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Deoxyribonucleic Acid Synthesis in Bacteriophage SPO1-Infected *Bacillus subtilis*

I. Bacteriophage Deoxyribonucleic Acid Synthesis and Fate of Host Deoxyribonucleic Acid in Normal and Polymerase-Deficient Strains

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The effect of bacteriophage SPO1 infection of *Bacillus subtilis* and a deoxyribonucleic acid (DNA) polymerase-deficient (pol$^-$) mutant of this microorganism on the synthesis of DNA has been examined. Soon after infection, the incorporation of deoxyribonucleoside triphosphates into acid-insoluble material by cell lysates was greatly reduced. This inhibition of host DNA synthesis was not a result of host chromosome degradation nor did it appear to be due to the induction of thymidine triphosphate nucleotidohydrolase. Examination of the host chromosome for genetic linkage throughout the lytic cycle indicated that no extensive degradation occurred. After the inhibition of host DNA synthesis, a new polymerase activity arose which directed the synthesis of phage DNA. This new activity required deoxyribonucleoside triphosphates as substrates, Mg^{2+} ions, and a sulfhydryl reducing agent, and it was stimulated in the presence of adenosine triphosphate. The phage DNA polymerase, like that of its host, was associated with a fast-sedimenting cell membrane complex. The pol$^-$ mutation had no effect on the synthesis of phage DNA or production of mature phage particles.

The process of bacteriophage multiplication in bacteria involves a number of mechanisms by which the host metabolism is modified to direct the synthesis of phage-specific macromolecules. Deoxyribonucleic acid (DNA) synthesis appears to be one of these functions which requires the induction of several new enzymes (6, 17). Phage-induced DNA polymerases in the virulent phages of *Escherichia coli* have been isolated and extensively characterized (2, 12, 18). However, the mechanism of viral DNA replication remains obscure.

The events following infection of *Bacillus subtilis* with some virulent phages are different from those of *E. coli* phage systems. Unlike the T-even phages (14), several *B. subtilis* phage systems (13, 23, 29, 30, 34) do not cause extensive degradation of the host chromosome. The inhibition of host DNA synthesis is followed by an induction of phage DNA synthesis (13, 34). As in the case of T2 and T4, each of which contains a substituted pyrimidine base in its DNA, new enzymes are induced which catalyze the incorporation of 5-hydroxymethyluracil (HMU) in place of thymine in these systems (6).

Further elucidation of the biochemistry of DNA replication in this system warranted the development of an in vitro assay for the phage-induced DNA polymerase activity. In the present report, we examine the events following infection of the wild type and a polymerase-deficient (pol$^-$) mutant of *B. subtilis* with the virulent phage SPO1. We describe the phenomenon of host polymerase inhibition, conservation of the host genome during the entire period of phage maturation, and the nature and specificity of a DNA polymerase activity that is induced after phage infection. A preliminary account of this work has been presented (C. O. Yehle and A. T. Ganesan, Bacteriol. Proc., p. 196, 1971).

MATERIALS AND METHODS

Bacterial strains and phage. This work was performed with *B. subtilis* 168S^r and strain 1420 pol$^-$, a mutant obtained from J. Gross, which is sensitive to methylmethanesulfonate and contains 10% of the wild-type DNA polymerase activity when assayed in the usual manner (31). Strains SB202 and Mu8u5u16 were used for the genetic linkage and marker frequency analyses, respectively (Table 1). Phage SPO1 was provided by K. Bott.

TABLE 1. *List of Bacillus subtilis strains*

Strain	Genotype	Growth response
168S^r	Prototrophic revertant of 168, streptomycin-resistant	
1420	*met$^-$leu$^-$ileu$^-$pol$^-$*	Methionine, leucine, isoleucine
SB202	*aro2$^-$trp2$^-$his2$^-$tyr1$^-$*	Shikimic acid, tryptophan, histidine, tyrosine
Mu8u5u16	*ade16$^-$leu8$^-$met5$^-$*	Adenine, leucine, methionine

Media. Cells were grown in TY broth (32) or the minimal medium of Spizizen (1), and the phage was assayed by using a base layer of a medium containing, per liter: tryptone, 10 g; NaCl, 5 g; agar, 15 g. The soft agar overlay was TY broth with 7 g of agar per liter. Transformants were assayed by plating on the appropriate supplemented minimal medium.

Preparation of cell extracts. Phage SPO1 infection was obtained by exposure of host cultures to a multiplicity of infection of five per bacterium. Phage development was stopped by the addition of sodium azide to a final concentration of 10^{-2} M, and the cells were collected by centrifugation. The cells were suspended at 20-fold concentration in 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (*p*H 8.0) and were lysed at 37 C with lysozyme (100 μg/ml) for use in the following assays. Protein determinations were made by the method of Lowry et al. (21).

DNA isolation. DNA was purified from the above extracts by the method of Marmur (23). Phage DNA was extracted and purified as described previously (35). DNA concentration was estimated by the method of Burton (5).

