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ERCC4 (*XPF*) Encodes a Human Nucleotide Excision Repair Protein with Eukaryotic Recombination Homologs

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***ERCC4* is an essential human gene in the nucleotide excision repair (NER) pathway, which is responsible for removing UV-C photoproducts and bulky adducts from DNA. Among the NER genes, *ERCC4* and *ERCC1* are also uniquely involved in removing DNA interstrand cross-linking damage. The ERCC1-ERCC4 heterodimer, like the homologous Rad10-Rad1 complex, was recently found to possess an endonucleolytic activity that incises on the 5′ side of damage. The *ERCC4* gene, assigned to chromosome 16p13.1-p13.2, was previously isolated by using a chromosome 16 cosmid library. It corrects the defect in Chinese hamster ovary (CHO) mutants of NER complementation group 4 and is implicated in complementation group F of the human disorder xeroderma pigmentosum. We describe the *ERCC4* gene structure and functional cDNA sequence encoding a 916-amino-acid protein (104 kDa), which has substantial homology with the eukaryotic DNA repair and recombination proteins MEI-9 (*Drosophila melanogaster*), Rad16 (*Schizosaccharomyces pombe*), and Rad1 (*Saccharomyces cerevisiae*). *ERCC4* cDNA efficiently corrected mutants in rodent NER complementation groups 4 and 11, showing the equivalence of these groups, and ERCC4 protein levels were reduced in mutants of both groups. In cells of an XP-F patient, the ERCC4 protein level was reduced to less than 5%, consistent with *XPF* being the *ERCC4* gene. The considerable identity (40%) between ERCC4 and MEI-9 suggests a possible involvement of ERCC4 in meiosis. In baboon tissues, *ERCC4* was expressed weakly and was not significantly higher in testis than in nonmeiotic tissues.**

Nucleotide excision repair (NER) is a universal DNA repair pathway that acts on most bulky chemical adducts and the major photoproducts (cyclobutane and [6-4] pyrimidine dimers) produced by UV radiation (19, 25, 52, 68). In xeroderma pigmentosum (XP) patients, who have a very high risk of skin cancer due to solar exposure, there is a partial or complete deficiency in NER (15). This observation provided a critical link between deficiency of an error-free repair process and enhanced mutagenesis and carcinogenesis in humans (15, 37). Genetic analysis of XP cell lines identified seven complementation groups of incision-defective XP patients (30, 79) and a common variant form of XP that is excision proficient (14). Parallel studies with rodent cell mutants isolated in culture demonstrated 11 complementation groups that are phenotypically similar to the XP lines (51). The rodent mutants proved well suited for cloning NER genes on the basis of functional complementation because of efficient DNA transformation (74, 76, 81, 83), and considerable overlap was found between the XP and rodent groups (68). Thus, the genes *XPA* (66), *XPB* (*ERCC3*) (83), *XPC* (34), *XPD* (*ERCC2*) (18, 81), and *XPG* (*ERCC5*) (36, 57) have been characterized, as has the *ERCC1* NER gene (76), which is not involved in any known human disorder (77).

Recently the NER process has been reconstituted by using recombinant proteins and highly purified factors (1, 43, 44). With the reconstituted systems, insights into the biochemical mechanism of NER have been obtained. Damage recognition likely involves XPA (2, 29), replication protein RPA (10, 24), and possibly other proteins. The biochemical overlap between transcription and NER is embodied in the transcription initiation factor TFIIH, which contains the XPB 3′-to-5′ helicase (16, 56) and the XPD 5′-to-3′ helicase (16, 55) as well as approximately six other proteins (17). These helicase activities are thought to participate in creating a preincision complex that contains a single-stranded region encompassing the DNA lesion. Asymmetrical dual incisions are made (27), first on the 3′ side of damage by the XPG endonuclease (45) and then on the 5′ side by the ERCC1-ERCC4 endonuclease (40, 41, 43). In the yeast *Saccharomyces cerevisiae*, the 5′ incision is performed by the homologous Rad10-Rad1 endonuclease complex (5). Following the excision of oligonucleotides predominantly 27 to 29 bases in length (27, 43, 65), synthesis and ligation complete the repair process (61).

Of the genes that are essential for the excision step of NER in the reconstituted systems (other than those encoding certain components of the TFIIH complex), all have been characterized at the level of the cDNA except for *ERCC4*. The human *ERCC4* genomic sequence was previously cloned by functional complementation by transfection of a chromosome-specific cosmid library (70). In this article we report characterization of the gene and functional *ERCC4* cDNA sequences, which correct the UV sensitivity of the CHO mutant UVS1 (22) assigned to group 11 (51) as well as that of the prototype mutant (UV41) in group 4 (71). These results imply that the two groups are genetically equivalent. Our results also support the

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conclusion that *ERCC4* is equivalent to *XPF*, on the basis of biochemical complementation studies (47, 49) and microinjection experiments combined with sequence analysis (62).

MATERIALS AND METHODS

Cells and culture conditions. CHO cell lines AA8 (wild type), UV41 (NER group 4), UV135 (group 5), UVS1 (group 11) (22, 51), and transformants were grown at 37°C in suspension or monolayer culture in α-modified minimum essential medium with 10% fetal bovine serum and antibiotics, without selection agents, as described previously (81). Population doubling times were determined in exponential growth by counting concentrations of cell suspension on a Coulter Multisizer II counter (Coulter Corporation). HeLa S3 and mouse L60WT4 cells were grown in suspension culture under conditions used for CHO cells. Human diploid fibroblasts XP3YO (XP-F, GM03542), immortalized XP2YOSV (XP-F, GM08437A), and WI38VA13 (simian virus 40 immortalized WI38) cells were maintained in monolayer culture in 15 to 20% fetal bovine serum.

cDNA library screening. A HeLa cDNA library in vector pEBS7 (48) was screened under conditions reported earlier (69). Replicates of 14 filters, each with 1.2×10^5 cells, were hybridized with ~18 kb of DNA derived from *ERCC4* cosmid clone pER4-5 (70). Repetitive elements were blocked by an initial association between 1 mg of sonicated human placental DNA and 10 to 100 ng of genomic probe, and hybridized filters were rinsed at 66°C to a final stringency of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate).

cDNA extension and construction of expression plasmids. To retrieve missing cDNA sequences, gene-specific PCR primers were derived from library clone sequences and used in reverse-transcribed PCR (RT-PCR) amplification of HeLa poly(A)$^+$ RNA (20). PCR amplifications were conducted with high-fidelity DNA polymerase *Pfu*I (Stratagene) for 20 to 30 cycles at temperatures ~5°C above primer-annealing temperatures. Primary products were expanded by using nested gene-specific and anchoring oligonucleotides in secondary and tertiary amplifications of 20 to 30 cycles. Products generated from cosmid pER4-6 (70) were obtained by a single course of 20 to 30 cycles. Intermediate and final constructs of the *ERCC4* cDNA were assembled in mammalian expression vector pcDNA3 (Invitrogen) containing a human cytomegalovirus (CMV) promoter, bovine growth hormone polyadenylation signal, and a *neo* gene for Geneticin selection. Sequence determination of cDNA fragments and full-length construct cER4-40, each subcloned into pBluescript II KS$^+$ (Stratagene), was performed by methods described below for cosmid sequencing.

