Nucleic Acids Research, 1996, Vol. 24, No. 7 **1267–1271**

Characterization of a UV endonuclease gene from the fisson yeast *Schizosaccharomyces pombe* and its bacterial homolog

Masashi Takao[1], Rie Yonemasu[1,2], Kazuo Yamamoto[2] and Akira Yasui[1,*]

[1]Department of Neurochemistry and Molecular Biology, Institute of Development, Ageing and Cancer, [2]Faculty of Science, Tohoku University, Sendai, 980-77, Japan

Received December 19, 1995; Revised and Accepted February 20, 1996

DDBJ accession no. D78571

ABSTRACT

From the fission yeast *Schizosaccharomyces pombe,* a cDNA fragment was isolated, which confers UV resistance on repair deficient *Escherichia coli* host cells. The cloned cDNA encodes a protein of 68 815 Da, which has a 36.6% identity of amino acid sequence with the previously identified 74 kDa UV endonuclease of the filamentous fungus *Neurospora crassa.* Analysis of several truncated gene constructs shows that only the C-terminal two thirds region, which has 54% identity of amino acid sequence with the C-terminal region of the *Neurospora* homolog, is necessary for complementing activity of UV-sensitivity in the *E.coli* host cells. Purified recombinant protein from *E.coli* host cells incises both UV-induced cyclobutane pyrimidine dimers and (6–4) photoproducts at the sites immediately 5′ to the DNA damage in the same fashion as the *Neurospora* protein. Furthermore, a bacterial homologous sequence was isolated from *Bacillus subtilis* and shows a similar complementing activity of UV sensitivity in *E.coli* host cells, indicating a wide distribution of this alternative excision repair mechanism in life.

INTRODUCTION

Ultraviolet light (UV) induces two major types of DNA damage: cyclobutane type pyrimidine dimers (CPDs) and (6–4) photoproducts [(6–4)PPs]. To ensure survival in a UV-rich environment, various DNA repair mechanisms exist to remove UV-induced DNA damage. One of the simplest and most effective ways to repair UV-induced damage is photoreactivation, in which a single enzyme named photolyase eliminates CPDs by transfer of absorbed visible light energy to CPDs as electrons to split CPDs by β-elimination (1). In contrast and in addition to this single enzyme process, another important and efficient repair mechanism for UV-induced DNA damage is nucleotide excision repair (NER) which requires a large protein complex, recently termed the ‘repairosome’. It contains more than ten independent gene products which recognize DNA damage and introduce incisions into the neighbourhood of the damaged sites on DNA (2,3).

Recently, we isolated a novel gene encoding a protein with an incision activity acting against both CPDs and (6–4)PPs from the filamentous fungus *Neurospora crassa* (4). Since *N.crassa* does not possess NER, this repair activity seems to play a substitutional role for NER in this organism. We designate this gene product, which is defective in the UV-sensitive mutant mus-18, as a *N.crassa* UV-damage endonuclease (NC-UVDE).

A similar enzymatic activity has been reported in an extract of the fission yeast *Schizosaccharomyces pombe* (5). To identify the gene responsible for this activity, we applied the same method as that used for isolation of NC-UVDE, namely, the complementation of repair-deficient *E.coli* cells after introduction of a foreign cDNA library and selection by UV irradiation of the transformants. We isolated a *S.pombe* homolog of the NC-UVDE gene. Herein we report the characterization of the gene and its product as well as a homolog of UVDE from the Gram positive bacterium *Bacillus subtilis.*

Cloning of *S.pombe* cDNA

Schizosaccharomyces pombe cDNA library (Clontech) in a vector pGADGH was introduced in *E.coli* SY2 (JM107 Δ*phr*::Cm^r Δ*uvrA*::Km^r Δ*recA*::Tet^r). Selection of UV-resistant clones was performed as previously reported (4). All UV-resistant transformants contained the same plasmid designated as pSpUVDE. The sequence of the insert was determined from both orientations by auto sequencer model DSQ-1000 (Simadzu).

