Nucleotide excision repair is a major pathway for repairing UV light-induced DNA damage in most organisms. Using insertional mutagenesis, we isolated a UV-sensitive mutant of Chlamydomonas reinhardtii that is blocked in the excision of cyclobutane pyrimidine dimers. The mutant is also sensitive to the alkylating agent, methyl methanesulphonate. We have cloned REX1, a novel gene that rescues the mutant. The gene is unusual in a eukaryotic organism in that it is predicted to encode two different proteins, a small protein (8.9 kDa) and a larger protein (31.8 kDa). Neither protein is homologous to known DNA repair proteins. Partial complementation is achieved with subclones of the gene encoding only the 8.9-kDa protein. The 8.9-kDa protein has homologues in many organisms including Saccharomyces cerevisiae, Arabidopsis, and humans. The 31.8-kDa protein appears to be less conserved. These findings may be of general importance for DNA repair in other organisms.

UV light induces DNA damage, which can be both mutagenic and toxic for living organisms. Plants, which are dependent on sunlight for growth and survival, use internal filters such as pigments and cuticular waxes to limit the hazardous effects of light. However, these protection mechanisms are not adequate, and like any other organisms, plants do get UV-induced DNA damage. Proteins involved in DNA repair pathways in plant systems have not been studied extensively compared with mammalian, yeast, and prokaryotic systems. The current research indicates that plants employ more than one mechanism to repair UV damage (1, 2).

Nucleotide excision repair is a common repair pathway that is employed to repair UV damage by both prokaryotes and eukaryotes. The evidence that plants have a repair system similar to nucleotide excision repair in human and yeast mainly comes from identification of many Arabidopsis homologues of the known yeast and human repair proteins (3). So far, mutations in only a few of these genes have been isolated and correlated with repair defects in Arabidopsis (4).

Chlamydomonas reinhardtii is a single-celled, photosynthesizing alga, which is widely used as a model system for plants.
using a germicidal lamp. After appropriate dilutions, the cells were spread on tri-acetate-phosphate plates using the starch embedding method, which enhances the plating efficiency of cell wall-deficient cells (14). Plates were wrapped in aluminum foil and kept in the dark for \( \sim 18 \) h, then grown under fluorescent light until colonies grew big enough to count. Percent survival was calculated comparing the number of colonies survived after UV exposure to the number of colonies formed by unirradiated cells.

**Spot Test for Survival after UV or MMS Treatment**—10 \( \mu l \) aliquots of 1 to 3 serial dilutions of each culture grown to approximately the same cell density were spotted on either normal plates or a plate supplemented with 1.5 mM MMS. One plate was exposed to 100 \( \text{J} \text{m}^{-2} \) UV light and kept in the dark for 18 h before growing in the light.

**Immunoblot assay for Detection of Removal of CPDs—***Chlamydomonas* cell cultures were normalized to an OD reading of 0.850 at 700 nm. For each cell line tested, 25 \( \mu l \) of liquid culture in a 14-cm diameter Petri dish was irradiated with 30 \( \text{J} \text{m}^{-2} \) using a germicidal UV lamp. 3 \( \mu l \) was removed at 0 h, 24 h, and 18 h for DNA extraction (11). UV irradiation, incubations, and DNA extraction were all done under non-photoactivating conditions. The DNA was suspended in 50 \( \mu l \) of 10 mM Tris and 1 mM EDTA, pH 8, plus 50 \( \mu g \text{ml}^{-1} \) RNase A and incubated for 1 h at 37 °C to hydrolyze RNA. DNA was reprecipitated and resuspended in 200 \( \mu l \) water. DNA concentration of the samples was quantitated based on fluorescence in the presence of 0.2 \( \mu g \text{ml}^{-1} \) ethidium bromide using a Chemi-Imager 4000 (Alpha Innotech Corp.). Known concentrations of \( \lambda \) DNA were used as standards. DNA (125 ng) in 200 \( \mu l \) denaturation buffer (1.5 mM NaCl, 0.5 mM NaOH) was spotted on a nylon transfer membrane (Nytran, Schleicher & Schuell) pretreated with 2 \( \times \) SSC. The membrane was treated with neutralization solution (1 \( \text{M} \) Tris, 2 \( \text{M} \) NaCl, pH 5) and baked at 80 °C, then incubated for one hour with anti-thymine dimer-specific mouse monoclonal antibody KTM53 (Kamiya Biomedical Co.) at a 1:2500 dilution, followed with one-hour incubation with horseradish peroxidase-conjugated secondary anti-mouse antibody (Bio-Rad) at a 1:2500 dilution. Chemiluminescence (ECL+ plus Western blot Detection System, Amersham Biosciences) was used for detection of the primary antibodies. The signal intensity was quantitated using a ChemiImager 4000 (Alpha Innotech Corp.).

