JOURNAL OF VIROLOGY, June 1975, p. 1308-1316
Vol. 15, No. 6

Printed in U.S.A.

Bacteriophage SPO1-Induced Macromolecular Synthesis in Minicells of *Bacillus subtilis*

JOHN N. REEVE* AND JAMES B. CORNETT[1]

Max-Planck-Institut für molekulare Genetik, Abt. Trautner, Berlin and Department of Microbiology and Medical Technology, University of Arizona, Tucson, Arizona 85721*

Received for publication 20 December 1974

SPO1 bacteriophage injects its DNA into minicells produced by *Bacillus subtilis CU403 divIVB1*. The injected DNA is partially degraded to small trichloroacetic acid-precipitable material and trichloroacetic acid-soluble material. The injected DNA is not replicated; however, it serves as a template for RNA and protein synthesis. The RNA produced specifically hybridizes to SPO1 DNA, and the amount of RNA hybridized can be reduced by competition with RNA isolated at all stages of the phage cycle from infected nucleate cells of the *B. subtilis CU403 divIVB1*. An unrelated phage, SPP1, also induces phage-specific RNA in infected minicells. Translation occurs in SPO1-infected minicells resulting in at least eight proteins which have been separated by gel electrophoresis, and two of these proteins have mobilities similar to proteins found only in infected *B. subtilis CU403 divIVB1* nucleate cells. A large proportion of the polypeptide material synthesized in infected minicells is very small and heterogeneous in size.

Minicells are small, quasi-spherical, anucleate cells produced in rod shaped bacteria by cell division occurring abnormally close to one cell pole (1, 24, 36). Macromolecular synthesis in anucleate minicells is extremely limited (4, 14, 23), although intermediary metabolism as shown by respiration, ATP production, and motility is present and can be maintained for extended periods of time (1, 20, 24).

The introduction of DNA into minicells of *Escherichia coli* by segregation of a plasmid DNA molecule (5, 8, 10-14, 17, 18, 27-29) produces a system capable of macromolecular synthesis and which permits the growth of T4 bacteriophage (28). The RNA and protein produced in plasmid-containing minicells have been shown to be plasmid coded products (5, 8, 10, 14, 28). Thus *E. coli* minicells have been shown to contain functional cellular components for transcription and translation if a source of genetic information is provided. RNA polymerase activity was found to be absent in plasmid-less minicells (1) so it was proposed that the segregated plasmid also carries functional RNA polymerase into the minicells (17). A more recent report has shown the presence of the β and β' polypeptide subunits of RNA polymerase in plasmid-less minicells of *E. coli* although no activity was demonstrated (26).

[1] Present address: Dept. of Microbiology, School of Medicine, Temple University, Philadelphia, Pa. 19140.

We have been investigating the properties of minicells produced by *Bacillus subtilis* (20, 22-24), and to demonstrate the capacity for macromolecular synthesis it was necessary to introduce DNA into the minicells. At present there is no known plasmid in *B. subtilis* so that the plasmid segregation system used in *E. coli* was unavailable. We have previously demonstrated that SPO1 bacteriophage readily adsorbs to minicells of *B. subtilis* (22) and here we have shown that SPO1 injects its DNA into the minicells and that this DNA can act as a template for RNA and protein synthesis within the minicells.

MATERIALS AND METHODS

Bacteria and bacteriophage. *B. subtilis CU403 divIVB1 thy*A, *thy*B, *met*B (24) was used throughout this study as a source of minicells and bacteriophage host. SPO1 bacteriophage was obtained from C. Yehle. SPO1 phage stocks were produced by infecting host bacteria at an adsorbance at 660 nm of (A_{660}) 0.5 in TY medium (2) containing 5×10^{-3} M $CaCl_2$ at 37 C with multiplicity of infection of 10. After cell lysis the lysate was cleared by centrifugation at 5,000 $\times$ *g* for 10 min. The supernatant was centrifuged at 27,000 $\times$ *g* for 3 h at 4 C and the phage pellet was allowed to resuspend overnight at 4 C in TBT buffer (2). The phage suspension was layered on top of a three-step CsCl gradient containing CsCl solutions in TBT of densities 1.3, 1.5, and 1.7 g/cc and the gradient was spun at 30,000 rpm for 20 h in an SW39 rotor at 15 C in a Spinco model L ultracentrifuge. The

main phage band was removed and dialyzed against Spizizen salts solution (32) containing 5×10^{-3} M $CaCl_2$.

SPO1 phage stocks containing ^3H- or ^{14}C-labeled DNA were obtained by adding [6-^{3}H]uridine (1 μCi/ml; 10 Ci/mM) or [2-^{14}C]uridine (0.02 μCi/ml; 5 mCi/mM), respectively, to the infected culture 10 min after phage addition (16).

SPP1 bacteriophage stock was kindly supplied by H. Esche.

Minicell production. *B. subtilis CU403 divIVB1 thy*A, *thy*B, *met*B was grown in 2-liter batches at 30 C in minimal medium as previously described (24). The cultures were rapidly chilled to 0 C at the end of exponential growth and the cells were harvested by centrifugation at 5,000 × *g* for 10 min at 0 C. The pellet was resuspended at 0 C in 20 ml of minimal medium lacking glucose, thymine, and methionine and was layered on 5 to 30% sucrose gradients (4 by 30 ml) containing the same salt solution. The gradients were centrifuged at 0 C for 20 min at 4,000 rpm in a HB4 rotor in a Sorvall RC2-B centrifuge. A broad minicell band resulted approximately ⅓ of the distance from the top of the gradient. This band was removed from the gradient, and the minicells were pelleted by centrifugation at 18,000 × *g* for 10 min at 0 C. The large nucleated cells formed a pellet at the bottom of the tube. The minicells were resuspended in the same salts solution and layered on top of a 30-ml 5 to 30% sucrose gradient. The gradient centrifugation, minicell removal, and centrifugation were repeated and the purified minicells so obtained were washed twice with minimal salts by centrifugation at 5,000 × *g* for 10 min. The minicells were finally resuspended in complete minimal medium (24) plus 5×10^{-3} M $CaCl_2$. A minicell suspension of $A_{660} = 0.2$ contained approximately 5×10^8 minicells per ml and a maximum of 10^4 colony-forming units per ml.

