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New Deoxyribonucleic Acid Polymerase Induced by *Bacillus subtilis* Bacteriophage PBS2

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The deoxyribonucleic acid (DNA) of *Bacillus subtilis* phage PBS2 has been confirmed to contain uracil instead of thymine. PBS2 phage infection of wild-type cells or DNA polymerase-deficient cells results in an increase in the specific activity of DNA polymerase. This induction of DNA polymerase activity is prevented by actinomycin D and chloramphenicol. In contrast to the major *B. subtilis* DNA polymerase, which prefers deoxythymidine triphosphate (dTTP) to deoxyuridine triphosphate (dUTP), the DNA polymerase in crude extracts of PBS2-infected cells is equally active whether dTTP or dUTP is employed. This phage-induced polymerase may be responsible for the synthesis of uracil-containing DNA during PBS2 phage infection.

Bacillus subtilis phage PBS2 is unique among known bacterial viruses (15) because its deoxyribonucleic acid (DNA) has been reported (24, 25) to contain uracil instead of thymine. Our interest in the role of unusual nucleosides in nucleic acids prompted this question: why do cells synthesize thymine-containing nucleotides from uracil-containing nucleotides (16), instead of simply using uracil for their DNA? We are therefore investigating the enzymatic mechanisms involved in the biosynthesis and function of uracil-containing PBS2 DNA in a cell which normally makes thymine-containing *B. subtilis* DNA. It has previously been reported by others that PBS2 phage induces deoxyuridine monophosphate (dUMP) kinase and deoxythymidine monophosphate (dTMP) phosphohydrolase activities (F. M. Kahan, 1963, Fed. Proc. **22**:406.) and also a deoxycytidine triphosphate (dCTP) deaminase (26). We report here that PBS2 phage induces a new DNA polymerase activity after infection of *B. subtilis*.

MATERIALS AND METHODS

Cells and phage. *B. subtilis* SB19E and PBS2 phage were obtained from I. Takahashi (26) through H. R. Warner. *B. subtilis* 1306 *leu⁻ met⁻ pol⁻* and *B. subtilis* 168-2 *leu⁻ trp⁻* (the recipient into which the *pol⁻* mutation was introduced to produce strain 1306) were isolated by Patricia Hempstead (doctoral thesis, University of London, 1968) and were kindly sent to us by Julian Gross.

Bacterial stocks. *B. subtilis* strains were stored on slants of 3.3% Difco Tryptose Blood Agar Base (TBB) supplemented when necessary with 100 μg of the required amino acids per ml. Since PBS2 phage adsorbs to active flagella (7), motile cells of each strain were isolated by picking cells which migrated most rapidly across a plate containing 0.5% Difco Penassay Broth in 0.4% agar.

Phage lysates. Methods for the growth and titering of PBS2 phage were modified from those of Takahashi (22, 26, and *personal communication*). Cultures of *B. subtilis* SB19 were shaken vigorously at 37 C in Penassay Broth until the density, as monitored by measuring the absorbance at 660 nm (A_{660}), equalled 1.0. This density corresponded to approximately 2×10^8 viable cells per ml. The culture was infected with a multiplicity of two PBS2 phage per cell, and shaking was continued for 2 hr. Cultures were held at 37 C overnight without shaking to allow completion of lysis. After centrifugation at 4 C for 15 min at 5,000 × *g* to remove debris, the lysates were titered and stored at 4 C. Typically, lysates contained 3×10^9 to 9×10^9 plaque-forming units per ml.

Phage titering. Lysates were appropriately diluted in adsorption medium containing, per liter: 1 g of yeast extract (Difco); 4 g of NaCl; 5 g of K_2SO_4; 1.5 g of KH_2PO_4; 3 g of Na_2HPO_4; 120 mg of $MgSO_4 \cdot 7H_2O$; 10 mg of $CaCl_2$; and 10 mg of $FeCl_3 \cdot 6H_2O$. Diluted phage (0.10 ml) were incubated with exponentially growing cells (0.2 ml in Penassay Broth) in 1.3 ml of adsorption medium for 5 min at room temperature. Then 1.5 ml of top agar (2.2% TBB at 52 C) was added, and the entire mixture was poured onto a fresh, dry-bottom agar plate (3.3% TBB). Plates were incubated in the inverted position overnight at 30 C to develop plaques. Careful adherence to this protocol is essential for the production of clear, visible plaques.

Phage infections. Penassay Broth was inoculated with cells from a TBB slant and shaken overnight at 37 C. Fresh Penassay Broth was inoculated with the overnight culture (5% by volume) and shaken vigorously at 37 C until the A_{660} reached 1.0. Then the

culture was titered for viable cells by dilution in adsorption medium and plating with TBB top agar by the overlay method (1). Then phage were added at a multiplicity of approximately five PBS2 phage per cell. Surviving cells were titered 10 min after infection.

