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Substrate specificity of ultraviolet DNA endonuclease (UVDE/Uve1p) from *Schizosaccharomyces pombe*

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ABSTRACT

Schizosaccharomyces pombe **ultraviolet DNA endonuclease (UVDE or Uve1p) has been shown to cleave 5′ to UV light-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine-pyrimidone (6–4) photoproducts (6–4PP). This endonuclease is believed to function in the initial step in an alternative excision repair pathway for the removal of DNA damage caused by exposure to UV light. An active truncated form of this protein, Δ228-Uve1p, has been successfully overexpressed, affinity purified and partially characterized. In the present study we present data from a detailed substrate specificity trial. We have determined that the substrate range of Uve1p is much greater than was originally believed. We demonstrate that this DNA damage repair protein is capable of recognizing an array of UV-induced DNA photoproducts (*cis-syn*-, *trans-syn* I- and *trans-syn* II CPDs, 6–4PP and Dewar isomers) that cause varying degrees of distortion in a duplex DNA molecule. We also demonstrate that Uve1p recognizes non-UV-induced DNA damage, such as platinum–DNA GG diadducts, uracil, dihydrouracil and abasic sites. This is the first time that a single DNA repair endonuclease with the ability to recognize such a diverse range of lesions has been described. This study suggests that Uve1p and the alternative excision repair pathway may participate broadly in the repair of DNA damage.**

INTRODUCTION

Ultraviolet (UV) light induces two major types of photodamage in cellular DNA, cyclobutane pyrimidine dimers (CPDs) and (6–4) photoproducts (6–4PPs) (1). Persistence of these lesions can interfere with essential processes such as transcription and DNA replication and may lead to mutation, cell death or neoplastic transformation (1,2). Various DNA repair mechanisms have evolved to correct the damage caused by UV light. In addition to direct reversal of the damage by DNA photolyases, various excision repair pathways have evolved that can either be highly specific or non-specific for CPDs and 6–4PPs. Photolyases specific for either CPDs or 6–4PPs have been identified and characterized (3–6) and these repair UV damage by light-dependent direct reversal of the DNA to the undamaged state. The nucleotide excision repair pathway has a wide species distribution and is comprised of a coordinated series of steps. The UV photoproduct is excised by a dual incision event that occurs at a distance 5′ and 3′ to the lesion by a multiprotein complex. Cleavage is followed by displacement of the damage-containing oligonucleotide, gap filling by DNA polymerase and finally ligation (1). Base excision repair (BER) involves an N-glycosylase/AP lyase cleaving the *N*-glycosidic bond of the 5′-pyrimidine of the CPD and subsequently incising the phosphodiester backbone at the abasic site (AP site) via a β-lyase mechanism (7,8). A third distinct excision repair pathway for the removal of UV damage has also been described (9–11). This ‘alternative excision repair’ pathway was first described in *Schizosaccharomyces pombe* cells where ultraviolet DNA endonuclease (UVDE or Uve1p, encoded by the *uve1+* gene) makes an initial incision directly 5′ to both CPDs and 6–4PPs (12,13). *Schizosaccharomyces pombe* Uve1p homologs have also been identified in *Neurospora crassa* (13) and *Bacillus subtilis* (14). Recently, we have reported the overexpression, purification to apparent homogeneity and initial enzymatic characterization of a truncated form of Uve1p (15). This protein (Δ228-Uve1p) contains a deletion of the N-terminal 228 amino acids, but remains active and stable over long periods of storage.

Uve1p is capable of cleaving both *cis-syn* CPDs (cs-CPD) and 6–4PPs (12,13). CPDs and 6–4PPs are the most frequently occurring forms of UV-induced damage but there are significant differences in the structural distortions induced in DNA by these two lesions. Incorporation of a cs-CPD into duplex DNA causes no significant bending or unwinding of the DNA helix (16–21) and destabilizes the duplex by ~1.5 kcal/mol (19). It has been demonstrated that this relatively small structural distortion allows

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CPD bases to retain most of their ability to form Watson–Crick hydrogen bonds (19,21). On the other hand, NMR studies have suggested that 6–4PPs bend the DNA to a greater extent than cs-CPDs (21,22) and there is a destabilization of ~6 kcal/mol (19) in the DNA duplex with a resulting loss of hydrogen bond formation at the 3′-side of the 6–4PP DNA adduct (22). The ability of Uve1p to recognize such different structural distortions suggested that it might also recognize other types of DNA damage.

CPDs can occur in DNA in four different isoforms [*cis-syn* I (csI), *cis-syn* II (csII), *trans-syn* I (tsI) and *trans-syn* II (tsII)] (23). Pyrimidine dimers exist predominantly in the csI form in duplex DNA whereas *trans-syn* (ts) dimers are found primarily in single-stranded regions of DNA (24). 6–4PPs are alkali-labile lesions at positions of cytosine (and much less frequently thymine) located 3′ to pyrimidine nucleosides (25). 6–4PPs are not stable in sunlight and are converted to their Dewar valence isomers upon exposure to 313 nm light (26,27). In this study we investigate the specificity of Δ228-Uve1p for a series of UV photoproducts: cs-CPD, tsI-CPD, tsII-CPD, 6–4PP and the Dewar isomers. We also investigated the possibility that Uve1p may recognize other types of non-UV photoproduct DNA damage. We describe the activity of Uve1p on DNA oligonucleotide substrates containing a variety of lesions, including a platinum–DNA GG diadduct (Pt-GG), uracil (U), dihydrouracil (DHU), 8-oxoguanine (8-oxoG), AP sites, inosine (I) and xanthine (Xn). This collection of substrates contains base lesions that induce a broad range of different DNA structural distortions. The results of these studies indicate that the substrate specificity range of Uve1p is much greater than was originally thought (11,13,14,28). Therefore we suggest that this endonuclease should no longer be referred to as UVDE, which implies a specificity for UV-induced DNA, but should be renamed in order to reflect its expanded substrate range. The role of this enzyme in the initiation of repair of a variety of DNA damages in addition to CPDs and 6–4PPs is also discussed in this context.