It has been previously reported that exposure of minicells to sucrose may be detrimental to minicell metabolism (28) and in the case of *B. subtilis* may cause parental cell lysis and subsequent contamination of the minicell preparation by lysed parental cells (24). The technique described above does not result in parental cell lysis, and in addition all experiments, except the competition RNA/DNA hybridization and protein gel electrophoresis, have been repeated using minicells purified by the ultrasonication technique (20). The results obtained with ultrasonically purified minicells are the same as those given below for sucrose gradient purified minicells.

Phage infection. Minicells or cells were infected at 0 C in minimal medium containing 5×10^{-3} M $CaCl_2$ at an input multiplicity of infection of approximately 5. After 10 min at 0 C the suspension was diluted to an $A_{660} = 0.2$, the temperature was raised to 37 C, and, when necessary, radioactive precursors were added.

Lysis of minicells and isolation of SPO1 DNA from infected minicells. Minicells infected with [6-^{3}H]uridine-labeled SPO1 were rapidly chilled to 0 C and pelleted by centrifugation at 18,000 × *g* for 5 min. The pellet was resuspended in 0.5 ml of lysis buffer (1 M NaCl; 0.05 M Tris; 0.005 M EDTA; 0.02 M NaCN, pH 6.9) containing 20 mg of lysozyme per ml at 37 C for 1 min and then held at 0 C for 10 min before addition of 0.5 ml of lysis buffer containing 2% sarkosyl. Slight warming gave total lysis of the minicells. This lysis procedure does not release DNA from unadsorbed phage.

The minicell lysate was layered on top of a 10-ml 10 to 25% sucrose gradient. The sucrose was dissolved in lysis buffer containing 0.1% sarkosyl. The gradient was spun at 20 C for 5 h at 30,000 rpm in a SW41 rotor by a Spinco model L65B centrifuge. The gradient was fractionated from the bottom directly onto Whatman glass fiber FG/C filters which had been presoaked in 5% trichloroacetic acid and dried. The filters were washed six times with 3 ml of 5% trichloroacetic acid and three times with 3 ml of ethanol before being dried. The radioactivity bound to each filter was measured in a liquid scintillation counting system.

RNA isolation. Two volumes of infected and uninfected cells and minicells were rapidly chilled by mixing with 1 volume of frozen minimal salts medium containing 0.02 M NaN_3. The suspension was then centrifuged for 5 min at 0 C at 18,000 × *g* and the pellet was resuspended in lysis buffer. Cell-minicell lysis was accomplished as described above. The lysate was mixed with CsCl dissolved in lysis buffer to give a final refractive index of 1.4048 at 15 C. The solution was centrifuged at 38,000 rpm at 15 C for 60 h in a 50 Ti rotor. The bottom 2 ml from a total of 10 ml was retained and dialyzed against 8.8 g of NaCl per liter plus 4.4 g of sodium citrate · $2H_2O$ per liter (SSC). The solution was used as the source of RNA for DNA/RNA hybridization experiments. The RNA content was measured by a modified orcinol procedure as described previously (24).

RNA and protein labeling. Minicells were infected as described above and uniformly labeled [^{14}C]proline (0.1 μCi/ml; 290 mCi/mmol) or 10^{-5} M unlabeled uridine plus [5-^{3}H]uridine (25 μCi/ml; 27 Ci/mmol) was added to label protein or RNA, respectively. Samples (0.2 ml) were removed at regular intervals and placed in 4 ml of 5% trichloroacetic acid containing 200 μg of unlabeled proline or unlabeled uridine per ml. The samples were kept at 0 C for at least 1 h before being filtered through 0.45-μm Sartorius filters (Membranfilter GmbH, 34 Göttingen, West Germany). The filters were washed six times with 3 ml of 5% trichloroacetic acid and once with 3 ml of ethanol before being dried. The radioactivity bound to each filter was counted as described above. Chloramphenicol and rifampin were added at final concentrations of 150 μg/ml and 100 μg/ml, respectively, where appropriate.

DNA/RNA hybridization. DNA was prepared by phenol extraction (31). The DNA was denatured by stirring in a solution of three parts 2× SSC plus two parts 1 N NaOH at room temperature for 1 h. The solution was brought to neutrality by the addition of HCl and made to a final volume of six parts by the addition of 2× SSC. The denatured DNA was filtered onto 0.2-μm Sartorius filters. The filters were dried at 60 C for 2 h and placed into small petri dishes, and 3.5 ml of 2× SSC was added, followed by 0.5 ml of RNA solution containing labeled RNA and, where necessary, the com-

petitor RNA. The petri dishes were placed at 69 C for 20 h. The filters were removed from the RNA solution, washed twice with 4 ml of 2× SSC, and incubated for 1 h at 37 C in SSC containing 20 μg of RNase A per ml. The filters were finally washed three times with SSC, once with 1% trichloroacetic acid, and once with ethanol before being dried. The radioactivity bound to each filter was counted as described above.

