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Synthesis of Bacteriophage ϕ29 Proteins in *Bacillus subtilis*

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Seventeen bacteriophage ϕ29 proteins were detected in ultraviolet light-irradiated *Bacillus subtilis* by autoradiography of polyacrylamide slab gels. The appearance of ϕ29 proteins occurred either before or concomitantly with viral DNA replication. Viral proteins detected early in the infectious cycle consisted of nine polypeptides ranging from 5,200 daltons to 54,000 daltons. Two of the early proteins were identified as, respectively, the major capsid protein and the protein comprising the filaments which extend from the head of the virus. Late ϕ29 proteins were composed of eight polypeptides ranging from 14,000 daltons to 95,000 daltons. Only three late proteins were noncapsid proteins. Among the early proteins, six were synthesized at diminishing rates late in the infectious cycle. One of the early proteins (protein 12) lacked histidine, whereas two (proteins 10 and 15) lacked tryptophan. Among the 17 proteins detected, 10 were viral noncapsid proteins. The amount of viral genetic information required to code for the 17 proteins detected in these experiments (81% of the potential genetic information of ϕ29 DNA) compares favorably with the genetic information detected as mRNA in a previous report, 85% of the potential information on the ϕ29 chromosome.

In previous communications from this laboratory, analysis of bacteriophage ϕ29 in vivo transcription was carried out by competition-hybridization techniques (3) and by isolation and characterization of ϕ29-specific RNA molecules (4). It was concluded that gene expression during the development of *Bacillus subtilis* bacteriophage ϕ29 occurred in two large blocks either as early functions or as late functions.

Phage ϕ29 early RNAs consisted of six components of molecular weights 0.75, 0.44, 0.37, 0.25, 0.09, and 0.04 $\times$ 10^6, accounting for 35% of the coding capacity of ϕ29 DNA. Although the synthesis of the major early component (0.75 $\times$ 10^6 daltons) stopped shortly after the onset of viral DNA synthesis, 10 min after infection, the synthesis of other early and late RNAs continued throughout the latent period. The synthesis of late ϕ29 RNAs began concomitantly with viral DNA replication, but late transcription was not found to be under the positive control of viral DNA replication. Late ϕ29 RNA was composed of three species with molecular weights 1.75, 0.93, and 0.07 $\times$ 10^6. In all, the sum of the molecular weight of ϕ29-specific RNAs identified accounted for approximately 85% of the potential RNA arising from one strand of ϕ29 DNA (11 $\times$ 10^6 daltons; reference 4).

Since ϕ29 virions are composed of seven polypeptides which require more than half of the genetic information to be found in the viral chromosome (6), few gene products remain to be identified.

In this paper we describe the detection of 17 ϕ29-specific proteins and their time course of synthesis in infected *B. subtilis*. In addition to the identification of the seven viral capsid proteins, these experiments reveal the presence of 10 viral noncapsid proteins.

MATERIALS AND METHODS

Bacteria and bacteriophages. *B. subtilis* 60084, a nonmotile prototroph derived from strain 168, was provided by Ernst Freese and used in all experiments. The efficiency of plating of bacteriophage ϕ29 on strain 60084 was 50% of that of its original host, *B. amyloliquefaciens* (8). As with *B. amyloliquefaciens*, infection of strain 60084 with ϕ29 results in the production of 200 to 300 phages per bacterium, with a latent period of approximately 40 min.

Media. Bacterial stocks were maintained on tryptose blood agar plates (Difco). This agar was also used

as a bottom agar for phage assays (1). The soft agar overlay consisted of nutrient broth medium with 0.4% agar (Difco). All media used for enumerating ϕ29 contained, per liter, 1 ml of a solution containing 1.5 M $MgCl_2 \cdot 6H_2O$, 1 M $CaCl_2$, and 0.035 M $MnCl_2 \cdot 4H_2O$.

