

## Molecular cloning of the human nucleotide-excision-repair gene *ERCC4*

(DNA repair/cell line UV41/excinuclease/genetic complementation/UV resistance)

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**ABSTRACT** *ERCC4* was previously identified in somatic cell hybrids as a human gene that corrects the nucleotide-excision-repair deficiency in mutant hamster cells. The cloning strategy for *ERCC4* involved transfection of the repair-deficient hamster cell line UV41 with a human sCos-1 cosmid library derived from chromosome 16. Enhanced UV resistance was seen with one cosmid-library transformant and two secondary transformants of UV41. Cosmid clones carrying a functional *ERCC4* gene were isolated from a library of a secondary transformant by selecting in *Escherichia coli* for expression of a linked neomycin-resistance gene that was present in the sCos-1 vector. The cosmids mapped to 16p13.13–p13.2, the location assigned to *ERCC4* by using somatic cell hybrids. Upon transfection into UV41, six cosmid clones gave partial correction ranging from 30% to 64%, although all appeared to contain the complete gene. The capacity for *in vitro* excision of thymine dimers from a plasmid by transformant cell extracts correlated qualitatively with enhanced UV resistance.

Nucleotide-excision repair is a major pathway that removes UV-radiation photoproducts, bulky monoadducts, cross-links, and oxidative damage from DNA by incising the damaged strand on both sides of the lesion (1–3). Seven human genes in this pathway have been identified and cloned by using mutants developed in rodent cells or derived from humans having the disorder xeroderma pigmentosum (XP) (4). Studies of hamster and mouse mutant lines first identified five genetic complementation groups (5, 6) having extreme UV-radiation sensitivity and excision-repair deficiency. From these groups of mutants, the complementing human genes *ERCC1* (7), *XPB/ERCC2* (8), *XPB/ERCC3* (9), and *XPG/ERCC5* (10) were cloned and shown to substantially overlap (9, 11–13) with the seven excision-deficient groups of XP (14). The mutants in rodent complementation groups 6–11 have lesser degrees of UV sensitivity than groups 1–5 (15–17), and of the former set, only the gene correcting group 6, *CSB/ERCC6*, has been isolated (18). By complementing XP cells, the *XPA* (19) and *XPC* (20) genes were isolated—genes so far not represented among the rodent groups.

Nucleotide-excision-repair complementation group 4 is represented by mutants UV41 (5) and UV47 (21), both of which are highly UV-sensitive ( $\approx 6$ -fold) and extremely sensitive to mitomycin C ( $\approx 100$ -fold) and other DNA cross-linking agents (21). These mutants and those in group 1, such as UV20 (5), are so far unique in their extreme sensitivities to both UV-radiation and cross-linking agents. These properties suggest that the complementing *ERCC1* and *ERCC4* genes are involved in distinct but overlapping pathways of monoadduct and cross-link removal. Previous transfection

studies using human genomic DNA resulted in corrected UV41 transformants without cloning of the *ERCC4* gene (22). Since *ERCC4* was assigned to chromosome 16 (23) and a cosmid library of this chromosome was available, we chose a cloning strategy in which UV41 was first corrected by transfection with that library. We report here the isolation of functional cosmid clones that map to the previously assigned chromosomal region of *ERCC4* (16p13.13–p13.2) and partially restore UV-resistance.

### MATERIALS AND METHODS

**Cells and Culture Conditions.** Mutant UV41 (5) and its transformants were grown in either monolayer or suspension culture in minimum essential medium,  $\alpha$  modification, supplemented with 10% (vol/vol) fetal bovine serum and antibiotics as reported (8). Colony-forming ability of transformants was determined after exposure to UV-radiation as reported (5). Triplicate 10-cm dishes were used for each dose point.

**Cosmid and Genomic DNA Transfections.** A chromosome 16 cosmid library in the vector sCos-1 (24) was kindly provided by Larry Deaven (Los Alamos National Laboratory). Cosmid DNA was introduced into UV41 cells by electroporation of three aliquots of  $5 \times 10^6$  cells; each aliquot was mixed with 5  $\mu$ g of DNA in a 1-ml cuvette of a GIBCO/BRL Cell Porator (300 V, 1600  $\mu$ F). Cells were plated at  $5 \times 10^5$  cells per 10-cm dish and allowed 48 h for expression. Mitomycin C to 10 nM and Geneticin to 1.7 mg/ml were added and incubation was continued for 10 days. One primary transformant (H41T1) was recovered, expanded in medium containing Geneticin, and shown to be cross-resistant to UV-radiation at 5 J/m<sup>2</sup>.

