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Phage ϕ29 protein p56 prevents viral DNA replication impairment caused by uracil excision activity of uracil-DNA glycosylase

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Protein p56 encoded by the *Bacillus subtilis* phage ϕ29 inhibits host uracil-DNA glycosylase (UDG) activity. In previous studies, we suggested that this inhibition is likely a defense mechanism developed by phage ϕ29 to prevent the action of UDG if uracilation occurs in DNA either from deamination of cytosine or the incorporation of dUMP during viral DNA replication. In this work, we analyzed the ability of ϕ29 DNA polymerase to insert dUMP into DNA. Primer extension analysis showed that viral DNA polymerase incorporates dU opposite dA with a catalytic efficiency only 2-fold lower than that for dT. Using the ϕ29 DNA amplification system, we found that ϕ29 DNA polymerase is also able to carry out the extension of the dA:dUMP pair and replicate past uracil. Additionally, UDG and apurinic-apyrimidinic endonuclease treatment of viral DNA isolated from ϕ29-infected cells revealed that uracil residues arise in ϕ29 DNA during replication, probably as a result of misincorporation of dUMP by the ϕ29 DNA polymerase. On the other hand, the action of UDG on uracil-containing ϕ29 DNA impaired *in vitro* viral DNA replication, which was prevented by the presence of protein p56. Furthermore, transfection activity of uracil-containing ϕ29 DNA was significantly higher in cells that constitutively synthesized p56 than in cells lacking this protein. Thus, our data support a model in which protein p56 ensures an efficient viral DNA replication, preventing the deleterious effect caused by UDG when it eliminates uracil residues present in the ϕ29 genome.

ϕ29 DNA polymerase | DNA repair | protein-primed replication | dUMP incorporation

Uracil residues are introduced into genomic DNA either by incorporation of dUMP in place of dTMP during replication or deamination of existing dCMP residues (1). *In vivo* studies have shown that misincorporation of dUMP during DNA synthesis introduces a significant amount of uracil residues into the DNA of many organisms (2–4). However, the levels of uracil actually remaining in DNA, ≈10–15 residues per human genome (5), are lower because of the DNA base excision repair (BER) process, which is responsible for the rapid uracil removal and repair (1). The BER pathway is initiated by uracil-DNA glycosylase (UDG), which cleaves the N-glycosidic bond, generating an apurinic-apyrimidinic (AP) site, which is then repaired through the sequential action of AP endonuclease (Ape), DNA polymerase, and DNA ligase. The first UDG activity reported was purified from *Escherichia coli* cells (6). Since then, enzymes highly homologous to the archetypal *E. coli* UDG have been identified in numerous organisms, including human cells, yeast, herpesvirus, and poxvirus. These UDGs (family 1) are able to eliminate uracil bases efficiently from both single-stranded and double-stranded DNA regardless of the partner base, U:A or U:G (7).

Bacteriophages have developed unique proteins that arrest critical cellular processes to commit bacterial host metabolism to phage reproduction. Phages PBS1 and PBS2, which infect *Bacillus subtilis* cells, contain uracil naturally instead of thymine in their DNA. This unusual base replacement is effected by the induction of a number of proteins that are expressed after phage infection to increase the size of the intracellular dUTP pool relative to that of the dTTP pool. In addition to these activities, phages PBS1 and PBS2 encode an inhibitor of the *B. subtilis* UDG, named Ugi (84 aa), which is essential for preservation of uracil residues incorporated into the phage DNA (8). The x-ray crystal structures of Ugi in complex with different UDGs revealed that Ugi mimics electronegative and structural features of duplex DNA (9).

Recently, we identified an inhibitor (protein p56) of the *B. subtilis* UDG encoded by bacteriophage ϕ29 (10). *In vitro* experiments showed that protein p56 blocks the DNA-binding ability of UDG, probably by mimicking the structure of DNA (11). The genome of the lytic phage ϕ29 is a linear dsDNA with a terminal protein (TP) covalently linked at both 5′ ends, which replicates by a protein-priming mechanism (12). Synthesis of the linear ϕ29 DNA starts at both ends, where the replication origins are located, and proceeds by a strand-displacement mechanism, generating replicative intermediates with long stretches of ssDNA. We have suggested that inhibition of the host UDG by protein p56 is likely a defense mechanism developed by ϕ29 and ϕ29-related phages, which also encode p56-like proteins, to prevent the damaging action of the BER pathway if uracils arise in their replicative intermediates (10). The presence of these residues in the ssDNA intermediates, arising either by deamination of cytosine or misincorporation of dUMP during the previous replication round, could recruit components of the cellular BER pathway, such as UDGs and Apes. As depicted in Fig. 1, the subsequent action of these activities would introduce a nick into the phosphodiester backbone with accompanying loss of the terminal region. In the present work, we show that ϕ29 DNA polymerase efficiently incorporates dUMP into DNA. This ability is probably responsible for the presence of most uracil residues found in the ϕ29 DNA molecules generated during the infective cycle. Furthermore, we have analyzed the consequences of the presence of uracil residues in the ϕ29 genome on *in vitro* viral DNA replication and virus production both in the presence of UDG and when UDG is inhibited by the ϕ29 protein p56.