For growth of bacteria prior to UV irradiation, the basic salts solution (BSS) described by Mendez et al. (6) was used. BSS consisted of (per liter): ammonium sulfate, 2 g; dipotassium phosphate, 14 g; monopotassium phosphate, 6 g; sodium citrate $\cdot 2H_2O$, 1 g; sodium chloride, 5.84 g; magnesium sulfate $\cdot 7H_2O$, 0.2; and manganous chloride (pH 7.0), 0.01 mM. After sterilization, BSS was supplemented with 0.5% glucose and a final concentration of 0.1 mM of 19 naturally occurring L-amino acids. Leucine was usually omitted.

Growth and irradiation of bacteria. Overnight cultures of *B. subtilis* 60084 growing in synthetic medium with 0.1 mM of 19 amino acids were diluted 1:10 in fresh warm medium and grown at 37 C with aeration until the cell density reached 8×10^7 bacteria per ml. Prior to infection, the cells were irradiated in 100-ml amounts in glass petri dishes with a diameter of 15 cm with continuous stirring under twin UV lights (Sylvania Germicidal lamps, G15T8) located 50 cm above the surface of the liquid for 10 min at room temperature. This dose of UV light resulted in the survival of 10^{-6} of the original population. After irradiation, the bacteria were placed in the dark and incubated at 37 C for 10 min with shaking to express the UV damage and infected as described below.

Adsorption procedure and synchronization of infection. After irradiation and expression of UV damage, the bacteria were centrifuged at 13,000 rpm in a Sorvall refrigerated centrifuge at 2 C for 6 min. The pellet was resuspended in one-tenth the original volume of BSS containing 10^{-2} M sodium azide. The bacteria were then infected with ϕ29 at a multiplicity of 50 phages per bacterium. Adsorption was carried out at 30 C for 10 min. The bacteria were chilled by the addition of cold BSS, centrifuged as above, and washed twice with cold BSS without azide. After washing, the infected cells were resuspended at 8×10^7/ml in BSS supplemented with glucose and 19 amino acids. Under these conditions, lysis of UV-irradiated infected bacteria began 40 min after infection and resulted in the production of 20 to 30 phages per infected cell.

Labeling of bacteria and processing of samples for electrophoresis. At regular time intervals after phage infection, 5-ml samples of irradiated infected bacteria were labeled with 2.5 μCi of ^{14}C-leucine (Amersham/Searle, 342 mCi/mmol). In these experiments the medium was supplemented with the mixture of 19 amino acids described above lacking leucine. In other experiments labeling was carried out in medium lacking the appropriate amino acid either with 0.5 μCi of L-[^{14}C]tryptophan (Amersham/Searle, 58 mCi/mmol) per ml or 0.5 μCi of L-[^{14}C]-histidine (Amersham/Searle, 327 mCi/mmol) per ml.

After uptake, the bacteria were chilled rapidly and incorporation was terminated by the simultaneous addition of a final concentration of 0.01 M sodium azide and 0.05 M of the appropriate amino acid. The bacteria were centrifuged as above and washed once with NCP (0.01 M sodium citrate $\cdot 2H_2O$, 0.08 M dibasic potassium phosphate, and 0.04 M monobasic potassium phosphate, pH 7.4). The pellet from 5 ml of bacteria was resuspended in 50 μliters of NCP containing 10^{-2} M sodium azide, 0.05 M of the appropriate amino acid, and 500 μg of egg white lysozyme per ml (Worthington Biochemical Corp., Freehold, N.J.). The bacteria were incubated at 37 C for 10 min and lysed by the addition of 0.2 ml of sample buffer (10% [wt/vol] glycerol, 5% [wt/vol] 2-mercaptoethanol, 3% [wt/vol] sodium dodecyl sulfate [SDS], and 0.625 M Tris, pH 6.8). The samples were boiled for 1 min and centrifuged at room temperature at 15,000 rpm. The supernatant solution was then subjected to electrophoresis on polyacrylamide gels as described below.