Cell extracts. Infected cultures were chilled in ice and centrifuged at 5,000 × *g* for 15 min at 0 C. The pellets were resuspended at 0 C in a buffer (1 ml per 100 ml of original culture) consisting of 10 mM tris (hydroxymethyl)aminomethane (Tris)-chloride, *p*H 7.5, plus 1 mM ethylenediaminetetraacetate (EDTA). Cells were lysed by incubation for 30 min at 37 C with 100 μg of lysozyme (Worthington) per ml, followed by sonic oscillation at 4 C for 10 sec at lowest power setting (Branson S75) to reduce the viscosity of the extracts. After centrifugation at 15,000 × *g* for 20 min at 0 C, the clarified supernatant extracts were made 0.1 mM in dithiothreitol and stored at −15 C. Extracts were dialyzed overnight at 4 C versus 100 volumes of 50 mM Tris-chloride (*p*H 7.5), 10 mM NaCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol, before assay of DNA polymerase activity.

Phage DNA. Using methods described by Yamamoto et al. (33), phage lysates were treated with nucleases and concentrated with polyethylene glycol and dextran sulfate. Phage DNA was extracted with warm, redistilled phenol as described by Mandell and Hershey (14), except that 2% sodium dodecyl sulfate was added. The DNA was dialyzed, concentrated by lyophilization, and quantitated by the indole method (21) with salmon sperm DNA as a standard.

Phage DNA bases and nucleosides. By using the methods of Wyatt (30), PBS2 DNA was hydrolyzed in formic acid, and its bases were separated and quantitated following paper chromatography in isopropanol-hydrochloride. In addition, by using methods described previously (19), PBS2 DNA was hydrolyzed with pancreatic deoxyribonuclease, venom phosphodiesterase, and alkaline phosphatase (all from Worthington), and its nucleosides were separated by paper chromatography with ethyl acetate-propanol-water. The nucleosides were eluted with 0.1 N HCl for identification and quantitation based on their spectra (5).

DNA polymerase assay. DNA polymerase activity was assayed by a modification of the method of Warner and Barnes (29). Reaction mixtures (0.250 ml) contained the following, unless otherwise indicated: 64 mM Tris-chloride, *p*H 8.5; 6.8 mM $MgCl_2$; 1.0 mM β-mercaptoethanol; 7.8 mM adenosine triphosphate (ATP); 1.6 mM deoxythymidine triphosphate (dTTP); 37 μM deoxyadenosine triphosphate (dATP); 32 μM deoxyguanosine triphosphate (dGTP); 33 μM dCTP-*2*-^{14}C (5.63 Ci per mole; 50,000 counts/min); and 100 μg of heat-denatured salmon sperm DNA. These mixtures were warmed to 37 C, and enzyme was added to start the reaction. Samples of 50 μliters were removed at 0.3, 5, 10, and 15 min of incubation and were transferred onto Whatman no. 3 paper discs for processing as described by Bollum (3). The dried discs were placed in 15 ml of scintillation fluid containing 0.5% 2,5-diphenyloxazole and 0.01% bis-(O-methyl-styryl)-benzene in toluene. They were counted in a Nuclear-Chicago Unilux counter at an efficiency of 55%. Unless otherwise noted, the activity of DNA polymerase is expressed as the initial rate of incorporation of ^{14}C-deoxycytidine monophosphate (counts per minute incorporated per minute of incubation) per milligram of protein. Assays were performed under conditions in which less than 5% of the available ^{14}C-dCTP was incorporated into acid-insoluble material. The initial rate of incorporation was linear with respect to the amount of uninfected or infected cell extract added, from 2 to 100 counts per min per min.

Other materials and methods. The dCTP-*2*-^{14}C and dATP-*8*-^{14}C were purchased from Schwarz/Mann. Other biochemicals were from Calbiochem or Sigma. Protein concentration was determined by the method of Lowry et al. (13) with crystalline trypsin as a standard. Ribonucleic acid (RNA) was measured by the colorimetric orcinol-$FeCl_3$ method (4). Nucleotides were separated when indicated by descending chromatography on Whatman no. 40 paper for 18 hr in isobutyric acid-0.1 M Na_2EDTA-1 M NH_4OH (100:1.6:60, v/v/v).

