A Uracil-DNA Glycosylase Inhibitor Encoded by a Non-uracil Containing Viral DNA*

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Summary

Uracil-DNA glycosylase (UDG) is an enzyme involved in the base excision repair pathway. It specifically removes uracil from both single-stranded and double-stranded DNA. The genome of the Bacillus subtilis phage ϕ29 is a linear double-stranded DNA with a terminal protein covalently linked at each 5’-end. Replication of ϕ29 DNA starts by a protein-priming mechanism and generates intermediates that have long stretches of single-stranded DNA. By using in vivo chemical cross-linking and affinity chromatography techniques, we found that UDG is a cellular target for the early viral protein p56. Addition of purified protein p56 to B. subtilis extracts inhibited the endogenous UDG activity. Moreover, extracts from ϕ29-infected cells were deficient in UDG activity. We suggested that inhibition of the cellular UDG by a defense mechanism developed by ϕ29 to prevent the action of the base excision repair pathway if uracil residues arise in their replicative intermediates. Protein p56 is the first example of a UDG inhibitor encoded by a non-uracil-containing viral DNA.

Uracil-DNA glycosylase (UDG) is a cellular enzyme involved in the base excision repair pathway. It specifically removes uracil from both single-stranded and double-stranded DNA. The genome of the Bacillus subtilis phage ϕ29 is a linear double-stranded DNA with a terminal protein covalently linked at each 5’-end. Replication of ϕ29 DNA starts by a protein-priming mechanism and generates intermediates that have long stretches of single-stranded DNA. By using in vivo chemical cross-linking and affinity chromatography techniques, we found that UDG is a cellular target for the early viral protein p56. Addition of purified protein p56 to B. subtilis extracts inhibited the endogenous UDG activity. Moreover, extracts from ϕ29-infected cells were deficient in UDG activity. We suggested that inhibition of the cellular UDG by a defense mechanism developed by ϕ29 to prevent the action of the base excision repair pathway if uracil residues arise in their replicative intermediates. Protein p56 is the first example of a UDG inhibitor encoded by a non-uracil-containing viral DNA.

Uracil in DNA may arise from spontaneous deamination of cytosine and from the occasional use of dUTP instead of dTTP during DNA replication. Hydrolytic deamination of cytosine generates G:U mismatches that cause G:C to A:T transition mutations. To maintain the genome integrity, most prokaryotic and eukaryotic cells rapidly eliminate such mismatches by the uracil-DNA glycosylase (UDG) enzyme. UDGs have been identified in numerous organisms, including human cells (Family-1 UDGs) (3). Studies on substrate specificity showed that Family-1 UDGs efficiently remove uracil residues from both single-stranded and double-stranded DNAs, often with preference for the single-stranded substrates (4, 5). The AP site generated by the UDG enzyme is further recognized by an AP endonuclease, which cleaves the phosphodiester bond of the DNA backbone 5’ to the AP site. Several AP endonucleases are active not only on double-stranded DNAs but also on single-stranded DNAs (6–8). Further repair can be accomplished via two pathways that involve different subsets of enzymes and result in replacement of one (short patch pathway) or more (long patch pathway) nucleotides (9).

Over the course of evolution, bacteriophages have developed unique protein families that bind to and inactivate critical cellular proteins, shutting off key processes. The DNA genome of phage PBS2 contains uracil in place of thymine residues (10). Following infection of Bacillus subtilis, this phage induces various enzymatic activities that ensure the use of dUTP rather than dTTP during viral DNA synthesis. In addition, phage PBS2 encodes an inhibitor of the B. subtilis UDG enzyme, named Ugi, which is essential for the preservation of uracil residues incorporated into phage DNA (11–13). Some studies showed that Ugi (84 amino acids) specifically inactivates Family-1 UDGs (13, 14). The x-ray crystal structures of Ugi in complex with different UDGs revealed that Ugi mimics the electropositive and structural features of duplex DNA (15–17).