Author contributions: G.S.-H., A.B., and M.S. designed research; G.S.-H. performed research; G.S.-H. and M.S. analyzed data; and G.S.-H., A.B., and M.S. wrote the paper.

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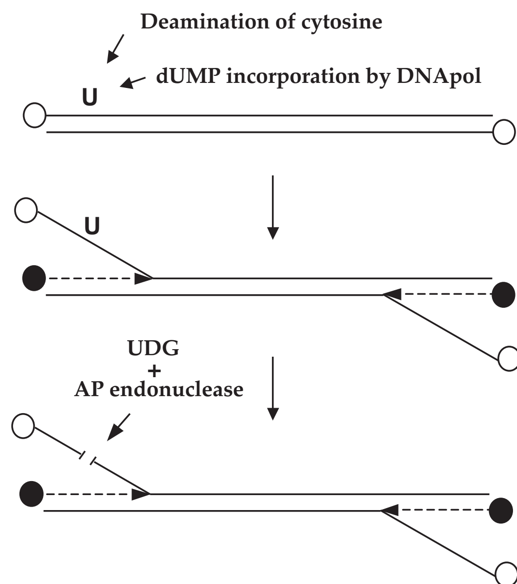

Fig. 1. Model of the effect of UDG on ϕ29 DNA replication when uracil residues appear in ssDNA regions of replicative intermediates either by deamination of cytosine or misinsertion of dUMP by ϕ29 DNA polymerase during the previous replication round. Parental TP (○) and primer TP (●) are indicated. Dashed lines indicate newly synthesized viral DNA. See Introduction for details. Figure was modified from Serrano-Heras *et al.* (10).

Results

ϕ29 DNA Polymerase Uses dUTP as a Substrate in Viral DNA Replication. Bacteriophage ϕ29 DNA polymerase is a protein-primed DNA-dependent replicase belonging to the eukaryotic-type family of DNA polymerases (family B). It contains both 5′-3′synthetic and 3′-5′degradative activities within a single polypeptide chain and accomplishes template-directed addition of dNMP units onto the 3′-OH group of a growing DNA chain, with intrinsic insertion discrimination of 10^4 to 10^6 (13), which is further improved 100-fold through proofreading by the exonuclease activity (14).

To analyze the ability of ϕ29 DNA polymerase to incorporate dUMP into DNA, primer extension reactions were carried out. Four synthetic primer/template structures (SP1/SP1c + 6) that differ in the first template base were used as substrates for DNA polymerization in the presence of either dTTP or dUTP. ϕ29 DNA polymerase displayed a clear preference to insert dU opposite dA with respect to other template nucleotides (dT, dC, and dG) as was the case for dT insertion (Fig. 2). Subsequently, we calculated insertion kinetic constants for dU as compared with dT, using the hybrid molecule SP1/SP1c + 6(A), which offers an A residue as the first nucleotide on the template. As shown in Fig. 3 and Table 1, ϕ29 DNA polymerase had a dUMP insertion efficiency only slightly lower than that for dTMP, mainly because of a moderately higher value of the K_m.

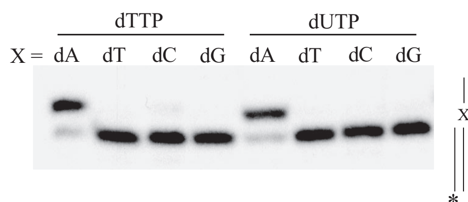

Fig. 2. Incorporation of dUMP by ϕ29 DNA polymerase. The assays were carried out as described in *Materials and Methods*, using as template four ^{32}P-labeled hybrid molecules SP1/SP1c + 6 (15/21 mer), which differ in the first template base, in the presence of either dTTP or dUTP (100 μM each). After incubation for 1 min at 4°C, samples were analyzed by 8 M urea–20% PAGE. Incorporation of dUTP or dTTP was detected as the appearance of the elongation product (16 mer) by autoradiography.