Expression of the cloned gene

A cDNA fragment was amplified with primers having appropriate restriction sites and inserted into the expression vector pFLAG2 (Kodak), generating pFDE. *E.coli* XL-1 Blue cells transformed with the expression construct pFDE were treated with 1 mM IPTG at OD = 0.6 for 5 h. Cells were collected, suspended in a buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM DTT and 1 mM PMSF, and sonicated. Supernatant of the cell lysate was loaded on a heparin–Sepharose column with a linear gradient of 0.1–1 M NaCl in buffer A (50 mM Tris–HCl, 1 mM EDTA and 1 mM

* To whom correspondence should be addressed

DTT, pH 7.5). Activity of UV-dimer endonuclease, which converts closed circular UV-irradiated plasmid into an open circular form, was assayed as detailed below. Fractions around 0.6 M NaCl were collected, diluted to 0.3 M NaCl with buffer A, and subsequently applied on a Hi-Trap Blue column (Pharmacia). Elution was made with a gradient of 0.3–2.0 M NaCl in buffer A containing 10% glycerol. Fractions at 1.5 M NaCl with activity peaks were collected and concentrated by centricon 30 (Amicon). Purity was around 35% as judged by SDS–PAGE. This fraction (BS-fraction) was used for *in vivo* plasmid nicking and incision assays.

Plasmid nicking assay

Close circular plasmid was exposed to UV (1 kJ/m^2) and incubated with BS-fraction or column fractions at 37°C for 30 min. The reaction was stopped by heating at 65°C for 10 min and analyzed on an agarose gel. Optimal conditions of the plasmid nicking activity for the BS-fraction were determined as 50 mM Tris–HCl (pH 7.9), 50 mM KCl, 15 mM $MgCl_2$ and 1 mM DTT, by varying the concentration of the respective ingredients and changing buffer pH.

Incision assay using synthetic oligonucleotides

Incision of UV-irradiated oligomer was assayed in essentially the same way as previously described (5). An oligonucleotide containing two dipyrimidine sequences (underlined), 5′-GTATACACACACGTATGCATCATGTTATACGCACACACAGTGCATACACATATAGC-3′, was either 5′-labelled with [γ-^{32}P]ATP by T4 polynucleotide kinase (Takara) or 3′-labelled with [α-^{32}P]ddATP by terminal deoxynucleotidyl transferase (Boehringer), annealed to its complementary oligomer and exposed to UV (3 kJ/m^2). The DNA (10 fmol) was incubated with BS-fraction, NC-UVDE or T4 PD-DNA glycosylase at 37°C for 60 min. The reaction was stopped by adding an equal volume of 90% formamide loading solution, heated at 95°C for 2 min, and analyzed on a 15% polyacrylamide denaturation gel. For the size marker, end-labelled, unirradiated DNA digested with *Nla*III or *Nsi*I was mixed and run on a lane. For preparing DNA containing CPD or (6–4)PP, photoreactivation of the 5′-labelled, UV-irradiated DNA with *Drosophila* phr or *Analycistis* phr was performed as described previously (4).

Construction of truncated UVDE gene

The cDNA was truncated from the N-terminus by digesting it at the *Sca*I or *Cla*I site and inserted into pUC18 or pGEM7Zf+ vectors (which generated a small portion of lacZ fusion), resulting in pDN101 or pDN349, respectively. To generate additional N-terminal deletion constructs, PCR was made with a 5′ primer starting at the 233th or 274th amino acid position and inserted in a modified pFLAG2 vector in which the FLAG peptide is fused to the C-terminus of the insert (pDN232 or pDN273, respectively). An antibody to the FLAG sequence (Kodak) was used to detect peptide production in transformants of pDN232 and pDN273. C-terminal deletion construct pDC564 was made by digesting pFDE with *Eco*O109I and *Bgl*II (in MCS of the vector), blunt-ended by the Klenow fragment of DNA polymerase and religated.

