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Induction of a mutant phenotype in human repair proficient cells after overexpression of a mutated human DNA repair gene

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ABSTRACT

Antisense and mutated cDNA of the human excision repair gene *ERCC-1* were overexpressed in repair proficient HeLa cells by means of an Epstein-Barr-virus derived cDNA expression vector. Whereas antisense RNA did not influence the survival of the transfected cells, a mutated cDNA generating an *ERCC-1* protein with two extra amino acids in a conserved region of its C-terminal part resulted in a significant sensitization of the HeLa transfectants to mitomycin C-induced damage. These results suggest that overexpression of the mutated *ERCC-1* protein interferes with proper functioning of the excision repair pathway in repair proficient cells and is compatible with a model in which the mutated *ERCC-1* protein competes with the wild-type polypeptide for a specific step in the repair process or for occupation of a site in a repair complex. Apparently, this effect is more pronounced for mitomycin C induced crosslink repair than for UV-induced DNA damage.

INTRODUCTION

One of the major DNA repair pathways in living cells is the nucleotide excision repair system. In *E.coli* this DNA repair pathway, mediated by the UvrABC endonuclease system, has a broad substrate specificity ranging from UV-induced photoproducts and bulky chemical adducts to DNA cross-links. (See 1,2 and 3 for recent reviews).

To unravel the excision repair mechanism in human cells, several laboratories have recently cloned human genes involved in this pathway. This was achieved either by DNA-mediated correction of rodent cells with a deficiency in DNA repair resulting in the cloning of the *ERCC-1, -2, -3, -5,* and *-6* genes (4–8) or by correction of the defect in human mutant cell lines from xeroderma pigmentosum (XP) patients which yielded the *XPAC* gene (9). For a recent review see Hoeijmakers and Bootsma (10).

The human DNA excision repair gene *ERCC-1* is the subject of this study. The gene restores the sensitivity to UV and mitomycin C (MMC) of excision deficient CHO mutants of complementation group 1 to almost wild-type levels and in addition fully compensates for the other repair parameters impaired in these mutants (4,11). The gene is unlikely to be involved in any of the known human excision repair disorders XP and Cockayne's syndrome (12). Hence, uptill now no human mutant cell lines, deficient in the *ERCC-1* gene have been identified. As a consequence, a direct involvement of the *ERCC-1* protein in human excision repair has formally not yet been demonstrated. As a first step in the elucidation of the functional role of *ERCC-1* in human cells we have studied the biological effects caused by overproduction of either antisense or mutated *ERCC-1* constructs in repair-proficient HeLa cells.

Recently, we have reported that a mutated *ERCC-1* cDNA obtained by linker insertion failed to correct the defect in CHO mutants of complementation group 1 (13). Construct pcDEMP2 harbouring this mutated cDNA generates an *ERCC-1* protein with two extra aminoacids (Ala-Tyr) distal from aminoacid 208. Transfection of another *ERCC-1* cDNA lacking the 72 bp of exon VIII did also not result in restoration of the repair defect of CHO group 1 mutants, despite the fact that this cDNA was isolated from a cDNA library and S1 mapping experiments suggested that it is generated by alternative splicing and hence might have a function in human cells (14).

For optimal overexpression of the cDNA constructs the Epstein-Barr virus derived cDNA expression vector pECV25 was used (15). This plasmid is stably maintained in human cells as an episome with a copynumber of about 25 to 50. High expression levels of stable mRNA are achieved by an expression cassette consisting of the RSV promoter, a rabbit β globin intron and a rabbit β globin polyadenylation signal.

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MATERIALS AND METHODS

Plasmid constructions

All DNA manipulations and cloning procedures are performed essentially as described previously (16). See figure 1 for more details.

Cell culture

Transfection of HeLa cells with the individual plasmids and hygromycin B selection was performed as described earlier (15).