**RESULTS**

**Cloning the REX1 Gene—**Using insertional mutagenesis as a tool to tag genes (12), we isolated several UV-sensitive mutants of *C. reinhardtii*. A plasmid (pSP1245), carrying selectable marker, the ble gene, which confers resistance against zeocin (9) was randomly inserted into the *Chlamydomonas* genome by transformation (8). Transformants were selected based on zeocin resistance and subsequently screened for UV sensitivity. One mutant, 72E2, had very low survival in the dark after UV exposure and was defective in excision of CPDs based on *in vivo* repair assays (15) (data not shown). Importantly, it had only one insert based on Southern blots (data not shown), and this insert was linked to the UV-sensitive phenotype.

We used a probe from the ble gene to map the flanking regions of the insert and subsequently used the bookshelf library technique (12) to isolate both flanking regions (Fig. 1). Based on the linkage analysis, the disrupted gene must be located in the proximity of the insert. Therefore, we used probes isolated from both flanking regions to screen a *Chlamydomonas* BAC genomic library. We found a BAC, 29e18, which contained both of the flanking regions (Fig. 1). An arginine requiring strain of 72E2 was co-transformed with pUC ARG7.8 and BAC 29e18 DNA. From multiple transformations, 2—7% of the transformants were UV-resistant (data not shown). Transformation with just pUC ARG7.8 plasmid gave no UV-resistant transformants. Thus, this BAC contains the gene that was disrupted or deleted in 72E2. Therefore, we used BAC 29e18 to isolate this gene. Starting with the right flanking region (arbitrarily designated as shown in Fig. 1), we did a small-scale chromosome walk toward the left flanking region. The chromosome walk revealed that the insert had caused an unusually large deletion, \( \sim 57.6 \text{ kb} \), in 72E2. Despite such a large deletion, which eliminated 12 or 13 predicted proteins, the mutant grows at about the same rate as wild-type strains in the absence of DNA damaging agents (data not shown). We eventually isolated a 6.7 \( \text{kb} \) Kpn1-EcoRV fragment (2944, Fig. 1) that was able to confer UV resistance to 72E2 (Fig. 2, A and B).

**Determining the Exon-Intron Boundaries**—We have found expressed sequence tags that mapped to this 6.7-kb region. We determined the exon-intron boundaries of the gene by using the available expressed sequence tags and doing PCR using a cDNA library. As shown in Fig. 1, the gene has 8 exons and 7 introns. The predicted transcript from this region surprisingly has two open reading frames (ORFs), which are separated from each other by 50 bp. The ORF nearest to the 5’ end of the transcript encodes a putative protein of 78 amino acids (8.9 kDa), whereas the other ORF encodes a protein of 305 amino acids (31.8 kDa). We called the putative proteins REX1-S (small protein) and REX1-B (big protein).

**Complementation Analysis of Deletion Constructs**—To more clearly define the region required for rescue of the UV-sensitive mutant 72E2, we made a series of constructs as outlined at the bottom of Fig. 1. These constructs were transformed into 72E2 and tested for complementation by the UV spot test. The subclone 2945, which lacks exon 1 and the promoter region, did not complement the mutant cells (data not shown). Construct 2949 complements as well as 2944 (Fig. 2B). Construct 2948 is the
smallest subclone that confers UV resistance to 72E2, although this complementation is only partial (Fig. 2, A and B). Subclone 2948 includes the entire ORF for REX1-S but includes only the first 7 amino acids for REX1-B (Fig. 1). This result shows that REX1-S is required for UV resistance. However, the fact that the complementation by 2948 was partial relative to the complementation by 2944, which includes both ORFs, raised some questions. Construct 2948 is missing about 500 bp at the 5′ end relative to 2949, which might be important for full promoter activity. Construct 2948 is missing about 500 bp at the 5′ end relative to 2949, which might be important for full promoter activity. Construct 2948 also lacks the 3′ UTR, which contains the polyadenylation signal that might result in an unstable mRNA. To address these questions we made a deletion construct, 2950, which has the same 5′ upstream region as 2949 and also includes 283 bp of the 3′-UTR containing the polyadenylation signal but deletes the REX1-B coding region except for the first 37 amino acids. Complementation with 2950 conferred partial UV resistance to 72E2 similar to 2948 (Fig. 2B), which indicated that lack of full complementation by 2948 was not due to an incomplete promoter region or lack of a poly(A) tail. These results indirectly suggest that expression of both REX1-S and REX1-B may be necessary for the full complementation of the repair defect.