MATERIALS AND METHODS

Uve1p preparations

Cultures of *Saccharomyces cerevisiae* (DY150) harboring pYex4T-1 plasmids (Clontech) encoding gluthathione *S*-transferase (GST)-tagged full-length (G-Uve1p) and truncated (GΔ228-Uve1p) forms of Uve1p were induced at mid log phase with 0.5 mM $CuSO_4$. Cells were harvested 2 h after induction and crude cell lysates were prepared in 50 mM Tris (pH 7.5), 100 mM EDTA, 50 mM NaCl, 10 mM β-mercaptoethanol and 5% glycerol in the presence of protease inhibitors (10 ng/ml pepstatin, 3 nM leupeptin, 14.5 mM benzamidine and 0.4 mg/ml aprotinin) using glass beads. Recombinant GΔ288-Uve1p was purified to apparent homogeneity using a gluthathione–Sepharose 4B (Pharmacia) affinity column and Δ228-Uve1p was prepared by thrombin cleavage on the affinity column as described previously (15).

GST preparation

Saccharomyces cerevisiae (DY150) cells were transformed with the pYex4T-1 expression vector without any insert (i.e. expressing GST alone). These cultures were induced with $CuSO_4$ and cell lysates were prepared as described for the Uve1p proteins. Purified recombinant GST was affinity purified on a gluthathione–Sepharose column in an identical manner to GΔ228-Uve1p (see above) and was included in all of the assays performed in this study as a control for trace amounts of potential contaminating endonucleases in the Uve1p protein preparations.

Table 1. Damaged oligonucleotide substrates used in this study

Substrate	Damaged oligonucleotide sequence (5′→3′)	Adduct	Opposite base(s)[a]
(A) cs-CPD 30mer	CATGCCTGCACGAA**T**^**T**AAGCAATTCGTAAT	cs-CPD	AA
(B) UD-30mer	CATGCCTGCACGAA**TT**AAGCAATTCGTAAT	Undamaged	AA
(C) cs-CPD 49mer	AGCTACCATGCCTGCACGAA**T**^**T**AAGCAATTCGTAATCATGGTCATAGCT	cs-CPD	AA
(D) tsI-CPD 49mer	AGCTACCATGCCTGCACGAA**T**^**T**AAGCAATTCGTAATCATGGTCATAGCT	tsI-CPD	AA
(E) tsII-CPD 49mer	AGCTACCATGCCTGCACGAA**T**^**T**AAGCAATTCGTAATCATGGTCATAGCT	tsII-CPD	AA
(F) 6–4PP 49mer	AGCTACCATGCCTGCACGAA**T**^**T**AAGCAATTCGTAATCATGGTCATAGCT	6–4PP	AA
(G) Dewar 49mer	AGCTACCATGCCTGCACGAA**T**^**T**AAGCAATTCGTAATCATGGTCATAGCT	Dewar	AA
(H) Pt-GG 32mer	TCCCTCCTTCCTTCC**G*G***CCCTCCTTCCCCTTC	Pt-GG	CC
(I) U 37mer	CTTGGACTGGATGTCGGCAC**X**AGCGGATACAGGAGCA	U	A/G
(J) DHU 37mer	CTTGGACTGGATGTCGGCAC**X**AGCGGATACAGGAGCA	DHU	A/G
(K) AP 37mer	CTTGGACTGGATGTCGGCAC**X**AGCGGATACAGGAGCA	AP	A/G
(L) I 31mer	TGCAGGTCGACT**X**AGGAGGATCCCCGGGTAC	I	T/C
(M) Xn 31mer	TGCAGGTCGACT**X**AGGAGGATCCCCGGGTAC	Xn	T/C
(N) 8-oxoG 37mer	CTTGGACTGGATGTCGGCAC**X**AGCGGATACAGGAGCA	8-oxoG	A/T/G/C

cs-CPDs, *cis-syn* cyclobutane pyrimidine dimers; tsI-CPD, *trans-syn* I CPD; tsII-CPD, *trans-syn* II CPD; 6–4PP, (6–4) photoproducts; Dewar, a Dewar isomer; Pt-GG, a platinum–DNA diadduct; U, uracil; DHU, dihydrouracil; AP, abasic site; I, inosine; Xn, xanthine ; 8-oxoG, 8-oxoguanine. ^, a UV-induced dimer between two adjacent thymines; *, a cisplatin-induced diadduct between two adjacent guanines; X, position at which the adducts U, DHU, AP, I, Xn and 8-oxoG are incorporated into the oligonucleotide substrates.

[a]The bases that are placed opposite to the lesions on the complementary DNA strand.