Northern analysis

Northern analysis was performed as described (15). All double-stranded (ds) probes were ^{32}P-labeled by the random hexamer primer method (17). For the preparation of single-stranded (ss) probes complete *ERCC-1* cDNA from pECVERCC1 was inserted into the polylinker of M13mp18 and M13mp19. Isolated (+) strand DNA from the resulting constructs M13mpERCC1 and M13mpCCRE1 was used as cold ss probes. Hybridization with ss probes was done in two steps. After hybridizing in 0.5M Na_2HPO_4/NaH_2PO_4 pH 7.0, 7% SDS at 65°C for 2h the filter was incubated overnight with cold ss M13mpERCC1 or M13mpCCRE1 DNA at 65°C in the same buffer. The filter was washed for 5 min in 2×SSPE (0.3M NaCl, 20mM NaH_2PO_4, 2mM EDTA pH 7.0) at 65°C and prehybridized again for 2h. Then hybridization was performed overnight in the same buffer with a ^{32}P-labeled double stranded 2.9 kb M13mp19 ClaI fragment. The filter was washed 2×10 min. in 2×SSPE at 65°C and autoradiographed. To determine differences in mRNA levels, autoradiograms were scanned with an LKB Ultrascan XL enhanced laser densiometer.

Western analysis

Western analysis was performed essentially as described earlier (15). Total cell extracts were applied on an 11% SDS-polyacrylamide gel, electrophoresed and transferred to a nitrocellulose membrane using the semi-dry blot procedure (18). The primary antibody used in this assay, was raised in rabbit against a protA-ERCC-1 fusion protein (ERCC-1 aminoacids 157−C-terminus) and purified by affinity chromatography. The specificity of the antibody was established in several ways:

i. The serum specifically precipitates labelled ERCC-1 protein synthesized *in vitro* in rabbit reticulocytes after administration of *in vitro* synthesized ERCC-1 mRNA

ii. The protein visualized by the antibody on western blots (see figure 3) exactly comigrates with the labelled *in vitro* synthesized ERCC-1 protein (apparent molecular weight of 39 kDa allthough the calculated molecular weight is 32.5 kDa).

iii. Finally, CHO cells containing an amplified transfected ERCC-1 gene show increased levels of protein reacting with the ERCC-1 antibody.

UV and MMC survival

Exponentially growing cultures were trypsinized, plated on 60 mm dishes (1000 cells/ dish) and left overnight at 37°C. Cells were subsequently rinsed two times with phosphate-buffered saline (PBS) and either exposed to UV light (0−12 J/m^2) or treated with mitomycin C (0−40 ng/ml for 24h). After cultivation in hygromycin B selective medium for 7 days, clones were fixed and stained with Giemsa. For each dose 3 dishes were counted.

RESULTS AND DISCUSSION

Introduction and expression of *ERCC-1* constructs in HeLa cells

Sense and antisense constructs used in this study are shown in figure 1. The individual plasmids were introduced into HeLa cells and hygromycin B selection was started after two days. All hygromycin B^R clones from one 10 cm dish (varying from 80

