the position of ERCC1.

### *ERCC1 antiserum depletes a repair-competent HeLa extract from ERCC1, 4, 11 and XPFC*

In view of the species specificity of the antiserum, a NER-competent extract from HeLa cells was used to study the effect of removal of ERCC1 protein in the *in vitro* repair reaction. To this end antiserum was immobilized on protein A–Sepharose beads prior to incubation with the HeLa extract. After removal of the beads by centrifugation the supernatant exhibited a significantly decreased *in vitro* NER activity (Figure 6A). No such effect was detected when preimmune serum was used even at a 50× higher concentration (Figure 6B). We conclude that ERCC1 protein can be removed from a human wild-type extract using specific antibodies, resulting in a lowered repair activity comparable with the level of a CG1 mutant.

The HeLa extract depleted of ERCC1 (HeLa$^{-ERCC1}$) was

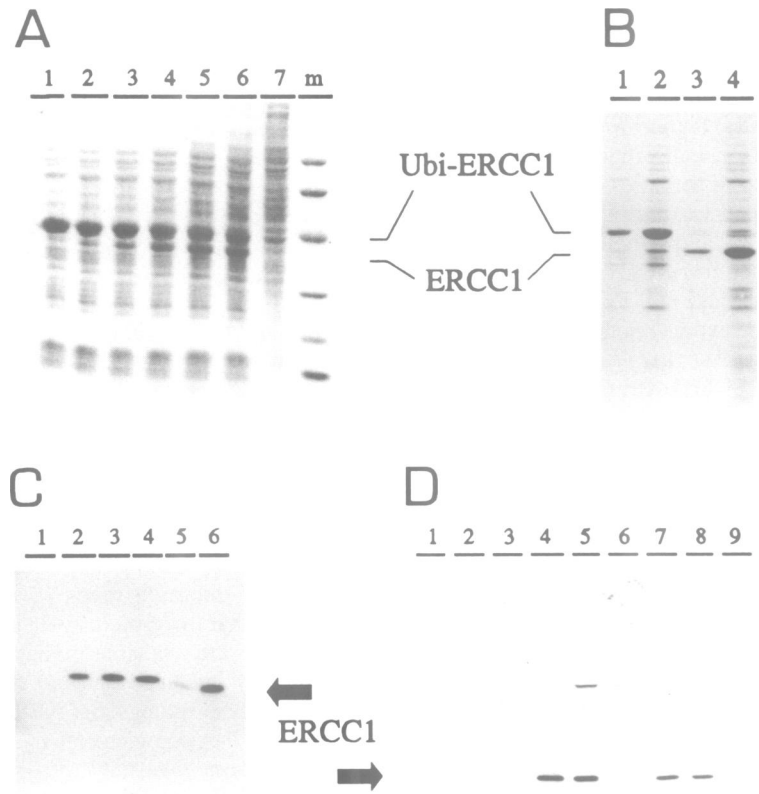


**Fig. 5.** Production of ubiquitin–ERCC1 fusion protein and characterization of anti-ERCC1 antibodies. (**A**) SDS–PAGE analysis of crude soluble lysate of ERCC1-overproducing *E.coli* incubated with increasing amounts of HeLa Manley-type extract. Lanes 1–6, lysate reacted for 2 h with 0, 15, 30, 45, 60 or 75 μg of HeLa extract; lane 7, 75 μg of HeLa extract alone; lane m, mol. wt markers: phosphorylase b (97 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). (NB the released ubiquitin of 8 kDa has run off the gel.) (**B**) SDS–PAGE analysis of lysate from ERCC1 fusion protein, partially purified by 33% saturated ammonium sulfate precipitation. Lanes 1–2, not treated with lyase, 0.7 and 35 μg of lysate; lanes 3–4, pre-treated with purified UBP1-lyase, 0.7 and 35 μg of lysate. (**C**) SDS–PAGE and autoradiography of immune-precipitated [$^{35}$S]ERCC1 translated *in vitro*. Lane 1, preimmune serum, undiluted; lanes 2–5, crude immune serum, diluted 1×, 10×, 100× and 1000×, respectively; lane 6, input amount of [$^{35}$S]ERCC1. (**D**) Specificity of ERCC1 antiserum on immunoblots of different cell extracts. Lanes 1–3, preimmune serum; lanes 4–6, crude immune serum; lanes 7–9, affinity-purified anti-ERCC1 antibodies; amounts used are equivalent to lanes 4–6. The extracts analysed are total HeLa cell sonicate (lanes 1, 4 and 7), Manley-type extracts of HeLa (lanes 2, 5 and 8) or CHO9 (lanes 3, 6 and 9).

