Translation of RNAs Synthesized In Vivo and In Vitro from Bacteriophage SP82 DNA

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The synthesis of 69 phage-specific polypeptides during the infection of *Bacillus subtilis* with bacteriophage SP82 was detected by pulse-labeling, one-dimensional electrophoresis, and autoradiography. SP82 virions were found to contain approximately 22 polypeptides, most of which were synthesized late in infection; evidence was obtained for the processing of the major virion protein. RNAs extracted at different times during infection were translated by using an *Escherichia coli* cell-free extract. Only smaller-molecular-weight peptides were produced efficiently in vitro; in the 9,000- to 60,000-molecular-weight range, 50 to 60% of the peptides synthesized in vivo were produced by translation of RNAs extracted from infected cells. Eight of the virion peptides were produced by in vitro translation of RNAs extracted from infected cells. RNAs were synthesized under defined conditions by RNA polymerase extracted from uninfected *B. subtilis* and by polymerases isolated from cells 8 and 20 min after infection with SP82. Translation of these RNAs yielded characteristic and different patterns of polypeptides. Nine of the 12 polypeptides produced by translation of RNAs synthesized by the host polymerase corresponded in mobility to peptides appearing in vivo in the 0 to 3 and 3 to 6 min intervals of pulse-labeling after infection; 12 of the 25 peptides synthesized from RNAs produced by polymerase extracted 8 min after infection corresponded in mobility to peptides detected in vivo 8 min after infection, and 15 of the 22 peptides directed by RNAs made by the polymerase isolated 20 min after infection corresponded to peptides present in vivo late in infection. Five of the peptides produced in vitro from the latter RNA corresponded to virion peptides.

After infection of *Bacillus subtilis* by either of two closely related bacteriophages, SP82 or SPO1 (26), the host RNA polymerase is modified by the addition of phage-specified polypeptides that confer transcriptional specificity for middle and late phage genes (3-8, 12, 16, 18-21, 25, 27). The evidence that changes in transcriptional specificity can be attributed to enzyme modification comes largely from competition-hybridization analyses of RNAs synthesized in vitro by polymerases extracted from uninfected or phage-infected cells. Although this method of analysis can demonstrate which temporal sequences have been transcribed preferentially, it does not provide a complete assessment of the fidelity of transcription. For example, competition-hybridization does not determine whether the initiation and termination sequences for individual mRNA's are recognized or whether all of the genes in a temporal class, or only a subset, are transcribed.

A measure of the fidelity of transcription was provided from previous studies that determined

the ability of RNA produced in vitro to direct the cell-free synthesis of dCMP deaminase, an SP82-specified enzyme (27). These investigations showed that SP82-modified polymerase was capable of synthesizing functional transcripts of the dCMP deaminase gene in vitro and that RNA produced by host enzyme directed the synthesis of only traces of dCMP deaminase; similar results were obtained with the SPO1-modified polymerase (24). In addition, an electrophoretic analysis (24) demonstrated that different polypeptides are produced in vitro from RNAs synthesized by polymerases from uninfected with SPO1-infected *B. subtilis*. However, these experiments, which were performed with a coupled transcription-translation system, did not correlate the polypeptides synthesized in vitro with those produced during SPO1 infection.

The present communication reports a more general assessment of the content of functional mRNA synthesized in vitro by host and SP82-modified polymerases isolated during the middle

(8 min) and late (20 min) stages of infection. RNAs synthesized in vitro by the three polymerases under defined conditions were extracted and added to an *Escherichia coli* S30 fraction for translation. The translation products were analyzed by one-dimensional electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide slab gels, and the arrays of polypeptides detected by autoradiography were compared with peptides produced by translation of RNAs extracted from SP82-infected cells and peptides synthesized in vivo during infection with SP82.

MATERIALS AND METHODS

Phage infection. Growth of *B. subtilis* strain 168 (Try⁻) and infection by SP82G were performed as described previously (13, 20), using a medium containing (per liter): sodium citrate, 0.5 g; NH_4SO_4 , 1 g; KH_2PO_4 , 3 g; K_2HPO_4 , 7 g; tryptophan, 10 mg; and Casamino Acids (Difco), 0.01%. The pH was adjusted to 7.0 with HCl, and, immediately before inoculation, the medium was supplemented with 0.5% glucose, 0.005 M MgSO_4 , 2×10^{-5} M MnCl_2 , and 5×10^{-4} M CaCl_2 . Lytic times were slightly longer in this medium, averaging 43 to 45 min, as compared with 36 to 38 min in M medium (20). A rifampin-resistant strain of *B. subtilis*, OSB420 (10; kindly provided by Lyle Brown), was grown and infected under the same conditions.

Labeling of SP82 proteins in vivo. At intervals before and during infection, 2-ml portions of the culture were incubated for 3 min at 37°C with 20 to 40 μCi of [^{35}S]methionine (approximately 100 Ci/mmol) per ml prepared by the method of Crawford and Gesteland (2). One milliliter of a solution of 2% unlabeled methionine was added, and the incubation was continued for an additional 2 min. Protein synthesis was stopped by adding 3 ml of an ice-cold solution containing 0.1 M NaCl, 0.05 M sodium citrate, 0.01 M sodium azide, 200 μg of chloramphenicol per ml, and 400 μg of phenylmethylsulfonyl fluoride (Sigma Chemical Co.) per ml; the samples were cooled in an ice bath; the cells were collected by centrifugation at 4°C; the supernatants were discarded; and the pellets were processed for electrophoresis as described below. Additional experiments were also performed with a mixture of 15 ^{14}C -labeled amino acids (New England Nuclear Corp.; NEC-445). These experiments employed the procedure described above except that 5 μCi of labeled precursor per ml was substituted for [^{35}S]methionine and a mixture of unlabeled amino acids was used in the 2-min chase.

