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Gene Expression During the Development of Bacteriophage φ29

III. Analysis of Viral-Specific Protein Synthesis with Suppressible Mutants

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Fifty-four suppressible mutants of bacteriophage φ29 have been isolated with a variety of mutagens and assigned to eight complementation groups. Viral-specific protein synthesis in UV light-irradiated, nonsuppressing *Bacillus subtilis* 60084 was analyzed with exponential acrylamide gels. Four additional φ29 proteins which were undetected on ordinary acrylamide gels are reported in this paper. Five phage φ29 proteins have been unambiguously assigned to specific cistrons. Two cistrons had pleiotropic effects on viral protein synthesis. Mutants in cistrons I or II were unable to synthesize DNA in nonsuppressing bacteria. Mutants in cistron I were unable to attach viral chromosomes to the host cell membrane, and the protein responsible for this function has been identified. The other viral protein playing a role in phage φ29 DNA synthesis is also identified and assigned to cistron II. Mutants in cistron II can attach viral chromosomes to membrane, but cannot synthesize DNA in nonsuppressing bacteria.

Recent work from this laboratory reported the detection and preliminary characterization of 17 proteins specified by bacteriophage φ29 in UV light-irradiated *Bacillus subtilis* (9). We have also reported (5) that the replication of φ29 DNA requires the synthesis of a viral protein which is synthesized early in the infectious cycle and participates in associating φ29 chromosomes with the host cell membrane.

In this paper, we report the detection of three additional early proteins (designated 3a, 8a, and 18) and one late protein (6a) which were resolved on gels containing exponential gradients of acrylamide. We describe further the isolation and characterization of 54 suppressible mutants of φ29 distributed in 8 complementation groups, the proteins affected by the mutations, and the identification of the φ29 protein required for the association of viral chromosomes with the cell membrane. Suppressible and temperature sensitive mutants of bacteriophage φ29 have also been reported recently (3, 10, 11).

MATERIALS AND METHODS

Bacteria and bacteriophages. *B. subtilis* 60084, a nonmotile prototroph derived from strain 168, was provided by Ernst Freese and used as the nonpermissive host. *B. subtilis su*$^+$3, isolated by Georgopoulos (2), was kindly provided by Kenneth Loveday and used in all experiments as the suppressing host bacterium. The efficiency of plating (EOP) of wild-type bacteriophage φ29 (φ29$^+$) on *B. subtilis su*$^+$3 was 1.6 times greater than its EOP on *B. subtilis* 60084, and infection of either strain with φ29$^+$ resulted in the production of approximately 250 phages per bacterium, with a latent period of 40 min. *Bacillus amyloliquefaciens* (12) was used as a nonpermissive host in recombination analysis involving two-factor crosses of φ29 *sus* mutants. The EOP of φ29$^+$ on this strain is the same as that on *B. subtilis su*$^+$3.

Mutagenesis. Several mutagenic agents were used to treat wild-type φ29 to isolate mutants capable of productive infection of *B. subtilis su*$^+$3 but not of *B. subtilis* 60084. Phage φ29$^+$ were treated with various mutagens, and the survivors were plated first on *B. subtilis su*$^+$3. Plaques were transferred to lawns of *B. subtilis* 60084 and *B. subtilis su*$^+$3 with toothpicks. Mutants were detected by their failure to propagate on the nonpermissive strain. Mutant stocks were prepared from single plaques after two consecutive plaque isolations on the permissive host *B. subtilis su*$^+$3. High-titered stocks were prepared from the confluent lysis of lawns of *B. subtilis su*$^+$3 and purified through cesium chloride gradients as described previously (5).

(i) Nitrous acid mutagenesis. A 10-ml solution containing 0.5 M sodium acetate and 1 mM $MgSO_4$ was adjusted to pH 4.2. Sodium nitrite was added to a final concentration of 0.1 M along with approximately 10^{11} PFU of φ29$^+$ in TM buffer (0.01 M Tris-0.01 M $MgSO_4$, pH 7.4). The mixture was incubated at room temperature (24 C) with gentle stirring. Samples (0.05 ml) were removed at 10-min intervals, and the reaction was stopped by diluting 1:100 in TMI buffer (TM containing 1 ml of a solution of 1.5 M $MgCl_2 \cdot 6H_2O$, 1 M $CaCl_2$, and 0.035 M $MnCl_2 \cdot 4H_2O$

per liter). The titer of surviving phage was determined by the soft agar overlay method (1) on media previously described (7). Mutants were isolated from samples taken at 20 to 40 min of treatment (survival 0.25 to 0.5%).

(ii) TEM mutagenesis in vitro. Phage $\phi 29^+$ (10^{10} PFU/ml) were incubated at 37 C in TMI buffer (pH 7.4) containing 100 μg of triethylene melamine (TEM) (Polyscience, Inc., Rydal, Pennsylvania) per ml. The titer of surviving phage was determined at 2-h intervals. Mutants were isolated from a sample taken after 8 h of treatment (2.5% survival).