Northern (RNA) blot analysis. A human and mouse RNA blot was generated as described earlier (69). Poly(A)$^+$ RNAs from HeLa S3 and mouse L60WT4 cells were electrophoresed on a 1% agarose–2.2 M formaldehyde gel, hybridized with a 1.5-kb mid-*ERCC4* cDNA PCR fragment, and rinsed with 2× SSC–1% sodium dodecyl sulfate (SDS) at 60°C. Baboon tissue poly(A)$^+$ RNAs were isolated from a 14-year-old male and separated on a 1% agarose–2.2 M formaldehyde gel as described previously (80, 87). The blot was probed first with a 1.4-kb *Sac*II-*Eco*RV *ERCC4* cDNA fragment and rinsed at 55°C to a final stringency of 0.1× SSC in 0.1% SDS. After removal of *ERCC4* sequences, the blot was probed with abundantly expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by using a 0.8-kb *Pst*I-*Xba*I fragment of cDNA insert from vector pHcGAP (American Type Culture Collection).

Transfection and transformant selection. After electroporation of lines UV41 and UVS1 at 300 V/1,600 μF with 5 μg of DNA in a volume of 1 ml, cultures were grown for 28 to 44 h for expression and plated in 20 nM mitomycin (UV41 only) or were given 5 to 7 J of UV-C per m^2 and then grown in 1.7 mg of Geneticin (Gibco BRL Life Technologies) per ml. Ten UV41-derived transformant colonies (41.cER4.30 through -34 and 41.cER4.40 through -44) and three UVS1-derived transformants (UVS1.cER4.1, -2, and -3) were isolated and grown to mass culture. For UV survival measurements, three 10-cm-diameter dishes were exposed at each fluence (71).

The XP lines XP3YO and XP2YOSV were electroporated at 0.3×10^7 to 1.0×10^7 cells per ml with 6 to 10 μg of linearized or supercoiled DNA. For assay of expression of stably integrated DNA, XP2YOSV cells were grown for 48 h and then plated at 0.8×10^5 or 8×10^5/100-mm-diameter dish in 0.8 mg of Geneticin per ml. Eight to 10 days after electroporation, surviving cells received 10 J of UV-C light per m^2, and seven colonies surviving double selection were isolated after an additional 7 days of growth. For assay of transient gene expression, electroporated XP cells were grown for 16 h and then trypsinized and plated into 6-well trays at 1×10^5 to 2×10^5 cells per well and irradiated after 2 h of incubation for attachment. Growth continued for 4 to 21 days, depending on UV fluence and cell line.

Excision nuclease assay. Cell extracts (38), excision substrate preparation (40), and repair assay conditions (46) were described earlier. An aliquot of 50 μg of cell extract (or 25 μg each for a mixture) was incubated for 60 min at 30°C with 6 fmol of substrate DNA, a double-stranded 140-mer containing a centrally positioned cholesterol adduct and a ^{32}P label at the sixth phosphodiester bond 5′ to the adduct. Products were resolved on a 10% denaturing polyacrylamide gel and, after autoradiography, excision products were quantified by scanning dried gels with an AMBIS Systems Scanner.

Immunoblot analysis. To produce anti-ERCC4 antibody, an N-terminal 260-amino-acid (aa) polypeptide of ERCC4 was overexpressed in bacteria by using an *Nco*I-*Bam*HI fragment of the *ERCC4* cDNA inserted into the same sites in the polylinker of the pET26b expression plasmid (Novagen). After induction using isopropyl-1-thio-β-D-galactopyranoside, cells were lysed, and protein was purified in 0.05% Sarkosyl on a column of Ni-nitrilotriacetic acid agarose (Qiagen). Mouse antiserum was raised against this antigen, and ascites fluid was used for the final collection of polyclonal antibody. Extracts from cell lines were prepared either by sonication of harvested cells in 50 mM Tris (pH 8.0)–1 mM EDTA–1 mM dithiothreitol–10% glycerol or by methods earlier described (38). Protein samples of 50 μg were electrophoresed in a 7.5% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and probed with anti-ERCC4 antibody by the enhanced chemiluminescence detection method as described by the manufacturer (Amersham).

Cosmid subcloning and sequencing. Human cosmid pER4-6 containing the *ERCC4* gene was sonicated, end repaired with Klenow and T4 polymerases, size selected, and cloned into M13mp18 as described earlier (32, 39). DNA templates were prepared using 96-well-format M13 kits (Qiagen). Single-stranded templates were sequenced on a Catalyst 800 Molecular Biology Labstation (Applied Biosystems Division [ABD]; Perkin Elmer) by using a fluorescently labeled universal −21m13 primer and *Taq* cycle sequencing (ABD). The resulting sequencing ladders were loaded onto 4.75% polyacrylamide gels, and data were collected on ABD 373A DNA Sequencers. Vector, ambiguous sequences, and clones containing *Alu* repetitive elements were identified prior to assembly by using a prescreening feature of the GENeration sequence assembly package (IntelliGenetics). Ambiguities in the assembled sequence were resolved by visual inspection of the chromatograms or resequencing with Sequenase Dye Deoxy Terminators (ABD). Areas containing compressions or other mobility artifacts were resequenced by using Taq Dye Deoxy Terminators with dITP. Cosmid pER4-6 sequence was obtained at a level of sevenfold redundancy and 90% coverage of both strands. PCR primers designed from cosmid sequences specifically for validation were tested with cosmid and human genomic DNA to verify the presence of a fragment of a predicted size in the human genome. Of 21 primer pairs tested, 14 gave products with both the cosmid and human genomic DNA, 5 gave a product with only the cosmid genomic DNA, and 2 gave no products. The last results most likely represent complications of the PCR rather than inaccurate sequence.