Next, the capacity of ϕ29 DNA polymerase to extend the dA:dUMP pair and replicate past uracil in the next replication round was evaluated by ϕ29 TP-DNA amplification assays. This amplification system (15) requires, in addition to TP and DNA polymerase, ϕ29 ssDNA binding protein (SSB) (to preclude the appearance of short palindromic DNAs) and ϕ29 dsDNA binding protein (DBP) (for optimal activation of the origins). Thus, in the presence of these four proteins, numerous consecutive replication rounds occur, mimicking the natural amplification of viral DNA. The experiments were performed by adding increasing amounts of dUTP and analyzing the DNA synthesized in alkaline agarose gel electrophoresis. Fig. 4 shows that ϕ29 DNA polymerase amplified at nearly optimal (in the absence of dUTP) levels when dUTP concentration was up to 0.25 μM; higher amounts of dUTP decreased the amplification reaction to ≈75% optimal levels. To confirm the incorporation of dUMP, DNA samples were treated with *E. coli* UDG and human ApeI. Such a treatment revealed that the amount of uracil residues incorporated into the synthesized DNA depended on the concentration of dUTP present in the reaction, as increasing amounts of dUTP caused the gradual shortening of the digested products under the denaturing conditions used. These results indicate that ϕ29 DNA polymerase is able to insert uracil residues into DNA when both dUTP and dTTP are present in the replication reaction. In addition, these findings clearly show the ability of ϕ29 DNA polymerase to elongate efficiently the dA:dUMP pair and read through uracil, giving full-length rather than truncated products.

Detection of Uracil Residues in ϕ29 DNA Molecules Synthesized During Infection. In bacteria, a considerable amount of dUTP is produced because it is an obligatory intermediate in the *de novo* synthesis of dTMP (16). This fact, together with the ability of numerous prokaryotic DNA polymerases to use dUTP in place of dTTP during DNA synthesis (17, 18), make incorporation of uracil into the bacterial chromosome unavoidable.

After determining that ϕ29 DNA polymerase incorporates dUMP into DNA *in vitro*, we next examined whether the viral DNA synthesized during the infective cycle contains uracil residues. Thus, *B. subtilis* cells were infected with wild-type phage ϕ29, and total intracellular DNA was isolated at different times, replication starting at ≈15 min after infection. DNA samples were treated with *E. coli* UDG followed by digestion with human ApeI to introduce nicks into DNA at sites of uracil incorporation. The amount of viral genome remaining intact after UDG/ApeI treatment was compared with that from untreated or ApeI-treated DNA samples by native agarose gel electrophoresis. At all times analyzed (25–50 min), the levels of full-length viral DNA after treatment with UDG and ApeI were significantly lower than those of undigested or ApeI-digested DNA samples (Fig. 5), revealing the presence of uracil residues, but not AP sites, in the ϕ29 genome during *in vivo* replication. Quantification of the unit-length phage DNA by densitometry of the gel showed that 28% of total DNA isolated was sensitive to UDG/ApeI digestion at early stages of infection and it increased up to 35% at late stages of infection. Thus, these data indicate that a significant number of the ϕ29 DNA molecules synthesized *in vivo* contain at least one uracil residue at each strand, one residue being close to the other. Taking into account the ability of ϕ29 DNA polymerase to use dUTP, the presence of uracils in ϕ29 DNA could be caused by the misincorporation of dUMP rather than cytosine deamination. In addition, isolated ϕ29 DNA from virions was also treated with *E. coli* UDG and human ApeI and analyzed by alkaline agarose gel electrophoresis. We ob-

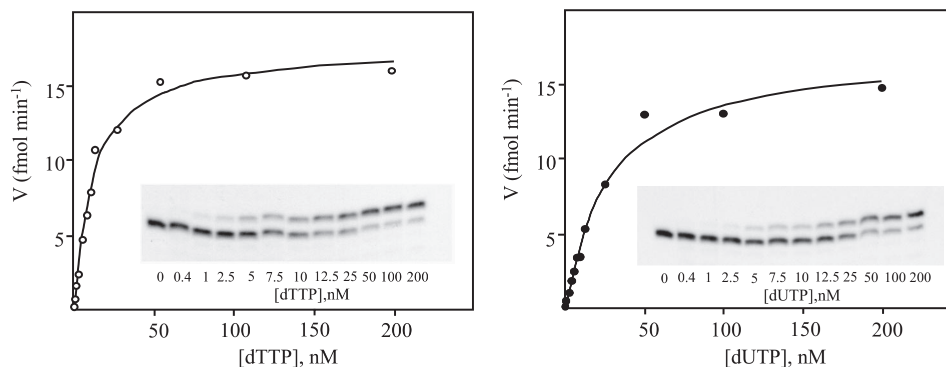

Fig. 3. dTMP and dUMP insertion opposite dA. ϕ29 DNA polymerase (27 nM) was incubated at 4°C for 1 min with 1.6 nM of the primer/template structure SP1/SP1c + 6(A), which offers an A residue as the first nucleotide on the template, and the presence of the indicated concentrations of either dTTP (*Left*) or dUTP (*Right*). Formation of the extended product was analyzed by electrophoresis in a 8 M urea–20% polyacrylamide gel followed by autoradiography and plotted against dTTP or dUTP concentration. (*Insets*) Electrophoretic analysis of a primer extension experiment at the indicated nucleotide concentrations.

served a 38% reduction of the amount of full-length ϕ29 DNA after treatment with UDG/ApeI compared with untreated or ApeI-treated samples, indicating that uracil-containing ϕ29 DNA molecules synthesized during infection are packaged into virions (data not shown).

