Host-Phage Interaction in Agrobacterium tumefaciens

IV. Phage-Directed Protein Synthesis

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Gel electrophoretic and autoradiographic techniques were used to detect the temporal sequence of protein synthesis after infection of the sensitive strain Agrobacterium tumefaciens with phage LV-1. Three classes of protein were detected: early proteins, class I, which include a protein capable of shutting off host protein synthesis; class II, proteins which are detected after 30 min; and late proteins, class III, which include the phage-directed endolysin and five additional proteins that appear 45 min after infection

Agrobacterium tumefaciens is a gram-negative rod which causes crown gall, a neoplastic disease affecting a variety of plants.

Lysogeny has been shown to be relatively common in strains of A. tumefaciens (12). Phage LV-1, a temperate phage isolated from A. tumefaciens V-1, is similar in size and morphology to colipage λ. DeLey et al. (4) have found that phage LV-1 is virtually indistinguishable from three other agrophyge, including omega, the first temperate agrophyge to be described (2). Phage LV-1 has a polyhedral head and a long flexible tail (12), which places it into class B of Bradley's phage classification (3). The linear, double-stranded LV-1 DNA has a molecular weight of $31 \times 10^6$ (5). A genome of this size should be able to encode 30 to 40 proteins. The phage is composed of four major structural proteins and possibly as many as seven minor ones. It is estimated that the structural proteins constitute less than 25% of the total coding capacity of the phage DNA.

One phage-coded protein, endolysin, a late enzyme responsible for lysis of the host cells after phage infection, has been described previously (10). Synthesis of endolysin can be detected at 45 min after infection, halfway through the latent period.

In the present study, gel electrophoretic and autoradiographic techniques were used to detect the temporal sequence of protein synthesis after infection of the sensitive strain A. tumefaciens B6 with phage LV-1.

MATERIALS AND METHODS

Bacterial and phage strains. A. tumefaciens B6, a phage-sensitive strain, originated from R. Klein, University of Vermont. Phage LV-1 was obtained by mitomycin C induction of the lysogenic strain A. tumefaciens V-1, which was originally isolated by R. Hamilton, the Pennsylvania State University. Phage were concentrated and purified by CsCl density gradient centrifugation prior to electrophoresis.

Media and growth conditions. Labelling experiments utilized modified Stonier medium consisting of (per liter): 0.1 g of CaSO4, 0.2 g of MgSO4·7H2O, 0.2 g of NaCl, 2.7 g of NH4NO3, 8.4 mg of Fe(NO3)3·9H2O, 0.1 mg of MnCl2, 0.5 mg of ZnCl2, 10 g of potassium citrate, 2 g of sodium glutamate, 0.34 g of NaH2PO4·H2O, and 0.9 g of KH2PO4 (8). Glucose, sterilized separately, was added to a final concentration of 0.2%. Cultures were incubated at 30°C in a gyratory water bath.

A glucose-yeast extract broth was used for all other experiments (7). These cultures were grown at 30°C on a reciprocal shaker.

Radioactive labeling of proteins. A. tumefaciens B6 was grown to mid-log phase (3 × 10⁹/ml) in Stonier medium. Before and after infection with phage LV-1 (multiplicity of infection of 15), portions of the culture were pulse-labeled for 3 min with 14C-labeled mixed amino acids (New England Nuclear) at a dosage of 2 μCi/ml. Incorporation of label was stopped by adding chilled Casamino Acids (Difco) to a final concentration of 3% and immediately placing the mixture on ice. The cells were then harvested by centrifuging for 10 min at 4300 × g.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. (i) Preparation of samples for disc gels. The cells from 2.5-ml portions of culture were harvested and lysed with 50 μl of sample buffer. The sample buffer, a modification of that described by Laemmli (6), consisted of 0.0625 M sodium phosphate buffer, pH 7.2, containing 2% SDS, 10% glycerol, and 5% β-mercaptoethanol. The samples were heated for 1 min at 100°C to facilitate dissociation of protein subunits.

(ii) Disc gels. Acrylamide gels (10%) were prepared and electrophoresis performed according to the methods of Weber and Osborn (11). Samples (25 to 40 μl) were applied to each gel.

After electrophoresis, gels for scintillation counting...
were frozen and sliced into 2-mm fractions. Each fraction was placed into a vial and solubilized in a 3% solution of Protosol (New England Nuclear). After incubation at 37°C for 48 h, the samples were counted in a liquid scintillation counter.

