

T2 DNA-DEPENDENT SYNTHESIS OF BACTERIOPHAGE-RELATED PROTEINS*

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The synthesis of proteins associated with the infection of *Escherichia coli* by virulent bacteriophage occurs in a regulated temporal sequence. Early events include the cessation of host protein synthesis¹ and the induction of enzymes concerned with the making of phage DNA,^{2, 3} while the later events deal mostly with the synthesis of structural proteins and phage lysozyme.^{4, 5} With the hope of learning more about the way in which phage-induced protein synthesis is regulated, we have developed an *in vitro* amino acid-incorporating system that is completely dependent on added T2 DNA and have used polyacrylamide gel electrophoresis⁶ to identify the products made by this system. The proteins synthesized in the cell-free system resemble those made early after phage infection.

Methods.—Preparation of cell-free extracts: Uninfected *Escherichia coli* B early log cells⁷ were used for the preparation of an amino acid-incorporating system similar to that of Nirenberg and Matthaei.⁸ Frozen cells (18 gm) were mixed with an equal volume of buffer containing 0.01 M Tris-Cl, pH 7.8; 0.01 M MgCl₂; 0.06 M KCl; 0.006 M 2-mercaptoethanol (standard buffer); and 54 μg of crystalline DNase free of RNase (it is important to add the DNase before the cells are broken). The cell suspension was forced through an ice-cold French pressure cell and centrifuged at 20,000 × *g* for 20 min. The pellet was washed with 18 ml of standard buffer, and the supernatant solutions combined and centrifuged at 30,000 × *g* for 30 min. The pellet was discarded and the soluble fraction (S-30) dialyzed against 500 ml of 60% sucrose in standard buffer for 2 hr. The dialyzate, concentrated 2–3 times to about 50 mg of protein/ml, was incubated for 40 min at 37° in 20 ml of a solution containing 50 μmoles of phosphoenolpyruvate (PEP), 300 μg of pyruvate kinase, 50 μmoles of adenosine 5'-triphosphate (ATP), 4 μmoles of guanosine 5'-triphosphate (GTP), 1.5 μmoles each of 20 C¹²-amino acids, and 20 mg of deacylated *E. coli* B transfer RNA. This mixture was dialyzed for 7 hr against 50 vol of standard buffer, divided into small aliquots, and stored in liquid nitrogen. Aliquots were not refrozen and used only once.

DNA-dependent amino acid incorporation: The incubation conditions were similar to those of Revel and Gros.⁹ A typical reaction mixture contained 5 μmoles of Tris-Cl, pH 7.4; 2 μmoles of MgCl₂; 24 μmoles of NH₄Cl; 0.7 μmoles of 2-mercaptoethanol; 1.25 μmoles of PEP; 5 μg of pyruvate kinase; 250 mμmoles of ATP; 50 mμmoles each of GTP, uridine triphosphate (UTP), and cytidine 5'-triphosphate (CTP); 25 mμmoles each of 19 cold amino acids; 16.8 mμmoles of C¹⁴-lysine (10 μc/μmole); 0.8–1.4 mg of S-30 protein; and 20–50 μg of T2 DNA, all in a final volume of 0.135 ml. Incubation was at 37°. Except for the samples which were analyzed by polyacrylamide electrophoresis or used for gel filtration, the reactions were terminated with 2 ml of 7% trichloroacetic acid (TCA), heated 10 min at 90°, filtered through a Millipore filter, washed with 20 ml of 7% TCA containing C¹²-lysine and arginine, placed in a glass vial, covered with 15 ml of Bray's solution,¹⁰ and counted in a Packard scintillation counter at 65% efficiency.

