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A eukaryotic gene encoding an endonuclease that specifically repairs DNA damaged by ultraviolet light

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Many eukaryotic organisms, including humans, remove ultraviolet (UV) damage from their genomes by the nucleotide excision repair pathway, which requires more than 10 separate protein factors. However, no nucleotide excision repair pathway has been found in the filamentous fungus *Neurospora crassa*. We have isolated a new eukaryotic DNA repair gene from *N.crassa* by its ability to complement UV-sensitive *Escherichia coli* cells. The gene is altered in a *N.crassa mus-18* mutant and responsible for the exclusive sensitivity to UV of the mutant. Introduction of the wild-type *mus-18* gene complements not only the *mus-18* DNA repair defect of *N.crassa*, but also confers UV-resistance on various DNA repair-deficient mutants of *Saccharomyces cerevisiae* and a human xeroderma pigmentosum cell line. The cDNA encodes a protein of 74 kDa with no sequence similarity to other known repair enzymes. Recombinant mus-18 protein was purified from *E.coli* and found to be an endonuclease for UV-irradiated DNA. Both cyclobutane pyrimidine dimers and (6–4)photoproducts are cleaved at the sites immediately 5′ to the damaged dipyrimidines in a magnesium-dependent, ATP-independent reaction. This mechanism, requiring a single polypeptide designated UV-induced dimer endonuclease for incision, is a substitute for the role of nucleotide excision repair of UV damage in *N.crassa*.

Key words: UV-endonuclease/*Neurospora crassa*/nucleotide excision repair/(6–4)photoproducts/cyclobutane pyrimidine dimer

Introduction

Throughout evolution, ultraviolet light (UV) has had a profound influence on living organisms, and the repair of UV-induced DNA damage has been of vital importance. The most important toxic and mutagenic UV-photoproducts are the cyclobutane pyrimidine dimers (CPDs) and (6–4)photoproducts [(6–4)PPs] (Friedberg, 1985; Mitchell and Nairn, 1989). Several different strategies for removing these photoproducts have been identified. Enzymatic photoreactivation (PHR) is a mechanism used in many microorganisms as well as in higher eukaryotes that specifically converts CPDs back to the monomeric form via a photolyase which uses the energy of visible light (see Sancar, 1990; Yasuhira and Yasui, 1992; Yasui *et al.*, 1994). A photoreactivating enzyme that acts on (6–4)PPs also exists in insects (Todo *et al.*, 1993). In a few known cases, as in the bacterium *Micrococcus luteus* and T4 phage-infected *Escherichia coli*, a pyrimidine dimer–DNA glycosylase/endonuclease (T4 endo) is found that specifically recognizes and removes CPDs by initiating base excision repair at sites where they occur (Grafstrom *et al.*, 1982). A versatile pathway that can remove both major UV photoproducts from DNA is nucleotide excision repair, which actually repairs (6–4)PPs more efficiently than cyclobutane dimers (Mitchell *et al.*, 1985; Svoboda *et al.*, 1993; Szymkowski *et al.*, 1993). Nucleotide excision repair (NER) is found in many prokaryotes and eukaryotes including many bacteria, yeast and humans.

Among the more than 30 mutagen-sensitive mutants of the filamentous fungus *Neurospora crassa*, NER-deficient mutants analogous to xeroderma pigmentosum (XP) in human cells or the *RAD3* epistasis group in the yeast *Saccharomyces cerevisiae* have not been found. Instead, an unusual phenotype was found in *mus-18* mutant cells, which are sensitive exclusively to UV but not to chemical mutagens (Ishii *et al.*, 1991). CPDs produced in the DNA of mutant cells by UV irradiation remained unrepaired even after 18 h of liquid holding (Ishii *et al.*, 1991). Originally we believed that *mus-18* mutant cells were defective in the repair of CPDs, possibly in a gene encoding an enzyme such as T4 endo. Since such a gene may complement the UV-sensitivity of NER-deficient *E.coli* cells, we introduced a *N.crassa* cDNA expression library into an *E.coli* strain, which is deficient in all three pathways for CPD repair in *E.coli* (NER, PHR and recombination repair). We report here that the gene defective in *mus-18* mutant encodes an endonuclease for both CPD and (6–4)PP sites.

Results

Cloning of a gene from N.crassa cDNA library complementing UV-sensitivity of E.coli strain SY2

We introduced a cDNA library of wild-type *N.crassa* into the repair-deficient *E.coli* SY2 strain and obtained a UV-resistant transformant harbouring a plasmid, pUVG31, which increased the UV resistance not only of SY2 but also of other *E.coli* host strains including *recA* (Figure 1). The nucleotide sequence of the 2.4 kb insert in pUVG31 was determined. The start codon was confirmed by sequence determination of a genomic fragment flanking

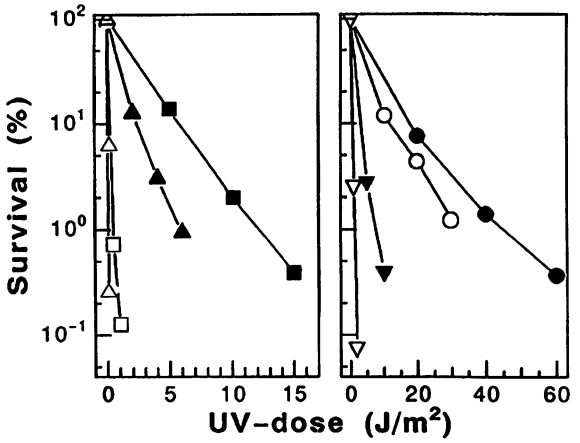

Fig. 1. Comparison of UV sensitivities of *E.coli* strains harbouring the cloned cDNA from *N.crassa* or vector plasmids. Survival curves of *E.coli* strains SY2 (*phr uvrA recA*) (triangles), SY1 (*phr uvrA*) (squares), KY29 (*phr recA*) (reverse triangles) and KY20 (*phr*) (circles) transformed with vector plasmid (open symbols) or with pUVG31 (closed symbols) after UV irradiation are shown.