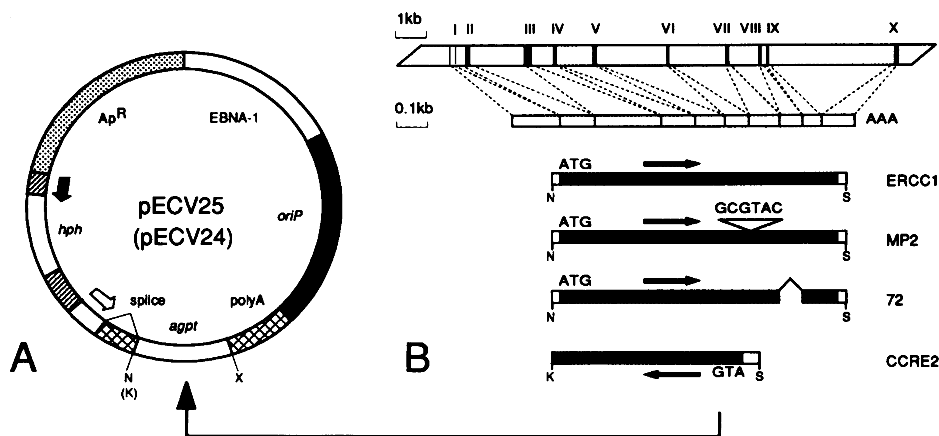

Figure 1. Schematic representation of the construction of the different plasmids harbouring various *ERCC-1* cDNA inserts. **A.** EBV-derived cDNA expression vector pECV25 with a transcription cassette consisting of the RSV promoter (open arrow), rabbit β globin intron, the *agpt* gene and rabbit β globin polyadenylation signal. Plasmid pECV24 is a derivative of pECV25 with a KpnI site instead of a NotI site. **B.** Upper part: Genomic organization of the *ERCC-1* locus. Lower part: The different *ERCC-1* inserts used in this study. cDNA inserts were exchanged with the *agpt* gene of pECV25. Black arrows indicate the orientation of the insert. Plasmid pECVERCC1 harbours the complete coding region of *ERCC-1* including a 5′ UTR of 21 basepairs and a 3′ UTR of 12 basepairs (lacking its own polyadenylation signal). The insert in pECVMP2 is derived from pCDEMP2 (13) and has 6 extra basepairs inserted into the KpnI site of *ERCC-1* cDNA by Tab-linker mutagenesis. pECV72, derived from pCDE72 (Van Duin et al., 1988) harbours *ERCC-1* cDNA lacking the 72 basepairs of exon VIII. There are no other differences between pECVERCC1 and pECVMP2 or pECV72. The antisense construct pECVCCRE2 has a 5′ UTR of 75 basepairs and harbours only 70% of the coding region of *ERCC-1* up to and including the KpnI site which was used for the insertion into pECV24. Abbreviations: *agpt*: aminoglycoside 3′-phosphotransferase gene conferring resistancy to G418; ; Ap^R: *E. coli* β-lactamase gene conferring resistancy to ampicillin; EBNA-1: Epstein Barr virus nuclear antigen 1; *hph*: hygromycin B phosphotransferase gene conferring resistancy to hygromycin B; K: KpnI site; N: NotI site; *oriP*: Epstein-Barr virus origin of replication ; polyA: rabbit β globin polyadenylation site; splice: second intron of the rabbit β globin gene; X: XhoI site.

to 200 clones) were pooled and grown to mass cultures. The pooled transformants were used in all subsequent experiments. Cells were maintained under continous selection pressure for hygromycin B to avoid loss of the episomal plasmids.

Northern analysis (Fig. 2.) revealed 30−60 times higher mRNA levels from all different constructs relative to the level of the endogenous *ERCC-1* mRNA.

A Western analysis using a monospecific polyclonal antiserum against the *ERCC-1* protein (see materials and methods for the specificity of this antibody) is shown in figure 3. In pECV25 transfected HeLa control cells (lane 4) the antibody detects a major band with an apparent molecular weight of 39 kDa which has been shown to correspond to the endogenous ERCC-1 gene product (Materials and Methods). The intensity of this band is increased in Hela cells transfected with the *ERCC-1* construct (lane 1), consistent with an significant overproduction of the ERCC-1 protein in these cells. Similarly , a clear overproduction of the ERCCMP2 mutant protein is observed in cells transfected with pECVMP2 (lane 2). In cells transfected with pECV72 only a faint band corresponding to the mutant protein is observed despite high expression at the mRNA level (figure 2). At present it is not clear whether this result is due to decreased stability of the mutant ERCC72 protein in HeLa cells or to a lower reactivity of the antibody with the mutant protein (ERCC72 lacks 24 aminoacids from exon VIII). Note that antibodies have been raised against aminoacids 157−297 (encompassing exon VIII) of ERCC-1 protein.