Preparation of T2L DNA: The bacteriophage T2L, grown in wild-type *E. coli* B, strain BO6, was isolated and purified according to the method of Herriot and Barlow.¹¹ A heavy suspension of the purified phage, 362 A₂₆₀ units, was washed twice with 0.9% NaCl, resuspended in 17 ml of 0.01 M Tris-Cl, pH 7.4, and deproteinized by extracting twice with two 15-ml portions of water-saturated phenol. The aqueous layer was dialyzed

for 48 hr against 3 changes of 0.9% NaCl, centrifuged at $34,000 \times g$ for 20 min to remove aggregated material, and divided into small aliquots for storage at -20° .

Polyacrylamide gel electrophoresis: The technique of Davis¹² was used with a water-cooled apparatus manufactured by Buchler Instruments. Electrophoresis was at pH 8.3 in 0.6×12 -cm columns of 7% polyacrylamide at 5 ma/tube. Radioactive samples of the soluble proteins, freed of ribosomes, were lyophilized to dryness and resuspended in 0.3 ml of the sample gel. The time for electrophoresis varies somewhat, and each experiment was stopped when the tracking dye, bromophenol blue, had moved 6.5 cm. The gels were removed from the tubes and allowed to stand overnight in 7% acetic acid to dissolve out the small molecules and short peptides. They were then sliced and dried according to the technique of Fairbanks *et al.*¹³ Kodak RP X-omat medical X-ray film was used to prepare the radioautographs of the dried gels.

Pulse-labeling experiments: Pulse labeling of phage-infected cells was performed as described by Levinthal *et al.*⁶ *E. coli* BO6 was grown at 37° in 50 ml of M-9 medium¹¹ to a concentration of 3×10^8 cells/ml at which point the culture was infected with T2L bacteriophage at a multiplicity of 10. Short pulses, at the times indicated in the figures, were given with C¹⁴-lysine (208 $\mu\text{c}/\mu\text{mole}$) and C¹⁴-arginine (178 $\mu\text{c}/\mu\text{mole}$) at a final concentration of 0.1 $\mu\text{c}/\text{ml}$ for each. Incorporation was stopped by pouring the culture onto crushed ice. The cells were pelleted at $20,000 \times g$, washed, and resuspended in 0.01 M Tris-Cl, pH 7.4, 0.01 M MgCl₂ buffer, and broken by sonic oscillation for 1–2 min. Ribosomes holding incomplete chains were sedimented, and the supernatant solution was concentrated by lyophilization and resuspended in 0.3 ml of sample gel.

Tryptic digestion of the radioactive protein: Labeled samples from *in vitro* and *in vivo* experiments were first purified by gel filtration on Sephadex G-75 (Fig. 5). The pooled low-molecular-weight fraction, region B in Figure 5, was supplemented with 3 mg of bovine serum albumin as carrier protein and enough 50% TCA added to give a final concentration of 7%. The mixture was heated for 10 min at 90° and the precipitate washed twice with 7% TCA and once with a mixture of ethanol:ether (1:1). The pellet was air dried and resuspended in 1.3 ml of 0.05 M (NH₄)₂CO₃. Crystalline trypsin, 100 μg , was added, and each preparation was incubated for 14 hr at 37° . The samples were lyophilized and dissolved in a small volume of water.

Fingerprints: Peptide separation by two-dimensional chromatography and electrophoresis was carried out according to Katz *et al.*¹⁴ Electrophoresis was performed at 2000 v in pyridine:acetic acid:water (1:10:289), pH 3.7. Descending chromatography in *n*-butanol:acetic acid:water (4:1:5) was for 16 hr. The dried papers were exposed to X-ray film for 30 days.

Materials.—Uniformly labeled C¹⁴-arginine and lysine were purchased from Schwarz BioResearch Inc. Nucleoside triphosphates were products of P-L Biochemicals, Inc., and *E. coli* B cells were purchased from Grain Processing Corp. Deacylated tRNA was obtained from General Biochemicals. Crystalline trypsin was from Worthington Biochemical Corp., and crystalline DNase was from Nutritional Biochemical Corp.