Purification of SP82 virions. SP82 was purified by sedimentation through CsCl as described previously (20).

Extraction of RNA from SP82-infected cells. Samples (100 ml) of the infected or uninfected cultures were poured on 100 g of crushed ice containing 1 ml each of sodium azide (100 mg/ml) and rifampin (2 mg/ml; Dow Chemical Co.) in 60% ethanol. The cells were harvested by centrifugation and washed with 20 ml of 0.01 M magnesium acetate-0.01 M Tris (pH

7.5), and the RNA was extracted as described by Hagen and Young (9).

RNA polymerase purification and in vitro RNA synthesis. RNA polymerase was purified from uninfected or SP82-infected *B. subtilis* by the procedure described previously (20, 27) except that gradient elution from a column of DNA-cellulose was omitted. The preparation of SP82 DNA has been described (20). The reaction mixtures for the synthesis of RNA were as described by Spiegelman and Whiteley (21) except that NaCl was added to a final concentration of 0.16 M. The molar ratio of polymerase to DNA in these syntheses varied from 120 to 180, assuming molecular weights of 5×10^5 and 90×10^6 for polymerase and SP82 DNA, respectively. The reaction mixtures were incubated at 37°C for 15 min, 20 μg of DNase (Worthington Biochemicals Corp.; RNase-free) per ml was added, and incubation was continued for an additional 15 min. The samples were then precipitated with 3 volumes of 95% ethanol overnight at -20°C. The precipitates were collected by centrifugation at 4°C, dissolved by incubation at 60°C for 5 min in 0.2 ml of sterile water, and used immediately as templates for cell-free protein synthesis.

Cell-free protein synthesis. The source of the S30 extract was *E. coli* MRE 600 (RNase I⁻) grown at 37°C in a medium containing (in grams per liter): tryptone, 10; yeast extract, 1; glucose, 1; and NaCl, 8. The S30 extract was prepared from cells in mid-log phase as described by Hagen and Young (9). Protein-synthesizing mixtures, prepared by the method of Hopper et al. (11), contained 20 to 40 μCi of [^{35}S]methionine (approximately 100 Ci/mmol) per 100 μl of reaction mixture. The reaction mixtures were preincubated for 20 min at 37°C in the presence of 5 μg of DNase per ml and then incubated for 20 min at 37°C with up to 1 mg of the exogenous RNA template per ml. Protein synthesis (determined by trichloroacetic acid-precipitable radioactivity) in response to increasing amounts of exogenously added RNA was linear in the range of 0 to 60 $\mu\text{g}/100 \mu\text{l}$ of reaction mixture. The reaction was terminated by adding 10 μl of a solution containing 0.05 M EDTA and 500 μg each of RNase and DNase per ml and incubating for 5 min at 37°C. The reaction mixtures were then processed for electrophoresis as described below.

To couple RNA and protein synthesis, the *E. coli* S30 protein synthesis reaction mixture was preincubated at 37°C for 45 min without DNase and in the presence of 100 μg of rifampin per ml. RNA and protein syntheses were initiated by the addition of 100 μg of SP82 DNA per ml, 0.06 mM CTP and UTP, and rifampin-resistant RNA polymerase extracted from *B. subtilis* OSB420 (10). The reactions were terminated as above, and the preparations were processed for electrophoresis.

Preparation of samples for electrophoresis. The cell pellets obtained from the in vivo labeling experiments were suspended in 25 μl of a solution containing 0.06 M Tris (pH 6.8), 500 μg of lysozyme per ml, 50 μg each of RNase and DNase per ml, and 150 μg of phenylmethylsulfonyl fluoride per ml and then incubated for 5 min at 37°C. Lysis was completed by the addition of 75 μl of sample buffer containing

0.1 M Tris (pH 6.8), 2% SDS (Accurate Chemical Co.), 2% β -mercaptoethanol, 20% glycerol, and 0.005% bromophenol blue and heating in a boiling-water bath for 5 min. The reaction mixtures resulting from cell-free protein syntheses were processed for electrophoresis by the addition of 3 ml of acetone, and the preparations were cooled on ice for 10 min. The precipitates were collected by centrifugation, air dried, and then dissolved in 0.2 ml of the above sample buffer diluted 1:1 with water, and the samples were heated in a boiling-water bath for 5 min.

Portions of 2 to 40 μ l of the samples were subjected to electrophoresis on SDS-polyacrylamide slab gels prepared by the method of Studier (23), except that the resolving gel contained a 14 to 20% exponential gradient of acrylamide (17). The volume of 20% acrylamide in the mixing chamber was 6 ml for gels 9 cm in length and 9 ml for 14-cm gels. An acrylamide/bis ratio of 30:0.174 was used for the resolving gel, whereas the stacking gel had an acrylamide/bis ratio of 30:0.8 and a final acrylamide concentration of 5%. The SDS used in the preparation of gels and in the running buffer was purchased from Matheson, Bell and Coleman.

Gels 9 cm in length were subjected to electrophoresis for approximately 3 h at 115 V, and 14-cm gels were subjected to electrophoresis for approximately 7.5 h at 125 V. The gels were stained and fixed in 10% acetic acid-25% isopropanol-0.05% Coomassie brilliant blue and then destained by diffusion in 10% acetic acid-5% methanol. The gels were then dried for autoradiography or processed for fluorography (1) and exposed to Kodak RP Royal X-Omat film at -70°C .