(iii) TEM mutagenesis in vivo. *B. subtilis* su^-3 growing in the basic salts solution (BSS) described by Mendez et al. (8), supplemented with 0.5% glucose and a final concentration of 0.1 mM of 19 naturally occurring L-amino acids, was harvested when the titer reached 5×10^7 bacteria per ml, and concentrated 10-fold in BSS. Phage φ29 at a multiplicity of 10 phages per bacterium was added and allowed to adsorb for 10 min at 37 C. The infected bacteria were washed once with BSS and suspended in 10 ml of BSS medium with glucose and L-amino acids. TEM was added at a final concentration of 25 μg/ml. The cells were incubated for 2 h at 37 C and treated with egg white lysozyme (10 μg/ml) for 15 min at 37 C. After low-speed centrifugation to remove bacterial debris, the supernatant solution was plated on *B. subtilis* su^+3.

(iv) EMS mutagenesis. Phage $\phi 29^+$ (10^{12} PFU/ml) were incubated at room temperature in TMI buffer (pH 7.0) containing 1% (vol/vol) ethyl methane sulfonate (EMS) (Eastman Organic Chemical, Rochester, N.Y.). The titer of surviving phage was determined at 30-min intervals, and mutants were isolated from a 150-min sample (0.03% survival).

(v) 5-Bromodeoxyuridine mutagenesis. *B. subtilis* su^+3 were grown and infected as described above for in vivo TEM mutagenesis. After being washed, the infected bacteria were suspended in 10 ml of BSS medium, supplemented with 19 L-amino acids, glucose, and 10 μg of trimethroprim (Burroughs Wellcome Co., Research Triangle Park, N.C.) per ml, to suppress endogenous methylation. The culture was incubated at 37 C. Fifteen minutes after infection, 100 μg of 5-bromodeoxyuridine per ml was added and incubation was continued for 2.5 h. At that time, the cells were treated with 10 μg of lysozyme per ml for 15 min at 37 C. After centrifugation, the supernatant solution was plated on *B. subtilis* su^+3.

Genetic analysis: (i) complementation. Exponentially growing *B. subtilis* 60084 (0.1-ml samples) at 4×10^7 bacteria per ml were infected with two φ29 *sus* mutants at a multiplicity of infection of 20 phages per bacterium. Adsorption was allowed to occur for 7 min at room temperature, and anti-φ29 antiserum was added to a final K value of 2 min^{-1} (1). After 5 min, the mixture was diluted 1:1,000 into prewarmed nutrient broth and incubated at 37 C for 90 min with shaking. An infection of *B. subtilis* 60084 with wild-type φ29 was carried out in parallel. Complementation values are expressed as (final phage titer of bacteria infected with *sus* mutants/final titer of $\phi 29^+$ infected bacteria) × 100.

(ii) Recombination. *B. subtilis* su^+3 (4×10^7 bacteria per ml), growing exponentially in nutrient broth, were centrifuged and washed in BSS. The bacteria were resuspended in 1/10 volume of BSS without glucose or amino acids. Bacteria (0.01 ml) were infected with a multiplicity of 25 of each of two φ29 *sus* mutants. After 15 min at room temperature, anti-φ29 antiserum was added to a final K value of 5 min^{-1}. The infected cells were diluted 1:10,000 into prewarmed nutrient broth and incubated at 37 C with shaking for 90 min. The number of phages resulting from these two-factor crosses was determined on both *B. amyloliquefaciens* strain H (12) and on *B. subtilis* su^+3. Recombination values are given as 2 × (titer on strain H)/(titer on su^+3) × 100.

Measurement of φ29 DNA synthesis and attachment of viral chromosomes to membrane. To measure φ29 DNA synthesis, *B. subtilis* 60084 was grown in nutrient broth until the cell density reached 7×10^7 bacteria per ml. At that time, the culture was treated with 100 μg of 6-(hydroxyphenylazo)-uracil (HPAU; Imperial Chemical Industries, Ltd., Macclesfield, England) per ml, a compound which stops bacterial DNA synthesis but does not interfere with φ29 DNA synthesis (7). After 5 min at 37 C, the culture was infected with 25 PFU of either $\phi 29^+$ or φ29 *sus* mutant stocks per bacterium. Immediately after phage addition, 200 μg of uridine per ml was added to the culture. Uridine presumably suppresses thymidine phosphorylase, since these conditions allow the linear incorporation of [H^3]thymidine for as long as an hour. After addition of uridine, the infected culture was treated with 5 μCi of tritiated thymidine (New England Nuclear Corp., Boston, Mass.; 52 Ci/mmol) per ml. Samples (2 ml) were removed at regular time intervals into cold, 10% trichloroacetic acid containing 1 mg of nonradioactive thymidine per ml. After the samples remained for an hour at 0 C, they were passed through glass-fiber filters and washed with 5% trichloroacetic acid containing 1 mg of thymidine per ml, rinsed with ethanol, dried, and counted in a liquid scintillation counter.