Enzyme assays. DNA polymerase was assayed as described by Richardson et al. (31), except that the reaction mixtures also contained 1 mM adenosine triphosphate (ATP). Measurement of the activity primed by A copolymer of deoxyadenylate and thymidylate (dAT) was performed by omission of the deoxyribonucleoside triphosphates of guanine and cytosine from the above reaction mixture. Lysates of cells made at different times after infection were assayed for polymerase activity without adding external DNA template. These lysates contained template in sufficient amounts to promote measurable in vitro synthesis with added substrates by the induced enzyme activity. This is referred to as endogenous template in this paper.

Thymidine triphosphate nucleotidohydrolase was assayed by the procedure of Roscoe (33). Amounts (150 nmoles each) of thymidine diphosphate (TDP), thymidine monophosphate (TMP), thymidine, and thymine were co-chromatographed with the samples to serve as markers. In this case, the reaction components were separated by descending chromatography on Whatman no. 1 paper by using a solvent system composed of isobutyric acid, 1 N NH_4OH, and 0.1 M ethylenediaminetetraacetate in ratios of 100:60:1.6, respectively. Thymidine and its nucleotides were separated after 16 hr of development. Approximately 1% of the total radioactivity on the chromatogram of the control sample was observed as TMP. The efficiency of counting for tritium on the paper, using a liquid scintillation counter (Packard Instrument Co., Inc.), was approximately 5%.

Transformation assays. Genetic linkage analyses to detect nuclease activity were performed with a strain of *B. subtilis* that carried four linked genes, controlling aromatic amino acid biosynthesis, distributed in a region of at least 10^7 daltons in molecular weight (26). Gene frequency analyses were performed as described by Yoshikawa and Sueoka (36).

Sucrose and CsCl gradient analyses. Cell extracts were subjected to zone centrifugation on 5 to 20% linear gradients of sucrose as described by Martin and Ames (25). CsCl density gradient centrifugation was performed with a fixed-angle 50 rotor in a Spinco L2-65 preparative ultracentrifuge at 34,000 rev/min at 5 C for 65 hr. Fractions were obtained from a total gradient volume of 8 ml by piercing the bottom of the tube. The tritium-labeled dAT copolymer used as a reference was the gift of P. Modrich.

Materials. [*8-^3H*] Deoxyadenosine-5'-triphosphate and the [*methyl-^3H*] thymidine-5'-triphosphate were purchased from Schwarz/Mann.

RESULTS

DNA polymerase in extracts of wild-type cells infected with SPO1. Cell extracts prepared at various times after infection of *B. subtilis* 168S^r with SPO1 were assayed for DNA polymerase activity. All assays were performed in the absence of externally added template. Upon infection, there was an initial increase in activity, after which the level of conversion of deoxyribonucleoside triphosphates into acid-insoluble material was reduced to a level 10 to 15% of that of uninfected cells by 6 to 9 min after infection (Fig. 1). Beginning between 12 and 15 min after infection, polymerase activity resumed and reached a level of incorporation equivalent to four times that found before infection. Although the DNA of bacteriophage SPO1 contains HMU in place of thymine (28), the DNA polymerase reaction mixture we used contained only the four usual deoxyribonucleoside triphosphates. As will be shown later, the product of this reaction with extracts of cells prepared after the initial decrease in DNA polymerase activity is characteristic of SPO1 DNA which has been partially substituted with thymine in place of HMU.

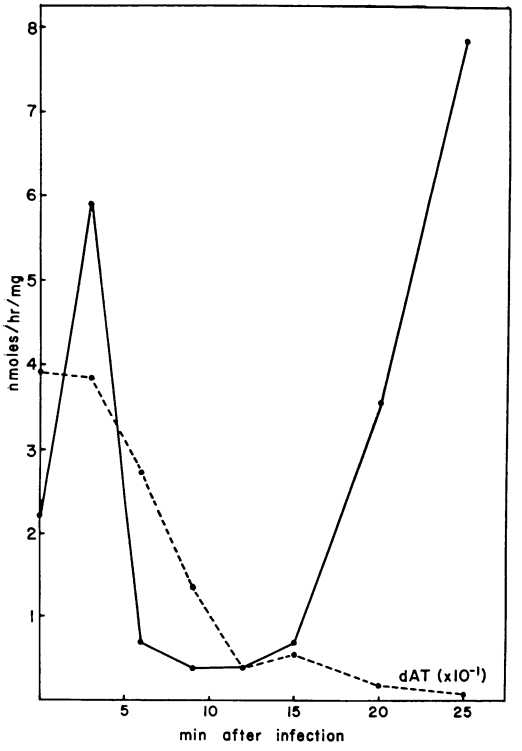

FIG. 1. *DNA polymerase activity of Bacillus subtilis 168S^r after infection with SPO1. Cells were grown in Spizizen's minimal medium supplemented with 0.5% glucose and 0.25% casein hydrolysate at 37 C and were infected with phage SPO1. At intervals, samples were withdrawn, treated, and assayed. The values for dAT-primed activity have been reduced 10-fold for ease of comparison.*