For the secondary transfection, high molecular weight ( $\approx 200$  kb) DNA from  $1.1 \times 10^9$  cells of the primary transformant grown in suspension culture was prepared (25). DNA in solution was concentrated  $\approx 5$ -fold by repeated extractions with 1-butanol, followed by dialysis against 0.01 M Tris-HCl/0.001 M Na<sub>2</sub>EDTA, pH 8.0. Calcium phosphate precipitates were prepared in 2 ml (26), and 20  $\mu$ g of DNA in 1 ml was added per dish to 28 10-cm dishes, each containing  $2 \times 10^6$  cells and 10 ml of medium. After 22 h, the dishes were rinsed and refed with 20 ml of fresh medium. After an additional 29-h expression, dishes were UV-irradiated at 3.5 J/m<sup>2</sup> and refed with 20 ml of medium containing Geneticin.

To test for correction of UV41 by cosmid clones, DNA samples were introduced into UV41 as calcium phosphate

Abbreviations: XP, xeroderma pigmentosum; neo, neomycin-resistance.

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precipitates (5  $\mu$ g of DNA per dish) or by electroporation (0.5–5  $\mu$ g per cuvette). After a 24-h expression, cells were exposed to UV-radiation at 3.5 J/m<sup>2</sup> and incubated  $\approx$ 8 days in medium containing Geneticin. Colonies were isolated using Pipetman tips and checked for stable UV-resistance.

**Cosmid Library Construction and Cloning.** High molecular weight DNA from  $2 \times 10^8$  cells of secondary transformant H41T2-1 was prepared as described for the primary transformant. Genomic DNA was sheared by aspirating twice in a Pipetman 1000 tip to produce a homogeneous solution and was partially digested with restriction endonuclease *Sau3A*I to convert  $\approx$ 30% of the DNA to 30–50 kb. Genomic DNA was ligated to vector p14B1 (8) at the unique *Bam*HI site using a vector/insert ratio of 32:1 at 0.22  $\mu$ g/ $\mu$ l. Ligation products were packaged with Gigapack II Gold extracts (Stratagene), and the resulting cosmids were transfected into *Escherichia coli* DH5 $\alpha$ MCR (GIBCO/BRL). After infection, cells were concentrated and plated onto agar dishes with Luria-Bertani medium containing kanamycin at 50  $\mu$ g/ml. Cosmid DNAs from kanamycin-resistant clones were purified using QIAwell-8 and plasmid purification kits (Qiagen, Chatsworth, CA).

**Southern Blot of *ERCC4* Cosmids.** Cosmid DNAs (see Fig. 6) were digested with *Eco*RI and size-fractionated on 1.0% agarose at 75 ng of DNA per lane. Two cDNA clones, cER4-4 and cER4-5 (L.H.T. and K.W.B., unpublished results), were digested with *Xba*I, which separates insert from vector, and were present in the gel at 25 ng per lane. A 19-nt oligomer, derived from sequence immediately upstream of the polyadenylation signal of cDNA clone cER4-4 and not found in cER4-5, was used as probe. DNA was transferred to nylon membranes (GeneScreenPlus; DuPont/NEN) and hybridized with 80 ng of <sup>32</sup>P-end-labeled probe (specific activity,  $1.4 \times 10^9$  cpm/ $\mu$ g) in 1 M NaCl/10% (wt/vol) dextran sulfate/1.0% SDS/sonicated herring sperm DNA (0.1 mg/ml) for 3 h at 42°C. The membrane was then rinsed with  $2 \times$  SSC at room temperature for 15 min and  $6 \times$  SSC at 42°C for 2 min ( $1 \times$  SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0).