The procedure to measure φ29 DNA attachment to the cell membrane has been published elsewhere (5). The samples were prepared as follows. A 5-ml amount of *B. subtilis* 60084 (6×10^7 bacteria per ml) growing in nutrient broth was treated with 100 μg of HPAU per ml for 5 min at 37 C. $\phi 29^+$ or *sus* mutants labeled with tritiated thymidine (about 10^{-5} counts per min per infectious particle) were added at a multiplicity of 10 phages per bacterium. Incubation was at 37 C for 27.5 min. Lysis and processing of these samples have been described (5). Centrifugation (5 h at 2 C) on linear (0 to 38%) Renografin gradients resolved parental DNA into a fast-sedimenting component (I), which represents membrane-bound DNA at equilibrium (5), and a slower-sedimenting component (II), which has been previously characterized as free DNA uncomplexed with membrane. After centrifugation, samples (0.65 ml) were precipitated with ethanol, rinsed with trichloroacetic acid on glass-fiber filters, dried, and counted in a liquid scintillation counter.

Preparation of exponential acrylamide slab gels. Acrylamide gel electrophoresis was performed as described by Laemmli (6). Exponential acrylamide gradients (9 to 18% acrylamide), which improve the

resolution of proteins with nearly identical migration on ordinary acrylamide gels, were prepared in slabs 0.08 cm in thickness, 12 cm in length, and 15.5 cm in width.

To the front chamber of a linear, lucite gradient maker was added 4 ml of a solution of 0.375 M Tris (pH 8.8), 0.1% (wt/vol) sodium dodecyl sulfate (SDS), 50% (wt/vol) glycerol; 18% (wt/vol) acrylamide (cross-linking ratio 37.5:1 acrylamide:*N,N'*-methylenebisacrylamide), 0.015% (wt/vol) ammonium persulfate, and 0.0625% *N,N,N',N'*-tetramethylethylenediamine (TEMED). This front chamber, containing a small magnetic stirring bar, was closed with a silicon stopper through which had been inserted a 21-gauge needle to bleed the pressure generated when the stopper is inserted in the chamber. The needle was then withdrawn, and the back chamber of the gradient maker was filled completely with a solution containing 0.375 M Tris (pH 8.8), 0.1% (wt/vol) SDS, 9% (wt/vol) acrylamide (cross-linking ratio 37.5:1 acrylamide:*N,N'*-methylenebisacrylamide), 0.015% (wt/vol) ammonium persulfate, and 0.0625% (wt/vol) TEMED.

The unit was placed on a magnetic stirring device to mix the contents of the front chamber. The two chambers were then connected, and the contents of the unit were withdrawn with a peristaltic pump connected to the front chamber, at the rate of 3 ml/min through tubing (1.3 mm in diameter) and delivered into a gel slab unit. This gel slab unit consisted of two glass plates (16.5 by 12.5 cm) separated by latex rubber strips (0.5 cm in width by 0.08 cm in thickness), greased lightly with silicon stop cock grease (Dow Corning Co., Midland, Mich.). During the preparation of the gradient, the contents of the front chamber (18% [wt/vol] acrylamide) remained constant and were continuously diluted by the incoming solution from the back chamber (9% [wt/vol] acrylamide), generating an exponentially decreasing gradient of acrylamide. When the liquid in the gel slab unit reached approximately 12 cm from the bottom rubber strip, pumping was stopped. The gel was sprayed with water with an atomizer and allowed to set. After hardening, the overlay water was removed and replaced with 0.375 M Tris (pH 8.8)-0.1% (wt/vol) SDS, and the gel was allowed to stand overnight.

The following equation can be used to calculate the concentration of acrylamide at any point in the gel:

$$A_c = (A_i - A_f)\, e^{\frac{V_c}{D_p}} + A_f$$

where A_c is the acrylamide concentration $\times$ 100, at a distance in centimeters (c) from the bottom of the gel; A_i is the initial acrylamide concentration in the front chamber $\times$ 100; A_f is the acrylamide concentration in the back chamber $\times$ 100; V is the total volume of the gel; D is the length of the gel in centimeters; and p is the pool size (milliliters in the front chamber). After polymerization, an upper stacking gel of 4.5% (wt/vol) acrylamide (cross-linking ratio 37.5:1 acrylamide:*N,N'*-methylenebisacrylamide), containing 0.125 M Tris (pH 6.8), 0.1% SDS (wt/vol), 0.03% ammonium persulfate, and 0.1% (wt/vol) TEMED, was poured and allowed to polymerize around a Teflon sample well-maker. The dimensions of each sample well were 1.25 cm in length, 0.6 cm in width, and 0.08 cm in thickness. The well-maker, machined out of a single piece of Teflon, could form 13 samples. The gels were run with a constant current of 10 mA/gel for 5 h at room temperature. The running buffer consisted of 0.025 M Tris (pH 8.3), 0.2 M glycine, and 0.1% (wt/vol) SDS. The slabs of acrylamide were stained for 10 min in 50% (wt/vol) trichloroacetic acid-0.1% (wt/vol) Coomassie blue; destained by diffusion overnight in 7% (vol/vol) acetic acid at room temperature; and equilibrated for 2 h against 7% (vol/vol) acetic acid-5% (vol/vol) methanol to prevent cracking during the subsequent drying step. The slabs were dried on Whatman no. 1 filter paper (under vacuum) under an infrared lamp and exposed in the dark to Kodak X-ray film.