Unlike phage PBS2, the genome of the B. subtilis phage ϕ29 does not contain uracil residues. It is a linear double-stranded DNA, with a terminal protein (TP) covalently linked at both 5’-ends. Replication of ϕ29 DNA starts nonsimultaneously at either DNA end, where the replication origins are located, by a protein-priming mechanism (18). Electron microscopy studies revealed two main types of ϕ29 replicative intermediates in infected cells, named type I and type II (19, 20) (Fig. 1A). Type I intermediates are unit-length linear double-stranded DNA molecules with one or more single-stranded branches of varying lengths. Type II intermediates are unit-length linear molecules in which a region of the DNA starting from one end is double-stranded, and the adjacent region containing the other end is single-stranded. Thus, both replicative intermediates have long stretches of single-stranded DNA. Most unexpectedly, during the functional characterization of the early viral protein p56 (56 amino acids), we found that p56 interacted with the B. subtilis UDG and inhibited its activity. Furthermore, we demonstrated that extracts from ϕ29-infected cells lacked UDG but not AP endonuclease activity. UDG inhibitors encoded by non-uracil containing viral DNAs were not identified previously. We propose that inhibition of the host UDG by protein p56 ensures the integrity of the ϕ29 replicative intermediates if uracil residues arise either by cytosine deamination or dUMP misincorporation.
EXPERIMENTAL PROCEDURES

Bacterial Strains, Bacteriophages, and Plasmids—B. subtilis 110NA (23), a nonsuppressor (su−) strain, and B. subtilis MO-101-P (24), a suppressor strain (su− su−), were used. Phage ϕ29 sus4 (56) (23) was used in cross-linking experiments. This phage carries a suppressor-sensitive mutation in gene 4, which encodes an activator of the late transcription (25). Phage ϕ29 sus4 (1242) (26) was used to measure UDG activity in extracts of infected cells. This phage contains a suppressor-sensitive mutation in gene 14, which encodes the holin protein (27). Thus, cell lysis is delayed. Phage stocks were prepared as reported (28).

To construct plasmid pCR2.1-TOPO, the TOPO TA cloning kit (Invitrogen) was used. Basically, a 292-bp DNA region, which contains gene 56, was amplified by the PCR using the oligonucleotides 5'-CGCTCTAGAGTCGAC-3' and 5'-GCGAGGAATCTGCAGTCAAAGACCTTTATC-3' as primers. The 267-bp PCR-synthesized fragment was cloned into the E. coli expression vector pCR2.1-TOPO (Invitrogen), which is based on the T7 promoter. Transformed cultures of the E. coli TOP10 strain were plated on LB plates containing 50 μg/ml of kanamycin. To construct plasmid pPR53, the 272-bp PstI restriction fragment of plasmid pCR2.1-TOPO, which carries gene 56, was inserted into the PstI site of the B. subtilis constitutive expression vector pPR53, which is based on the P5 promoter of phage λ (29). The B. subtilis strain YB886 (30) was used for cloning, and transformants were selected for phleomycin resistance (0.8 μg/ml). Gene 56 was engineered to encode a FLAG-tagged p56 protein (p56FLAG). To this end, a two-step mutagenesis method was used. In the first step, gene 56 was amplified by PCR using plasmid pPR53.p56 as template and the oligonucleotides A (5'-CTCTAGAGTCGACGG-3') and B (5'-GTATCTGACTCTTATAGTGGGACCTT-3') as primers. In the second step, the 298-bp PCR-amplified fragment was further digested with PstI and the 267-bp digestion product was inserted into the PstI site of the B. subtilis vector pPR53 (plasmid pPR53.p56FLAG).

Phage Growth under One-step Conditions—B. subtilis 110NA cells were exponentially grown at 30 °C in LB medium supplemented with 5 mM MgSO4 to an absorbance at 560 nm (A560) equivalent to ~10^8 colony-forming units (cfu) per ml. The culture was then infected with ϕ29 at a multiplicity of 5–10. After 10 min of incubation with gentle shaking, unadsorbed phages were eliminated by centrifugation of the infected culture. Cells were resuspended in the same volume of medium and incubated with vigorous shaking for the indicated time.