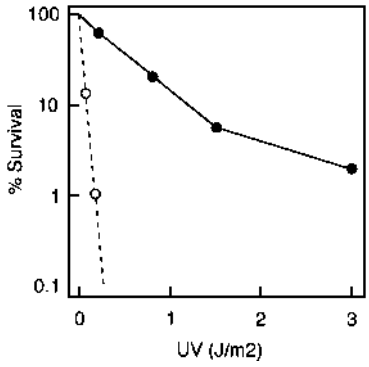

Figure 1. Survival curve of *E.coli* cells transformed with cDNA selected from the *S.pombe* library. UV-sensitive *E.coli SY2 (phr uvrA recA)* was transformed with vector plasmid (○) and pSpUVDE containing cloned cDNA (●). Colony forming abilities after UV irradiation are shown.

Cloning of *Bacillus subtilis* homolog of eukaryotic UVDE

A homology search of the database with the conserved amino acid sequence between NC-UVDE and SP-UVDE was performed by TBLAST (6), predicting a homologous sequence in *B.subtilis* genomic sequence (Z49782). The bacterial genomic fragment showing UVDE homology was amplified with primers (5′-CTATGATTTTCAGATTCGGGTTCGTTT-3′ and 5′-CATTTATGACTTCCATTGCAGCGCACC-3′ for the 5′- and 3′-end of the ORF, respectively) and cloned into pT7-Blue (Novagen). The plasmid was introduced into SY2 cells and examined for UV resistance.

RESULTS

Cloning of a cDNA complementing UV sensitivity of repair deficient *E.coli* host cells

We introduced a cDNA library made from *S.pombe* into the *E.coli* strain SY2, which lacks DNA repair activity in all the three pathways essential for repair of UV-induced DNA damages, namely, recombination, NER and photoreactivation. UV-resistant transformants were isolated after three rounds of UV-selection procedures (Fig. 1) and a plasmid harbouring a 2 kb insert with the longest open reading frame (ORF) consisting of 598 amino acid (aa) was isolated. The presence of an in-frame stop codon upstream of the first start codon for translation yielding the longest ORF, suggests that it is the start codon for translation of this gene. The deduced aa sequence was compared with that of the previously isolated NC-UVDE gene (Fig. 2) and was found to have 36.6% identity. The similarity between the two sequences was found in the C-terminal two-thirds region (around 54%), while only 16% identity was found in the N-terminal one-third of the proteins. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databases with the accession number D78571.

Characterization of a recombinant protein obtained from *E.coli* host cells

We expressed the cloned cDNA from the putative start codon using the *tac* promoter in *E.coli* and analyzed whether the *S.pombe*

