3370–3380 *Nucleic Acids Research, 1996, Vol. 24, No. 17*

Mutational analysis of the human nucleotide excision repair gene *ERCC1*

Anneke M. Sijbers, Peter J. van der Spek, Hanny Odijk, Joke van den Berg, Marcel van Duin[+], Andries Westerveld[§], Nicolaas G. J. Jaspers, Dirk Bootsma and Jan H. J. Hoeijmakers*

Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands

Received May 17, 1996; Revised and Accepted July 16, 1996

ABSTRACT

The human DNA repair protein ERCC1 resides in a complex together with the ERCC4, ERCC11 and XP-F correcting activities, thought to perform the 5′ strand incision during nucleotide excision repair (NER). Its yeast counterpart, RAD1–RAD10, has an additional engagement in a mitotic recombination pathway, probably required for repair of DNA cross-links. Mutational analysis revealed that the poorly conserved N-terminal 91 amino acids of ERCC1 are dispensable for both repair functions, in contrast to a deletion of only four residues from the C-terminus. A database search revealed a strongly conserved motif in this C-terminus sharing sequence homology with many DNA break processing proteins, indicating that this part is primarily required for the presumed structure-specific endonuclease activity of ERCC1. Most missense mutations in the central region give rise to an unstable protein (complex). Accordingly, we found that free ERCC1 is very rapidly degraded, suggesting that protein–protein interactions provide stability. Survival experiments show that the removal of cross-links requires less ERCC1 than UV repair. This suggests that the ERCC1-dependent step in cross-link repair occurs outside the context of NER and provides an explanation for the phenotype of the human repair syndrome xeroderma pigmentosum group F.

INTRODUCTION

Repair of damaged DNA prevents accumulation of lesions that give rise to mutations, chromosomal instability, carcinogenesis or cell death. A wide variety of DNA lesions caused by exposure to UV light and numerous chemical agents are removed via the nucleotide excision repair (NER) pathway. This repair process involves specific damage recognition, dual incision of the damaged strand, followed by lesion removal, gap filling and finally strand ligation (for a recent review see 1). Most of the proteins engaged in NER have been identified by making use of UV-sensitive mutant rodent cells (ERCC1–ERCC11) and cells derived from patients suffering from xeroderma pigmentosum (XP-A–XP-G), Cockayne's syndrome (CS-A and CS-B) or trichothiodystrophy (TTD-A). XPA protein is thought to play an important role in the damage recognition step, as it specifically binds to damaged DNA (2–4) and interacts with several other repair proteins, including the RPA heterotrimer (5,6), XPG (5), the basal transcription factor TFIIH (7) and the ERCC1 complex (8–11). Following damage recognition the helicase activities of XPB and XPD (12,13), present in the TFIIH complex (14–16), are thought to convert the damaged site into a substrate for XPG and the ERCC1 complex, likely to be responsible for dual strand incision around the lesion. Further action of RPA, PCNA, RF-C, DNA polymerase δ and/or ε and ligase are needed to complete the full NER reaction (17).

Although *ERCC1* was the first human NER gene cloned (18), information on its enzymatic function is still very limited. The protein exists in a complex together with the ERCC4, ERCC11 and XP-F correcting activities (19–21). Largely due to the difficulty of purifying it to homogeneity (22), the exact composition of the complex has not yet been fully resolved, although recently a heterodimeric ERCC1 complex was reported (23). By homology with its *Saccharomyces cerevisiae* counterpart RAD10 (24), which associates with the RAD1 protein (25,26), the ERCC1 complex is expected to mediate endonucleolytic incision at the 5′ side of the lesion (27–31). The nature of this putative activity, however, remains to be established. The domain of ERCC1 involved in the transient interaction with XPA extends from residue 93 to 120 (8), in a region that is strongly conserved in RAD10 (24). Further, on the basis of this conservation, the area could be involved in association with the human homolog of RAD1 (26), ERCC4 and ERCC11 and/or XPF.

Beyond the central region, towards the C-terminus, significant homology with the C-terminus of the *Escherichia coli* NER protein UvrC is observed (see Fig. 9). This domain is conserved in the *Schizosaccharomyces pombe* ERCC1 homolog Swi10 (32), but absent in RAD10 from *S.cerevisiae* (33). Both homologs have an additional function in a mitotic recombination pathway. In *S.cerevisiae* this pathway involves recombination between

* To whom correspondence should be addressed

Present addresses: [+]Department of Immunology, N.V. Organon, PO box 20, 5340 BH Oss, The Netherlands and [§]Department of Antropogenetics, University of Amsterdam, AMC, Meibergdreef 15, 1105 AZ Amsterdam, The Netherlands

direct repeats (34–36) in which *RAD1* is required for removal of non-homologous sequences from the 3′-ends of recombining DNA (37–39). In *S.pombe* this pathway entails mating type switching (40). The mammalian ERCC1 complex may have such a function as well. This idea is supported by the extreme hypersensitivity to DNA cross-linking agents that is unique to ERCC1- and ERCC4-deficient rodent mutants (41). Interstrand cross-links probably require recombination for their elimination. In order to obtain more information on the significance of various ERCC1 domains for both the NER and recombination functions, we have constructed *ERCC1* cDNAs with specific mutations and measured their ability to correct the mutagen hypersensitivity of rodent ERCC1 mutant 43-3B.

MATERIALS AND METHODS

Plasmids

The *E.coli* expression construct pETUbi..ERCC1 described earlier (42) encodes a ubiquitin–ERCC1 fusion protein, in which the ubiquitin moiety is thought to protect the N-terminus of ERCC1 against proteolytic degradation. The N-terminal ubiquitin part can be cleaved off by the enzyme ubiquitin lyase (see 42). Plasmid pSVL5 is a modification of the pSVL eukaryotic expression vector (Pharmacia) in which the *Eco*RI, *Sal*I, *Kpn*I and *Hin*dIII sites have been removed. Subsequently, the *ERCC1* cDNA, isolated via PCR, was cloned behind the strong SV40 late promoter, giving rise to plasmid pSVL5E (5.8 kb). The PCR-derived *ERCC1* cDNA insert was verified by sequence analysis. Plasmid pUCPROMH-1 (4.2 kb), containing the wild-type *ERCC1* cDNA under the control of its own genomic promoter, has been described previously (43). Plasmids pRSVneo and pSV3gptH respectively harbour the dominant selectable marker genes *neo* and *gpt* (18,44). Plasmid pHG containing the *DHFR* gene (45) was used to drive gene amplification in mammalian cells.

Construction of mutant cDNAs

Missense and C-terminal deletion mutations in *ERCC1* were introduced using site-directed mutagenesis (46). The complete *ERCC1* cDNA together with its promoter region was inserted in M13mp18 (Pharmacia), giving rise to Mp18PROM. After mutation induction this insert was used to replace the wild-type *ERCC1* cDNA in plasmid pUCPROMH-1.

The *ERCC1–UvrC* hybrid construct consists of the *ERCC1* cDNA in which the C-terminus, conserved between ERCC1 and the *E.coli* NER protein UvrC, is replaced by the C-terminus of *UvrC* [*ERCC1*(1–708)–*UvrC*(1600–1767)]. The *ERCC1* part was amplified using a forward primer containing an optimal translation initiation sequence and a reverse primer containing *ERCC1*(697–708) and *UvrC*(1600–1617) sequences. The complementary oligonucleotide was used (as forward primer) to amplify the C-terminus of *UvrC*. The two amplified fragments were used as template in a subsequent PCR to amplify the *ERCC1–UvrC* hybrid gene.

N-Terminal deletion mutations were made via PCR using sense primers containing an optimal translation initiation sequence. The *ERCC1–UvrC* hybrid gene and N-terminal deletion mutants were cloned into plasmid pSVL5E, replacing the wild-type *ERCC1* cDNA.

All mutations were verified by sequence analysis. Furthermore, at least two separate cDNAs were used to assess the biological effects.

DNA transfections

Wild-type and mutated *ERCC1* cDNAs were co-transfected with pRSVneo (in some cases after *in vitro* ligation). 43-3B (ERCC1-deficient CHO) cells (47) were transfected using either the calcium phosphate DNA precipitation procedure (48) or lipofectin (BRL) as described (49). Stable transfectants (mass populations or single clones) selected on G418 (800 μg/ml; Gibco) were checked for the presence of the intact human *ERCC1* cDNA by PCR as described earlier (50).