Gel electrophoresis. Cells (A_{660} = 0.4) and minicells (A_{660} = 4) were infected with SPO1 and suspended at 37 C in minimal medium containing ^{14}C-labeled amino acid mix (10 μCi/ml of cells; 30 μCi/ml of minicells; New England Nuclear, NEC −445). Casamino Acid (0.1%) was added 2 min before terminating the incorporation of labeled amino acids by addition of NaCN (final concentration 0.01 M). The cells or minicells were washed once by addition of 10 ml of ice cold lysis buffer and centrifugation at 10,000 × *g* for 5 min at 0 C. The pellet was resuspended in 50 μl of lysis buffer containing 1 mg of lysozyme per ml and incubated at 37 C for 1 min followed by 10 min at 0 C. Lysis and polypeptide disaggregation were effected by addition of an equal volume of a solution comprised of 0.05 M Tris-hydrochloride (pH 6.8), 1% sodium dodecyl sulfate, 1% mercaptoethanol, 0.002 M EDTA, 10% glycerol, and heating to 100 C for 3 min.

Gel electrophoresis separation of the protein was accomplished by the technique of Studier (33, 34) using the buffer system of Laemmli (15). Kodak RB 54 film was exposed to the dried gel for 48 h before being developed to produce autoradiograms. The autoradiograms were scanned using a double-beam recording microdensitometer Model Mk 111 c (Joyce, Loebl and Co.; Gateshead-on-Tyne, England).

RNA polymerase assay. Ten milliliters of cells or minicells (A_{660} = 0.15) were pelleted and resuspended in 40 μl of 25% sucrose in 0.25 M Tris (pH 8.1), plus 5 μl of EDTA (20 μg/ml) plus 5 μl of lysozyme solution (6.4 mg/ml in 0.25 M Tris, pH 8.4). The suspension was incubated at 37 C for 15 min before addition of 7.5 μl of 15% Brij in 10 mM Tris (pH 7.2), plus 7.5 μl of 0.1 M $MgCl_2$. Complete lysis was obtained by two 30-s exposures to sonic treatment using a MSE sonicator at maximum setting. RNA polymerase activity within the lysate was assayed by the technique of Burgess (3) and protein by the Lowry technique (19).

Protein molecular weight determination. The molecular weights of radioactively labeled proteins after polyacrylamide gel electrophoresis were estimated by comparison with ^{14}C-labeled SPP1 proteins of known molecular weight (H. Esche, Ph.D. Thesis, Freie Universität, Berlin, 1974). The molecular weights of the SPP1 proteins were initially ascertained by coelectrophoresis with cytochrome *c* (molecular weight 12,000), chymotrypsin (molecular weight 25,000), ovalbumin (molecular weight 45,000), bovine serum albumin (molecular weight 67,000), and aldolase (molecular weight 158,000) obtained from Boehringer Mannheim GmbH, West Germany.

Source of materials. [2-^{14}C]uridine, [6-^{3}H]uridine, [5-^{3}H]uridine, and [^{14}C]proline were obtained from The Radiochemical Centre, Amersham, England; lysozyme and RNase A were from Worthington Biochem. Co., Freehold, N. J.; chloramphenicol from Sigma Chemical Co., St. Louis, Mo.; and rifampin from Calbiochem, San Diego, Calif.

RESULTS

Minicells produced by *E. coli* have been reported to lack RNA polymerase activity (1), so we assayed this activity in minicells produced by *B. subtilis*. Extracts of minicells were found to contain an activity assayed over a 5-min linear incorporation as 0.018 trichloroacetic acid-precipitable counts/min per mg of protein. Extracts of parental cells contained an activity in the same assay system giving 0.051 trichloroacetic acid-precipitable counts/min per mg of protein.

The presence of active RNA polymerase in minicells of *B. subtilis* suggested that were we to supply the minicells with DNA then RNA-synthesis and possibly protein synthesis could occur within the minicells. We had previously shown that SPO1 adsorbs readily to minicells (22) and Fig. 1 demonstrates that SPO1 injects

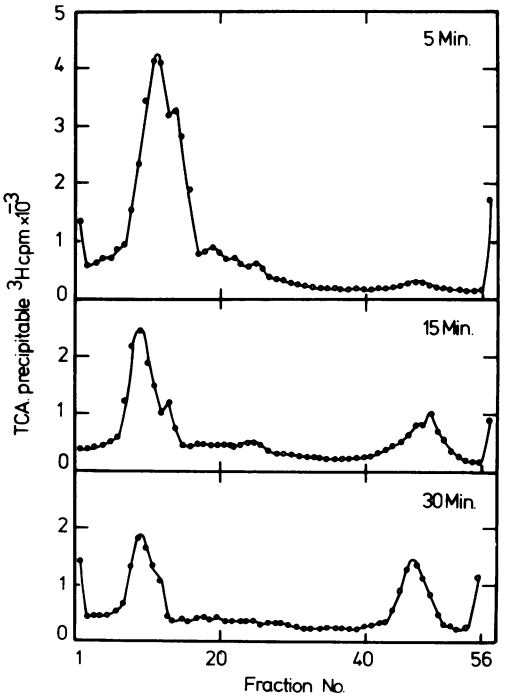

FIG. 1. *Sucrose gradient analysis of ^{3}H-labeled SPO1 DNA from phage infected minicells. Minicells were infected with ^{3}H-labeled SPO1 at 0 C and after 10 min the mixture was placed at 37 C. After 5, 15, and 30 min at 37 C, the minicells were lysed and the lysate was spun through a 10 to 25% sucrose gradient. The gradients were fractionated and the radioactivity contained in the trichloroacetic acid (TCA)-precipitable material in each fraction was measured. Fraction 1 is the bottom of the gradient.*

its DNA into minicells. At 5, 15, and 30 min (37 C) after ^{3}H-labeled SPO1 had adsorbed to the minicells (82% of the ^{3}H-labeled SPO1 adsorbed), the minicells were washed free of unadsorbed phage and lysed. The lysate was centrifuged through a 10 to 25% sucrose gradient. The DNA 5 min after injection formed one peak at the position that intact SPO1 DNA is found which has been extracted by phenol from intact phage. With subsequent incubation the DNA is degraded to smaller, albeit trichloroacetic acid-precipitable, material which forms a second peak close to the top of the sucrose gradient. Although there is a small amount of material between the two peaks, there is effectively no increase in this material over the 30-min incubation period. The trichloroacetic acid-precipitable, radioactive material found in the 5-min gradient accounts for essentially 100% of the ^{3}H-labeled SPO1 material adsorbed to the minicells. The trichloroacetic acid-precipitable, radioactive material recovered from the gradients after 15 and 30 min demonstrated net losses of 17.5 and 39% as compared to the amount of trichloroacetic acid-precipitable material recovered from the 5-min gradient. Thus degradation of injected SPO1 DNA to non-trichloroacetic acid-precipitable material also occurs within minicells.