RESULTS

PBS2 DNA properties. Takahashi and Marmur (24, 25) reported that acid hydrolysis of PBS2 DNA yielded guanine, cytosine, adenine, and uracil (14, 14, 36, and 36 moles per cent, respectively). Enzymatic digests of PBS2 DNA were shown (24) to contain a nucleotide with the spectral and chromatographic properties of deoxyuridine-5′-monophosphate. The discrepancy between the chemically determined base composition of PBS2 DNA and the value calculated from its density or its denaturation profile (24) apparently is not caused by glucosylation of the DNA (2, 28) but may be explained entirely by the substitution of uracil for thymine in phage DNA (P. Cassidy, F. Kahan, and A. Alegria, 1965, Fed. Proc. **24**:226). The X-ray diffraction pattern of PBS2 DNA is typical of double-stranded DNA in the B configuration (12). PBS1 phage, from which PBS2 phage segregated as a clear-plaque variant, has been found to contain DNA with a molecular weight of 190 million daltons (11) with single-strand interruptions (31).

We have confirmed and extended some of the above observations. PBS2 DNA displayed a spectrum typical of DNA, with absorption ratios of 2.14 for 260 nm/280 nm and 0.40 for 230 nm/260 nm in neutral solution. On boiling and rapid cooling to 0 C, a solution of PBS2 DNA showed a 33% hyperchromic shift at 260 nm, identical to that found with similarly treated native salmon sperm DNA.

Formic acid hydrolysates of PBS2 DNA contained a base (37 moles per cent) with a chromatographic mobility and an ultraviolet absorption spectrum identical to that of authentic uracil. Thymine and 5-hydroxymethyluracil were not detected (less than 1 mole per cent). Furthermore, enzymatic hydrolysates of PBS2 DNA contained a compound which behaved like deoxyuridine (34 moles per cent), and no thymidine nor 5-hydroxymethyl-deoxyuridine was detected (less than 1 mole per cent).

Colorimetric analysis of PBS2 DNA by the indole-hydrochloride method gave results similar to a salmon sperm DNA standard (which was 1.2 A_{490} per A_{260} unit). In the orcinol-$FeCl_3$ assay for RNA, both salmon sperm DNA and PBS2 DNA gave 11% of the color development of the yeast RNA standard (0.97 A_{660} per A_{260} unit). Since chromatographically pure deoxyribonucleosides also give a similar amount of color, these results suggest that PBS2 DNA does not contain ribonucleotides (less than 1%). All of the above data are consistent with PBS2 phage containing only DNA, in a complementary, double-stranded form, with deoxyuridine substituting for thymidine.

Characteristics of PBS2 infection. Infections performed under these conditions have the following characteristics. At a multiplicity of five phage per cell, 85 to 90% of the original cells are killed after 10 min, and 30 to 50% of these cells form infective centers (see also reference 22). The phage-infected cells enter a 20-min eclipse period, during which less than 0.5% of the original phage are recoverable by lysis with lysozyme in cyanide (10). The concentration of colorimetrically determined DNA (29) remains constant at 11 μg per ml of culture from 0 to 20 min after infection; then it rises gradually to a level of 25 μg/ml by 120 min. The titer of intracellular phage rises abruptly from 25 to 45 min after infection. Phage are released as lysis begins with the A_{660} of the culture dropping from 1.3 at 30 min to 0.2 at 120 min of infection. The free phage titer increases to 2×10^9 to 5×10^9 per ml, giving an average burst size of 10 to 20 phage per cell (see also reference 22).

PBS2 DNA polymerase induction. The results of typical DNA polymerase assays on cell-free extracts of uninfected or phage-infected cells are shown in Fig. 1. *B. subtilis* SB19 cells show a DNA polymerase activity, as described by Okazaki and Kornberg (17). After infection for 45 min by PBS2 phage, these cells exhibit a larger amount of DNA polymerase activity. Extracts of strain SB19 prepared at various times after PBS2 infection show (Fig. 2) an increase in DNA polymerase specific activity beginning at about 20 min and reaching a maximum after 45 min of infection. A similar induction profile was observed during PBS2 infection of a *B. subtilis* mutant defective in DNA polymerase, strain 1306 *pol*$^-$ (*see below*). Thus, the induction of DNA polymerase, the increase in DNA content, and the appearance of new phage occur in rapid succession, just before lysis begins. Two other PBS2-induced enzymes, dTMP phosphohydrolase (A. Price and S. Fogt, *manuscript in preparation*) and dCTP deaminase (26), are induced with a time course similar to DNA polymerase.