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed as described by Laemmli (5), modified by L. Gold for slab gels. Gel slabs (15.5 cm wide by 10 cm high and 0.08 cm in thickness) were poured between glass plates. All experiments were performed with running gels of 12.5% acrylamide. The cross-linking ratio of acrylamide to bisacrylamide was 37.5:1. After polymerization an upper running gel of 4% acrylamide was poured and allowed to polymerize around a plastic sample well-maker. The gels were run with a constant current of 10 mA/gel for 5 h at room temperature. The slab of acrylamide was then stained for 10 min in 50% trichloroacetic acid-0.1% Coomassie Blue and destained by diffusion overnight in 7% acetic acid at room temperature. The gel slabs were dried on Whatman no. 1 filter paper under vacuum and infrared lamp and exposed in the dark to Kodak X-ray film.

RESULTS

When irradiated with UV as described in Materials and Methods, the rate of protein synthesis of *B. subtilis* 60084 was found to be approximately 10% of control unirradiated bacteria. Figure 1 shows the incorporation of ^{14}C-leucine in unirradiated, irradiated infected, and irradiated uninfected bacteria. When infected with ϕ29 at a multiplicity of 50 phages per bacterium, the rate of ^{14}C-leucine incorporation in irradiated bacteria was usually three to four times that of irradiated uninfected controls.

Figure 2 shows the results of an experiment designed to detect viral-specific proteins labeled throughout the latent period of bacteriophage ϕ29 in irradiated host bacteria. Samples of infected UV-irradiated bacteria were allowed to incorporate ^{14}C-leucine for 5 min at regular time intervals. Control samples consisting of UV-irradiated uninfected bacteria were prepared at 5, 15, and 25 min. Samples were prepared as described in Materials and Methods and subjected to electrophoresis on SDS slab gels. Seventeen proteins present in infected but not in control uninfected samples were

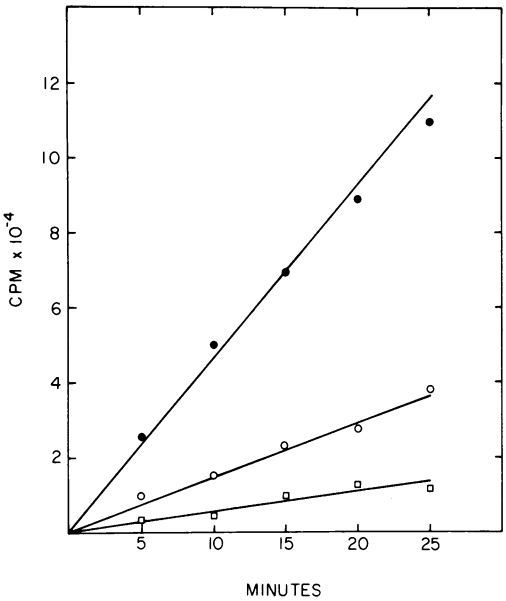

FIG. 1. *Incorporation of ^{3}H-leucine in UV-irradiated and φ29-infected B. subtilis. B. subtilis 60084 was grown, UV irradiated, and infected with φ29 at a multiplicity of 50 phages per bacterium, as described in Materials and Methods. The bacteria were resuspended in BSS medium supplemented with a mixture of 19 amino acids (0.1 mM each) and ^{3}H-leucine (New England Nuclear Corp.; 2.5 μCi/ml; 1 Ci/mmol). Samples (2 ml) were removed at regular time intervals into 10% trichloroacetic acid and kept at 0 C for 1 h. The material was filtered on Whatman GFC glass fiber filters, washed with 5% trichloroacetic acid, and counted in a liquid scintillation spectrometer. Symbols: ●, control, unirradiated uninfected bacteria; ○, irradiated infected bacteria; and □, irradiated uninfected bacteria.*