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1    MPSRKSKAAALDTPQSESSTFSSTLDSSAPSPARNLRRSGRNILQPSSEK
51   DRDHEKRSGEELAGRMMGKDANGHCLREGKEQEEGVKMAIEGLARMERRL
101  QRATKRQKKQLEEDGIPVPSVVSRFPTAPYHHKSTNAEEREAKEPVLKTH
151  SKDVEREAEIGVDDVVKMEPAATNIIEPEDAQDAAERGAARPPAVNSSYL
201  PLPWKGRLGYACLNTYLRNSKPPIFSSRTCRMASIVDHRHPLQFEDEPEH
251  HLKNKPDKSKEPQDELGHKFVQELGLANARDIVKMLCWNEKYGIRFLRLS
301  SEMFPFASHPVHGYKLAPFASEVLAEAGRVAAELGHRLTTHPGQFTQLGS
351  PRKEVVESAIRDLEYHDELLSLLKLPEQQNRDAVMIIHMGGQFGDKAATL
401  ERFKRNYARLSQSCKNRLVLENDDVGWTVHDLLPVCEELNIPMVLDYHHH
451  NICFDPAHLREGTLDISDPKLQERIANTWKRKGIKQKMHYSEPCDGAVTP
501  RHRRKHRPRVMTLPPCPPDMDLMIEAKDKEQAVFELMRTFKLPGFEKIND
551  MVPYDRDDENRPAPPVKAPKKKKGGKRKRTTDEEAAEPEEVDTAADDVKD
601  APEGPKEVPEEERAMGGPYNRVYWPLGCEEWLKPKKREVKKGKVPEEVED
651  EGEFDG
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Fig. 2. Deduced amino acid sequences of the cloned gene. The amino acid sequence deduced from the 1986 bp ORF is shown. For underlined amino acid sequence see Discussion.

the 5′-side of the cDNA (accession number D11392). The sequence contains an open reading frame (ORF) of 1968 bases. The deduced amino acid sequence predicted a protein of M_r 74 412 with no significant similarity to any previously reported DNA repair enzymes including T4 endo (Figure 2).

Partial complementation of UV sensitivity in yeast rad mutants and human xeroderma pigmentosum cell line

To investigate the influence of the cloned gene on UV sensitivity in other organisms, the cDNA was introduced behind a yeast promoter and transformed *S.cerevisiae* strains of various DNA repair defects. Two mutants deficient in NER genes, *rad1* and *rad2*, as well as a post-replicative repair-deficient strain *rad18* were converted to UV-resistant cells by the introduced plasmid, whereas the UV sensitivity of the wild-type strain increased slightly by the transformation (Figure 3A). Similarly, human XP complementation group A (XPA) cells harbouring the cDNA obtained UV resistance at a level ~50% of that of HeLa cells (Figure 3B). The UV resistance of HeLa cells was not influenced by the introduction of the plasmid (not shown). Thus, the cloned gene provided DNA repair-

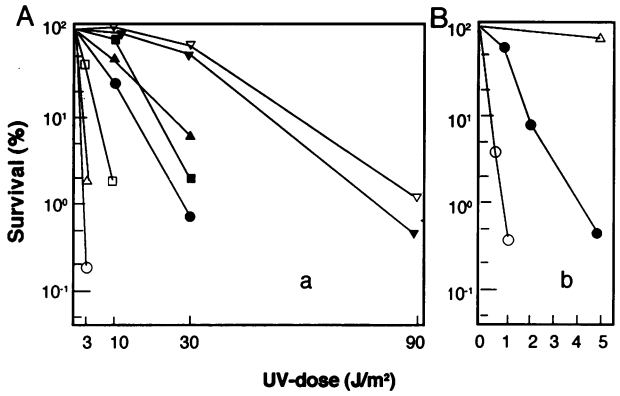

Fig. 3. Survival curves of yeast and human cell lines. (**A**) *S.cerevisiae* strains of GRF18 (wild-type) (reverse triangles), ya1031 (*rad1*) (circles), ya2-2 (*rad2*) (triangles), and ya18-1 (*rad18*) (squares) transformed with vector plasmid pKT10-*LEU2* (closed symbols) or with cloned cDNA in the vector (open symbols) after UV irradiation. (**B**) XPA cell lines transformed with a vector pcDNA (open circles) or plasmid with cloned cDNA behind CMV promoter in the vector (closed circles); level of UV-resistance for HeLa cells transformed with the vector plasmid (open triangles).

deficient cells from various species with increased resistance to UV irradiation. This strongly suggests that the ORF encodes a factor which acts independently of other cellular proteins to support repair of UV damage, possibly at an initiation step of repair processes.

