Identification of Some Bacteriophage T4 Prereplicative Proteins on Two-Dimensional Gel Patterns

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Profiles of bacteriophage T4 early proteins resolved by a two-dimensional nonequilibrium pH gradient electrophoresis system (P. Z. O'Farrell, H. M. Goodman, and P. H. O'Farrell, *Cell* 12:1133-1142, 1977) are presented. Over 65 phage-induced proteins were resolved. Amber or deletion mutants were used to identify 17 proteins in the gel patterns as the products of specific genes.

The high-resolution, two-dimensional electrophoretic methods of O'Farrell (16) and O'Farrell et al. (18) have provided comprehensive profiles of cellular proteins from a variety of biological systems. Modifications of these methods have been used to analyze membrane proteins (2, 21) and bacteriophage T4 coat proteins (8). Recently, another two-dimensional method has been used for the detection of labile protein phosphorylation modifications (3). The use of two-dimensional electrophoretic methods to monitor the regulation of protein synthesis and in vivo modifications of proteins is well documented and has been summarized (5).

Although two-dimensional electrophoretic separations of bacteriophage T4-coded early proteins have been reported (1, 16), the electrophoretic method has not been widely used for the analysis and identification of proteins in the T4 system. As a part of a study of bacteriophage T4-induced enzymes which synthesize deoxyribonucleotides and T4 DNA, we have applied a two-dimensional, nonequilibrium pH gradient electrophoretic procedure (18) to the separation of the T4-coded prereplicative proteins. Over 65 bacteriophage T4 proteins are resolved; 17 of these proteins have been identified thus far as the products of specific genes. The conditions used to label the T4-coded prereplicative proteins and minor modifications of the electrophoresis procedure are described.

MATERIALS AND METHODS

Bacteria and phage. The sources of *Escherichia coli* B and *E. coli thyA deoB* (GM201), a low-thymine-requiring strain derived from strain B, have been described previously (13). Table 1 lists bacteriophage T4 mutant strains and their sources.

Materials. Materials were obtained from the following sources: ampholines, pH 3.5 to 10, 40% (wt/vol), LKB Instruments Inc., Rockville, Md.; urea, Gold Label grade, Aldrich Chemical Co., Milwaukee, Wis.; acrylamide and bisacrylamide, Bio-Rad Laboratories, Rockville Centre, N.Y.; sodium dodecyl sulfate (SDS)

and Tergitol Nonidet P-40, Sigma Chemical Co., St. Louis, Mo.; $^{35}\text{SO}_4^{2-}$, Amersham Corp., Arlington Heights, Ill.; Millex-HA 0.45- μm filters, Millipore Corp., Bedford, Mass.; and XAR-5 film, Eastman Kodak Co., Rochester, N.Y. The first-dimension gel tubes were cut from Kimble 0.2-ml disposable serological pipettes (inner diameter, 1.5 mm) and were purchased from Arthur H. Thomas Co., Philadelphia, Pa. Molecular weight marker proteins were purchased from the following sources: *E. coli* β -galactosidase, rabbit muscle phosphorylase a, and rabbit muscle glyceraldehyde 3-phosphate dehydrogenase, Boehringer Mannheim Biochemicals, Indianapolis, Ind.; bovine serum albumin (fraction V), rabbit muscle pyruvate kinase, ovalbumin, rabbit muscle aldolase, bovine pancreas DNase I, and β -lactoglobulin B, Sigma Chemical Co.; and bovine pancreas chymotrypsinogen A, Worthington Biochemicals Corp., Freehold, N.J.