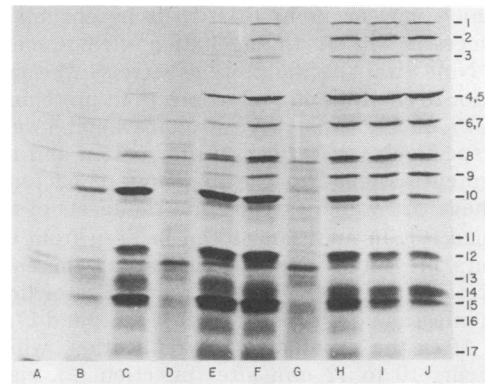

FIG. 2. *Synthesis of φ29 proteins in B. subtilis. B. subtilis 60084 was grown to a density of 8 × 10^7 bacteria per ml in BSS medium supplemented with 0.5% glucose and 0.1 mM each of 19 L-amino acids. The bacteria were irradiated with UV light, and the radiation damage was allowed to express itself for 10 min at 37 C. The bacteria were concentrated 10-fold by centrifugation, and a sample was infected with φ29 in BSS medium without glucose or amino acids but with 10^{-2} M azide at a multiplicity of 50 phages per bacterium, as described in Materials and Methods. After adsorption (10 min, 30 C), the bacteria were centrifuged in the cold, washed free of azide, and resuspended in prewarmed BSS medium with glucose and amino acids. At 5-min intervals, 5-ml samples of infected bacteria were labeled with 2.5 μCi of ^{14}C-leucine (342 μCi/mmol). After 5 min of incorporation, uptake was stopped with excess nonradioactive leucine, 10^{-2} M azide, and rapid chilling to 0 C. The samples were processed and subjected to electrophoresis on SDS acrylamide slab gels as described in the text. The figure presented is an autoradiogram of samples of infected bacteria and uninfected control samples. Samples designated A, D, and G are proteins from control, uninfected irradiated bacteria labeled, respectively, from 0 to 5 min, 10 to 15 min, and 20 to 25 min after resuspension in warm medium. Samples B (0–5 min), C (5–10 min), E (10–15 min), F (15–20 min), H (20–25 min), I (25–30 min), and J (30–35 min) were prepared from irradiated bacteria infected with φ29. The numbers on the right-hand side of the figure were assigned to viral-specified proteins which were detected in the infected samples but not in the controls. They are arranged in order of decreasing molecular weight. The amount of each sample subjected to electrophoresis was 25 μliters. This corresponds to the proteins found in 0.5 ml of infected or uninfected bacteria. In infected samples 0.5 ml of bacteria would produce approximately 8 × 10^8 phages. Trichloroacetic acid-precipitable radioactivity in the samples ranged from 10,000 to 15,000 counts/min. The dried gel slab was exposed to Kodak X-ray film for 10 days. Lysis of infected bacteria began 40 min after resuspension in warm medium.*

detected and numbered consecutively in order of decreasing molecular weight. Proteins 2 through 8 correspond to the viral capsid proteins (see Fig. 4). Similar results were obtained when infected bacteria were labeled with ^{14}C-amino acid mixture, but the yield of viral proteins was low and lysis was considerably delayed. In these experiments it has proved difficult to identify protein 5, the minor capsid protein of φ29, since its mol wt (48,000) is close to the major capsid protein, the protein which forms the bulk of bacteriophage φ29 and which is intensely labeled. Autoradiography of proteins isolated from purified virions or from infected UV-irradiated bacteria after prolonged electrophoresis revealed the presence of the minor capsid protein. But under these conditions, proteins 16 and 17 were lost to the lower buffer tank (data not shown). In UV-irradiated bacteria, φ29 DNA replication began 10 to 12 min after resuspending the infected bacteria in warm medium, a time similar to previous determinations (3). Several host-specific bands, but predominantly one near position 12, continued to be synthesized in uninfected UV-treated cells. These may represent protein synthesis

which may occur in *B. subtilis* by enzymatic processes without the mediation of ribosomes.

Nine viral-specific proteins were synthesized prior to the 10th min after infection: proteins 4, 8, 10, and 12 through 17. Proteins 4 and 8 were, respectively, the major capsid protein and the protein assigned to the filaments of bacteriophage ϕ29 which extend from the facets of the head (6). In addition, it can be seen from the data presented in Fig. 2 that six of the early proteins are synthesized at reduced rates later in infection: proteins 10, 12, 13, 15, 16, and 17.