Survival assays

To determine the colony forming ability (CFA), DNA constructs (5–10 μg) were co-transfected with pSV3gptH (2–5 μg) into 5×10^5 43-3B cells in three 90 mm dishes, as described previously (18). After 10–14 days of selection on mycophenolic acid (MPA; Gibco) and mitomycin C (MMC; Kyoma) the cells were fixed, stained and colonies were counted, providing a rough estimate of the survival. To more precisely determine the correcting ability of the mutated *ERCC1* cDNAs, cells of 43-3B, its parental cell line CHO9 and stable transfectants were plated at densities varying from 200 to 1000 cells/60 mm dish. After attachment, cells were either rinsed with phosphate-buffered saline (PBS) and UV irradiated at various doses (Philips TUV low pressure mercury tube, 15 W, 0.45 J/m^2/s, predominantly 254 nm) or incubated with different doses of MMC. The numbers of surviving colonies were counted in triplicate dishes.

In some experiments the presence of non-proliferating giant cells hampered accurate colony counting. Therefore, S phase-dependent [^{3}H]thymidine incorporation, as a measure of the number of proliferating cells, was determined as well. To this end, 500–5000 cells were seeded in 30 mm wells and either rinsed and UV irradiated or incubated with MMC or cisplatin [*cis*-diammine-dichloroplatinum(II); Lederle] for 1 h. Seven days later, before reaching confluency, the cells were incubated with [^{3}H]thymidine (2 μCi/ml) and 20 mM HEPES for 1 h, rinsed twice with PBS and incubated for a further 1 h in unlabelled medium to deplete radioactive precursor pools. Then, cells were lysed in alkali and radioactivity was quantified by scintillation counting. The two methods used to determine mutagen sensitivity, the classical CFA assay and the rapid and simple [^{3}H]thymidine incorporation assay, gave essentially the same results.

Immunoblotting

Total cell extracts of stable transfectants (90 μg) were checked for the presence of (mutant) ERCC1 protein on immunoblots using affinity purified anti-ERCC1 antiserum (19).

Two-dimensional electrophoresis was carried out as described by O'Farrell (51). The proteins were first separated according to their isoelectric point (pI) and subsequently at right angles en masse by SDS electrophoresis in a polyacrylamide gradient (7.5–20%) gel.

ERCC1 amplification

Cosmid 43-34 carrying the *ERCC1* gene, the *gpt* and the *agpt* markers (18) was ligated to pHG containing the *DHFR* gene and transfected into 43-3B cells. Initially, the transfected cells were grown in medium containing MPA (25 μg/ml) and MMC (10^{-8} M) to select for the presence of the *gpt* and the *ERCC1* genes respectively. In parallel, a part of the transfected cells was

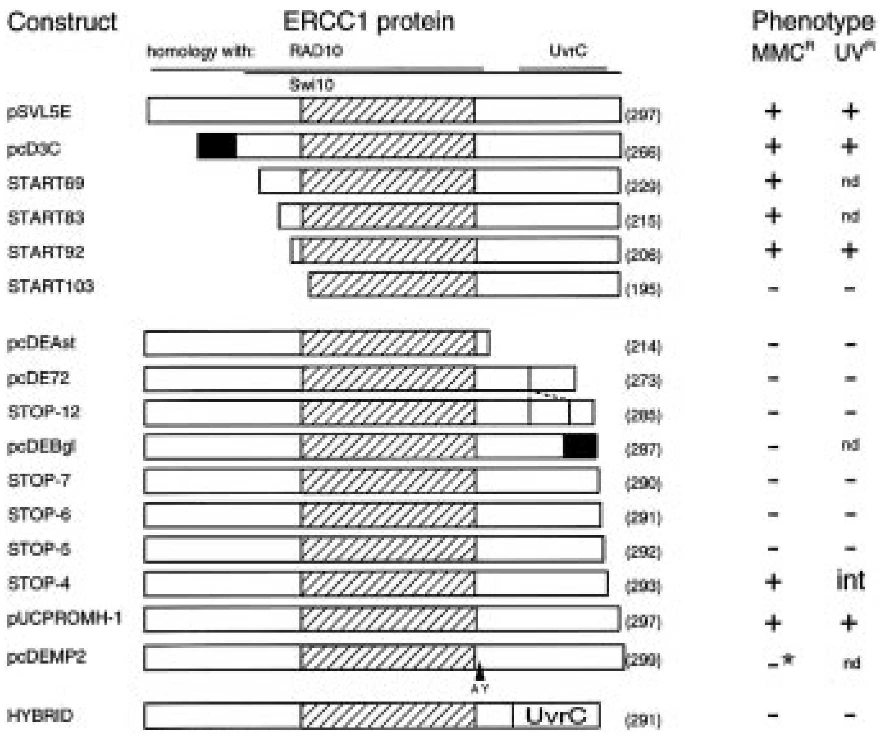

Figure 1. Schematic representation of *ERCC1* deletion mutants and their correcting abilities. +, correction; int, intermediate correction; –, no correction of the 43-3B mutant phenotype; nd, not determined; *, dominant negative when overexpressed in wild-type cells. Numbers in brackets, number of amino acid residues. The hatched region illustrates the most strongly conserved sequence between ERCC1 and RAD10 and the black regions indicate nonsense sequences. In pcDE72, ERCC1 is lacking exon VIII, as indicated by the dashed lines, whereas in pcDEMP2 an extra alanine and tyrosine (AY) are inserted distal from residue 208. For several mutants the CFA was determined as described by Westerveld *et al.* (18).

treated with UV light and MPA. Both were followed by selection on 10 µg/ml methotrexate (MTX; Lederle). By stepwise increasing the MTX concentration from 10 to 500 µg/ml amplification of the *DHFR* gene together with its flanking sequences was induced. Stably transfected clones were analysed for amplification of the *ERCC1* gene, transcript and protein.

Microinjection

ERCC1 and ubiquitin–ERCC1 proteins (0.1 pg), purified from overproducing *E.coli* (42), were injected into the cytoplasm of human primary fibroblasts (XP-G cells were used). Rat serum albumin (RSA) was used as a control. Cells were fixed 10 min or 1 h after injection. Immunofluorescence was carried out using either anti-RSA or anti-ERCC1 antisera.

In situ hybridization

Metaphase spreads of 41D cells were used for *in situ* hybridization with AAF-modified pHG as a probe as described elsewhere (52). Hybridization was visualized using rabbit anti-AAF and peroxidase-conjugated pig anti-rabbit antisera.

Immunofluorescence

Cells grown on slides were rinsed with PBS and fixed in PBS containing 2% paraformaldehyde for 10 min and in methanol for 20 min. After extensive washing with PBS supplemented with 0.15% glycine and 0.5% BSA the slides were incubated with pre-immune or affinity purified anti-ERCC1 antiserum (1:100 dilution in PBS) for 1.5 h at room temperature, rinsed and stained with goat anti-rabbit FITC-conjugated antiserum (1:80 dilution) for 1.5 h. Finally, the slides were rinsed and sealed in Vectashield mounting medium (Vector) containing 4′,6′-diamidino-2-phenylindole and propidium iodide as a nuclear marker.

RESULTS

To identify the regions in ERCC1 essential for its function in NER and cross-link repair, mutated *ERCC1* cDNAs were assayed for correction of the rodent group 1 mutant 43-3B. Like other mutants in this complementation group and in group 4, this UV-sensitive cell line also exhibits an extreme sensitivity to cross-linking agents such as MMC and cisplatin. The latter feature is not displayed by other UV-sensitive NER-deficient complementation groups and probably reflects the role of ERCC1 in recombination needed for elimination of interstrand cross-links. The requirement for ERCC1 for UV resistance corresponds with its function in NER. Stably transfected neomycin-resistant mass populations were examined for their responses to UV irradiation and MMC. To validate the findings two separate cDNAs for each mutation were tested. Since a negative result can have trivial reasons we studied in addition, when indicated, individual clones which were verified to contain one or more copies of intact mutated or wild-type *ERCC1* cDNA. Transfection into Chinese hamster 43-3B cells of a wild-type human *ERCC1* cDNA (encoding 297 residues) almost fully complements both repair defects of these cells (see Figs 1 and 2).