Inhibition of dAT-primed synthesis in extracts of infected cells. The ability of DNA polymerase to promote template-dependent dAT copolymer synthesis rapidly declined after SPO1 infection (Fig. 1). The increase in activity with endogenous template beginning between 12 and 15 min after infection was not paralleled by a concomitant increase in dAT copolymer-primed synthesis. The failure of the synthetic copolymer to serve as a template was not due to a specific degradation of the polymer. Incubation of tritium-labeled dAT copolymer template in the presence of infected cell extracts did not cause a loss of acid-insoluble radioactivity. In a more stringent test, unlabeled dAT, having been incubated in the presence of infected cell extracts, did not lose its ability to prime a subsequent reaction catalyzed by the addition of an excess of purified *E. coli* DNA polymerase (Table 2). Thus, a nuclease activity capable of using dAT copolymer as its substrate was not detected by this assay. This conclusion is also strengthened by the observation that the host chromosome and its genetic activity are predominantly conserved during the period of infection and phage maturation.

Effect of thymidine triphosphate nucleotidohydrolase induction on the DNA polymerase assay. The inhibition of host DNA replication was associated with a number of other metabolic changes which indirectly affected the synthesis of DNA that contains thymine. Within 6 to 8 min after SPO1 infection, host DNA polymerase activity was reduced to approximately 10% of its original activity. Both the endogenous and dAT-primed activities were suppressed by this phage-induced function. It was shown earlier (34) that ϕe, a phage similar to SPO1, also contained HMU in its DNA and induced a number of changes in the nucleotide metabolism of the cell to promote the synthesis of phage DNA and prevent host DNA replication. Roscoe (34) suggested that the thymidine triphosphate nucleotidohydrolase induced upon infection is at least in part responsible for the arrest of bacterial DNA synthesis. We measured the level of thymidine triphosphate nucleotidohydrolase activity in SPO1-infected cell extracts to determine whether this enzyme can seriously affect the in vitro DNA polymerase

TABLE 2. *Effect of uninfected and infected cell lysates on the ability of dAT copolymer to prime a reaction with Escherichia coli DNA polymerase*[a]

Sample	Acid-precipitable counts/min	
	+dAT	−dAT
Control	1,381	67
Uninfected cell lysate	1,083	135
Infected cell lysate	1,235	136

[a] A 2.7-nmole amount of dAT copolymer was incubated in a DNA polymerase assay mixture containing unlabeled deoxyribonucleoside triphosphates of adenine and thymine in the presence and absence of extracts of uninfected and infected cells containing 19 μg of protein. After incubation, the solutions were heated at 70 C for 15 min. Tritium-labeled deoxyadenosine triphosphate (10^4 counts per min per nmole) and thymidine triphosphate were then added along with 0.9 or 1.8 units of purified *E. coli* DNA polymerase I to insure that saturating levels of enzyme were used. The mixtures were then incubated at 37 C for 30 min. Both levels of polymerase produced similar levels of incorporation, and the results are presented as an average. *E. coli* DNA polymerase was a gift of D. Brutlag.

reaction. The maximum rate of TMP formation, 28 μmoles per hr per mg of protein, was obtained from extracts of cells prepared 25 min after infection. No measurable radioactivity was found in the regions of the chromatogram where TDP or thymidine was found. Total recovery of radioactivity for each sample compared well with that for the control. Under these conditions and those of the DNA polymerase assay, a maximum of 15% of the thymidine triphosphate (TTP) in the DNA polymerase reaction mixture could be degraded during the assay period. We thus concluded that the thymidine triphosphate nucleotidohydrolase activity in extracts of SPO1-infected cells would not appreciably affect the assay for DNA polymerase.

Maintenance of host chromosome integrity after phage infection. The host chromosome of *B. subtilis* remained unimpaired throughout the latent period after SPO1 infection. The intactness of the bacterial chromosome can be judged by its molecular weight and genetic integrity as assayed in the *B. subtilis* transformation system. This can be accomplished by assaying the activities of genes that are distributed along the chromosome and, more rigorously, by studying the conservation of genes that are linked in a known region of the chromosome. These genes control the biosynthesis of aromatic amino acids and are clustered in the terminal region of the *B. subtilis* chromosome. There are at least 15 genes mapped in this region (26) which has a molecular weight in the range of 10×10^6 to 15×10^6 daltons. It is known that a DNA preparation with an average molecular weight of 40×10^6 to 60×10^6 daltons is capable of transforming all of the four linked, but well separated, genes of the above linkage group with 40% efficiency. A preparation with an average molecular weight of 20×10^6 daltons showed only approximately 20% cotransfer of the linked markers. Therefore, the ability of a DNA preparation to transform, with high frequency, a multiple auxotroph carrying several linked mutations reflects the intactness of the molecules in a given DNA preparation.