Immunoblotting—Gel-separated proteins were transferred electrophoretically to Immobilon-P membranes (Millipore) using a Mini Trans Blot (Bio-Rad) at 100 mA for 4 h. Transfer buffer contained 25 mM Tris, 192 mM glycine, 20% methanol. Membranes were probed with anti-p56 serum for 60 min. Antigen-antibody complexes were detected using anti-rabbit horseradish peroxidase-conjugated antibodies and ECL Western blotting detection reagents (Amersham Biosciences). For quantitative immunoblotting (29), cell extracts were prepared from a known number of viable cells, which was determined before phage addition. Increasing amounts of the cell extract and known amounts of purified protein p56 were run in the same gel.

In Vivo Chemical Cross-linking—Bacteria were washed with 50 mM Heps, pH 8.0, and concentrated 20-fold in buffer P (50 mM Heps, 10 mM EDTA, 20% sucrose, pH 8.0). The cross-linker dithiobis(succinimidylpropionate) (DSP) (Pierce) was dissolved in Me2SO just before use. DSP was added to the culture at the indicated concentration. After incubation at room temperature for 20 min, Tris-HCl, pH 7.5, was added at a final concentration of 150 mM to quench the reaction. Cells were harvested by centrifugation, resuspended in loading buffer without β-mercaptoethanol (60 mM Tris-HCl, pH 6.8, 2% SDS, 30% glycerol), and disrupted by sonication.

Isolation of p56FLAG Complexes—B. subtilis 110NA cells carrying plasmid pPR53.p56FLAG were exponentially grown in LB medium containing phleomycin (0.8 μg/ml) at 30 °C to an A560 equivalent to ~10^8 cfu/ml. Cells were concentrated 10-fold in buffer TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and disrupted by French pressure treatment (20,000 psi). The whole-cell extract was centrifuged at 7,000 rpm and 4 °C in a Sorvall SS34 rotor for 10 min. The supernatant was loaded onto an anti-FLAG M2 affinity column (Sigma) under gravity flow. Later, the column was extensively washed with buffer TBS. Proteins bound to the column were eluted with buffer TBS containing FLAG-peptide (500 μg/ml) (Sigma). Eluted proteins were precipitated with acetone and resuspended in loading buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 30% glycerol).

Peptide Mass Fingerprinting—Gel-separated proteins were stained with Sypro Ruby and subjected to in situ digestion with trypsin, as described (31). Peptide masses were measured with a matrix-assisted laser desorption/ionization time of flight mass spectrometer Autoflex (Bruker Daltonic; Bremen, Germany) equipped with a reflector and employing 2,5-dihydroxybenzoic acid as matrix and a Anchor-Chip surface target. The mass spectra were fitted to data bases by the program Mascot (32).

Purification of Protein p56—E. coli BL21(DE3) cells carrying plasmid pCR2.1-TOPO.p56 were grown in LB medium containing 50 μg/ml of kanamycin at 34 °C. When the culture reached an A560 of 0.9, isopropyl-1-thio-β-D-galactopyranoside was added to a final concentration of 0.5 mM. After 30 min, cells were incubated with rifampicin (120 μg/ml) for 75 min. Cells were collected by centrifugation and frozen at −70 °C before being used. Protein p56 was purified under ice-cold conditions using the following protocol. Cells were ground with alumina in buffer A (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 7 mM β-mercaptoethanol, 5% glycerol) containing 0.65 M NaCl. After removal of alumina and cell debris by centrifugation, the cleared lysate was mixed with polyethyl entine (0.3%), incubated on ice for 20 min, and centrifuged at 12,000 rpm in a Sorvall GSA rotor for 10 min. The supernatant was made 0.3 M NaCl with buffer A and centrifuged as before for 20 min. The resulting pellet was washed with buffer A containing 0.7 M NaCl. After centrifugation (12,000 rpm in a Sorvall SS34 rotor for 20 min), the supernatant was processed by stepwise ammonium sulfate precipitation at 65, 45, and finally 30%. Proteins were recovered from the 30% ammonium sulfate supernatant by raising it to 50% ammonium sulfate. This last pellet was resuspended in buffer A to a final salt concentration of 55 mM (estimated by conductivity measurements). The protein preparation was then loaded onto a Mono Q column equilibrated with buffer A containing 55 mM NaCl. Protein p56 was eluted from the column with 0.3 M NaCl. This fraction was further loaded onto a 15–30% glycerol gradient and subjected to centrifugation at 62,000 rpm in a Beckman SW65 rotor for 20 h. Fractions containing p56 were pooled, precipitated with ammonium sulfate to 70% saturation, resuspended in buffer A containing 50% glycerol, and stored at −70 °C.

