Template Properties of Bacteriophage T4 Vegetative DNA

I. ISOLATION AND CHARACTERIZATION OF TWO TEMPLATE FRACTIONS FROM GENTLY LYSED T4-INFECTED BACTERIA*

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G. STANLEY COX+ AND THOMAS W. CONWAY

From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

The synthesis and template properties of T4 vegetative DNA were studied. The DNA-containing material in lysates of cells taken 20 min past T4 infection sediments in sucrose gradients as two major components. Both fractions function as templates for amino acid incorporation in a DNA-dependent in vitro system (coupled transcription-translation).

The slower sedimenting activity is not present in uninfected cells and appears in wild type T4-infected cells only after 12 min at 30°, shortly after DNA synthesis starts. It is dependent for its activity on an added S-30 fraction from either uninfected or T4-infected cells and is completely inhibited by deoxyribonuclease or rifampin. On a weight basis the slower sedimenting template is about 30 to 70% as active as mature T4 DNA when supplemented with S-30 extracts from uninfected cells.

The spectrum of proteins synthesized in response to the slower sedimenting template is different from that produced in response to mature T4 DNA. In contrast to mature DNA, this template is capable of directing the synthesis of material that precipitates with antiserum directed against whole T4 particles. Thus, it appears capable of directing the synthesis of mRNA for phage structural proteins, i.e. late proteins.

The faster sedimenting component is about 8-fold less active for stimulating amino acid incorporation than mature DNA. Significant amounts of RNA polymerase are associated with this DNA in active transcription complexes, yet polyacrylamide gel electrophoresis of the proteins synthesized in response to this fraction show a pattern that resembles the early proteins made from mature T4 DNA in extracts from uninfected cells.

EXPERIMENTAL PROCEDURE

Growth and Purification of Bacteriophage

Bacteriophage T4D was grown by conventional methods (16) on Escherichia coli B06 at 37° in force-aerated cultures of M-9 medium (16) supplemented with 0.5 g of NaCl/liter. Phage were isolated from lysates by differential centrifugation (17) or by the polyethylene glycol-NaCl method (18).

Infection of Cultures

Cells of E. coli B, strain B06, were grown in the M-9 medium with shaking or forced aeration at 30° to a density of 5 to 107 cells/ml approximated from the optical density at 650 nm. Aeration was

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†Present address, Laboratory of Molecular Biology, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014.
Preparation of Cell-free Extracts

E. coli B cells were grown at 37° in 15 liters of M-9 medium (16) with vigorous aeration, harvested during the early logarithmic phase of growth (1 to 4 x 10^6 cells/ml) by pouring over crushed ice, collected in a continuous flow centrifuge, washed once in standard buffer (0.01 M Tris-Cl, pH 7.4, 0.01 M MgCl2, 0.05 M KCl, and 0.005 M 2-mercaptoethanol), and used for the preparation of an amino acid incorporating system (S-30) similar to that described by Nirenberg and Matthaei (19) or Capocchi (20).

Ribosomes were pelleted by centrifugation of the S-30 fraction (19) at 145,000 x g for 3 hours. The top two-thirds of the supernatant solution was removed (S-145 fraction), dialyzed against standard buffer, and stored in aliquots at -70°. The remaining supernatant fluid was discarded, and the ribosomal pellet resuspended in a few milliliters of standard buffer (NH4Cl replaced KCl at the same concentration) and centrifuged at 20,000 x g for 15 min to remove aggregated material. The particles were further purified by two more cycles of high and low speed centrifugation and finally stored in the above buffer at

The protein concentration in extracts was estimated by the biuret method (21) using bovine serum albumin as the standard.

Preparation of Mature DNA

DNA was extracted from phage suspensions (30 to 50 A260/A280) with redistilled phenol saturated with buffer (0.01 M Tris-Cl, pH 7.4, and 0.10 M KCl) at room temperature and then dialyzed exhaustively against the same buffer. The final preparations showed A260/A280 ratios of 1.80 to 1.95. The concentration of DNA was determined by the method of Dische as described by Ashwell (22) using calf thymus or salmon sperm DNA as standards.