(iii) Slab gels for autoradiography. Polyacrylamide gels and buffers were prepared using the methods of Studier (9). Samples (10 to 20 μl) were placed into each well of the slab gel. Electrophoresis was carried out at a constant voltage of 35 V for 12 to 14 h. Staining and destaining were the same as for the disk gels. The slab gels were dried under vacuum and placed on Kodak Blue brand medical X-ray film for 10 to 14 days before being developed.

RESULTS

Synthesis of phage LV-1 structural proteins in infected cells. The four major structural phage proteins, A1, A2, A3, and A4, described by De Ley et al. (4) are readily detected by SDS-polyacrylamide gel electrophoresis of purified phage LV-1 (Fig. 1). Only two of these proteins, A2 and A3, can be detected in infected cell extracts against the heavily stained background of host protein (Fig. 2). These two proteins are first observed at 45 min after infection, halfway through the latent period.

Synthesis of major phage-directed proteins in infected cells. Pulse labeling of cells for 3 min with 14C-labeled mixed amino acids (2 μCi/ml) at intervals after infection revealed the pattern of phage protein synthesis in infected A. tumefaciens B6. Attempts were made to shut off host protein synthesis to insure incorporation of the labeled amino acids into phage protein rather than into host protein. UV irradiation, even in low doses, rendered A. tumefaciens B6 virtually incapable of supporting phage-directed protein synthesis. Another indirect attempt to suppress host synthesis was to treat the cells with nalidixic acid (10 μg/ml) shortly before phage infection, but nalidixic acid only partially suppressed host cell synthesis.

Fortunately, one of the early functions of phage LV-1 is to reduce the protein synthesis of the host strain B6. This inhibition is evident as early as 1 min after infection and is quite pronounced by 30 min (Fig. 3), as demonstrated by gel electrophoresis of the labeled samples. By 60 min postinfection, 10 to 12 protein peaks appear, and the major structural proteins are evident.

Synthesis of minor phage-directed proteins in infected cells. Autoradiography was used to detect phage-directed proteins which are synthesized in smaller quantities than could be readily detected by scintillation counting of fractionated disk gels. 14C-labeled extracts of uninfected and phage-infected A. tumefaciens B6 were run on 10 and 15% polyacrylamide slab gels according to the procedures of Studier (9). An autoradiogram of a 10% gel is shown in Fig. 4. Uptake of label was lowest at 15 min after infection, and it was slightly higher in later samples. The intensity of the exposure of the autoradiogram (Fig. 4) indicates that protein synthesis did not return to the preinfection level.

Since host protein synthesis was not entirely stopped, it was sometimes difficult to distinguish phage proteins from residual host proteins. Thirteen new proteins appear to be definitely of phage origin. An additional seven proteins, P1, P3, P5, P8, P10, P11, and P15 (Fig. 5a), could be either phage or host directed. Figure 5a provides an analysis of the time sequence of appearance of the phage proteins. Synthesis of eight of the 13 phage proteins had begun by 15 min after infection. Phage proteins P4, P7, P9, P13, P17, and P18 exhibited intervals of maximal synthesis followed by decreased synthesis, which suggests the existence of control mechanisms.

The major phage structural proteins, A2 and A3, are indicated in Fig. 4. Synthesis of A3 had begun by 15 min, whereas A2 was first detected in the 30-min sample. Either P12 or P13 (Fig. 4) probably corresponds to phage structural protein A1. A4 could not be located because the Tris-glycine buffer system does not allow separation of proteins having molecular weights less than 25,000 on a 10% gel (9).

When the proteins were separated by electrophoresis on a 15% slab gel, four new proteins migrating in advance of P18 could be distinguished on the autoradiogram (Fig. 6). These were designated P19a, P19b, P20, and P21. The times of their appearance are presented in Fig. 5b. Protein P19a is an early protein, which is being synthesized by 15 min postinfection. The other three proteins did not appear until halfway through the latent period.

DISCUSSION

We have found that, after infection of A. tumefaciens B6 with phage LV-1, one of the early functions of the phage is to reduce host protein synthesis. This suppression is evident as early as 1 min after infection, and it is very pronounced by 30 min (Fig. 3). This was a fortuitous discovery since our attempts to artificially suppress host synthesis either had little effect or were so effective that phage protein synthesis was also blocked. However, since this phage-directed suppression was not complete, some of the proteins being synthesized after infection could still represent residual host
synthesis. Such doubtful proteins are designated by a question mark in Fig. 5.

Sixteen, and possibly as many as 24, phage-directed proteins were detected by autoradiography. This represents a substantial portion of the estimated 30 to 40 proteins which the phage DNA should be able to encode. The major structural proteins described by De Ley et al. (4) are tentatively identified on the autoradiograms (Fig. 4 and 6).

