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Viral Protein Synthesis in Bacteriophage φ29-Infected *Bacillus subtilis*

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Twenty-three ^{14}C-labeled phage φ29-specific proteins in lysates of UV-irradiated *Bacillus subtilis* have been resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis and identified by autoradiography. Included in this group of proteins are the six major structural proteins of the virion. Analysis of the temporal sequence of viral protein synthesis indicates that three groups of proteins can be identified by time of appearance, beginning at 2 to 4, 4 to 6, or 8 to 10 min after infection, respectively. These proteins account for approximately 90% of the coding capacity of the φ29 genome.

The *Bacillus subtilis* phage φ29 has a unique complex morphology (1) and an infectious double-stranded DNA of 11,000,000 molecular weight (2, 16). DNA-RNA hybridization-competition experiments have defined "early" and "late" classes of φ29-specific mRNA molecules (10, 18), and analysis of the temporal sequence of mRNA synthesis indicates "light" and "heavy" DNA strand transcription with at least one transcriptional switch (13, 18). These features make φ29 an attractive subject for studies of gene expression and viral morphogenesis.

In this report we have identified 23 φ29-specific proteins by polyacrylamide gel electrophoresis and have examined the temporal sequence of viral protein synthesis in *B. subtilis*.

MATERIALS AND METHODS

Phage and bacteria. The growth of φ29 in *Bacillus amyloliquefaciens* strain H and purification of the virus have been described (2). *B. subtilis* SpoA12 (17) was used for studies of protein synthesis.

Growth and infection conditions. Bacteria were grown to 2 × 10^8 cells/ml in minimal medium M40 (15) supplemented with tryptophan (50 μg/ml). Cells were sedimented (5,000 × *g*, 5 min, 25 C) and suspended at 2 × 10^9 cells/ml in M40 medium. To reduce host protein synthesis by UV irradiation, a 2-ml suspension of cells was irradiated for 10 min at a dose of 50 ergs per mm^2 per s in a 35 × 10 mm petri dish, with mixing every 15 s. Photoreactivation was minimized by performing all subsequent operations in the light of the germicidal lamp. Phage were added at a multiplicity of infection (MOI) of 50. The amount of viral protein synthesized is directly proportional to MOI from 5 to 50 (Mario Tosi, personal communication). Infected cells were diluted to 2 × 10^8 cells/ml in prewarmed M40 medium and incubated at 37 C on a reciprocal shaker (125 linear excursions/min). A mixture of ^{14}C-amino acids (see below) was added to the infected culture to prepare ^{14}C-labeled φ29.

Preparation of ^{14}C-labeled φ29 proteins. Specific details of the protocols are described in the Results section. Proteins were labeled with a mixture of ^{14}C-amino acids (New England Nuclear Corp., NEC 445, 2 or 20 μCi/ml, about 200 μCi/mmol). Labeling was terminated by pipetting the infected culture into an equal volume of an iced solution containing 0.1 M NaCl, 0.05 M sodium citrate, 0.01 M sodium azide, chloramphenicol (200 μg/ml), and the serylprotease inhibitor phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co., St. Louis, Mo.; 600 μg/ml).

For protein extraction, cells were sedimented (10,000 × *g*, 20 min, 4 C) and the supernatant fluid was transferred to one volume of iced 10% trichloroacetic acid. The trichloroacetic acid precipitate was collected by centrifugation (13,000 × *g*, 20 min, 4 C), covered with anhydrous ether to remove the trichloroacetic acid and stored at 4 C overnight. After decanting the ether, the precipitate was dissolved in electrophoresis sample buffer (9). This solution of the trichloroacetic acid precipitate of the cell-free culture fluid is referred to as the supernatant fraction. The cells were suspended and lysed in one-tenth the culture volume of a solution containing 0.06 M Tris-hydrochloride (pH 6.8), lysozyme (200 μg/ml), and PMSF (300 μg/ml). The lysate was treated with DNase (DNase 1, Calbiochem, 20 μg/ml) and mixed with 0.05 ml of 3× electrophoresis sample buffer (9). This preparation is referred to as the cell pellet.

Electrophoresis. Proteins were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. The system was basically that of Laemmli (9), except that a slab gel apparatus was used (21). Acrylamide, *N*,*N*′-methylenebisacrylamide, *N*,*N*,*N*′*N*′-tetramethylenediamine, and 2-mercaptoethanol were purchased from Eastman Kodak Co., Rochester, N.Y.

Lower gels contained 8 or 12% acrylamide, or were 12 to 16% linear polyacrylamide gradients made with

a Buchler gradient mixer. Gels were run at a constant current of 17 mA. Gels were fixed in 50% trichloroacetic acid overnight, stained with 0.1% Coomassie blue (Schwartz Mann, Orangeburg, N.Y.) in 50% trichloroacetic acid for 15 to 30 min, and destained with 7% acetic acid overnight before drying. Kodak no-screen medical X-ray film or Kodak blue-sensitive X-ray film type SB54 were used for autoradiography. Tracings of the autoradiographs were made using a Joyce, Loebl MK III CS microdensitometer.