Figure 2 shows the results of such an experiment. Host DNA isolated from cells after various periods of phage infection was used to transform the four linked mutations in a multiple auxotroph. This linkage group is demonstrated in Fig. 3. In these experiments, trp^+ transformants were selected on plates supplemented with the other required metabolites. These transformants were then scored for the other three gene activities. The decline in trp^+ activity between 15 and 20 min after infection was due to the synthesis of phage genomes. Phage DNA synthesis interfered with

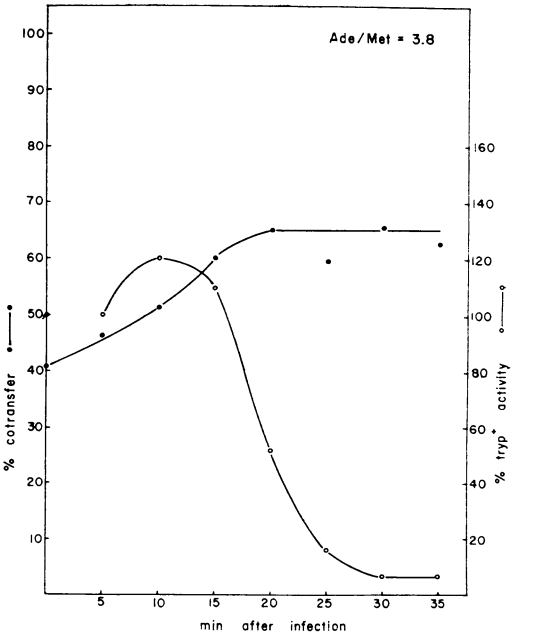

FIG. 2. *Genetic analyses of the host genome after SPO1 infection. DNA purified from extracts of cells at various periods after infection was assayed for genetic linkage and marker frequency. Trp^+ recombinants were selected and scored for the three remaining markers of the linkage group.*

the transformation system since both DNA species were taken up by competent cells. Cells which have taken up and integrated host DNA occur as transformants on selective media. However, cells which have taken up phage DNA or host and phage DNA will appear as infectious centers and will be observed by cell lysis. As low a concentration of DNA as possible was used for the transformation assays to minimize the occurrence of coincidental transformation and transfection. Also, as the proportion of phage DNA to host DNA becomes greater, the host gene pool becomes diluted by the accumulating phage DNA, resulting in a falsely low value. Nevertheless, when the surviving trp^+ recombinants from samples taken at the latter stages of the lytic cycle were examined for cotransfer of the other markers of the linkage group, it was found that the cotransfer remained quite high throughout the lytic cycle. The 40 to 60% level of cotransfer is equivalent to the value obtained with DNA molecules 40×10^6 to 70×10^6 daltons in molecular weight (9). This is comparable to the size of molecules normally isolated from uninfected cells as a result of normal shear effects. For compari-

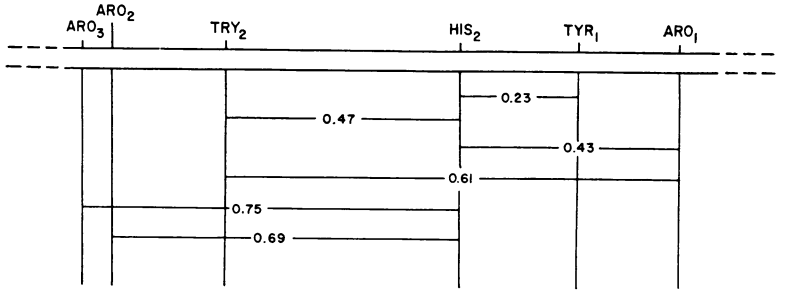

FIG. 3. *Linkage map of loci of aromatic amino acid biosynthesis in Bacillus subtilis.*

son, shear experiments, which resulted in a reduction of the molecular weight to 8×10^6 daltons, caused greater than 95% loss of linkage (9). Further, host chromosome analyses (*manuscript in preparation*) with CsCl density gradients to achieve a separation of host and phage DNA revealed a quantitative recovery from infected cells of host DNA which retained a high level of genetic linkage. It was thus concluded that the host chromosome is not extensively degraded as a result of infection by SPO1.

Marker frequency analyses of the DNA preparations revealed that the ratio of activity for a gene near the origin to that of one near the terminus (ade/met) remained unaltered throughout the entire period of infection and phage multiplication. This suggests that no selective degradation of either the origin or terminus of the chromosome occurs as a result of infection. Attempts to distinguish the host polymerase activity from that induced upon phage infection by fractionation of cell extracts on sucrose density gradients yielded a bimodal distribution of polymerase activities. Further attempts to detect a change in DNA polymerase in infected wild-type strains compared to that in uninfected cells were complicated by the excessive repair-type synthesis which affected both host and phage DNA template. We decided to continue these studies in a mutant which contained low levels of DNA polymerase I.