Biological consequences of overexpressing *ERCC-1* constructs in HeLa cells

To investigate the biological consequences of the introduction of the various *ERCC-1* constructs into repair proficient cells, survival studies were performed. Since CHO cells of complementation group 1 are corrected by *ERCC-1* for their UV and MMC sensitivity, the colony forming ability of the pooled HeLa transformants was determined after graded exposures to UV or MMC. Transfection of the pECV25 vector alone did not have any significant effect on the UV or MMC resistance of HeLa cells (results in figure 4 only shown for the pECV25 transfected cell population).

1) *Antisense constructs.* Introduction into the HeLa cells of the pECVCCRE2 antisense construct had no measurable effect on the sensitivity to UV or MMC (figure 4 panels A and D). This observation is in agreement with results from both Northern and Western analysis. Degradation of the endogenous transcript as found in a number of other antisense studies (19, 20) was not apparent, using a single stranded antisense *ERCC-1* cDNA as a probe (see Fig. 2B, lanes 4−6). In addition, there is no detectable decrease in the amount of endogenous *ERCC-1* protein (see Fig. 3, lanes 4 and 5). It appears that the antisense approach, as followed under the present conditions and with this particular construct, is not suitable for investigating the involvement of *ERCC-1* in the human excision repair process.

2) *Overproduction of naturally occuring ERCC-1* transcripts. Besides the full-length *ERCC-1* mRNA, repair proficient human cells produce as well transcripts lacking exon VIII (14). However, this naturally occuring mutant form of the ERCC-1 protein does not correct the repair defect in CHO 43-3B cells (13). Therefore it was of interest to investigate whether ERCC72 contributes to the repair process in human cells. We have found in all experiments (three independent survival experiments) a slight but reproducible increase in survival after MMC treatment of transfectants harbouring the pECV72 construct (Fig. 4, panel B) relative to the pECV25 control and the *ERCC-1* transfectants. For UV also a slight increase in resistance is observed with the pECV72 construct when compared to the pECV25 control vector, which is however less apparent when compared to the ERCC-1 transfected cells (figure 4, panel E). Taken together these findings may indicate a possible protective effect at least for MMC of the ERCC-1 protein lacking exon VIII (ERCC72). However, the observed differences are small and it is therefore difficult to draw any definite conclusions.

3) *Overexpression of ERCCMP2.* In contrast to the marginal effects observed with pECVERCC1 and pECV72 transfectants, Hela cells transfected with pECVMP2 generating a protein with 2 extra amino acids (*ERCCMP2*) show a clear decrease in survival after MMC treatment (D_{10} = 25.5 ng/ml and D_{37} = 15 ng/ml) as compared to pECV25 transfected control cells (47 and 26.5 ng/ml for D_{10} and D_{37} respectively) (Fig. 4, panel C). This effect is not seen, at least to a significant extent after UV treatment (D_{10} = 10 J/m^2 for ERCCMP2 and 11.2 J/m^2 for control cells

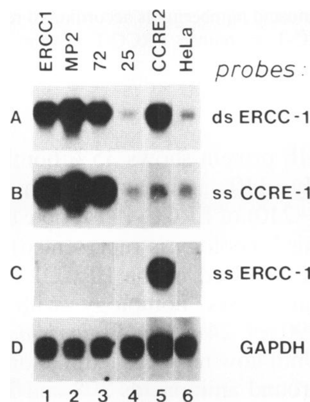

Figure 2. mRNA expression from the different cDNA constructs in HeLa cells. Lanes: Total RNA from HeLa cells transfected with: 1. pECVERCC1, 2. pECVMP2, 3. pECV72, 4. pECV25, 5. pECVCCRE2, 6. control HeLa cells. Panels: Probes used: A. ds ERCC-1 cDNA, B. ss M13mpCCRE1 detecting sense *ERCC-1* transcripts, C. ss M13mpERCC1 detecting antisense *ERCC-1* transcripts, D. ds GAPDH probe (22).