RESULTS AND DISCUSSION

To assess the functional mRNA content of RNA synthesized by the purified polymerases, the cell-free translation products of *in vitro* RNA were compared by electrophoretic mobility with polypeptides synthesized *in vivo*. The latter were identified only by mobility and times of synthesis since SP82 nonsense or deletion mutants were not available.

These comparisons are based on the assumption that the appearance and intensity of labeling of peptides produced *in vivo* and *in vitro* measure the activities of the corresponding mRNA's. This assumption is valid only if there is little or no processing of synthesized peptides and if there are no significant changes in protein turnover or in the specific activities of the methionine pool during the lytic cycle. Processing will be discussed below. The specific activity of the methionine pool and protein turnover have not been measured directly, but, as shown below, the arrays of peptides, intensities of labeling, and temporal sequences of peptides synthesized *in vivo* are similar to those obtained by *in vitro* translation of RNAs extracted from phage-infected cells. Since *in vitro* translation is not affected by changes in pool size or protein turn-

over, we have assumed that the presence and intensity of labeling of peptides *in vitro* and *in vivo* reflect the activities of various mRNA's.

The assignment of times of synthesis of peptides produced *in vivo* (shown below) also assumes that stable mRNA's are not produced. Levinthal et al. (15) found that the half-life of mRNA in uninfected and SP01-infected *B. subtilis* was 2 to 4 min. No evidence was obtained for the existence of long-lived mRNA. Preliminary experiments also failed to demonstrate the presence of stable mRNA during SP82 infection although differences in the functional half-lives of different messages were apparent (J. S. Downard and H. R. Whiteley, unpublished data).

Comparison of polypeptides synthesized during infection with polypeptides synthesized *in vitro* from RNAs extracted during infection. Polypeptides synthesized *in vivo* were analyzed by pulse-labeling portions of infected cells with [^{35}S]methionine at intervals throughout the lytic cycle. As shown in the autoradiographs of Fig. 1A and 4A and diagrammatically in Fig. 2A, these experiments revealed a complex pattern: synthesis of most of the host peptides ceased by the 9 to 12 min interval of labeling, and new polypeptides were synthesized. Only one host peptide (the band located immediately below P27) appeared to be synthesized throughout infection; as discussed later, this band may represent a host peptide and a virion peptide of identical mobilities. A phage-specific peptide was defined as a radioactive band that appears after the addition of SP82, whose electrophoretic mobility differed from that of host peptides or a peptide that showed an increase in labeling after infection. This definition would include any host peptide whose synthesis is induced by phage regulatory proteins. Comparisons with the patterns of labeling in unirradiated cells infected with SP82 at 37 and 33 $^{\circ}\text{C}$ (data not shown) confirmed that peptides having mobilities of host peptides which showed increased labeling in the 0 to 3 and 3 to 6 min intervals after infection represent SP82-directed products. Only two SP82-directed peptides could be distinguished that were synthesized at different times in the lytic cycle and had an apparently identical mobility: P44, which was produced in the 0 to 6 min interval, and P45, whose synthesis began in the 9 to 12 min interval and continued to lysis. Thus, electrophoretic mobility could be used to identify all translation products formed from RNAs synthesized *in vivo* and *in vitro* except for the latter two peptides and the peptide discussed above, whose mobility apparently coincided with that of a host peptide.

Although newly synthesized peptides can be detected in almost every interval of labeling

shown in Fig. 1, the majority of the SP82-specific peptides first appeared during the 3 to 6 and 9 to 15 min intervals of labeling. A portion of the peptides whose synthesis began 3 to 6 min after infection continued to be synthesized through the 21 to 24 min interval, whereas the synthesis of others stopped at earlier times. As discussed below, a number of peptides synthesized in the 9 to 15 min interval have electrophoretic mobilities that coincide with the mobilities of virion peptides.

Several points concerning the *in vivo* pattern of peptide synthesis deserve comment. First, the *in vivo* pattern is reproducible—i.e., repeated

experiments have shown only minor variations in the intensities of a few bands (e.g., compare Fig. 1A and 4A). Second, preliminary experiments comparing peptides labeled 10 to 20 min after infection with [³⁵S]methionine or a mixture of 15 ¹⁴C-amino acids lacking methionine demonstrated the presence of only one methionine-deficient peptide. Third, the amount of [³⁵S]methionine incorporated in each interval of infection increased progressively so that incorporation in the late stages of infection was 2.5 times greater than that in the early stages. A comparison of autoradiograms obtained by electrophoresis of equal volumes of samples and equal

FIG. 1. Comparison of polypeptides synthesized during infection of *B. subtilis* by SP82 with polypeptides synthesized *in vitro* from RNA extracted during infection. (A) Autoradiograph of 9-cm SDS-polyacrylamide gel showing polypeptides pulse-labeled at the indicated intervals of infection. Approximately 50,000 cpm of incorporated [³⁵S]methionine was added to each sample well; the gel was exposed for 1 week at -70°C . (B) Fluorograph of 9-cm SDS-polyacrylamide gel showing polypeptides synthesized *in vitro* from RNA extracted at the indicated times of infection. Approximately 40 μg of each RNA preparation was added to cell-free protein synthesis reaction mixtures that had a final volume of 0.1 ml. The lane designated (-RNA) shows the products of cell-free synthesis without the addition of exogenous RNA. Approximately 75,000 cpm of incorporated [³⁵S]methionine from each reaction mixture was added to each sample well; the gel was exposed for 24 h at -70°C . Molecular weights were estimated from the mobilities of the subunits of RNA polymerase from uninfected and SP82-infected *B. subtilis*. The polymerase preparations were subjected to electrophoresis on the same gel with the samples.