Viral Protein p56 Prevents the ϕ29 DNA Replication Impairment Caused by UDG Activity. In earlier studies, we reported the identification of the first example of a UDG inhibitor (named protein p56) encoded by a nonuracil containing viral DNA (10, 11). We proposed that inactivation of the host UDG by such inhibitor would ensure an efficient ϕ29 DNA replication because it would avoid the deleterious effect produced by UDG on viral DNA synthesis if uracil residues arise in the single-stranded replicative intermediates. As an approach to test the role suggested for protein p56 in ϕ29 DNA replication, we first examined the effect of the presence of *E. coli* UDG on phage DNA synthesis when uracil residues are incorporated by using *in vitro* ϕ29 TP-DNA amplification assays. Alkaline agarose gel analysis revealed that ϕ29 TP-DNA amplification in the presence of UDG when dUTP was added to the reactions was severely affected, whereas it reached nearly normal levels of replication activity in the absence of dUTP (Fig. 6 *Center* and *Left*). Specifically, in the presence of UDG, more than a 90% reduction in the amount of synthesized DNA was observed at the lower dUTP concentration assayed (0.08 μM). With higher amounts of dUTP, the effect of UDG on the ϕ29 DNA amplification was even more severe, reducing up to 0.7% the amount of full-length ϕ29 DNA, with most of the replicated phage DNA remaining as short elongation products. Taken together, these results indicate that the excision of transiently incorporated uracil by UDG diminishes the viral DNA replication, most likely because of an impairment of replication fork progression at the abasic sites generated by UDG, as it has been described for many family B DNA polymerases (19). Interestingly, the simultaneous addition of viral protein p56 prevented the harmful effect of UDG on ϕ29 DNA replication even at dUTP amounts as high as 0.8 μM. Even at 2.5 μM dUTP, the amplification activity in the presence of p56 was 42-fold higher that in its absence (Fig. 6 *Right*). Although this amplification was only 30% of the level obtained in the absence of dUTP, the majority of the detected DNA was completely replicated. Therefore, our findings demonstrate that viral protein p56 is able to inhibit the damaging effect caused by UDG on ϕ29 DNA replication when uracil residues are incorporated into the viral genome. These results are in agreement with previous studies, which showed that the interaction between p56 and UDG blocked DNA binding by UDG (11).

Inhibition of Host UDG by Protein p56 Enhances Virus Production of Uracil-Containing DNA. After finding that excision of uracil residues from ϕ29 by UDG causes a severe impairment of *in vitro* ϕ29 DNA replication, we performed *in vivo* experiments to assess the effect of the presence of uracil residues in ϕ29 DNA on the phage production. To this purpose, we measured the infectivity of uracil-containing ϕ29 DNA as its ability to produce phage ϕ29 particles, compared with that of a DNA synthesized *in vitro* in the absence of dUTP. The availability of the *in vitro* ϕ29 TP-DNA amplification system allowed us to generate viral genomes with uracil residues incorporated into the newly synthesized DNA. Using various concentrations of dUTP (0.08, 0.8, and 8 μM), we obtained amplified ϕ29 DNA containing increasing amounts of uracil residues. Subsequently, similar amounts of

Table 1. Kinetic constants for dTTP and dUTP incorporation of Φ29 DNA polymerase

dNTP	K_m, nM	K_{cat}, min^{-1}	K_{cat}/K_m, nM^{-1}·min^{-1}
dTTP	12.2 ± 1.3	0.055	4.5×10^{-3}
dUTP	28.2 ± 4.1	0.053	1.9×10^{-3}