After the onset of DNA replication, which occurs 10 to 12 min after infection (3), eight viral-specific proteins were synthesized: proteins 1, 2, 3, 5, 6, 7, 9, and 11. The late ϕ29 proteins were, in general, of higher molecular weight than the early ϕ29 proteins. Proteins 6 and 7 were also difficult to resolve in experiments where leucine was used as the label, since a relatively large amount of protein had to be applied to the gels to detect proteins 16 and 17. These proteins were defined better when tryptophan or histidine was used to label the infected bacteria, since proteins 16 and 17 contained relatively large amounts of these amino acids, a property which allowed less total protein to be applied to the gel. Applying less total protein to the gels improved the resolution of proteins 6 and 7, which are close in molecular weight (see Table 1 and Fig. 3 and 4).

Figure 3 shows the results of an experiment in which ϕ29 proteins were labeled with ^{14}C-histidine. It can be seen from these data that protein 12, a noncapsid protein, was missing when histidine was used to label the infected bacteria.

To determine which viral-specific proteins contained tryptophan, bacteria were irradiated and infected with ϕ29 and labeled from the 5th to the 35th min after infection with ^{14}C-tryptophan. The data presented in Fig. 4 show that proteins 10 and 15, both of which are viral noncapsid proteins, were not labeled with tryptophan. A preparation of the structural proteins prepared from ϕ29 purified through cesium