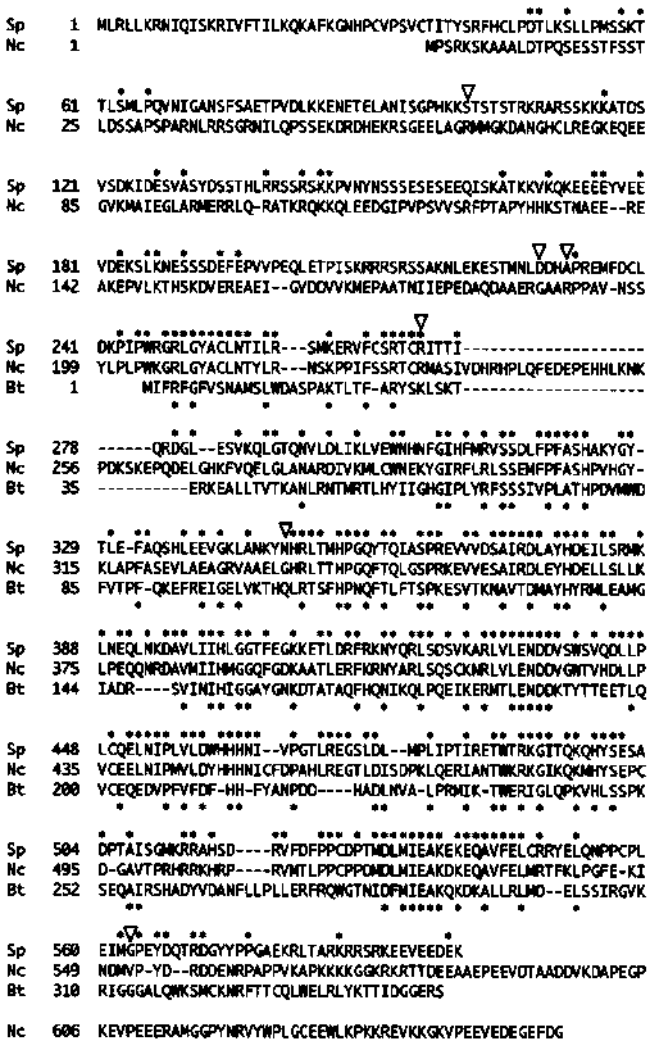

Figure 2. Alignment of amino acid sequences deduced from cloned *S.pombe* cDNA, *N.crassa* UVDE, and a homologous gene from *B.subtilis.* Asterisks above or below the alignment show identical residues between *S.pombe* and *N.crassa* or among three sequences, respectively. Positions of truncated cDNA constructs (Fig. 5) are indicated by arrowheads. *B.subtilis* sequence was translated from a genomic sequence in the database (Z49782).

protein has an activity similar to that of NC-UVDE to cleave UV-irradiated DNA. Since cell lysate of a SY2 transformant was found to convert UV-irradiated supercoiled plasmid DNA to a relaxed form, the purification process was monitored by the nicking activity of UV-irradiated supercoiled DNA. Applying cell lysate on a heparin–Sepharose and subsequently to a blue-Sepharose column, ~35% homogeneity of the protein without bacterial nuclease activity was obtained (not shown). Since further purification led to an immediate inactivation of the enzymatic cleavage activity, we used the blue-Sepharose fraction (BS-fraction) in all *in vitro* assays reported herein.

First, the requirements of *S.pombe* UVDE for plasmid nicking activity were examined. The BS-fraction was mixed with UV-irradiated and non-irradiated supercoiled plasmid DNA of two different sizes. Only the UV-irradiated DNA was incised when appropriate concentrations of magnesium and potassium were included in the reaction (Fig. 3A). The effect of ATP on the

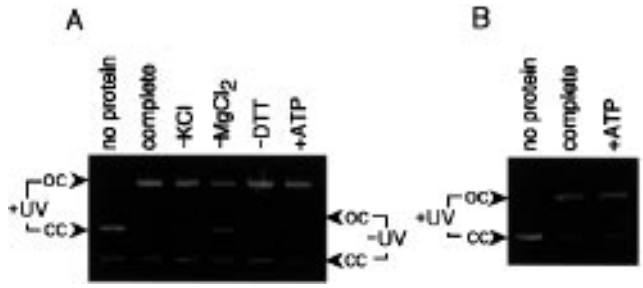

Figure 3. Requirements of UVDE reaction for plasmid nicking activity. Blue-Sepharose fraction of *S.pombe* UVDE was mixed with UV-irradiated plasmid (+UV) and non-irradiated plasmid (–UV) in a optimized reaction buffer (complete), which had been determined in separate experiments, or in a buffer with or without the ingredients shown above. The UVDE fraction added was diluted so that the reaction resulted in nicking +UV plasmid to 95% (**A**) and 60% (**B**). Addition of –UV plasmid was omitted in (B).

plasmid nicking activity was also examined using the amount of the BS-fraction which converted 60% of UV-irradiated plasmid to a relaxed form in the complete reaction. As shown in Figure 3B, the addition of ATP did not enhance the activity. The required presence of magnesium and salt as well as ATP independence of SP-UVDE for its activity are the same as found for NC--UVDE.