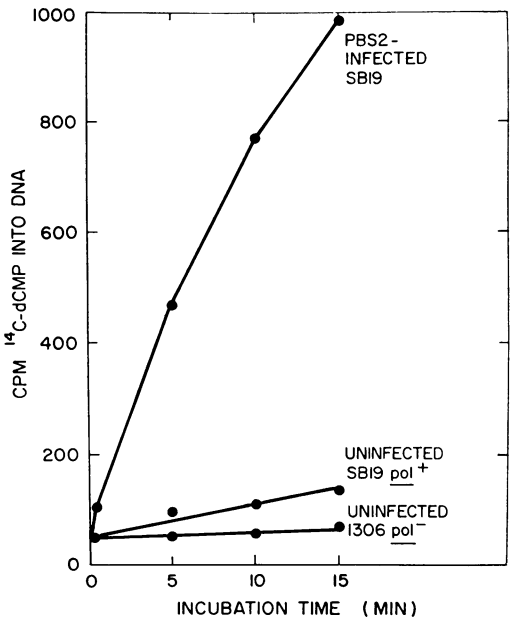

FIG. 1. *DNA polymerase assays by using ^{14}C-dCTP incorporation into acid-precipitable material (see Materials and Methods). Incubation mixtures contained dialyzed extracts from B. subtilis 1306 pol$^-$, B. subtilis SB19 pol$^+$, or B. subtilis SB19 infected for 45 min with PBS2 phage (1.26, 0.90, and 1.50 mg of protein, respectively). The counter background level (40 counts per min) has not been subtracted from the data presented.*

Although DNA polymerase activity (1,100 counts per min per min per ml) was detected in a stock of PBS2 phage (2×10^{11} per ml) concentrated by the polyethylene glycol method (see Materials and Methods), the activity does not appear to be associated with phage. Centrifugation of this phage stock at 25,000 × *g* for 1 hr pelleted over 99% of the viable phage, but left all of the DNA polymerase activity in the supernatant fluid. The source of this activity is still under investigation.

Induction in a polymerase-deficient strain. To eliminate the possibility that the observed increase in DNA polymerase activity in infected cells was

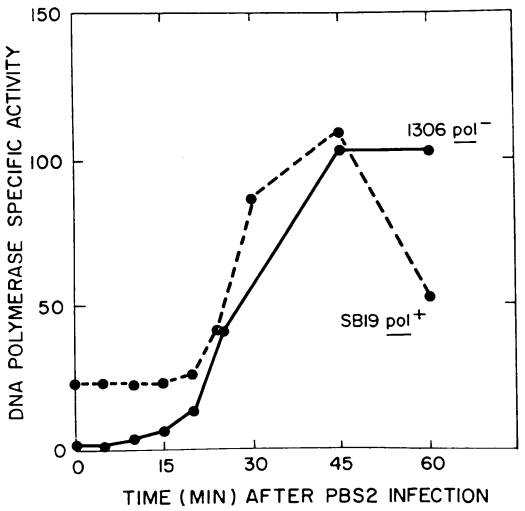

FIG. 2. *Time course of DNA polymerase induction during PBS2 phage infection of B. subtilis SB19 pol⁺ or B. subtilis 1306 pol⁻. Assays for DNA polymerase specific activity were performed as described in Materials and Methods.*

due to an increased level of the normal *B. subtilis* DNA polymerase (6, 17), we employed a strain, *B. subtilis* 1306 *pol⁻*, deficient in DNA polymerase activity (Patricia Hempstead, Ph.D. thesis, University of London, 1968). A similar strain has been described recently by Gass et al. (9). PBS2 forms plaques on a lawn of strain 1306 with the same efficiency as on strain SB19, and the timing of phage synthesis and lysis is similar in both strains. As shown in Fig. 1 and 2, the specific activity of DNA polymerase in extracts of strain 1306 *pol⁻* is only one-tenth that of strain SB19 *pol⁺*. It was, therefore, easier to detect the beginning of phage DNA polymerase induction in strain 1306. Figure 2 shows that the DNA polymerase level in PBS2-infected strain 1306 increased at about 10 min after infection, reaching a maximum after 45 min, at a level similar to that found in PBS2-infected strain SB19.

That the polymerase activities above are not affected by other differences between strain SB19 *pol⁺* and strain 1306 *pol⁻* was indicated by the observation that the specific activity of DNA polymerase in strain 168-2 *pol⁺* (from which strain 1306 *pol⁻* was derived) was identical to that in strain SB19 *pol⁺*, both in uninfected cells and in cells infected for 45 min by PBS2 phage.

Alternative extraction methods. Since the above extracts were prepared by lysozyme treatment at 37 C, the time course of PBS2 DNA polymerase induction in strain 1306 was determined again by preparing extracts by different methods as described below.

Pelleted cells were frozen for 1 hr, thawed in buffer (see Materials and Methods), incubated for 2 hr at 0 C with 100 μg of lysozyme per ml, subjected to vigorous sonic oscillation (four times for 20 sec at 2-min intervals) to disrupt the cells, and then centrifuged and treated as described in Materials and Methods. Alternatively, frozen pelleted cells were suspended in buffer containing 0.1 mM dithiothreitol with 100 μg of lysozyme per ml and were allowed to lyse at 4 C overnight followed by treatment as in Materials and Methods. The specific activity of DNA polymerase was found to increase after PBS2 infection with a time course similar to that shown in Fig. 2, independent of the method employed for preparing extracts.