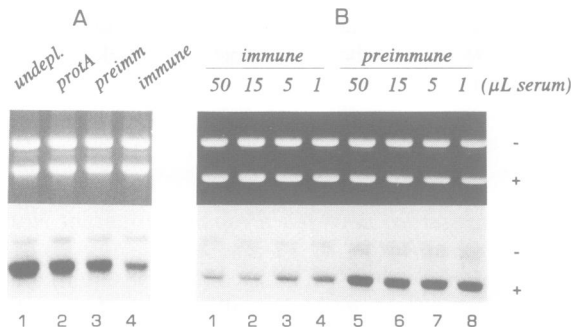


**Fig. 6.** Depletion of HeLa extract using anti-ERCC1 antibodies. Serum was immobilized on protein A−Sepharose beads; after incubation with repair-competent HeLa extract and centrifugation, the supernatant was tested for repair activity *in vitro*. (**A**) 10 μl of serum was used for depletion of 150 μg HeLa extract. Lane 1, undepleted extract (100 μg); lane 2, extract treated with empty protein A beads (90 μg); lane 3, extract depleted with pre-immune serum (80 μg); lane 4, extract depleted with immune serum (110 μg). (**B**) Various concentrations of sera were used to deplete 150 μg of HeLa extract. Lanes 1−4, anti-ERCC1 serum (50, 15, 5 and 1 μl respectively); lanes 5−8, pre-immune serum (50, 15, 5 and 1 μl respectively).

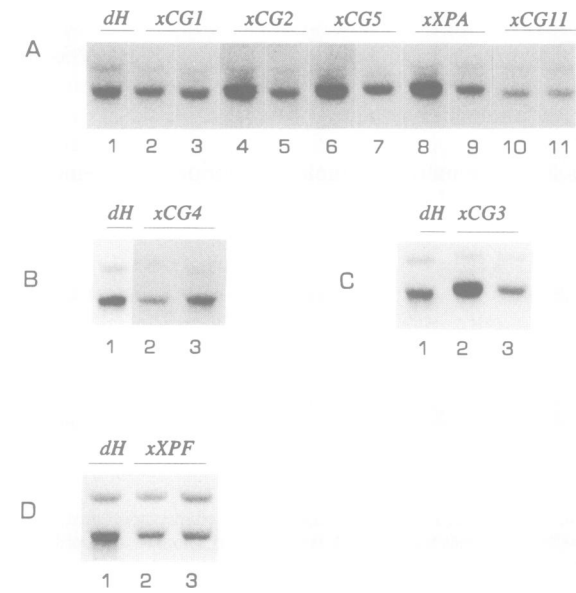


**Fig. 7.** Complementation analysis with ERCC1-depleted HeLa extract. 7.5 μl of anti-ERCC1 serum used for depletion. Different panels represent independent experiments. Plasmid recoveries are similar within 10% in each experiment. (**A**) Lane 1, HeLa$^{-ERCC1}$ alone; lane 2, 1:1 mix with UV85 (CG1); lane 3, UV85 alone; lane 4, mix with UV5 (CG2); lane 5, UV5 alone; lane 6, mix with UV135 (CG5); lane 7, UV135 alone; lane 8, mix with XP12RO (XPA); lane 9, XP12RO alone; lane 10, mix with UVS1 (CG11); lane 11, UVS1 alone. (**B**) Lane 1, HeLa$^{-ERCC1}$ alone; lane 2, 1:1 mix with UV47 (CG4); lane 3, UV47 alone. (**C**) Lane 1, HeLa$^{-ERCC1}$ alone; lane 2, 1:1 mix with 27-1 (CG3); lane 3, 27-1 alone. (**D**) Lane 1, HeLa$^{-ERCC1}$ alone; lane 2, 1:1 mix with XP2YO (XP-F); lane 3, XP2YO alone. In (B) and (C) controls for positive complementation taken along in these experiments are not shown, but gave results identical to lanes A4 and D2.