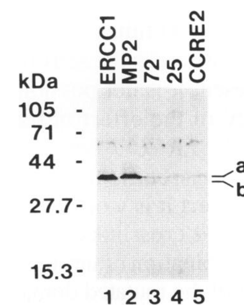

Figure 3. *ERCC-1* protein expression in HeLa transfectants. Molecular weight markers are indicated on the left. Arrows points to specific *ERCC-1* protein bands: a: full length *ERCC-1* protein, b: *ERCC-1* protein lacking 24 amino acids encoded by exon VIII due to alternative splicing (see fig. 1B). HeLa cells have been transfected with: pECVERCC1 (lane 1), pECVMP2 (lane 2), pECV72 (lane 3), pECV25 (lane 4) and pECVCCRE2 (lane 5).

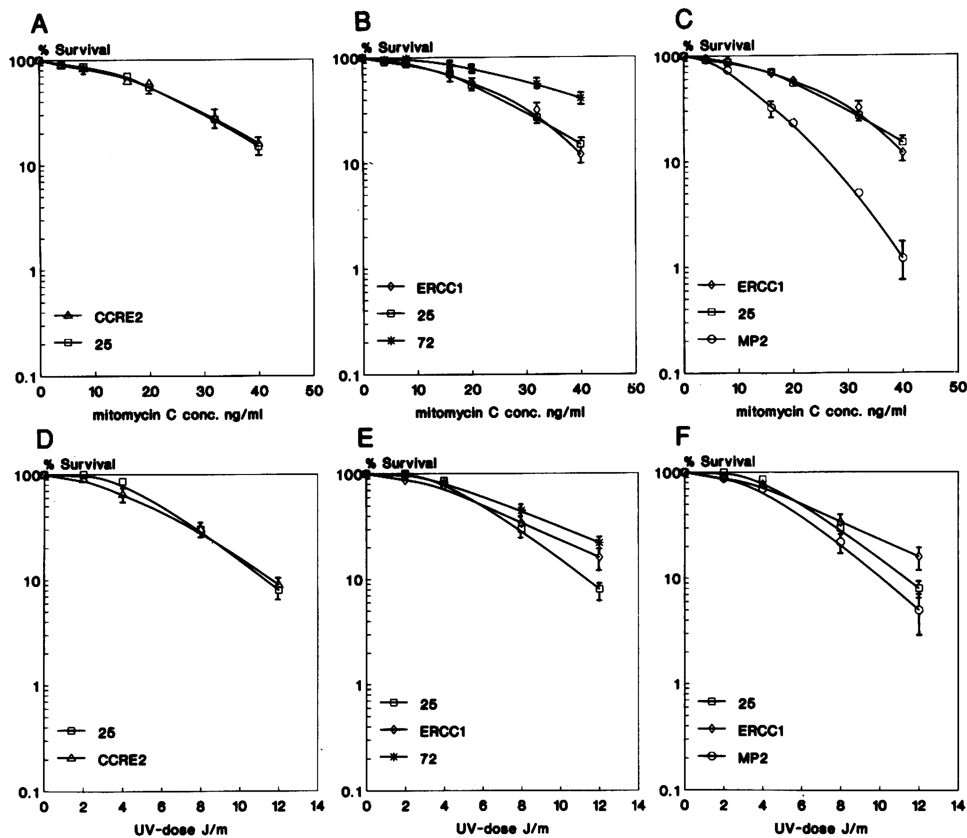

Figure 4. Survival of HeLa transfectants after UV or MMC treatment. For each dose 3 dishes were counted. Results are from at least 3 separate experiments. Symbols are indicated in the legend of each figure. ERCC-1: cells transfected with pECVERCC1; 72: pECV72; MP2: pECVMP2; 25: pECV25 (control); CCRE2: pECVCCRE2.