Fig. 2B illustrates that the mutant 72E2 is not only sensitive to UV but is also sensitive to the methylating agent, MMS. The recovery of resistance to MMS by 72E2 transformed with the constructs shown in Fig. 1 parallel closely the results found using UV as the damaging agent; i.e. 2944 gives nearly complete resistance, whereas constructs 2948 and 2950, which encode only REX1-S, give only partial complementation. Thus, the REX1 gene is required for resistance to both UV and MMS.

In the initial characterization of the mutant, 72E2, we monitored the repair of CPDs by alkaline agarose gel electrophoresis analysis of the DNA after digestion with T4 endonuclease V, which specifically cleaves next to CPDs (data not shown). These results showed that 72E2 was severely deficient in the rate of removal of CPDs in the dark. Using a more quantitative antibody spot test, we confirm this deficiency and show that 72E2 transformed with the constructs shown in Fig. 1 (except 2945) recover the ability to repair CPDs in the dark (Fig. 3). These results correlate very well with the survival results in that 2944 transformants, which give nearly wild-type resistance to UV, show a rate of removal of CPDs close to that of wild-type. The constructs 2948 and 2950, which encode only REX1-S, give partial UV resistance and have a rate of removal of CPDs slower than wild-type or 2944 transformants.

Homologues of REX1 Proteins in Other Organisms—A BLAST analysis using the REX1-S protein shows that it has homologues in many organisms, including Arabidopsis, Drosophila, S. cerevisiae, mouse, and human (Fig. 4A). Surprisingly, it does not have homology to any known repair protein. REX1-B protein also has homology to Arabidopsis and rice proteins of unknown function (Fig. 4B) and lower homology to human and mouse proteins. Interestingly, there is no S. cerevisiae homologue of REX1-B. In the middle of REX1-B there is a
A. REX1-S (8.9 kDa)

B. REX1-B (31.9 kDa)

FIG. 4. Alignment of the C. reinhardtii (C.r.) REX1 proteins with related protein sequences. Abbreviations and accession numbers of the REX1-S related proteins are as follows: Arabidopsis thaliana (A.t.), NP172702; Drosophila melanogaster (D.m.), Q9VM77; Homo sapiens (H.s.), AAH38848; Schizosaccharomyces pombe (S.p.), NP596155; S. cerevisiae (S.c.), NP768866; Mus musculus (M.m.), BAC25362; Neurospora crassa (N.c.), CAD21494. Abbreviations and accession numbers of the REX1-B-related proteins are as follows: A. thaliana (A.t.), NP196111; Oryza sativa (O.s.), BA990353; M. musculus (M.m.), NP79853; H. sapiens (H.s.), BAC30773. Amino acids that are identical to the REX1 sequences are shaded in black and similar amino acids are shaded in gray.

long stretch of mainly repetitive amino acids, which is not present in Arabidopsis and rice homologues.

DISCUSSION

We have isolated a novel gene that is required for resistance to both UV light and MMS. The prediction that this gene encoded two different proteins was unexpected for a eukaryotic organism. Although we have yet to show that both of these proteins are made in vivo, the fact that homologues of both proteins exist in other organisms lends credence to their existence. The two proteins are not coded by the same gene in other organisms. In Arabidopsis, the gene for the REX1-S homologue is on chromosome 1 and the REX1-B homologue gene is found on chromosome 5. The apparent bicistronic nature of the Chlamydomonas REX1 transcript is very unique in eukaryotes.

Whether REX1-S and -B proteins have any relationship in addressing this question, other than the data showing that subclones lacking REX1-B cannot complement as well as a subclone (2944), which includes the coding regions for both proteins (Figs. 2 and 3). We do not know what the precise function of REX1 might be in DNA repair. Nevertheless, we know that, whatever its function is, it must be before the excision step of the CPDs as our assays show that the mutant 72E2 cannot remove CPDs under non-photoreactivating conditions (Fig. 3, A and B). CPDs are known to be repaired by the nucleotide excision repair pathway in well studied species such as human, S. cerevisiae, and Escherichia coli (16). The yeast, S. cerevisiae, without doubt has been the most intensively studied model eukaryotic system. Recently, an international consortium has systematically deleted up to 95% of the known ORFs (17). A systematic screening of about 5000 of these deletion mutants has identified 103 genes that are required for resistance to MMS (18). A similar screen identified 31 genes that conferred sensitivity to UVC when deleted (19). The S. cerevisiae REX1 homolog is one of the about 5% of the ORFs that were not deleted in this project. Obviously, the effect on S. cerevisiae of deleting this gene will be of considerable interest. We believe that the REX1 homologs may prove to be of general importance in DNA repair.

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