To demonstrate substrate specificity and position of the nicks, a synthetic oligomer was prepared, which contained one TC and one TT dipyrimidine sequence, major potential sites for UV-induced (6–4)PP and CPD, respectively (Fig. 4A). Restriction sites for *Nsi*I and *Nla*III which cleave 5′ of the first thymines of TC and TT, respectively, were included so that the restriction digests work as size markers. The oligomers were either 5′- or 3′-labelled, annealed to its complementary strand oligomer and UV irradiated. This DNA was mixed with the BS-fraction and the reaction products were analyzed by polyacrylamide gel electrophoresis (PAGE) as shown in Figure 4B and C. The reaction produced two fragments at the TC and TT sites (Fig. 4B and C, lane 1) with the same migration as the DNA fragments resulting from the reaction with NC-UVDE (Fig. 4B and C, lane 2) and those of marker fragments (i.e. mixture of *Nsi*I and *Nla*III digests). In contrast, the reaction of the DNA with T4 phage-derived CPD-DNA glycosylase showed a major band near the TT site created by its AP-lyase action (Fig. 4B and C, lane 3). Thus, the sites cleaved by SP-UVDE are different from those of T4 CPD-DNA glycosylase and identical to those of NC-UVDE. Judging from the positions of the nicked DNA fragments in Figure 4, SP-UVDE introduced a single nick immediately 5′ to the damage leaving 3′-OH and 5′-phosphate termini at the nicked site as is the case for NC-UVDE (4).

In order to further confirm the substrate specificity, 5′-labelled, UV-irradiated DNA was photoreactivated with either photolyase from *Anacystis nidulans* or *Drosophila melanogaster* prior to the incision reaction. As shown in Figure 4B, when oligomer was photoreactivated with *Drosophila* photolyase, which reverts (6–4)PP lesions, the band intensity at the TC site was significantly reduced for the SP-UVDE reaction (lane 4) and the NC-UVDE reaction (lane 5). Photoreactivation of the DNA with *Anacystis* photolyase, which reverts CPD lesions, diminished the band intensity at the TT site of the SP-UVDE reaction (lane 7) and the NC-UVDE reaction (lane 8). Reasonably, only photoreactivation with *Anacystis* photolyase eliminated the substrates for T4

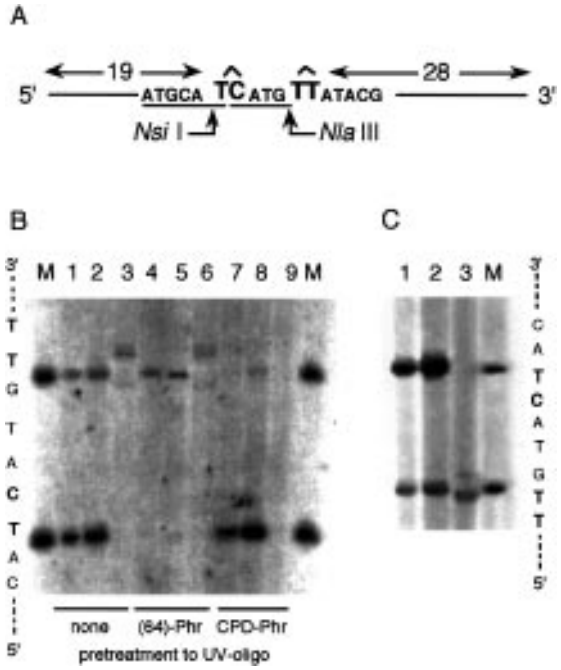

Figure 4. Substrates of UVDE and incision sites. (**A**) A 54mer oligomer, which has single TC and TT dipyrimidine sequences, was used. (**B**) PAGE analysis of 5′-labelled oligomers nicked by UVDEs or T4 PD-DNA glycosylase. (**C**) PAGE analysis of 3′-labelled oligomers nicked by UVDEs or T4 PD-DNA glycosylase. The 54mer was ^{32}P-end-labelled, double-stranded and UV irradiated. The DNA was incubated with *S.pombe* UVDE (lanes 1, 4 and 7), *N.crassa* UVDE (lanes 2, 5 and 8) or T4 PD-DNA glycosylase (lanes 3, 6 and 9). A digest of the reaction was analyzed on a denatured 15% gel. To selectively revert (6–4)PP or CPD from the substrate, the UV-irradiated DNA was photoreactivated with (6–4) photolyase (lanes 4–6) or with CPD photolyase (lanes 7–9) prior to the incision reaction. A mixture of unirradiated DNA digests with *Nla*III and *Nsi*I was loaded on the marker lanes (M).