Sequence homology analysis. The intron-exon structure of *ERCC4* was determined by comparison of cDNA sequences with the genomic sequence by using ALIGN (IntelliGenetics) and assessment of the coding region predictions made by XGRAIL versions 1.2 and 1.3 (75). By using ALIGN, repetitive elements were identified by comparison to a subset of known human repeats: *Alu*, L1, transposon-like human element, and long terminal repeat. Additional searches were performed against the Genome Sequence Data Base (GSDB) (daily update), dbEST (daily update), and SWISSPROT (release 32; December 1995). Protein alignment was generated by GeneWorks software (IntelliGenetics).

Nucleotide sequence accession numbers. GSDB designations: cosmid pER4-6, L76568; cDNA cER4-40, L77890.

RESULTS

Construction of functional *ERCC4* cDNAs. Initially, a HeLa cell cDNA expression library in vector pEBS7 was screened by using a mixture of *Eco*RI fragments of a functional cosmid, pER4-5. Three of five positive clones (cER4-1, cER4-4, and cER4-5, with 0.7- to ~2-kb inserts) were electroporated into UV41 cells, but none conferred UV resistance. Nucleotide sequences subsequently derived for the five clones confirmed their identity with *ERCC4* by alignment with sequences from cosmid pER4-6 (70). All clones were found to carry considerable intronic material in addition to various lengths of coding sequence. Clones cER4-4 and cER4-5 overlapped and provided 1,795 bp of *ERCC4* open reading frame (ORF) representing the 5′ half of the coding region, and clone cER4-2 contained 354 bp of the final third of the ORF (Fig. 1). Using cDNA-derived sequences, RT-PCR was used to amplify missing 5′-end, middle, and 3′-end regions. Subcloned 5′-end RT-PCR products obtained from HeLa cell cDNA extended only 12 bp upstream from the first of two closely spaced in-frame ATG codons; the downstream ATG was present in clone cER4-5. Surprisingly, subcloning of the major 3′ RT-PCR product generated two distinct species. Eleven of 14 subclones had a splicing variation, which is shown as clone cER4-22 (Fig. 1), which terminated the ORF in exon 11 at position 3180 (see Fig. 6). However, three clones, represented by cER4-40 in Fig. 1, were not spliced. They extended beyond the splice donor site to the poly(A) region, with an ORF ending at position 2749.

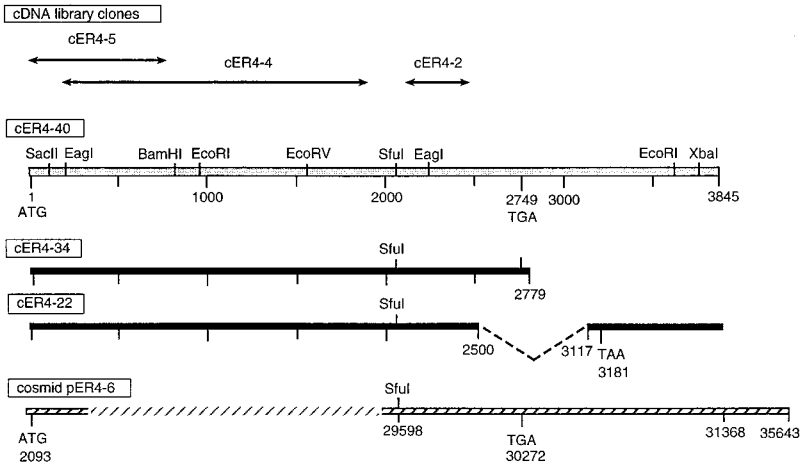

FIG. 1. Construction of *ERCC4* cDNAs. cER4-40 and cER4-34 represent two functional cDNA clones, constructed as composites from cER4-22 (mRNA leader sequence to *Sfu*I) and cosmid pER4-6 (*Sfu*I to bp 31368), in expression vector pcDNA3 (Invitrogen). Clone cER4-22 is an alternatively spliced, nonfunctional variant assembled from library clones cER4-4 and cER4-5 and RT-PCR fragments.

Constructs cER4-22 (spliced) and cER4-34 and cER4-40 (unspliced) were assembled in the pcDNA3 CMV expression vector from a combination of library and PCR-derived sequences.

To test for the minimum functional transcript, clone cER4-34 was constructed by using only 30 bp downstream of the stop codon. Both cER4-34 and cER4-40 have the same 2.75-kb ORF, which conferred UV resistance to the group 4 mutant UV41 (see below). Clone cER4-22, with a 2.58-kb ORF representing the predominantly amplified transcript, was not functional. A Northern blot of human and mouse poly(A)$^+$ RNA, hybridized with a 1.5-kb mid-cDNA PCR fragment, had a major band of ~3.8 kb in both species, slightly smaller in the mouse RNA (Fig. 2). A transcript of that length is consistent with the ~3,850-bp construct cER4-40. Two additional fragments of ~7 and 2.4 kb, seen predominantly in human RNA, likely resulted from improperly processed or alternatively polyadenylated transcripts.

Correction of UV sensitivity in CHO transformants. The functionality of plasmids cER4-34 and cER4-40 was measured in stable transformants of UV41 in response to UV irradiation. Transformants 41cER4.34 and 41cER4.40 showed ~90% restoration of UV resistance compared with wild-type AA8 (based on D_{10}, the UV fluence reducing survival to 10%) (Fig. 3). In addition, the transformants had significantly enhanced growth rates. During 5 weeks of exponential growth in suspension culture, the doubling times of the two transformants were 13.3 to 13.4 ± 0.17 h (standard error of the mean) compared to 14.1 ± 0.14 h for UV41 and 12.8 ± 0.22 h for AA8 cells, representing a 54% increase toward the wild-type growth rate. *ERCC4* cDNAs were also transferred into cell line UVS1, which was previously assigned to complementation group 11 (22, 51). *ERCC4* and control transformants of UVS1 were generated, and their UV sensitivity was found to be fully corrected compared with that of wild type AA8 cells (Fig. 3). This result implies identity between complementation groups 4 and 11, and this was confirmed in excision nuclease assays in which UVS1 was complemented by purified ERCC1-ERCC4 complex (6). However, UV resistance was not transferred to UVS1 cells by transfection with genomic *ERCC4* clone pER4-6 under selection for both UV resistance and *neo* expression (results not shown). Thus, correction of UVS1 by the human *ERCC4* cDNA may require overexpression from the CMV promoter to overcome the effects of a possible dominant negative mutation.