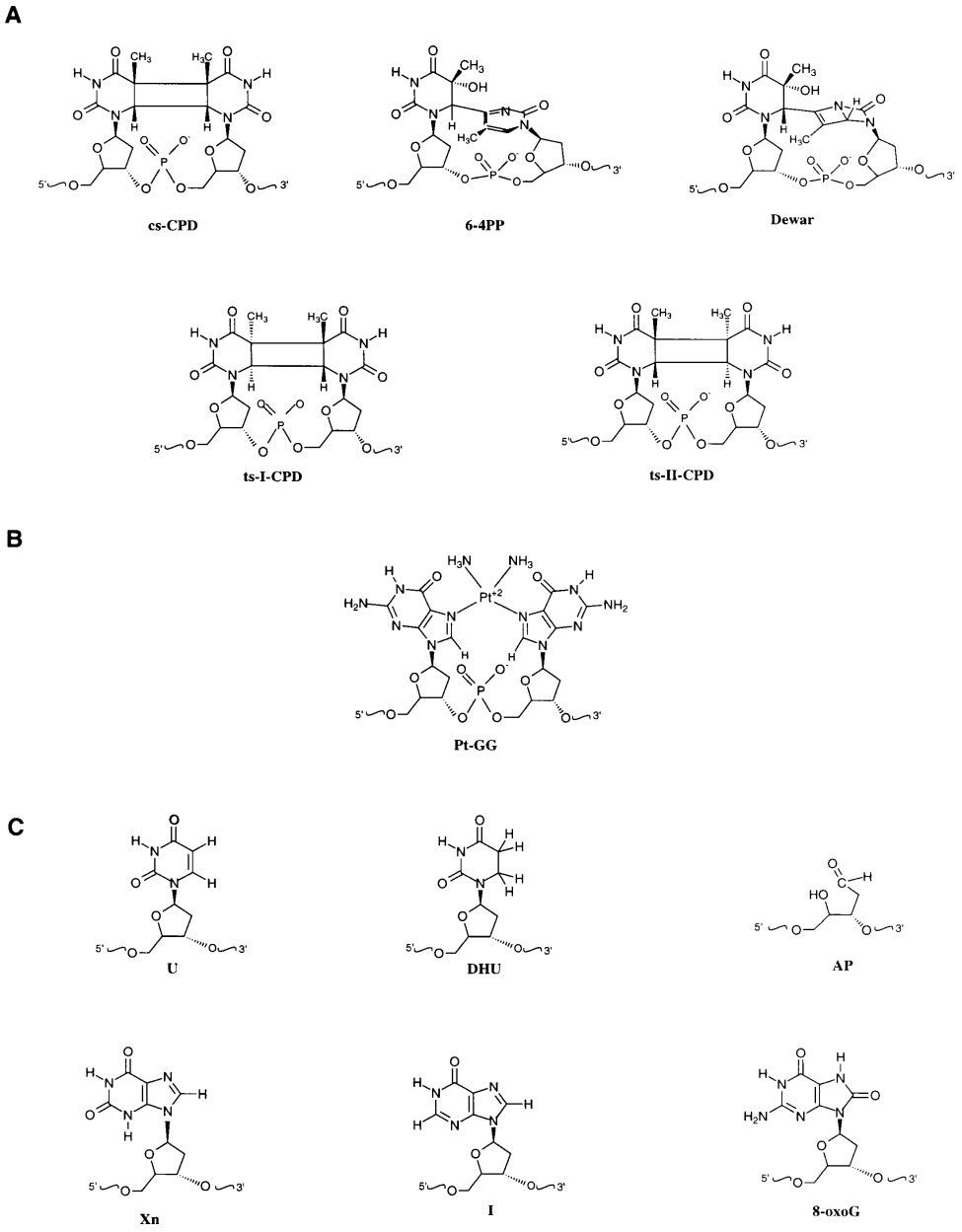

Figure 1. Structures of DNA damage lesions incorporated into the synthetic oligonucleotides used in this study. (**A**) UV-induced lesions. *cis-syn* cyclobutane dimer (cs-CPD), (6–4) photoproduct (6–4PP), Dewar valence isomer (Dewar), *trans-syn* I cyclobutane dimer (ts-I-CPD) and *trans-syn* II cyclobutane dimer (ts-II-CPD). (**B**) Platinum–DNA diadduct. *cis*-[Pt(NH_3)$_2${d(GpG)-N7(1),-N7(2)}] (Pt-GG). (**C**) Non-bulky DNA damage. U, uracil; DHU, dihydrouracil; AP, abasic site; Xn, xanthine; I, inosine; 8-oxoG, 8-oxoguanine.

Oligonucleotides containing DNA damage

The DNA damage-containing oligonucleotides used as substrates in this study are presented in Table 1. The structure of each damaged lesion is presented in Figure 1. The 30mer cs-CPD-containing oligonucleotide (cs-CPD 30mer) was prepared as described previously (29). The 49mer oligonucleotides containing a cs-CPD (cs-CPD 49mer), a tsI-CPD (tsI-CPD 49mer), a tsII-CPD (tsII-CPD 49mer), a 6–4PP (6–4PP 49mer) and a Dewar isomer (Dewar 49mer) were synthesized as described previously (29). The oligonucleotide containing a platinum–DNA GG diadduct (Pt-GG 32mer) and its complementary strand were

prepared as previously described (30). The uracil-containing oligonucleotide (U 37mer), the undamaged oligonucleotides and the complementary strand oligonucleotides for all the substrates were synthesized by the Emory University Microchemical Facility. The DHU-containing oligonucleotide (DHU 37mer) was synthesized by Research Genetics (Birmingham, AL). The oligonucleotides containing inosine (I 31mer) and xanthine (Xn 31mer) and their complementary strand were a gift from Dr Yoke Wah Kow (Emory University, Atlanta, GA). The 8-oxoguanine-containing 37mer (8-oxoG 37mer) was synthesized by National Bioscience Inc.

Preparation of labeled substrates

The cs-CPD 30mer, the 49mer UV photodamage-containing oligonucleotides and the Pt-GG 32mer were 5′-end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; Amersham) using polynucleotide kinase (31). The oligonucleotides U 37mer, DHU 37mer, I 31mer, Xn 31mer and 8-oxoG 37mer were 3′-end-labeled using terminal transferase and [α-^{32}P]ddATP (3000 Ci/mmol; Amersham) (32). End-labeled duplex oligonucleotides were gel purified on a 20% non-denaturing polyacrylamide gel. DNA was resuspended in ddH_2O and stored at –20°C.

Preparation of AP substrate

5′-End-labeled duplex U 37mer (20–50 pmol) was incubated with 6 U of uracil DNA glycosylase (UDG) for 30 min at 37°C in UDG buffer (30 mM HEPES–KOH, pH 7.5, 1 mM EDTA and 50 mM NaCl) to generate the AP site-containing oligonucleotide (AP 37mer). The DNA was extracted with PCIA (phenol:chloroform:isoamylalcohol, 29:19:1 v/v/v) equilibrated with HE buffer (10 mM HEPES–KOH pH 8.0 and 2mM EDTA) with 0.1% 8-hydroxyquinoline and was evaluated for its AP site content by cleavage with 0.1 M piperidine at 90°C for 20 min.

Uve1p assays

Reactions with various Uve1p preparations were carried out in a total volume of 20 µl and contained reaction buffer (20 mM HEPES pH 6.5, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM $MnCl_2$) and end-labeled oligonucleotide substrate (10–30 fmol). The substrate/buffer mix was incubated for 20 min at 37°C with Uve1p. In the case of G-Uve1p and GΔ228-Uve1p crude cell lysates 5 µg of protein were used for all assays. Fifty nanograms of affinity-purified GΔ228-Uve1p (0.75 pmol) and Δ228-Uve1p (1.2 pmol) were incubated with all of the UV-induced photoproducts. For all other assays 0.5 µg of affinity-purified GΔ228-Uve1p (7.5 pmol) and Δ228-Uve1p (12 pmol) were incubated with the substrates. Two micrograms of affinity-purified recombinant GST (72 pmol) was incubated with each substrate under identical reaction conditions to control for potential contaminating nucleases which may have co-purified with the Uve1p preparations. DNA repair proteins [*Escherichia coli* exonuclease III, *E.coli* endonucleases III and IV, *E.coli* uracil DNA glycosylase and *S.cerevisiae* endonuclease III-like glycosylase (Ntg)] specific for each oligonucleotide substrate were also incubated with these substrates under their individual optimum reaction conditions, as a means to determine the specific DNA cleavage sites of Uve1p. The reaction products were analyzed on 20% denaturing (7 M urea) polyacrylamide gels (DNA sequencing-type gels) as described previously (33). The DNA bands corresponding to the cleaved and uncleaved substrate were analyzed and quantified by PhosphorImager analysis (Molecular Dynamics model 445SI) and autoradiography.