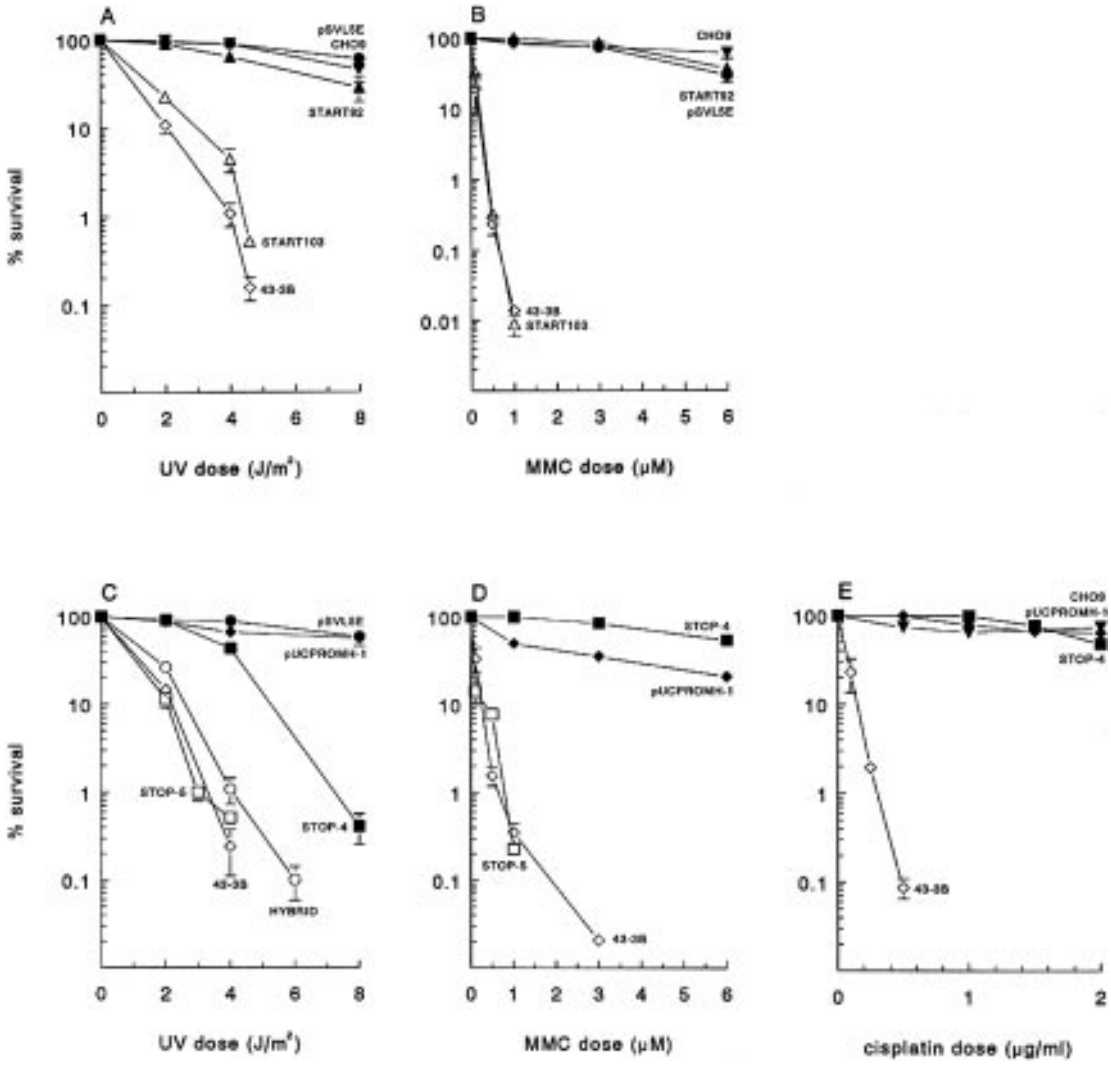

Figure 2. Survival of 43-3B transfectants containing N-terminal deletion mutants after treatment with (**A**) UV and (**B**) MMC and C-terminal deletion mutants after treatment with (**C**) UV, (**D**) MMC and (**E**) cisplatin. ♦, pUCPROMH-1 (mass population) and ●, pSVL5E (single clone) both containing the wild-type *ERCC1* cDNA; ▲, START92 (mp); Δ, START103 (mp); ■, STOP-4 (sc); □, STOP-5 (sc); ○, HYBRID (mp). ▼, the parental wild-type cell line CHO9 (mp); ◊, the mutant 43-3B (mp). The number of proliferating cells was measured as [^{3}H]thymidine incorporation. Points are average values for duplicate wells (or four for the untreated cells) and the error bars represent standard errors of means.

N-Terminal deletion mutants of *ERCC1*

Construct pcD3C encoding a truncated ERCC1 protein lacking the first 54 amino acids (see Fig. 1) has been shown to confer MMC resistance on 43-3B cells (24). We have further shortened the protein by constructing *ERCC1* cDNAs containing the start codon at amino acid positions 69, 83, 92 and 103 (see Fig. 1), preceded by an optimal translation initiation sequence.

Whereas the constructs START69, START83 and START92 all corrected both the UV and MMC sensitivity of recipient cells, START103 could not do so (see Fig. 2A and B for the START92 and START103 mutants; for others data not shown). We conclude that an N-terminal deletion of 91 residues comprising almost one third of the protein does not interfere with its repair functions.

It was not possible to verify the effect of the START92 and START103 mutations at the protein level by immunoblot analysis as our affinity purified ERCC1 antiserum mainly recognizes epitopes in the N-terminus of the protein. Therefore, it remains uncertain whether a deletion of 102 amino acids results in an unstable protein or interferes with the protein activity itself.

C-Terminal deletion mutants of *ERCC1*

Previous studies have suggested that the strongly conserved C-terminal part of ERCC1 is crucial for its function. pcDEAst, in which the *ERCC1* cDNA contains a premature stop codon at amino acid position 214 coding for a 'RAD10-like' ERCC1 protein (see Fig. 1), could not correct MMC sensitivity. Neither could pcDEBgl, encoding a truncated protein of 287 amino acids with 17 unrelated C-terminal residues due to a frameshift mutation (33), nor pcDE72, a splice mutant lacking exon VIII (24) (see Fig. 1).

To more precisely determine the extent of the C-terminal functional area, a premature stop codon was introduced at amino acid position 286 resulting in a C-terminal deletion of 12 residues (STOP-12). In addition, STOP-7, STOP-6, STOP-5 and STOP-4

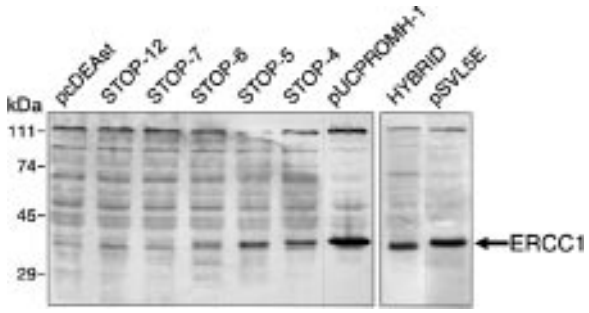

Figure 3. Expression of wild-type and ERCC1 C-terminal deletion mutant proteins. Equal amounts (90 µg) of whole cell extracts were loaded (pcDEAst, STOP-12, -7, -6 and -5, pSVL5E and HYBRID mass populations; STOP-4 and pUCPROMH-1 single clones). Blots were incubated with affinity purified anti-ERCC1 antiserum. ERCC1 protein migrates at 39 kDa. Untransfected 43-3B is shown in Figure 6. Note that this antiserum is specific for human ERCC1 and does not recognize the endogenous Chinese hamster protein.

were constructed (Fig. 1). Of these C-terminal truncations only STOP-4 was able to correct the sensitivity of 43-3B cells to MMC, whereas for UV only a partial correction was found (Fig. 2C and D). The normal survival of STOP-4-transfected cells after exposure to cisplatin (Fig. 2E) showed that the observed resistance to MMC reflects correction of cross-link sensitivity in general and rules out the possibility of deficient drug uptake or metabolism. Thus, only four residues can be deleted from the C-terminus without losing the cross-link repair function, although the UV damage repair function is already slightly affected. The various truncated proteins in whole cell extracts from stable transfectants were analysed by immunoblotting (Fig. 3). STOP-6, STOP-5, STOP-4 and STOP-7 (weakly) proteins could be visualized, indicating that they are stable *in vivo* though, with the exception of STOP-4, non-functional. Further shortening of the protein apparently induces instability, as no ERCC1 could be detected in STOP-12 and pcDEAst (Fig. 3; note that the endogenous Chinese hamster ERCC1 protein is not recognized by our affinity purified anti-ERCC1 antiserum; see also Fig. 6).

To assess whether the sequence homology of the C-terminus with the *E.coli* UvrC repair protein extends to the functional level, a hybrid construct was generated, with the human part substituted for its bacterial equivalent (Fig. 1). This ERCC1–UvrC hybrid protein is properly expressed (HYBRID in Fig. 3), however, it failed to correct the sensitivity to UV and MMC (Figs 1 and 2C).