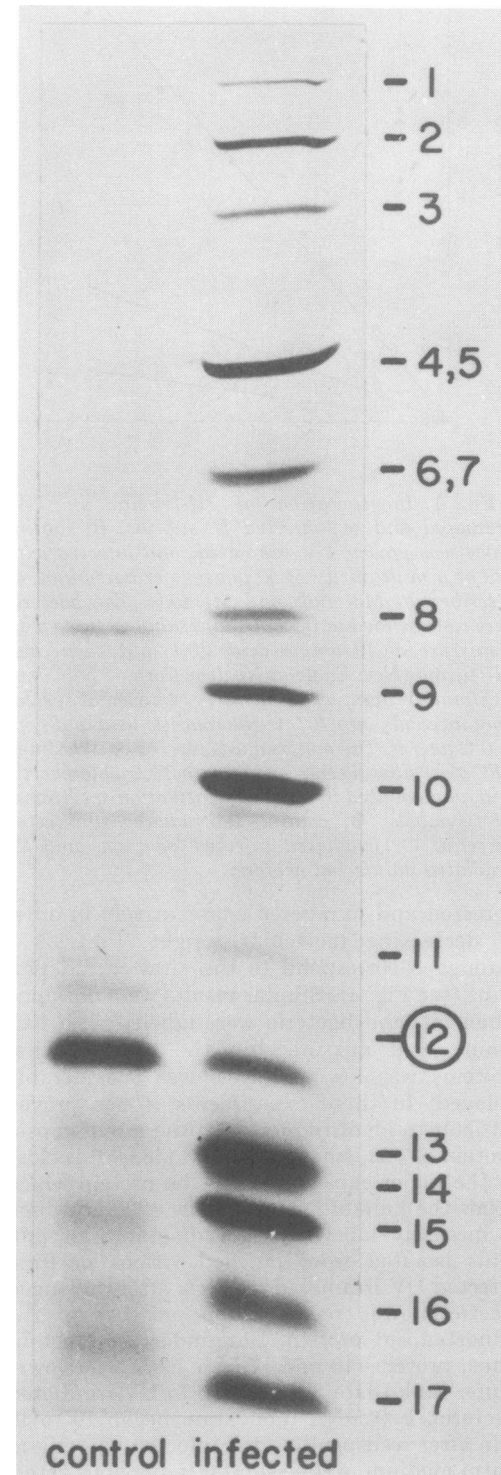

FIG. 3. *Phage ϕ29 proteins in B. subtilis labeled with ^{14}C-histidine. After UV irradiation and infection of B. subtilis 60084 with ϕ29 (see Materials and Methods), infected bacteria and control uninfected bacteria were resuspended in BSS medium supplemented with 0.5% glucose and a mixture of 0.1 mM each of 19 occurring amino acids lacking histidine. Samples were labeled with L-[^{14}C]histidine (0.5 μCi/ml; 327 mCi/mmol) from 5 to 35 min after resuspension in warm medium. Samples corresponding to 0.20 ml of bacteria were subjected to electrophoresis on SDS acrylamide slabs, as described in the text. Input radioactivity was 13,000 counts/min for the uninfected control sample and 47,000 counts/min for the infected sample. The dried gel slab was exposed to Kodak X-ray film for 6 days. The circled number corresponds to the ϕ29 protein which was not detected when infected bacteria were labeled with histidine.*

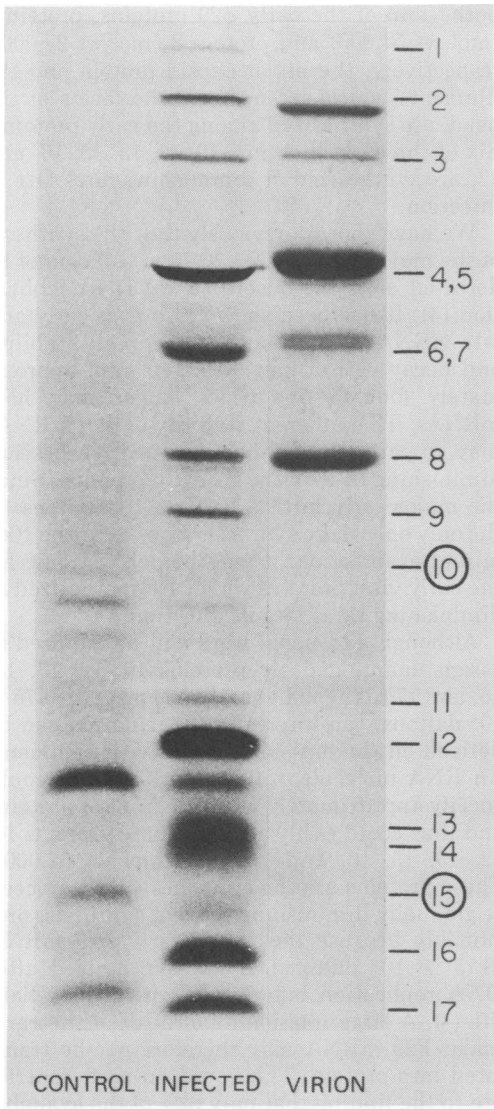

FIG. 4. *Phage φ29 proteins labeled with ^{14}C-tryptophan. UV-irradiated infected and uninfected B. subtilis 60084 were resuspended in BSS medium supplemented with 0.5% glucose and a mixture of 0.1 mM each of 19 naturally occurring* L*-amino acids, without tryptophan. Samples were labeled from 5 to 35 min after infection with 0.5 μCi of* L*-[^{14}C]tryptophan per ml (58 mCi/mmol). Samples corresponding to 0.2 ml of bacteria were subjected to electrophoresis in SDS 12.5% acrylamide gels, as described in Materials and Methods. Input radioactivity was 2,000 counts/min for the uninfected control and 8,000 counts/min for the infected sample. The gel was exposed to X-ray film for 14 days. The circled numbers correspond to φ29 proteins 10 and 15 which were not detected when infected bacteria were labeled with ^{14}C-tryptophan. The sample labeled "virion" represents proteins from approximately 2 × 10^{10} phages stained with Coomassie Blue from CsCl-purified φ29 and juxtaposed to identify φ29 capsid proteins.*

chloride and stained with Coomassie Blue has been placed next to the autoradiogram to identify viral capsid proteins in the sample extracted from infected bacteria. Under the conditions of this experiment, the 13 and 14 doublet protein band was resolved better.