Having demonstrated that DNA is injected into minicells by SPO1, we wished to determine whether the DNA was replicated. ^{3}H-labeled SPO1 were allowed to adsorb to minicells which were subsequently placed at 37 C for 45 min in the presence of [^{14}C]uridine. The minicells were lysed and the lysate was analyzed by equilibrium CsCl centrifugation (Fig. 2). The trichloroacetic acid-precipitable material within the gradient showed almost no overlap of ^{14}C and ^{3}H, indicating that little or no [^{14}C]uridine was incorporated into the ^{3}H-labeled SPO1 DNA. The density of the ^{14}C-labeled trichloroacetic acid-precipitable material and the fact that it was found to be totally alkali soluble (pH 13, 10 h, 37 C) indicated strongly that the [^{14}C]uridine had been polymerized in RNA. The density of the ^{3}H-labeled SPO1 DNA was found to be 1.74 g/cc (Fig. 2), in agreement with the previously published value (21).

We further investigated the macromolecular synthetic capacities of infected and uninfected minicells using [^{3}H]uridine and [^{14}C]proline. Figure 3A shows that infected minicells polymerized both uridine and proline into trichloroacetic acid-precipitable material. Uninfected minicells apparently could polymerize uridine into trichloroacetic acid-precipitable material to a small extent, but were totally incapable of converting proline to trichloroacetic acid-precipitable material. The material produced by uninfected minicells from [^{3}H]uridine was investigated by CsCl equilibrium centrifugation; however, after minicell lysis and centrifugation (as described in Fig. 2) essentially no trichloroacetic acid-precipitable material was recovered (results not shown). The fate of uridine in uninfected minicells is still under investigation. Figure 3B demonstrates that uridine polymerization in infected minicells was not inhibited by the presence of chloramphenicol, whereas proline polymerization was completely blocked. The addition of rifampicin 10 min after phage infection blocked subsequent uridine polymerization in infected minicells, whereas addition of rifampin at the same time to uninfected minicells had little effect on uridine conversion to trichloroacetic acid-precipitable material (Fig. 3C). The addition of rifampin 10 min after infection depressed the rate of proline conversion to trichloroacetic acid-precipitable material, whereas when rifampin was added at the time of phage infection (Fig. 3D) the conversion of proline to trichloroacetic acid-precipitable material was completely inhibited. Uridine polymerization in infected minicells was re-

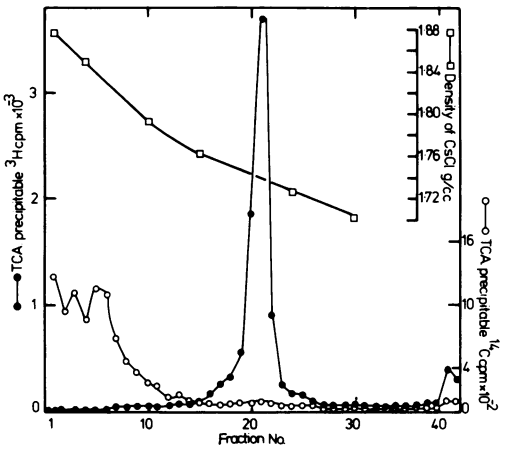

FIG. 2. *CsCl gradient analysis of trichloroacetic acid (TCA)-precipitable material liberated from minicells which had been infected by ^{3}H-labeled SPO1 in the presence of [^{14}C]uridine. Minicells were infected with ^{3}H-labeled SPO1 and after 10 min at 0 C were placed at 37 C in the presence of [2-^{14}C]uridine (2.5 μCi/ml; 59 mCi/mmol) for 45 min. The minicells were lysed and the lysate was brought to a refractive index of 1.4048 with CsCl dissolved in lysis buffer. After centrifugation (60 h, 38,000 rpm, 15 C) in a 50 Ti rotor, the gradient was fractionated, and the radioactivity contained in the TCA-precipitable material in each fraction was measured. Fraction 1 is the bottom of the gradient.*

duced to a very low level by the addition of rifampin at the time of phage infection (Fig. 3D).

The trichloroacetic acid-precipitable material produced from uridine by infected minicells was further investigated by means of DNA/RNA hybridization to determine whether the RNA produced in minicells after phage infection was specifically transcribed from the phage DNA. ^{3}H-labeled RNA was isolated from both SPO1- and SPP1-infected minicells (SPP1 is a phage unrelated to SPO1 which also induces RNA and protein synthesis in minicells, although approximately 10-fold less efficiently than SPO1) and hybridized against SPO1, SPP1, and *B. subtilis* DNAs (Table 1). The hybridization is very specific, namely SPO1-induced ^{3}H-labeled RNA binds almost exclusively to SPO1 DNA and SPP1-induced ^{3}H-labeled RNA binds exclusively to SPP1 DNA. Hybridization was also attempted between SPP1-induced ^{3}H-labeled RNA and heavy and light, separated, single-stranded SPP1 DNA, as it has been previously shown that early SPP1-RNA is exclusively transcribed from the heavy strand and throughout the SPP1 growth cycle transcription is mostly from the heavy strand (6). The ^{3}H-labeled RNA induced in minicells by SPP1 hybridized 18-fold more to the heavy strand than to the light strand (Table 1). The almost exclusive hybridization between ^{3}H-labeled RNA induced by SPP1 and isolated heavy single-stranded DNA of SPP1 suggested that transcription of phage DNA in minicells may be limited to early genes (6). To test this hypothesis using SPO1, RNA was isolated before and 6, 10, 20, 30 min after infection of a culture of *B. subtilis CU403 divIVB1* with SPO1