Bacteriophage T4 infection and radioactive labeling of T4 early proteins. Cultures of *E. coli* B or strain GM201 were grown at 37°C to a density of 5.0×10^8 cells per ml in Fraser-Jerrel medium (7) supplemented with 5 μg of thymine per ml for GM201. The cells were infected at 30°C at a multiplicity of 8 phage per cell. Infections were terminated after 8 min by pouring the cultures over crushed ice. The cells were collected by centrifugation at 4°C. T4 early proteins were labeled in parallel cultures grown in the minimal salts medium of Vogel and Bonner (22) in which MgCl_2 was substituted for MgSO_4 and Na_2SO_4 was added to a final concentration of 10^{-5} M. Glucose was added to the medium at a final concentration of 0.2%. $^{35}\text{SO}_4^{2-}$ was added at 0.1 mCi per ml of culture 2 min after infection. Infected cells incorporate 8 to 20% of the $^{35}\text{SO}_4^{2-}$ into protein in 8 min under these conditions.

Preparation of sonic extracts and samples for electrophoresis. Cell pellets from 100 ml of unlabeled T4-infected culture and 15 ml of the corresponding $^{35}\text{SO}_4^{2-}$ -labeled T4-infected culture were suspended in 20 mM potassium phosphate buffer, pH 7.0, containing 1 mM dithiothreitol in a final volume of 1.5 ml. Cell suspensions at 0°C were disrupted by six 10-s bursts of sonic oscillation from a Branson 75-W sonicator at setting 3, at 1-min intervals. Unbroken cells and debris were removed by centrifugation at $5,000 \times g$ for 5 min. The protein concentrations in the sonic extracts were determined by the method of Kalb and Bernlohr (10).

TABLE 1. Identification of T4 early proteins on two-dimensional gels

Gene	Protein	(x, y) coordinate, Fig. 2	No. Fig. 3	Mutant strain	Source ^a
43	DNA polymerase	84, 89	1	<i>amb22</i>	a
<i>nrdA</i>	Ribonucleoside diphosphate reductase, α subunit	83, 85	2	<i>nrdAam67</i>	b
<i>rIIA</i>	pRIIA	87, 82	3	<i>rNB2226</i> and <i>r1272</i> (deletions)	c
46	p46	58, 73	4	<i>amN130</i>	a
30	DNA ligase	77, 71	5	<i>amH39X</i>	d
41	RNA priming protein (14, 15)	91, 71	6	<i>amN81</i>	d
39	DNA topoisomerase subunit	71, 70	7	<i>amN116</i>	e
52	DNA topoisomerase subunit	53, 63	8	<i>amH17</i>	a
<i>nrdB</i>	Ribonucleoside diphosphate reductase, β subunit	99, 59	9	<i>nrdBam55</i>	f
32	DNA helix-destabilizing protein	102, 50	10	<i>amA453</i>	a
44	DNA replication protein (19, 20)	71, 50	11	<i>amN82</i>	a
<i>rIIB</i>	pRIIB	82, 48	12	<i>rNB2226</i> and <i>r1272</i> (deletions)	c
<i>td</i>	Thymidylate synthetase	48, 44	13	<i>farP22</i> and <i>farC61</i> (deletions)	g
42	Deoxycytidylate hydroxy- methylase	86, 37	14	<i>amN122</i>	h
1	Deoxyribonucleoside monophosphate kinase	96, 33	15	<i>amC42</i> $\times$ 3	h
<i>ipIII</i>	Internal protein III	22, 32	16	<i>amHA9</i>	i
60	DNA topoisomerase subunit ^b (12)	55, 16	17	<i>amE594</i>	j

^a (a) R. S. Edgar, California Institute of Technology Collection; (b) this laboratory (6); (c) C. Goff, Haverford College, Haverford, Pa.; (d) J. D. Karam, Medical University of South Carolina, Charlotte; (e) L. Snyder, Michigan State University, East Lansing; (f) Y.-C. Yeh, University of Arkansas for Medical Science, Little Rock; (g) D. H. Hall, Georgia Institute of Technology, Atlanta; (h) J. S. Wiberg, University of Rochester, Rochester, N.Y.; (i) L. Black, University of Maryland, Baltimore; (j) H. Bernstein, University of Arizona, Tucson.

^b A. F. Seasholtz and G. R. Greenberg, manuscript in preparation.