then tested for the ability to correct NER-deficient Chinese hamster mutants in mixing experiments. As expected, no complementation was detected when HeLa$^{-ERCC1}$ was mixed with a representative of CG1. However, when an extract of CG2, CG3, CG5 or XP-A was added to HeLa$^{-ERCC1}$ a significantly higher repair synthesis was observed (Figure 7A and C), indicating that the antibodies cannot remove the correcting activities of these groups. In contrast, HeLa$^{-ERCC1}$ failed to restore repair in mixtures with extracts of CG4, CG11 and XP-F (Figure 7B, A and D), showing that in addition to ERCC1 protein, correcting activities of CG4, CG11 and XP-F are concomitantly removed from the wild-type extract. Both crude and affinity-purified antisera were equally capable of depleting HeLa extract from ERCC1, 4, 11 and XPFC.

## Discussion

### *In vitro repair studies in Chinese hamster NER-deficient mutants*

In agreement with findings of others (Biggerstaff and Wood, 1992) we have observed that extracts isolated from cultured Chinese hamster cells can support NER of DNA damage *in vitro*. DNA substrates with a well-defined and narrow damage spectrum can be used in this assay, provided they are extensively purified. Clearance of UV-damaged plasmids from oxidative thymine species by pretreating with bacterial Nth protein proves to be an absolute requirement when Chinese hamster cell extracts are used, due to an apparently high endonuclease III-like activity in these cells.

The NER deficiency of CG1, 2, 3, 4, 5 and 11 *in vitro* agrees with the low unscheduled DNA synthesis levels (Van Duin *et al.*, 1988) and low incision capabilities of these cell strains in culture. In CG6, which is only moderately sensitive to UV, the repair activity is in the range of NER-proficient extracts. The ERCC6 gene is defective in CS-B (Troelstra *et al.*, 1992), where preferential repair of actively transcribed strands is impaired, but overall genome repair, which is responsible for the bulk of repair synthesis, is unaffected (Venema *et al.*, 1990). The normal *in vitro* repair rates are likely to reflect overall repair, since transcription is not taking place in the reaction conditions. Our finding that, *in vitro*, human and hamster repair activities are similar, contrasts with the low activity of overall repair in cultured rodent cells in comparison with human cells (Bohr and Hanawalt, 1987; Lommel and Hanawalt, 1991). It follows that the factors responsible for this difference do not play a role in the *in vitro* reaction, perhaps because they are related to chromatin structure.

Extracts of CG2, 3 and 5 are able to complement each other and representatives of all other CGs tested. This suggests that the proteins which are missing or defective in these CGs are available in the complementing extracts and can freely interact to ensure restoration of normal repair activity. The findings that XP-F can correct CG2 and CG3 and that XP-A complements CG3 and CG5 show that human and Chinese hamster repair proteins are sufficiently conserved and interchangeable *in vitro*. This is in line with the correction *in vivo* by transfection of human genes into the Chinese hamster mutants.

### *Evidence for a repair enzyme complex*

Mixtures of CGs 1, 4, 11 and XP-F do not show restoration of repair activity *in vitro*, whereas they do *in vivo* after cell hybridization. Since non-complementation occurred with independent mutants from CG1 and CG4, this unexpected phenomenon is unlikely to be related to an allele-specific behaviour. Our data indicate that the normal gene products needed for correction are either unable to exchange *in vitro* or are not present in the mixtures. Such a situation can occur

when these correcting proteins are part of a pre-existing stable complex. Depletion of a wild-type extract using specific anti-ERCC1 antibodies not only results in the loss of CG1 complementing activity, but concomitantly removes the activity which is capable of correcting CG4 and CG11 or even XP-F. This strongly suggests that such a complex does exist in wild-type extracts. This is further supported by the finding that human ERCC1 protein, which has an apparent mol. wt of 39 kDa in denaturing SDS gels, sediments in glycerol gradients as a much larger species.