These cells are rather resistant to disruption by sonic oscillation in buffer. Severe sonic treatment (eight times for 20 sec at no. 3 setting on Branson S75) did not completely break open the cells. The DNA polymerase specific activity in such extracts was less than 40% of that observed with the three extraction methods described above.

Finally, cells of strain 1306 which had been infected by PBS2 phage for 30 min were treated with 100 μg of chloramphenicol per ml, followed by the normal extract preparation method, except that the extract buffer contained 100 μg of chloramphenicol per ml. The specific activity of DNA polymerase in this extract was identical to that found in extracts of these cells prepared by lysozyme treatment at 37 or 4 C as described above. Thus, the DNA polymerase specific activities observed in PBS2-infected cells are independent of these four methods of extract preparation.

Inhibitors and DNA polymerase induction. The data in Table 1 indicate that the fourfold increase in DNA polymerase specific activity which occurs after PBS2 infection of strain SB19 can be prevented by the addition at 1 min after infection of actinomycin D or chloramphenicol. When these inhibitors were added 10 or 25 min after infection, the polymerase induction was stopped at levels below the normal maximum level of induction. These results suggest that RNA and protein biosynthesis are required for PBS2 DNA polymerase induction.

Treatment of uninfected cells with mitomycin C under conditions known to induce defective phage in *B. subtilis* SB19 (27) did not result in an increase in DNA polymerase activity (Table 1). This culture lysed several hours after removal of mitomycin C, but no plaque-forming particles were released. This suggests that the observed PBS2 DNA polymerase induction is not the result of the induction of defective phage in the host cell.

TABLE 1. *DNA polymerase activity in extracts of B. subtilis SB19 pol⁺ following various treatments*

Treatment[a]	Specific activity[d]
Uninfected cells	23
PBS2-infected cells (30 min)	87
PBS2 + chloramphenicol:[b]	
at 1 min	28
at 10 min	37
at 25 min	47
PBS2 + actinomycin D:[b]	
at 1 min	7
at 10 min	38
Uninfected cells + mitomycin C for 10 min[b]	20
Cells mock-infected by lysate supernatant[c]	22

[a] All extracts were prepared 30 min after infection (or after treatment for uninfected cells).

[b] Antibiotics were added at the indicated times after infection at the following concentrations: chloramphenicol, 100 μg/ml; actinomycin D, 10 μg/ml; or mitomycin C, 3 μg/ml.

[c] PBS2 lysate was centrifuged at 25,000 × *g* for 1 hr to pellet more than 99% of the phage, and the supernatant fluid was used for a "mock infection."

[d] Activity is expressed as counts per minute of ^{14}C-dCMP incorporated into DNA per minute of incubation per milligram of protein.

A "mock infection" performed with a lysate from which the phage had been pelleted also did not result in increased DNA polymerase activity (Table 1), indicating that phage are required for DNA polymerase induction.

Finally, rifampin was added at a level of 20 μg/ml at the time of infection to prevent *B. subtilis* RNA synthesis by inhibiting RNA polymerase activity (10). PBS2 infection in the presence of rifampin results in lysis with a normal burst of new phage as described above. During infection by PBS1 phage under these same conditions, Bert Rima and I. Takahashi (*personal communication*) have found that rifampin blocks the synthesis of RNA hydridizable to *B. subtilis* DNA while permitting normal synthesis of RNA hybridizable to PBS1 DNA. We have observed that the level of PBS2-induced DNA polymerase after 45 min of infection of either strain SB19 *pol*⁺ or strain 1306 *pol*⁻ is identical in the presence and absence of rifampin. These results suggest that phage RNA is responsible for the phage-induced increase in DNA polymerase activity. The basis for the rifampin resistance of PBS2 infection is now under investigation.

When added directly to assay mixtures at levels of 200 to 500 μg/ml (much greater than the levels which could be present in dialyzed extracts of treated cells), these antibiotics inhibited the PBS2-induced DNA polymerase less than 50%, suggesting that their effects described above are due to their inhibitory action on macromolecule synthesis in infected cells.

Mixed extracts activity. The activity of PBS2-induced DNA polymerase in crude extracts was not reduced by the presence of an equal volume of extract from uninfected cells of strain SB19 or strain 1306.

Thus, the lower level of activity in these uninfected cells does not appear to be the result of an excess of a diffusable inhibitor, which, if present, may have been expected to reduce the PBS2 DNA polymerase activity.