Results.—**The incorporating system:** Background incorporation due to cellular DNA was reduced by treating the frozen cells with DNase and by using radioactive arginine and lysine to measure incorporation instead of the commonly used leucine which is incorporated in the absence of added mRNA or DNA.¹⁵ The addition of pancreatic DNase appears necessary despite the presence in these extracts of relatively much larger amounts of *E. coli* DNase activity. Unlike previously described systems,^{16–18} ours is virtually dependent on added DNA, is not stimulated by added purified RNA polymerase, and contains a full complement of the ribosomal initiation factors,^{9, 19, 20} essential for protein synthesis directed by natural mRNA or DNA. T-even bacteriophage DNA efficiently stimulates amino acid incorporation, and the 70-fold increase over the background shown in Table 1 is typical. Conditions that allow RNA synthesis are

TABLE 1. Conditions for the T2 DNA-dependent incorporation of C¹⁴-lysine.

Reaction mixture	C ¹⁴ -Lysine Incorporation	
	Cpm	μμMoles
Complete	6184	423.0
- T2 DNA	94	6.3
- UTP, CTP	556	38.7
- PEP	1930	132.0
+ DNase, 10 μg	78	5.3

The composition of the complete reaction mixture is given in *Methods*. Incubation time was 60 min.

necessary since incorporation was reduced over 90 per cent when UTP and CTP were omitted; addition of DNase completely inhibited incorporation; and, in an experiment not shown, 3.8 μμmoles of actinomycin D gave an 80 per cent inhibition of protein synthesis. Other DNA's, such as salmon sperm, calf thymus, and *Bacillus subtilis*, were only slightly active.

The nature of the proteins synthesized in vitro: Attempts were made to detect the synthesis of phage structural proteins by precipitation with sera from rabbits immunized with T2 bacteriophage. Our experiments, like those of Zillig and co-workers,¹⁷ were negative. As a result, we focused on an examination of the proteins made early after infection.

A detailed analysis of these proteins was accomplished by the elegant poly-

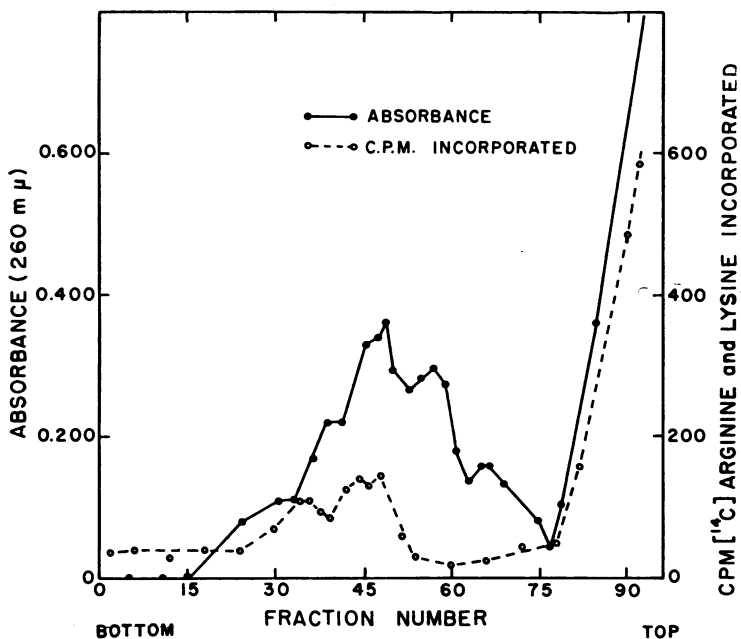


FIG. 1.—Sucrose-density gradient centrifugation of the proteins produced in response to T2 DNA. The reaction mixture (see *Methods*) contained 1.5 mg of S-30 protein and 51 μg of T2 DNA. It was incubated for 20 min at 37° and layered onto a linear gradient of 5–20% (w/v) sucrose in 0.01 M Tris-Cl, pH 7.4, 0.01 M MgCl₂, and 0.05 M KCl. The A₂₆₀ and the hot TCA-precipitable material were determined on alternate fractions.