A plot of the times of appearance of new proteins suggests that control mechanisms are important in directing synthesis of phage LV-1 proteins (Fig. 5). Proteins P17, P18, and P19a are actively synthesized from 15 to 45 min after infection, after which their synthesis appears to decline steadily. P14 and P16 appear at 30 min whereas P2, P6, P20, and P21 are delayed until 45 min. It is interesting to note that on stained gels (Fig. 2), the two most abundant structural proteins, A2 and A3, were not distinguishable until 45 min after phage infection. However, by autoradiographic methods, A3 can be detected at 15 min and A2 at 30 min (Fig. 4 and 6).

Proteins P19a and P19b, seen in Fig. 6, may be two distinct proteins, or they may represent different forms of the same protein. P19b is vaguely discernible at the leading edge of P19a in the 45-min sample. By 60 min, P19b is predominant, and P19a is very indistinct. Only P19b is evident at 90 min. This suggests that P19b may be a cleavage product of P19a. As P19a is being cleaved at 45 min, the new, smaller protein, P19b, is able to migrate farther, thus appearing at the leading edge of P19a. As more P19a is cleaved and no more is synthesized, it disappears and P19b predominates. Either P19b or P20 is probably equivalent to A4, one of the phage structural proteins.

Although the exact size of agrophage endolysin has not yet been determined (10), it is probably similar to the lytic enzymes coded by the T phage and by coliphage λ. If this is the case, the enzyme probably has a molecular weight of 15,000 to 20,000, and it would be expected to migrate in the lower third of the 15% slab gel. The in vitro assay revealed that endolysin begins to be synthesized at 45 min after phage infection (10), the same time that P19b, P20, and P21 are first detected (Fig. 6).

Different classes of protein synthesis can be detected in phage-infected cells. The early proteins, class I, include one capable of shutting

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**Fig. 1.** Structural proteins of phage LV-1. Phage particles, purified by banding in CsCl, were denatured, and the component proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. The gels were stained with Coomassie Blue. MP, Minor protein.
FIG. 2. The appearance of phage LV-1 structural proteins in extracts of infected A. tumefaciens B6. Proteins in the crude cell extracts were separated by electrophoresis on 10% SDS-polyacrylamide gels and stained with Coomassie Blue. (a) Uninfected; (b) 45 min after infection; (c) 60 min; (d) phage LV-1 structural proteins.
Fig. 3. Protein synthesis in A. tumefaciens B6 before and after infection with phage LV-1 (MOI of 15). Samples were pulsed for 3 min with 14C-labeled mixed amino acids (New England Nuclear) at a dosage of 2 μCi/ml. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels. The gels were then sliced and counted.

Fig. 4. Protein synthesis in A. tumefaciens B6, before and after phage infection, as determined by autoradiography on a 10% slab gel. Cultures were pulsed for 3 min with 14C-labeled mixed amino acids (2 μCi/ml). After electrophoresis, the gel was dried and placed on Kodak X-ray film for a 13-day exposure period. (a) Uninfected; (b) 15 min after infection; (c) 30 min; (d) 45 min; (e) 60 min; (f) 90 min.
Fig. 5. Time sequence of appearance of new proteins after infection of A. tumefaciens B6 with phage LV-1. Dotted, dashed, and solid lines represent increasing amounts of protein being synthesized. Question marks indicate the proteins which could be either host or phage coded. (a) Analysis of phage proteins seen in Fig. 4; (b) analysis of the four smallest proteins separated on the 15% slab gel (Fig. 6).

Fig. 6. Protein synthesis in A. tumefaciens B6, before and after phage infection, as determined by autoradiography of a 15% slab gel. Cultures were pulsed for 3 min with \textsuperscript{14}C-labeled amino acids (2 \textmu Ci/ml). The gel was placed on Kodak X-ray film and exposed for 14 days. (a) and (b) Uninfected; (c) 15 min after infection; (d) 30 min; (e) 45 min; (f) 60 min; (g) 90 min.
off the host protein synthesis as early as 1 min after infection. Fourteen proteins can be detected by radioautography within the first 15 min after infection. An additional five proteins appear in a second class, class II, which are detected after 30 min. The late proteins, class III, which include the endolysin, consist of an additional five proteins that appear 45 min after infection and these continue to be synthesized until the end of the latent period. One of the class I proteins and one of the class II proteins appear to be shut off after 48 min which indicates a regulatory mechanism for turning off protein synthesis as well as initiation. From the concentrations of the bands, periods of increased and decreasing amounts or protein synthesis are observed with the suggestion of the cleavage of one of the proteins, P19.

Further experiments with temperature-sensitive mutants will be needed to determine the function of these proteins.

LITERATURE CITED