RESULTS

Uve1p cleavage of CPD-containing substrates

Uve1p isolated from *S.pombe* was first described as catalyzing a single ATP-independent incision event immediately 5′ to the UV photoproduct and generating termini containing 3′-hydroxyl and 5′-phosphoryl groups (12). We have recently described the overexpression and purification to apparent homogeneity of recombinant Uve1p as a GST-tagged, truncated protein, GΔ228-Uve1p, and its corresponding thrombin cleavage product, Δ228-Uve1p (15). The purified GΔ288-Uve1p, Δ288-Uve1p and crude cell lysates of recombinant G-Uve1p and GΔ288-Uve1p make an incision directly 5′ to CPDs similar to that observed with the native protein (12,15).

In this study, we have used both 5′- and 3′-end-labeled duplex CPD 30mer (cs-CPD 30mer) to demonstrate the ability of Uve1p to cleave a CPD-containing substrate at two sites (Fig. 2A and B). The primary product (arrow a) accounted for ~90% of the total product formed and resulted from cleavage immediately 5′ to the damage. The second incision site was located 1 nt upstream and yielded a cleavage product (arrow b) which represented the remaining 10% of the product formed. This minor product is 1 nt shorter or longer than the primary product depending on whether 5′- or 3′-end-labeled substrate is being examined. The longer minor product formed when the substrate was 3′-end-labeled (Fig. 3B) is indicative of a secondary incision site rather than the presence of a contaminating 3′-exonuclease activity. The same cleavage pattern was observed for each type of Uve1p preparation used, i.e. crude extracts of cells expressing GΔ228-Uve1p and affinity-purified GΔ228-Uve1p and Δ228-Uve1p (Fig. 2A and B, lanes 2–4, respectively), as well as extracts of cells expressing G-Uve1p (data not shown). No cleavage products were observed when the cs-CPD 30mer substrates were incubated with buffer only or purified recombinant GST prepared and affinity purified in an identical manner to the purified Uve1p proteins (Fig. 2A and B, lanes 1 and 5, respectively). This control eliminates the possibility that these DNA strand scission products are formed as a result of the presence of trace amounts of non-specific endonuclease contamination. We conclude that Uve1p recognizes a duplex cs-CPD-containing oligonucleotide substrate and cleaves this substrate at two sites. The primary site is immediately 5′ to the damage and the secondary site is 1 nt 5′ to the site of damage.

Uve1p activity on bipyrimidine UV photoproducts

Uve1p is capable of cleaving both CPDs and 6–4PPs when they are incorporated into oligonucleotide substrates (12,13). These lesions induce substantially different distortions in duplex DNA. The ability of native Uve1p to recognize both of these damages prompted us to investigate whether this endonuclease was also capable of recognizing other forms of UV-induced photodamage. To determine the substrate range of recombinant Δ228-Uve1p for UV-induced bipyrimidine photoproducts, various Uve1p preparations were incubated with synthetic 49mer oligonucleotides containing different forms of UV damage (Fig. 1A). The substrates used in these experiments were 5′-end labeled duplex

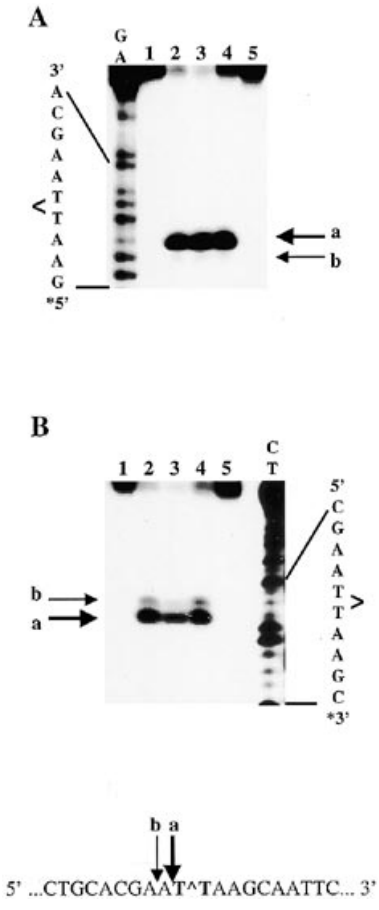

Figure 2. Sites of Uve1p cleavage of CPD-containing substrates. Various Uve1p preparations were incubated with 5′- or 3′-end-labeled (*) cs-CPD 30mer. Cleavage products corresponding to Uve1p-mediated strand scission of cs-CPD 30mer were visualized on a DNA sequencing-type gel. (**A**) 5′-End labeled cs-CPD 30mer duplex was incubated with buffer only (lane 1), an extract of cells overexpressing GΔ228-Uve1p (5 µg) (lane 2), affinity-purified GΔ228-Uve1p (lane 3), affinity-purified Δ228-Uve1p (50 ng of each) (lane 4) and affinity-purified GST alone (2 µg) (lane 5). (**B**) 3′-End labeled cs-CPD 30mer duplex was incubated with the same Uve1p preparations. Order of lanes is the same as for (A). Arrows a and b indicate the primary and secondary cleavage sites. The photoproduct (T^T corresponds to CPD) containing a section of cs-CPD 30mer is shown at the bottom of the figure. For simplicity the complementary strand is not shown.