Relationship between the cloned gene and the mus-18 mutation

We examined the relationship between the cloned gene and the *mus-18* mutation of *N.crassa*. We isolated a DNA fragment covering the whole cDNA region from a genomic library of *N.crassa* and introduced this genomic fragment into *mus-18* mutant cells. Two stable transformants showed the same resistance as the wild-type to UV (Figure 4A). Furthermore, we inactivated the *mus-18* gene in the wild-type strain by using repeat-induced point mutation (RIP). RIP utilizes a general response of *N.crassa* that pre-meiotically inactivates duplicated DNA sequences by introduction of point mutations (Selker *et al.*, 1987; Selker and Garrett, 1988). Two offspring from a cross between wild-type cells and wild-type cells transformed with the cloned genomic gene showed the same sensitivity to UV as the *mus-18* mutant (Figure 4B), indicating that both endogenous and introduced *mus-18* genes were inactivated by RIP in these clones. Finally, Southern hybridization analysis of wild-type and *mus-18* mutant genomic DNAs demonstrated alterations including a deletion in the mutant genome (Figure 4C). From these results, we concluded that the cloned gene is defective in the *N.crassa* mutant *mus-18*. We designated the cloned gene as the *mus-18* gene.

Purification of mus-18 protein from E.coli

A tag encoding eight amino acid residues called FLAG was fused to the *mus-18* gene at the 3′-end and the recombinant gene was expressed in repair-deficient SY2 host cells. The recombinant gene provided the host cells with the same UV resistance as the gene without the tag (not shown). The recombinant protein was purified with the help of antibody against FLAG as well as nicking activity to UV-irradiated plasmid. Since the deduced amino

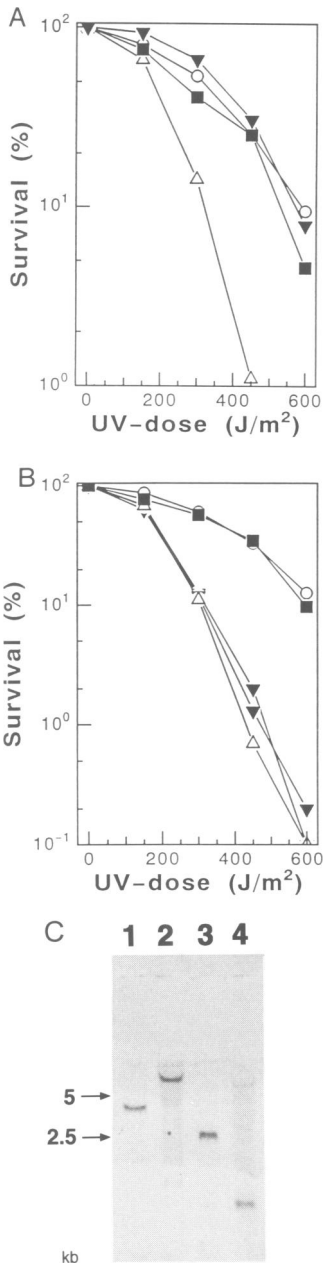

Fig. 4. Influence of the introduction of the cloned gene into *mus-18* mutant or wild-type cells of *N.crassa* on UV survival.
(**A**) Complementation of UV sensitivity of the *mus-18* mutant cells by introduction of a genomic fragment of *N.crassa* covering the cloned cDNA in the plasmid pUVEE1. UV sensitivities of the *mus-18* mutant cells transformed with a pUVEE1 derivative carrying a hygromycine-resistant marker (hygB) (closed squares) or by co-transfection of pUVEE1 with plasmid pCSN44 carrying hygB (closed reverse triangles) as well as those of the wild-type (open circles) and the *mus-18* mutant (open triangles) are shown. (**B**) UV sensitivities of the cells with *mus-18* gene inactivated with RIP. UV sensitivities of the wild-type (open circles), wild-type cells transformed with pUVEE1 (closed squares), the *mus-18* mutant (open triangles) and two offspring obtained from the cross (closed reverse triangles). (**C**) Rearrangement of genomic DNA in *mus-18* mutant cells. Southern hybridization of genomic DNA prepared from wild-type (lanes 1 and 3) and *mus-18* mutant (lanes 2 and 4) digested with *Eco*RI (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4) to a genomic DNA covering the cloned gene.

acid sequence in the ORF of the cloned cDNA contained a number of successive histidine sequences (see underlined sequences in Figure 2), we first applied extract of the cells to Ni^{2+}-nitrilotriacetic acid (Ni–NTA) agarose. Purification was followed by phosphocellulose and heparin sepharose affinity chromatography. SDS-gel electrophoresis of the fractions which possessed nicking activity against UV-irradiated plasmid are shown in Figures 5A and B. Fractions of the same elution from the cells transformed with vector plasmid (Figure 5A) did not show such activity (Figure 5B). More than 95% of the final fraction contained the product of the *mus-18* gene, including its degradation product as judged from the gel (Figure 5A), as well as from the Western blot of the gel using antibody against FLAG (Figure 5A). Thus, we confirmed that the gene product of *mus-18* alone introduces nicks into UV-irradiated DNA. The purified protein requires Mg^{2+} but not ATP in the nicking reaction against UV-irradiated plasmid (Figure 5D).

Determination of substrates and sites for nicking activity of purified mus-18 protein in UV-irradiated plasmids and synthetic oligonucleotides

To identify nucleotide sequences nicked by *mus-18* protein (*Mus-18*), we treated a linearized plasmid with *Mus-18*. The enzyme introduced nicks only when the plasmid had been irradiated with UV (Figure 6). DNA bands with different intensities were produced at TC, TT, CC and CT sequences by treatment of UV-irradiated DNA with *Mus-18*. As shown by the migration speed of DNA fragments on the polyacrylamide gel electrophoresis (PAGE) in Figure 6, those produced with *Mus-18* migrated more slowly than the sequence markers. The positions of the bands at the TC and TT sequences indicated by arrows a and b were close to the first nucleotides of the dipyrimidine sequence markers at the top of the PAGE and behind the second nucleotides at the bottom of the PAGE (shown by c and d). This suggests that, in contrast to the DNA fragments of sequence markers with 3′-phosphate produced by chemical destruction of bases, *Mus-18* nicked DNA at the site immediately 5′ to the dipyrimidines, leaving 3′-OH.