Preparation of T4 Vegetative DNA Fraction S-5

Procedure A—Cells were grown and infected as described above. In some experiments chloramphenicol (50 μg/ml) was added at 10 min past infection to prevent lysis at late times. Generally, at 20 min past infection the culture was chilled by pouring it over crushed ice (0.5 volume) so that the temperature dropped to 0° within a few seconds. The chilled cells were collected by centrifugation at 10,000 x g for 10 min. The supernatant solution was discarded and the pellets resuspended in a small volume of standard buffer to approximately 3 to 6 x 10^6 cells/ml. Egg white lysozyme and EDTA were added to final concentrations of 300 μg/ml and 5 x 10^-4 M, respectively, and the mixture was incubated at 37° for 30 min. If detergent was used (see legends for details) to extract the DNA, it was added at this time and incubation continued for 5 min. Otherwise DNA was extracted by three cycles of freezing and thawing in a Dry Ice-acetone bath. In either case, the resulting lysate was centrifuged at 5,000 x g for 10 min, and the supernatant fluid, designated S-5, was dialyzed overnight at 4° against 200 volumes of 0.01 M Tris-Cl (pH 7.4) containing 0.15 M NaCl. These preparations were generally stored at 0° and used within 1 week as they tended to lose activity if stored frozen.

Procedure B— Cultures of infected cells were chilled as in Procedure A. Then 1/10 volume of 0.01% sucrose in 0.06 M NaCl was added and the cells pelleted by centrifugation at 8700 x g for 10 min. The addition of sucrose greatly reduced clumping and spontaneous lysis of infected cells. Next, the cells were resuspended in a buffer composed of 0.01 M NaCl and 0.10 M Na2S2O3 in 0.01 M Tris-Cl, pH 7.8, which in some experiments also included 20% sucrose. The cell suspension was adjusted so that a 100-fold dilution gave an A260 of about 0.3. To 0.8 ml of this cell suspension was added 0.2 ml of lysis medium (10.1 M Tris-Cl, pH 8.4, 4 mg/ml of egg white lysozyme, and 0.05 mM EDTA, pH 8) and the mixture incubated at 0° for 10 min. Then 1.0 ml of lysis medium 2 (0.2 M KCl/0.01 mM EDTA) was added and incubation continued for another 10 min at 0°. The cells were then subjected to three cycles of freezing and thawing. In some experiments lysis medium 2 also contained detergent, either 0.4% Na deoxycholate-SO4 or

\[\text{1.0% Brij 58 (Atlas Chemical Ind.)}\]. In these instances the second incubation was carried out at 30° for 10 min. For S-5 preparation the lysate was centrifuged and the supernatant solution dialyzed as described in Procedure A. For sucrose gradient analysis the lystate was treated as described below.

Sucrose Gradient Analysis

Infected cells were treated as described above in Procedure B through the freeze-thaw step, and the lysates layered directly onto sucrose gradients. If the gradient fractions were to be assayed for template activity, the cell concentration for lystate preparation was about 3 to 6 x 10^6 cells/ml. If the DNA was radioactive, the cell concentration was less than 2 x 10^6 cells/ml.

Sucrose gradients were constructed from neutral solutions of 10% and 40% sucrose made up in buffer containing 0.01 M Tris-Cl, pH 7.8, 0.1 M KCl, 1 mM EDTA, and 2 mM 2-mercaptoethanol. Underlying the gradient (24 ml) was a bottom layer (4 ml) of 70% sucrose to prevent the pelleting of fast sedimenting material at the bottom of the tube. About 1.0 ml of lystate was layered on top of the gradient using a wide bore pipette. Centrifugation was for 30 min at 15,000 rpm in a Spincos SW25.1 rotor at 15°. If the fractions were to be assayed in the in vitro system, the tubes were pierced with a 1.5-mm (inner diameter) cannula in order to reduce mechanical shearing of the DNA. In some experiments the visible material was carefully inserted into a Pasteur pipette through the upper sucrose solution and gently sucking up the DNA layered on the 70% sucrose shelf (pad DNA). It was either used directly or dialyzed against 0.01 M Tris-Cl (pH 7.5) prior to the remove the sucrose. Samples containing radioactivity were either counted directly or precipitated with ice-cold 7% trichloroacetic acid and filtered and counted as described below for RNA samples.

Thymidine Incorporation

E. coli B cells were grown in M-9 medium at 30° and infected as described above. At 4 min after infection, a portion of the culture was transferred to an aerated tube containing 2 μg/ml of [CH3-3H] thymidine (18.1 Ci/mmol), 1 μg/ml of unlabeled thymidine, and 500 μg/ml of uridine. At the desired times 0.5 ml of culture was transferred to 0.5 ml of 10% trichloroacetic acid at 0°. The resulting precipitates were filtered on Millipore filters, washed with 20 ml of ice-cold 5% trichloroacetic acid, and counted in scintillation fluid (23). For sucrose gradient analysis the cultures were treated as described above.