acrylamide gel electrophoresis technique devised by Fairbanks, Levinthal, and Reeder.¹³ Since an appreciable amount of labeled material remains attached to the ribosome as incomplete protein chains (Fig. 1), the ribosomes were removed by centrifugation at $150,000 \times g$ for one hour and the supernatant solution used for analysis on the polyacrylamide gels. The results of one such experiment are shown in Figure 2. As can be seen from strip C, about 90 per cent of the radioactivity incorporated *in vitro* consists of a single protein well separated from the other phage proteins. Strips D and E show proteins labeled *in vivo* when infected cells are pulsed with radioactive amino acids at the indicated times after infection. These controls bear a resemblance to those of T4-infected extracts similarly analyzed by Levinthal *et al.*⁶ It should be noted that a protein with an electrophoretic mobility like the major band occurs in uninfected *E. coli* B (strip B). However, synthesis of the fast-moving protein is substantially increased early after infection (strip D) and even appears after 13–15 minutes, indicating that its synthesis, though reduced, is not completely turned off. Other proteins made *in vitro* match well with some of the proteins made during the first few minutes of infection.

In order to separate the fast-moving protein from other proteins synthesized *in vitro*, samples were chromatographed on a calibrated Sephadex G-75 column.²¹ The elution pattern is shown in Figure 3. Peak A contains proteins having molecular weights above 60,000, and B contains proteins with molecular weights of about 9,000–10,000. The separated A and B regions were pooled and concentrated before analysis by polyacrylamide gel electrophoresis (Fig. 4). A sample

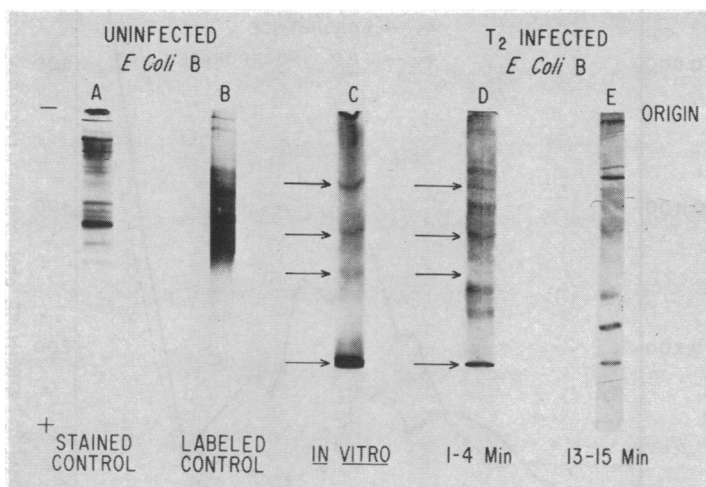


FIG. 2.—(A) *E. coli* B S-30 protein stained with amido black. (B) Uninfected *E. coli* B proteins pulse-labeled for 2 min with C^{14} -arginine and lysine (91,264 cpm). (C) C^{14} -arginine and lysine-labeled proteins from the *in vitro* incubations of a mixture 20 times larger than that described in *Methods* (98,997 cpm). (D) 93,760 cpm and (E) 44,960 cpm of C^{14} -soluble proteins derived from the pulse labeling of *E. coli* B cells with T2L bacteriophage as described in *Methods*.

Dried slices of the gels B, C, and D were exposed to X-ray film for 8 days, and E for 16 days.