The absence of complementation in some combinations indicates that the *in vitro* assay cannot replace classical cell fusion as a system for complementation analysis. To explain these non-complementation patterns *in vitro*, several mechanisms can be envisaged. (i) Reconstitution of a complex from its components may not be possible under *in vitro* conditions. An inactive complex harbouring a tightly bound defective protein in one mutant extract will be unable to complement another mutant, in which the same complex is crippled by the presence of another impaired component. (ii) Alternatively, or in addition, dependent on the type of mutation in the cell strain, the complex may be required for stability of its components *in vivo*. In that case cell-free extracts from CG1 lacking ERCC1 would be also deficient in ERCC4 and the other proteins, and vice versa. Indirect support for this idea comes from studies with 41D, a CG1 cell strain containing an amplified correcting human *ERCC1* gene. Whereas at DNA and mRNA levels *ERCC1* is amplified in this strain 100- to 1000-fold, the amount of ERCC1 protein is enhanced only by a factor of three and many degradation products become apparent on immunoblots with anti-ERCC antibodies. In addition, micro-injection into human fibroblasts of an excess of ERCC1 protein, over-produced in *E.coli*, results in unusually rapid (< 1 h) degradation *in vivo* as judged by immunofluorescence (our unpublished results). These data suggest that ERCC1 protein on its own may not be very stable in the cell, which is in agreement with our observation that addition of an excess of purified ERCC1 protein fails to restore repair activity in CG1 extracts.

### *Involvement of the xeroderma pigmentosum group F gene product*

Extracts from a human XP-F cell strain fail to complement Chinese hamster extracts of CG1, CG4 and CG11 *in vitro*. This pattern of non-complementation is group specific, as efficient correction of repair is found with other CGs. In addition, the XP-F extract is not able to correct the ERCC1-depleted HeLa extract, indicating that the XPF-correcting activity is also part of the postulated complex. Well-controlled cell fusions *in vivo* between human XP-F cells and Chinese hamster NER-deficient mutants, being complicated by poor interspecies fusion and chromosome loss in the resulting hybrids, have not been done so far, so there is no *in vivo* correlation of our *in vitro* results. Direct involvement of ERCC1 in XP-F is ruled out by the inability of the *ERCC1* gene to correct XP-F cells *in vivo* (Van Duin *et al.*, 1989). Therefore, ERCC4 and ERCC11 are possible candidates for the gene defective in XP-F. This would represent another overlap between Chinese hamster mutants and human repair disorders. Alternatively, XPFC might also encode a distinct protein, which may be less likely in view of gradient sedimentation data.

### *The relation between ERCC4 and ERCC11*

The complex between the ERCC1 and ERCC4 proteins also contains the correcting activities of CG11 and XP-F. The latter two mutants do not show the extreme sensitivity to DNA crosslinking agents characteristic for representatives of CG1 and 4. However, absence of mitomycin C sensitivity has also been noted in some ERCC1 mutants, whereas the sensitivity to UV has been retained (J.Van de Berg and J.H.J.Hoeijmakers, unpublished results). It is possible that such relatively mild mutations are present in CG11 and XP-F as well. Since the strain UVS1 is the sole representative of CG11 isolated so far, one has to consider also the theoretical possibility that the complementation in cell hybrids of CG11 with CG1 or CG4 mutants is of the intra-allelic type. Our (unpublished) observation that transfected *ERCC1* cDNA is unable to correct UVS-1 cells definitely rules out involvement of *ERCC1*. On the other hand, complementation has been shown after cell fusion with UVS1 and the two independent CG4 mutants UV47 (Hata *et al.*, 1991) and UV41 (D.Busch, personal communication), which makes intra-allelic complementation less likely. Although such patterns of complementation have never been observed in the numerous fusions between either Chinese hamster or human (XP) repair-deficient mutant cells, a definitive answer must await the isolation of the *ERCC4* or *ERCC11* gene and its transfection into the reciprocal mutants.

### *Possible functions of the complex*

The homologue of *ERCC1* in *S.cerevisiae* is the *RAD10* gene (Van Duin *et al.*, 1986). This protein can form a very stable complex with the *RAD1* gene product, both *in vivo* and *in vitro* (Bailly *et al.*, 1992; Bardwell *et al.*, 1992), explaining the similar phenotypes of the yeast *RAD1* and *RAD10* mutants, having a defect in NER as well as in mitotic recombination (Schiestl and Prakash, 1988, 1990). Also *ERCC1* and *ERCC4* mutants share unique features, such as extreme sensitivity to crosslinking agents. Therefore, it is conceivable that the RAD1–RAD10 complex and the mammalian repair complex identified here are equivalent and have similar functions. The studies in yeast do not exclude the possibility that additional proteins are part of the RAD1–RAD10 complex, which is relevant in view of our findings that ERCC11 and/or XPFC correcting activities also appear to be present in the mammalian complex. In this idea, the yeast *RAD1* gene becomes a good candidate for being the homologue of one of the mammalian genes *ERCC4*, *ERCC11* or *XPFC*.