Assay for Protein and RNA Synthesis

The composition of reaction mixtures used for protein synthesis was as follows: Tris-Cl (pH 7.8), 50 mM; MgCl2, 10 mM; NH4Cl, 33 mM; KCl, 50 mM; dithiothreitol, 3.0 mM; 2-mercaptoethanol, 4.7 mM; ATP, 2.0 mM; GTP, CTP, UTP, 0.5 mM each; 19 °C-serine, 0.2 mM each; S-30 protein, DNA, and [14C]labeled amino acid as indicated in the table and figure legends. Reactions were carried out in a final volume of 0.150 ml and incubation was for 20 min at 37°. Except for the samples to be analyzed by polyacrylamide gel electrophoresis or those incubated with antisem, the reactions were terminated with 2 ml of 7% trichloroacetic acid and radioactivity determined as described previously (24).

Reaction mixtures for RNA synthesis contained the following: Tris-Cl (pH 7.8), 50 mM; MgCl2, 10 mM; KC1, 167 mM; 2-mercaptoethanol, 4.7 mM; ATP, CTP, UTP, 0.50 mM each; S-30 or S-145 protein, DNA, and [14C]GTP as indicated in the table and figure legends. The final volume of incorporation mixtures was 0.150 ml and incubation was at 37° for 5 to 10 min. The reaction was stopped by adding 2 ml of ice-cold 7% trichloroacetic acid and placing the tubes in ice. The precipitates were collected on 2.4 cm Whatman glass fiber filters (GF/C) and washed with 20 ml of ice-cold 7% trichloroacetic acid. The filters were placed in scintillation vials, dried in an oven, and counted in a toluene-based scintillation fluid.

Immune Assay

The source of antibody against T4 phage was unfractionated serum prepared from rabbits receiving several injections of a purified phage suspension. Incubations were terminated by adding 10 μg of chloramphenicol to in vitro reaction mixtures, and radioactivity which could be precipitated with antiserum against whole T4 phage was determined in the following manner. In vitro extracts or in vitro reaction mixtures were incubated with diluted anti-T4 serum and 2 to 4 A260 units of T4 DNA were added.
phage as carrier in a total volume of 0.50 ml at 37° for 1 hour and then at 4° overnight. Sufficient antiserum (diluted with buffer containing 10 mM Tris-Cl (pH 7.4) /0.15 M NaCl) was added to precipitate the carrier phage. Exact details are given in the table and figure legends. The visible precipitate was centrifuged for 10 min at half speed in a clinical bench top centrifuge. The pellet was washed 3 times in 0.5 ml of 0.01 M Tris-Cl (pH 7.4) buffer containing 0.15 M NaCl and 1 mg/ml each of [14C]-arginine and [14C]-lysine. The final pellet was allowed to dissolve overnight in 0.20 ml of 0.1 M NaOH at room temperature. This was neutralized by adding 0.20 ml of 10 M HCl followed by precipitation with 2 ml of 7% trichloroacetic acid. The tubes were heated at 90° for 10 min, filtered on Millipore filters, washed with 20 ml of 7% trichloroacetic acid, and counted in Bray's (23) scintillation fluid.

**Polyacrylamide Gel Electrophoresis**

The 14C-labeled proteins synthesized in vitro were analyzed in two different electrophoresis systems. The first system was that of Davis (25) as described by Celis (26). Reaction mixtures were scaled up 4-fold (0.60 ml) and incubation stopped by adding 1.4 ml of an ice-cold buffer containing 0.01 M Tris-Cl (pH 7.4), 0.01 M MgCl2, 0.001 M [14C]-lysine, and 0.001 M [14C]-arginine. After the ribosomes were removed by centrifugation at 145,000 x g for 2.5 hr at 4°, the supernatant fluid was dialyzed overnight against 0.01 M Tris-Cl (pH 7.4)/0.01 M MgCl2 buffer. It was then frozen and lyophilized to dryness. The residue was dissolved in 0.2 to 0.3 ml of the above buffer containing 10% sucrose.

The stacking and separating gels, electrode buffer, and other details were as described (25), with the exception that samples were layered onto the stacking gel in 10% sucrose instead of being polymerized in a sample gel. Electrophoresis was carried out toward the anode at 30 V/cm. After the bromphenol blue marker had migrated 10 cm, the proteins were fixed by placing the gel in a 10% acetic acid, and left overnight at room temperature. Gels were frozen and sliced laterally into 1.5-mm sections with a wire jig. The sections were placed in scintillation vials with 0.2 ml of scintillation mixture containing 10% acetic acid, 20% 2-mercaptoethanol, and 40% glycerol in toto reaction mixtures that were scaled up 2-fold (0.300 ml). Extracts from infected cells were treated similarly. Samples were heated in boiling water for 90 s just before analysis.