amounts of incorporated radioactivity showed that the same patterns of peptides were produced and that the increase in incorporation during infection was due to an increased rate of synthesis of peptides that first appeared 9 to 15 min after infection. Fourth, the genome of SP82 is sufficiently large (molecular weight, 90×10^6 ; J. M. Lawrie and H. R. Whiteley, *Gene*, in press) to encode approximately 150 polypeptides of molecular weight 40,000, indicating that only a portion of the peptides that could be directed by SP82 have been detected under the conditions of electrophoresis and autoradiography shown in Fig. 1. A larger number of peptides have been detected by two-dimensional gel electrophoresis (M. J. Milhausen and H. R. Whiteley, unpublished data). Fifth, the times of synthesis of the more intensely labeled peptides (shown as thick lines in Fig. 2A) can be corre-

lated with the six temporal RNA classes detected earlier by competition-hybridization experiments (21). The six peptide classes shown in Table 1 account for approximately 80% of the 69 phage-specified peptides shown in Fig. 1. A total of eight RNA classes were defined by competition-hybridization experiments using isolated heavy (H) and light (L) strands of SP82 DNA (13), but although peptides corresponding to each of these classes are shown in Fig. 1A and 4A, a number of peptides remain that do not fit the various RNA classes. This is to be expected since competition-hybridization is a relatively insensitive method of analysis. However, it should be noted that the numbers of RNA and peptide classes derived from these labeling experiments depend in part on the labeling intervals and that more classes might be detected by use of shorter labeling intervals.

FIG. 2. Diagrammatic representation of phage polypeptides synthesized during infection of *B. subtilis* by SP82 and polypeptides synthesized in vitro from RNA extracted during infection. (A) Pattern of polypeptides synthesized in vivo. Representation derived from visual inspection of Fig. 1A and 4A. SP82-directed peptides have been numbered P1 to P69. SP82-directed peptides were defined as those bands in the 0 to 6 min intervals of labeling that showed an increased intensity of labeling compared with the pattern shown in the first lane (host peptides) and all bands in the 6 to 24 min intervals that showed an increase in intensity. (B) Pattern of polypeptides synthesized in vitro. Polypeptides that correspond in times of synthesis and mobilities to peptides synthesized in vivo have been given the same number as the peptides shown in (A). For (A) and (B), synthesis is represented by solid lines for periods of increasing or maximum synthesis and broken lines for periods of decreasing synthesis. Polypeptides predominantly labeled have been designated by heavy lines. Peptides in (B) were given two numbers when the bands were not sufficiently well resolved to permit identification with one of two peptides of very similar mobilities shown in (A). The six peptides that were synthesized in vitro that did not correspond to peptides produced during infection have been lettered a to f.

TABLE 1. Correspondence of temporal classes of proteins and RNAs

Intervals of synthesis of major peptides ^a			Intervals of synthesis of RNA classes ^b	
Class	Interval (min)	% Total peptides	Class	Interval (min)
A	0-3 to 3-6	7.5	1	3-7
B	0-3 to 12-15	3	2	4-13
C	0-3 to 18	6	3	4-17
D	0-3 to lysis	25	4	4-lysis
E	9-12 to lysis	18	5	7-lysis
F	12-15 to lysis	18	6	13-lysis

^a Class derived from heavy lines shown in Fig. 2; the percentage of total peptides includes all peptides detected in each interval.

^b Defined by competition-hybridization experiments (20).

Figure 1B and the corresponding diagram shown in Fig. 2B demonstrate the pattern of polypeptides produced by cell-free synthesis in response to RNAs extracted at the indicated times of infection. Immediately apparent is the absence of peptides with molecular weights greater than 56,000 and the accumulation of radioactivity near the bottom of the gel. Part of the latter represents the translation of mRNA provided by the *E. coli* S30 fraction (lane designated -RNA). In the range of 9,000 to 20,000 molecular weight, good correlation was found between the in vitro and in vivo patterns with respect to mobilities and times of synthesis of the peptides (polypeptides smaller than P65 could not be resolved despite the use of briefer periods of autoradiography). In the range of 20,000 to 60,000 molecular weight, the translation of extracted RNAs yielded measurable quantities of those polypeptides that were synthesized in relatively large amounts in vivo. Six peptides were found (Fig. 1B) that did not correspond to peptides shown in Fig. 1A: these have been labeled a to f, and peptides a to d were present in trace amounts. The mechanisms that might account for the production of these peptides are discussed below. A comparison of Fig. 1A and B suggests that preferential translation can occur in vitro. For example, some early peptides (produced in the 0 to 3 min interval; e.g., P22, P44, and P55) were more intensely labeled by in vitro translation than by in vivo synthesis, whereas some middle peptides (produced in the 6 to 9 min interval; e.g., P25 and P43) either were less efficiently translated in vitro than in vivo or mRNA's coding for these peptides were preferentially degraded during extraction. Overall, Fig. 18 indicates greater trans-

lation of smaller mRNA's. In addition, it should be noted that the time of appearance of some peptides produced by the translation of in vivo RNAs was delayed slightly, suggesting that an accumulation of larger quantities of mRNA's was required for efficient translation in vitro.