Combining these data, we postulate that the mammalian enzyme complex has a dual function, as has its homologue in yeast. Deficiency of the NER function results in a ~5- to 10-fold increased sensitivity to UV light and other chemical agents inducing bulky DNA adducts. Such a sensitivity is also found in XP-A cells, where NER is fully impaired due to the complete inactivation of the XPAC protein (Tanaka *et al.*, 1990). On top of the moderate sensitivity for crosslinks (such as found in XP-A cells), the additional participation of ERCC1 and ERCC4 proteins in a recombinational repair pathway might explain the extreme (80- to 100-fold) sensitivity of the corresponding mutants to crosslinking agents. Obviously, interstrand crosslinks may require additional recombination steps for their removal.

### Note added

In the course of manuscript preparation we became aware of recent results obtained by M.Biggerstaff, D.E.Szymkowski and R.D.Wood, indicating an association between CG1, CG4 and XP-F correcting activities and showing non-complementation in extracts mixtures obtained from these CGs (Biggerstaff *et al.*, 1993).

## Materials and methods

### Plasmid DNA

Plasmids pBluescript KS$^+$ (pBKS, 3.0 kb), pTZ19R (3.0 kb), pHM14 (3.7 kb) and pSLM (4.3 kb) were isolated from *E.coli* RecA$^-$ hosts by alkaline lysis and purified twice on a CsCl gradient. pBKS and pTZ19R were either exposed to 450 J/m$^2$ of UV light of predominantly 254 nm or treated with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R.Baan, TNO, Rijswijk), inducing *N*-(guanin-8-yl) AAF adducts. pBKS plasmid was treated with Nth protein from *E.coli* (generously provided by C.J.Jones, ICRF, London) after UV irradiation and extensively purified as closed circular DNA on neutral sucrose gradients as described by Wood *et al.* (1988) and Biggerstaff *et al.* (1991).

AAF-modified plasmids were collected by repeated di-ethyl-ether extractions and ethanol precipitation (Landegent *et al.*, 1984) and also repurified on a sucrose gradient. In both cases pHM14 or pSLM plasmids were mock-treated in parallel. The average numbers of lesions per damaged plasmid molecule were 10–12 pyrimidine dimers and 15–20 AAF–guanine adducts, respectively.

### Cell lines and extracts

Mutant Chinese hamster cells, hypersensitive to UV irradiation or other mutagenic agents, were isolated from either the wild-type CHO strains AA8 (Busch *et al.*, 1980) or CHO9 (Wood and Burki, 1982). These mutants have been assigned to complementation group 1 (43-3B, UV85), group 2 (UV5), group 3 (27-1), group 4 (UV41, UV47), group 5 (UV135) (Busch *et al.*, 1989), group 6 (14-60-23B) (D.Busch, personal communication) and group 11 (UVS1) (Hata *et al.*, 1991; Riboni *et al.*, 1992; Numata *et al.*, 1993). XP12RO(SV40) and XP3YO(SV40) are SV40-transformed lines belonging to XP CGs A and F, respectively. HeLa and CHO9 cells served as repair-competent controls.

Cells were cultured in 850 cm$^2$ plastic roller bottles or in 265 cm$^2$ glass Petri dishes in a 1:1 mixture of Ham's F10 and DMEM medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS). Extracts were prepared from 2–5 ml of packed cell pellet by the method of Manley *et al.* (1983) as modified by Wood *et al.* (1988), dialysed in buffer A and stored at −80°C. Buffer A contained 25 mM HEPES/KOH pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 17% (v/v) glycerol.

### In vitro repair assay

The standard reaction mixture (50 μl) contained 250 ng damaged pBKS and 250 ng pHM14 plasmid DNA, 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 20 μM each of dCTP, dGTP and TTP, 8 μM dATP, 74 kBq of [α-$^{32}$P]dATP, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.45% glycerol, 18 μg bovine serum albumin and 200 μg of cell-free extract. The mixtures were incubated for 3 h at 30°C. Plasmid DNAs were isolated, linearized by restriction and electrophoresed on an agarose gel. Data were analysed by autoradiography and quantified by scintillation counting of DNA bands excised from dried gel.