The final concentrations in the stacking gel were 2.5% (w/v) acrylamide, 0.625% (w/v) N,N'-methylenebisacrylamide, 0.37% (w/v) N,N',N',N'-tetramethylethylenediamine, 0.1% (w/v) Na dodecylSO4, 0.125 M Tris-Cl (pH 6.7), 2.0% (w/v) sucrose, and 0.0005% (w/v) riboflavin to catalyze polymerization. The separating gels contained 0.125 M Tris-Cl (pH 6.7), 20% (w/v) sucrose, and 0.0005% (w/v) Na dodecylSO4, 0.375 M Tris-Cl (pH 8.8), and 0.7% (w/v) ammonium persulfate to catalyze polymerization. The electrode buffer (pH 8.3) was 0.25 M Tris, 0.192 M glycine, and 1% (w/v) Na dodecylSO4.

Electrophoresis was carried out at 30 V/cm. After the bromphenol blue marker had migrated 10 cm, the proteins were fixed by placing the gel in a 10% acetic acid, and left overnight at room temperature. Gels were frozen and sliced laterally into 1.5-mm sections with a wire jig. The sections were placed in scintillation vials with 0.2 ml of scintillation mixture containing 10% acetic acid, 20% 2-mercaptoethanol, and 40% glycerol in toto reaction mixtures that were scaled up 2-fold (0.300 ml). Extracts from infected cells were treated similarly. Samples were heated in boiling water for 90 s just before analysis.

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**Observation of Template Activity in Crude Extracts from Bacteriophage T4-infected Cells**—Initial attempts to incorporate amino acids with extracts prepared according to Snyder and Geiduschek (15) were unsuccessful. It was found that virtually all of the template activity was removed by a low speed centrifugation (20,000 x g for 15 min) used to clear cellular debris from the lysate before analyzing it on a sucrose gradient (Table I, Experiment 1). However, by using a slower centrifugation (Table I, Experiment 2) it was possible to leave at least some of the template activity in solution.

The modified procedure consists of a lysozyme-freeze-thaw treatment of cells harvested 18 to 20 min after infection at 30°, followed by centrifugation of the lysate at 5,000 x g for 5 min to remove cellular debris and unlysed cells, instead of the 20,000 x g centrifugation used by Snyder and Geiduschek (15). The supernatant fraction is then dialyzed as described under “Experimental Procedure” and used as template in the transcription-translation system (Table I, Experiment 2). This 5,000 x g supernatant fraction will be designated as the S-5 fraction.

To determine if the template activity in the S-5 fraction was phase-specific or also existed in extracts from uninfected cells, similar lysates were prepared from uninfected cells and assayed for amino acid incorporation. Table II shows that the activity from T4-infected cultures is over 30-fold higher than that from uninfected cells. The lack of activity in extracts of the uninfected cells is in agreement with the observation that, unlike T4 DNA, Escherichia coli DNA does not serve as an efficient DNA template in the coupled in vitro system (26, 30).

To further clarify the nature of the template component in the S-5 and to determine whether the activity was a consequence of DNA or mRNA species present in the extracts, DNase and rifampin (an inhibitor of E. coli DNA-dependent RNA polymerase initiation) were added to in vitro reaction mixtures.

**TABLE I**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fraction incorporated (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>(a) Pellet of whole lysate centrifuged through sucrose gradient</td>
<td>2211</td>
</tr>
<tr>
<td>(b) Pellet of whole lysate centrifuged at 20,000 x g for 15 min</td>
<td>4647</td>
</tr>
<tr>
<td>(c) Pellet of 20,000 x g supernatant centrifuged through sucrose gradient</td>
<td>182</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Supernatant fraction of whole lysate centrifuged at 5,000 x g for 15 min</td>
<td>1873</td>
</tr>
</tbody>
</table>

**Materials**

The [U-14C]-Lysine (310 mCi/mmol), [U-14C]-Arginine (312 mCi/mmol), [8-14C]-GTP (38 mCi/mmol), and [14C]-Histidine (18 Ci/mmol) were purchased from Schwarz-Mann. Polyethylene glycol (M, 6000 to 7500) was from J. T. Baker Chemical Co. Eastman Organic Chemicals was the source of all reagents used for polyacrylamide gel electrophoresis. Nucleoside triphosphates were obtained from P-L Biochemicals. Pyruvate kinase, egg white lysozyme, phosphoenolpyruvate, and unlabeled amino acids were purchased from Sigma Chemical Co. All other chemicals were reagent grade and purchased from commercial sources.