Electron microscopy. Phage ϕ29-infected unirradiated or UV-irradiated bacteria were prepared for electron microscopy by a modification (E. W. Hagen, manuscript in preparation) of the in situ lysis technique of Kellenberger et al. (7). Phage in lysates were adsorbed to carbon-coated Formvar films and negatively stained with 2% (wt/vol) phosphotungstate (PTA) solutions adjusted to pH 7.0 with 1 N KOH (3). Micrographs were taken with a Siemens Elmiskop 1A electron microscope.

RESULTS

Phage ϕ29 infection of irradiated cells. During ϕ29 infection of *B. subtilis* SpoA12, transcription of host DNA continues at the preinfection rate and this mRNA is continuously used to produce protein (19). We have employed UV irradiation to suppress host protein synthesis during infection. The capacity for phage production of the infected irradiated cells is about 5% of the unirradiated control culture, and the average burst size is 10 PFU per cell. Application of the in situ lysis technique (7) to ϕ29 infection reveals more than 100 DNA-containing particles of typical ϕ29 morphology in bursts from single irradiated cells. Figures 1 and 2 show typical progeny of unirradiated and irradiated cells.

Protein synthesis during viral infection. We have used SDS polyacrylamide gel electrophoresis to examine the size distribution of the polypeptide chains coded by the ϕ29 genome. The molecular weight of ϕ29 proteins was determined by relating the electrophoretic mobility of viral proteins to the mobility of 13 reference proteins in the same gel. The relative electrophoretic mobility of the reference proteins in a 12 to 16% linear polyacrylamide gradient as a function of molecular weight is presented in Fig. 3. The molecular-weight range of the reference proteins is from 12,400 to 96,000.

To examine viral protein synthesis, UV-irradiated cells were infected with ϕ29 (MOI of 50) and labeled with a mixture of ^{14}C-amino acids (2 μCi/ml) from 10 to 45 min after infection. Autoradiographs of the ^{14}C-labeled proteins of both the cell pellet and the supernatant fractions separated by SDS gel electrophoresis are presented in Fig. 4. Very little radioactive protein is detected in the pellet and supernatant fractions of the uninfected, irradiated control culture. The trivial names of the viral protein bands (Fig. 4) reflect in part our current knowledge of the ϕ29 genetic map and gene functions (manuscript in preparation). The order of 10 genes has been determined by three-factor crosses, and these genes have been designated by letters (17). The protein band that represents the product of gene H is P(H). Other bands are identified as virion components (e.g., Ap for tail appendage, etc.) or by position in the profile (e.g., LM3 for low-molecular-weight protein 3).

Six of the 20 viral-specific proteins visible in Fig. 4 are also present in purified ϕ29 virions. Mendez et al. (12) have identified Hd and F as head proteins; Ap, C1, and C2 as neck proteins; and P(H) as a tail protein of the virus. We have confirmed their results by using purified components of disassembled ϕ29 virions, including heads, head fibers, and tail appendages (D. Hickman, unpublished data). The Fig. 4 insert provides a clearer view of the major head (Hd) and collar protein (C1, C2) region of the autoradiograph and indicates that the protein P(L) present only in infected cells also bands in this region.

Our analysis of the ^{14}C-labeled proteins in infected cells (Fig. 4) is complicated by a partitioning of the ϕ29 proteins. By 45 min the bulk of the ϕ29 protein has been released from the cells, but proteins A2, LM4, and LM7 remain associated primarily with the cell pellet. At this time most of the cells appear intact when examined by phase contrast microscopy.

The assigned molecular weights of the ϕ29 proteins are listed in Table 1. The molecular-weight range for each protein results from pooling data from 8 and 12% polyacrylamide gels and 12 to 16% linear gradients of polyacrylamide, each containing reference proteins.

Time course of protein synthesis. The results of pulse-labeling experiments to reveal the temporal sequence of viral protein synthesis in unirradiated and irradiated cells are presented in Fig. 5 and 6. The early events were examined by use of 2-min pulses with 20 μCi of the ^{14}C-amino acid mixture per ml and later events by use of 3-min pulses. The first pulse occurred 2 min after the addition of ϕ29 (MOI of 50) to the cells.

In unirradiated cells (Fig. 5), the bands LM3 and LM6 appear in the pellet in quantity from 4 to 6 min (profile c) and begin to leak into the supernatant fraction at about 8 min after infection (profile e'). The synthesis of head fiber (F) protein can also be followed, beginning at about 8 min after infection (Fig. 5, profile e). The

FIG. 1. *Electron micrograph of a negatively stained ϕ29 infected unirradiated B. subtilis cell lysed at 60 min after infection by the in situ technique.* $\times$*86,000.*