To identify the DNA damages nicked by *Mus-18* and to examine how it introduces nicks at the damaged sites, a 54mer oligonucleotide containing only two dipyrimidine sequences of TC and TT and an oligonucleotide with its complementary sequence were synthesized and purified (Figure 7A). Considering the above results obtained with labelled plasmids, cleavage sites for two restriction enzymes of *Nsi*I and *Nla*III were introduced immediately 5′ to the TC and TT sequences, respectively. This oligonucleotide, which had been labeled at 5′-sites or 3′-ends and annealed to its complementary oligonucleotide, was used as substrates for *Mus-18* after UV irradiation. 5′-end-labelled DNAs, which were nicked at TC or TT sites with *Mus-18*, migrated faster than those cleaved with T4 endo (Figure 7B, lanes 1 and 2). To determine whether the substrates for *Mus-18* were CPDs n(6–4)PPs, UV-irradiated DNA was exposed to visible light after addition of *Anacystis* photolyase for CPDs or *Drosophila* photolyase for (6–4)PPs before incubation with *Mus-18* (Figure 7, lanes 3–6). Removal of UV-induced (6–4)PP and CPD lesions by PHR led to an almost complete disappearance of the band at TC (lane 3) or TT (lane 5), whereas PHR with photolyase for CPDs repaired all the substrates for T4 endo (lane 6). These results indicate that, in contrast

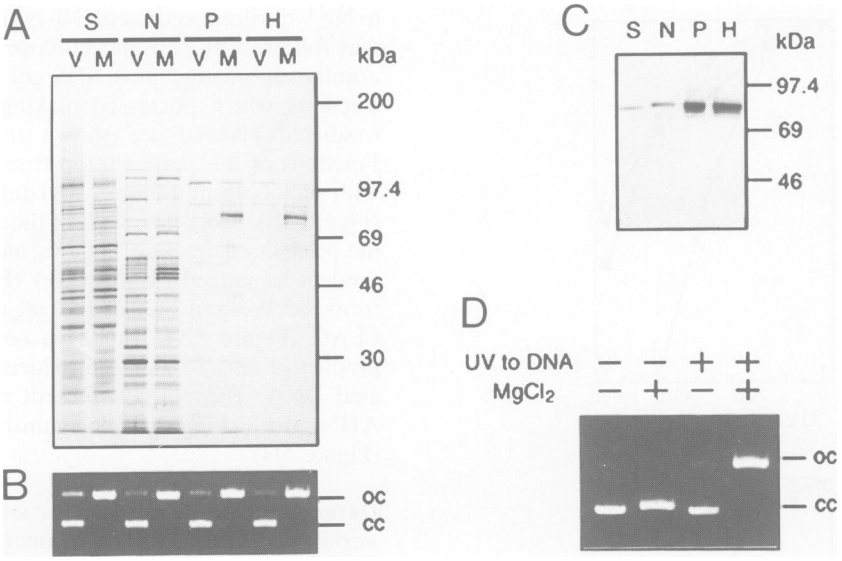

Fig. 5. Purification of *mus-18* gene product from *E.coli* transformant cells and its nicking activity against UV-irradiated plasmids. (**A**) SDS–PAGE of the supernatant of crude extract (S) or the fractions with nicking activity prepared from Ni-NTA (N), phosphocellulose (P) and heparin–sepharose (H) of SY2 cells harbouring pK*mus-18*FLAG are shown on lanes M. The same fractions as well as the supernatant of crude extract from SY2 cells harbouring vector plasmids are shown on lanes V. (**B**) Nicking activities of the fractions against UV-irradiated plasmids. (**C**) Western blot of each fraction with nicking activity using antibody raised against FLAG. (**D**) Mg^{2+} dependency of nicking activity in phosphocellulose fraction of *Mus-18*. No ATP was included in this reaction mixture.

to T4 endo, *Mus-18* recognized both CPDs and (6–4)PPs and cleaved DNA at the sites of damage. 3′-end-labelled DNA nicked with *Mus-18* migrated slightly more slowly than that nicked with T4 endo (Figure 7C, lanes 1 and 2), which corresponds to the results with 5′-end-labelled DNA and indicates that *Mus-18* introduces a single nick at a site 5′ to the sites nicked with T4 endo.

Figure 7D shows that 5′-end-labelled DNAs nicked with *Mus-18* at TC or TT sequences (lane 2) migrates to the same positions as those created after digestion with *Nsi*I or *Nla*III, respectively (lanes 1–3). The cleaved DNAs served as substrates for terminal deoxytransferase with ddTTP, resulting in an addition of a nucleotide (lanes 4–6), indicating the existence of a 3′-OH terminus. Migrations of 3′-labelled and *Mus-18*-nicked DNA fragments were also the same as those produced by the restriction enzymes (Figure 7E, lanes 1–3). Subsequent treatment of cleaved DNA with calf intestinal phosphatase (CIP) slightly retarded the migrations of all DNA (lanes 4–6), indicating the existence of a phosphate at the 5′-end of DNA nicked with *Mus-18*.