### Anti-ERCC1 antibodies and immunoblotting

Human ERCC1 protein was overproduced as a ubiquitin–ERCC1 fusion protein in *E.coli*. The ubiquitin part is thought to protect the N-terminal part of the ERCC1 protein against degradation. The pETUBI..ERCC1 vector contained a strong inducible T7 promoter and was transformed to the bacterial host strain BL21(DE3)LysS (M.H.M.Koken *et al.*, submitted).

A polyclonal anti-ERCC1 antiserum was raised in rabbits by injection of gel-purified ubiquitin–ERCC1 fusion protein. For immunoblot analysis protein samples were separated on 11% SDS–polyacrylamide gel and transferred to a PVDF membrane in 25 mM Tris–HCl, pH 8.3 containing 20% methanol and 0.2 M glycine. The membranes were treated with non-fat milk containing 0.1% Tween 20 and sodium azide for at least 1 h and then incubated for 16 h with 1000× diluted antiserum at 4°C. Then the blots were washed with PBS containing 0.5% Tween 20 and incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG. The blots were washed again and developed with the coloured substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

The ERCC1 antiserum was affinity-purified using a protein A–Sepharose column for isolating the IgG fraction of the serum and an Affigel® 10 column (Bio-Rad) carrying immobilized purified fusion protein.

### In vitro translation and immunoprecipitation

ERCC1 protein was translated *in vitro* using a rabbit reticulocyte lysate system as described by the manufacturer (Promega) using 50 μCi of [$^{35}$S]methionine (1 mCi/mmol). Antiserum was incubated with ERCC1 protein for 2 h at 4°C in 100 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM $Na_2EDTA$ and 0.5% Triton (NETT buffer). The 10% protein A–Sepharose beads in NETT buffer containing 2% BSA and 0.02% sodium azide were added and tumbled for 1 h at 4°C. After centrifugation and washing four times in NETT buffer the immunoprecipitate was separated on 11% SDS–PAGE and the gel was dried and exposed to X-ray film.

### Glycerol gradient sedimentation

The molecular weight of ERCC1 was determined on a 10–30% non-denaturing glycerol gradient in buffer A. NER-proficient HeLa extract (650 μg) was loaded, together with three markers: aldolase (147 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa) and sedimented in a Beckman SW41 rotor at 280 000 *g* at 4°C for 24 h. After fractionation the presence of ERCC1 protein was monitored on immunoblots using anti-ERCC1 antibodies and horseradish peroxidase-conjugated second antibody, using a phosphorescence detection method.

### Antibody depletion of NER-proficient extract

HeLa cell extract was treated with polyclonal anti-ERCC1 antiserum. Protein A–Sepharose CL-4B beads (protA) (70 μg) were washed three times in PBS and incubated with 10 μl anti-ERCC1 antibodies or preimmune serum for 15 min at 0°C. Then the beads were washed three times in buffer A and added to a repair-competent HeLa extract for 30 min at 0°C. The supernatant obtained after spinning down the beads was used as a depleted HeLa extract and tested in the *in vitro* repair assay.

## Acknowledgements

The authors wish to express their gratitude to Drs Wood and Biggerstaff for their vital help in the beginning of this project. In addition, M.H.M.Koken, J.van den Berg, W.Vermeulen, J.de Wit and A.P.M.Eker are acknowledged for preparing the ubi–ERCC1 construct, mutation analysis, microinjections, transfections and help with protein purification, respectively. Dr D.Busch is thanked for making his mutant cell strains and unpublished data available to us. This research was supported in part by the Netherlands Foundation for Chemical Research (SON) and Medical Sciences (GMW, 900-501-113) with financial aid from the Netherlands Organization for Scientific Research and by the Commission of European Communities (CEC).

## References

Bailly,V., Sommers,C.H., Sung,P., Prakash,L. and Prakash,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8273–8277.

Bardwell,L., Cooper,A.J. and Friedberg,E.C. (1992) *Mol. Cell. Biol.*, **12**, 3041–3049.

Biggerstaff,M. and Wood,R.D. (1992) *J. Biol. Chem.*, **267**, 6879–6885.

Biggerstaff,M., Robins,P., Coverley,D. and Wood,R.D. (1991) *Mutat. Res.*, **254**, 217–224.

Bohr,V.A. and Hanawalt,P.C. (1987) *Carcinogenesis*, **8**, 1333–1336.

Busch,D.B., Cleaver,J.E. and Glaser,D.A. (1980) *Somat. Cell Genet.*, **6**, 407–418.