An analysis of a series of experiments such as those illustrated in Fig. 1B showed that 50 to 60% of the peptides synthesized in vivo with molecular weights from 9,000 to 60,000 can be produced by cell-free translation of in vivo RNA. With longer exposures of autoradiograms, trace amounts of additional peptides can be detected, including a few with molecular weights of up to 100,000, accounting for approximately 70% of the in vivo products. The lack of complete correspondence in the patterns of in vivo and in vitro products, including the synthesis of peptides a to f, may arise from procedures used for the translation and/or extraction of RNAs, although, as stated earlier, our methods yield at least one functional mRNA, that coding for dCMP deaminase (27). It is also possible that small quantities of peptides produced in vivo or in vitro were not detected due to the conditions of autoradiography used in these experiments. It seems more likely that the incomplete fidelity of translation arises from the use of the *E. coli* S30 fraction as the source of the translation components. Several investigations (14, 22) have shown that the source of the ribosomes can have a significant effect on the quantities of different peptides produced in vitro, particularly when ribosomes from gram-negative cells are used to translate mRNA's from gram-positive cells. Whatever the cause(s) for discrepancies noted between Fig. 1A and B and 2A and B, errors attributable to the translation system can be distinguished by comparing the translation products of RNA synthesized in vitro with peptides generated by the translation of RNAs extracted from phage-infected cells and also with peptides synthesized in vivo.

SP82 virion proteins. To determine which of the polypeptides numbered in Fig. 2B were virion proteins, cells infected with SP82 were continuously labeled with [³⁵S]methionine from 5 min after infection until lysis. Phages were purified from the resulting lysate, denatured by boiling in sample buffer, and subjected to electrophoresis. As shown in Fig. 3, lanes b and c, approximately 24 SP82 polypeptides can be distinguished; most are present in trace amounts. Figure 3, lane a, shows the polypeptides labeled during the 19 to 22 min interval of infection; those that correspond in mobilities to phage peptides have been numbered according to Fig. 2. At least 15 polypeptides labeled during this interval corresponded to virion proteins, and the

FIG. 3. Autoradiograph of SDS-polyacrylamide gel comparing polypeptides synthesized late during infection with SP82 virion proteins. (a) Polypeptides pulse-labeled 19 to 22 min after infection. Polypeptides were pulse-labeled from 19 to 22 min after infection as described in the text. (b) Polypeptides from labeled purified phage. *B. subtilis* was labeled with 30 μCi of [^{35}S]methionine per ml from 5 min after infection until lysis. SP82 was purified from the resulting lysate and denatured by boiling for 5 min in sample buffer. Approximately 40,000 cpm of incorporated [^{35}S]methionine was added to each sample well; a 9-cm gel was used, and the gel was exposed for 5 days at -70°C . (c) Scan of the autoradiograph shown in (b).

majority of these were synthesized predominantly during the later stages of infection. However, polypeptides P24, P28, and P35 were synthesized throughout infection, and the mobility of one virion peptide matched that of an apparent host polypeptide (H). As noted earlier, synthesis of this "host peptide" apparently continued throughout infection. Possibly, band H is a composite of two peptides (a host and a phage-specific peptide) of identical mobility that are synthesized at early and late times, respectively, during infection. However, additional experiments will be required to determine whether this is so. From reference to Fig. 2, it appears that eight of the polypeptides (P13, P25, P28, P35, P41, P51, P58, and P61) synthesized *in vitro* from extracted RNA had mobilities that matched those of virion peptides.

Processing of polypeptides during infection. An examination of the times of synthesis and intensity of labeling of polypeptides 20 and 21 in Fig. 1 to 3 (in Fig. 3, P21 is shown as a heavy line just above P23) suggested the possibility that P21 was a cleavage product of P20. Thus, both polypeptides appeared 9 to 12 min after infection and subsequently increased in amount in a coordinated manner. Although both were present in relatively large amounts during infection, RNA extracted from phage-infected cells supported the *in vitro* synthesis of only P20. As shown in Fig. 3, P21 had the same mobility as the major polypeptide present in lysed virions (presumably the major capsid protein) and had a molecular weight of approximately 45,000. However, P20, with a molecular weight of approximately 46,000, was not present in equivalent amounts in the lysed preparations of labeled phage (Fig. 3, lane b). To determine whether P20 was a precursor of P21, a culture of *B. subtilis* was labeled with [^{35}S]methionine from 9 to 12 min after infection, an interval when only a trace of P21 was detected in the experiments shown in Fig. 1, and then chased by the addition of excess unlabeled methionine. Portions of the culture were taken at 2-min intervals thereafter and analyzed by electrophoresis and autoradiography. The resulting pattern is shown in Fig. 4B and is compared with the normal pattern of protein synthesis shown in Fig. 4A. In agreement with Fig. 1, only a trace of P21 was detected in the 9 to 12 min sample during normal infection. Taking the 9 to 12 min interval as the starting time (0 min in Fig. 4B), it is apparent that a substantial quantity of P21 appeared 4 min after the addition of unlabeled methionine and that the amount of this peptide increased concomitant with a decrease in the amount of labeled P20. Thus, the major structural polypeptide of SP82 appears

FIG. 4. Autoradiographs of SDS-polyacrylamide gels demonstrating processing of polypeptide P20. (A) Autoradiograph of a gel showing polypeptides pulse-labeled at the indicated times of infection. (B) Autoradiograph of a gel showing the labeling of peptides in the following pulse-chase experiment. A culture of SP82-infected *B. subtilis* was labeled with 25 μCi of [^{35}S]methionine per ml during the 9 to 12 min interval after infection; 5 mg of nonradioactive methionine per ml was added (onset of chase); portions were removed at the indicated times after the addition of the nonradioactive methionine and processed as described in the text. Polypeptides that changed in the intensity of labeling during the chase have been identified with the polypeptide numbers shown in Fig. 2. Approximately 150,000 cpm of incorporated [^{35}S]methionine (A and B) was applied to each sample well on a 14-cm SDS-polyacrylamide gel and subjected to electrophoresis as described in the text. The gel was exposed for 3 days at -70°C .

to be synthesized first as a 46,000-molecular-weight precursor and is then cleaved to a peptide of molecular weight 45,000.