Samples for electrophoresis were prepared according to the method of O'Farrell (16) with the following modifications. The sonic extracts were not treated with DNase, and the lysis buffer contained 2% (wt/vol) ampholines in the pH range 3.5 to 10. Sample volumes of 2 to 30 μ l containing 10 to 100 μ g of protein and approximately 0.1 μ Ci of trichloroacetic acid-precipitable ³⁵S activity were loaded on the first-dimension gels.

Two-dimensional electrophoresis. Electrophoresis in the first dimension was carried out with the nonequilibrium pH gradient electrophoresis method of O'Farrell et al. (18) modified as follows. The amount of urea in 10 ml of gel mixture was reduced from 5.5 to 5.1 g to prevent crystallization of urea in the gel. Ampholines in the pH range 3.5 to 10 were added to give the final 2% ampholine concentration. The first-dimension gel mixture was filtered through Millex-HA 0.45- μ m filters to remove possible particulate matter, and the gels were poured batchwise to a height of 10.5 to 11.0 cm. Electrophoresis was carried out for 4 h at 500 V.

After equilibration, the first-dimension gels were transferred to the top of 1.5-mm-thick 10% polyacrylamide gels and immobilized with melted agarose, 1% (wt/vol) in SDS sample buffer (16). The separating and

stacking gels were 9.6 and 2.3 cm, respectively. The buffer system and gel mixtures for the second dimension were those described by Laemmli (11). The gels were stained with Coomassie blue to detect *E. coli* proteins, dried, and exposed to X-ray films for 48 to 90 h. To reduce the background haze on the autoradiograms, 5.25% NaOCl (Clorox) was used to remove the emulsion from the side of the developed X-ray film that had not been in direct contact with the gel. This step improves the contrast between the protein spots and the background.

The pH gradient formed during electrophoresis in the first dimension was measured directly after suspending 0.5-cm segments of the gel in 1.0 ml of deionized water. The samples were mixed vigorously at least three times during 1 h, and the pH was measured with a glass electrode on a Radiometer pH meter, using an expanded scale. Figure 1 shows the pH gradient formed.

Assignments of molecular weights were made for the protein products of genes 43, 46, 32, and 1 in crude extracts and for the purified products of genes *nrdA*, 39, 52, and *nrdB* by single-dimensional SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels. The proteins used as markers and their assigned molecular

FIG 1. pH gradient in the first-dimension gel after electrophoresis for 2,000 volt-hours. Measurements were taken of 0.5-cm segments suspended in 1 ml of deionized water after one hour.

weights in SDS electrophoresis were β -galactosidase, 135,000; phosphorylase a, 92,500; bovine serum albumin, 67,000; pyruvate kinase, 57,000; ovalbumin, 45,000; aldolase, 40,000; glyceraldehyde 3-phosphate dehydrogenase, 36,000; DNase I, 31,000; and chymotrypsinogen A, 25,700. The eight T4-coded proteins listed above were then identified in the two-dimensional gel patterns, and their relative mobilities and assigned molecular weights were used to establish the molecular weight axis presented in Fig. 2.

RESULTS AND DISCUSSION

Identification of phage T4 early proteins. Figures 2 and 3 present the profile of proteins synthesized after infections by *amN122* (gene 42⁻) and *amA453* (gene 32⁻), respectively. Following the convention suggested by O'Farrell (16), the autoradiograms are presented with the basic proteins on the left side. Table 1 lists the T4 proteins identified in the two-dimensional gel

FIG 2. Two-dimensional gel pattern of ³⁵S-labeled early bacteriophage T4 proteins synthesized after infection by *amN122* (gene 42⁻). Conditions for infection, sample preparation, and electrophoresis are described in Materials and Methods. The autoradiogram is presented with the basic proteins on the left side. The pH of the gradient is shown on the top axis. The (x, y) coordinates of identified proteins are presented in Table 1.

FIG 3. Two-dimensional gel pattern of ^{35}S -labeled early bacteriophage T4 proteins synthesized after infection by *amA453* (gene 32 $^{-}$). The numbered protein spots are identified in Table 1. The spots are numbered on the upper left side except where arrows have been used.

system, the mutant strains used, (x,y) coordinates for Fig. 2, and number assignments for Fig. 3. Figure 2 is presented on a grid to facilitate the identification of additional proteins.