Missense mutations in *ERCC1*

To further examine the presence of functional domains involved in one or both repair functions of ERCC1, specific amino acids were substituted in the region most strongly conserved between human ERCC1, *S.cerevisiae* RAD10 and *S.pombe* Swi10 (see Fig. 4). This part may contain the binding site for the human homolog of RAD1 (26) and/or it may harbour a DNA binding site (24). The types of changes made and the effects on UV and MMC survival after transfection into 43-3B cells are summarized in Table 1. For a number of ERCC1 mutants we found a considerable difference in correction of the UV and MMC sensitivity. This was also observed in individual clones containing intact mutated cDNA. For instance, in clone $P_{150}\rightarrow V$(I) the UV sensitivity was complemented only partially, whereas the extreme sensitivity to both MMC and cisplatin was almost fully restored (Fig. 5). In contrast, other mutant *ERCC1* transfectants retained the sensitivity to UV and showed partial or no correction of the MMC sensitivity. Immunoblot analysis revealed that most mutations gave rise to no or hardly detectable ERCC1 protein (Fig. 6), suggesting that they cause protein instability. In those cases where protein was detected a partially corrected phenotype was seen (Table 1), as shown for the $P_{150}\rightarrow V$ and $L_{141}\rightarrow H$ substitutions (see also Fig. 6). These observations suggest that a reduced amount of (mutated) ERCC1 is sufficient for the repair of MMC damage but not for the repair of UV damage. This interpretation is strongly supported by the isolation of two clones carrying the same mutated *ERCC1* cDNA ($P_{150}\rightarrow V$), but which were found to express the encoded protein to a different level in repeated experiments. Clone $P_{150}\rightarrow V$(II) exhibits only partial correction of the MMC sensitivity and no correction of UV sensitivity, in contrast to $P_{150}\rightarrow V$(I) (Fig. 5A and B). Immunoblot analysis revealed that the level of correction correlated with the amount of mutated ERCC1 protein detectable (determined by copy number and site of integration, which differs in each transfectant; Fig. 6).

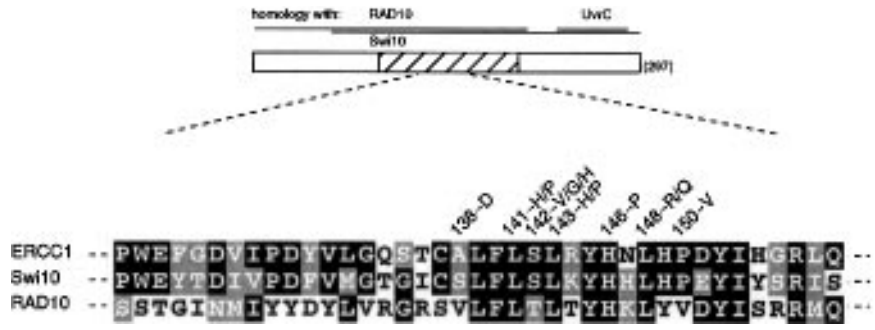

Figure 4. Homology between ERCC1, Swi10 and RAD10 proteins in their most strongly conserved region. Identical amino acids are in black boxes, physicochemically related residues are in grey boxes and missense mutations introduced are indicated. Displayed are part of ERCC1 (297 amino acids) amino acids 120–159, Swi10 (252 amino acids) amino acids 62–101 and RAD10 (210 amino acids) amino acids 117–156 (numbers according to 24,32,70).

Table 1. Summary of ERCC1 missense mutations, their expression and correcting abilities

Missense mutation	Checked in mass population or single clone	UV^R	MMC^R	Protein expression level
$C_{76}\rightarrow W$	mp	nd	–	nd
$Q_{107}\rightarrow R$	mp	nd	–	nd
$A_{138}\rightarrow D$	mp	–	–	Not detectable
$L_{141}\rightarrow H$	mp	int	++	Reduced
$L_{141}\rightarrow P$	sc	–	–	Not detectable
$S_{142}\rightarrow G$	mp	nd	–	nd
$S_{142}\rightarrow H$	mp	nd	++	nd
$S_{142}\rightarrow V$	sc	–	int	Strongly reduced
$L_{143}\rightarrow H$	mp	int	+	Strongly reduced
$L_{143}\rightarrow P$	mp	–	int	nd
$H_{146}\rightarrow P$	mp	–	int	Strongly reduced
$L_{148}\rightarrow R$	mp	nd	–	nd
$L_{148}\rightarrow Q$	mp	nd	–	nd
$P_{150}\rightarrow V$ I	sc	int	++	Reduced
$P_{150}\rightarrow V$ II	sc	–	int	Strongly reduced
$W_{200}\rightarrow S$	mp	nd	+	nd
$Q_{251}\rightarrow K$	mp	nd	+	nd

++, wild-type correction; +, correction close to wild-type; –, no correction; int, intermediate correction of the 43-3B mutant phenotype; nd, not determined. For most mutants the CFA was determined as described by Westerveld *et al.* (18). (I) and (II) refer to two different populations containing the $P_{150}\rightarrow V$ mutated cDNA.

ERCC1 expression was also analysed at the single cell level by immunofluorescence. In rodent cells transfected with pUC-PROMH-1, a human wild-type *ERCC1* construct, ERCC1 is expressed in the nucleus of every cell, but the expression level seems somewhat lower than in HeLa cells (Fig. 7). Although variation is seen in transfectants expressing mutated ERCC1 protein (even in cells derived from one single clone), the overall expression levels are consistently lower than in cells containing the wild-type cDNA. As with immunoblot analysis, no ERCC1 protein could be detected in transfectant containing the $S_{142}\rightarrow V$ construct that failed to correct.

Amplification and microinjection

The remarkable absence of ERCC1 protein in most point mutants can be explained in two ways. Either the mutation renders the ERCC1 mRNA or protein unstable or the mutation interferes with correct folding of the protein and prevents it from proper association with the other component(s) of the complex. Uncomplexed (aberrant or wild-type) ERCC1 protein is then rapidly degraded. To investigate these possibilities we assessed the fate of an excess of wild-type ERCC1 protein obtained by *DHFR*-driven amplification of the wild-type gene and by microinjection of purified ERCC1 protein in primary fibroblasts.

In an attempt to overproduce ERCC1 protein a construct containing the *DHFR* gene and the wild-type human *ERCC1* cosmid (Fig. 8A) together with two dominant selectable marker genes, *gpt* and *agpt*, as positive controls, was transfected into 43-3B cells. Transformants containing the different dominant markers and a functional *ERCC1* gene (as determined by wild-type UV and MMC resistance) were treated with stepwise increasing methotrexate concentrations inducing amplification of the *DHFR* gene together with its flanking sequences. Southern blot analysis and *in situ* hybridization to metaphase chromosomes revealed a massive (100- to >1000-fold) amplification of the *ERCC1* gene in all stable transfectant clones analysed (e.g. clone 41D in Fig. 8B and C respectively). A corresponding dramatic increase in ERCC1 transcripts was found as well (Fig. 8D, compare first lane with last lane). In contrast, ERCC1 induction at the protein level was only ~4-fold as estimated by immunoblotting (Fig. 8E, arrow points to full-length ERCC1). Two-dimensional protein analysis of the transfected cells clearly shows enhanced levels of DHFR and co-amplification of *gpt* and *agpt* (two genes not selected for), whereas no protein spot corresponding to ERCC1 could be seen (Fig. 8F). Similar results were obtained with a number of other transformants carrying the amplified functional *ERCC1* gene (data not shown). Apparently, it is not possible to overexpress human ERCC1 protein in mammalian cells.

To analyse the stability of wild-type ERCC1 protein in another manner, purified full-length ERCC1 protein and a ubiquitin–ERCC1 fusion product (42), both overproduced in *E.coli*, were directly injected into the cytoplasm of human primary fibroblasts with the aid of a glass microneedle. Injection of RSA as a control resulted in a clear cytoplasmic immunostaining 10 min following injection, which remained fully stable for 1 h at least. In contrast, a similar number of ERCC1 molecules (representing more than five times the amount normally present in a cell) produced a very weak cytoplasmic staining early (within 10 min) after injection with occasional nuclear staining above background. No exogenous protein could be seen after 1 h. These microinjection results strongly suggest that an excess of free ERCC1 is rapidly degraded