**In Vitro Assay of Excision Repair.** Cell extracts were prepared as described (27), dialyzed overnight against storage buffer [25 mM Hepes, pH 7.9/100 mM KCl/12 mM MgCl<sub>2</sub>/0.5 mM EDTA/2 mM dithiothreitol/17% (wt/vol) glycerol], and stored in small aliquots at  $-80^\circ\text{C}$ . The plasmid DNA substrate (pUNC1991-4) is a semisynthetic covalently closed plasmid containing four thymine dimers at unique sites. It was prepared with <sup>32</sup>P label at the 11th phosphodiester bond 5' to each thymine dimer as described (28). We used  $> 2 \times 10^5$  cpm per reaction mixture ( $\approx$ 0.6  $\mu$ g). The plasmid and a total of 150  $\mu$ g of extract were incubated for 2 h at 30°C in 60- $\mu$ l reaction mixtures, and the reaction products were analyzed as described (28).

In two of the experiments, recovered cpm were determined by Cerenkov counting and equal cpm were loaded on the gel. Quantitation was by scanning the region of the gel containing the excision products by using a Zeineh soft laser scanning densitometer. For the third experiment, loaded cpm were normalized after scanning of the dried gel with a Fuji BAS1000 Bio-Imaging Analyzer.

## RESULTS

**Production of Primary and Secondary Transformants of UV41 Cells.** The procedure used to clone *ERCC4* is outlined in Fig. 1. Transfection of  $\approx 10^7$  UV41 cells by electroporation with the sCos-1 library of human chromosome 16 resulted in one primary transformant that was resistant to both Geneticin and 10 nM mitomycin C, a concentration that is nontoxic to wild-type AA8 cells. This clone (H41T1) was shown to be cross-resistant to UV-radiation, implying that it was a genuine *ERCC4* transformant and not merely altered in mitomy-

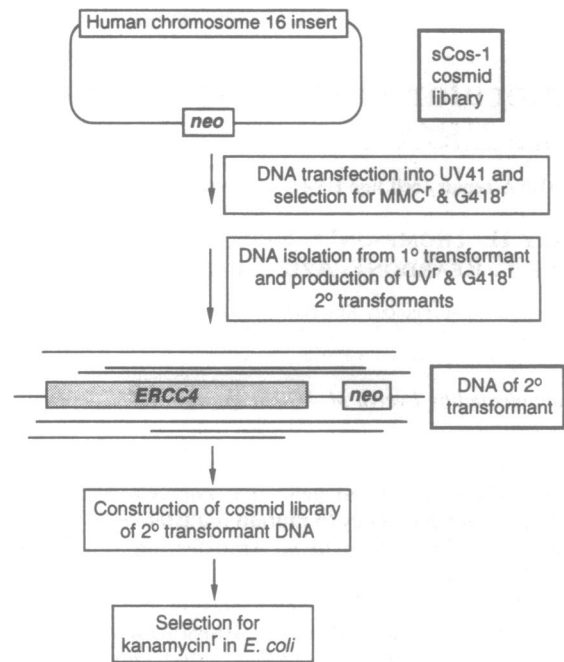


Fig. 1. Strategy for cloning *ERCC4* by using a chromosome 16 cosmid library.

cin C metabolism (29). DNA isolated from H41T1 cells was used in a secondary transfection that employed calcium phosphate precipitates to maximize the amount of DNA transferred. Two transformants resistant to both Geneticin and UV-radiation were obtained from  $5.6 \times 10^7$  cells. All three transformants were analyzed on a Southern blot by using as the probe an *Apa*LI fragment of sCos-1 that spanned a 1.25-kb region starting just upstream of the origin of replication and extending into the ampicillin-resistance gene (results not shown). The findings suggested that (i) the *ERCC4* circular sCos-1 cosmid had broken near the origin of replication when integrating into the primary transformant and (ii) the primary transformant contained two copies of sCos-1 while the secondary transformants contained one copy.