Restoration of excision nuclease activity in CHO transformants. To confirm that the nearly normal UV sensitivity and growth rate of the UV41 transformants resulted from enhanced DNA repair, we analyzed transformants using a cell-free assay for damage excision activity. Figure 4A shows an

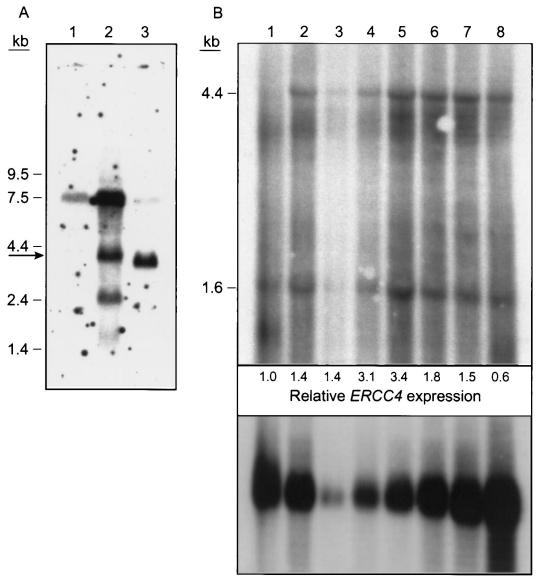

FIG. 2. Northern blot analysis of *ERCC4* poly(A)$^+$ RNA. (A) Human (HeLa) and mouse (L60WT4) RNA. The blot was hybridized with a 1.5-kb PCR fragment from the middle region of *ERCC4* cDNA. Lane 1, 10 μg of total human RNA; lane 2, 5 μg of human poly(A)$^+$ RNA; lane 3, 5 μg of mouse poly(A)$^+$ RNA. Arrow indicates a 3.8-kb band hybridizing in both human and mouse RNA. (B) Baboon tissue RNA. Ten micrograms of poly(A)$^+$ RNA from baboon tissues was hybridized first with a 1.4-kb *Sac*II-*Eco*RV *ERCC4* cDNA fragment and then with a probe for GAPDH. Lane 1, testis; lane 2, spleen; lane 3, lymph node; lane 4, lung; lane 5, liver; lane 6, kidney; lane 7, heart; lane 8, brain. Markers indicate 4.4 and 1.6-kb bands appearing in all tissues. *ERCC4* and GAPDH hybridizing bands were quantified with Molecular Analyst version 1.4 software on a Molecular Imager (BioRad), and the ratio of 4.4-kb band values to GAPDH band values was calculated for each tissue. Comparative levels of expressed *ERCC4* (individual tissue ratios normalized to the testis ratio) are shown below the *ERCC4* lanes. Photographs of *ERCC4* hybridization images and the GAPDH autoradiogram were scanned and processed with Adobe Photoshop 3.0 software.

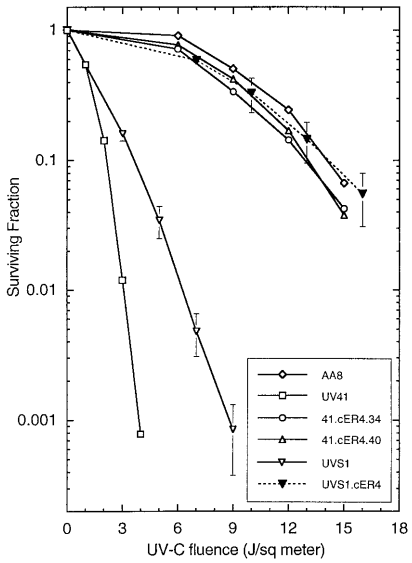

FIG. 3. UV-C survival curves of NER group 4 and 11 *ERCC4* transformants. One experiment is shown for AA8, UV41, and transformants 41.cER4.34 and 41.cER4.40. Error bars for UVS1 curves show the standard deviations of measurements for two Geneticin-resistant vector transformants and three *ERCC4* transformants (UVS1.cER4).

autoradiogram comparing the excision products (24- to 32-nucleotide [nt] fragments released from a 140-bp adducted substrate) of wild-type, mutant, and transformant cell extracts. Combined extracts of group 5 mutant UV135 and UV41 (lane 4) served as a positive control, to demonstrate the level of activity obtainable with complementing extracts. No detectable product was released by either mutant line (UV41 and UV135 in lanes 2 and 3, respectively), but excision products were generated by the transformant extracts (lanes 5 and 7). The absolute levels of excision are summarized in Fig. 4B. Wild-type AA8 and the complementing mutant extracts (Fig. 4B, UV41 + UV135) had similar excision activities. In the transformants, the activity was restored to ~15 to 20% that of the

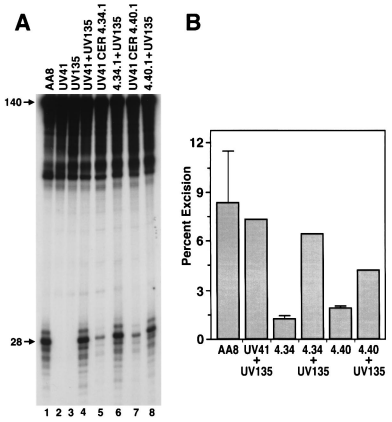

FIG. 4. Excision activity of UV41 transformants. (A) Excision assay autoradiogram. The prominent ~28-nt excision product and unexcised 140-mer substrate are indicated. Lanes 1 to 3, control cell lines; lanes 5 and 7, transformants; lane 4, UV41 mixed with UV135, showing complementation of the repair defect; lanes 6 and 8, transformants mixed with UV135. (B) Quantitation of correction in *ERCC4* transformants shown in panel A. Excision is expressed as percent substrate released. Error bars are standard errors of the means for duplicate experiments with AA8, 41cER4.34 (4.34), and 41cER4.40 (4.40).

Exon	Length, bp	Intron	Length, bp
1	207		
2	181	1	1,658
3	196	2	4,349
4	208	3	1,271
5	181	4	2,475
6	130	5	1,265
7	111	6	1,905
8	598	7	843
9	93	8	2,023
10	113	9	6,864
11	731	10	2,778
ORF length	2,749	Distance spanned	28,180

FIG. 5. Intron and exon structure of the human *ERCC4* gene region. Sequence of cosmid pER4-6 was generated from a total of 585 M13mp18 subclones spanning 35.6 kb. In addition to the *ERCC4* coding region, nine *Alu* family repeats (0.25 Alu per kbp) were identified. Also, two genes, a ribosomal gene and a zinc finger gene, were located on the inverse strand.

wild type. However, complementation of the transformant extracts with UV135 did not reach the excision level observed in the UV41 + UV135 control, an effect attributable to variation in the quality of the extract preparations. With this effect taken into account, excision in the transformants can be normalized to ~20 to 40% that of the wild type.