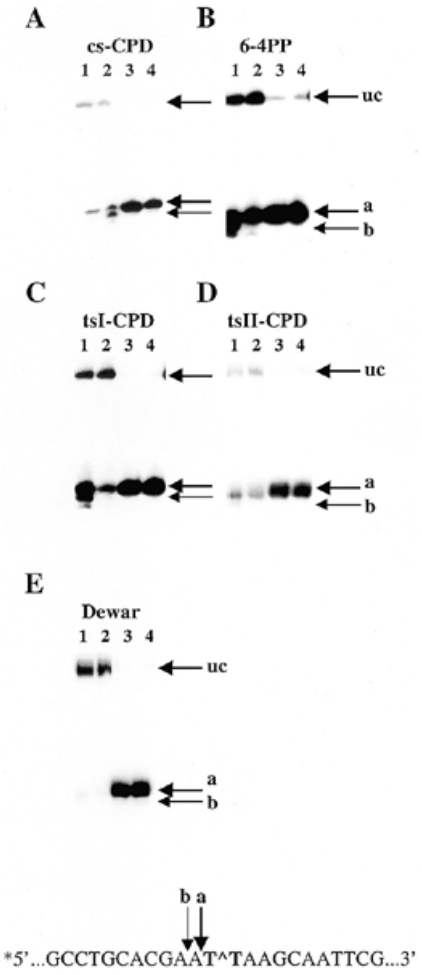

Figure 3. Uve1p activity on bipyrimidine UV-induced photoproducts. To determine if Uve1p was capable of recognizing a broad spectrum of UV-induced photoproducts, crude extracts from cells expressing GΔ228-Uve1p (lane 1) and G-Uve1p (lane 2) (5 µg of each) and affinity-purified Δ228-Uve1p (lane 3) and GΔ228-Uve1p (lane 4) (50 ng of each) were incubated with the following 5′-end-labeled (*) duplex oligonucleotide substrates (**A**) cs-CPD 49mer, (**B**) 6–4PP 49mer, (**C**) tsI-CPD 49mer, (**D**) tsII-CPD 49mer and (**E**) Dewar 49mer. The UV photoproduct (T^T) containing a section of the sequence is shown at the bottom of the figure. Arrows a and b indicate the major and minor products formed by Uve1p-mediated cleavage. Arrow uc indicates the uncleaved substrate.

cs-CPD 49mer, tsI-CPD 49mer, tsII-CPD 49mer, 6–4PP 49mer and Dewar 49mer (Table 1). Generally, purified GΔ228-Uve1p and Δ228-Uve1p cleaved all of the bipyrimidine photoproduct substrates in a similar manner with respect to both the site and extent of cleavage (Fig. 3A–E, lanes 3 and 4). The cleavage pattern observed when crude cell lysates of G-Uve1p and GΔ228-Uve1p were incubated with the substrates was less consistent (Fig. 3A–E, lanes 1 and 2). Very low levels of product were observed when these extracts were incubated with the Dewar isomer (Fig. 3E, lanes 1 and 2). No cleavage products were detected when the damaged substrates were incubated with buffer alone or purified recombinant GST, demonstrating that no other DNA repair proteins were responsible for the cleavage of the substrate (data not shown). In addition, incubation of Uve1p with end-labeled undamaged substrate (UD 30mer) did not result in the formation of any cleavage products (data not shown). We conclude that Uve1p recognizes and cleaves these five UV-induced bipyrimidine photoproducts in a similar manner and that they are substrates for this enzyme. This is the first time that a single protein endonuclease capable of recognizing such a broad range of UV-induced photoproducts has been described.

Uve1p activity on an oligonucleotide substrate containing a platinum–DNA GG diadduct

We have shown that Uve1p is capable of cleaving a range of UV-induced photoproducts. All of these lesions cause different helical distortions when present in duplex DNA. To determine whether Δ228-Uve1p was capable of recognizing distortions caused by non-UV photoproduct diadducts, we investigated

whether Uve1p recognized an oligonucleotide containing a platinum–DNA lesion. *cis*-Diamminedichloroplatinum(II) (cisplatin) is a widely used antitumor drug that induces several types of mono- and diadducts in DNA. One of the major, biologically relevant adducts formed results from the coordination of N-7 of two adjacent guanines to platinum to form the intrastrand crosslink *cis*-[Pt(NH_3)$_2${d(GpG)-N7(1), -N7(2)}] (*cis*-Pt-GG) (Fig. 1B; 34). A 5′-end-labeled duplex 32mer oligonucleotide with a single platinum intrastrand crosslink between positions 16 and 17 (Pt-GG 32mer) (Table 1) was incubated with either GΔ228-Uve1p or Δ228-Uve1p and the reaction products were visualized on a denaturing polyacrylamide gel (Fig. 4). The 3′→5′ exonuclease activity of *E.coli* exonuclease III was used to identify the specific site of cleavage of Uve1p, as a platinum–DNA diadduct will terminate or stall the digestion of the duplex DNA at this site (35,36). Incubation of 5′-end-labeled Pt-GG 32mer with exonuclease III (Fig. 4, lane 3) generates 5′-end-labeled oligonucleotide fragments with 3′-hydroxyl termini. Base-specific chemical cleavage (Fig. 4, lane 1) of the same substrate generates 5′-end-labeled fragments with 3′-phosphoryl termini which consequently migrate faster than the exonuclease III product on DNA sequencing-type gels. (Due to over-reaction with hydrazine all of the nucleotides are revealed in the sequencing lane.) GΔ228-Uve1p cleaved Pt-GG 32mer 5′ to the GpG adduct position at two adjacent sites (Fig. 4, lane 4, arrows c and d). The products (c and d) migrate with the exonuclease III products confirming that they have 3′-hydroxyl termini. Comparison with the Maxam–Gilbert sequencing ladder (Fig. 4, lane 1) indicates that the GΔ228-Uve1p-mediated cleavage products are generated by cleavage at sites located 2 and 3 nt 5′ to the platinum–DNA GG diadduct. The GΔ228-Uve1p-mediated cleavage products were quantified by PhosphorImager analysis and it was determined that cleavage at the primary site c (arrow c) accounted for ~90% of the total product formed, while cleavage at the secondary site (arrow d) accounted for the remaining 10%. In contrast, Δ228-Uve1p appeared to cleave Pt-GG 32mer only at the primary site c (i.e. 2 nt 5′ to the damage) (Fig. 4, lane 5). When the quantity of protein used and the total amount of product formed is taken into account it is estimated that the cleavage of Pt-GG 32mer by Uve1p is ~40-fold less efficient than cleavage of the UV-induced photoproducts. Despite this significant decrease in efficiency it can be concluded that Pt-GG 32mer is a substrate for Uve1p, albeit a poor one, and more importantly that Uve1p is capable of recognizing and cleaving a non-UV photoproduct dimer lesion.