transfected with pECV25) (panel F). Since overproduction of intact *ERCC-1* does not have any significant influence on survival after MMC treatment, this effect is likely to be due to the mutated geneproduct and not a result of the overproduction itself. Apparently the mutated *ERCC-1* protein interferes with the normal repair process in human cells, possibly by competing with the wild-type polypeptide for a specific step in the repair process or for occupation of a site in a repair complex. It is interesting in this respect to note that overproduction of *ERCCMP2* does only detectably sensitize HeLa cells to MMC treatment, as compared to UV irradiation. This phenomenon is in agreement with the phenotype of CHO cells of complementation group 1. These mutant cells are 100 times more sensitive to MMC than wild type CHO cells, whereas the sensitivity to UV is only 7–8 times higher. At present it is not possible to decide whether the substrate dependency of the effect observed here is due to the specific function of the *ERCC-1* domain mutated in *ERCCMP2* or whether it reflects the specific function of the *ERCC-1* protein as a whole. In this respect it is worth noting that repair of MMC damage (i.e. interstrand crosslinks) is more complex (possible involvement of recombination) than the repair of UV damage. Specific involvement of the mutated domain in the former process would be in agreement with higher sensitization to MMC as compared to UV damage.

The MP2 mutation (insertion of two aminoacids between Tyr208 and Leu209) is localized in a region of the protein which is highly conserved in human and mouse (ref. 13 and fig. 5). This region is aswell conserved in the yeast *rad10* gene: the C-terminal

Figure 5. Homology between h-ERCC-1, m-ERCC-1, RAD10 and UVRA proteins in the region of the MP2 mutation. Identical aminoacids are boxed with solid lines, physico-chemically related residues are indicated by open boxes. The triangle indicates the position of the insertion of the two extra aminoacids in pECVMP2. The aminoacid numbering is according to references 13, 23 and 24 respectively (h-ERCC-1 = human ERCC-1 protein; m-ERCC-1 = mouse ERCC-1 protein).

half of the RAD10 protein shows 35% homlogy over a region of approximately 110 aminoacids with the middel part (aminoacids 103–210) of ERCC-1 (14, note that in figure 5 only a small part of this homology is represented). Additionally, the MP2 mutation is localized in the ERCC1 region (aminoacids 203–245) which shows homology with a small section (aminoacids 49–90 ref. 24) of the *Escherichia coli* UvrA protein (13) (figure 5). Until now no specific function has been ascribed to the segment around aminoacids 208 and 209 in the ERCC1 protein. However, the conservation of this area in both the yeast *rad10* gene and the *Escherichia coli uvrA* gene suggests that it may play an important function in the repair process. It would be interesting to construct the same 'MP2' mutation in both *rad10* and *uvrA* or to exchange the homologous segments between the different proteins. Such experiments might help to elucidate the

specific function of this particular domain, especially in view of the substrate specificity discussed above.

The results presented here are to our knowledge the first example of induction of a mutant phenotype in repair proficient mammalian cells by overproducing a mutated repair gene. Recently, similar experiments were performed in *Escherichia coli* by overproducing mutated UmuD protein from a multicopy plasmid in wildtype cells, which resulted in the generation of a dominant phenotype with respect to UV mutagenesis due to heterodimer formation between mutated and wildtype UmuD proteins (21).

We propose that the approach presented here might constitute a valuable method to investigate the function of (specific domains in) cloned genes, in cases where no mutant cell lines are available, especially in mammalian cells and perhaps also in transgenic animals where site-directed mutagenesis of endogenous genes is a difficult task.

The present analysis emphasizes the actual involvement of the *ERCC-1* gene in the DNA repair process of human cells and indicates that the contribution of *ERCC-1* in this process might be more important for the repair of MMC induced−(i.e. crosslinks) than UV induced DNA damage.

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