CPD-DNA glycosylase (lane 6 and 9). These results indicate that the incision occurs immediately 5′ to both CPDs and (6–4)PP lesions as previously found for NC-UVDE (4). Furthermore, the manner of the incision of the recombinant SP-UVDE is consistent with the activity reported for the extract of *S.pombe* cells (5).

Minimum size of the cDNA indispensable for enzymatic activity

The N-terminal one-third of the SP-UVDE is different in aa sequence from that of the *Neurospora* homolog. We next investigated, whether this part of the protein is necessary for the complementing activity of UV sensitive *E.coli* host cells. Several truncated SP-UVDE genes were constructed and introduced into *E.coli* SY2 host cells. No change in UV resistance of the *E.coli* transformants were observed until the N-terminal sequence was deleted up to the 232th residue (Fig. 5A). Deletions from the N-terminus to the 273th as well as the 349th residue led to a phenotype as UV-sensitive as that of cells harbouring vector plasmid (Fig. 5B). In order to confirm protein production in the transformants, whole cell lysates were analyzed by Western blotting using antibody against the tag fused at the C-terminus of the recombinant protein. The transformants harbouring pDN232 and pDN273 produced truncated soluble proteins of reasonable sizes (Fig. 5C), suggesting that the deletion up to 273th residue of the gene first inactivates the enzymatic activity of the gene product. On the other hand, even a small deletion of 35 residues at the C-terminal sequence of the protein influenced the enzymatic activity (Fig. 5A and B). From these results, it is concluded that the C-terminal two-thirds of the protein, where aa sequences are well conserved between two UVDEs, almost coincides with the region indispensable for the enzymatic activity.

A bacterial homolog of UVDE

In a recently published database of the genomic nucleotide sequence from the Gram positive bacterium *Bacillus subtilis*, an ORF was found which showed a remarkable similarity in its deduced aa sequence to the NC- and SP-UVDE (Fig. 2). We obtained the genomic fragment of the ORF by polymerase chain reaction and introduced it into *E.coli* SY2 cells. Figure 6 depicts the complementing activity of UV sensitivity by introduction of the bacterial gene. Thus, UVDE is distributed not only in eukaryotes but also in eubacteria.