Interaction between p56 and E. coli UDG in Vitro—The reaction mixture (10 μl) contained 20 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 1 mM EDTA, 20 mM NaCl, 20% glycerol, 2 μg of protein p56, and 0.2 μg of E. coli UDG (New England Biolabs). After incubation at room temperature for 15 min, the sample was kept at 4 °C for 15 min and analyzed by
nondenaturing PAGE (16% polyacrylamide). Gel electrophoresis was performed at 4 °C.

UDG and AP Endonuclease Activities in B. subtilis Extracts—B. subtilis strain 110NA was grown to mid-log phase in LB medium at 30 °C (−10° cfu/ml). Cells were concentrated 10-fold in buffer U (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 12 mM β-mercaptoethanol, 1 mM EDTA) containing a protease inhibitor mixture (1 tablet of Complete, Mini, EDTA-free per 10 ml) from Roche Applied Science. Cells were disrupted by French pressure treatment (20,000 p.s.i.). After centrifugation at 7,000 rpm and 4 °C in a Sorvall SS-34 rotor for 10 min, the supernatant (extract) was kept at 4 °C for up to 2 weeks. Total protein concentration (1.35 mg/ml) was determined by the Lowry method using bovine serum albumin as standard.

RESULTS

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A & Pendonuclease (AP) Endonuclease Activity

A. Type I

B. Type II

FIGURE 1. A types of φ29 replicative intermediates. The genome of φ29 is a linear double-stranded DNA with a TP covalently linked at each 5'-end (parental TP; white circle). A free molecule of the TP (primer TP; black circle) provides the hydroxyl group needed by the viral DNA polymerase to start DNA synthesis at both φ29 DNA ends (protein-priming replication mechanism) (21). During elongation, φ29 DNA polymerase catalyzes highly processive polymerization coupled to strand displacement (22), and consequently, complete replication of both strands proceeds continuously from each priming event. See Introduction for more details. B. genetic and transcriptional map of the left end of the φ29 genome. Only relevant features are shown. White arrows indicate the location of genes previously identified. The black arrow indicates the position of gene 56, which has been identified in this work. Genes 4 (double-stranded DNA binding protein), 3 (single-stranded DNA binding protein), 2 (TP), and 1 (DNA polymerase) are essential for in vivo φ29 DNA replication (18). Gene 1 encodes a membrane-localized protein involved in the membrane association of φ29 DNA replication. This protein enhances the rate of viral DNA replication in vivo (33). Wavy lines represent transcripts from the indicated early promoters. The position of the transcriptional terminator TA1 is indicated.
these cells were incubated with DSP, the ~35-kDa p56 complex was again detected. Hence, the viral protein p56 was able to interact with a host protein both during the infective process and in the absence of viral components. Taking into account the molecular mass of p56 (6.6 kDa) and DSP (0.4 kDa), the molecular mass of the host protein would be ~28 kDa.