PBS2 DNA polymerase requirements. The effects of omission of various assay components on PBS2 DNA polymerase activity in crude extracts of strain 1306 *pol*⁻ were investigated (Table 2, extract A). The reaction is completely dependent on the presence of magnesium ions. It does not require ATP, which had been added (29) in an attempt to maintain the deoxyribonucleotides in their triphosphate form in the presence of hydrolytic enzymes in crude extracts. The PBS2

TABLE 2. *Dependence on assay components of PBS2 phage-induced DNA polymerase activity in crude extracts*

Assay mixture[a]	Relative activity (%)	
	Extract A[b]	Extract B[c]
Complete	100	100
No $MgCl_2$	0	0
No ATP	100	190
No dGTP	7	8
No dATP (or dCTP)	27	5
No dTTP	0	6
No DNA	51	5
No β-mercaptoethanol	103	99
plus 0.1 μmole of PHMB[a]	33	
plus 0.7 μmole of PHMB	30	38

[a] See Materials and Methods. PHMB = *p*-hydroxymercuribenzoate in *p*H 8.5 solution.

[b] Extract A is dialyzed extract prepared by lysozyme-sonic treatment (see Materials and Methods) of *B. subtilis* 1306 *pol*⁻ infected for 45 min by PBS2 phage. The initial rate for the complete mixture was 21 counts per min of ^{14}C-dCMP incorporated per min using 390 μg of protein.

[c] Extract B is dialyzed extract prepared by freeze-lysis (see text) of *B. subtilis* SB19 *pol*⁺ infected for 45 min by PBS2 phage. The initial rate for the complete mixture was 57 counts per min of ^{14}C-dAMP incorporated per min using 310 μg of protein.

DNA polymerase is dependent on the presence of all four deoxyribonucleoside triphosphates.

Paper chromatography of reaction mixtures showed that ^{14}C-dCTP was hydrolyzed during the incubations to produce dCDP and dCMP, with uninfected and infected cell extracts showing similar rates. All measurements of DNA polymerase initial rates of synthesis were performed under assay conditions in which less than 5% of the available dCTP was incorporated into DNA and less than 20% of the dCTP was degraded.

When 33 μM dTTP was employed in assays, uninfected cell extracts incorporated ^{14}C-dCTP linearly for 1 hr. In contrast, extracts of cells infected with PBS2 phage for 30 min stopped incorporation within 5 min (R. Ruettinger, *unpublished data*). However, infected cell extracts displayed linear kinetics of ^{14}C-dCTP incorporation when the dTTP concentration was raised to 1.6 mM (Fig. 1). Chromatography of reaction mixtures containing ^{3}H-dTTP [or ^{3}H-deoxyuridine triphosphate (dUTP)] demonstrated that this nucleoside triphosphate was incorporated into DNA, but it was also rapidly hydrolyzed to nucleoside diphosphates and monophosphates (plus some nucleosides) in the presence of infected cell extracts. Therefore, the concentration of dTTP (or dUTP) in assays was routinely made 50-fold greater than the dCTP, dATP, and dGTP concentrations (see Materials and Methods), so that less than 25% of the dTTP (or dUTP) was degraded during initial rate measurements.

While the PBS2 DNA polymerase activity was not diminished by the omission of β-mercaptoethanol from the assay mixture, addition of *p*-hydroxymercuribenzoate reduced its activity by 70% (Table 2). In contrast, the DNA polymerase activity in crude extracts of uninfected strain SB19 *pol*$^+$ and strain 168-2 *pol*$^+$ is inhibited less than 15% by *p*-hydroxymercuribenzoate (*unpublished data*), suggesting that the PBS2 DNA polymerase is different from the major *B. subtilis* DNA polymerase.

PBS2 polymerase dependence on DNA. The dependence on added DNA of the PBS2 DNA polymerase activity is highly variable among different extracts. Activity values were reduced by 29, 38, 49, and 60% when denatured salmon sperm DNA was omitted from assays of PBS2-infected cell extracts prepared as described in Materials and Methods. We ascribe this difference to the variable amounts of host or phage DNA present in crude extracts prepared by this method (which typically contained 85–100% of the total DNA in lysed cells).

To eliminate the effects of endogenous DNA on assays, a modified procedure was used to prepare extracts which were essentially free of DNA (Harold Sadoff, *personal communication*). Cultures were centrifuged at 5,000 *g* for 15 min at 0 C. The pellets were resuspended (in 1% of the original volume) in 10 mM Tris-chloride (*p*H 7.5) containing 1 mM EDTA, and again centrifuged at 0 C. The pelleted cells were frozen overnight at −15 C. They were thawed by rapid stirring at 0 C in 20 mM Tris-chloride (*p*H 8.5), 2 mM EDTA, 200 mM NaCl, 10 mM β-mercaptoethanol, and 1 mM phenylmethyl-sulfonyl fluoride (a protease inhibitor). After standing at 0 C for 3 hr, the viscous suspension of lysed cells was centrifuged at 30,000 × *g* for 25 min. The clear supernatant fluid contained only 2 to 10% of the total DNA in lysed cells. Such extracts showed the same relative increase in DNA polymerase specific activity after PBS2 infection of strains SB19 and 1306 as described above for alternative extraction methods.