DNA polymerase in extracts of a pol⁻ mutant infected with SPO1. We obtained from J. Gross (*unpublished data*) a polymerase-deficient mutant of *B. subtilis* similar to that of *E. coli* isolated by DeLucia and Cairns (7). The mutant is sensitive to methylmethanesulfonate and contains 10 to 15% of the activity of the wild-type strain as assayed in vitro. Use of this strain allowed us to examine the effect of phage infection on polymerase activity in the presence of a relatively low level of repair-type synthesis. Assays of polymerase activity on lysates of these cells after infection

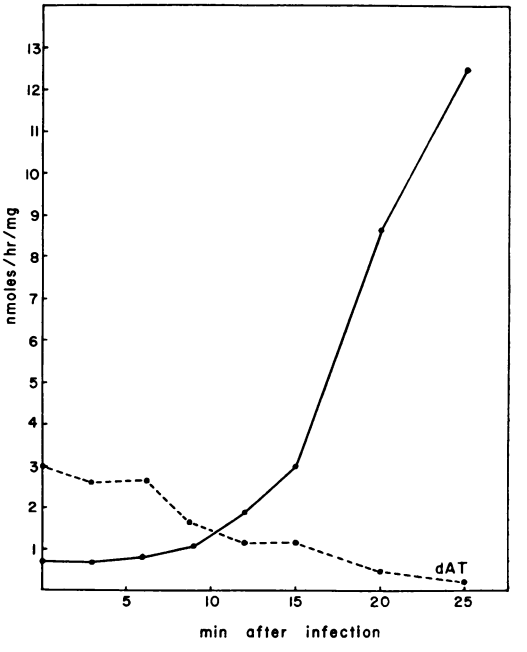

FIG. 4. *DNA polymerase activity of Bacillus subtilis 1420 pol⁻ after infection with SPO1. The experiment followed the procedure described in the legend of Fig. 1.*

indicated a remarkable increase in activity by 25 min after infection (Fig. 4). Again, assays were performed with only the endogenous template. The rapid increase in such an activity suggests that the induction of a DNA polymerase occurred 8 to 10 min after infection. Alternatively, the increased activity could be due to a modification of the endogenous DNA to make it a more efficient template. A comparison of the initial activity or that found during the first several minutes of the latent period to that after 25 min of infection indicated an 18-fold increase. As shown for the wild-type infected cells (Fig. 1), the dAT-primed synthesis, which initially showed poor activity in

TABLE 3. *Effect of added DNA templates on SPO1 DNA polymerase*[a]

Source of DNA template	Priming activity
Endogenous	100
+ dAT copolymer	44
+ Native SPO1	34
+ Denatured SPO1	46
+ Native *B. subtilis*	44
+ Denatured *B. subtilis*	77
+ Activated salmon sperm	88
+ Denatured salmon sperm	96
+ Activated calf thymus	17

[a] Reaction mixtures contained the standard assay system with 20 nmoles of the added DNA primers and samples containing 17.5 μg of protein of a lysate of strain 1420 pol⁻ prepared 25 min after infection. The endogenous template (1.8 nmoles) directed the incorporation of 1 nmole of deoxyribonucleoside triphosphate and was given a value of 100. Incorporation in the presence of added primers is expressed relative to the endogenous incorporation.

this mutant, again decreased several-fold after infection. These observations suggest that this activity is suppressed by a phage-induced function, possibly caused by the destruction or modification of the host enzyme to block its accessibility to dAT after infection. We have already ruled out nucleases as causative agents for this loss of activity.

The large increase in DNA polymerase activity after infection of the polymerase-deficient mutant indicates that a polymerase is being induced by the phage genome. Assays of DNA polymerase in the linear range of increasing activity versus protein concentration indicate a 25- to 30-fold increase in specific activity in phage-infected cells. Thus, the activity increased from 0.11 nmole of deoxyribonucleoside triphosphates incorporated per mg of protein to 3.3 nmoles per mg of protein in strain 1420. Wild-type cells have activity in the range of 1.4 nmoles per mg of protein (27). The phage DNA polymerase required the presence of deoxyribonucleoside triphosphates, Mg^{2+}, and β-mercaptoethanol and was stimulated several-fold by the addition of ATP (1 mM). This activity, as will be shown later, specifically directed the synthesis of phage DNA. Addition of various DNA templates to the reaction mixture failed to stimulate incorporation more than the addition of the endogenous template alone (Table 3). In fact, externally added templates tended to reduce the level of normal incorporation. The reason for this inhibition is unknown but may be due to nonparticipating competition by the added DNA at the site of DNA synthesis. Thus, there appears to be a strong preference of the phage polymerase for the endogenous template.