Only minor additional changes in the composition of polypeptides pulse-labeled 9 to 12 min after infection were evident during the chase period. A trace of a polypeptide (directly above P5 in Fig. 4B) appeared during the chase but was not seen in Fig. 4A, suggesting that this peptide may be produced artifactually or could not be resolved in Fig. 4A. Comparisons of Fig. 4A and B show that a faintly labeled band (P11) disappeared at approximately the same rate in both, perhaps because of selective degradation. As shown in Fig. 4A, P33 was synthesized coor-

dinately with P34. Figure 4B shows that P33 decreased in amount after the addition of unlabeled methionine. Only P33 appeared to be produced *in vitro* from extracted RNA. However, the present experiment does not permit any conclusions concerning possible precursor-product relationships between these two peptides.

Structure of RNA polymerases used for *in vitro* RNA synthesis. The subunit compositions shown in Fig. 5 are in general agreement with the structures reported for polymerase isolated from *B. subtilis* infected with the related phage SPO1 (4, 8, 18, 19). Most notably, polymerase preparations extracted 8 min after infection with SP82 contained a decreased amount

FIG. 5. Subunit structures of RNA polymerases from uninfected and SP82-infected *B. subtilis*. Polymerase was extracted as described in the text; molecular weights are given in thousands. (a) RNA polymerase from uninfected *B. subtilis*; (b) polymerase extracted 8 min after infection; (c) polymerase extracted 20 min after infection.

of the σ subunit and had additional polypeptides of molecular weights 28,000 (28K) and 18,000 (18K). Trace amounts of peptides of molecular weights 26,000 (26K) and 13,000 (13K) were also present in some preparations. Enzyme isolated 20 min after infection contained only a trace of the 18K peptide and a reduced amount of the 28K peptide, but had substantially increased quantities of the 26K and 13K polypeptides. The presence of small amounts of the 28K subunit in the 20-min enzyme and of the 13K subunit in the 8-min enzyme could be interpreted as indicating that partial dissociation had occurred or that the preparation contained different amounts of two enzymes. It is not known

whether the trace amounts of additional polypeptides that can be seen in Fig. 5 represent loosely bound components of RNA polymerase or whether they are contaminants or arise from degradation of polymerase.

Comparisons of SP82 polymerase preparations with preparations of SPO1 polymerase (kindly supplied by J. J. Duffy and E. P. Geiduschek, data not shown) demonstrated that the 28K and 13K SP82-specified polypeptides had the same mobilities as did polypeptides v^{28} and v^{13} , respectively, of the SPO1-modified enzyme. A polymerase preparation isolated 20 min after SPO1 infection also contained a polypeptide that corresponded to the 26K peptide associated with the SP82 polymerase. On the basis of these and other comparisons (16), it is tentatively concluded that the 28K, 26K, and 13K subunits of the SP82-modified polymerases also correspond to peptides IV, V, and VI, respectively, present in the SPO1-modified enzymes isolated by Pero et al. (18).

It is of interest that Fig. 2 shows pulse-labeled polypeptides that have mobilities similar to those of the polypeptides that copurify with RNA polymerases extracted 8 and 20 min after infection (Fig. 5). The intervals of synthesis of peptides corresponding to the 28K, 26K, and 13K polymerase subunits (P37 or P38, P41 or P42, and P60, respectively) are in general agreement with the presence of these peptides in polymerases isolated 8 and 20 min after infection and also with the times of synthesis reported for the SPO1-associated polypeptides IV, V, and VI (6, 8). However, studies of the subunit structure of polymerases extracted 6, 8, 10, 15 (27), and 20 min after infection (Milhausen and Whiteley, unpublished data) showed that the 18K peptide is associated with the core subunits early after infection and is greatly diminished or absent from polymerases extracted 15 to 20 min after infection. The only 18K peptide detected in Fig. 1A (P52) is synthesized in trace amounts in the 3 to 6 min interval and in larger amounts in the middle of the infection; synthesis of small amounts of this peptide continues to lysis. The time of appearance of P52 suggests either that this peptide is not the 18K peptide found in association with RNA polymerase 8 min after infection or that the 18K peptide is synthesized but does not become associated with polymerase isolated at later times in infection possibly because of additional modification of the enzyme.

Comparisons of polypeptides directed by RNA synthesized *in vivo* and *in vitro*. The following experimental conditions of RNA synthesis have been investigated with regard to the effect on the patterns of polypeptides produced by subsequent translation: varying NaCl concen-

trations, the effect of removing the 21K peptide, varying the time of RNA synthesis and coupling with protein synthesis. Of these, the concentration of NaCl and the presence of the 21K peptide produced the greatest effect; coupling of RNA synthesis to protein synthesis had a minor effect. As detailed elsewhere (G. B. Spiegelman, W. R. Hiatt, and H. R. Whiteley, *J. Biol. Chem.*, in press), the presence of the 21K peptide and the ionic strength had a significant effect on transcription. Concentrations of NaCl greater than 0.2 M inhibited RNA synthesis by host or 8-min polymerase, but yielded RNAs that were translated more efficiently. Electrophoresis and autoradiography of reaction mixtures indicated that changes in the NaCl concentration affected transcription by altering the quantities of specific transcripts rather than the kinds of transcripts. Based on these observations, a concentration of 0.16 M NaCl was selected for a comparison of RNAs produced by different enzymes. As shown previously (16, 18, 27), the 21K peptide also has a major effect on transcription; the polymerase preparations used in the present investigation contained the 21K peptide.