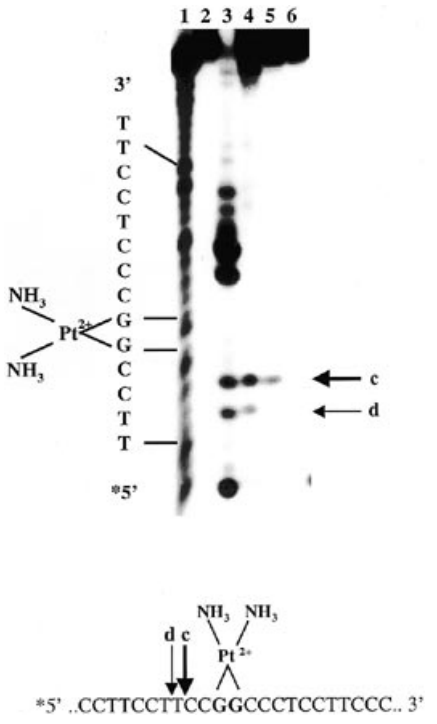

Figure 4. Uve1p activity on a platinum–DNA GG diadduct-containing substrate. Affinity-purified GΔ228-Uve1p (lane 4) and Δ228-Uve1p (0.5 μg) (lane 5) were incubated with 5′-end-labeled duplex (*) Pt-GG 32mer. This substrate was also incubated with buffer alone (lane 2), *E.coli* exonuclease III (150 U; Promega) (lane 3) and affinity-purified GST (2 μg) (lane 6). Maxam and Gilbert sequencing (lane 1) of the oligonucleotide was carried out to identify the site of cleavage. Arrows c and d indicate the major and minor cleavage sites, respectively. The platinum–DNA GG diadduct containing a section of the substrate is shown at the bottom of the figure. The sequence of the complementary strand is omitted.

Uve1p activity on substrates containing non-bulky DNA damage

The ability of Uve1p to recognize and cleave non-UV photoproduct DNA diadducts prompted us to investigate whether other types of base damage could also be recognized by this versatile endonuclease. These damages included abasic sites (AP sites), uracil (U), dihydrouracil (DHU), inosine (I), xanthine (Xn) and 8-oxoguanine (8-oxoG) (Fig. 1C). For these studies, we utilized 37mer oligonucleotide substrates with the damages placed near the center of the molecule and within the same DNA sequence context (Table 1). These oligonucleotides, AP 37mer, U 37mer, DHU 37mer and 8-oxoG 37mer, were incubated with various Uve1p preparations and the reaction products were analyzed on DNA sequencing-type gels. In addition, 31mer oligonucleotides containing inosine (I 31mer) and xanthine (Xn 31mer) were also tested as potential Uve1p substrates (Table 1).

AP sites arise in DNA from the spontaneous hydrolysis of N-glycosyl bonds (37) and as intermediates in DNA glycosylase-mediated repair of damaged bases (38). AP endonucleases cleave hydrolytically 5′ to the site to yield a 3′-hydroxyl terminus, AP lyases cleave by a β-elimination mechanism leaving a 3′-αβ-unsaturated aldehyde (39). To determine if Uve1p were able to recognize and cleave AP sites we incubated affinity-purified GΔ228-Uve1p and Δ228-Uve1p and crude extracts of cells expressing GΔ228-Uve1p with a 5′-end-labeled oligonucleotide substrate containing an AP site placed opposite a G residue (AP/G 37mer). The products were analyzed on a DNA sequencing-type gel as before (Fig. 5A, lanes 3–5, respectively). *Escherichia coli* endonuclease III (which has an associated AP lyase activity) and *E.coli* endonuclease IV (a hydrolytic AP endonuclease) were used in order to determine if the cleavage products formed during incubation with Uve1p preparations were as a result of a β-elimination mechanism or hydrolytic cleavage (Fig. 5A, lanes 2 and 6, respectively). Uve1p recognized the AP site in this oligonucleotide substrate and cleaved it in a similar manner to *E.coli* endonuclease IV. Incubating the Uve1p proteins with an oligonucleotide substrate where the AP site was placed opposite to an adenine residue (AP/A 37mer) resulted in no significant

change in the amount of cleavage product formed (Table 2). To further support the notion that Uve1p recognizes AP sites, we used unlabeled cs-CPD 30mer as a specific competitor for Uve1p. Addition of 40× unlabeled CPD 30mer to reactions of a 5′-end-labeled AP/G 37mer with the purified GΔ228-Uve1p resulted in an ~60% decrease in the amount of product formed (Fig. 5B, lane 5). The addition of 40× unlabeled undamaged 30mer (UD 30mer) had no effect on the amount of product observed (Fig. 5B, lane 7). We conclude that Uve1p is capable of recognizing AP sites and that changing the complementary base to G or A has little effect on the extent of cleavage.

Table 2. Activity of Uve1p on oligonucleotide substrates containing uracil, dihydrouracil and AP sites

Protein	U/G	U/A	DHU/G	DHU/A	AP/G	AP/A
[a]Positive control	90–100	50–60	70–80	15–20	90–100	90–100
GΔ228-Uve1p	8–12	1–5	37–42	10–15	90–100	90–100
GST	1–5	1–5	1–5	1–5	1–5	1–5

The percent of substrate converted into total DNA cleavage products formed when the DNA damage lesion is base paired with a G or an A in the complementary strand. Details of experiments are outlined in Materials and Methods.
[a]Positive control: when analyzing U 37mer, uracil DNA glycosylase (UDG) was used as a positive control; for assays involving DHU 37mer, the *S.cerevisiae* endonuclease III-like homolog Ntg1 was used as a positive control; *E.coli* endonuclease IV was used as a positive control for AP endonuclease activity.