***ERCC4* gene structure.** The complete sequence was determined for cosmid pER4-6 (Fig. 1). Comparison of cosmid and cDNA sequences showed that the gene is composed of 11 exons and spans a genomic distance of 28.2 kb (Fig. 5). No polymorphic differences were observed between the cDNA and cosmid sequences, which were derived from different sources. A composite nucleotide sequence showing the cDNA and flanking genomic regions is depicted in Fig. 6. Promoter consensus elements such as TATA, CAAT, and GC boxes are absent, and the 3′ untranslated region (UTR) has no AAT AAA polyadenylation signal (see Fig. 6 legend). The region beginning 275 bp upstream of the translation start site is rich in recurring elements. In particular, the heptamer TTCGGC(T/C) is repeated 10-fold. The 3′ UTR contains a 93-bp dyad repeat with 89% identity (positions 2937 to 3029 and 3045 to 3137), which may have functional significance. Two candidate ATG translation start codons, separated by 30 nt, are present. Although the downstream ATG, with a purine at position −3, more closely matches the optimal setting (31), sequences surrounding both codons may provide an acceptable initiation context, and our translation begins with the first ATG. The polypeptide predicted by this sequence has 916 amino residues, with a calculated M_r of 104,491 and pI of 6.49.

By the procedure for 5′ rapid amplification of cDNA ends (20), 5′ poly(A)-tailed HeLa mRNAs were amplified between poly(T)-anchoring primers and *ERCC4*-specific primers either 289 or 399 bp downstream of the first ATG. The resulting single products were ~320 and ~425 bp, respectively, i.e., only ~10 to 30 bp longer than predicted for the coding sequence alone. Four subcloned 5′ products of rapid amplification of cDNA ends had 5′-ward sequences beginning at positions −12 (Fig. 6) to +22. RT-PCR amplification of the region between positions −87 and +289 failed to generate a product, arguing that the *ERCC4* transcript does not extend that far upstream and that the leader sequence is short.

ERCC4-related proteins. The conceptual translation of the *ERCC4* ORF gives a protein of 916 aa (Fig. 6). ERCC4 protein exhibits notable homology with *S. cerevisiae* Rad1 (50), *S.*

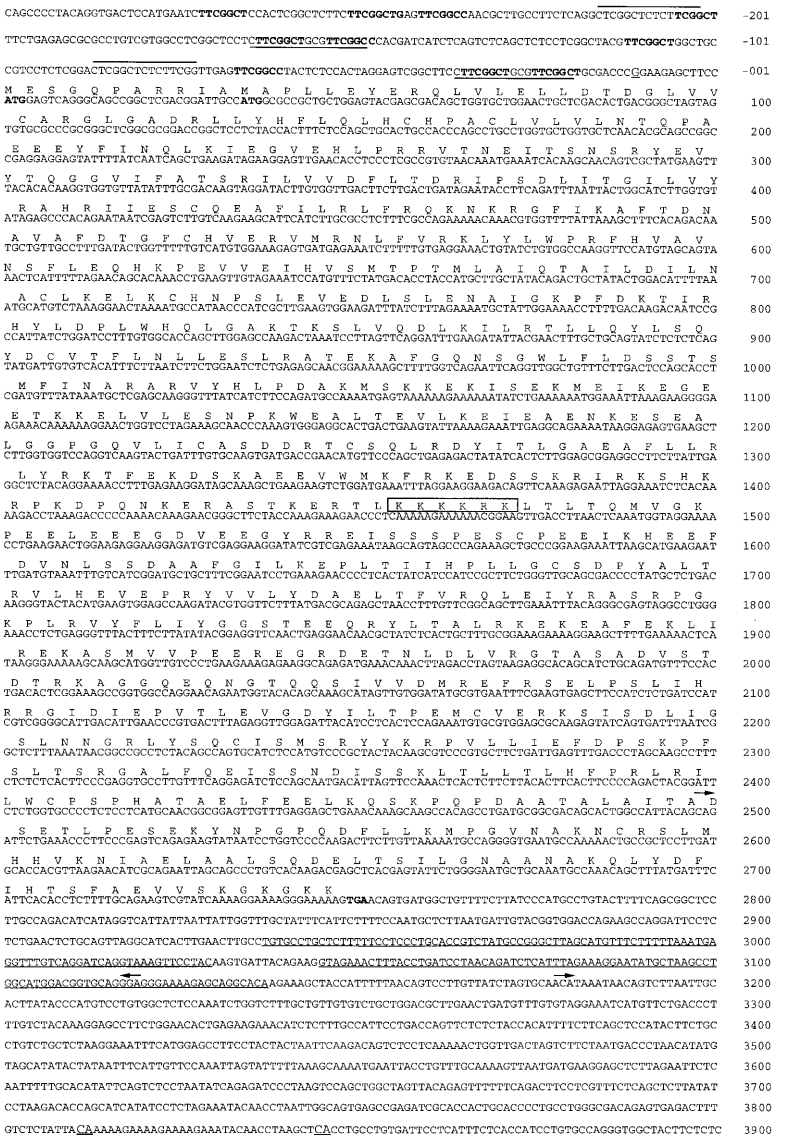

FIG. 6. Composite of cDNA sequence, translated amino acid sequence, and flanking genomic regions. The ORF spans positions 1 to 2748. Sequences at positions −300 to −13 and 3845 to end are from genomic cosmid pER4-6; positions −12 to 3844 are derived from poly(A)$^+$ RNA. Two translation start codons (ATG) and the stop codon (TGA) are marked in bold. In the promoter region, a core repeat, TTCGGC(T/C), is shown in bold. A 15-bp repeat is overlined, and a 17-bp repeat is underscored. In the leader region at position −12 (G), the start position of the most 5′-ward RT-PCR product is shown underlined and in a larger type size. Within the ORF, a potential nuclear localization signal, KKKKRK (boxed, residues 486 to 491), is encoded from positions 1456 to 1473. In the 3′ UTR, a 93-bp dyad repeat is underlined, and two polyadenylation sites (CA) found in 3′-end PCR products are shown underlined and in a larger type size. In the cloned 3′ RT-PCR products, polyadenylation began at positions 3813 and 3846. Although this region contains no consensus poly(A) signal, a 5.9-kb linear sequence (CMV promoter and cDNA sequence extending to the *Sfu*I site of cER4-22 [Fig. 1], joined with cosmid pER4-6 from *Sfu*I to position 31760), which contained no other polyadenylation signals, was functional upon transfection. Alternative splicing represented in cER4-22 is indicated by arrows at positions 2500 (splice donor), 3117 (splice acceptor), and 3181 (translation stop codon TAA).

pombe Rad16 (13), and *Drosophila* MEI-9 (60), all of which are implicated in NER (Fig. 7). The Rad1 protein is 184 aa longer than ERCC4, and for optimum alignment we removed the Rad1 N- and C-terminal regions. Overall identity of ERCC4 with Rad1 is 28% (51% similarity); identity is 33% with Rad16 (57% similarity) and 40% (61% similarity) with MEI-9. In the middle region of ~250 aa (not shown in Fig. 7), ERCC4 is not conserved. The degree of homology is highest for ERCC4 residues 16 to 304 and 570 to 830, suggesting that the protein consists of at least two functional domains. In the N-terminal region, ERCC4 has two consensus leucine zippers (33), which might promote interaction with other proteins.