Table 2 shows that the DNA polymerase activity in an extract (Extract B) of PBS2-infected cells prepared by the above freeze-lysis method is almost completely dependent upon added DNA. Furthermore, the activity in such an extract (B) has a dependence on the other assay components similar to that described earlier for an extract prepared by lysozyme treatment (Extract A in Table 2), except that ATP was inhibitory to Extract B activity. The basis for this inhibition is being investigated.

PBS2 DNA polymerase DNA specificity. The results of assays employing different DNA species are shown in Table 3. Although an extract (Extract A) of PBS2-infected strain 1306 *pol*$^-$ prepared by lysozyme lysis was not dependent upon added DNA, the activity was stimulated by exogenous DNA, especially denatured DNA. An extract (Extract B) prepared by freeze-lysis, however, was almost completely dependent upon added DNA. The activity with denatured salmon sperm DNA was more than twice that with native salmon sperm DNA, whether dTTP or dUTP was employed in the assay. Both types of extract contained PBS2 DNA polymerase activity which preferred PBS2 DNA. Extract B had over three times more activity with PBS2 DNA, native or denatured, than with denatured salmon sperm DNA. In all cases, the PBS2 DNA polymerase utilized dUTP as well as (or better than) dTTP.

The activity of the PBS2 DNA polymerase with denatured calf thymus DNA, or with salmon sperm DNA activated by treatment with deoxyribonuclease, was similar to that obtained with denatured salmon sperm DNA.

PBS2 DNA polymerase nucleotide specificity. Extracts of uninfected and phage-infected cells were compared with respect to their DNA polymerase activity when dTTP or dUTP was used in

TABLE 3. *Relative activity of PBS2 DNA polymerase in crude extract with various DNA species and dTTP or dUTP*

DNA added[a]	Relative activity (%)			
	Extract A[b]		Extract B[b]	
	dTTP	dUTP	dTTP	dUTP
Denatured salmon sperm	100	95	100	105
Native salmon sperm	35	50	35	50
Denatured PBS2	140	150	320	380
Native PBS2	115	110	310	400
None	40	50	<2	<2

[a] All DNA species were employed at 400 μg/ml in assays. DNA was denatured, when indicated, by heating solutions at 100 C for 5 min, followed by rapid cooling in ice.

[b] Assay mixtures contained 1.6 mM dTTP or dUTP (see Materials and Methods). Extracts were prepared from *B. subtilis* 1306 *pol*⁻ infected for 45 min by PBS2 phage, by lysozyme lysis for Extract A (see Materials and Methods) or freeze-thaw lysis for Extract B (see text). The results are expressed for each extract as a percentage of the activity found with denatured salmon sperm DNA and dTTP. These activities were 80 counts per min per min with 600 μg of protein of Extract A, and 37 counts per min per min with 250 μg of protein for Extract B.

TABLE 4. *B. subtilis and PBS2 DNA polymerase activity with various deoxyribonucleoside triphosphates*

Extract[a]	Radioactive nucleotide[b]	Activity[c] with	
		dTTP	dUTP
Uninfected strain SB19	^{14}C-dCTP	14	8
	^{14}C-dATP	17	11
PBS2-infected strain 1306	^{14}C-dCTP	123	117
	^{14}C-dATP	153	155

[a] Assays contained extracts (see Materials and Methods) of uninfected *B. subtilis* SB19 (1.00 mg protein) or *B. subtilis* 1306 *pol*⁻ infected for 45 min by PBS2 phage (0.30 mg of protein).

[b] Assays contained 8 nmoles of ^{14}C-dCTP or ^{14}C-dATP, and 400 nmoles of dTTP or dUTP, as indicated with other components as described in Materials and Methods.

[c] Activity is expressed as picomoles of ^{14}C-nucleotide incorporated per minute of incubation per milligram of protein.

the assay. To eliminate the possibility of artifacts arising from dCTP deaminase activity (26) in PBS2-infected cells, the assays were performed with ^{14}C-dATP as well as ^{14}C-dCTP (see Table 4). The specific activity of DNA polymerase in extracts of PBS2-infected strain 1306 *pol*⁻ was about nine times greater than that of uninfected strain SB19 *pol*⁺, independent of whether radioactive dATP or dCTP was employed.