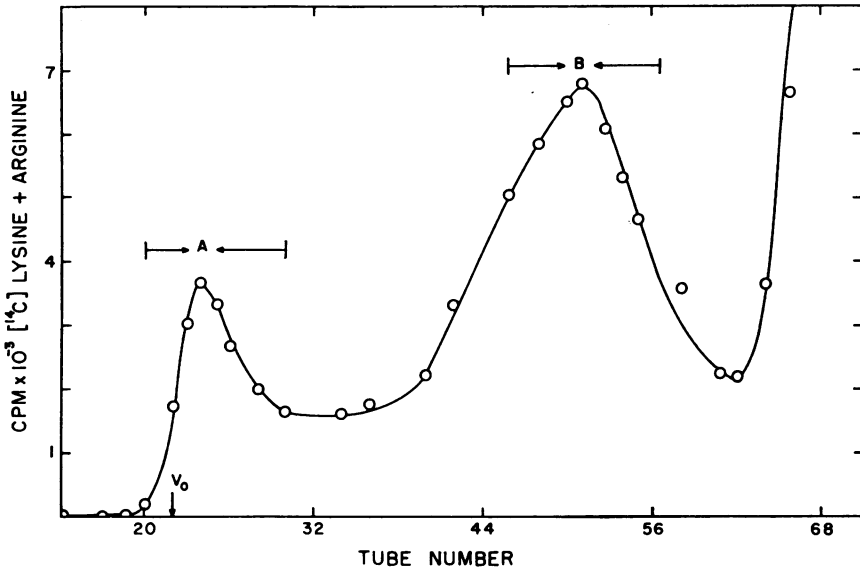


FIG. 3.—Sephadex G-75 chromatography of the soluble radioactive proteins. The reaction mixture described in *Methods* was scaled up 40-fold, incubated 20 min, and stopped by adding 5 ml of chilled 0.01 *M* Tris-Cl, pH 7.4, 0.01 *M* MgCl₂. The ribosomes were pelleted and the supernatant solution applied to a Sephadex G-75 column (51 × 2.8 cm) equilibrated with 0.05 *M* Tris-Cl, pH 7.5, 0.1 *M* KCl. The column was eluted with the same buffer at room temperature, 4.5-ml fractions collected, and the total radioactivity determined for 0.2-ml. aliquots.

of high-molecular-weight C¹⁴-protein derived from the infected cells (1 to 4-min pulse) was included for comparison with the *in vitro* synthesized products. Again good agreement of some bands was found. The high-molecular-weight sample contained some of the low-molecular-weight protein as a contaminant; however, the low-molecular-weight material migrated as a single band (strip C, Fig. 4).

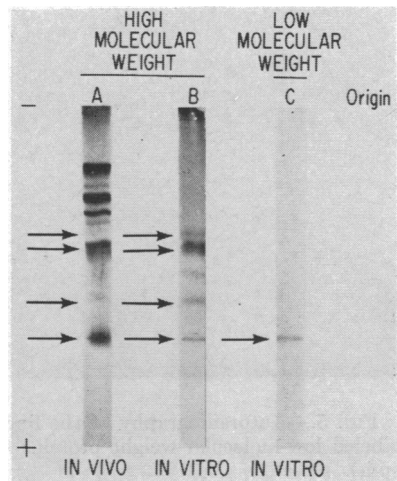


FIG. 4.—(A) Polyacrylamide gel analysis of the high-molecular weight C¹⁴-protein from pulse-labeled phage-infected cells separated by Sephadex filtration (60,000 cpm).

(B) High-molecular-weight C¹⁴-protein made *in vitro* and isolated from region A, Fig. 3 (37,060 cpm).

(C) Low-molecular weight radioactive protein labeled *in vitro* and isolated from region B, Fig. 3 (26,000 gm).

All dried gel slices were exposed to X-ray film for 10 days.