Protein p56 Interacts with UDG—To identify the cell target for protein p56, gene 56 was engineered to encode a FLAG-tagged p56 protein (p56FLAG). This variant of p56 carries the peptide DYPKDDDDKK fused to its C-terminal end. The mutant gene was cloned into the B. subtilis expression vector pPR53 (plasmid pPR53.p56FLAG). By immunoblotting, we verified that B. subtilis cells carrying the recombinant plasmid constitutively expressed protein p56FLAG at levels similar to those detected for p56 at late stages of −29 infection (not shown). Extracts from these cells were applied to an anti-FLAG affinity column. The pure FLAG peptide was then used to elute p56FLAG. As a negative control, extracts from B. subtilis cells harboring plasmid pPR53 were used. The eluted proteins were separated by SDS-Tricine-PAGE and stained with SyproRuby (Fig. 3A). Protein p56FLAG co-eluted with five proteins (named A to E) that were absent in the control sample. With the exception of protein E, such proteins were identified by peptide mass fingerprinting using the MASCOT search program (32). Protein A and B were identified as the E2 (47.5 kDa; dihydrolipoamide acetyltransferase) and E3 (49.7 kDa; dihydrolipoamide dehydrogenase) subunits of the pyruvate dehydrogenase multienzyme complex (PDH), respectively.

To investigate whether protein p56 was an inhibitor of the B. subtilis UDG, we set up an assay to measure UDG activity in B. subtilis extracts. In the first experiment, a 34-mer single-stranded oligonucleotide containing a single uracil residue at position 16 (ssDNA−U16) was incubated with UDG for 15 min at room temperature, and the reaction mixture was analyzed by non-denaturing PAGE. As shown in Fig. 3B, free protein p56, but not free UDG, was detected. Moreover, a protein band migrating faster than UDG was visualized (p56−UDG−coli). This band contained p56 and UDG, as confirmed by immunoblot analysis and peptide mass fingerprinting, respectively (not shown). In conjunction with the above, this result demonstrated that protein p56 forms a complex with UDG.

Protein p56 Functions as an Inhibitor of the B. subtilis UDG—Family-1 UDGs are able to excise uracil base efficiently from both single-stranded and double-stranded DNAs. Furthermore, they are specifically sensitive to Ugi (3), a UDG inhibitor encoded by phage PBS2. Removal of uracil by UDGs generates an AP site that can be processed by hydrolytic AP endonucleases. In the absence of an AP endonuclease activity, chemical cleavage of the DNA at the AP site can be achieved by treatment with heat and alkali.

To determine whether protein p56 was an inhibitor of the B. subtilis UDG, we set up an assay to measure UDG activity in B. subtilis extracts. In the first experiment, a 34-mer single-stranded oligonucleotide containing a single uracil residue at position 16 (ssDNA−U16) was incubated with UDG for 15 min at room temperature, and the reaction mixture was analyzed by non-denaturing PAGE. As shown in Fig. 3B, free protein p56, but not free UDG, was detected. Moreover, a protein band migrating faster than UDG was visualized (p56−UDG−coli). This band contained p56 and UDG, as confirmed by immunoblot analysis and peptide mass fingerprinting, respectively (not shown). In conjunction with the above, this result demonstrated that protein p56 forms a complex with UDG.

The B. subtilis UDG is highly homologous to the E. coli UDG (Family-1 UDGs) (3, 40). Therefore, we analyzed whether protein p56 was able to interact with UDG purified from E. coli. To this end, protein p56 was incubated with E. coli UDG for 15 min at room temperature, and the reaction mixture was analyzed by non-denaturing PAGE. As shown in Fig. 3B, free protein p56, but not free UDG, was detected. Moreover, a protein band migrating faster than UDG was visualized (p56−UDG−coli). This band contained p56 and UDG, as confirmed by immunoblot analysis and peptide mass fingerprinting, respectively (not shown). In conjunction with the above, this result demonstrated that protein p56 forms a complex with UDG.
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We next examined whether the viral protein p56 was able to inhibit the UDG activity of the *B. subtilis* extract (Fig. 4C). To this end, the ssDNA-U16 substrate was incubated with 1.6 μg of extract in the presence of different amounts of purified protein p56 (from 0.5 to 16 ng). After 10 min, NaOH was added to the reactions. A decrease in the amount of the cleavage product was detected with 2 ng of p56. Moreover, cleavage of the substrate did not take place in the presence of 8 ng of p56. Therefore, these results showed that protein p56 acts as an inhibitor of the *B. subtilis* UDG.