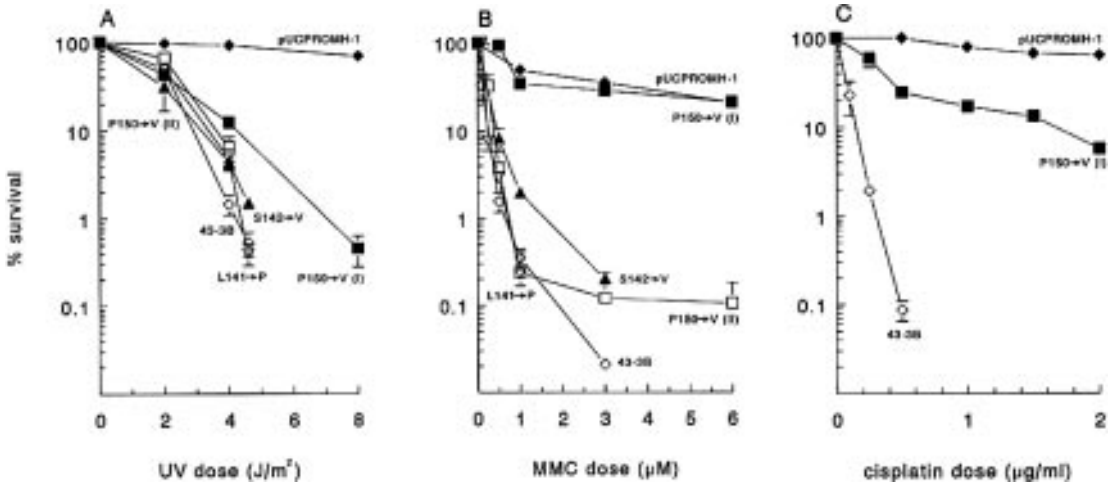

Figure 5. Survival of 43-3B transfectants containing missense mutants following (**A**) UV, (**B**) MMC and (**C**) cisplatin treatment. ◊, the mutant cell line 43-3B (mass population) and 43-3B transfected with: ▽, L_{141}→P (single clone); ▲, S_{142}→V (sc); ■, P_{150}→V(I) (sc); □, P_{150}→V(II) (sc); ◆, pUCPROMH-1 containing the wild-type *ERCC1* cDNA (sc). The number of proliferating cells was measured as either CFA or overall [^{3}H]thymidine incorporation. Points are average values for duplicate wells (or four for the untreated cells) and the error bars represent standard errors of means.

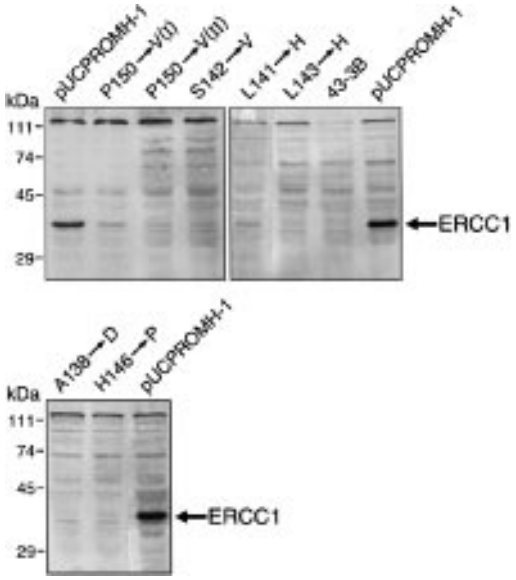

Figure 6. Expression of wild-type and missense mutated ERCC1 proteins. Equal amounts (90 μg) of whole cell extracts were loaded [pUCPROMH-1, P_{150}→V(I), P_{150}→V(II), S_{142}→V single clones and others mass populations]. Blots were incubated with affinity purified anti-ERCC1 antiserum. ERCC1 protein migrates at 39 kDa. Note that the antiserum does not recognize the endogenous Chinese hamster ERCC1. (I) and (II) refer to two different populations containing the P_{150}→V mutated cDNA.

in the cell. Similar results were obtained for the XPB and XPD repair proteins, known to be part of the basal transcription factor TFIIH (our unpublished results).

DISCUSSION

ERCC1 mutations were assayed for complementation of the UV sensitivity (NER defect) and MMC sensitivity (recombination defect) of the rodent group 1 mutant 43-3B. In this mutant endogenous ERCC1 protein is hardly detectable (R.D.Wood, personal communication) and will not compete with the human counterpart for complex formation in the transfectants. By deletion analysis of ERCC1, the minimal essential size of the protein for both of its repair activities could be deduced. From the N-terminus, one third of the ERCC1 protein (91 amino acids) can be removed without loss of correcting ability. This finding indicates that this region (24) is not required for the NER or cross-link repair function of ERCC1. Consistently, this region is poorly conserved when compared with *S.cerevisiae* RAD10 (24) and largely absent in the *S.pombe* homolog Swi10 (32). However, a cysteine to tryptophan substitution (C_{76}→W) within this non-essential part results in a non-functional protein (Table 1), pointing to a possible role in protein folding. Removal of 102 N-terminal amino acids fully inactivates ERCC1. This deletion may affect the transient association of ERCC1 with the damage recognition protein XPA, since this interaction involves amino acids within the region of residues 93–120 of ERCC1 (8). In addition or alternatively, based on the homology between RAD10 and ERCC1, removal of the 102 residues may abolish the formation of a complex of ERCC1 with the human homolog of RAD1. The stretch of residues 90–210 in RAD10 has been implicated in the binding of RAD1 (26).

Within the central area, missense mutations were introduced affecting the best conserved part between amino acid positions 138 and 150. Most of these mutated *ERCC1* cDNAs produced reduced amounts of protein and could not fully complement the repair defect of the recipient cells. The most plausible interpretation of these findings is that all the different point mutations affect protein stability, probably by interfering with complex formation with ERCC4/ERCC11/XPF. Free ERCC1 molecules are highly unstable inside the cell, as was shown for an excess of wild-type ERCC1 introduced transiently by microinjection or by continuous overexpression in stable amplificants. In line with this observation, the amount of ERCC1 protein in human XP-F and rodent group 4 and 11 cells is strongly reduced (20,22), whereas the *ERCC1* gene itself does not carry any mutation and is properly expressed at the mRNA level (our unpublished observations).

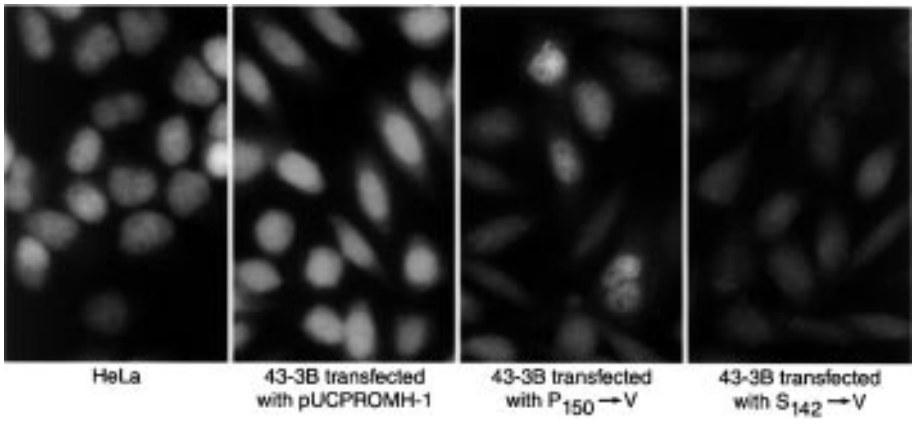

Figure 7. Expression of (mutated) ERCC1 in single cells. Cells from transfectant $P_{150}\rightarrow V$ are derived from clone (I). Immunofluorescence was carried out using affinity purified anti-ERCC1 antiserum. HeLa mass population; pUCPROMH-1, $P_{150}\rightarrow V$ and $S_{142}\rightarrow V$ single clones. Like $S_{142}\rightarrow V$, mock-transfected 43-3B cells displayed no fluorescent staining.

The transfectants expressing detectable (but lowered) levels of ERCC1 protein showed a partial correction. This is consistent with the idea that this central area is needed for interaction with ERCC4 and stability of the protein. Interestingly, the UV sensitivity of the *S.cerevisiae rad1-20* mutant is caused by a mutation in the RAD10 binding domain of RAD1 and is partially corrected by overexpression of RAD10 protein, presumably increasing the concentration of active RAD1–RAD10 protein complex (53).

In those cases where diminished amounts of mutated protein were detected, the repair of UV damage (NER) was consistently more impaired than the repair of cross-links (recombination). No mutation was found that affected cross-link repair and not NER. It appears that lower levels of the ERCC1 complex are required for cross-link elimination than for UV lesion removal. Either the number of interstrand cross-links is very low, such that small amounts of ERCC1 complex are sufficient, or the ERCC1 complex is more active or not the rate limiting step in cross-link repair. Some exceptional rodent group 1 and 4 mutants exhibit only moderate cross-link sensitivity combined with full UV impairment (54). We have found the same for cells from XP-F patients (our unpublished results), explaining why XP group F presents a NER deficiency rather than a deficiency in cross-link repair.

Several groups reported that increased levels of ERCC1 transcripts correlate with increased cisplatin resistance of human cells (55–57). However, we found only an ~4-fold increase in ERCC1 protein, despite a massive increase in ERCC1 transcripts (Fig. 8), and no elevated resistance to mitomycin C in over-producing cells (our unpublished results; 58). Thus, ERCC1 protein levels should be determined before conclusions can be drawn with respect to involvement of this protein in cisplatin resistance. Consistent with this cautious note and with our idea that small amounts of ERCC1 complex are sufficient for cross-link repair function, no elevated ERCC1 protein levels were found in nitrogen mustard-resistant cells (59), indicating that increased ERCC1 levels are not involved in resistance to this cross-linking agent.