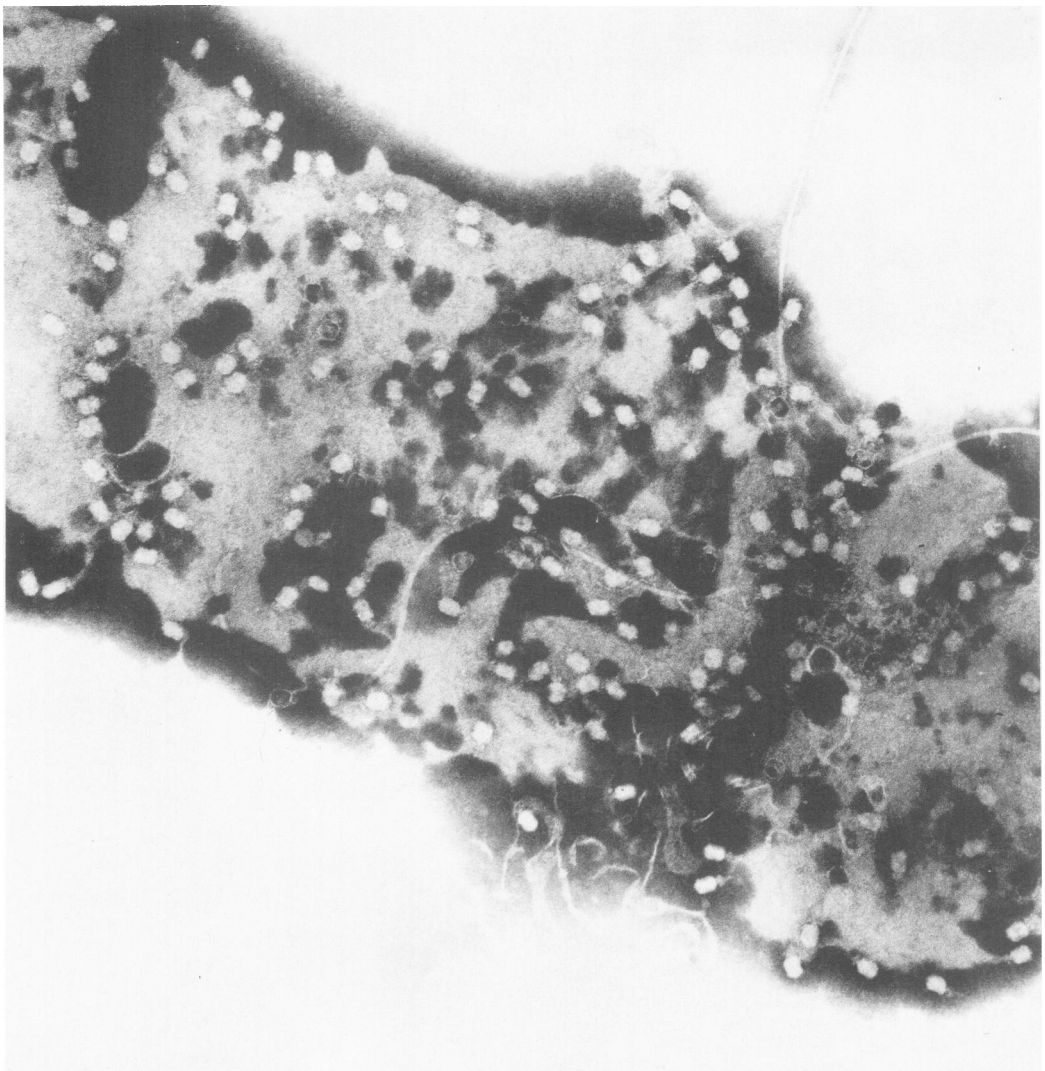

FIG. 2. *Electron micrograph of a negatively stained φ29 infected UV-irradiated B. subtilis cell lysed at 60 min after infection by the in situ technique.* ×*70,000.*

presence of other viral specific bands are best observed in the supernatant fractions (e.g., proteins Ap, Hd, P(N), LM2, LM5, LM5B, and LM8).

B. subtilis was irradiated with UV light for 10 min at 50 ergs per mm^2 per s prior to infection, and the reduction in cell protein synthesis is revealed by comparison of Fig. 5 with Fig. 6. The onset of φ29 protein synthesis is not delayed by irradiation. In Fig. 6, three "early" proteins can be detected that are not evident in Fig. 4. Proteins AF and BF band above and below the head fiber protein (F). These bands are visible from 4 to 10 min after infection (Fig. 6, profiles b′, c′, and d′), but are obscured by F synthesis between 10 and 15 min (Fig. 6, profiles e′ and f′). Careful inspection of Fig. 6 suggests that synthesis of AF and BF is reduced at 10 min (profile e′) and 15 min (profile g′), respectively. The protein LM6B is present in the 2- to 10-min interval (Fig. 6, profiles a′, b′, c′, and d′), but then disappears from the autoradiograph.

The temporal sequence of φ29 protein synthesis is summarized in Fig. 7. First to appear are seven proteins of unknown function (A1, A2, LM3, LM4, LM5, LM6, and LM6B) that can be detected 2 to 4 min after infection. The synthe-

sis of six proteins (Hd, AF, BF, LM7, LM8, and LM9) begins at about 4 min after infection. At about 8 min, a time just prior to the initiation of φ29 DNA synthesis in unirradiated cells (10, 20), eight more protein bands appear, including P(J), P(H), C1, P(L), C2, F, P(N), and LMSB. The appearance of proteins Ap and LM2 is delayed until 10 min after infection.