Sample preparation for electrophoresis. Protein samples of UV light-irradiated *B. subtilis* 60084 infected with $\phi 29^+$ or *sus* $\phi 29$ mutants for acrylamide gel analysis were prepared as described previously (9). In all experiments presented, the multiplicity of infection was 50 phages per bacterium. Infected bacteria (5 ml) were allowed to incorporate [^{14}C]leucine (2.5 μCi; Amersham/Searle; 342 mCi/mmol) from 5 to 35 min after infection.

Nomenclature. Suppressible mutants of $\phi 29$ are designated by Arabic numerals preceded by a capital letter which indicates the mutagen used to induce the mutant. Bu refers to bromodeoxyuridine mutagenesis, N refers to nitrous acid, T stands for triethylenemelamine, and E refers to ethyl methane sulfonate. $\phi 29$ proteins are numbered with Arabic numerals in order of decreasing molecular weight. The new proteins detected in this paper are designated 3a, 6a, 8a, and 18. Complementation groups are referred to by Roman numerals.

RESULTS

Fifty-four suppressible mutants of bacteriophage $\phi 29$ were isolated with four different mutagens. The approximate numbers of plaques tested to obtain one mutant were as follows: bromodeoxyuridine, 2,800; nitrous acid, 600; triethylene melamine in vitro, 2,300; TEM in vivo, 2,500; EMS, 2,500. Table 1 presents the distribution of mutants obtained with various mutagens.

Table 2 presents the results of complementation analysis. Table 3 presents the recombination values obtained from all possible two-factor crosses of $\phi 29$ *sus* mutants in complementation groups I through VIII. The mutants are arranged in their order on the genetic map. The order and position of the cistrons are based upon results involving adjacent pairs and not upon the recombination values of distant pairs, since, as had been noticed by other workers (10), recombination values involving distant pairs are not additive in this sytem. The order of

sus N6 and *sus* N8 is not apparent from the data presented in Table 3. However, three-factor crosses (*sus* N14 *sus* N8 × *sus* N6, 0.5% recombination; *sus* N6 × *sus* N8 *sus* N4, 2.2% recombination) indicate that the order of these mutants is N14, N6, N8.

Figure 1 presents data on the ability of mutants in ϕ29 groups I, II, and VI to synthesize viral DNA in the nonsuppressing host *B. subtilis* 60084. In these experiments, *B. subtilis* 60084, growing logarithmically in nutrient broth, were treated with 100 μg of HPAU per ml for 5 min at 37 C and then infected with either ϕ29$^+$ or *sus* ϕ29 mutants at a multiplicity of 25 phages per bacterium. Immediately after phage infection, 200 μg of uridine per ml and 5 μCi of [H^3]thymidine per ml were added to the infected bacteria. Samples were removed at regular intervals, and incorporation of radioactivity into whole cells was measured as described in Materials and Methods. As can be seen from the data of Fig. 1, incorporation of radioactivity in ϕ29$^+$-infected bacteria begins 10 min after phage addition. We have previously reported (5, 7) that uninfected bacteria treated with HPAU incorporate insignificant amounts of [H^3]thymidine into DNA under these conditions (<1% of the incorporation of ϕ29$^+$-

TABLE 1. *Distribution of ϕ29 sus mutants*[a]

Representative mutant[b]	Cistron	Nitrous acid	5-Bromodeoxyuridine	Ethyl methane sulfonate	Triethylene melamine	
					In vivo	In vitro
N14	1	2				
N6	II	2			1	
N8	III	2				
Bu2	IV		1		1	1
N4	V	10				6
T12	VI	1				1
Bu1, Bu4	VII	12	4	2	1	
N3	VIII	4			2	1

[a] The number of mutants in each complementation group isolated after treatment of ϕ29$^+$ with the indicated mutagens is presented. The complementation groups are listed in their order on the genetic map.

[b] Mutants chosen from each group for complementation and recombination analysis, as well as for acrylamide gel analysis of viral proteins.

TABLE 2. *Complementation of ϕ29 sus mutants*[a]

Mutants	N14	N6	N8	Bu2	N4	T12	Bu1	N3
N14	0.18	21.1	32.1	84.6	4.1	19.2	20.0	65.2
N6		2.6	11.1	42.9	17.5	50.0	72.7	43.7
N8			0.28	10.1	13.2	47.5	55.2	24.3
Bu2				1.3	15.7	60.0	55.2	27.7
N4					0.99	11.5	17.0	6.2
T12						0.53	21.5	18.2
Bu1							0.69	23.3
N3								0.15

[a] The numbers represent complementation values calculated as described in Materials and Methods.

TABLE 3. *Recombination between φ29 sus mutants*[a]

Mutants	N14	N6	N8	Bu2	N4	T12	Bu1	N3
N14	—	3.3	4.3	8.7	15.9	18.3	19.8	22.2
N6			2.4	8.6	8.6	17.8	8.6	14.7
N8				9.4	8.7	13.4	16.2	17.6
Bu2					5.7	7.6	9.3	11.7
N4						3.6	6.6	12.9
T12							5.4	7.3
Bu1								5.8
N3								—

[a] Recombination analysis was done as described in Materials and Methods. Each number is the average percent recombination of two crosses performed between the mutant at the top of the column and the mutant at the left of the row.