**Cosmid Cloning of *ERCC4* by Linkage to the Neomycin-Resistance (neo) Gene.** DNA from secondary transformant clone H41T2-1 was used to construct a cosmid library with the p14B1 vector, which contains no neo gene (8). Cosmids were transfected into *E. coli*, and a selection for kanamycin resistance (conferred by the neo gene of sCos-1 present in the insert) was performed to rescue *ERCC4* sequences (see Fig. 1). Thirty cosmid clones were recovered from  $2.4 \times 10^6$  transfected bacteria, and six clones were capable of conferring UV resistance when introduced into UV41 cells by electroporation. The six functional cosmids (pER4-2, -3, -4, -5, -6, and -7) contained six common *Eco*RI restriction fragments ranging from 0.7 to 7.5 kb, and three of these fragments were common to six nonfunctional cosmids. For each functional cosmid, one or more UV41 transformant clones that survived UV-irradiation 24 h after transfection were isolated for further analysis.

**Chromosomal Mapping of Functional Cosmid Clones.** To test whether the recovered cosmids contained the *ERCC4* gene as defined by localization to chromosome 16p13.13–p13.2 in cell hybrids (23), several functional cosmid clones were mapped to human metaphase chromosomes by fluorescence *in situ* hybridization. These cosmids specifically hybridized to the predicted region, as shown for cosmid pER4-2 in Fig. 2. Because of the linkage of this chromosome 16 insert

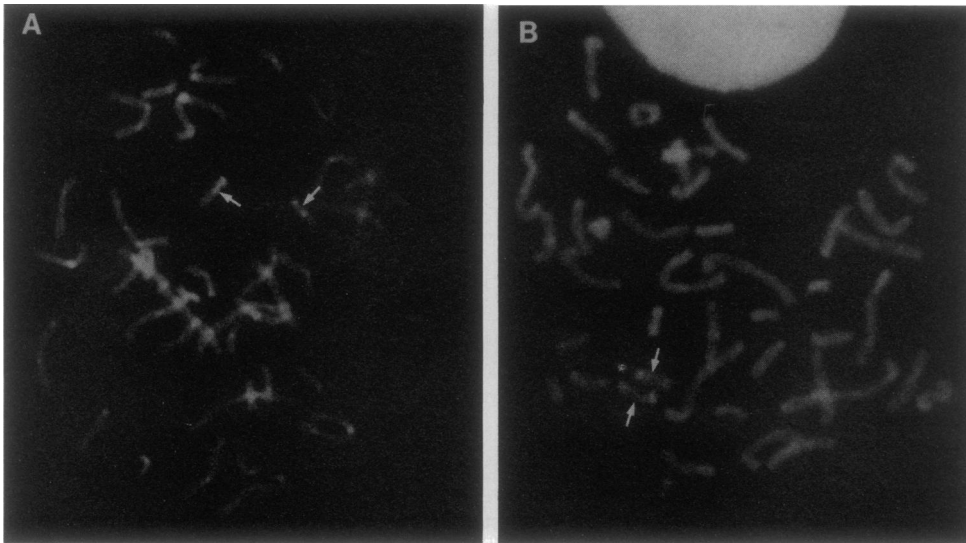


FIG. 2. Localization of *ERCC4* cosmid clones on two normal human metaphases (A and B) by fluorescence *in situ* hybridization. Cosmid clone pER4-2 was biotinylated and hybridized to human metaphase preparations as described (23). The arrows mark the position of the centromere of chromosome 16. Hybridization is telomeric of the midpoint of the p arm (it appears to be at the end of the p arm of the chromosome on the left in A because the end of the p arm is folded back).

sequence to the neo gene, we must have cloned a human sequence rather than a reverted hamster *ERCC4* gene.

**Restoration of UV Resistance in Transformants.** To evaluate the level of correction obtained in the transformants, UV-radiation survival curves were obtained as shown in Fig. 3. All transformants except one (clone HER4.5.1) conferred UV-resistance. This clone probably survived the initial screening because of transient correction by the cosmid DNA. For all other transformants, two aspects of the correction are immediately apparent. (i) Restoration of UV