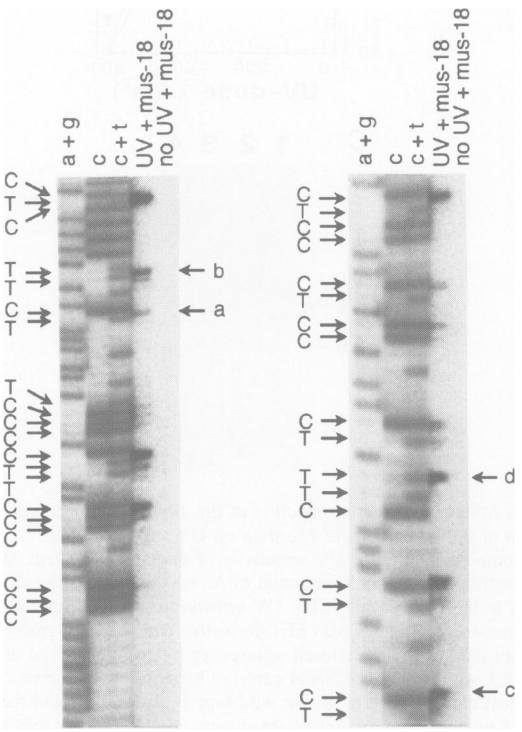

Fig. 6. Determination of substrates for nicking activity of *Mus-18*. PAGE of 5′-end-labelled plasmid (with or without UV) treated with *Mus-18*. PAGE of one experiment is shown from the left panel to the right. For a, b, c and d, see text.

Discussion

mus-18 gene and mutation in N.crassa UV-sensitive mutant

We have herein reported the isolation of a unique eukaryotic DNA repair gene by complementation of UV-sensitive *E.coli* mutant cells with an introduced cDNA library from *N.crassa*. The *mus-18* gene encodes a novel protein with a highly hydrophilic carboxy terminus. There are many sequences consisting of successive basic and/or acidic amino acid residues. Among them the sequence 4×Lys-2×Gly-2×(LysArg) (underlined in Figure 2) is similar to that found at the carboxy terminus of protamine, 6×Arg-2×Gly-4×Arg in the protamine IA of rainbow trout (Ando and Watanabe, 1969), a peptide which interacts closely with DNA. *mus-18* genes with various deletions in this sequence influenced the complementing activity of UV-sensitivity for *E.coli* host SY2 cells (not shown), suggesting important role(s) of the sequence in the enzymatic function of the gene product.