Since the transcriptional specificity of the SPO1-modified polymerase has been studied with a coupled transcription-translation system (24), a comparison was made of the specificity of SP82 transcription under coupled and uncoupled conditions. For these experiments, RNA polymerase was extracted from an uninfected and SP82-infected rifampin-resistant strain of *B. subtilis*; the polymerase was then added together with SP82 DNA to a reaction mixture containing the *E. coli* S30 fraction preincubated with rifampin. As shown in Fig. 6, patterns of polypeptides obtained from uncoupled (lane a) and coupled (lane b) conditions with the host polymerase were superficially similar, although differences were found in the quantities of some peptides, and one peptide seen in lane a was not synthesized by the coupled system. Similar patterns of small-molecular-weight peptides were obtained from uncoupled (lane c) and coupled (lane d) conditions, using the 20-min polymerase; in addition, five peptides of higher molecular weight were produced by the coupled system. These studies suggest that coupling transcription and translation does not have a major effect on the specificity of transcription.

Uncoupled conditions were used to compare the transcriptional specificities of the isolated host, 8-min, and 20-min polymerases in order to duplicate conditions used in earlier competition-hybridization experiments (12, 21, 27). These results are shown in Fig. 7. Bands labeled in the left margin identify those peptides directed by RNA synthesized in vitro (lane c)

FIG. 6. Autoradiographs of SDS-polyacrylamide gels comparing polypeptides synthesized in vitro under coupled and uncoupled conditions. RNA was synthesized in vitro under coupled conditions, using the same concentrations of polymerase and DNA as in uncoupled synthesis as described in the text. Approximately 100,000 cpm of incorporated [35 S]methionine was applied to a sample well of 9-cm gels; the gels were exposed for 1 week at -70°C . Polypeptides synthesized under (a) uncoupled and (b) coupled conditions from RNA synthesized by *B. subtilis* RNA polymerase. Polypeptides synthesized under (c) uncoupled and (d) coupled conditions from RNA produced by polymerase extracted 20 min after infection by SP82.

whose mobilities correspond to peptides produced in vivo (lane a) and synthesized by the translation of in vivo RNA (lane b). The same conventions were used in labeling the other two sets of radioautographs shown in this figure.

Nine of the approximately 15 peptides produced from RNA synthesized by the host polymerase have mobilities matching those of peptides synthesized in vivo. Figure 2 shows that there are five peptides (P22, P44, P55, P59, and P69)

FIG. 7. Autoradiographs of SDS-polyacrylamide gels comparing cell-free translation products of *in vitro* RNA with polypeptides synthesized *in vitro* from RNA extracted from infected cells or pulse-labeled *in vivo*. Polypeptides were pulse-labeled (a) 3 to 6, (d) 6 to 9, and (g) 21 to 24 min after infection, and approximately 50,000 cpm of incorporated [35 S]methionine was applied to the sample wells of a 9-cm gel. These are compared with the translation products of *in vivo* RNA extracted (b) 4, (e) 8, and (h) 20 min after infection. Cell-free reaction mixtures were supplemented with approximately 100 μ g of each RNA preparation, and about 130,000 cpm of incorporated [35 S]methionine was applied to each lane of the gel. The translation products of RNA synthesized *in vitro* by (c) host, (f) 8-min, and (i) 20-min RNA polymerases are compared with the products of *in vivo* RNA. RNA was synthesized *in vitro* as described in the text, and equal quantities of each preparation were used to direct cell-free protein synthesis. Approximately 180,000 cpm of incorporated [35 S]methionine was applied to each lane of the gel. Exposure of the gels was for 1 week at -70°C .

whose synthesis *in vivo* begins immediately after infection and stops at 6 min. The time of synthesis of these peptides agrees with the time of synthesis of the earliest RNA class detected by competition-hybridization (Table 1) and specifically with the time of synthesis of class H1, an early class transcribed from the heavy strand of SP82 DNA (13). As reported earlier (27), competition-hybridization experiments showed that RNA synthesized *in vitro* by the host polymerase contains H1 RNA. Four of the peptides seen in Fig. 7, lane b, correspond in mobility to four of the early peptides (P22, P44, P55, and P59) shown in Fig. 2; as shown in Fig. 7, the synthesis of P69 could not be detected because of peptides

produced from mRNA in the *E. coli* S30 fraction. These four peptides are also produced in relatively large amounts by the translation of RNA synthesized *in vitro* by the host enzyme (lane c). In toto, these observations suggest that class H1 RNA contains the transcripts for the P22, P44, P55, and P59 peptides.

Trace amounts of five additional peptides can be seen in Fig. 7, lane c; their mobilities correspond to early peptides whose synthesis continues beyond 6 min (P23, P32, P35, P46, and P52). Lane c also shows three bands (identified with a bar on the right side of lane c), which are produced in relatively large amounts, whose mobilities do not correspond to any phage-specific

peptide seen in Fig. 1, 2, and 4; the band in lane b that corresponds to the largest of the three bands marked with a bar is a host peptide. These peptides and three additional bands shown in Fig. 7, lane f, may reflect errors in the initiation and/or termination of transcription in vitro. It is also possible that they represent an overproduction of transcripts that are synthesized in few copies in vivo and hence are not detected by pulse-labeling under our conditions or that some RNAs undergo cleavage in vivo and that such processing does not occur in vitro.

It is somewhat surprising that host polymerase did not produce substantial quantities of transcripts coding for the 28K peptide, which becomes associated with polymerase after SP82 infection. As shown in Fig. 2, there are two peptides (P37 and P38) of approximately 28,000 molecular weight that are synthesized in vivo soon after infection. As discussed below, P38 is produced by the translation of RNAs synthesized by the 8- and 20-min polymerases but not from RNA synthesized by the host enzyme. This may indicate that transcription of the gene for this peptide requires modification of the host polymerase. The 18K peptide may be a candidate for this role since this polypeptide copurified with polymerase isolated from cells soon after infection.