Inhibition of UDG Activity in ϕ29-Infected Cells—If protein p56 functions as a UDG inhibitor during ϕ29 infection, extracts from infected cells should be deficient in UDG activity. To test this hypothesis, *B. subtilis* cells were infected with ϕ29 under one-step growth conditions, and extracts were prepared at 30 min of infection. Then the ssDNA-U16 substrate was incubated, in the absence of Mg2+, with increasing amounts of a *B. subtilis* extract. After 10 min, NaOH was added to the reaction mixtures. As shown in Fig. 4A, a cleavage product was generated using 0.2 μg of extract. Moreover, the substrate was almost totally cleaved when 1.6 μg of extract were used. The same cleavage product was detected when the substrate was incubated with UDG purified from *E. coli*. Therefore, the *B. subtilis* extract was capable of removing uracil from single-stranded DNA generating an AP site (UDG activity). However, under the assayed conditions, the extract lacked AP endonuclease activity, because cleavage of the substrate did not occur when the reactions were not treated with NaOH (Fig. 4A).

Furthermore, the UDG activity of the *B. subtilis* extract was sensitive to the presence of Ugi (Fig. 4B) and was able to excise uracil from double-stranded DNA bearing a G/U mismatch (not shown). Collectively, these results demonstrated the presence of Family-1 UDG in the *B. subtilis* extract.

We have measured UDG activity in *B. subtilis* extracts. This enzymatic activity was Mg2+-dependent and excised uracil residues from both single-stranded and double-stranded DNAs. Addition of purified protein p56 to the *B. subtilis* extract inhibited the endogenous UDG activity. Moreover, a drastic reduction in UDG activity was observed in extracts from ϕ29-infected cells, whereas the AP endonuclease activity remained intact. Therefore, the early viral protein p56 functions as an inhibitor of the cellular UDG during ϕ29 infection. This conclusion was supported by protein-protein interaction experiments. A complex formed by p56 and a host protein of ~28 kDa was detected in infected cells using chemical cross-linking techniques. Moreover, UDG (26 kDa) co-eluted with p56FLAG when extracts of *B. subtilis* cells producing p56FLAG and anti-FLAG affinity columns were used. Protein p56 also formed a complex with *E. coli* UDG in vitro, as determined by native PAGE. *B. subtilis* UDG has strong sequence homology to *E. coli* UDG (40). Both enzymes belong to a family of highly conserved DNA glycosylases, which remove uracil from both single-stranded and double-stranded DNAs (3).

By affinity chromatography, we found that in addition to UDG, E2 and E3 co-eluted with protein p56FLAG. E2 and E3 are components of the PDH multienzyme complex, which catalyzes the irreversible oxidative decarboxylation of pyruvate to acetyl-coenzyme A. PDH complexes of Gram-positive bacteria have a core of 60 E2 subunits with icosahedral symmetry (41). Besides its role in oxidative metabolism, the E2 subunit appears to have DNA binding activity (42, 43). The low isoelectric point of protein p56 suggests that it might act as a double-stranded DNA mimic, like the UDG inhibitor Ugi and the highly acidic protein HI1450 from *Haemophilus influenzae* (15, 16, 44). If this were the case, co-
elution of the E2 subunit with p56FLAG could be related to the ability of E2 to interact with DNA. Nevertheless, at present, it is unknown whether a p56-E2 complex is formed during φ29 infection.