Expression of *ERCC4* cDNAs in XPF cells. We attempted to demonstrate functionality of the *ERCC4* cDNA constructs in cells of the XP2YO lineage (23, 85), the only lines of the XP-F complementation group that were available to us. We found that immortalized XP2YOSV (GM8437A) cells, which are only ~two- to threefold sensitive to killing by UV-C, were also exceptionally heterogeneous in UV sensitivity. Using a relatively UV-sensitive subclone of XP2YOSV, we observed slightly enhanced UV resistance from cER4-40 transfection in a transient correction assay that measures cell growth (data not shown). Surprisingly, confirmation of this result was prevented by a sudden cessation of growth, a phenomenon seen with

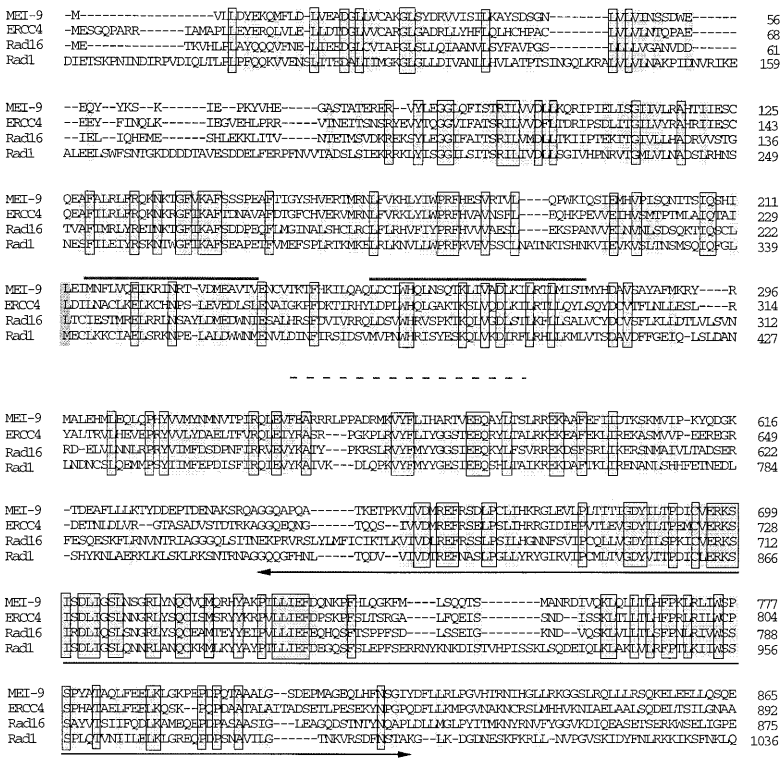

FIG. 7. Alignment of conserved regions of the ERCC4 family of proteins, *Drosophila* MEI-9, *S. pombe* Rad16, and *S. cerevisiae* Rad1. ERCC4 protein sequence was derived from the ORF of functional clones cER4-34 and cER4-40, which were confirmed by comparison with genomic sequence. Rad1 residues 1 to 69 and 1037 to 1100 were deleted for optimal alignment. Identical residues are shown in shaded boxes; conserved residues are shaded only. Two consensus leucine zipper regions found in ERCC4 (at residues 233 to 254 and 270 to 298) are indicated by overlining, and the Rad10 binding domain of Rad1 is underscored.

about 70% of the subclones. Transfections of XP2YOSV with cER4-40 that was linearized by cutting within the vector sequence, which we expected to transfer both *ERCC4* and the linked *neo* gene, efficiently produced stable Geneticin-resistant transformants at a frequency of $\sim 10^{-3}$. However, several Geneticin-resistant clones initially surviving exposure to 10 J of UV-C per m^2 exhibited neither elevated UV resistance nor excision activity nor increased ERCC4 protein levels (results not shown). Transfection of diploid fibroblasts from the closely related XP-F line XP3YO (GM3542) with cER4-40 and cER4-34 also conferred no transient resistance to UV-C. This behavior of the *ERCC4* cDNAs suggests that expression may be suppressed in the XP2YO lineage.

ERCC4 protein levels in human and CHO cells. To identify the ERCC4 protein and evaluate its status in repair-proficient and -deficient cells, immunoblot analysis was performed with antibodies recognizing an N-terminal ERCC4 polypeptide. In extracts from repair-competent HeLa and WI38VA13 cells, a protein with a mass of ~110 kDa was detected; this value correlates well with the calculated value of 104 kDa deduced from the cDNA sequence (Fig. 8a). In immortalized XP-F cells, this protein was present at a level less than 5% that in the WI38VA13 cells (Fig. 8). Extracts from CHO mutants UV41 and UVS1 also showed reduced levels of ERCC4 compared with that of AA8 (Fig. 8b). In the cDNA transformant, 41cER4-34 (lane 4), the expressed protein was detected at the same position as the human control (lane 1), confirming that construct cER4-34 has a full-length sequence. The intermediate level of ERCC4 expressed in the transformant (~40% of wild-type AA8) suggests instability of human ERCC4 protein in the hamster cells, an observation that is consistent with the relatively low level of excision measured in cell extracts of this transformant. Although significantly diminished, the amount was sufficient to restore survival after UV-C almost completely.