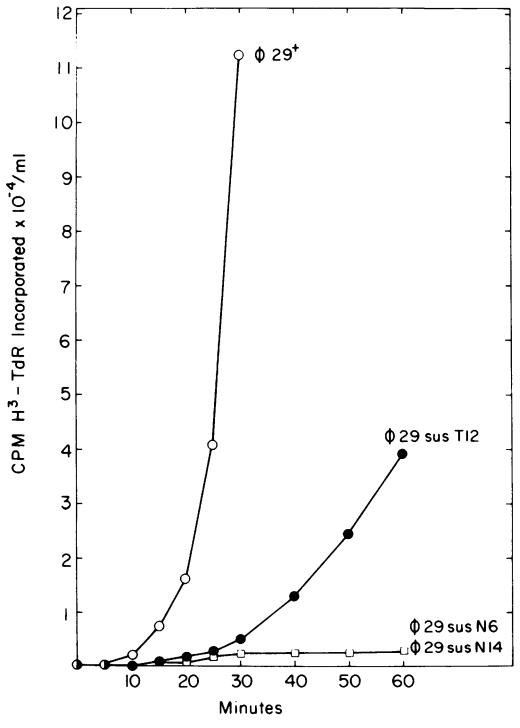

FIG. 1. *DNA synthesis in nonpermissive B. subtilis 60084 infected with φ29 sus mutants. The incorporation of [H³]thymidine was measured in B. subtilis 60084 treated with the bacterial DNA synthesis inhibitor 6-(hydroxyphenylazo)-uracil (as described in Materials and Methods) after infection with φ29⁺ and φ29 sus mutants. Mutants in complementation groups III, IV, V, VII, and VIII were found to synthesize DNA at rates comparable to the data presented in this figure for φ29⁺.*

infected bacteria 30 min after infection). Mutants in complementation groups III, IV, V, VII, and VIII were found to synthesize DNA at rates comparable to that of $\phi 29^+$ (data not shown). Mutants in groups VI, as shown by $\phi 29^+$ *sus* T12, were found to synthesize DNA at considerably lower rates than $\phi 29^+$-infected bacteria. Mutants in complementation groups I and II (respectively, *sus* N14 and *sus* N6) were incapable of synthesizing DNA in the nonsuppressing host *B. subtilis* 60084.

Mutants *sus* N14 and *sus* N6 were examined further for their capacity to attach viral chromosomes to the cell membrane of the nonsuppressing host *B. subtilis* 60084. Bacteria were infected at a multiplicity of 10 phages per cell with [H³]thymidine-labeled *sus* N14 or *sus* N6. Samples were removed at 27.5 min after infection and incubation at 37 C. Membrane-bound and free DNA were resolved on Renografin gradients as described previously (5). In Fig. 2, component I represents membrane-bound DNA and component II represents free DNA. It can be seen from these results that mutant *sus* N6 parental DNA is found in approximately equal amounts as free and membrane-bound DNA. Similar results have been reported for bacteria infected with $\phi 29^+$ (5). ³H-labeled *sus* N14 parental DNA, on the other hand, does not become complexed with the bacterial membrane. Similar results were presented previously with a temperature-sensitive mutant of φ29, designated φ29 TS35 (5).

Figure 3 presents results of viral protein synthesis in the UV-irradiated, nonsuppressing host, *B. subtilis* 60084, infected with $\phi 29^+$ and

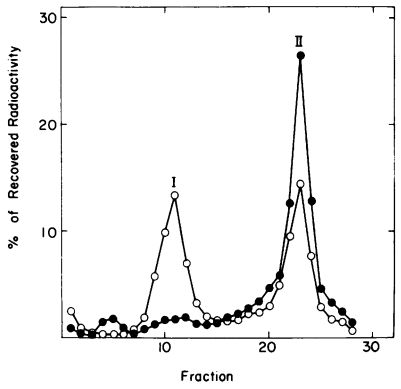

FIG. 2. *Association of parentally labeled DNA from φ29 sus N14 and φ29 sus N6 with the cell membrane in the nonpermissive B. subtilis 60084. Bacteria were infected at a multiplicity of 10 phages per bacterium with [H³]thymidine-labeled sus N14 (●) or sus N6 (○). Samples were removed 27.5 min after infection and incubation at 37 C. Membrane-bound DNA is designated in the figure by Roman numeral I. Free DNA is designated by Roman numeral II. Resolution of membrane-bound and free DNA was accomplished as described in a previous communication (5).*

sus mutants of groups I through VIII. To make comparisons possible, similar amounts of radioactivity were placed in each sample well. Missing viral proteins are indicated in the figure by arrows. It should be noted that, in most cases, the amount of high-molecular-weight proteins synthesized is reduced.

The use of exponential acrylamide gels allowed the resolution of four new proteins specified by bacteriophage φ29, which were not detected in a previous communication (9). They have been designated proteins 3a, 6a, 8a, and 18. Proteins 3a, 8a, and 18 are early φ29 proteins and are synthesized prior to the onset of viral DNA replication (data not shown).