Furthermore, the PBS2-induced DNA polymerase uses dUTP and dTTP equally well, as observed above. In contrast, the *B. subtilis* SB19 *pol*⁺ DNA polymerase uses dUTP only about 60% as well as dTTP. This ratio has been observed consistently, and it is identical to the value obtained with uninfected strain 168-2 *pol*⁺. These ratios are independent of the ^{14}C-deoxyribonucleotide employed. The observed dUTP/dTTP ratio is in good agreement with the value of 69% obtained by Okazaki and Kornberg (17), who used the partially purified *B. subtilis* SB19 DNA polymerase. These results suggest that the PBS2-induced DNA polymerase is probably different from the major host DNA polymerase activity.

Polymerase product. The acid-insoluble products of reactions employing extracts of uninfected strain SB19 *pol*⁺ or PBS2-infected strain 1306 *pol*⁻ with dUTP or dTTP were subjected to hydrolytic conditions. All products were stable to 3-hr incubations at 37 C with KOH, ribonuclease, or Pronase, but they were 90% hydrolyzed by deoxyribonuclease alone and were completely hydrolyzed by deoxyribonuclease plus snake venom phosphodiesterase (*unpublished data*), indicating that the product of these reactions is DNA.

DISCUSSION

We have presented evidence that the uracil-containing DNA phage PBS2 induces a new DNA polymerase after infection of *B. subtilis*. The specific activity of DNA polymerase in crude extracts increases five to tenfold after 45 min of PBS2 infection of wild-type cells, or 50- to 100-fold after infection of a strain of *B. subtilis* deficient in DNA polymerase I. Similar observations during infection of *B. subtilis* by the hydroxymethyluracil-containing DNA phage SPO1 have recently been reported by C. Yehle and A. T. Ganesan (Bacteriol. Proc., 1971, p. 196).

The increase in DNA polymerase activity after PBS2 infection can be prevented, or stopped once induction has begun, by inhibitors of RNA or protein biosynthesis, suggesting that at least part of the PBS2 DNA polymerase may be made de novo after infection. Furthermore, the level of DNA polymerase activity in wild-type cells does not increase after treatment with mitomycin C nor after a "mock infection" by a lysate freed of phage. Thus, the increase in DNA polymerase activity after PBS2 infection does not appear to result from the induction of defective phage by

PBS2 (20) nor from the presence of a nonsedimentable inducer in PBS2 lysates.

The PBS2-induced DNA polymerase appears to be different from the major host enzyme, DNA polymerase I (9, 17). The phage enzyme is induced normally in a host which seems to lack DNA polymerase I. The phage enzyme is considerably more sensitive to inhibition by *p*-hydroxymercuribenzoate than the host DNA polymerase I. Finally, the PBS2-induced DNA polymerase utilizes dUTP as well as it does dTTP, whereas the *B. subtilis* DNA polymerase I used dUTP only 60% as well as dTTP (see also reference 17). Proof that the PBS2-induced DNA polymerase is unique to PBS2 infection will require the separation of the phage enzyme from all host DNA polymerases (9) and the isolation of phage mutants which make a defective DNA polymerase.

We plan to purify and characterize the PBS2 DNA polymerase with respect to its molecular weight, metal ion and initiation requirements, nuclease activities, and ability to copy a DNA template. Of special interest is the question whether the PBS2 DNA polymerase prefers as a primer-template its own uracil-containing DNA (as suggested in Table 3). Since the synthesis of *B. subtilis* DNA stops, but it remains physically stable after PBS2 infection as judged by transforming activity (18, 23), the phage must have some mechanism for the selective replication of phage DNA in the cell. This selectivity may arise, for example, from a preference of the phage DNA polymerase for phage DNA, from localization of enzyme and DNA in some membrane complex (8), or from specificity for dUTP rather than dTTP of the phage DNA polymerase. The SP01 phage-induced DNA polymerase also utilizes dTTP and selectively replicates its own phage DNA (Yehle and Ganesan, *personal communication*). To prove that the PBS2-induced DNA polymerase is indeed responsible for viral DNA synthesis in vivo, we are trying to find a PBS2 mutant defective in the DNA polymerase.

We have noted that the PBS2 DNA polymerase can use dTTP and dUTP equally well, at least at high concentrations in crude extracts. We must purify the PBS2 polymerase free from nucleotidohydrolases in order to study the affinity of dTTP and dUTP for the enzyme. Presumably only dUTP is available in PBS2-infected cells, since only uracil-containing DNA is synthesized (see reference 32; also P. Berget and H. R. Warner, *personal communication*). However, appropriate phage mutants defective in deoxyribonucleotide metabolism may allow the accumulation of dTTP (instead of dUTP) as occurs in uninfected cells, so that the PBS2 DNA polymerase might make thymine-containing phage DNA. Such mutant thymine DNA would allow us to determine whether uracil plays an essential role for PBS2 phage, or whether uracil and thymine are interchangeable in the replication and function of viral DNA.

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