Sucrose gradient analyses of DNA polymerase in uninfected and infected cell extracts. To characterize further the nature of the enzyme activity, lysates of 168S^r, 1420 pol⁻, and 1420 pol⁻ after 25 min of infection with SPO1 were subjected to zone centrifugation through a 5 to 20% sucrose gradient. The resulting fractions from the gradient were assayed for polymerase both in the absence of externally added template and in the presence of added dAT copolymer. In the absence of externally added template, only fractions containing both the DNA template and DNA polymerase registered any incorporation. The distribution of the endogenous activity (Fig. 5a) in the gradient containing the 168S^r extract was similar to that observed earlier (11). Although each gradient showed the same distribution of host DNA, no discernible profile was observed for the gradient of the uninfected pol⁻ mutant extract. The total activity was approximately 10-fold lower than that of the wild type. However, the profile of activity for extracts of the mutant after SPO1 infection was similar to that of the uninfected wild type. Quantitative recovery of the activity in fractions of the gradient containing the extract of the phage-infected mutant showed that, like the host DNA polymerase, the activity which arises after phage infection is also associated with a fast-sedimenting fraction.

When fractions of the three gradients were assayed for activity primed by dAT copolymer (Fig. 5b), a preference of the fast-sedimenting fraction of the phage-infected extract for its endogenous template was displayed. The polymerase activity associated with the fast-sedimenting fractions from cell lysates of the wild type and the uninfected 1420 pol⁻ was increased several-fold in the dAT-primed assay. In addition, there was also polymerase activity in the slow-sedimenting top fractions which presumably contained enzyme but no template. This activity, like DNA polymerase I, was able to use dAT, SPO1, and SB168 DNA as templates for synthesis. When similar assays were performed on the fast-sedimenting fraction of the lysate of the phage-infected pol⁻ mutant, they did not show any dAT-primed synthesis. Again, polymerase was found in the slow sedimenting fractions which utilized the templates mentioned above.

CsCl density gradient analysis of the product of the DNA polymerase in extracts of infected cells. Since there was a several-fold increase after the activity of the DNA polymerase which was mainly bound to a fast-sedimentable membrane fraction, it was of interest to examine the product of synthesis. Although the base ratios are similar, the

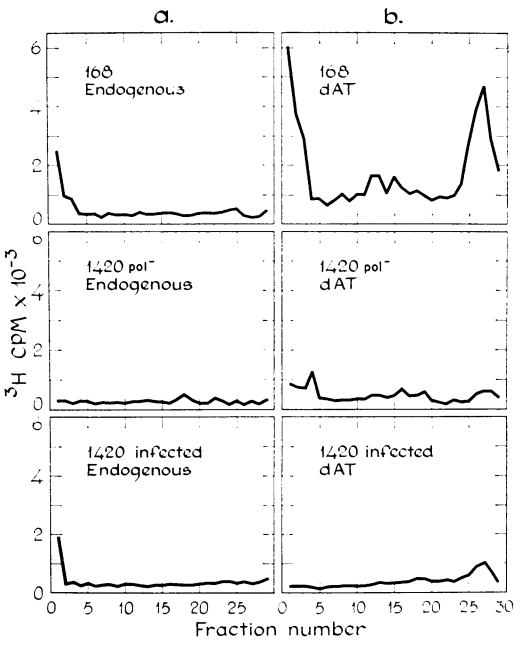

FIG. 5. *Sucrose gradient fractionation of crude extracts of uninfected Bacillus subtilis 168S^r, uninfected 1420 pol$^-$, and infected 1420 pol$^-$. Lysates of uninfected cells and cells collected 25 min after infection with SPO1 were prepared. Amounts (200 μliters) of each extract were layered on 4.4 ml of a 5 to 20% linear gradient of sucrose in 0.01 M tris(hydroxymethyl)-aminomethane (pH 7.4), 0.01 M $MgCl_2$, and 0.001 M β-mercaptoethanol, with 0.3 ml of a cushion of 60% sucrose. The samples were centrifuged at 30,000 rev/min in the SW39 rotor of a Spinco L2-65 preparative ultracentrifuge for 3 hr at 5 C. Under these conditions, host and phage DNA are found in the pellet fractions. Fractions (0.15-ml) were collected by piercing the bottom of the tube with a Buchler fraction-collecting apparatus. A 10-μliter sample of each fraction was assayed for polymerase activity by using the standard reaction mixture in the absence and presence of dAT copolymer template (6 nmoles). The recovery of activity from each gradient was quantitative.*

DNA of SPO1 could be separated from that of its host on CsCl density gradients, since the buoyant density of *B. subtilis* DNA is 1.703 g/ml and that of SPO1 DNA is 1.740 g/ml (28). The density increase is presumably due to the replacement of thymine by HMU. The nature of the product being synthesized and of the template being used in the polymerase reaction could be determined by the position of the labeled product in a CsCl density gradient. Label banding in the region of phage DNA density would indicate that phage DNA has acted as the template for synthesis. The DNA polymerase assay mixtures contained only the four usual deoxyribonucleoside triphosphates. If the new polymerase activity specifies SPO1 DNA as its template, the incorporation of thymine in place of HMU during the polymerase reaction should cause a shift in density toward that of host DNA. To determine the nature of the DNA product of the in vitro assay of extracts prepared during the latter half of the latent period, we subjected the product to isolation on a CsCl equilibrium density gradient. As shown in Fig. 6, the label incorporated during the polymerase reaction occurred at two different densities. One coincided with the density region at which normal SPO1 DNA bands. The predominant peak of the label occurred at a density intermediate between that of SPO1 DNA and *B. subtilis* DNA. The density of 1.726 g/ml corresponding to this peak was significantly greater than that of single-stranded *B. subtilis* DNA (1.718 g/ml), which rules out the possibility that host DNA containing the label could occur in the denatured state. The sharp distribution of label in both density regions is an indication that the product is of high molecu-