It can be seen (Fig. 3) that φ29 mutants in complementation group I, as exemplified by φ29 *sus* N14, are missing protein 3a (67,000 daltons; see Fig. 4). A very small amount of this protein is synthesized even in φ29⁺-infected bacteria. Since this mutant is unable to attach viral chromosomes to the cell membrane, we conclude that protein 3a functions in the attachment of φ29 chromosomes to the bacterial membrane.

Mutants in complementation group II, as exemplified by φ29 *sus* N6, were found to be missing protein 8a (27,000 daltons; see Fig. 4). Protein 8a is therefore required for the synthesis of φ29 DNA. Mutations in cistron III (φ29 *sus* N8) apparently involve a regulatory gene of φ29 which acts as a positive control for late proteins. Infection of *B. subtilis* 60084 with φ29 *sus* N8 results in the synthesis of most early viral proteins (3a, 8a, 10, 12, 13, 15, 16, 17, and 18) in normal amounts, reduced amount of early viral protein 14, and delayed early structural viral protein 8 (9), but affects late proteins which are synthesized in amounts considerably reduced (proteins 1, 2, 3, 6, 6a, 7, and 9). Viral protein 11 appears to be missing in φ29 *sus* N8-infected bacteria. Another mutant in complementation group III was examined by gel analysis and gave protein patterns which were similar to bacteria infected with φ29 *sus* N8.

Infection of nonsuppressing bacteria with mutants in complementation group IV (φ29 *sus* Bu2) gave patterns which indicated that the primary defect lies in the absence of protein 3, which has previously been identified as the protein comprising the tail of bacteriophage φ29 (8, 9).

Mutants of complementation group V (φ29 *sus* N4) appear to be missing protein 6, previously identified as a collar protein (8, 9). In a mutant of cistron VI (φ29 *sus* T12), early protein 13 was missing and protein 14 (9; early) was synthesized in reduced amounts. Late proteins 1 and 2 were not detectable. Protein 14 is an early noncapsid viral protein which is synthesized throughout infection (9). Infection of *B. subtilis* 60084 with mutants in complementation group VI apparently also affects other viral proteins which are synthesized in very reduced amounts (Fig. 3). Phage φ29 *sus* T12 apparently adsorbs and injects normally, as evidenced by the complementation data presented in Table 2. Complementation group VII (φ29 *sus* Bu4) gave essentially normal viral protein patterns.

Cistron VIII (φ29 *sus* N3) appears to control the synthesis of protein 6a, a newly identified late noncapsid viral protein (38,000 daltons; see Fig. 4).

Figure 4 presents a curve, showing the migration of previously characterized φ29 proteins on exponential acrylamide gels, to estimate the molecular weight of the newly identified viral proteins (3a, 6a, 8a, and 18).

DISCUSSION

Fifty-four suppressible mutants of bacteriophage φ29 have been isolated with a variety of mutagens and assigned to eight complementation groups. Representative mutants from each group were used to infect the nonsuppressing host *B. subtilis* 60084 for identification of the viral protein(s) affected by the mutations. It