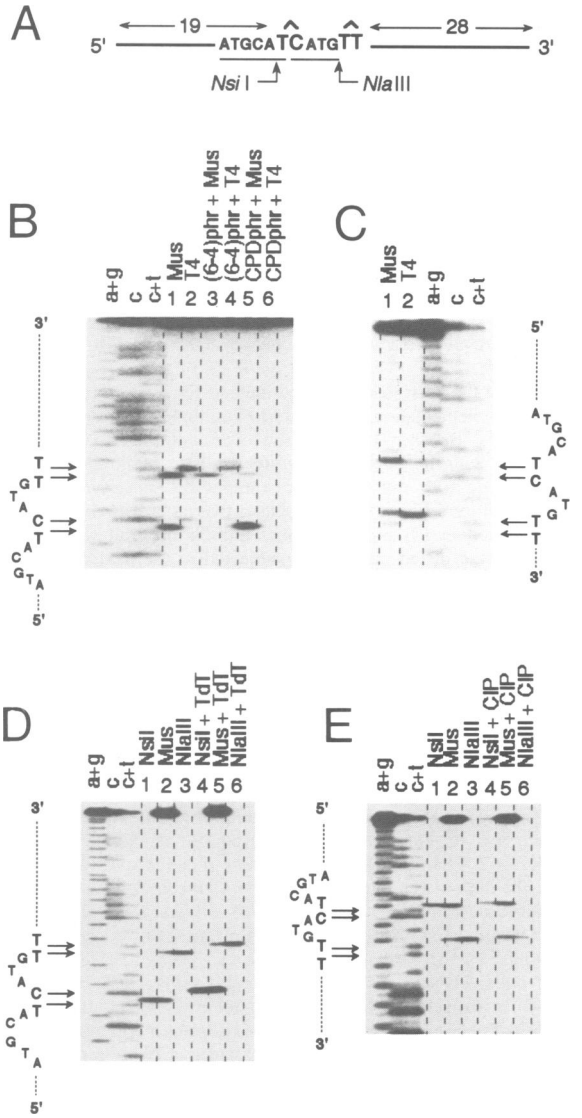

Fig. 7. Determination of substrates and nature of nicks introduced by *Mus-18*. (**A**) Structure of synthetic oligonucleotide NN3 containing two dipyrimidine sequences. Recognition sequences and cleavage sites for *Nsi*I and *Nla*III enzymes are shown by underlines and arrows, respectively. Numbers of nucleotide are indicated. (**B**) PAGE analysis of 5′-labelled NN3 nicked by *Mus-18* (Mus) or T4 endo (T4) with and without PHR. NN3 annealed with its complementary oligo was UV-irradiated and treated with *Mus-18* (lane 1) or with T4 endo (lane 2). UV-irradiated and annealed DNA was subsequently photoreactivated with (6–4)photolyase (lanes 3 and 4) or with CPD photolyase (lanes 5 and 6) before treatment with *Mus-18* (lanes 3 and 5) or with T4 endo (lanes 4 and 6). (**C**) PAGE analysis of 3′-end-labelled NN3 nicked with *Mus-18* or T4 endo. After UV irradiation, DNA was treated with *Mus-18* (lane 1) or with T4 endo (lane 2). (**D**) PAGE analysis of 5′-end-labelled NN3 nicked with *Mus-18* or with restriction enzymes. UV-irradiated DNA was nicked with *Mus-18* (lane 2) and treated with TdT in the presence of ddTTP (lane 5). For comparison of migration in PAGE, undamaged DNA was digested with *Nsi*I (lane 1) or with *Nla*III (lane 3), and the ddTTP-incorporated (lanes 4 and 6, respectively). (**E**) PAGE analysis of 3′-end-labelled NN3 nicked by *mus-18* protein. UV-irradiated DNA was treated with *mus-18* protein (lane 2) and subsequently with CIP (lane 5). For comparison of migration, undamaged DNA was digested with *Nsi*I (lane 1) or *Nla*III (lane 3), and dephosphorylated (lanes 4 and 6, respectively).

We showed the relationship between the *mus-18* mutant of *N.crassa* and the cloned gene in three different ways: complementation of UV-sensitivity in mus-18 mutant by

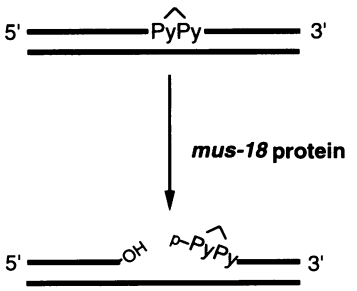

Fig. 8. Model for incision introduced with *Mus-18* at a UV-induced pyrimidine dimer.

introduction of the cloned gene, RIP and a comparison of Southern analyses between wild-type and mutant genomic DNA using the cloned gene as the probe. Judging from the Southern analysis, the mutation includes a deletion at a part of genomic fragment containing the *mus-18* gene. These results indicate that the intermediate UV sensitivity of the *mus-18* mutant is due to the deficiency of *Mus-18*.

Incisions introduced by purified Mus-18

The *mus-18* gene encodes a protein which possesses nicking activity against both CPDs and (6–4)PPs. Using plasmid and synthetic DNAs, we showed that *Mus-18* introduced nicks at the sites immediately 5′ to UV-induced CPDs as well as (6–4)PPs, resulting in a single strand break on the DNA strand with damage (Figure 8). Substrates for *Mus-18* include UV-induced dimers produced at di-pyrimidine sequences of TT, TC, CC and CT. The difference of intensities of the nicked bands shown in Figure 6 are thought to be due to either different production of damage by UV and/or different preference of repair by *Mus-18*. Because of high UV-doses (3 kJ/m^2) given to the plasmid as well as to the oligonucleotides, a relatively large amount of (6–4)PPs at TC sequences was produced. After a low dose of UV irradiation to DNA, TT sites were predominantly cleaved by the protein (not shown). Although we do not presently know the preference of nicking activities among UV-induced dimers by the protein, there seems to be a difference in recognition by the protein between CPDs and (6–4)PPs. To introduce a nick at a cyclobutane-type thymine–thymine dimer, *Mus-18* would need a longer flanking sequence at the 3′-side to the damage, when compared with (6–4)PPs at the same place as judged from analysis with synthetic DNAs of various lengths (unpublished results). This may suggest the importance of the flanking region of the damage for its recognition by *Mus-18*.

Complementation of UV sensitivities in host cells by mus-18 gene

Another interesting question which arose from our findings on *Mus-18* is how the nicked sites are further processed in the cells. The *mus-18* gene introduced in *E.coli*, yeast and human cells similarly increased the UV-survival of these host cells, suggesting that there exists repair pathway(s) processing the DNA nicked by Mus-18 protein in these organisms. In this respect, it resembles T4 endo, which can confer UV resistance on yeast rad mutants and human XP cells (Valerie *et al.*, 1986, 1987). In yeast cells, a post-replicative repair-deficient *rad18* mutant with

complete NER activity as well as two mutants of NER, *rad1* and *rad2*, which are defective in nicking activities at 5′ and 3′ to DNA damage, respectively (Bardwell *et al.*, 1994; Harrington and Lieber, 1994), were provided with a similar increase of UV resistance by the *mus-18* gene (Figure 3). These results suggest that nicks introduced by *Mus-18* are further processed by repair pathway(s) independent of incision machinery of NER in these organisms. However, complementation of UV sensitivities in various repair-deficient host cells except *N.crassa mus-18* mutant was only partial and did not attain the UV resistance of wild-type cells. Nicked sites with dimers at the 5′-ends may not be suitable substrates for 5′–3′ exonucleases. Whether *N.crassa* possesses a special exonuclease(s) for this purpose remains to be determined.