Fourteen proteins were identified by the disappearance of a spot in gel profiles from amber mutant strains. For example, the spot corresponding to deoxycytidylate hydroxymethylase, no. 14 in Fig. 3, is clearly missing at (x,y) coordinates (86, 37) in Fig. 2 (infection by *amN122*, a gene 42 mutant). Likewise, only a trace of gene 32 protein is detectable at no. 10 in Fig. 3. The trace quantity of protein may be due to revertants in the *amA453* phage stock or to read-through of the amber mutation. The protein products of *rIIA*, *rIIB*, and *td* were identified by the absence of a protein of the appropriate molecular weight in strains carrying deletion mutations. Although the deletions in these mutant strains extended through several other T4 genes and the gel profiles from these mutants showed some changes, no additional protein identifications could be made. In using amber or deletion mutants for protein identification, it is advantageous to know the molecular weight of

the wild-type gene product, since it is possible that a polar effect by a mutation could alter the synthesis of additional gene products.

The identified T4 proteins were well resolved from nearby proteins and appeared as single discrete spots with the exception of gene 41 protein (no. 6 in Fig. 3), which routinely appeared as a doublet. Both portions of the doublet were absent in the gel pattern from a T4 strain carrying an amber mutation in gene 41 (data not shown). A few additional proteins could be detected when the X-ray films were exposed for longer times. These spots may be T4 proteins that are present in very low amounts or have low levels of sulfur-containing amino acids. Two major wall proteins of the *E. coli* cell continue to be synthesized after infection by T4 (9) and may be present in the two-dimensional gel patterns.

A comparison of Fig. 2 and 3 illustrates the reproducibility of protein locations between two samples. The positions of proteins larger than the product of gene 60 (no. 17 in Fig. 3) were highly reproducible on 10% polyacrylamide gels in the second dimension. The use of higher-percentage polyacrylamide gels resolved some

of the small proteins that ran into the front on 10% polyacrylamide gels and improved the reproducibility of their positions.

The T4 proteins between γ coordinates 75 and 62 in Fig. 2 showed apparent molecular weights which were considerably lower than values reported in the literature (17) for T4 proteins in this range. The assignments of molecular weights were made by comparison with molecular weight markers as described in Materials and Methods and were confirmed by identifying some *E. coli* proteins of known molecular weights in the two-dimensional gels. It may be mentioned here that the product of the *nrdB* gene migrates to an apparent molecular weight of 43,500 (K. S. Cook and G. R. Greenberg, manuscript in preparation), which is somewhat higher than previously reported (4).

The procedure for preparing electrophoresis samples included a low-speed centrifugation step to remove debris from the sonic extract, and the resulting pellet contained only 4% of the total radioactivity. Gel profiles from the pellet fraction showed no proteins which were not found in the supernatant gel profile, though the proteins at coordinates (32, 54), (29, 40), (81, 38), (79, 30), and (105, 23) appeared enriched in the pellet. The procedure for sample preparation also included the addition of unlabeled infected cells to prevent possible loss of any ^{35}S -labeled proteins which were present in low amounts. When ^{35}S -labeled samples were prepared without carrier protein, the gel profiles contained all of the proteins detectable in Fig. 2 and 3, but the intensity of some protein spots was decreased. An alternative method of sample preparation which involves the solubilization of proteins with SDS before the addition of lysis buffer (2) has also been tested. Although this method increased the intensity of certain spots, it resulted in the exclusion of some acidic proteins from the first-dimension gel, even when the ratio of the final percentages of Nonidet P-40 to SDS was 8 to 1. As a result of this difficulty, sample preparation by sonication was used to identify gene products.

The identification of phage T4 proteins presented in this study is being applied to the analysis of the components and structure of the T4 deoxyribonucleotide synthetase complex (G. R. Greenberg and C.-S. Chiu, Fed. Proc. 37:1409, 1978, and manuscript in preparation).

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