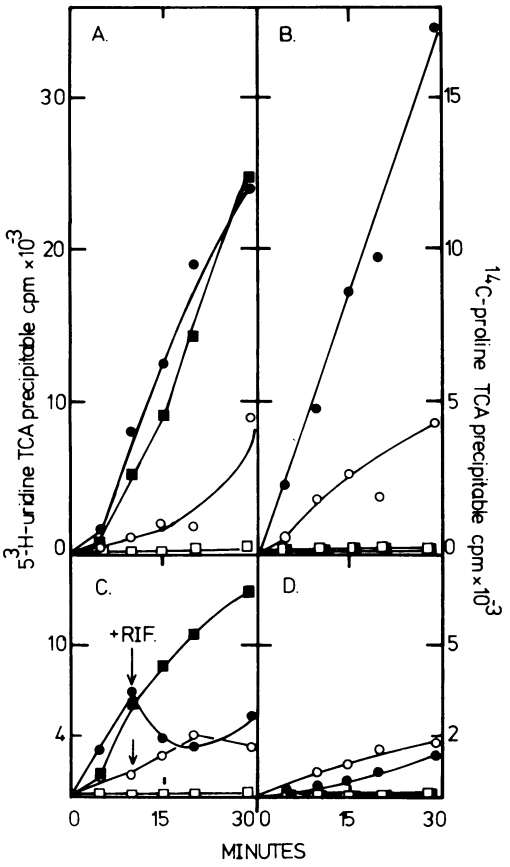

FIG. 3. *Incorporation of [5-^{3}H]uridine and uniformly labeled [^{14}C]proline into trichloroacetic acid (TCA)-precipitable material by infected and uninfected minicells. Minicells were infected at 0 C with SPO1 and after 10 min infected and uninfected minicells were suspended at 37 C in a mixture of unlabeled uridine (10^{-5} M) plus [5-^{3}H]uridine (25 μCi/ml; 27 Ci/mmol) and uniformly labeled [^{14}C]proline (0.1 μCi/ml; 290 mCi/mmol). At regular intervals, 0.2-ml samples were removed and placed in ice-cold 5% TCA. Radioactivity in the TCA-precipitable material in each sample was measured. 0 time is the time of transfer from 0 to 37 C. Infected (●) and uninfected (○) minicells in the presence of [5-^{3}H]uridine. Infected (■) and uninfected (□) minicells in the presence of [^{14}C]proline. (A) No additions; (B) chloramphenicol at 0 min; (C) rifampin (RIF) at 10 min; (D) RIF at 0 min.*

TABLE 1. *DNA/RNA hybridization using ^{3}H-labeled RNA isolated from SPO1- and SPP1-infected minicells*[a]

DNA bound to filter (5 μg/filter)	^{3}H-labeled SPO1 trichloroacetic acid-precipitable material bound to filter[b] (counts/min)	^{3}H-labeled SPP1 trichloroacetic acid-precipitable material bound to filter[c] (counts/min)
	20	15
SPO1	6,041	13
SPP1	74	591
SPP1-L[d]	89	38
SPP1-H[d]	91	426
B. subtilis	64	25

[a] Minicells at an A_{650} of 2 were infected with SPO1 or SPP1 at an input multiplicity of infection of 5 and subsequently incubated for 30 min at 37 C in minimal medium containing 100 μCi of [5-^{3}H]uridine per ml (27 Ci/mmol). Minicell lysis and RNA extraction was as described.

[b] 13,500 counts/min added to each reaction mixture.

[c] 600 counts/min added to each reaction mixture.

[d] SPP1-L and SPP1-H are the light and heavy single strands obtained from SPP1 DNA by strand separation (6, 25).

(9). The isolated RNA was used to compete with ^{3}H-labeled RNA made in SPO1-infected minicells in DNA/RNA hybridization experiments (Fig. 4). RNA isolated from uninfected cultures of *B. subtilis CU403 divIVB1* competed with the RNA made in minicells to reduce the binding of radioactive material by 30% at very high concentrations of competing RNA, but showed no competition at lower concentrations. RNA isolated from cells 6 min after infection competed with the RNA from minicells to reduce the binding by 56%, whereas RNA isolated from cells 10, 20, and 30 min after infection reduced the binding of ^{3}H-labeled RNA by 78, 75, and 76%, respectively. Thus the competing RNA isolated from cells 10, 20, and 30 min after infection must contain competing SPO1 RNA not present in RNA extracted 6 min after infection. All combinations of RNA extracts, e.g., 6 min plus 10 min, 10 min plus 20 min, etc., resulted in further reduction in bound ^{3}H-labeled RNA (results not shown), indicating that the RNA extracted at each time during the phase cycle contained different RNAs capable of competing with ^{3}H-labeled RNA made in infected minicells. The RNA made in infected minicells was therefore definitely not limited to early mRNA.