At the C-terminal end, no more than four residues appear to be dispensable for both ERCC1 functions. An ERCC1 protein lacking the C-terminal five amino acids, although stable, failed to correct the UV and MMC sensitivity of 43-3B cells. Residue –5 is close to the point where the homology of ERCC1 with the C-terminus of the *E.coli* UvrC repair protein ends (33). Interestingly, the C-terminus of UvrC itself is also essential for its endonuclease function (60), though residues that are thought to be directly involved in the incision activity of UvrC may be located elsewhere (61). It was shown that the *Bacillus subtilis* UvrC protein can substitute for the *E.coli* UvrC protein in the uvrABC excinuclease, despite their low homology (38%) (62). Interestingly, residues conserved between these two proteins are also present in ERCC1 and are therefore likely to be important for nuclease activity. A database search revealed the presence of two small subdomains homologous to this essential C-terminal part in a large group of proteins implicated in either DNA break induction or sealing. Representatives of each class of proteins are aligned in Figure 9. In addition to the known prokaryotic UvrC homologs, inducing 5′ (and possibly also 3′) incision during NER (60,61,63), this group includes homologs of RadC, a protein active in recombination-dependent repair of DNA breaks (64), and NAD-dependent DNA ligases. Furthermore, residues within subdomain 1 were found to be conserved in a number of other nucleases, among which were the 5′ nuclease domain of *Taq* polymerase (65), the human flap-endonuclease FEN-1, equivalent to the 5′→3′ endonuclease of *E.coli* DNA polymerase I (66), and many of the bacterial members of the 5′ nuclease family described by Gutman and Minton (*E.coli* polymerase I amino acids 188–212; 67). The latter region constitutes the last part of the strongly conserved I region in FEN-1 shared with the XPG and *S.cerevisiae* RAD2 nucleases (68) generating the 3′ incision in the eukaryotic NER reaction. The crystal structure of *Taq* polymerase reveals that this area adopts a specific α-helix–turn–α-helix conformation followed by a long loop and two helices (69). Its role in the catalysis of the nuclease reaction has not yet been resolved.

This evolutionary evidence strongly suggests that the domain homologous to UvrC is somehow involved in the activity of the ERCC1 protein, supporting a direct role of ERCC1 in the incision 5′ of the DNA lesion. An *ERCC1–UvrC* hybrid gene, however, failed to complement the repair defect of the rodent group 1 mutant, indicating that the C-terminal regions of UvrC and ERCC1 have diverged too much to allow domain swapping. In

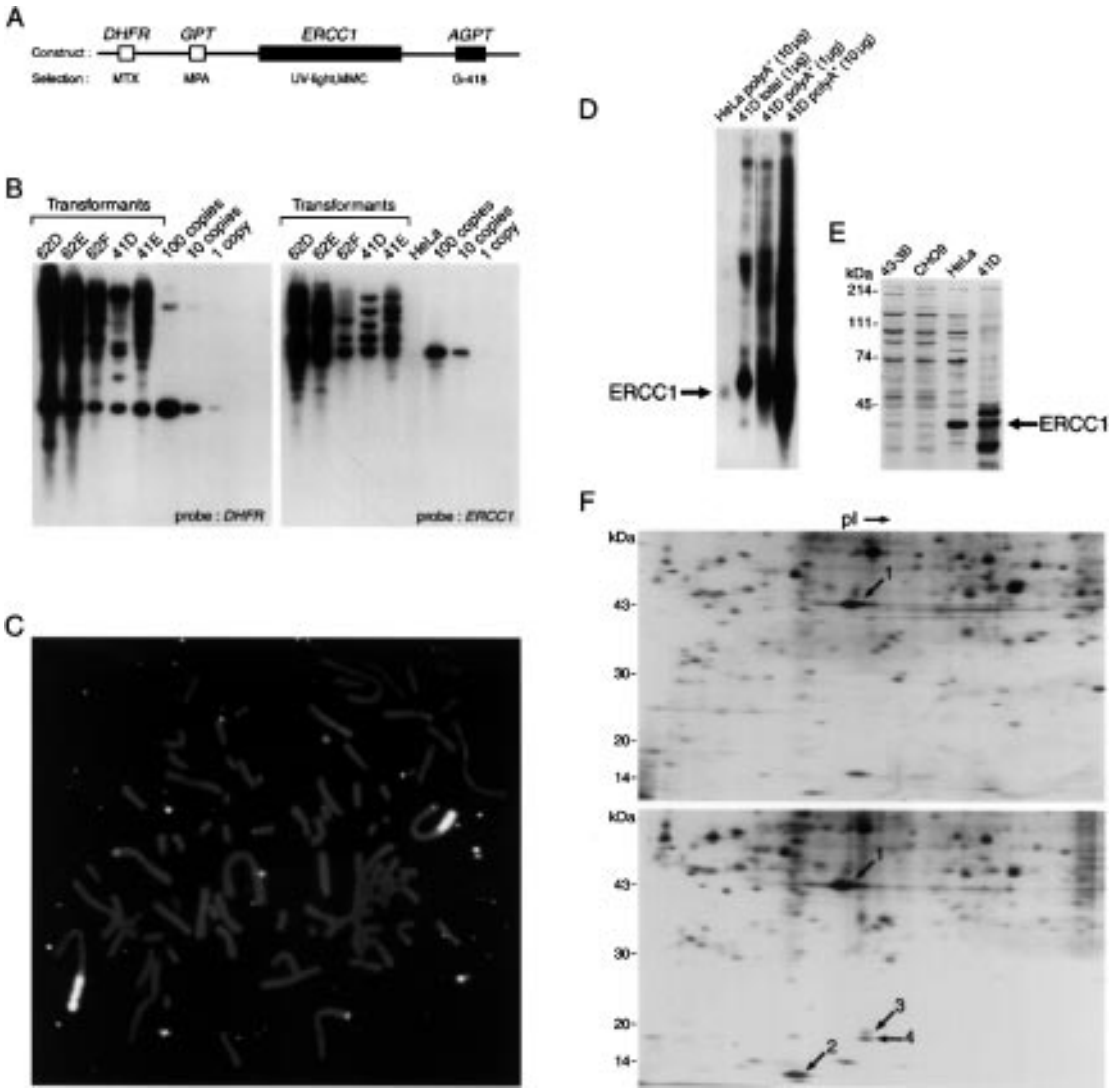

Figure 8. Characterization of transformant 41D. (**A**) Construct used for the *DHFR*-driven gene amplification. Black boxes indicate the sequences derived from the *ERCC1* gene on cos34-34 and the pTCF plasmid (containing the *agpt* gene), whereas open boxes represent regions derived from plasmid pHG (carrying the *DHFR* gene) and pSV3gptH (carrying the *gpt* gene). (**B**) Southern blot analysis of *Eco*RI-digested DNA (15 µg) from various amplified transformants and 1, 10 and 100 copies of the transfected construct/genome with a ^{32}P-labelled *ERCC1* or *DHFR* probe (pHG) to quantify the amplification. (**C**) *In situ* hybridization on 41D metaphase spreads with a *DHFR* probe. (**D**) Northern blot analysis of total and poly(A)$^+$ RNA from HeLa and 41D cells. For hybridization a ^{32}P-labelled *ERCC1* probe was used. Arrow indicates mature ERCC1 transcript, but note that precursor and incompletely spliced ERCC1 transcripts hybridize as well. Compare the first and last lanes, both containing equal amounts of poly(A)$^+$ RNA from HeLa cells and amplificant 41D. (**E**) Immunoblot analysis of 43-3B, CHO9, HeLa and 41D extracts (20 µg) using crude anti-ERCC1 antiserum. The arrow indicates the full-length ERCC1 protein. (**F**) Two-dimensional protein analysis of 43-3B and 41D whole cell extracts. The two-dimensional gel was silver stained. (1) actin; (2) DHFR; (3) agpt; (4) gpt. Note that (co-amplified) ERCC1 (mol. wt 39 kDa), supposed to be present in the area between these indicated proteins, is not detectable.

this regard it should be noted that the C-terminus of UvrC stops at the –6 position in ERCC1 (24), i.e. just beyond the –4 residue critical for both ERCC1 repair functions. The presence of detectable levels of the crucial C-terminally truncated proteins further supports the idea that this area is required for catalysis rather than for stabilization. An ERCC1 protein with two extra residues at position 208 (see pcDEMP2 in Fig. 1) is also stable in the cell and when strongly overexpressed it exerts a dominant negative effect (58). Poisoning of the ERCC1 complex by this mutant protein suggests that the catalytic domain may extend from the C-terminal end to residue 208 at least. The conservation of the UvrC homology in mammalian ERCC1 and *S.pombe* Swi10 contrasts with its complete absence in *S.cerevisiae* RAD10 (see Fig. 4). Nevertheless, purified RAD1–RAD10 is capable of incision (29–31). A possibility is that the RAD1–RAD10 nuclease can do without this domain. Perhaps more likely, cryptic sequences from the distinct N-terminal part of RAD10 can provide this function or, alternatively, stretches in RAD1 that have no match in its *S.pombe* homolog Rad16.