Busch,D., Greiner,C., Lewis,K., Ford,R., Adair,G. and Thompson,L. (1989) *Mutagenesis*, **4**, 349–354.

Cleaver,J.E. and Kraemer,K.H. (1989) *Xeroderma pigmentosum. The Metabolic Basis for Inherited Disease. Vol. II.* McGraw-Hill Book Co., New York, pp. 2949–2971.

Collins,A.R. (1993) *Mutat. Res.*, **293**, 99–118.

Coverley,D., Kenny,M.K., Munn,M., Rupp,W.D., Lane,D.P. and Wood,R.D. (1991) *Nature*, **349**, 538–541.

Coverley,D., Kenny,M.K., Lane,D.P. and Wood,R.D. (1992) *Nucleic Acids Res.*, **20**, 3873–3880.

Flejter,W.L., McDaniel,L.D., Johns,D., Friedberg,E.C. and Schultz,R.A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 261–265.

Friedberg,E.C. (1992) *Cell*, **71**, 887–889.
Grossman,L. and Yeung,A.T. (1990) *Photochem. Photobiol.*, **51**, 749–755.
Gulyas,K.D. and Donahue,T.F. (1992) *Cell*, **69**, 1031–1042.
Hansson,J., Munn,M., Rupp,W.D., Kahn,R. and Wood,R.D. (1989) *J. Biol. Chem.*, **264**, 21788–21792.
Hansson,J., Keyse,S.M., Lindahl,T. and Wood,R.D. (1991) *Cancer Res.*, **5**, 3384–3390.
Hata,H., Numata,M., Tohda,H., Yasui,A. and Oikawa,A. (1991) *Cancer Res.*, **51**, 195–198.
Hoeijmakers,J.H.J. (1991) *J. Cell Sci.*, **100**, 687–691.
Hoeijmakers,J.H.J. (1993) *Trends Genet.*, **9**, 211–217.
Hoeijmakers,J.H.J. and Bootsma,D. (1990) *Cancer Cells*, **2**, 311–320.
Huang,J.C., Svoboda,D.L., Reardon,J.T. and Sancar,A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3664–3668.
Landegent,J.E., Jansen in de Wal,N., Baan,R.A., Hoeijmakers,J.H.J. and Van der Ploeg,M. (1984) *Exp. Cell Res.*, **153**, 61–72.
Lehmann,A.R. (1982) *Mutat. Res.*, **106**, 347–356.
Lehmann,A.R. (1987) *Cancer Rev.*, **7**, 82–103.
Lehmann,A.R. *et al.* (1992) *Mutat. Res.*, **273**, 1–28.
Lommel,L. and Hanawalt,P.C. (1991) *Mutat. Res.*, **255**, 183–191.
Manley,J.L., Fire,A., Samuels,M. and Sharp,P.A. (1983) *Methods Enzymol.*, **101**, 568–582.
Nance,M.A. and Berry,S.A. (1992) *Am. J. Med. Genet.*, **42**, 68–84.
Nichols,A.F., Schmidt,W.J., Chaney,S.G. and Sancar,A. (1992) *Chem. Biol. Interact.*, **81**, 223–231.
Numata,M., Hata,H., Shiomi,T., Matsunaga,T., Mori,T., Nikaido,O., Yasui,A. and Oikawa,A. (1993) *Cancer Res.*, **53**, 495–499.
O'Donovan,A. and Wood,R.D. (1993) *Nature*, **363**, 185–188.
Park,E., Guzder,S., Koken,M.H.M., Jaspers-Dekker,I., Weeda,G., Hoeijmakers,J.H.J., Prakash,S. and Prakash,L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11416–11420.
Riboni,R., Botta,E., Stefanini,M., Numata,M. and Yasui,A. (1992) *Cancer Res.*, **52**, 6690–6691.
Robins,P., Jones,C.J., Biggerstaff,M., Lindahl,T. and Wood,R.D. (1991) *EMBO J.*, **10**, 3913–3921.
Schaeffer,L., Roy,R., Humbert,S., Moncollin,V., Vermeulen,W., Hoeijmakers,J.H.J., Chambon,P. and Egly,J. (1993) *Science*, **260**, 58–63.
Scherly,D., Nouspikel,T., Corlet,J., Ucla,C., Bairoch,A. and Clarkson,S.G. (1993) *Nature*, **363**, 182–185.