We further investigated the protein synthesizing capability of infected minicells by gel electrophoresis of the material synthesized after SPO1 infection of minicells. Infected and uninfected minicells were incubated at 37 C for 45 min in the presence of a mixture of ^{14}C-labeled amino acids. The radioactively labeled material synthesized in infected minicells was separable into at least eight bands (Fig. 5C), whereas we were unable to demonstrate protein bands in uninfected minicells. Figure 5A and B show protein separation patterns from infected and uninfected cells. Three large peaks, x, y, z, are only present in infected cells as compared to uninfected cells. Peaks 6 and 7 produced in minicells (Fig. 5C) have the same mobility as peaks x and y, respectively. A high background was found in the gels from infected minicells,

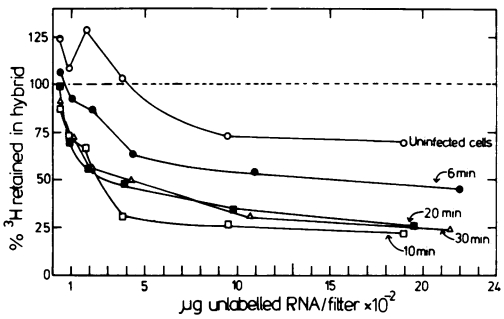

FIG. 4. *DNA/RNA competition hybridization between ^{3}H-labeled RNA isolated from SPO1-infected minicells and RNA isolated from parental cells at various stages of the SPO1 infective cycle. ^{3}H-labeled RNA was isolated from infected minicells after 30 min of incubation at 37 C in the presence of 100 µCi of [5-^{3}H]uridine (27 Ci/mmol) per ml. RNA was isolated from uninfected B. subtilis CU 403 divIVB1 (○) and 6 (●), 10 (□), 20 (■), and 30 (△) min after SPO1 infection. A constant amount of ^{3}H-labeled RNA (12,500 counts/min) from minicells was competed with increasing amounts of nonlabeled RNA from cells. The number of trichloroacetic acid-precipitable counts retained in the hybrid molecules bound to each filter is plotted as the percentage of the counts bound in the absence of competitor, which in this experiment was 4,782 counts/min. Each filter had 5 µg of SPO1 DNA and essentially no radioactivity was retained on control filters without DNA.*

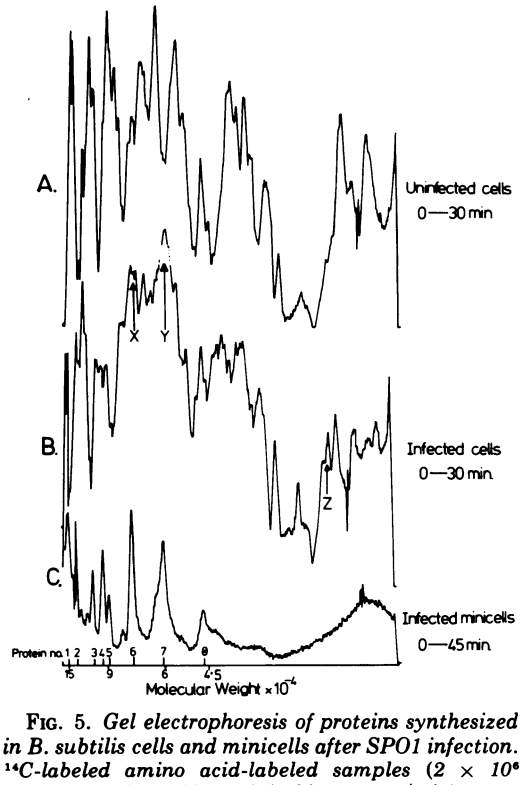

FIG. 5. *Gel electrophoresis of proteins synthesized in B. subtilis cells and minicells after SPO1 infection. ^{14}C-labeled amino acid-labeled samples (2 × 10^6 trichloroacetic acid-precipitable counts/min) were run in parallel through a 15% polyacrylamide gel for 16 h at 55 V, and autoradiographs were prepared and scanned. (A) Uninfected cells incubated with ^{14}C-labeled amino acids for 30 min; (B) infected cells incubated with ^{14}C-labeled amino acids for 30 min; (C) infected minicells incubated with ^{14}C-labeled amino acids for 45 min.*

especially towards the lower-molecular-weight range. As the same number of trichloroacetic acid-precipitable radioactive counts were loaded onto all three gels in Fig. 5, it can be concluded that much of the material synthesized in minicells was low-molecular-weight polypeptides of irregular size, much of which passed completely through the gel.

DISCUSSION

We have demonstrated the presence of active RNA polymerase within minicells of *B. subtilis* and, as it was previously shown that SPO1 phage was unable to grow on minicells (22), we have investigated the extent of phage-induced macromolecular synthesis within minicells.

SPO1 phage was shown to inject its DNA into minicells (Fig. 1), however, the DNA within the minicells apparently does not replicate (Fig. 2) and is partially degraded to produce small-molecular-weight, trichloroacetic acid-precipitable material (Fig. 1) and trichloroacetic acid-soluble material. The injected DNA is not degraded into increasingly smaller molecules, but once a molecule is attacked it is degraded to very small pieces as the little amount of material found of intermediary size between whole SPO1 genome size molecules and the small degradation product does not increase with time. Similar results have been obtained for degradation of conjugally transferred DNA in minicells of *E. coli* (G. G. Khachatourians, R. J. Sheeny, and R. Curtiss III, Bacteriol. Proc., p. 31, 1972). Agarose gel electrophoresis of the degraded material indicates a molecular weight of approximately 10^5 for the small DNA fragments, i.e., approximately 0.1% of an intact SPO1 genome (Hill and Reeve, unpublished data). The inability of SPO1 DNA to replicate in minicells, as demonstrated by failure to incorporate uridine into DNA (Fig. 2), was not unexpected although it is not understood. It is known, however, that SPO1 DNA replication is a complex process (16) which requires the synthesis of phage-specific DNA polymerase (37, 38) and probably hydroxymethyluracil synthesizing enzymes (9, 30). The ability of DNA molecules to replicate within minicells of *E. coli* has only been convincingly demonstrated for small plasmid molecules, e.g., ColE1 (11), CloDF13 (35) and several small R-factors (17), which presumably use the host cell DNA replication enzymes which must be present in the minicells.