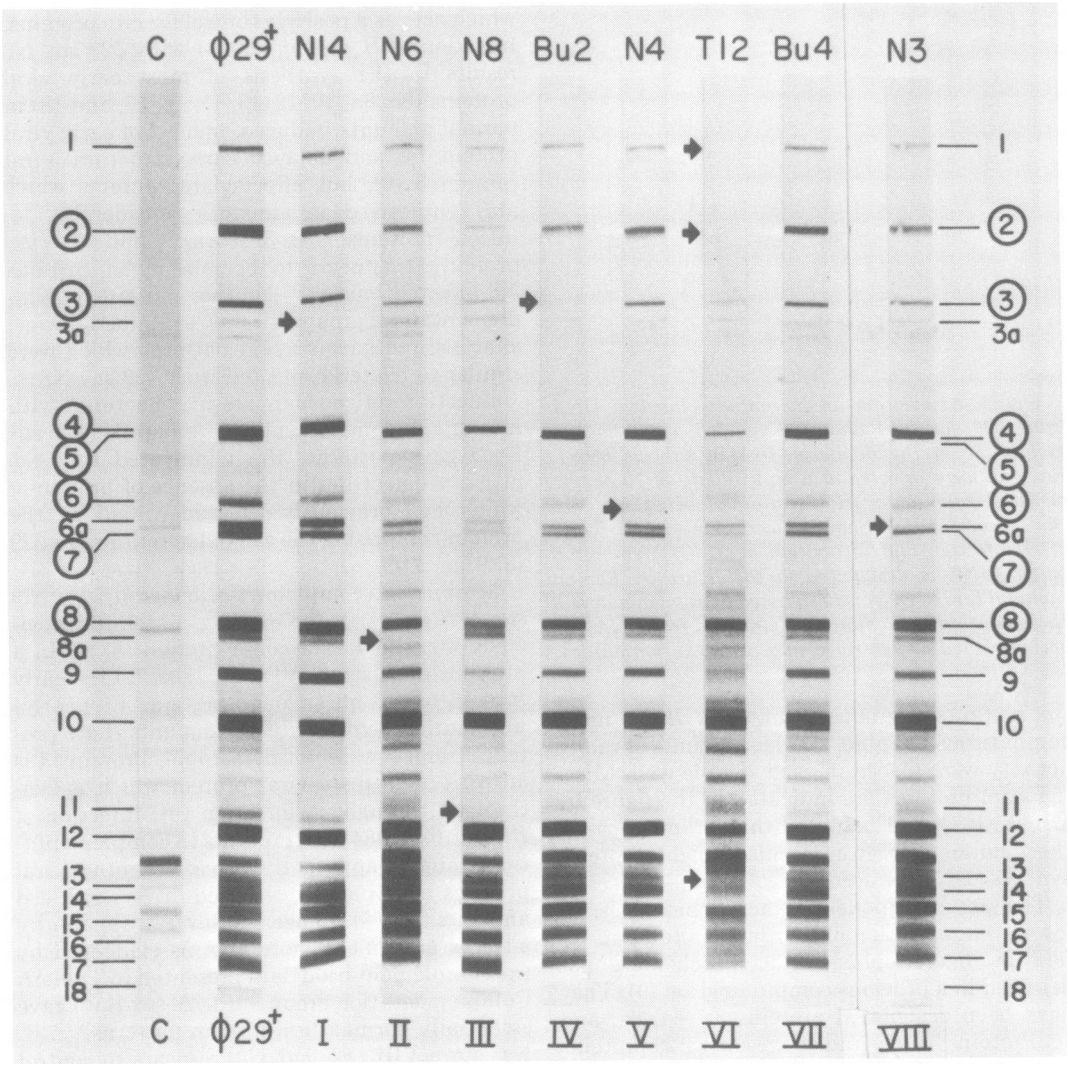

FIG. 3. *Synthesis of viral proteins in UV light-irradiated nonpermissive B. subtilis 60084 infected with wild-type phage and φ29 sus mutants. Bacteria (8 × 10⁷/ml) growing exponentially in BSS medium, supplemented with 0.5% glucose and 0.1 mM each of 19 L-amino acids, were irradiated with UV light. Irradiation damage was allowed to express itself for 10 min at 37 C. The bacteria were concentrated 10-fold by centrifugation, and samples were infected with φ29 and φ29 sus mutants in BSS medium which lacked glucose or amino acids and with 10^{-2} M azide, at a multiplicity of 50 phages per bacterium. After adsorption (10 min, 30 C), the bacteria were centrifuged in the cold, washed free of azide, and suspended in prewarmed BSS medium with glucose and amino acids. Five minutes after resuming incubation at 37 C, 5-ml samples of the infected bacteria were labeled with 2.5 μCi of [C¹⁴]leucine (342 Ci/mmol). After 35 min in incubation at 37 C, incorporation was stopped with excess nonradioactive leucine, 10^{-2} M azide, and rapid chilling to 0 C. The samples were processed as described previously (9) and subjected to electrophoresis on 9 to 18% exponential acrylamide slab gels prepared as described in Materials and Methods. Viral proteins are indicated by the numbers found on each side of the figure. The circled numbers are viral capsid proteins which have been previously identified (9). The gel labeled C is a control (UV light-irradiated, uninfected sample). At the top of the figure, each gel sample is designated by a symbol which refers to the mutant used. At the bottom of the figure, the Roman numerals refer to the complementation groups to which each of the mutants has been assigned. Missing proteins are identified for each sample by arrows. Each sample presented contained approximately 35,000 counts/min of radioactive proteins to make comparisons possible. The control uninfected sample contained 12,500 counts/min of radioactive proteins.*

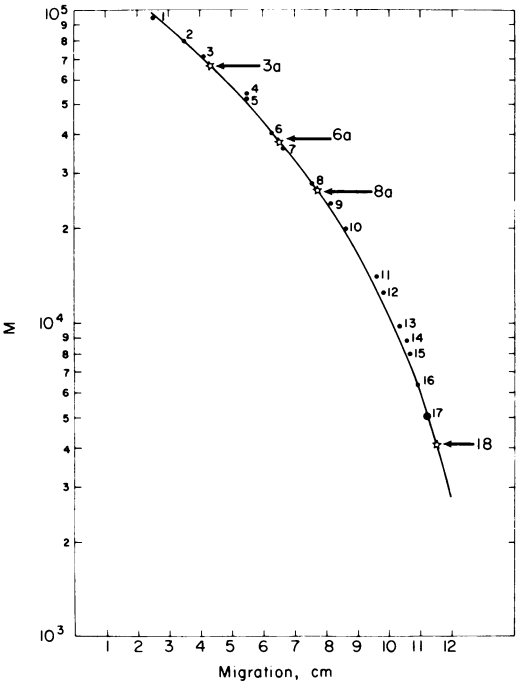

FIG. 4. *Migration of ϕ29 proteins on exponential acrylamide gels. The figure represents the migration and molecular weight of 17 ϕ29 proteins which have been identified previously (9), and the migration of the 4 proteins identified in this paper on exponential acrylamide gels. Previously identified proteins are represented by filled circles, newly discovered proteins (3a, 6a, 8a, and 18) are represented by stars.*

would appear from our results that *B. subtilis sus*$^+$3 carries a nonsense suppressor mutation since many of the ϕ29 mutants are unable to synthesize a single protein in the nonsuppressing host *B. subtilis* 60084. The data also suggest that in bacteriophage ϕ29 there is some clustering of phage functions (10). Cistrons I and II which control viral DNA synthesis map to the left of the ϕ29 chromosome, whereas cistrons controlling the synthesis of virion structural proteins are found near the center of the map.