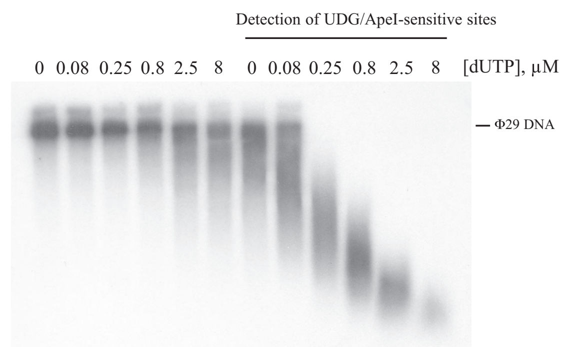

Fig. 4. Viral DNA amplification in the presence of increasing amounts of dUTP. ϕ29 TP-DNA amplification assays were carried out essentially as described in *Materials and Methods*, in the presence of 80 μM of the four dNTPs and the indicated concentration of dUTP. After incubation for 90 min at 30°C, parallel amplification samples from each dUTP concentration were subjected to digestion with *E. coli* UDG and human ApeI to analyze incorporation of uracil residues. The amount and size of untreated (*Left*) and UDG/ApeI-treated viral DNAs (*Right*) were analyzed by alkaline agarose gel electrophoresis followed by autoradiography. The migration position of unit length ϕ29 DNA (19,285 bases) is indicated.

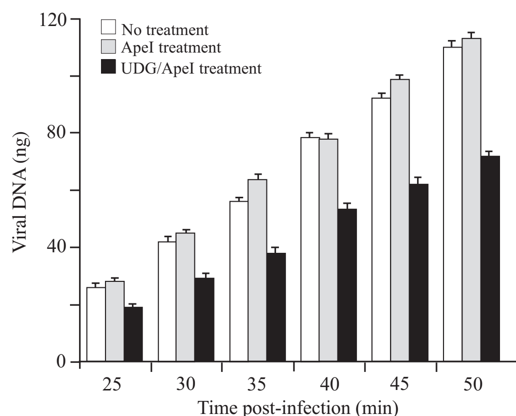

Fig. 5. Detection of uracil in ϕ29 DNA during viral infection. *B. subtilis* 110NA strain, grown in defined minimal medium at 37°C, was infected with wild-type phage ϕ29. At the indicated times, total intracellular DNA was isolated and divided into three aliquots: one part was untreated (white columns) and the other were digested either with human ApeI alone (gray columns) or *E. coli* UDG plus human ApeI (black columns). These DNA samples were analyzed by native agarose gel electrophoresis and stained with ethidium bromide. The same amount of total DNA was loaded. Bands corresponding to unit-length ϕ29 DNA were quantified by densitometry and plotted as a function of time. Data represent average values of four independent experiments with standard deviations.

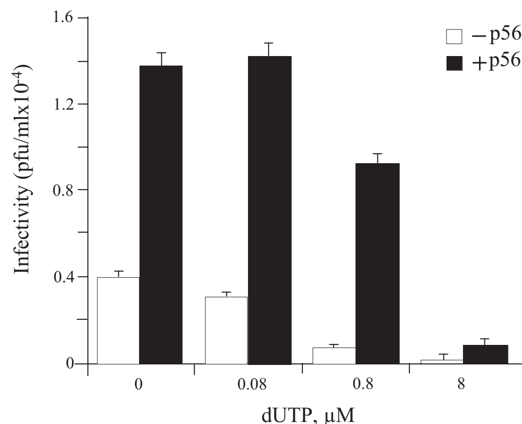

Fig. 7. Infectivity of viral DNAs containing uracil residues. *In vitro* ϕ29 TP-DNA amplification assays were performed in the presence of 0.08, 0.8, or 8 μM dUTP to obtain ϕ29 DNA containing increasing amounts of uracil. Subsequently, equal amounts of such ϕ29 DNAs were used to transfect either p56-producing (black columns) or nonproducing (white columns) *B. subtilis* competent cells. After transfection, infectivity was determined. Data represent average values of three independent experiments with standard deviations.

these DNA preparations were used to transfect *B. subtilis* competent cells carrying the plasmid vector pPR53 (p56 nonproducing cells) (20), and the infectivity was determined. As a control, DNA amplified *in vitro* in the absence of dUTP was used. As shown in Fig. 7, as the uracil content of the DNA was higher the infectivity of such DNA decreased.

To study the *in vivo* role of protein p56, we used the same DNA preparations to transfect p56-producing cells to find out the effect of inhibiting the host UDG on the infectivity of uracil-containing ϕ29 DNA. These cells, which carry the recombinant plasmid pPR53.p56, constitutively synthesize protein p56 at levels only slightly lower than those detected at 40 min of phage infection (10). The results revealed that the infectivity of uracil-containing DNAs increased substantially in *B. subtilis* cells whose UDG activity was inhibited because of the presence of p56, in comparison with p56 nonproducing cells (Fig. 7). The number of infective units formed by ϕ29 DNAs amplified *in vitro* in the presence of 0.08 and 0.8 μM dUTP in p56-producing cells was