Although SPO1 DNA is not replicated in minicells, we have been able to demonstrate that it is transcribed and translated (Fig. 3). The transcription in infected minicells is rifampin sensitive (Fig. 3C, D), and transcription is continually being reinitiated as rifampin added 10 min after infection blocks subsequent RNA synthesis (Fig. 3C). Polymerization of [^{3}H]uridine in uninfected minicells was consistently observed, however we were unable to isolate large-molecular-weight RNA synthesized in uninfected minicells. A similar result was recently reported for minicells produced by *E. coli* in which only 3% of the counts incorporated by uninfected minicells were extractable as large-molecular-weight RNA (14). Also, in agreement with this report, we found that a large percentage of the incorporation of uridine in uninfected minicells was rifampin resistant (Fig. 3D). Protein synthesis, as shown by proline conversion to trichloroacetic acid-precipitable material, occurs only in infected minicells and is completely chloramphenicol and rifampin sensitive when the inhibitors are added at the time of infection (Fig. 3B and D). If rifampin is added 10 min after infection then protein synthesis continues, indicating the presence of reusable mRNA synthesized during the first 10 min of infection. Incorporation of uridine is linear for approximately 30 min after infection, whereas proline incorporated is linear for at least 60 min (Reeve, unpublished observation). The linear incorporation of uridine could be limited by the rate of degradation of the DNA template (Fig. 1). We considered it possible that only early mRNA was synthesized in SPO1-infected minicells, as it has been previously reported that early mRNA is transcribed by host RNA polymerase (9). The host polymerase is modified later in the infection cycle (7), and six distinct classes of mRNA are successively produced (9). In addition early mRNA is synthesized in the presence of chloramphenicol, and chloramphenicol had no inhibitory effect on the RNA synthesized in infected minicells (Fig. 3B). We therefore isolated RNA before and 6, 10, 20, and 30 min after infection of parental cells with SPO1 phage, and, by using DNA/RNA hybridization, we were able to show that the RNA made in minicells is not limited to early mRNA (Fig. 4), as it is competed by RNA made at all stages throughout the phage cycle and it is known that early mRNA synthesis is terminated 4 min after infection (9). Some competition is found with RNA isolated from uninfected cells (Fig. 4), however the RNA made in SPO1-infected minicells hybridizes only to SPO1 DNA (Table 1) and not to isolated *B. subtilis* DNA. This specificity of hybridization of phage-induced

RNA to phage DNA was confirmed by the use of a second phage, SPP1, which also induces RNA and protein synthesis in minicells (Reeve, unpublished observation). The RNA produced after SPP1 infection hybridizes only with SPP1 DNA and specifically to the heavy strand of SPP1 DNA (Table 1). This strand specificity is also found in SPP1 infection of normal cells (6).

The proteins synthesized in infected minicells were analyzed by gel electrophoresis (Fig. 5). It was found that at least eight distinct bands were present when the minicells were allowed to incorporate high specific activity ^{14}C-labeled amino acids for 45 min after infection. At least two of the bands are clearly visible in the gel scans of protein from infected cells. No bands were found in uninfected minicells. The demonstration of bands representing proteins synthesized in plasmid-containing minicells of *E. coli* has been reported (5, 13, 14, 28), and in one case the synthesis of a functional enzyme has been demonstrated (8). A high background of irregularly sized polypeptide material synthesized in minicells of *E. coli* has been reported (14), similar to the high background found in Fig. 5C. The fragments of DNA produced by the degradation of the injected SPO1 DNA (Fig. 1) could possibly act as templates to produce the irregularly sized polypeptides as found in Fig. 5C. We are currently extending the comparison of proteins synthesized in infected minicells with those synthesized in infected and uninfected cells to conclusively show them to be phage products. Infection of minicells by amber phage mutants may also give further information on the extent and types of proteins synthesized in infected minicells. These projects, together with the assay for known phage-specific enzymes in infected minicells, are currently under investigation.

ACKNOWLEDGMENTS

This work was initiated in the laboratory of N. H. Mendelson and supported at that stage by research grant 1 Ro 1 GM 18735 from the National Institute of General Medical Science.

We would like to thank H. Esche and J. E. Womack for help and instruction in many of the techniques employed.