Translation of RNAs synthesized in vitro by the 8-min polymerase (Fig. 7, lane f) yielded approximately 25 peptides. A comparison of lanes c, f, and i shows that a larger number of peptides were directed by RNAs synthesized by the 8- and 20-min polymerases than by RNAs produced by the host enzyme. This observation agrees with data obtained by hybridizing RNAs synthesized in vitro by the three polymerases with the 37 DNA fragments produced by treatment of SP82 DNA with the restriction enzyme *Hpa* II (Lawrie, Spiegelman, and Whiteley, Gene, in press). The latter studies showed that the host polymerase transcribed approximately 10% of the genome (three of the DNA fragments hybridized to RNA produced by the host enzyme), whereas most of the genome was transcribed by the phage-modified polymerases (34 of the DNA fragments hybridized to RNAs synthesized by the 8- and 20-min enzymes).

As shown in Fig. 7, 12 of the peptides of lane f had mobilities matching peptides produced in vivo 8 min after infection (lane d) and from RNA extracted 8 min after infection (lane e); 5 of these were produced in trace amounts by the host enzyme (lane c). Some of the 13 additional peptides seen in lane f can be correlated with peptides synthesized from other in vivo RNAs; 2 have mobilities corresponding to early peptides (P22 and P59), 1 corresponds to P44-P45 which,

as noted elsewhere, may be either a late or an early peptide, and 3 bands match peptides produced in vivo late in infection (P20, P33, and P53). Two of the remaining bands match peptides d and e described earlier, and five of the bands are labeled with bars, indicating that they do not correspond to any peptides shown in Fig. 1, 2, and 4. A total of 11 peptides, including 2 labeled with bars, were also directed by RNAs synthesized by the host polymerase (lane c).

A comparison of lanes c and f indicates that approximately the same amounts of three of the four early peptides (bands corresponding to P22, P44, and P59) were synthesized by the host and 8-min polymerases. In part, the synthesis of these peptides in the experiment shown in lane f can be attributed to the presence of small amounts of unmodified polymerase in the 8-min enzyme preparation since, as seen in Fig. 5, the latter contained a trace of the σ subunit. However, the translation of RNA produced by the 8-min polymerase lacking σ (obtained by chromatography of the enzyme through phosphocellulose [data not shown]) also yielded trace amounts of the same three peptides, and, as noted below, two early peptides were produced from RNA synthesized by the 20-min polymerase. Possibly, the modified enzymes may recognize some of the "strong" early promoters. When the translation products of in vivo and in vitro RNAs are compared, Fig. 2 and 7, lane e, show that approximately 17 phage-specific peptides were synthesized from RNA extracted 8 min after infection; as judged by electrophoretic mobility, 13 of these peptides were produced by the translation of RNA synthesized by the 8-min polymerase in vitro.

RNAs synthesized in vitro by the 20-min polymerase (Fig. 7, lane i) directed the production of approximately 22 peptides, 15 of which were produced in vivo (lane g) and by the translation of RNA extracted from cells 20 min after infection (lane h). A comparison of lanes f and i shows that some of these peptides were also directed by RNAs synthesized by the 8-min polymerase. As stated earlier, Lawrie et al. (Gene, in press) have also demonstrated similar patterns of in vitro transcription by 8- and 20-min SP82-modified polymerases by hybridizing in vitro RNAs to *Hpa* II restriction fragments of SP82 DNA. It is not known whether the similarity in peptides seen in lanes f and i of Fig. 7 is because both modified enzymes are able to recognize the same promoters or because two of the phage-specified subunits are present in both enzymes. As shown in Fig. 5, the 20-min polymerase contained some of the 28K peptide, and the 8-min enzyme contained traces of the 13K peptide. Of the nine bands shown in Fig. 7,

lane i, which have been labeled in the right margin, two correspond to early proteins (P22 and P59), five match peptides shown in lane f, and two (bands e and f) were produced by the translation of RNA extracted 20 min after infection but were not synthesized *in vivo*. Four of the peptides (P41, P51, P58, and P61) correspond in mobility to virion peptides; P20, which is also seen in lane i, is the precursor to the virion peptide P21. All of the peptides seen in lane i have mobilities that correspond to peptides synthesized *in vivo* or by *in vitro* translation of RNAs extracted from infected cells. A comparison of lanes h and i shows that approximately 22 peptides were produced by the translation of RNA extracted from cells 20 min after infection; 17 of the peptides directed by RNA synthesized by the 20-min polymerase *in vitro* match peptides shown in lane h.

In summary, an analysis of translation products indicates that the polymerases extracted from uninfected and SP82-infected *B. subtilis* 8 and 20 min after infection synthesized *in vitro* many of the transcripts that are synthesized *in vivo* at early, middle, and late stages of infection, respectively. The autoradiographs shown in Fig. 7 suggest that different relative amounts of functional mRNA's are synthesized *in vivo* and *in vitro*. This may be due to the conditions of RNA synthesis or may indicate the presence *in vivo* of additional mechanisms that modulate the quantities of transcripts produced from different genes at different times of infection. The polypeptide patterns of Fig. 7 indicate that the host and 20-min polymerases possess enhanced affinities for the early and late regions of the genome, respectively, and display a greater accuracy *in vitro* with respect to the transcription of sequences coding for peptides in the 9,000- to 60,000-molecular-weight range than does the 8-min polymerase. Despite the fact that the analysis was restricted to the lower-molecular-weight peptides, the data in Fig. 7 support the conclusion that *in vitro* translation provides a useful method of monitoring the fidelity of transcription *in vitro* by the unmodified and SP82-modified RNA polymerases.

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