Cistrons I and II control the synthesis of proteins required for the replication of ϕ29 DNA. Mutants in cistron II, such as ϕ29 *sus* N6, do not synthesize viral DNA in the nonsuppressing host. The protein responsible for this function is protein 8a (27,000 daltons). However, attachment of viral chromosomes to the cell membrane occurs normally in mutants in cistron II. Mutants in cistron I, such as ϕ29 *sus* N14, are also unable to synthesize viral DNA in the nonsuppressing host (Fig. 1). The results presented in Fig. 3 show that cistron I controls the synthesis of protein 3a (67,000 daltons), which is required for the attachment of ϕ29 chromosomes to the cell membrane. We have previously reported similar properties for a temperature-sensitive mutant of ϕ29 TS35 (5). The results presented in this paper indicate that in bacteriophage ϕ29 not only is attachment of phage chromosomes to membrane required for viral DNA synthesis, but also that the action of at least another viral protein is essential for phage DNA synthesis after attachment to membrane. Talavera et al. (11) have identified two early functions and one late function which are required for ϕ29 DNA synthesis. The early functions may be under the control of cistrons which correspond to cistrons I and II of this communication. The results also show that attachment of ϕ29 chromosomes to the cell membrane can occur in the absence of viral DNA synthesis. In infections of the nonsuppressing host by mutants in cistron I or II, late viral functions were detected by acrylamide gel electrophoresis. This confirms our previous conclusion that in ϕ29 the synthesis of late viral functions is not under the positive control of phage DNA replication (7). The low rate of viral DNA synthesis by ϕ29 *sus* T12 (cistron VI) apparently reflects the low amounts of many viral proteins.

Cistrons I, II, IV, V, and VIII control the synthesis of single proteins which are not synthesized when mutants in these complementation groups infect the nonsuppressing host. Mutations in cistron III (ϕ29 *sus* N8) and cistron VI (ϕ29 *sus* T12) have pleiotropic effects on many ϕ29 proteins. We have recently isolated another suppressible mutant of bacteriophage ϕ29, designated ϕ29 *sus* N34, which maps within two recombination units of ϕ29 *sus* T12 and which does not complement this mutant. Preliminary analysis of *B. subtilis* 60084 infected with ϕ29 *sus* N34 revealed that viral proteins 1, 11, and 13 were missing. Since ϕ29 *sus* T12 is affected for many other proteins, it would appear that this mutant may carry another mutation which is either nonsuppressible or incompletely suppressible by *B. subtilis su*$^+$3. This suggests, however, that viral protein 13 may be a positive control element for the efficient synthesis of some late, high-molecular-weight proteins. The primary defect of mutations in cistron III (ϕ29 *sus* N8) has not been identified. Infection of nonsuppressing bacteria with mutants in this complementation group result in the normal synthesis of early proteins, with the exception of protein 14 which is made

in reduced amounts. Protein 11 was not detectable when mutants in cistron III infect nonsuppressing bacteria. However, protein 11 is made late in infection in very small amounts (9), and we cannot conclude that it plays any role as a positive control element for the efficient synthesis of late viral proteins. It is more likely that protein 14, a low-molecular-weight early ϕ29 protein, may play a role in the control of the synthesis of late high-molecular-weight viral proteins. Additional work will be necessary to establish the mechanisms which control the synthesis of late high-molecular-weight proteins in ϕ29.

The protein specified by cistron VII (ϕ29 *sus* Bu4) has not been identified since infection of nonpermissive *B. subtilis* 60084 gave protein patterns which were essentially similar to $\phi29^+$ infection. It is possible that, in the mutants examined, the position of the nonsense codon does not affect drastically the molecular weight of the protein involved. It is also possible that this cistron controls the synthesis of viral protein 5, the minor capsid protein of ϕ29. The detection of protein 5 is difficult because its migration is very close to the migration of the major ϕ29 capsid protein 4. Moreover, the molar ratio of the major capsid protein (protein 4) to the minor capsid protein (protein 5) in completed ϕ29 particles is at least 16:1, and one to two copies of protein 5 are found per completed viral particle (unpublished observations). For these reasons, protein 5 is not identifiable in autoradiograms of ϕ29 proteins synthesized in vivo.

We have been unable so far to find mutations affecting viral proteins 12 through 18 which are low-molecular-weight early proteins (except ϕ29 *sus* T12, which apparently lacks protein 13). Since the molecular weights of these proteins span the range of 4,200 to 12,500 daltons, it is possible that the small size of DNA coding for these proteins presents a small target for the mutagens employed. It is also possible that these low-molecular-weight proteins are not required for productive infection.

It is surprising that we have not been able to detect amber peptides on acrylamide gels of the ϕ29 *sus* mutants examined. The conditions under which these mutants were isolated may be such that the location of nonsense condons is near the origin of the DNA sequences specifying these proteins. Amber peptides may also be of low molecular weight, run off the acrylamide gels, or be masked in the very crowded region spanning the ϕ29 low-molecular-weight proteins.

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