DISCUSSION

Twenty-three ^{14}C-labeled viral specific proteins in lysates of φ29 infected UV-irradiated *B. subtilis* can be resolved by SDS polyacrylamide gel electrophoresis and identified by autoradiography.

We believe that φ29 infection of UV-irradiated cells is a reasonable model for analysis of protein synthesis during φ29 infection of *B. subtilis*. The capacity of irradiated cells to make φ29 is about 5% that of the unirradiated cultures, and the average burst size is 10 infectious particles/cell. The onset of protein synthesis and the temporal sequence is similar in irradiated and unirradiated cultures (Fig. 5 and 6). An analysis of densitometer tracings of autoradiographs indicates that ^{14}C-labeled structural proteins are present in infected irradiated cells and in virions from unirradiated cells in nearly the same proportions (e.g., head protein, 49 and 50%; head fibers, 15 and 20%; and appendages, 18 and 19%, respectively; data not presented).

Examination of the progeny phage of single cell bursts by the in situ lysis technique reveals more than 100 phage particles containing DNA and having typical φ29 morphology (Fig. 1 and 2). Because it has been demonstrated that "late" mRNA synthesis occurs in the absence of φ29 DNA synthesis (10) and that all of the φ29 structural proteins are synthesized in irradiated cells, we have not examined the effect of UV irradiation on φ29 DNA synthesis.

Defective phage SPα and SPβ particles have been observed in *B. subtilis* lysates (F. Eiserling, Ph.D. thesis, UCLA, 1964), but electron microscopy of irradiated cell lysates and of in situ cell bursts demonstrates only φ29 particles. If these or other defective phage were induced by irradiation but not assembled, the proteins would appear in the control gel prepared with uninfected irradiated cells (Fig. 4, profiles c and d) and would not be considered φ29 proteins.

If our image of protein synthesis is reasonable, it must be consistent with the results of our genetic analysis of φ29 infection and with the coding capacity of the φ29 genome. We have isolated temperature-sensitive (*ts*) mutants

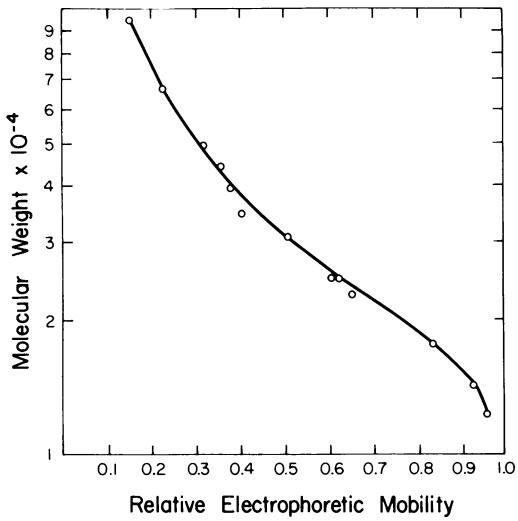

FIG. 3. *Relationship between relative electrophoretic mobility (with respect to the tracking dye) and molecular weight for reference proteins in a 12 to 16% linear SDS polyacrylamide gel gradient. Points are given for the following proteins: phosphorylase A (96,000), bovine serum albumin (67,000), heavy chain of immunoglobulin G (50,000), ovalbumin (45,000), creatine phosphokinase (40,000), pepsin (35,000), DNase 1 (31,000), light chain of immunoglobulin G and chymotrypsinogen (25,000), trypsin (23,000), myoglobin (17,800), lysozyme (14,000), and cytochrome c (12,400).*

that map in 13 cistrons (5) and (*sus*) mutants of 13 cistrons (17). The integration of these sets of mutants by quantitative complementation is in progress. We believe we have identified conditional lethal mutations in at least 15 cistrons (unpublished data). If any proteins in the gel profiles represent cleavage products of precursor proteins, or if some viral functions can be partially supplied by the host (i.e., are not conditionally lethal), there is even better agreement between the protein analysis and the genetic data.

Analysis of phage T4 infection has indicated that some precursor proteins are cleaved during maturation (4, 6, 8, 9). In our evaluation of coding capacity, we must consider this possibility for φ29 infection. We have observed that both the P(J) and the Ap proteins are resolved into two bands (Fig. 4). Suppressor-sensitive (*sus*) mutants that map in gene J (17) do not produce proteins P(J), Ap, and LM2 during infection of the nonpermissive host. Polypeptide fragments presumed to result from premature chain termination also give the double band in the gel. Preliminary pulse-chase experiments with wild-type φ29 indicate that protein