Uracil lesions can occur in DNA by the spontaneous deamination of a cytosine residue (1). Dihydrouracil is a pyrimidine photoproduct that is formed by the deamination of cytosine with subsequent ring saturation upon exposure to ionizing radiation under anoxic conditions (40). To determine if Uve1p recognized uracil and dihydrouracil lesions we incubated GΔ228-Uve1p with 3′-end-labeled 37mer oligonucleotides containing uracil and DHU residues placed opposite to a G (U/G 37mer and DHU/G 37mer). The results of this set of experiments are summarized in Table 2. Purified GΔ228-Uve1p cleaved U/G 37mer and DHU/G 37mer in a typical Uve1p-mediated fashion: immediately 5′ to the position of the lesion to form a major product and again 1 nt 5′ to the damaged site to form a minor product (data not shown). The major product accounted for ~90% and the minor product ~10% of the total Uve1p-mediated cleavage products formed.

Persistence of uracil and DHU lesions through replication may lead to the incorporation of adenine residues opposite the damaged base. To examine if Uve1p was equally efficient at recognizing uracil and DHU when they were base paired with an adenine residue we constructed the substrates U/A 37mer and DHU/A 37mer. The results obtained from the analysis of Uve1p cleavage of these substrates are summarized in Table 2. No Uve1p-mediated cleavage products were observed when purified GΔ228-Uve1p was incubated with the U/A 37mer (Table 2). Incubating purified GΔ228-Uve1p with DHU/A 37mer rather than DHU/G 37mer resulted in a 4-fold decrease in the amount of Uve1p-mediated cleavage products observed (Table 2). Uve1p recognizes and cleaves uracil and DHU when they are placed opposite a G (U/G or DHU/G). However, when the lesions are placed in a situation where Watson–Crick hydrogen bonding is maintained (U/A or DHU/A), Uve1p either fails to recognize the lesion completely (U/A) or the extent of cleavage is significantly decreased (DHU/A).

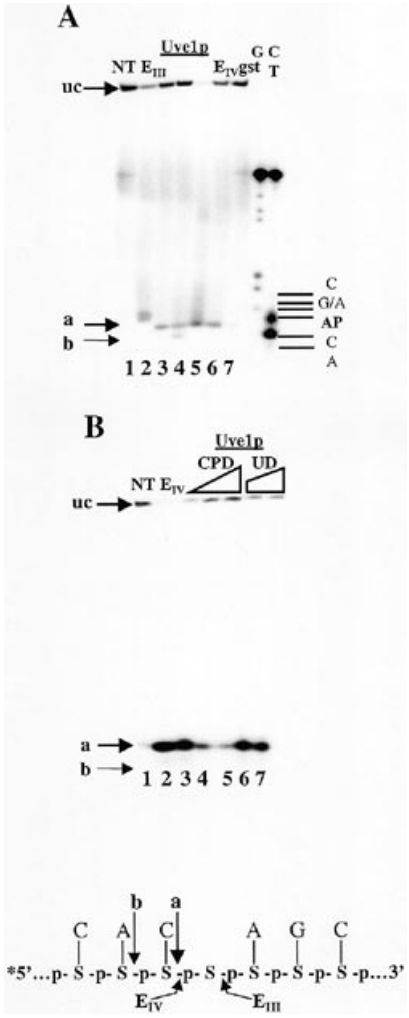

Figure 5. (**A**) Cleavage of an oligonucleotide substrate containing an AP site by Uve1p. To investigate if Uve1p was capable of cleaving an abasic site in a hydrolytic manner we prepared a 5′-end-labeled (*) abasic substrate, AP 37mer, and incubated this substrate with buffer alone (lane 1), *E.coli* endonuclease III (AP lyase, lane 2), affinity-purified GΔ228-Uve1p and Δ288-Uve1p (0.5 μg of each) (lanes 3 and 4), extracts of cells overexpressing GΔ288-Uve1p (5 μg) (lane 5), *E.coli* endonuclease IV (hydrolytic AP endonuclease, lane 6) and purified recombinant GST (2 μg) (lane 7). (**B**) Competitive inhibition of AP site recognition and cleavage. To demonstrate that the products generated are a result of Uve1p-mediated cleavage at the AP site, AP 37mer was incubated with buffer alone (lane 1), *E.coli* endonuclease IV (lane 2) and affinity-purified GΔ228-Uve1p (0.5 μg) (lane 3) with 10× and 40× unlabeled cs-CPD 30mer (lanes 4 and 5, respectively) and 10× and 40× unlabeled UD 37mer (lanes 6 and 7, respectively). Arrows a and b indicate the primary and secondary Uve1p-mediated cleavage products, respectively. Arrow uc indicates the uncleaved substrate. A portion of the sequence of the AP substrate is shown at the bottom of the figure. S corresponds to deoxyribose and p corresponds to phosphate. The location of the cleavage sites of endonuclease III (E_{III}) and endonuclease IV (E_{IV}) are also indicated. For simplicity the complementary strand is omitted from the figure.

Additionally, the Uve1p preparations were incubated with the following substrates to determine if these lesions were capable of being cleaved by Uve1p: inosine (the deamination product of

adenine) and xanthine (the deamination product of guanine) placed opposite a T or C (I/T 31mer, I/C 31mer, Xn/T 31mer and Xn/C 31mer) and 8-oxoguanine (a product of oxidative damage) placed opposite all four bases (8-oxoG/G 37mer, 8-oxoG/A 37mer, 8-oxoG/T 37mer and 8-oxoG/C 37mer). The amounts of Uve1p-mediated cleavage products formed for each of the substrates examined have been quantified as a percentage of the original substrate and are presented in Table 3. High levels of products are observed when Uve1p is incubated with substrates containing UV photoproducts, while low to moderate levels are observed when Uve1p is incubated with substrates containing U, DHU, Pt-GG DNA diadduct and Ap sites. Uve1p cleavage of the complementary strand was not observed for any of the substrates examined in this study.

Table 3. Uve1p cleavage efficiency on different substrates

Substrate	Percent cleavage[a]
cs-CPD 49mer	89
tsI-CPD 49mer	75
tsII-CPD 49mer	75
6–4PP 49mer	71
Dewar	83
AP 37mer	12.5
DHU 37mer	3
Pt-GG 32mer	2.5
U 37mer	1
8-oxoG 37mer	0
I 31mer	0
Xn 31mer	0

[a]The percent cleavage was calculated by quantifying the amount of Uve1p-mediated cleavage product formed when 300 ng of affinity-purified GΔ228-Uve1p was incubated with ~150 fmol of each substrate.