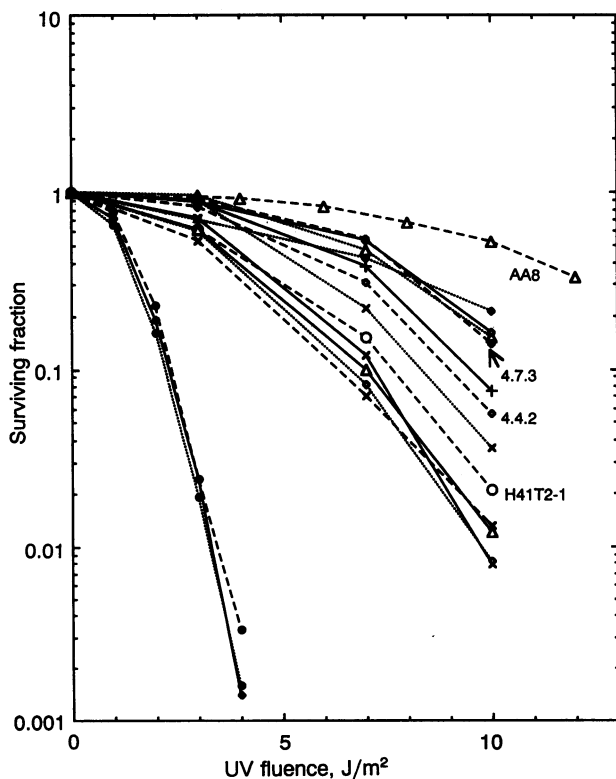


FIG. 3. UV-radiation survival curves of cosmid transformants of UV41. ●/dashed and dotted lines, UV41, two experiments; △/dashed line, wild-type AA8; ◇/dotted line, primary transformant H41T1; ○, secondary transformant H41T2-1; △/solid line, HER4.2.3; △/dotted line, HER4.3.3; ◇/dashed line, HER4.4.2; ◇/solid line, HER4.4.3; ◆/solid line, HER4.5.1; ×/solid line, HER4.5.2; ×/dashed line, HER4.5.3; ×/dotted line, HER4.5.4; +, HER4.6.3; ◆/dotted line, HER4.7.1; ◆/dashed line, HER4.7.3.

resistance was incomplete and ranged from 30% to 64% based on  $D_{37}$  (the fluence that reduces survival to 37%). Many of the transformants derived with cosmid clones had significantly higher survival than either the primary or secondary transformant. Thus, if the complete gene is present in the transformants, then it appears that human *ERCC4* does not fully correct the hamster-cell mutation. (ii) For the transformants derived from the six cosmid clones, there was variability in the level of correction, both among the transformants for a given cosmid (e.g., transformants of cosmid pER4-5) and among those of different cosmids. Two sources of such variability could be the number of copies of *ERCC4* and the chromosomal site(s) of integration.

**Restoration of Excinuclease Activity in Transformants.** The enhanced UV-survival of the transformants implied partial restoration of repair. Increased UV-survival could be caused by transfection with genes involved in cell cycle regulation (30). We wished to confirm the restoration of excision activity by using an assay specific for excision. Thus, we measured the repair capacity of several transformants with the recently developed excinuclease assay in which excised thymine dimers are detected in radiolabeled oligonucleotides that are 27–29 nt long (28). Fig. 4 shows an autoradiogram comparing transformants and parental cell lines. Human HeLa cells had more activity than CHO AA8 cells, whereas the UV41 mutant had no detectable activity. Intermediate levels of repair were seen with the secondary transformant H41T2-1 and the cosmid transformants HER4.4.2 and HER4.7.3. Very efficient complementation was seen in mixed extracts of UV41 and the group 5 mutant UV135, indicating that UV41 extract can be fully complemented. The repair levels of the cell lines shown in Fig. 4 are summarized in quantitative form in Fig. 5. The relative levels of repair of wild-type AA8 and the three transformants were in the same order as the levels of cellular resistance measured by survival curves (e.g.,  $D_{37}$  values). The  $D_{37}$  values taken from Fig. 3 are as follows: AA8, 11.6 J/m<sup>2</sup>; HER4.7.3, 7.9 J/m<sup>2</sup>; HER4.4.2, 6.3 J/m<sup>2</sup>, and H41T2-1, 4.5 J/m<sup>2</sup>. Thus there is a qualitative correlation between repair measured *in vitro* and cell survival after UV-irradiation.