Expression of *ERCC4* mRNA in baboon tissue. To determine whether transcript regulation shows tissue-specific variation, we analyzed a panel of primate tissues for the level of *ERCC4* mRNA (Fig. 2B). Abundantly expressed GAPDH was also measured to standardize the level of mRNA present in each lane. All baboon tissues had two species, ~4.4 and 1.6 kb, while those in human RNA were ~3.8 and 2.4 kb. The differences in transcript lengths between human and baboon RNAs may be a consequence of variability in untranslated regions of the transcripts. A larger transcript (~7 kb), seen in human RNA, had no apparent representation in baboon tissues and only a minor presence in mouse RNA. Expression of *ERCC4* was seen in all tissues, with levels in testis (lane 1) and brain (lane 8) lower than those in spleen, lymph node, lung, liver, kidney, and heart when normalized to GAPDH.

DISCUSSION

Starting with probes from the previously cloned *ERCC4* genomic sequence, we have reconstructed a functional, composite cDNA from a combination of cDNA library clones and RT-PCR products derived from mRNA and cosmid genomic sequences. The ORF encodes an ERCC4 protein of 916 aa; comparative values are 1,100 aa for *S. cerevisiae* Rad1, 892 aa for *S. pombe* Rad16, and 946 aa for *Drosophila* MEI-9. The degree of conservation between ERCC4 and its homologs ranges from 28% identity for Rad1 to 40% identity for MEI-9 (Fig. 7). Rad1 and Rad16 proteins are essential components of

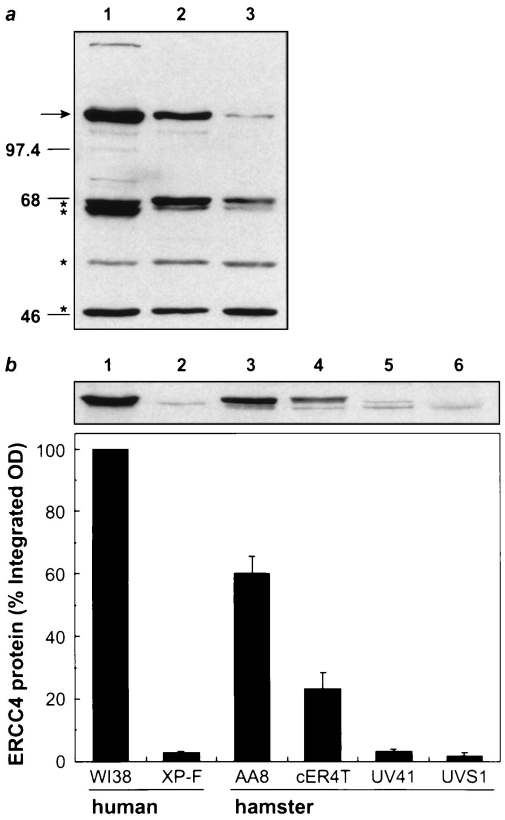

FIG. 8. Immunoblot analysis of cell extracts using anti-ERCC4 antibody. (a) Detection and estimation of molecular mass of the ERCC4 protein. Lane 1, HeLa S3; lane 2, WI38VA13; lane 3, XP-F (XP2YOSV). The arrow indicates the full-length ERCC4 protein with a mass estimated at 110 kDa; asterisks are adjacent to ERCC4 degradation products or cross-reacting proteins. Molecular masses of protein standards are indicated to the left of the image. (b) Detection and quantitation of ERCC4 protein in mutant cell lines. After the film was developed, the immunoblot image was captured using a video camera linked to the NIH-Image software program (version 1.58) run on an Apple Quadra 650 computer. The integrated optical density for each band at the 110-kDa position was subsequently determined with the RFLPscan program (version 2.01; Scanalytics, CSP Inc.). The values, normalized to that of human WI38VA13, are relative and are the standard errors of the means from four experiments. Lanes are identified at the bottom of the panel; lane 4, cER4T, is transformant 41cER4.34. For data presentation, the immunoblot was scanned and processed with Adobe Photoshop 3.0 software.

NER in yeasts (13, 50, 86), and MEI-9 is essential for efficient meiotic recombination as well as NER (8, 12). *mei-9* was first identified in a screen for mutations causing meiotic nondisjunction (3), and it is required for efficient reciprocal exchange (12). Subsequently, *mei-9* was identified as a gene involved in mutagen sensitivity and excision repair (8). Thus, *mei-9* differs from *RAD1*, *rad16*, and other NER genes in yeasts, which have not been found to have a role in meiosis. The relatively high level of conservation between ERCC4 and MEI-9 suggested the possibility that ERCC4 might be secondarily involved in meiosis. Our examination of baboon tissues showed no elevation in whole testis compared with nonmeiotic tissues. However, the possibility remains that ERCC4 may have a recombination function in meiotic cells.

The ERCC1-ERCC4 heterodimer purified from HeLa cells (47) is the human homologous counterpart of the *S. cerevisiae* Rad10-Rad1 endonuclease complex (64, 73), which had incision activity 5′-ward of the lesion in NER, as inferred from model substrates (5). The purified ERCC1-ERCC4 complex had analogous incision activity (40, 41, 47) and promoted repair in a highly purified reconstituted system (43). During repair, the ERCC1-ERCC4-mediated 5′ incision appears to require prior 3′ incision by the XPG endonuclease (40, 43, 45). In Rad1, the region of interaction with Rad10 lies between residues 809 and 997 (Fig. 7) (4), and dimeric association was also demonstrated for the C terminus of Rad16 with Swi10, a homolog of Rad10 (13). The substantial identity among ERCC4 and its three homologs (52 of 188 residues; Fig. 7) in this region suggests that it also contains the ERCC1 binding domain in ERCC4. The nonfunctional cDNA construct cER4-22 (Fig. 1) is expected to encode a truncated ERCC4 protein that diverges after aa 833 and is missing the 83 normal C-terminal aa. This truncation overlaps with the region that involves the Rad1-Rad10 interaction. In HeLa cells, a prevalent *ERCC4* transcript corresponds to this truncated sequence, but it is unclear whether the truncated protein of 856 aa has physiological significance.

We found that ERCC4 cDNA efficiently corrected the CHO UVS1 mutant (Fig. 3) that was assigned to complementation group 11 (22, 51). Previously, a hypothetical ERCC11 protein was postulated to reside within the ERCC1-ERCC4 complex, on the basis of biochemical complementation studies (78). Moreover, our previous attempt to correct UVS1 cells by transfection with an *ERCC4* cosmid that corrected the group 4 mutant UV41 gave no evidence for restoration of UV resistance, which suggested that UVS1 represented a distinct gene. From this result it would appear that UVS1 may contain a mutant hamster *ERCC4* allele that behaves in a codominant manner in the presence of human *ERCC4* and that is not complemented by *ERCC4* unless it is expressed by a strong promoter. We have no explanation for the reported complementation between UVS1 and UV41 (22). Both UVS1 (22) and the CHO mutant UV140 (11), which is also corrected by ERCC4 cosmid pER4-6 (70), exhibit only weak sensitivity (two- to threefold) to the cross-linking agent mitomycin, compared to the 90-fold hypersensitivity of the prototype mutants in groups 1 and 4 (26). UV140 and UVS1 are ~fourfold sensitive to killing by UV-C (11, 22). This differential hypersensitivity to UV-C versus mitomycin between the mutants suggests either that different functional domains of ERCC4 are involved in UV-C versus cross-link repair or that cross-link repair has less stringent requirements for the level of ERCC4 activity. CHO group 1 mutants that are relatively resistant to mitomycin have also been identified (11).