In conclusion, analysis of mutations introduced throughout the coding area of ERCC1 has revealed dispensability of the poorly conserved N-terminal third of the protein, contrasting with a much more stringent need for the C-terminus. Mutant protein stabilities and local sequence conservation in many DNA break

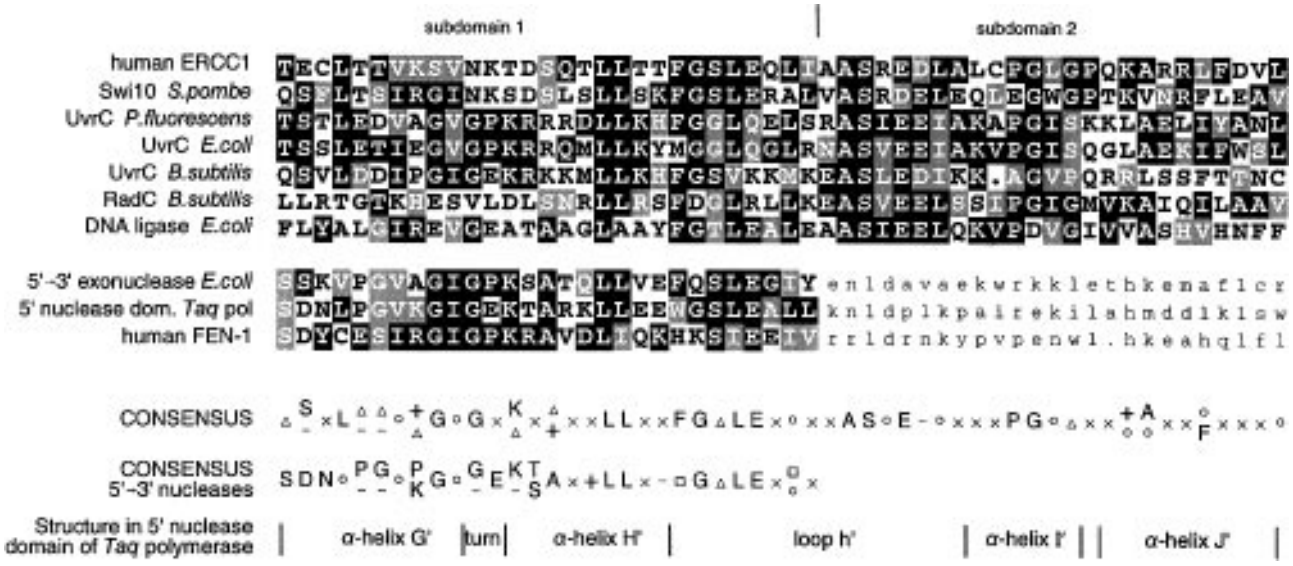

Figure 9. Homology of the UvrC-like C-terminal domain of ERCC1 with other proteins. Shown is part of human ERCC1 (297 amino acids) amino acids 239–290, *S.pombe* Swi10 (252 amino acids) amino acids 181–232, *P.fluorescens* UvrC (607 amino acids) amino acids 554–605, *E.coli* UvrC (588 amino acids) amino acids 536–587, *B.subtilis* UvrC (598 amino acids) amino acids 539–589, *B.subtilis* RadC (231 amino acids) amino acids 43–94, *E.coli* NAD-dependent DNA ligase (671 amino acids) amino acids 514–565, an *E.coli* potential 5′→3’ exonuclease (251 amino acids) amino acids 179–230, *Taq* polymerase (832 amino acids) amino acids 193–244 and human FEN-1 protein (380 amino acids) amino acids 235–295 (numbers according to 24,32,65,71–77). Identical amino acids are in black boxes and physicochemically related residues are in grey boxes. The consensus sequence is indicated. ×, any residue; ○, L V I M; Δ, S T A G P; –, D E; +, K R H; □, W Y F.

processing proteins suggest that the C-terminal domain is primarily required for enzymatic activity of ERCC1, presumed to be a structure-specific endonuclease. The central region of the protein appears to be involved in protein–protein interactions needed for protection against degradation. The repair of cross-links requires lower amounts of ERCC1 than does NER, which could explain the cross-link resistance of XP-F cells and may indicate that the ERCC1-dependent step in this process occurs outside the context of NER. To confirm these findings at the protein level the isolation of the other complex components is underway.

NOTE

During the preparation of this manuscript the gene encoding the XPF protein (the equivalent of ERCC4 and ERCC11) was cloned and the purified ERCC1–XPF complex has been shown to indeed have structure-specific endonuclease activity (Sijbers *et al*., *Cell*, in press).

ACKNOWLEDGEMENTS

We are grateful to M. H. M. Koken, J. de Wit, W. Vermeulen and G. Weeda for preparing the ubiquitin–ERCC1 construct and for help with transfections and microinjections. In addition, we acknowledge J. van den Tol, H. Meijers-Heijboer, P. Warmerdam, E. Kootwijk and A. Overmeer-Graus for construction of several mutated *ERCC1* cDNAs and for generation and characterization of the amplificants. M. Kuit is acknowledged for photography. This research was supported in part by the Netherlands Foundation for Medical Sciences (GMW, 900-501-113), and by the EEC (project PL00950056).

REFERENCES

1 Hoeijmakers,J.H.J. (1994) *Eur. J. Cancer*, **30A**, 1912–1921.
2 Robins,P., Jones,C.J., Biggerstaff,M., Lindahl,T. and Wood,R.D. (1991) *EMBO J.*, **10**, 3913–3921.
3 Jones,C.J. and Wood,R.D. (1993) *Biochemistry*, **32**, 12096–12104.
4 Asahina,H., Kuraoka,I., Shirakawa,M., Morita,E.H., Miura,N., Miyamoto,I., Ohtsuka,E., Okada,Y. and Tanaka,K. (1994) *Mutat. Res.*, **315**, 229–237.
5 He,Z., Henricksen,L.A., Wold,M.S. and Ingles,C.J. (1995) *Nature*, **374**, 566–569.
6 Matsuda,T. *et al*. (1995) *J. Biol. Chem.*, **270**, 4152–4157.
7 Park,C.-H., Mu,D., Reardon,J.T. and Sancar,A. (1995) *J. Biol. Chem.*, **270**, 4896–4902.
8 Li,L., Elledge,S.J., Peterson,C.A., Bales,E.S. and Legerski,R.J. (1994) *Proc. Natl. Acad. Sci. USA*, 91, 5012–5016.
9 Park,C.-H. and Sancar,A. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 5017–5021.
10 Nagai,A. *et al*. (1995) *Biochem. Biophys. Res. Commun.*, **211**, 960–966.
11 Li,L., Peterson,C.A., Lu,X. and Legerski,R.J. (1995) *Mol. Cell. Biol.*, **15**, 1993–1998.
12 Sung,P., Bailly,V., Weber,C., Thompson,L.H., Prakash,L. and Prakash,S. (1993) *Nature*, **365**, 852–855.
13 Roy,R., Schaeffer,L., Humbert,S., Vermeulen,W., Weeda,G. and Egly,J.-M. (1994) *J. Biol. Chem.*, **269**, 9826–9832.
14 Schaeffer,L., Roy,R., Humbert,S., Moncollin,V., Vermeulen,W., Hoeijmakers,J.H.J., Chambon,P. and Egly,J. (1993) *Science*, **260**, 58–63.
15 Schaeffer,L., Moncollin,V., Roy,R., Staub,A., Mezzina,M., Sarasin,A., Weeda,G., Hoeijmakers,J.H.J. and Egly,J.M. (1994) *EMBO J.*, **13**, 2388–2392.
16 Drapkin,R., Reardon,J.T., Ansari,A., Huang,J.C., Zawel,L., Ahn,K., Sancar,A. and Reinberg,D. (1994) *Nature*, **368**, 769–772.
17 Shivji,M.K.K., Podust,V.N., Hubscher,U. and Wood,R.D. (1995) *Biochemistry*, **34**, 5011–5017.
18 Westerveld,A., Hoeijmakers,J.H.J., van Duin,M., de Wit,J., Odijk,H., Pastink,A., Wood,R.D. and Bootsma,D. (1984) *Nature*, **310**, 425–429.
19 van Vuuren,A.J., Appeldoorn,E., Odijk,H., Yasui,A., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers,J.H.J. (1993) *EMBO J.*, **12**, 3693–3701.
20 Biggerstaff,M., Szymkowski,D.E. and Wood,R.D. (1993) *EMBO J.*, **12**, 3685–3692.