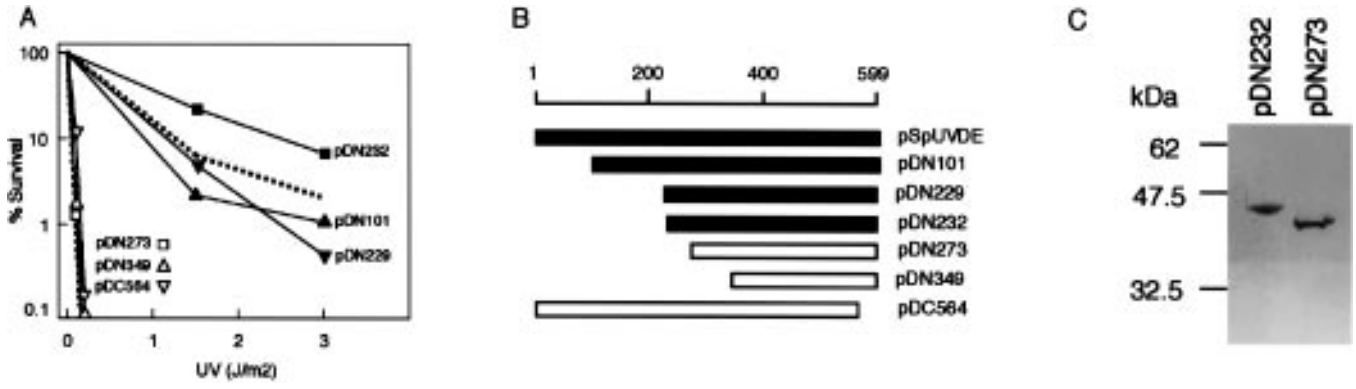

Figure 5. Expression and survival of truncated *S.pombe* UVDE. Survival curves for SY2 transformants of truncated constructs are shown in (**A**). Curves of the full-length construct pSpUVDE and vector from Figure 1 are shown as broken lines. Amino acid sequence regions of the plasmid constructs are depicted in (**B**) with plasmid names. The number in the name represents the position of the truncated residue. Expression of truncated peptides from pDN232 and pDN273 in the host cells was examined by Western analysis (**C**).

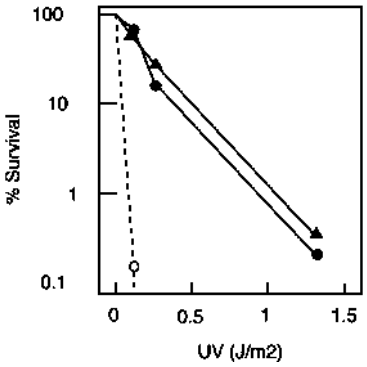

Figure 6. Survival curve for SY2 cells transformed with the *B.subtilis* genome fragment. A plasmid containing an open reading frame of the *B.subtilis* UVDE homology (●), *S.pombe* UVDE clone pFDE (▲) and vector pFLAG2 (○) were transformed in SY2 host. Survival curves after UV-exposure are shown.

DISCUSSION

The presence of a second excision repair pathway for UV-induced DNA damage was first suggested in the fission yeast *Schizosaccharomyces pombe* (7) and recently, it was shown that this pathway is distinct from NER (8). This alternative excision repair pathway involves an enzyme similar to the UV endonuclease, which we identified in *N.crassa* (9). We showed here that *S.pombe* has a homolog of the *N.crassa* gene, which encodes an endonuclease for UV-induced CPDs as well as (6–4)PPs. Since the enzymatic activity of the recombinant protein is identical to the reported enzymatic activity in the extract of *S.pombe* cells (5), we concluded that the reported UV endonuclease activity in *S.pombe* is derived from the cloned gene. Thus, we showed the existence of a second excision repair pathway, besides the well-known NER, for UV-induced DNA damage in this widely-studied yeast species.

We previously found a number of consecutive hydrophilic aa sequences in the NC-UVDE, including a sequence 4Xlys-2Xgly-2X(LysArg) found near the C-terminal region with similarity to a part of protamine (4). Alignment of the aa sequences of NC- and SP-UVDEs (Fig. 2) indicates that this sequence is not well conserved, although several deletions in this sequence inactivates the complementing activity of the NC-UVDE (4). From the experiments using N- or C-terminal deletion constructs, only the carboxyl two-thirds of the SP-UVDE turned out to be essential for the complementing activity. We do not know the function of the little conserved N-terminal region of the UVDE proteins. This region, which is abundant in Ser and Glu residues for both proteins, may have functions to interact with other proteins.

The aa sequence alignments between the eukaryotic UVDEs and the possible bacterial homolog found in *Bacillus subtilis* show a 27% identity. Although this is a rather low similarity for the protein with specific functions, judging from the complementing activity of UV sensitivity in *E.coli* host cells, the bacterial protein is probably structurally and functionally similar to the eukaryotic homologs.

Lac promoter expression of the *Bacillus* gene in forward orientation is toxic for the host, especially for the cells in the stationary phase, as shown by our finding that almost all cells from the overnight culture were not viable. This phenomenon was previously observed in an *E.coli* culture harbouring the *Neurospora* UVDE gene (unpublished data). Reproducible survival curves of transformants with the *Bacillus* gene were obtained when the coding sequence was introduced in the reverse orientation to the promoter in the plasmid, suggesting that high expression of the gene product is disadvantageous for the *E.coli* host cells.