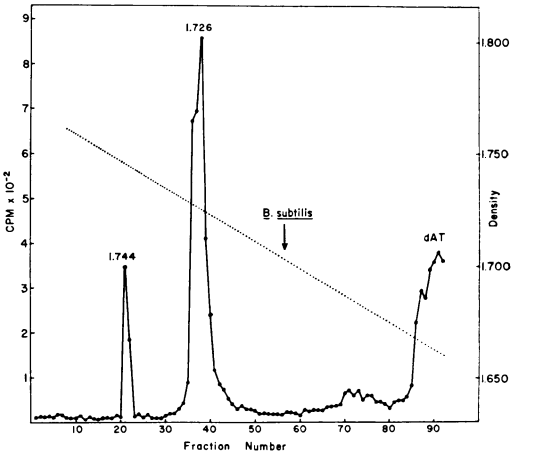

FIG. 6. *CsCl equilibrium density gradient of product of SPO1-induced DNA polymerase of cell extract. A 100-μliter sample of an extract of strain 1420 prepared 20 min after infection with SPO1 was added to 700 μliters of the standard DNA polymerase reaction mixture containing tritium-labeled thymidine triphosphate (10^4 counts per min per nmole) and incubated at 37 C. Tritium-labeled dAT was added as a density marker, and the solution was brought to a total volume of 9.0 ml and 1.710 g/ml by the addition of 0.01 M tris(hydroxymethyl)aminomethane (pH 8.0) and solid CsCl. The solution was centrifuged and fractionated. Fractions (100-μliter) were collected, and the refractive index of every tenth tube was recorded. The fractions were then precipitated with cold 10% trichloroacetic acid with 50 μg of herring sperm DNA added as the carrier.*

lar weight. It appears that the endogenous phage DNA is acting as a template for the new polymerase and that this activity is capable of inserting thymine in place of HMU in the progeny molecules.

DISCUSSION

The effect of phage infection, as pertains to DNA synthesis, on both the wild-type and polymerase-deficient mutant differs only in the initial peak of activity observed immediately after infection of the wild type but not the mutant. The nature of this initial activity in the wild type is being examined. The incorporation of deoxyribonucleoside triphosphates with either endogenous DNA or dAT copolymer templates decreases rapidly during the first 10 min after infection. Although the pol$^-$ mutant contains only 10% of the activity of the wild type, the residual dAT-primed activity is still suppressed upon infection. The mutation which occurs in the pol$^-$ strain appears to have no effect on the synthesis of phage DNA or on induction of the phage polymerase activity. The levels of phage polymerase activity in both strains are similar. The activity increase observed beginning 12 to 15 min after infection is directed toward the synthesis of phage DNA as shown by the fractionation of the labeled product on CsCl density gradients (Fig. 6). The dAT copolymer no longer acts as a template for the phage-induced enzyme activity arising after infection. Several other templates previously used to assay phage-induced polymerases were examined for priming activity and were found to be unable to stimulate incorporation above that of the level primed by endogenous DNA (Table 3). This may be due to the fact that the phage polymerase and endogenous template are closely associated with a membrane complex, which excludes the involvement of any additional DNA template in the polymerase assay system used here. Once released from this complex, the polymerase molecule may lose its template preference. Indeed, preliminary studies with the DNA polymerase from SPO1-infected cells after elimination of the endogenous DNA indicate that the enzyme has lost its specificity. We are attempting to obtain the phage-induced activity free from endogenous DNA, but which will still require SPO1 DNA as a template.

The suggestion by Roscoe (34) that the induction of thymidine triphosphate nucleotidohydrolase upon ϕe infection is not exclusively responsible for the arrest of bacterial DNA synthesis has been supported by our results. The levels of thymidine triphosphate nucleotidohydrolase induced by ϕe and SPO1 are similar. In spite of the excessive levels of TTP in our polymerase assay mixtures, based on a rate of TTP degradation of 28 μmoles per hr per mg of protein, reversal of the inhibition of host DNA polymerase does not occur (Fig. 1 and 4). Thus, it can be concluded that the induction of thymidine triphosphate nucleotidohydrolase is not responsible for the inhibition of host DNA replication after phage infection as previously suggested by Hayward (13). Our conclusion is supported by the recent work of Marcus and Newlon (22) who, with the use of a ϕe mutant defective in thymidine triphosphate nucleotidohyrolase, showed that this activity is not necessary for the arrest of host DNA synthesis. The nature of the phage control over host DNA replication is currently under study.