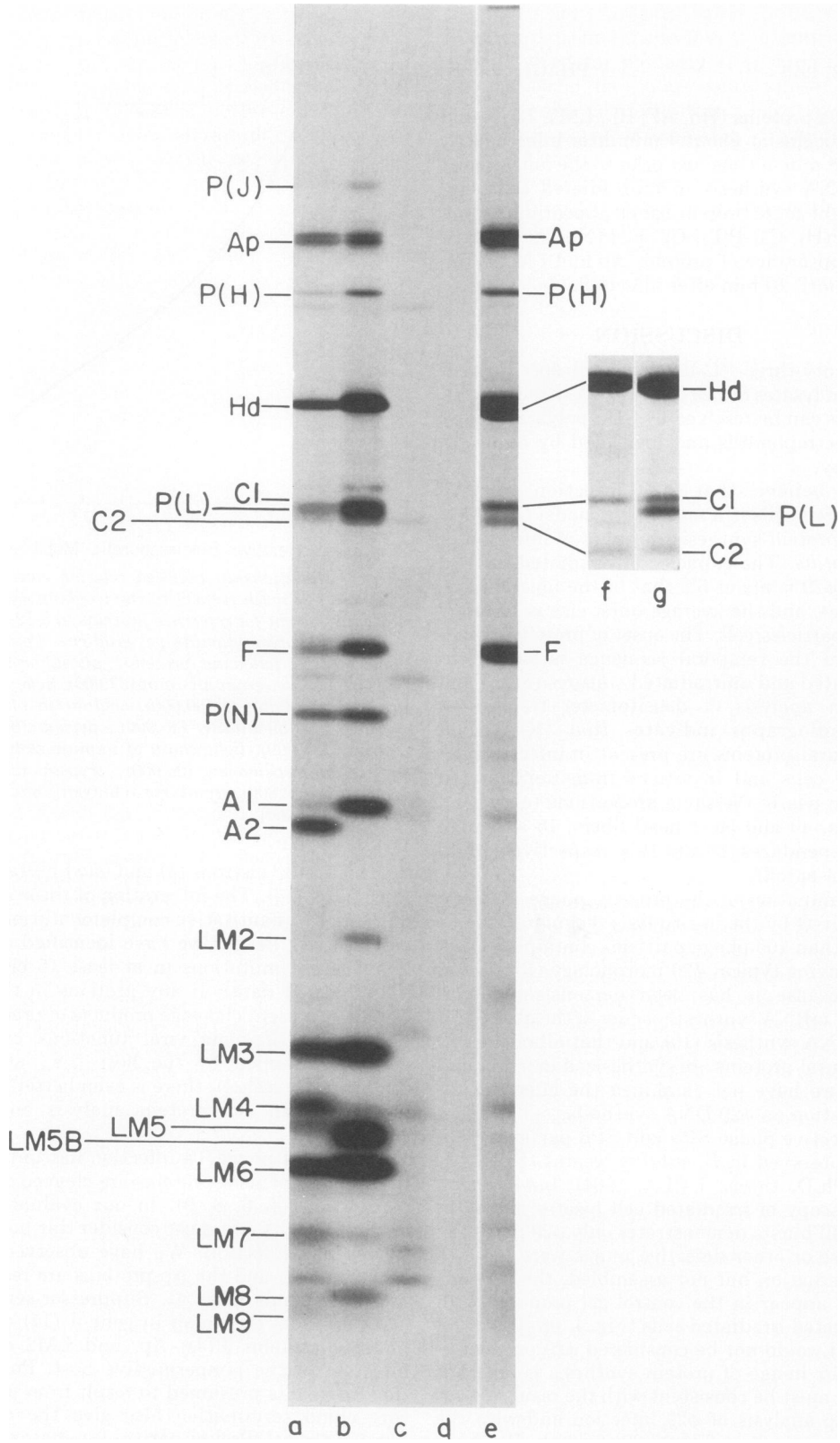

FIG. 4. *Autoradiographs of ^{14}C-labeled ϕ29 proteins produced in UV-irradiated B. subtilis and separated by SDS polyacrylamide gel electrophoresis. Cells were labeled with a mixture of ^{14}C-amino acids from 10 to 45 min after infection. a, Cell pellet fraction of infected cells; b, trichloroacetic acid precipitate of the supernatant fraction from the infected cells; c, cell pellet fraction of an irradiated, uninfected control; d, trichloroacetic acid*

P(J) is the precursor of the appendage protein (Ap) and the protein LM2 (data not shown). We have no explanation for the double-band phenomena, but submit that putative cleavage products Ap and LM2 can be eliminated from our consideration of coding capacity.

The combined molecular weights of proteins Ap and LM2 listed in Table 1 do not equal that of protein P(J). Also, our estimates of molecular weights of the φ29 structural proteins are generally lower than those of Mendez et al. (12). Although there is close agreement on the molecular weight of the head fibers (F), there is about a 6% difference in the estimates for the appendage protein (Ap).