LITERATURE CITED

1. Adler, H. I., W. D. Fisher, A. Cohen, and A. A. Hardigree. 1967. Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. U.S.A. **57**:321-326.
2. Biswal, N., A. K. Kleinschmidt, H. Ch. Spatz, and T. A. Trautner. 1967. Physical properties of the DNA of bacteriophage SP50. Mole. Gen. Genet. **100**:39-55.
3. Burgess, R. R. 1969. A new method for the large scale purification of *Escherichia coli* deoxyribonucleic acid-dependent ribonucleic acid polymerase. J. Biol. Chem. **244**:6160-6167.
4. Cohen, A., W. D. Fisher, R. Curtiss III, and H. I. Adler. 1968. The properties of DNA transferred to minicells during conjugation. Cold Spring Harbor Symp. Quant. Biol. **33**:635-640.
5. vanEmbden, J., and S. N. Cohen. 1973. Molecular and genetic studies of an R factor system consisting of independent transfer and drug resistance plasmids. J. Bacteriol. **116**:699-709.
6. Esche, H., and H. Ch. Spatz. 1973. Asymmetric transcription of SPP1 *in vitro*. Mol. Gen. Genet. **124**:57-63.
7. Fox, T. D., and J. Pero. 1974. New phage-SPO1-induced polypeptides associated with *Bacillus subtilis* RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. **71**:2761-2765.
8. Frazer, A. C., and R. Curtiss III. 1973. Derepression of anthranilate synthatase in purified minicells of *Escherichia coli* containing the Col-*trp* plasmid. J. Bacteriol. **115**:615-622.
9. Gage, L. P., and E. P. Geidushek. 1971. RNA synthesis during bacteriophage SPO1 development: six classes of SPO1 RNA. J. Mol. Biol. **57**:279-300.
10. Hori, H., R. Takata, A. Muto, and S. Osawa. 1974. Ribosomal RNA synthesis in the F' 14 episome-containing minicells of *Escherichia coli*. Mole. Gen. Genet. **128**:341-347.
11. Inselburg, J. 1970. Segregation into and replication of plasmid DNA in chromosomeless segregants of *Escherichia coli*. J. Bacteriol. **102**:642-647.
12. Kass, L. R., and M. B. Yarmolinsky. 1970. Segregation of functional sex factor into minicells. Proc. Natl. Acad. Sci. U.S.A. **66**:815-822.
13. Kool, A. J., M. Pranger, and H. J. J. Nijkamp. 1972. Proteins synthesized by a non-induced bacteriocinogenic factor in minicells of *Escherichia coli*. Mole. Gen. Genet. **115**:314-323.
14. Kool, A. J., M. S. van Zeben, and H. J. J. Nijkamp. 1974. Identification of messenger ribonucleic acids and proteins synthesized by bacteriocinogenic factor CloDF13 in purified minicells of *Escherichia coli*. J. Bacteriol. **118**:213-224.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
16. Levner, M. H., and N. R. Cozzarelli. 1972. Replication of viral DNA in SPO1-infected *Bacillus subtilis*. I. Replicative intermediates. Virology **48**:402-416.
17. Levy, S. B. 1971. Physical and functional characteristics of R-factor deoxyribonucleic acid segregated into *Escherichia coli* minicells. J. Bacteriol. **108**:300-308.
18. Levy, S. B., and P. Norman. 1970. Segregation of transferable R factors into *E. coli* minicells. Nature (London) **227**:606-607.
19. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
20. Mendelson, N. H., J. N. Reeve, and R. M. Cole. 1974. Physiological studies of *Bacillus subtilis* minicells. J. Bacteriol. **117**:1312-1319.
21. Okubo, S., B. Strauss, and M. Stodolsky. 1964. The possible role of recombination in the infection of competent *Bacillus subtilis* by bacteriophage deoxyribonucleic acid. Virology **24**:552-562.
22. Reeve, J. N., and N. H. Mendelson. 1973. Minicells of *Bacillus subtilis*: a new phage blocking agent. J. Virol. **12**:944-945.
23. Reeve, J. N., and N. H. Mendelson. 1974. Minicells of *Bacillus subtilis*: a unique system for transport studies. Biochim. Biophys. Acta **352**:298-306.
24. Reeve, J. N., N. H. Mendelson, S. I. Coyne, L. L. Hallock, and R. M. Cole. 1973. Minicells of *Bacillus subtilis*. J. Bacteriol. **114**:860-873.

25. Riva, S. C., M. Polsinelli, and A. Falaschi. 1968. A new phage of *B. subtilis* with infectious DNA having separable strands. J. Mol. Biol. **35**:347-356.
26. Rogerson, A. C., and J. E. Stone. 1974. β-β' subunits of ribonucleic acid polymerase in episome-free minicells of *Escherichia coli*. J. Bacteriol. **119**:332-333.
27. Roozen, K. J., R. G. Fenwick, D. P. Allison, and R. Curtiss III. 1971. Segregation of λdv DNA into minicells of *E. coli* K12. Genetics **68**:548-549.
28. Roozen, K. J., R. G. Fenwick, and R. Curtiss III. 1971. Synthesis of ribonucleic acid and protein in plasmid-containing minicells of *Escherichia coli* K-12. J. Bacteriol. **107**:21-33.
29. Roozen, K. J., R. G. Fenwick, S. B. Levy, and R. Curtiss III. 1970. Characterization of DNA isolated from minicells of plasmid-harboring minicell-producing strains of *E. coli* K12. Genetics **64**:s 54.
30. Roscoe, D. H., and R. G. Tucker. 1966. The biosynthesis of 5-hydroxymethyldeoxyuridylic acid in bacteriophage-infected *Bacillus subtilis*. Virology **29**:157-166.
31. Spatz, H. Ch., and T. A. Trautner. 1970. One way to do experiments on gene conversion: transfection with heteroduplex SPP1 DNA. Mole. Gen. Genet. **109**:84-106.
32. Spizizen, J. 1958. Transformation of biochemically deficient strains of *B. subtilis* by desoxyribonucleate. Proc. Natl. Acad. Sci. U.S.A. **44**:1072-1078.
33. Studier, F. W. 1973. Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237-248.
34. Studier, F. W., and J. Maizel. 1969. T7 directed protein synthesis. Virology **39**:575-585.
35. Veltkamp, E., W. Barendsen, and H. J. Nijkamp. 1974. Influence of protein and ribonucleic acid synthesis on the replication of the bacteriocinogenic factor CloDF13 in *Escherichia coli* cells and minicells. J. Bacteriol. **118**:165-174.
36. Voros, J., and R. N. Goodman. 1965. Filamentous forms of *Erwinia amylovora*. Phytopathology **55**:876-879.
37. Yehle, C. O., and A. T. Ganesan. 1972. 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. J. Virol. **9**:263-272.
38. Yehle, C. O., and A. T. Ganesan. 1973. Deoxyribonucleic acid synthesis in bacteriophage SPO1-infected *Bacillus subtilis*. II. Purification and catalytic properties of a deoxyribonucleic acid polymerase induced after infection. J. Biol. Chem. **248**:7456-7463.