Figure 5 presents a summary of the time of detection of φ29-specific proteins derived from many experiments similar to those presented in Fig. 2. In general, viral-specific proteins appeared in infected *B. subtilis* as early or late proteins. Early proteins were defined as those synthesized prior to the onset of φ29 DNA replication, 10 min after infection (3). Late proteins were defined as those whose synthesis began to be detected in conjunction with the onset of viral DNA replication.

Table 1 presents data on the molecular weight of the φ29 capsid and noncapsid proteins detected in UV-irradiated bacteria. The molecular weight standards used in these determinations were the seven viral capsid proteins (6). Most of the early proteins were of low molecular weight. Phage φ29 proteins range from mol wt 5,200 to 95,000.

DISCUSSION

Seventeen proteins specified by bacteriophage φ29 in UV-irradiated *B. subtilis* were detected by electrophoresis on SDS acrylamide slab gels. In general, the expression of φ29 proteins occurs either as early or late during the infectious cycle. Early proteins defined as those

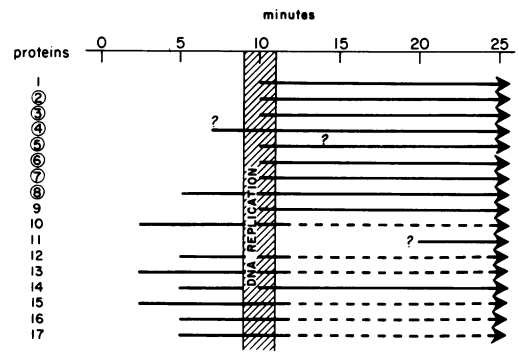

FIG. 5. *Time course of synthesis of φ29 proteins in B. subtilis. This figure is a representation of information derived from several experiments similar to the one described in Fig. 2. Proteins are represented by numbers 1 through 17 and by arrows. They are arranged in order of decreasing molecular weight. The beginning of each arrow represents the approximate time when the synthesis of each protein was detected. Broken lines indicate proteins whose synthesis proceeds at diminishing rates late in the infectious cycle. The question marks indicate uncertainities about the precise onset of synthesis of the particular protein involved. Circled numbers correspond to the seven φ29 capsid proteins.*

TABLE 1. *Molecular weight of φ29 proteins*[a]

Protein no.	M × 10^{-4}	Remarks
Early φ29 proteins		
④	54	Major capsid
⑧	28	Horns
10	20	Shut-off
12	12.5	Shut-off
13	9.8	Shut-off
14	8.8	
15	8	Shut-off
16	6.4	Shut-off
17	5.2	Shut-off
Late φ29 proteins		
1	95	
②	80	Appendages
③	71	Tail
⑤	48	Minor capsid
⑥	40	Collar
⑦	36	Collar
9	24	
11	14	

[a] The molecular weight of each φ29 protein detected in UV-irradiated infected bacteria was determined by using the relationship devised by Shapiro et al. (7) and the molecular weights of the seven φ29 capsid proteins published by Mendez et al. (6) which internally standardize the gels. Early proteins were those detected prior to onset of φ29 DNA replication (10 min after infection). Late proteins were those whose synthesis began concomitantly with or subsequent to phage DNA replication. The circled numbers are the viral capsid proteins. The term "shut-off" refers to those proteins whose synthesis proceeds at diminishing rates late in the viral infectious cycle.

proteins whose synthesis was detected prior to the 10th min after infection, a time which marks the onset of φ29 DNA replication, ranged from mol wt 5,200 to 54,000. The sum of the mol wt of early φ29 proteins was 152,700. Assuming the average mol wt of an amino acid to be 125 and that of a nucleotide to be 330, and assuming further that three nucleotides code for each amino acid, the early φ29 proteins, therefore, would be coded by 1.2 × 10^6 daltons of φ29 single-stranded DNA.