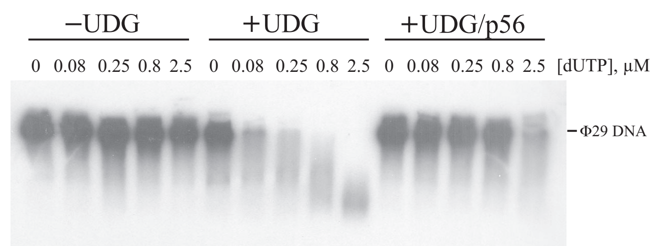

Fig. 6. Effect of UDG on *in vitro* viral DNA replication in the absence or presence of viral protein p56. (*Center* and *Right*) ϕ29 DNA amplification reactions containing the indicated concentrations of dUTP were carried out in the presence of UDG alone (120 ng) (*Center*) or as a mixture with protein p56 (2.4 μg) at a molar ratio of 80 (*Right*). After 90 min at 30°C, the reactions were stopped and analyzed by alkaline agarose gel electrophoresis followed by autoradiography. (*Left*) As control, ϕ29 DNA amplification assays were performed with the same concentrations of dUTP in the absence of UDG. The amount of amplified viral DNA (full-length DNA and elongation products) was measured by densitometry. The migration position of unit length ϕ29 DNA (19,285 bases) is indicated.

4.5- and 9-fold higher, respectively, than those in p56 nonproducing cells, and the transfection efficiency reached a value of $\approx 1.5 \times 10^4$ pfu/ml. Plaque production of DNA amplified in the presence of 8 μM dUTP also showed a significant enhancement (3.5-fold) when p56-containing cells were transfected. However, the transfection efficiency of such DNA was much lower than that obtained by using DNAs with a lower uracil load, suggesting that high levels of uracil residues in the viral genome are not well tolerated by phage ϕ29. We also observed a 3.5-fold improvement of plaque production of a DNA amplified *in vitro* in the absence of dUTP when p56-producing cells were infected compared with p56 nonproducing cells, probably because of the insertion of uracil residues in the phage genome during DNA replication. Taken together, these results demonstrate that the presence of uracil residues in the ϕ29 genome causes a considerable decrease in the production of phage particles, likely as a result of host UDG activity. In addition, this study also shows that viral protein p56, which inhibits host UDG activity, favors to a large extent the infectivity of uracil-containing DNA.

Discussion

Nucleotide selection by DNA polymerases has been reported to depend primarily on base-pair geometry (21). Uracil is roughly equivalent to thymine in terms of hydrogen-bonding number and strength when it interacts with template dA to form the dA:dUMP base pair. This fact would explain that a great number of polymerases insert dTMP and dUMP into DNA with similar efficiencies. *In vitro* experiments have illustrated that *E. coli* DNA polymerases I, II, and III competently use dUTP in addition to dTTP for DNA polymerization (2). In mammalian cells, DNA polymerases α and β, which function predominantly in chromosomal replication and repair, respectively, have been reported to exhibit similar kinetic constants for dTTP and dUTP (22). In contrast, DNA polymerase γ, the principal replication enzyme in mitochondria, showed a significantly higher affinity for dTTP than for dUTP (17). Moreover, several thermostable DNA polymerases use dUTP highly inefficiently during PCR amplification (23). Therefore, in these cases, dTTP and dUTP are not necessarily equally well used as a substrate in DNA replication. In this work, we have analyzed the ability of bacteriophage ϕ29 DNA polymerase to incorporate dUMP during DNA synthesis. We showed that the viral DNA polymerase inserts dUMP opposite template dA, with a catalytic efficiency

only ≈2-fold lower than that for dTMP. Furthermore, this DNA polymerase was able to perform the extension of base-paired uracil and to use dU as template during *in vitro* viral DNA replication. This behavior has been also described for many DNA-dependent replicases belonging to the same family as ϕ29 DNA polymerase (family B), such as eukaryotic DNA polymerases ε and δ, and prokaryotic DNA polymerases purified from *E. coli* and *B. subtilis* cells (18, 24).

The *in vivo* studies reported here revealed the presence of uracil residues in the ϕ29 DNA molecules synthesized during the infective cycle. It seems likely that most of the uracils arise from misinsertion of dUMP rather than by deamination of cytosines, because viral DNA polymerase efficiently incorporates dUMP into DNA. Misincorporation of dUMP into DNA has been also observed in a number of viruses, including polyomavirus (25) and adenovirus (26), as well as in bacteria (2, 3). It has been estimated that under normal physiological conditions *E. coli* incorporates one uracil residue into DNA for every 300 thymine nucleotides polymerized (2). In eukaryotic cells, incorporation of dUMP by replicative DNA polymerases is also thought to be the most abundant source of genomic uracil (4), although the content of such residue appears to be at least 10^3-fold lower than that reported for prokaryotic systems, mainly because these cells lack dCTP deaminase, which converts dCTP directly to dUTP, and produce abundant dUTPase (5, 16).