A protein linker that holds φ29 DNA in a supercoiled form in the virion (14) and a component of the purified φ29 head, described as a minor band immediately below the major head protein in gel profiles (12), must be considered. We have not observed the protein linker in the disrupted virus, but it may be hidden in a protein band of comparable molecular weight, e.g., the major head protein (14). If it remains bound to the DNA of the virion and does not enter the gel, then it might be one of the "nonstructural" proteins in the infected cells (Fig. 4). We can detect the minor band in ^{14}C-labeled φ29 purified by equilibrium sedimentation in CsCl, but we have never detected this protein in lysates of φ29-infected *B. subtilis* SpoA12. Furthermore, we can resolve four minor bands of equal density directly below the major head protein in examining the stained SDS gel profile of CsCl-purified φ29 grown on *B. amyloliquefaciens* strain H. We have noted that virus yield in *B. subtilis* and *B. amyloliquefaciens* is quite variable and that satellite bands and aberrant structures are present in CsCl density gradients of most φ29 lysates (data not presented). For these reasons the minor head protein band could be an anomaly reflecting malfunction during phage morphogenesis. Alternatively, we may miss this band during our analysis because of nonspecific protease activity. We have tried to minimize proteolysis by incorporation of the serylprotease inhibitor PMSF into our lysis mixture and extraction method. Therefore, if we include protein P(J), but not proteins Ap and LM2, consider the protein linker (14) to be present but unidentified in the gel profile, and ignore the minor head protein (12), we can estimate the coding capacity invested in the remaining 21 proteins. We assume the average molecular weight of the amino acids in phage protein to be 115 and have estimated the molecular weight of small proteins by extrapolation of the calibration curve in Fig. 3. Early proteins LM6B, AF, and BF are assigned molecular weights of 12,000, 28,700, and 28,300, respectively. These values are added to those of Table 1 and the sum is about 560,000, reflecting an estimated 90% of the coding capacity of the φ29 genome.

Viral-specific mRNA has been detected between 2 and 7 min after infection (11). "Early" RNA is synthesized from the L strand of viral DNA and is produced throughout the infectious

TABLE 1. *Molecular weights of φ29 proteins as determined by SDS polyacrylamide gel electrophoresis*

φ29 Protein[a]	No. of gels[b]	Mol wt range[c]	Avg mol wt[d]	Mol wt (previous determination)[e]
P(J)	1		87,500	
Ap	6	73,900–76,100	75,300	80,000
P(H)	7	61,000–63,700	62,300	71,000
Hd	8	43,500–46,000	45,000	54,000
C1	8	34,000–37,700	36,400	40,000
P(L)	1		36,000	
C2	8	33,000–36,500	35,200	36,000
F	7	27,200–29,800	28,500	28,000
P(N)	5	24,100–26,500	25,100	
A1	4	21,900–22,800	22,400	
A2	1		21,800	
LM2	5	18,100–20,000	18,800	
LM3	4	16,200–17,700	16,700	
LM4	4	14,400–16,600	15,200	
LM5	3	14,400–15,000	14,700	
LM5B	1		14,200	
LM6	1		13,000	
LM7	1		8,500	
LM8	1		5,300	
LM9	1		4,500	

[a] Two proteins, AF and BF, with molecular weights within the range reported for protein F, appear early at 4 min after infection (see Fig. 6). A third protein, LM6B, with a molecular weight slightly less than 13,000, is detected at 2 min after infection (Fig. 6).

[b] Gels were 8 or 12% polyacrylamide or 12 to 16% linear polyacrylamide gradients.

[c] Molecular weights were determined by comparing the relative electrophoretic mobilities of φ29 proteins with mobilities of reference proteins run on each gel.

[d] Values for proteins LM7, LM8, and LM9 were derived by extrapolation of the curve in Fig. 3.

[e] Determined by Mendez et al. (12).

precipitate of the supernatant fraction from the irradiated, uninfected control; and e, purified φ29 virions. Profiles a, b, c, d, and e were from a 12 to 16% linear polyacrylamide gradient. Insert profiles f and g from a 16 to 20% linear polyacrylamide gradient show the collar region more clearly; f, purified φ29 virions; g, trichloroacetic acid precipitate of the supernatant fraction from infected cells.