It has been shown previously (4) that six φ29 mRNAs are produced prior to φ29 DNA replication. The sum of the mol wt of φ29 early mRNAs was 1.94 × 10^6 (4). Therefore, the experiments described in this paper reveal that only 62% (1.2 × 10^6/1.94 × 10^6 × 100) of the information available as mRNA is translated into proteins prior to the onset of φ29 DNA replication. This observation suggests that translation of some early φ29 mRNAs may be delayed or that a fraction of early RNA may not be translated, or both. Two of the early φ29 proteins, protein 4 (mol wt 54,000) and protein 8 (mol wt 28,000), respectively, the major capsid protein and the filaments which extend from the facets of the head, are synthesized among the early proteins. Six of the early proteins, 10, 12, 13, 15, 16, and 17, are synthesized at diminishing rates late in infection.

We have shown previously that the synthesis of the major early mRNA (0.75 × 10^6) cannot be detected after the onset of viral DNA replication (4). It has been shown also that early and late mRNAs of φ29 have considerable stability and decay with a half-life of 10 min, approximately four to five times longer than host mRNAs. This inherent stability of φ29 mRNAs may, in part, explain the continued synthesis at diminishing rates of the six early proteins. Since the major early mRNA (0.75 × 10^6 daltons) is the only one whose synthesis stops after the 10th min after infection, it may be polycistronic for the early viral proteins which are synthesized at diminishing rates late in infection.

Although additional work will be required to assign unambiguously proteins 10, 12, 13, 15, 16, and 17 to the major early φ29 mRNA (0.75 × 10^6 daltons), an interesting speculation can be derived on the basis of a tentative assignment. An RNA molecule of 0.75 × 10^6 daltons could specify approximately 95,000 daltons of proteins and thus could easily accommodate proteins 10, 12, 13, 15, 16, and 17 (total mol wt 62,000). These proteins are the only ones which proceed at gradually diminishing rates of synthesis, presumably because the major early φ29 mRNA (0.75 × 10^6 daltons) is not synthesized after DNA replication but decays with a long half-life (4). A large fraction (one-third) of the early major F29 mRNA may therefore not be translated into protein. This could, in part, contribute to the finding that only 62% of the available early φ29 RNA is translated into proteins which appear prior to DNA replication.

Phage φ29 late proteins consisted of eight polypeptides, only three of which were viral noncapside proteins. The mol wt of late φ29 proteins ranged from 8,800 to 95,000. Among these late products, the synthesis of protein 2 (80,000 daltons; appendages), protein 3 (71,000 daltons; tail), and proteins 6 and 7 (respectively, 40,000 and 36,000 daltons; collar proteins) were detected. The presence of the minor capsid protein, protein 5, a late protein (48,000 daltons), was difficult to detect in most experiments. The reason for this is probably that this component is present in very small amounts in completed particles and its molecular weight is close to the most abundant component in φ29

virions, protein 3, or the major capsid protein. It has been shown previously that two very large ϕ29 mRNAs (1.75×10^6 and 0.93×10^6 daltons) and a relatively small RNA molecule (0.07×10^6 daltons) are synthesized after DNA replication (4). The sum of the mol wt of all late ϕ29 proteins was 408,000, or approximately 3.2×10^6 of single-stranded DNA. Thus, there appears to be an excess of ϕ29-specific proteins synthesized late in infection and compared with the amount of genetic information available as mRNA ($3.2 \times 10^6/2.75 \times 10^6 \times 100 = 117\%$). This observation may reflect the fact that not all early ϕ29 mRNA may be translated as early proteins, but that translation may occur later in infection.

The sum of the mol wt of all ϕ29 proteins detected was 561,000. This amount of protein requires 81% of the information available on ϕ29 DNA and compares favorably with the 85% of potential ϕ29 information obtained as the sum of the ϕ29 mRNAs detected previously (4).

Since it is possible that comigration of viral proteins with nearly identical molecular weights might occur in these experiments, the number of ϕ29 proteins reported here must be considered a low estimate of the total number of viral functions. The most direct way of resolving doublet protein bands or comigration of two proteins within a single band remains infection by suppressible nonsense mutants of ϕ29. Work along these lines is in progress.

ACKNOWLEDGMENT

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