Role of Mus-18 and its distribution

In so far as we investigated, we obtained no evidence that *Mus-18* introduces nicks at DNA damage other than UV-induced damage; this coincides well with the phenotype of the *mus-18* mutant, which has exclusive sensitivity to UV. Although *Mus-18* may recognize the structural changes of DNA, which are caused by chemical agents and are similar to those produced by UV-induced dimers at dipyrimidine sequences, the natural substrates for *Mus-18* are apparently UV-induced dimers. *N.crassa* has little, if any, inefficient NER against UV damage (Ishii *et al.*, 1991). Therefore, the *mus-18* gene may have substituted the role of NER for UV-induced damage in this organisms. Recently, it was reported that mutants of *Schizosaccharomyces pombe* with a complete defect in NER retain considerable capacity for repair of CPDs and (6–4)photoproducts in DNA (McCready *et al.*, 1993). More recently, an enzymatic activity in a cell extract of *S.pombe* was reported, which introduces nicks at TT and TC dimers in a fashion similar to *Mus-18* (Bowman *et al.*, 1994). Since *S.pombe* does not possess any CPD photolyase activity (Yasui *et al.*, 1989), it would appear that a homologue of *Mus-18* may support the repair of UV-induced CPDs in *S.pombe*. In contrast to *S.pombe*, *N.crassa* is characterized by very efficient CPD photolyase activity (Yajima *et al.*, 1991; Eker *et al.*, 1994). *Mus-18*, therefore, plays important roles in removal of UV-induced (6–4)PPs, which are the main target of NER in many eukaryotes. Thus, *Mus-18* and its homologue seem to compensate for the lack of NER or PHR of UV-induced DNA damage in evolutionary closely related organisms of *N.crassa* and *S.pombe*, respectively. A similar enzyme may also be present in other organisms.

The purified protein of *mus-18*, which is now designated 'UV-induced dimer endonuclease' of *Neurospora*, will be a useful tool for identifying UV-induced damage in cells, as T4 endo is used for analysis of remained CPDs. All UV-induced dimers at dipyrimidine sequences including CPDs and (6–4)PPs or, in combined use with CPD photolyase, unrepaired (6–4)PPs can be detected.

Materials and methods

Strains and cell line

For expression cloning of cDNA from *N.crassa*, *E.coli* strain SY2 (JM107 Δ*phr::Cm*^{r} Δ*uvrA::Km*^{r} Δ*recA::Tet*^{r}) (Yasuhira and Yasui, 1992) was used. SY1 (SY2 without Δ*recA*), KY29 (SY2 without Δ*uvrA*) and KY20 (Δ*phr::Cm*^{r}) were also transformed with the cloned gene. KY20 and KY29 were obtained from Dr K.Yamamoto. Yeast *S.cerevisiae* strains used in this study were: GRF18 (wild-type, *leu2*), ya1031 (*rad1 leu2*), ya2–2 (*rad2 leu2*) and ya18-1 (*rad18 leu2*). These strains except for GRF18 (a gift of Dr G.R.Fink) were established by A.Yasui. The XPA cell line, XP12ROSV, was obtained from Dr K.Tanaka.

cDNA and genomic libraries

The cDNA library of *N.crassa* constructed in lambda ZAP (Orbach *et al.*, 1990) was obtained from Dr M.S.Sachs. It was converted by *in vitro* excision to an expression library for *E.coli*. A genomic library was made from wild-type *N.crassa* by introduction of *Eco*RI-digested DNA into λEMBL3.

Cloning and sequencing of N.crassa cDNA and genomic DNA

We followed the previously reported method for isolation of photolyase gene by complementation in SY2 cells (Yasuhira and Yasui, 1992) with modifications. Briefly, 100 μl of the overnight culture of SY2 cells transformed with the cDNA library was irradiated with UV of 0.1 J/m^2 on a LB plate with four antibiotics (ampicillin, kanamycin, chloramphenicol and tetracycline) and incubated overnight. Surviving colonies were collected in LB plus antibiotics and further cultured. The cell-suspension was plated on LB and subjected to the next round of UV irradiation. After three rounds of selection, a number of surviving colonies were examined to test their UV resistance.

The genomic library was screened with the cloned cDNA probe, and a 4.7 kb *Eco*RI fragment containing the whole sequence of the cloned cDNA was isolated. Nucleotide sequences of the entire cDNA containing 2422 bp as well as of a 1 kb genomic fragment containing the upstream sequence of the ORF were determined on both DNA strands.

Plasmids construction and transformation for expression of the cloned gene in E.coli, yeast and human XPA cell lines

For expression of the *mus-18* gene product in *E.coli* strains, the coding sequence from the cloned cDNA was introduced behind the *trc* promoter of pKK233-2 (Pharmacia). For expression of the *mus-18* gene in yeast strains, the cDNA was introduced behind a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter in pKT10 plasmid (obtained from Dr M.Nakafuku) with a *LEU2* selection marker. Yeast cells were transformed by the method of Hinnen *et al.* (1978). For expression in human cells, cDNA was introduced behind the CMV promoter of pcDNAI (Invitrogen). Cells were transfected by Lipofectin (Gibco BRL) together with plasmid harbouring a G418-resistant marker. Selection was made with 800 mg/ml of G418 (Wako, Japan). Colony-forming ability after UV irradiation with various doses was determined for transformed *E.coli*, yeast and human cells. The data present mean values of at least two independent experiments. For purification of the *mus-18* gene product, a tag of eight amino acid residues, FLAG (Kodak), were attached by PCR at the end of the coding sequence before the stop codon. The cDNA with the tag was introduced behind the tac promoter of pKKENS, a derivative of pKK 223-3 (Pharmacia), resulting in pK*mus-18*FLAG.

Transfection of N.crassa and repeat induced point mutation (RIP)

A 4.5 kb *Eco*RI genomic DNA fragment harbouring the whole *mus-18* gene was attached with a hygromycin resistant marker (HygB) and introduced into *mus-18* mutant cells. Normally, several copies of DNA are integrated into the genome of *N.crassa*. To inactivate the *mus-18* gene by the repeat induced point mutation (RIP) phenomenon, the *N.crassa* wild-type strain carrying two *mus-18* genes was crossed to the wild-type strain of the opposite mating type, using a previously reported method (Selker and Garrett, 1988).