**Presence of 3'-End Sequence in Functional *ERCC4* Cosmids.** Incomplete correction might be due to having an incomplete gene in the original sCos-1 library; either end could be truncated. However, if the 5' end were deleted, one would expect extremely low transfection efficiencies with the cosmid clones (see *Discussion*). Since truncation of the 3' end of *ERCC4* was a more likely possibility, we tested the cosmids by using an oligonucleotide probe for a noncoding

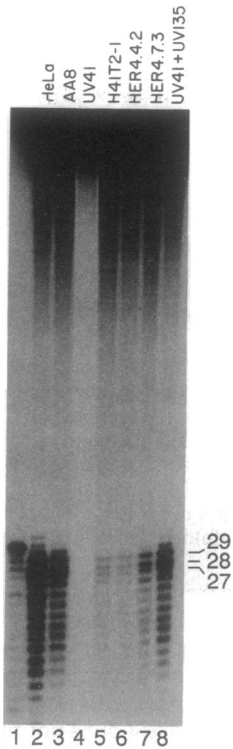


FIG. 4. Autoradiogram of excision products in the excinuclease assay. Lanes: 1, size markers; 2-4, control cell lines; 5, secondary transformant; 6 and 7, cosmid transformants of differing UV sensitivity; 8, UV41 extract combined with extract from UV135 showing efficient correction of the repair deficiency. The positions of the main excision products (27-29 nt) are indicated.

region just upstream of the polyadenylation site in one cDNA clone. As shown in Fig. 6 (lanes 8-13), all six cosmids that restored UV-resistance were clearly positive for a 4.8-kb *EcoRI* fragment. At the same time, six cosmids that did not confer UV resistance were all negative (lanes 2-7). The probe also detected a cDNA clone containing its complementary sequence (lane 1, arrow) but gave a weak signal with a cDNA clone that lacked this sequence (lane 14). The faint bands seen for some nonfunctional cosmids (lanes 2, 4, 5, and 7) appear to be due to nonspecific hybridization. These results argue strongly that the 3' end of *ERCC4* is present in the functional cosmids and support our interpretation that the incomplete correction we observe (Figs. 3-5) is most likely not due to an incomplete gene.

## DISCUSSION

We have presented the cloning of the human *ERCC4* gene, which was earlier identified by genetic analysis as one of a

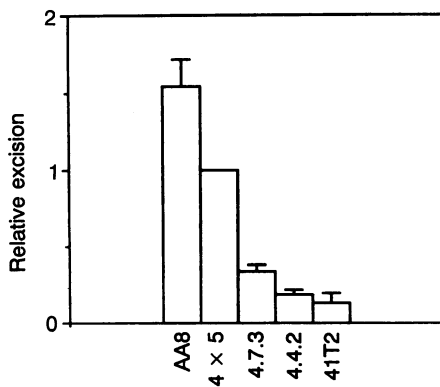


FIG. 5. Quantitative level of correction in *ERCC4* transformants. Excision is expressed relative to UV41 + UV135 complementation (bar 4 × 5), which was present in all three experiments. For the data set,  $n = 3$  for H41T2-1, HER4.4.2, HER4.7.3, and UV41 + UV135 and  $n = 2$  for AA8 and UV41. The value for UV41 was at background (0.018) and was subtracted from the other values. Error bars are the SEM.

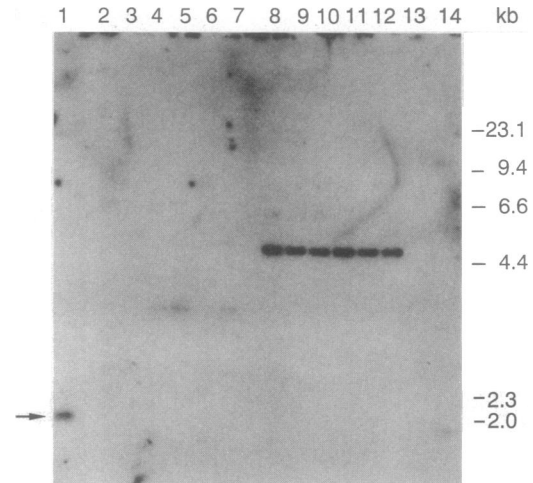


FIG. 6. Test of cosmid clones for the presence of the 3' end of *ERCC4*. An autoradiogram of DNAs digested with *EcoRI* (cosmids) or *Xba I* (cDNAs) to release inserts of vector pEBS7 and hybridized with a  $^{32}\text{P}$ -labeled oligonucleotide probe for the 3' end of *ERCC4* is shown. Lanes: 1, positive control (cDNA clone cER4-4); 2-7, six nonfunctional cosmids (pER4-11, -12, -14, -16, -17, and -18, respectively); 7-13, six functional cosmids (pER4-2, -3, -4, -5, -6, and -7, respectively); 14, negative control (cDNA clone cER4-5).