Because the major product of the cell-free system could be obtained easily and in a relatively pure radiochemical form, we isolated large amounts for comparison with similarly isolated protein derived from phage-infected cells pulsed early after infection with C^{14} -arginine and lysine. The labeled low-molecular-weight fraction from the extracts of the infected cells was slightly contaminated with proteins of differing electrophoretic mobilities but consisted mostly of a component that migrated identically with the *in vitro* product. A tryptic digest of each of the proteins was fingerprinted with the results shown in Figure 5. The numbered peptides seem to be clearly present in both the *in vitro* and the *in vivo* preparation. Corresponding peptides vary somewhat in shape and size since two separate fingerprints are compared. Noncorresponding peptides may reflect the extent of contamination of the *in vivo* product with other proteins as well as the presence of some free arginine and lysine in the peptide mixture obtained from the protein synthesized *in vitro*.

Discussion.—Because the system we have developed is capable of making recognizable phage-induced proteins in response to phage DNA, it should prove useful in determining whether regulation of protein synthesis at a particular stage of development occurs during transcription or translation or during both. Other DNA-dependent systems where protein products have been identified^{22, 23} may be less suited for such an analysis since they are designed to measure the synthesis of single proteins.

Considering the experiments of Geiduschek and co-workers,²⁴ who found that only early mRNA is made *in vitro* by the RNA polymerase in response to T-even phage DNA, and the finding that late structural proteins are not made in a T4 DNA-stimulated amino acid-incorporating system,¹⁷ we were not surprised to

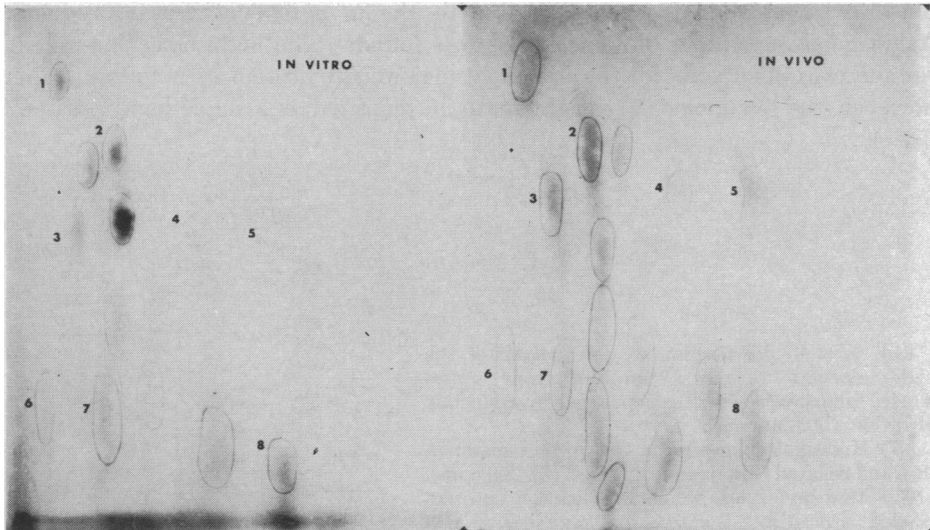


FIG. 5.—Autoradiography of the fingerprints of tryptic digests of C^{14} -arginine and lysine-labeled low-molecular-weight proteins synthesized *in vivo* (106,000 cpm) and *in vitro* (73,190 cpm).

find that the electrophoretic pattern of the proteins made *in vitro* resembled the early rather more than the late proteins. Further restriction of the synthesis of these proteins may be the result of the very active *E. coli* DNases or the added pancreatic DNase.

As yet we have been unable to find a function for any of the proteins made *in vitro*. A comparison of fingerprints of the tryptic peptides from the major *in vitro* product and a similar protein induced *in vivo* suggests that at least this protein is phage-related. We are continuing to look for enzymatic activity associated with these proteins and are using amber mutants of T4 in attempting to locate their position on the genetic map.

Summary.—We have developed a T2 DNA-dependent amino acid-incorporating system which synthesizes several proteins with electrophoretic mobilities similar to phage-related proteins made early after infection. A low-molecular-weight protein represents 80–90 per cent of the *in vitro* product, and its tryptic peptides resemble peptides derived from low-molecular-weight early phage-induced protein.

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