21 Reardon, J.T., Thompson, L.H. and Sancar, A. (1993) *Cold Spring Harbor Symp. Quant. Biol.*, **58**, 605–617.
22 van Vuuren,A.J., Appeldoorn,E., Odijk,H., Humbert,S., Moncollin,V., Eker,A.P.M., Jaspers,N.G.J., Egly,J.-M. and Hoeijmakers,J.H.J. (1995) *Mutat. Res.*, **337**, 25–39.
23 Park,C.-H., Bessho,T., Matsunaga,T. and Sancar,A. (1995) *J. Biol. Chem.*, **270**, 22657–22660.
24 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.
25 Bailly,V., Sommers,C.H., Sung,P., Prakash,L. and Prakash,S. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 8273–8277.
26 Bardwell,L., Cooper,A.J. and Friedberg,E.C. (1992) *Mol. Cell. Biol.*, **12**, 3041–3049.
27 Matsunaga,T., Mu,D., Park,C., Reardon,J.T. and Sancar,A. (1995) *J. Biol. Chem.*, **270**, 20862–20869.
28 Bardwell,A.J., Bardwell,L., Tomkinson,A.E. and Friedberg,E.C. (1994) *Science*, **265**, 2082–2085.
29 Tomkinson,A.E., Bardwell,A.J., Tappe,N., Ramos,W. and Friedberg,E.C. (1994) *Biochemistry*, **33**, 5305–5311.
30 Sung,P., Reynolds,P., Prakash,L. and Prakash,S. (1993) *J. Biol. Chem.*, **268**, 26391–26399.
31 Tomkinson,A.E., Bardwell,A.J., Bardwell,L., Tappe,N.J. and Friedberg,E.C. (1993) *Nature*, **362**, 860–862.
32 Rödel,C., Kirchhoff,S. and Schmidt,H. (1992) *Nucleic Acids Res.*, **20**, 6347–6353.
33 van Duin,M., van den Tol,J., Warmerdam,P., Odijk,H., Meijer,D., Westerveld,A., Bootsma,D. and Hoeijmakers,J.H.J. (1988) *Nucleic Acids Res.*, **16**, 5305–5322.
34 Klein,H. (1988) *Genetics*, **120**, 367–377.
35 Schiestl,R.H. and Prakash,S. (1988) *Mol. Cell. Biol.*, **8**, 3619–3626.
36 Schiestl,R.H. and Prakash,S. (1990) *Mol. Cell. Biol.*, **10**, 2485–2491.
37 Fishman-Lobell,J. and Haber,J.E. (1992) *Science*, **258**, 480–484.
38 Ivanov,E.L. and Haber,J.E. (1995) *Mol. Cell. Biol.*, **15**, 2245–2251.
39 Saffran,W.A., Greenberg,R.B., Thaler,M.S. and Jones,M.M. (1994) *Nucleic Acids Res.*, **22**, 2823–2829.
40 Gutz,H. and Schmidt,H. (1985) *Curr. Genet.*, **9**, 325–331.
41 Busch,D., Greiner,C., Lewis,K., Ford,R., Adair,G. and Thompson,L. (1989) *Mutagenesis*, **4**, 349–354.
42 Koken,M.H.M., Odijk,H., van Duin,M., Fornerod,M. and Bootsma,D. (1993) *Biochem. Biophys. Res. Commun.*, **195**, 643–653.
43 van Duin,M., Koken,M.H.M., van den Tol,J., ten Dijke,P., Odijk,H., Westerveld,A., Bootsma,D. and Hoeijmakers,J.H.J. (1987) *Nucleic Acids Res.*, **15**, 9195–213.
44 Gorman,C., Padmanabhan,R. and Howard,B.H. (1983) *Science*, **221**, 551–553.
45 O'Hare,K., Benoist,C. and Breathnach,R. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1527–1531.
46 Kunkel,T.A., Robetts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
47 Wood,R.D. and Burki,H.J. (1982) *Mutat. Res.*, **95**, 505–514.
48 Graham,F.L. and van der Eb,A. (1973) *Virology*, **52**, 456–467.
49 Troelstra,C., Landsvater,R.M., Wiegant,J., van der Ploeg,M., Viel,G., Buys,C.H.C.M. and Hoeijmakers,J.H.J. (1992) *Genomics*, **12**, 745–749.
50 Troelstra,C., van Gool,A., de Wit,J., Vermeulen,W., Bootsma,D. and Hoeijmakers,J.H.J. (1992) *Cell*, **71**, 939–953.
51 O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
52 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.
53 Siede,W., Friedberg,A.S. and Friedberg,E.C. (1993) *J. Bacteriol.*, **175**, 6345–6347.
54 Busch,D., Greiner,C., Rosenfeld,K.L., Ford,R., de Wit,J., Hoeijmakers,J.H.J. and Thompson,L.H. (1994) *Mutagenesis*, **9**, 301–306.
55 Dabholkar,M., Vionnet,J., Bostick-Bruton,F., Yu,J.J. and Reed,E. (1994) *J. Clin. Invest.*, **94**, 703–708.
56 Dabholkar,M., Bostick-Bruton,F., Weber,C., Bohr,V.A., Egwuagu,C. and Reed,E. (1992) *J. Natl. Cancer Inst.*, **84**, 1513–1517.
57 Geleziunas,R., McQuillan,A., Malapetsa,A., Hutchinson,M., Kopriva,D., Wainberg,M.A., Hiscott,J., Bramson,J. and Panasci,L. (1991) *J. Natl. Cancer Inst.*, **83**, 557–564.
58 Belt,P.B., van Oosterwijk,M.F., Odijk,H., Hoeijmakers,J.H. and Backendorf,C. (1991) *Nucleic Acids Res.*, **19**, 5633–5637.
59 Bramson,J., McQuillan,A. and Panasci,L.C. (1995) *Cancer Lett.*, **90**, 139–148.
60 Lin,J.J. and Sancar,A. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 6824–6828.
61 Lin,J.J. and Sancar,A. (1992) *J. Biol. Chem.*, **267**, 17688–17692.
62 Lin,J.J. and Sancar,A. (1990) *J Biol Chem.*, **265**, 21337–21341.
63 Moolenaar,G.F., Franken,K.L.M.C., Dijkstra,D.M., Thomas-Oates,J.E., Visse,R., van de Putte,P. and Goosen,N. (1995) *J. Biol. Chem.*, **270**, 30508–30515.
64 Felzenszwalb,I., Boiteux,S. and Laval,J. (1992) *Mutat. Res.*, **273**, 263–269.
65 Lawyer,F.C., Stoffel,S., Saiki,R.K., Myambo,K., Drummond,R. and Gelfand,D.H. (1989) *J. Biol. Chem.*, **264**, 6427–6437.
66 Robins,P., Pappin,D.J.C., Wood,R.D. and Lindahl,T. (1994) *J. Biol. Chem.*, **269**, 28535–28538.
67 Gutman,P.D. and Minton,K.W. (1993) *Nucleic Acids Res.*, **21**, 4406–4407.
68 Harrington,J.J. and Lieber,M.R. (1994) *Genes Dev.*, **8**, 1344–1355.
69 Kim,Y., Eom,S.H., Wang,J., Lee,D.-S., Suh,S.W. and Steitz,T.A. (1995) *Nature*, **376**, 612–616.
70 Reynolds,P., Prakash,L., Dumais,D., Perozzi,G. and Prakash,S. (1985) *EMBO J.*, **4**, 3549–3552.
71 Gaffney,T.D. *et al.* (1994) *Mol. Plant Microbe Interact.*, **7**, 455–463.
72 Sancar,G.B., Sancar,A. and Rupp,W.D. (1984) *Nucleic Acids Res.*, **12**, 4593–4608.
73 Chen,N., Zhang,J. and Paulus,H. (1989) *J. Gen. Microbiol.*, **135**, 2931–2940.
74 Levin,P.A., Margolis,P.S., Setlow,P., Losick,R. and Sun,D. (1992) *J. Bacteriol.*, **174**, 6717–6728.
75 Ishino,Y., Shinagawa,H., Makino,K., Tsunasawa,S., Sakiyama,F. and Nakata,A. (1986) *Mol. Gen. Genet.*, **204**, 1–7.
76 Shao,Z. and Newman,E.B. (1993) *Eur. J. Biochem.*, **212**, 777–784.
77 Murray,J.M., Tavassoli,M., Al-Harithy,R., Sheldrick,K.S., Lehmann,A.R., Carr,A.M. and Watts,F.Z. (1994) *Mol. Cell. Biol.*, **14**, 4878–4888.