Protein p56 from phage ϕ29 inhibits the DNA-binding ability of the cellular UDG enzyme. We recently proposed that this inhibition is essential for an efficient infection, because the action of UDG on ϕ29 ssDNA molecules that contain uracils would be deleterious for ϕ29 DNA replication (10, 11). To test this hypothesis, we have studied the consequences of uracil misincorporation on *in vitro* ϕ29 DNA replication when UDG is present. Removal of the uracil residues incorporated into the phage genome by UDG caused a drastic reduction in the efficiency of ϕ29 DNA replication. Furthermore, it is interesting to note that the presence of ϕ29 DNA binding proteins (SSB and DBP) during viral DNA amplification did not inhibit uracil excision by UDG. In fact, this activity could have been enhanced by viral SSB binding to ssDNA, as interactions between SSBs and UDGs from *E. coli* and *Mycobacterium tuberculosis* have been reported to result in increased efficiency of uracil excision from structured substrates (27). On the other hand, we have demonstrated that protein p56 prevents the ϕ29 DNA replication impairment caused by the action of UDG on the uracil-containing ϕ29 DNA. In agreement, transfection activity of uracil-containing ϕ29 DNA was significantly higher in cells that constitutively synthesized protein p56 than in cells lacking this protein.

Uracilation of genomes represents a constant threat to survival of many organisms, including viruses. The presence of uracil in DNA can affect DNA dynamic conformation as well as sequence-specific protein binding (28). Nonetheless, it has been reported that the major measurable consequences of high levels of uracil in DNA are lesions created by the attempt of cells to remove this nucleotide (29). For example, it has been found that a vicious circle of excision and repair of the DNA, caused by high intracellular levels of dUTP, led to strand breaks, strand exchanges, and eventually cell death as a result of chromosomal aberrations (29). *In vivo* studies revealed that *E. coli* with dUTPase mutations transiently accumulates small DNA fragments during replication fork progression, probably because of the UDG-based repair process (30, 31). In the case of viruses, such as polyoma virus and adenovirus, the removal of uracil causes strand breakage and may contribute to the formation of Okazaki fragments in the viral genome (25, 26). Moreover, a mutant of phage T5, containing significant amounts of uracil in its DNA, failed to produce plaques unless the plating host was deficient in UDG activity (32).

In summary, we have demonstrated that uracil residues accumulate in the ϕ29 genome during DNA replication, probably because of misincorporation of dUMP into DNA by the viral DNA polymerase. In addition, the results shown in this work provide an important validation of the model proposed for the *in vivo* role of viral protein p56 in DNA synthesis. According to this model, bacteriophage ϕ29 encodes an UDG inhibitor (protein p56) to prevent the impairment of DNA synthesis produced by excision of uracils from the viral genome.

Materials and Methods

Nucleotides, DNA Templates, and Proteins. Unlabeled nucleotides, [γ-^{32}P] ATP (3,000 Ci/mmol) and [α-^{32}P]dATP (3,000 Ci/mmol) were purchased from Amersham Pharmacia Biochemicals. Oligonucleotide SP1 (5′-GATCACAGTGAGTAC) was 5′-labeled with [γ-^{32}P] ATP and phage T4 polynucleotide kinase (obtained from New England Biolabs) and purified electrophoretically on 8 M urea-20% polyacrylamide gels. Labeled SP1 was hybridized to oligonucleotides SP1c + 6(A) (5′-TCTATAGTACTCACTGTGATC), SP1c + 6(T) (5′-TCTATTGTACTCACTGTGATC), SP1c + 6(C) (5′-TCTATCGTACTCACTGTGATC), and SP1c + 6(G) (5′-TCTATGGTACTCACTGTGATC). Hybridizations were performed in the presence of 0.2 M NaCl and 50 mM Tris·HCl, pH 7.5, resulting in primer/template structures. Oligonucleotides were obtained from Invitrogen. TP-DNA from ϕ29 *sus14*(1242) virions, isolated as described (33), was used as input template for *in vitro* amplifications experiments. ϕ29 DNA polymerase, TP, protein p6 (DBP), and protein p5 (SSB) were overproduced in *E. coli* cells and purified essentially as described (34–37). *In vitro* and *in vivo* incorporation of uracil residues was determined by treatment with *E. coli* UDG and human ApeI from New England Biolabs and Trevigen, respectively. Protein p56 (56 aa) was overproduced in *E. coli* cells and purified as described (10).

Phages and Bacterial Strains. *B. subtilis* 110NA (38), a nonsuppresor (su^-) strain, was infected with wild-type phage ϕ29 to study the uracil content in the ϕ29 genome during infection. *B. subtilis* YB886 (39), carrying empty vector (pPR53) or recombinant vector (pPR53.p56), and *B. subtilis* MO-101-P (40), a suppressor strain (su^{+44}), were used for transfection experiments.