Southern analysis of wild-type and mus-18 mutant DNA of N.crassa

Genomic DNA from wild-type and *mus-18* mutant cells was isolated by the standard method, digested with restriction enzymes, and blotted on a nitrocellulose filter. A DNA probe was prepared from the cloned genomic DNA containing the whole *mus-18* gene by digestion with *Eco*RI. Hybridization was performed by using a non-radioactive DNA labelling and detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

Purification of mus-18 gene product from E.coli host cells

SY2 cells harbouring PK*mus-18*FLAG or vector plasmid were grown until an OD of 0.5 in 1 l of LB medium supplemented with carbenicillin

was attained. After expression had been induced by 1 mM IPTG for 5 h at 37°C, cells were collected in 20 ml sonication buffer (300 mM NaCl, 50 mM NaH_2PO_4, 1 mg/ml lysozyme and 1 mM PMSF, pH 8.0) and disintegrated by sonication for 5 min at 0°C. Cell debris was removed by centrifugation (20 min, 43 000 *g*, 4°C). Crude extract was applied on a 3 ml Ni–NTA agarose (Qiagen) column and fractions eluted from 15 mM imidazole containing 0.3 M NaCl were collected. The fraction was diluted with buffer A (50 mM Tris–HCl pH 7.5 and 1 mM EDTA) to adjust NaCl concentration to 0.2 M and loaded on a phosphocellulose column (1.5 ml bed volume), which had been pre-equilibrated with 0.2 M NaCl–buffer A. *Mus-18* was eluted at 0.35 M NaCl–buffer A. The fraction was then loaded on a heparin sepharose column (0.3 ml bed volume) pre-equilibrated with 0.2 M NaCl–buffer A. The *mus-18*-FLAG protein was eluted at 0.4 M NaCl–buffer A. Yield was 72 mg of *mus-18*-FLAG protein with 97% homogeneity (Figure 5A). The purified protein was stored in a solution of 25 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 200 mM NaCl, 1 mM DTT, 200 mg/ml BSA and 40% glycerol at –20°C, and used for all experiments, unless otherwise mentioned.

Plasmid incision assay and unit definition of purified Mus-18

Closed circular plasmid DNA was UV-irradiated with 0.1 kJ/m^2 or 1 kJ/m^2 at a DNA concentration of 0.2 μg/μl and used as a standard assay or unit definition, respectively. The *mus-18*-containing fraction was mixed with 0.1 μg DNA in a total 10 μl volume of an optimized reaction buffer (50 mM Tris–HCl pH 7.9, 0.1 M NaCl, 20 mM $MgCl_2$, 1 mM DTT and 100 mg/ml BSA) at 37°C for 30 min. The reaction was stopped by heating at 65°C for 10 min and analysed on 0.75% agarose gel. One unit of the *mus-18* nicking activity is defined as the amount required to convert UV-irradiated ccDNA (1 kJ/m^2) to ocDNA in 60 min at 37°C in a 100 ml reaction volume. Specific activity of the purified *Mus-18* was 780 units/mg. For T4 endo, buffer used for *Mus-18* was used with 20 mM EDTA and without $MgCl_2$.

Incision assay using plasmid DNA and synthetic oligonucleotides for substrate and site determination of nicks

Anacystis nidulans photolyase for CPDs (Eker *et al.*, 1990) and *D.melanogaster* photolyase for (6–4)PPs (Todo *et al.*, 1993) were generous gifts from Drs A.P.M.Eker and T.Todo, respectively. T4 endo was purified from *E.coli* strain harbouring the den V gene behind the *tac* promoter obtained from Drs K.Valerie and K.de Riel. PHR was performed in transparent tubes with fluorescent light at a distance of 10 cm for 1.5 h at room temperature. *Mus-18* protein as well as T4 endo were used at 37°C for 1 h. For analysis of nicked sites shown in Figure 6A, pBluescript plasmid was digested with *Xba*I, dephosphorylated and labelled with [γ-^{32}P]ATP. Labelled DNA was digested with *Sac*I and isolated from 5% neutral gel after PAGE. Half of the DNA was irradiated with 3 kJ/m^2 UV (254 nm, 5J/m^2 s) and treated with *Mus-18*. Chemical destruction of bases after the method of Maxam–Gilbert (Maxam and Gilbert, 1980) was performed using the other half of the labelled DNA.

An oligonucleotide with two dipyrimidine sequences (underlined): 5′-GTA TAC ACA CAC GTA TGC ATC ATG TTA TAC GCA CAC CAC AGT GCA TAC ACA TAT AGC-3′ was synthesized and purified by PAGE using 15% acrylamide gel. It was labelled at 5′- or 3′-sites with [γ-^{32}P]ATP using polynucleotide kinase (Takara) or with [α-^{32}]P-ddATP using terminal deoxy-transferase (TdT, Boehringer Mannheim), respectively. DNA of correct size was once more purified by PAGE (15% gel) and annealed with a complementary strand. After UV irradiation (3 kJ/m^2), DNA was treated with *Mus-18* and analysed by PAGE (15% gel) together with unirradiated DNA digested with restriction enzymes. TdT or calf intestine phosphatase (Takara) was used to add a nucleotide at the 3′-end with cold ddTTP or to remove a phosphate at the 5′-end of the nicked DNA, respectively. We always used 35 U of *Mus-18* for 10 fmol DNA. Sequence and nicked sites were analysed after PAGE by a BAS 2000 Image Analyser (Fuji Film).

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