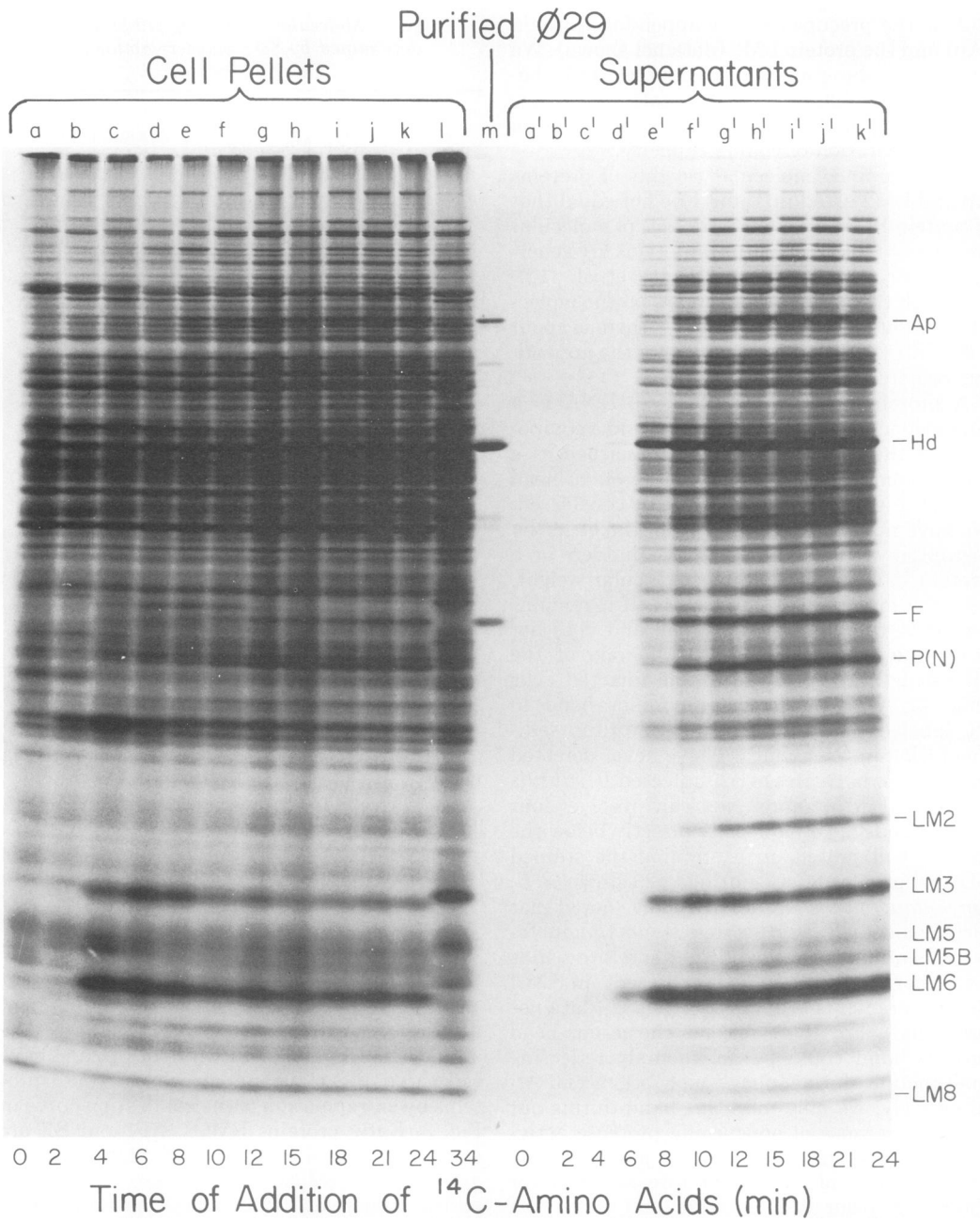

FIG. 5. *Autoradiograph of ^{14}C-labeled φ29 proteins showing the time course of protein synthesis in unirradiated B. subtilis. Cells were infected with wild-type φ29 at a MOI of 50. Beginning at 2 min after infection, culture samples were removed at intervals and pulse labeled for 2- or 3-min periods with a mixture of ^{14}C-amino acids. Labeling was terminated as described in the Materials and Methods, and both the cell and supernatant fractions were analyzed by SDS gel electrophoresis. Times indicate the beginning of consecutive pulses. Uninfected control cells were pulse labeled early (profiles a and a', 0 to 2 min) and late (profile L, 34 to 37 min). Profiles b through k represent pellet fractions of infected cells, and profiles b' through k' are corresponding trichloroacetic acid precipitates of supernatant fractions of the infected cells. Profile m is of purified φ29 virions.*