UV41 cells were corrected, to various degrees, by transfection with either cDNA construct cER4-34 (having a truncated 3′ UTR) or cER4-40 (representing the native mRNA) (Fig. 1). Incomplete correction was seen with all endpoints for the two transformants. (i) Population doubling times in culture were shortened significantly. This effect in UV41 might be due to the restoration of NER capacity through its action on spontaneous damage, such as oxidative lesions (53). Unrepaired oxidative damage leading to disruption of the normal cell cycle regulation and p53 response was invoked to explain the severe abnormalities in and early death of *ERCC1* knockout mice, which have abnormal p53 expression (42). Alternatively, ERCC4 may have a role in cell cycle progression that does involve repair. (ii) Cell survival after UV-C exposure gave an ~90% level of correction based on D_{10} fluences (Fig. 3), which was a significant improvement over the 30 to 64% level of correction seen earlier with the *ERCC4* genomic sequence (70). These partial corrections presumably reflect the inefficient functioning of human ERCC4 protein complexed with hamster ERCC1. Consistent with this idea is the finding that overexpression of human *ERCC1* in wild-type AA8 cells conferred increased sen-

sitivity to cross-linking agents (9). (iii) Excision nuclease activity, which was undetectable in UV41 extracts, was present in transformants at <40% of the AA8 level (Fig. 4), suggesting that the heterologous human protein may function even less efficiently in vitro than in vivo. A similar partial level of correction was seen in excision nuclease assays with *ERCC4* genomic transformants (70). (iv) The hamster ERCC4 protein level in UV41 was very low, while the transformants had total ERCC4 levels that were ~40% of the AA8 level, or lower if the antibody recognized human ERCC4 more efficiently than hamster ERCC4 (Fig. 8 and results not shown). ERCC4 appears to be unstable in hamster cells in the absence of overexpressed ERCC1.

It was previously suggested on the basis of biochemical complementation studies that *ERCC4* might be equivalent to *XPF* (7, 49, 78). The purified complex containing ERCC1 and the 112-kDa protein was shown to complement UV41 and XP-F cell extracts (47). More recently, anti-ERCC4 antibody detected the 112-kDa protein that copurifies with ERCC1, and recombinant ERCC1-ERCC4 complex isolated from the baculovirus system corrected the repair deficiency in XP-F cells using the excision nuclease assay (6). In the latter assay, the ERCC4 protein was encoded by the sequence from cDNA plasmid cER4-34 (Fig. 1). Mutations were also identified in *ERCC4* in XP-F cells, providing genetic evidence that *ERCC4* equals *XPF* (62).

Using our cDNA constructs, we were unable to convincingly demonstrate correction of the related XP-F cell lines XP2YOSV and XP3YO in both transient and stable transfections. We attribute these negative results in part to the relatively mild UV-C sensitivity and partial repair capacity (21) of the immortalized XP-F cells combined with unpredictable growth upon subcloning and also to an apparent lack of stable expression of the *ERCC4* cDNA. The latter inference is suggested by the uncorrected UV sensitivity of six stable *neo* transformants of XP2YOSV resulting from cER4-40 transfection. Joint integration and coexpression of *neo* and *ERCC4* were expected for at least some transformants using linearized molecules. The failure of the overexpressing *ERCC4* cDNA to confer UV resistance was consistent with results of our attempt to transfer the *ERCC4* gene using cosmid pER4-7, which also gave no clear correction of Geneticin-resistant XP2YOSV cells. Thus, *ERCC4* expression controlled either by the CMV promoter or by its native promoter did not restore repair capability. Aberrant XP-F regulation or a dominant negative effect of a mutant allele might block expression of *ERCC4* transgenes in cells of the XP2YO lineage.

In an earlier study, somatic cell hybrids between diploid XP3YO and UV41 cells were produced by selecting for complementation of mitomycin sensitivity (72). Since significant restoration of both mitomycin and UV resistance was observed, it was concluded that the UV41 and XP-F mutations were in different genes. However, in retrospect we realize this interpretation was incorrect, since the results can be explained by the partial repair capacity of the XP-F mutant allele(s) (21). In a study involving microcell-mediated chromosome transfer, it was concluded that human chromosome 15 gave partial correction to UV resistance in XP2YOSV cells (54). This conclusion is at odds with the localization of *ERCC4* to chromosome 16p13.13-p13.2 (35) and can be attributed to the extreme population heterogeneity we observed for UV sensitivity with XP2YOSV.

Among the NER genes, *ERCC1* and *ERCC4* are uniquely involved in interstrand cross-link repair, as evidenced by the extreme sensitivity of a subset of the CHO mutants in groups 1 and 4 to cross-linking agents (26, 63, 84). Mutants in NER groups 2, 3, and 5 are apparently relatively proficient in cross-link repair (but not the repair of monoadducts accompanying cross-links), since they are much less sensitive to cross-linking agents. Thus, ERCC1 and ERCC4 proteins must possess a function, which is likely involved in homologous recombination, that assists in removing cross-links. A unique role for the Rad10 and Rad1 proteins in mitotic recombination (28, 58, 59) parallels the putatively unique roles of ERCC1 and ERCC4 in cross-link repair (see Discussion in reference 67). The cloned *ERCC4* cDNA and recombinant protein should help to elucidate ERCC4's putative recombinational repair function as well as help with further investigation into its possible role in recombination during meiosis.

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ADDENDUM IN PROOF

Fresh preparations of baboon poly(A)$^+$ RNA and human *ERCC4* cDNA probe were made and used in two additional Northern blots like those shown in Fig. 2B. In these new long-exposure blots, 7.5-kb hybridizing bands were detected in all of the tissues, which included ovary tissue; however, the 7.5-kb band was slightly more abundant in the lane containing RNA from testes. In addition, hybridizing bands of 4.4, 3.7 (most intense), and 2.0 kb were detected only in the testes.

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