The presence of the NC-UVDE gene homolog in yeast and bacteria increases the significance of this alternative DNA repair pathway. We previously pointed out the possibility that NC-UVDE may play a substitutional role for NER in *N.crassa* which does not possess efficient NER activity (4). Although *S.pombe* has NER, photolyase activity for CPD has not been found (10). Similarly, photolyase gene seems to be missing in *Bacillus subtilis* (11,12). Thus, SP-UVDE in *S.pombe* and its homolog in the *B.subtilis* could substitute for the photoreactivation of CPDs in these organisms.

The function and interaction of the *S.pombe* UVDE with other repair systems in the well characterized yeast system are more easily analyzed than in *Neurospora.* It is now of interest to identify the processing of the nicked UV damage. We previously introduced the NC-UVDE gene into various repair deficient mutants of the budding yeast *Saccharomyces cerevisiae* and found that the repair pathway initiated with the nicking by UVDE can be completed in the rad2 mutant which is deficient in the 3′ nicking activity of NER. There are most probably other proteins involved in damage processing in this alternative DNA repair pathway.

ACKNOWLEDGEMENTS

We would like to thank Drs Takeshi Todo and Andries P.M. Eker for providing us with *Drosophila* and *Anacystis* photolyases, respectively. The presence of nucleotide sequence in the *Bacillus* database homologous to a part of the UVDE gene of *N.crassa* was first informed by Dr Paul W. Doetsch, for which we are very grateful. Technical assistance by Ms Izumi Chiba and Ms Junko Kikuchi is acknowledged. This work was supported by a Grant-in-Aid for Scientific Research on Priority Area No. 07270101 from the Ministry of Education, Science, Sports and Culture of Japan to A. Yasui.

REFERENCES

1 Sancar,A. (1994) *Biochemistry,* **33**, 2–9.
2 Aboussekhra,A., Biggerstaff,M., Shivji,M.K.K., Vilpo,J.A., Moncollin,V., Podust,V.N., Protic,M., Hubscher,U., Egly,J.-M. and Wood,R.D. (1995) *Cell*, **80**, 859–868.
3 Hoeijmakers,J.H.J. (1993) *Trends Genet.*, **9**, 173–177.
4 Yajima,H., Takao,M., Yasuhira,S., Zhao,J.H., Ishii,C., Inoue,H. and Yasui,A. (1995) *EMB0 J.*, **14**, 2393–2399.
5 Bowman,K.K.,Sidik,K., Smith,C.A., Taylor,J.-S., Doetsch,P.W. and Freyer,G.A. (1994) *Nucleic Acids Res.*, **22**, 3026–3032.
6 Altschul,S.F., Gish,W., Miller,W., Myers,E.W. and Lipman,D.J. (1990) *J. Mol. Biol.* **215**, 403–410.
7 Birnbolm,H.C. and Nasim,A. (1975) *Mol. Gen. Genet.* **136**, 1–8.
8 McCready,S., Car,A.M. and Lehmann,A.R. (1993) *Mol. Microbiol.* **10**, 885–890.
9 Freyer,G.A., Davey,S., Ferrer,J.V., Martin,A.M., Beach,D. And Doetsch,P.W. (1995) *Mol. Cell. Biol.* **15**, 4572–4577.
10 Yasui,A., Eker,A.P.M. and Koken,M. (1 989) *Mutat. Res.*, **217**, 3–10.
11 Quirk,P.G., Hicks,D.B. and Krulwich,T.A. (1993) *J. Biol. Chem.*, **268**, 678–685.
12 Malhotra,K., Kim,S.-T. and Sancar,A. (1994) *Biochemistry,* **33**, 8712–8718.Malhotra,K., Kim,S.-T. and Sancar,A. (1994) *Biochemistry,* **33**, 8712–8718.