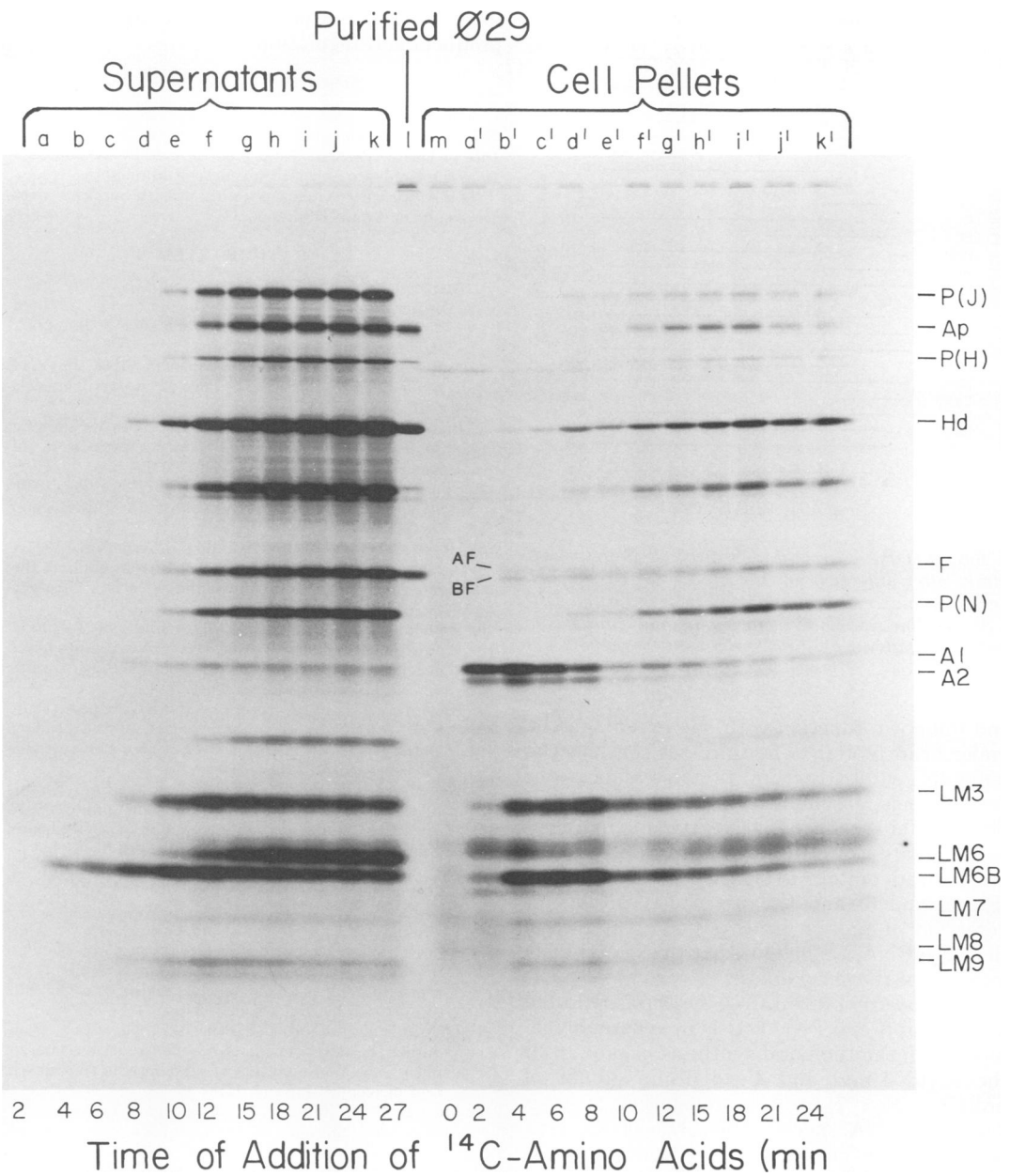

FIG. 6. *Autoradiograph of ^{14}C-labeled φ29 proteins showing the time course of protein synthesis in UV-irradiated B. subtilis. Cells were infected with wild-type φ29 at a MOI of 50. Beginning at 2 min after infection, culture samples were removed at intervals and pulse labeled for 2- or 3-min periods with a mixture of ^{14}C-amino acids. Labeling was terminated as described in Materials and Methods, and both the cell and supernatant fractions were analyzed by SDS gel electrophoresis. Times indicate the beginning of consecutive pulses. Uninfected control cells were pulse labeled early (profile m, 0 to 2 min). Profiles a through k represent trichloroacetic acid precipitates of supernatant fractions of the infected cells and profiles a' through k' are corresponding pellet fractions of infected cells. Profile L is of purified φ29 virions.*

cycle. Late RNA synthesis is independent of viral DNA synthesis (10) and is transcribed from the H strand of φ29 DNA (18). We have defined three groups of φ29 proteins by their first appearance during the time course experiments (Fig. 7), namely 2 to 4 min, 4 to 6 min, and 8 to 10 min after infection. The first two groups contain proteins of low molecular weight

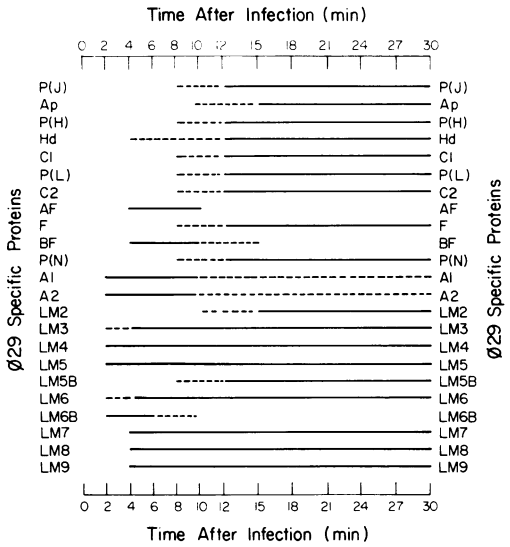

FIG. 7. *Time course of φ29 directed protein synthesis after infection of UV-irradiated B. subtilis. Less than maximal protein synthesis is indicated with a dashed line; maximal or near maximal synthesis is indicated with a solid line.*

and unknown function with one exception, the major head protein. The third set contains the remaining viral structural proteins among others, and it is reasonable to conclude that group three proteins reflect H DNA strand transcription and late RNA translation (18). Because the major head protein is synthesized in quantity (Fig. 5 and 6) and because the sensitivity of detection of protein synthesis is greater than that of mRNA, we believe that this protein is a member of the third group.

The pattern of φ29 mRNA synthesis reported by Loskutoff and Pene (11) is in general agreement with the temporal synthesis of protein. If the 2- to 4-min and 4- to 6-min groups of proteins (Fig. 7) reflect translation of their "early" mRNA species, then 37% of the estimated coding capacity of the genome is expressed in translation of six mRNA species that comprise 35% of the RNA transcription potential. The combined molecular weight of group three proteins reflects 50% of the coding capacity and the late mRNA species reflect 50% of the potential transcripts.

We are examining protein synthesis following infection of the nonpermissive host by *sus* mutants (manuscript in preparation) and are using pulse-chase experiments to validate our observations of precursor cleavage. It will be useful to correlate these observations with a more extensive examination of transcription products and regulation.

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