Primer Extension Analysis. The incubation mixture contained 12.5 μl of 50 mM Tris·HCl (pH 7.5), 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 10 mM MgCl, 27 nM of ϕ29 DNA polymerase, and 100 μM of either dTTP or dUTP. As substrate, 1.6 nM of one of the four 5′-labeled hybrid molecules, SP1/SP1c + 6(X) that differ in the first template base, were used. After incubation for 1 min at 4°C to minimize 3′-5′exonuclease activity of ϕ29 DNA polymerase, reactions were stopped by adding EDTA up to 10 mM. Samples were analyzed by 8 M urea-20% PAGE and autoradiography. To determine insertion kinetic constants for dU as compared with dT, primer extension reactions were performed as described above, using 5′-labeled SP1/SP1 c + 6(A) as substrate and in the presence of the indicated concentrations of either dTTP or dUTP. Apparent values for Michaelis-Menten constant K_m and V_{max} for dUMP or dTMP incorporation opposite dA were obtained by least-squares nonlinear regression to a rectangular hyperbola by using Kaleidagraph 3.6.4 software. K_{cat} was calculated by dividing the V_{max} by the enzyme concentration. Catalytic efficiency was obtained by dividing K_{cat} by K_m. Assays were performed at least three times to guarantee reproducibility.

ϕ29 TP-DNA Amplification Assay. The incubation mixture contained 30 μl of 50 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM ammonium sulfate, 1 mM DTT, 4% glycerol, 0.1 mg/ml BSA, 80 μM each dCTP, dGTP, dTTP and [α-^{32}P]dATP (2μCi), 5 ng of ϕ29 TP-DNA, 10 ng of ϕ29 DNA polymerase, 25 ng of TP, 10 μg of ϕ29 SSB, and 10 μg of ϕ29 DBP. When indicated, reactions also contained different amounts of dUTP, and the concentration of dTTP was adjusted so that the amount of dUTP plus dTTP remained constant (80 μM). To determine the incorporation of uracil residues during *in vitro* ϕ29 TP-DNA amplification, the reactions were incubated for 1.5 h at 30°C, followed by treatment with *E. coli* UDG (100 ng) and human ApeI (2.5 units). Then, the samples were heated at 72°C for 10 min and filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The excluded volume was analyzed by alkaline agarose gel electrophoresis followed by autoradiography. To study the effect of UDG alone or together with viral protein p56 on *in vitro* ϕ29 TP-DNA amplification, reactions were carried out in the presence of either UDG (120 ng) or UDG (120 ng) plus p56 (2 μg). After incubation for 1.5 h at 30°C, the samples were processed and the amplified DNA was analyzed by electrophoresis in alkaline agarose gels as described above.

Detection of Uracil Residues in the ϕ29 Genome During Viral DNA Replication. *B. subtilis* 110NA cells were exponentially grown at 37°C in defined medium prepared as described (41) and supplemented with tryptophan (40 μg/ml), to 10^8 cells/ml. Then, cells were infected with wild-type phage ϕ29 at a multiplicity of infection of 10. At the indicated times after infection, total intracellular DNA was isolated as described (42). To determine the presence of uracil in the ϕ29 genome, isolated DNA was treated with *E. coli* UDG (100 ng) and human ApeI (2.5 units) and analyzed by native agarose gel electrophoresis followed by staining with ethidium bromide. The use of native instead of alkaline agarose gel allowed us to run the DNA samples for 16 h to separate the ϕ29 genome and chromosomal DNA of *B. subtilis.* Bands corresponding to unit-length ϕ29 DNA were quantified by densitometry, using proteinase K-treated ϕ29 DNA (150 ng) as an internal marker.

In addition, TP-DNA isolated from phage particles was treated with *E. coli* UDG (100 ng) and human ApeI (2.5 units) and analyzed by alkaline agarose gel electrophoresis followed by staining with ethidium bromide.

Infectivity Assay for the *in Vitro*-Amplified ϕ29 DNA. *In vitro* ϕ29 TP-DNA amplification reactions in the presence of various concentrations of dUTP (0.08, 0.8, and 8 μM) were carried out as described above to obtain amplified ϕ29 DNA, containing increasing amounts of uracil. After incubation for 1.5 h at 30°C, the reactions were stopped with 10 mM EDTA and quantified by alkaline agarose gel electrophoresis and ethidium bromide staining. Similar amounts of DNA amplified in the absence or the presence of dUTP were used to transfect pPR53 or pPR53.p56-carrying YB886 competent cells, prepared as described (43). After transfection, infectivity, expressed as pfu, was determined by plating on suppressor strain *su*$^{+44}$.

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