The nature of the infectious cycle in the SPO1 system differs in one important respect from that of the lytic systems of *E. coli*. The arrest of host DNA replication is not accompanied by any appreciable degradation of the host chromosome. It has been shown previously that the chromosome of *B. subtilis* is not extensively degraded upon infection by several bacteriophages (24, 30, 34). These studies have involved examination of the physical integrity of the host chromosome or the retention of transforming activity for a single marker. The work of Roscoe (34) showed that no double-strand breaks occurred in the host DNA after infection. In this case, however, no conclusion could be made about the possibility of single-strand breaks resulting from infection. The transformation system in *B. subtilis* serves as an assay for both double- and single-strand breaks in the chromosome. The genetic activity of *B. subtilis* transforming DNA is lost upon the introduction of single-strand breaks in the molecule (19). Reduction of the single-strand average molecular weight of transforming DNA from 9.5×10^6 to 3.8×10^6 by deoxyribonuclease treatment results in a 82 and 91% loss of transforming activity for a single marker and linkage group, respectively. The examination of transforming activity for a single marker would have detected single-strand scissions only within a small region of the chromosome. Our assay was more inclusive since we examined a much larger segment of the host chromosome by looking for a loss of linkage in the aromatic linkage group of SB202. Our results do not exclude the possibility that a few selective breaks, which may be responsible for the inhibition of host DNA replication, occur in the host chromosome. However, no such nucleolytic activity has yet been described which could perform this specific function.

The arrest of host DNA replication upon in-

fection by SPO1 is followed by a rapid increase in a DNA polymerase activity which is specific for phage DNA synthesis. This is shown by the fact that label incorporated during the polymerase reaction occurs predominantly in a DNA product having a density similar to that of SPO1 DNA and phage DNA which has had HMU partially replaced by thymine (Fig. 6). Conclusive proof that a completely new DNA polymerase is induced by the phage must await its further characterization. It is possible that the phage induces a modification of the existing host DNA polymerase by a mechanism similar to that proposed by Losick and Sonenshein (20) for the RNA polymerase in differentiating cells. Although the large increase in specific activity of DNA polymerase after infection suggests that a new enzyme is being induced, the possibility remains that an alteration of the endogenous phage DNA to make it an efficient template could explain the increase. The formation of multiple sites of phage DNA replication could also be responsible for a large increase in activity.

As previously observed for host DNA replication (9), it appears that SPO1 phage DNA synthesis is associated with the cellular membrane (Fig. 5). Similar observations have been made in other phage systems such as T4 (8), P22 (4), and ϕX174 (16). Since the membrane-associated activity seems to retain its specificity for a native DNA template, it is of interest to learn whether such a structure provides a necessary component(s) required for semiconservative DNA replication. A system has recently been reported (10) which lends further support to the involvement of membrane-associated functions in bacterial DNA replication. The stimulation of phage polymerase activity by ATP suggests that similar functions may be supporting phage DNA replication. The function of the cell membrane in energy metabolism and a possible relationship to the replication of DNA in uninfected cells are currently being examined.

Since we have not been able easily to obtain hydroxymethyldeoxyuridine triphosphate (dHMUTP), all of our assays of DNA polymerase activity have been made with only the four usual deoxynucleoside triphosphates. Present evidence suggests that thymine can efficiently replace HMU in this system of incorporation. This is similar to the purified DNA polymerase of T2 which can efficiently use deoxycytidine triphosphate in place of 5-hydroxymethyldeoxycytidine triphosphate, the normal substrate of T2 DNA polymerase (2, 3). It was shown that the level of thymidine triphosphate nucleotidohydrolase induced upon infection by SPO1 is not high enough to affect significantly in vitro incorporation in a reaction mixture containing TTP. However, it is quite possible that this level of thymidine triphosphate nucleotidohydrolase is alone sufficient to prevent the incorporation of thymidine monophosphate into phage DNA in vivo. If, for example, the enzyme is localized near the site of replication, it could be capable of blocking the availability of TTP to the polymerase molecule. The increasing pool of dHMUTP produced after infection would also serve to exclude thymine from phage DNA. As is presently suggested, there may be no need for a specificity for dHMUTP on the part of the DNA polymerase molecule itself. Careful measurement of the rates of reaction would be afforded by use of a purified phage DNA polymerase.

The DNA polymerase found after phage infection appears to be using endogenous phage DNA as a template, judging from the buoyant densities of the labeled reaction product on CsCl density gradients (Fig. 6). The nature of the product of the in vitro DNA polymerase reaction after induction of the phage activity has not been thoroughly characterized. We do not know whether the template is being replicated in a semiconservative manner or whether we are only observing repair-type synthesis. However, we do know that the activity is restricted to the phage chromosome. Preliminary attempts to recover host biological activity in a transformation assay with the labeled product from the 1.726 g/ml density region have failed. SPO1 mutants have been used in marker rescue experiments similar to those performed by Kaiser and Hogness (15) in an attempt to recover phage biological activity from the intermediate density region. Successful recovery of SPO1 markers from these fractions has indicated that phage DNA was present. These results are the subject of a subsequent paper.

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