Schiestl,R.H. and Prakash,S. (1988) *Mol. Cell. Biol.*, **8**, 3619–3626.
Schiestl,R.H. and Prakash,S. (1990) *Mol. Cell. Biol.*, **10**, 2485–2491.
Selby,C.P. and Sancar,A. (1990) *Mutat. Res.*, **236**, 203–211.
Shivji,M.K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.
Sibghat-Ullah,S.A. (1990) *Biochemistry*, **29**, 5711–5718.
Sibghat-Ullah,S.A., Husain,I., Carlton,W. and Sancar,A. (1989) *Nucleic Acids Res.*, **17**, 4471–4484.
Stefanini,M., Collins,A.R., Riboni,R., Klaude,M., Botta,E., Mitchell,D.L. and Nuzzo,F. (1991) *Cancer Res.*, **51**, 3965–3971.
Stefanini,M., Giliani,S., Nardo,T., Marinoni,S., Nazzaro,V., Rizzo,R. and Trevisan,G. (1992) *Mutat. Res.*, **273**, 119–125.
Stefanini,M., Vermeulen,W., Giliani,S., Nardo,T., Mezzina,M., Sarasin,A., Harper,J.I., Arlett,C.F., Hoeijmakers,J.H.J. and Lehmann,A.R. (1993) *Am. J. Hum. Genet.*, in press.
Tanaka,K., Satokata,I., Ogita,Z., Uchida,T. and Okada,Y. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 5512–5516.
Tanaka,K., Miura,N., Satokata,I., Miyamoto,I., Yoshida,M.C., Satoh,Y., Kondo,S., Yasui,A., Okayama,H. and Okada,Y. (1990) *Nature*, **348**, 73–76.
Thompson,L.H., Shiomi,T., Salazar,E.P. and Stewart,S.A. (1988) *Somat. Cell. Mol. Genet.*, **14**, 605–612.
Thompson,L.H., Mitchell,D.L., Regan,J.D., Bouffler,S.D., Stewart,S.A., Carrier,W.L., Nairn,R.S. and Johnson,R.T. (1989) *Mutagenesis*, **4**, 140–149.
Tobias,J.W. and Varshavsky,A. (1991) *J. Biol. Chem.*, **266**, 12021–12028.
Troelstra,C., van Gool,A., de Wit,J., Vermeulen,W., Bootsma,D. and Hoeijmakers,J.H.J. (1992) *Cell*, **71**, 939–953.
Van Duin,M., De Wit,J., Odijk,H., Westerveld,A., Yasui,A., Koken,M.H.M., Hoeijmakers,J.H.J. and Bootsma,D. (1986) *Cell*, **44**, 913–923.
Van Duin,M., Janssen,J.H., de Wit,J., Hoeijmakers,J.H., Thompson,L.H., Bootsma,D. and Westerveld,A. (1988) *Mutat. Res.*, **193**, 123–130.
Van Duin,M., Vredeveldt,G., Mayne,L.V., Odijk,H., Vermeulen,W., Klein,B., Weeda,G., Hoeijmakers,J.H.J., Bootsma,D. and Westerveld,A. (1989) *Mutat. Res.*, **217**, 83–92.
Venema,J., Mullenders,L.H.F., Natarajan,A.T., Van Zeeland,A.A. and Mayne,L.V. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4707–4711.
Vermeulen,W., Stefanini,M., Giliani,S., Hoeijmakers,J.H.J. and Bootsma,D. (1991) *Mutat. Res.*, **255**, 201–208.
Weber,C.A., Salazar,E.P., Stewart,S.A. and Thompson,L.H. (1990) *EMBO J.*, **9**, 1437–1447.
Weeda,G., Van Ham,R.C.A., Vermeulen,W., Bootsma,D., Van der Eb,A.J. and Hoeijmakers,J.H.J. (1990) *Cell*, **62**, 777–791.
Wood,R.D. and Burki,H.J. (1982) *Mutat. Res.*, **95**, 505–514.
Wood,R.D., Robins,P. and Lindahl,T. (1988) *Cell*, **53**, 97–106.
Zdzienicka,M.Z., Mitchell,D.L., Venema,J., Van Hoffen,A., Van Zeeland,A.A., Mullenders,L.H., De Wit,J. and Simons,J.W. (1991) *Mutagenesis*, **6**, 179–183.

*Received on April 14, 1993; revised on May 17, 1993*