Inhibition of the cellular UDG activity after phage infection was established previously in two systems. The first example was the inhibition of the *B. subtilis* UDG after infection with phage PBS2 (11). The need for this inhibition was obvious, because the DNA genome of PBS2 contains uracil instead of thymine residues. Thus, phage PBS2 has developed a defensive mechanism against the action of the host UDG. The UDG inhibitor encoded by phage PBS2 was identified as an acidic protein of 84 amino acids (Ugi) (13). Less obvious was the inhibition of the *E. coli* UDG after infection with phage T5, because its DNA genome does not contain uracil residues. In this case, the UDG inhibitor has not yet been identified, although early studies suggested that it could be a pre-early phage-encoded protein (45). In fact, the pre-early region of phage T5 contains six small open reading frames that presumably encode host function inhibitors (46).

Why does phage φ29 encode an inhibitor of the cellular UDG? We think that this inhibition is related to the mechanism of φ29 DNA replication. As depicted in Fig. 6, replication of the linear φ29 DNA starts nonsimultaneously at both ends, where the replication origins are located, using a free molecule of the TP as primer. Once initiated, replication proceeds by a strand displacement mechanism, and consequently, replicative intermediates (type I and type II) with long stretches of single-stranded DNA are generated (18). If uracil arises in the φ29 genome, either by misincorporation of dUMP or by cytosine deamination, and the damage is not repaired before DNA replication, type I replicative intermediates carrying a uracil residue on single-stranded DNA could appear. The presence of uracil in the replicative intermediate could recruit components of the cellular BER pathway, such as UDGs and AP endonucleases. As shown in this work, *B. subtilis* cells synthesize an enzymatic activity that efficiently removes the aberrant base uracil from single-stranded DNA, generating an AP site. The subsequent action of an AP endonuclease activity would introduce a nick into the phosphodiester backbone with accompanying loss of the terminal DNA region. It has been shown that the ExoA protein of *B. subtilis* has AP endonuclease activity on single-stranded DNA (7). When two replication forks moving in opposite directions merge, type II replicative intermediates are formed. Then DNA synthesis would continue, and a shorter viral DNA molecule lacking one parental TP would be generated. Therefore, the action of the cellular UDG on single-stranded DNA regions of the φ29 replicative intermediates would be harmful for viral replication.

Uracilation of DNA represents a constant threat to the survival of many organisms, including viruses. More than likely, phage φ29 has developed alternative strategies to protect its genome from uracilation.

One possibility may be the recruitment of the cellular deoxyuridine triphosphatase enzyme (dUTPase), which maintains a low dUTP:dTTP ratio and, consequently, minimizes the misincorporation of uracil into DNA. Most interestingly, herpesviruses, poxviruses, and certain retroviruses encode dUTPase, whose function is thought to be associated with the ability of these viruses to replicate in cells that produce low levels of dUTPase (1). Another possible strategy of φ29 to counteract the accumulation of uracil in its genome may be the recruitment of a mismatch-specific UDG activity. For example, *E. coli* encodes a double-strand specific enzyme capable of removing uracil residues from G:U mismatches arising through spontaneous cytosine deamination. Such an enzyme is related to the human thymine-DNA glycosylase and is insensitive to inhibition by Ugi (47).

The generation of replicative intermediates containing long stretches of single-stranded DNA is a feature of the protein-primed mechanism...
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of DNA replication. Therefore, it is expected that gene 56 is conserved in the genome of other \( \phi 29 \)-related phages. In the case of phage B103, there is an open reading frame that would encode a 56-amino acid protein, whose deduced sequence has a high level of homology to protein p56 (64% identity and 75% similarity) (48). Furthermore, in phage GA-1, which is the most distantly \( \phi 29 \)-related phage, there is an open reading frame that would encode a 130-amino acid protein. The region of this putative protein spanning amino acids 27 and 82 shows 23% identity and 27% similarity with protein p56, suggesting that they may have similar functions.3

To conclude, protein p56 of phage \( \phi 29 \) functions as an inhibitor of the cellular Family-1 UDG. This inhibition is likely a defense mechanism developed by \( \phi 29 \) and \( \phi 29 \)-related phages to prevent the action of the BER pathway if uracil residues arise in the replicative intermediates.

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