DISCUSSION

Uve1p is believed to initiate the first step in the alternative excision repair pathway for UV photoproducts and is the only known single protein endonuclease capable of recognizing and cleaving such a wide range of UV photoproducts (9–14). Uve1p has been cloned, overexpressed and affinity purified in an active stable truncated form, Δ228-Uve1p (15). The results presented here provide evidence that the substrate specificity range of Uve1p is much broader than was originally suspected. Based on these results, it is likely that Uve1p and its repair pathway may be involved in the repair of a number of different types of DNA damage.

Uve1p cleaves immediately 5′ to CPDs and 6–4PPs (12). In this study we demonstrate a second minor cleavage site (accounting for ~10% of the product formed) 1 nt upstream of the major site. This minor product is only observed when greater amounts of protein are utilized, as is the case in the assays described here. It is possible that this secondary product was unobserved previously because of the limited quantities of partially purified native *S.pombe* Uve1p available.

We have demonstrated that Uve1p generates cleavage products when it is incubated with oligonucleotides containing a variety of UV photoproducts: cs-CPDs, tsI-CPDs, tsII-CPDs, 6–4PPs and Dewar valence isomers. Recently, a BER N-glycosylase which recognizes tsII-CPDs has been described (41). This glycosylase also cleaves substrates containing cs-CPDs and to a lesser extent tsI-CPDs, but it does not recognize 6–4PPs or Dewar isomers. The Uve1p studies reported here are the first description of a single DNA repair endonuclease capable of recognizing such a broad spectrum of UV-induced photoproducts. Another group has also observed Uve1p-mediated cleavage of a Dewar-containing photoproduct substrate (A.Yasui, personal communication). Kinetic studies should determine which of these substrates are preferentially processed by Uve1p.

All of the UV-induced photoproducts used in this study cause substantially different distortions when they are incorporated into duplex DNA (19,21,27,42–44). It is possible that Uve1p is recognizing the structural distortion caused by these DNA damages as opposed to a specific lesion. To determine if this were the case we investigated if Uve1p was capable of recognizing a non-UV-induced dimer-like lesion. Formation of the *cis*-Pt-GG intrastrand crosslink causes a bending in the double helical structure towards the major groove with a partial unwinding of the DNA (45). This bending is not believed to prevent the platinated strand from base pairing to its complement to form a duplex, although there may be some effects on base stacking and the stability of the duplex DNA (45,46). DNA cleavage products were observed when Uve1p was incubated with the platinum-containing substrate Pt-GG 32mer. Uve1p was less efficient (~40-fold) at recognizing and cleaving this substrate than it was at cleaving the cs-CPD 30mer substrate. We also observed a change in the preferred site of cleavage when Uve1p was incubated with Pt-GG 32mer. This change may be due to the conformational structure of the platinum–DNA diadduct–Uve1p complex. It is also possible that the presence of the metal and appended ammine ligands in the major groove caused the observed shift in cleavage site. In addition, it is also noteworthy that the 5′-deoxyribose of the adduct is fixed in the C(3′)*endo* conformation (47), which is non-standard for B-DNA. It is possible that this structural asymmetry on the 5′-side of the adduct affects the position of cleavage. These factors, taken together, may also be responsible for the decrease in cleavage efficiency of Uve1p when it is incubated with Pt-GG 32mer. Although Uve1p is unique among UV repair enzymes in recognizing cs- as well as ts-CPDs and 6–4PPs, it is not unique in recognizing a platinum adduct as well as either a CPD or a 6–4PP. Photolyases from *S.cerevisiae* and *E.coli* have been shown to bind to platinum–DNA GG diadducts as well as CPDs (48,49) and nuclease SP isolated from spinach leaves recognizes and cleaves DNA containing a platinum–DNA GG diadduct as well as 6–4PPs (50).

We have also demonstrated that Uve1p is capable of cleaving a number of non-bulky DNA lesions: AP sites, uracil and dihydrouracil. The cleavage efficiency of Uve1p decreased dramatically when U/G and DHU/G 37mer substrates were replaced by U/A and DHU/A 37mer substrates. It is possible that Uve1p is recognizing a distortion caused by the formation of a wobble base pair between the uracil and DHU and the opposite G rather than the actual lesion itself. Changing the opposite base to an adenine had no detectable effect on Uve1p-mediated cleavage of an AP site, however, this does not rule out the possibility that changing the base to either cytosine or thymine may affect Uve1p cleavage of this substrate. We have recently reported that Uve1p is capable of recognizing and cleaving mismatched base pairs, indicating that the loss of hydrogen bond formation is a significant factor in determining the substrate

specificity of Uve1p (51). It is not surprising therefore that Uve1p failed to recognize the inosine-containing substrates as it has been reported that inosine is able to form hydrogen bonded base pairs with all four normal bases (52). In contrast, in substrates containing xanthine placed opposite a T or C (where hydrogen bond formation is unstable; 53) and 8-oxoguanine when it was placed opposite all four bases no Uve1p-mediated cleavage products were observed. It is clear therefore that the loss of hydrogen bond formation is not the sole factor in determining the activity of Uve1p on uracil- and DHU-containing substrates. It is also apparent that the degree of DNA bending is not solely responsible, as Uve1p recognizes lesions that induce minimal bending, such as cs-CPDs, and ones that induce a much greater degree of bending, 6–4PPs, to a similar extent. The nature of the damaged lesion itself must play an important role in directing Uve1p to the site of damage. Perhaps it is a combination of factors that determines the substrate preference of Uve1p, for example: the size and structure of the lesion, the distorting effect of the DNA damage giving rise to bending of the DNA backbone and/or a loss of hydrogen bonding ability possibly resulting in extrahelical protrusions. Determination of the structure of Uve1p and its interactions with DNA may elucidate the most important factors as well as the mechanism underlying Uve1p activity.

It has been shown recently that the nucleotide excision repair enzyme XPG serves as a cofactor for the efficient function of hNth1 by promoting binding of hNth1 to the damaged DNA (54). It is possible that *in vivo* other accessory proteins may contribute to the efficiency of Uve1p, thereby enhancing its ability to initiate the repair of a wide variety of DNA damages. Reconstitution of the Uve1p pathway *in vitro* would allow us to examine the effects of other proteins on the activity of Uve1p and to investigate their roles as cofactors in the Uve1p-mediated recognition and repair of different types of DNA damage.

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