series of genes that are required for the functioning of nucleotide-excision repair in mammalian cells (5, 6). In a previous study, transfection of CHO UV41 cells with human DNA resulted in correction of cells to repair proficiency. Cloning of the gene was hampered by the apparent absence of repetitive sequences (22), which had been used to clone other genes such as *ERCC2* (8). We used the sCos-1 chromosome 16-specific library for our initial transfection of UV41 because the *ERCC4* gene had been assigned to this chromosome using CHO-human somatic cell hybrids (23). The sequences that we cloned map to the expected region of chromosome 16, confirming the previous chromosomal assignment.

From a technical standpoint, the ability of the neo gene of sCos-1 to confer kanamycin resistance in *E. coli* eliminated the need to perform hybridization screening of the cosmid library prepared from the secondary transformant. The main limitation of this approach for cloning other genes by transfection/complementation is set by the size of the gene. Genes up to  $\approx 30$  kb can be accommodated by cosmid vectors with adequate efficiency. A survey of the sizes of known human repair genes (4, 31-35) suggests that  $\approx 50\%$  of the genes remaining to be cloned might be suitable for this approach as cosmid libraries for most human chromosomes are available in the National Gene Library Project.

The partial correction we observed with six cosmid clones was as high as 64% based on survival of UV41 transformants. The CHO mutant UV140 (36), which has intermediate UV sensitivity, was corrected to a similar maximum extent (13-63%) by cosmid clone pER4-6 (L.H.T. and K.W.B., unpublished results). Transfection efficiencies for UV resistance would be expected to be much lower than we observed ( $1 \times 10^{-4}$  for UV140 transfection) if the 5' end of the gene were missing, requiring integration near a promoter. Sequence corresponding to the 3' end of the gene was present in all six functional cosmids (Fig. 6). The incomplete correction we observed might result from inefficient gene expression, and the possibility remains that the cloned *ERCC4* sequences are incomplete in the upstream region or rearranged. Partial correction might also occur even if normal *ERCC4* protein levels are present in transformants. Several studies suggest that *ERCC4* may not complement a CHO-cell

deficiency as efficiently as some other nucleotide-excision repair genes such as *ERCC2*, which gave full correction of UV5 cells (8), and *XRCC1*, which gave full correction of EM9 cells (31). Studies using cell extracts to test for complementation suggest that both the *ERCC1* and *ERCC4* proteins, as well as the XP-F and *ERCC11* proteins (if indeed these proteins are distinct from *ERCC4*), are present in a complex (37–39). This complex may impose constraints on the efficiency of complementation by a heterologous protein. The human *ERCC4* protein may not integrate well into the hamster complex, particularly when an altered hamster protein is also present. The partial correction ( $\approx 60\%$ ) also seen with the intact *ERCC1* gene present in a group 1 transformant is consistent with this idea (40).

The variability among the UV-survival curves of the genomic and cosmid transformants (Fig. 3) suggests differences in the repair capacity of the cells. Three of the cell lines were examined in the recently introduced excinuclease assay, which directly measures the removal of the major UV photoproduct, thymine dimers, in the excised oligonucleotide. These experiments confirmed that restoration of repair capacity had occurred in the transformants and that there was a correlation between the levels of *in vitro* repair and relative UV resistance.

Using the pER4-5 cosmid as probe, we have isolated cDNA sequences that map to chromosome 16 (L.H.T., K.W.B., and M.J.S., unpublished results). Overexpressed *ERCC4* protein will now be needed to reconstitute the entire nucleotide-excision-repair process *in vitro*.

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