**RESULTS**

**Observation of Template Activity in Crude Extracts from Bacteriophage T4-infected Cells**—Initial attempts to incorporate amino acids with extracts prepared according to Snyder and Geiduschek (15) were unsuccessful. It was found that virtually all of the template activity was removed by a low speed centrifugation (20,000 x g for 15 min) used to clear cellular debris from the lysate before analyzing it on a sucrose gradient (Table I, Experiment 1). However, by using a slower centrifugation (Table I, Experiment 2) it was possible to leave at least some of the template activity in solution.

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To further clarify the nature of the template component in the S-5 and to determine whether the activity was a consequence of DNA or mRNA species present in the extracts, DNase and rifampin (an inhibitor of E. coli DNA-dependent RNA polymerase initiation) were added to in vitro reaction mixtures.
mixtures primed with mature T4 DNA or the S-5 fraction. The results of this experiment are presented in Table III. Pancreatic deoxyribonuclease (10 µg) or rifampin (1 µg) inhibited 

\[ \text{DNA template activity in S-5 fractions from uninfected and T4-infected cells} \]

Cells of Escherichia coli B06 were grown in M-9 medium at 30°. At a concentration of 6 to 8 x 10⁸ cells/ml the culture was divided and one-half was infected with T4 as described under "Experimental Procedure." The S-5 extracts were prepared from both cultures according to Procedure A (including 0.2% Na dodecyl-SO₄). The reaction mixtures for protein synthesis contained 1.6 nmol of \[^{14}C\text{Lysine (20 µCi/µmol), 0.95 mg of S-30 protein, and 0.025 ml of the indicated extract, corresponding to 125 µg and 140 µg of protein for the uninfected and infected extracts, respectively. Incubation was at 37° for 20 min. Incorporation in the absence of S-5 was 2.3 pmol of \[^{14}C\text{Lysine.} \]

\begin{table}[h]
  \centering
  \begin{tabular}{|c|c|c|}
    \hline
    \textbf{Source of S-5 extract} & \textbf{[^{14}C\text{Lysine incorporated}}} & \textbf{Relative specific activity\textsuperscript{a}} \\
    \hline
    Uninfected E. coli B06, \text{2.0 µg of DNA} & 2.6 & 0.03 \\
    T4-infected E. coli B06, \text{7.5 µg of DNA} & 318 & 1.00 \\
    \hline
  \end{tabular}
  \caption{DNA template activity in S-5 fractions from uninfected and T4-infected cells}
  \textsuperscript{a}Specific activities (picomoles of [^{14}C\text{Lysine incorporated}/µg of DNA) were normalized to the T4 extract.}
\end{table}

The S-5 fractions were prepared from T4-infected cells according to Procedure A. Reaction mixtures for protein synthesis contained 1.6 nmol of \[^{14}C\text{Lysine (103 µCi/µmol), 0.90 mg of S-30 protein, and 10 µg of mature T4 DNA or 5 µg of S-5 DNA in a final volume of 0.150 ml. Pancreatic deoxyribonuclease or rifampin was added at time zero as indicated. Incubation was at 37° for 25 min.

\begin{table}[h]
  \centering
  \begin{tabular}{|c|c|c|}
    \hline
    \textbf{Template} & \textbf{Additions} & \textbf{[^{14}C\text{Lysine incorporated}}} \\
    \hline
    None & & 820 \\
    Mature T4 DNA & & 164,000 \\
    Mature T4 DNA & DNase, 10 µg & 790 \\
    Mature T4 DNA & Rifampin, 1 µg & 910 \\
    S-5 & & 21,190 \\
    S-5 & DNase, 10 µg & 350 \\
    S-5 & Rifampin, 1 µg & 640 \\
    \hline
  \end{tabular}
  \caption{DNA nature of S-5 template component}
  \end{table}

FIG. 1. The kinetics of appearance of intracellular S-5 template compared to the rate of DNA synthesis in T4-infected Escherichia coli B. Cells were grown in M-9 medium at 30° and infected with T4 phage when the density reached 5.3 x 10⁸ cells/ml. At the times indicated, 400-ml portions of the culture were chilled and the cells collected for preparation of S-5 fractions according to Procedure A (freeze-thaw only). Protein synthesis (△), 5-ml samples of the culture were withdrawn and pipetted into 0.6 ml of 50% trichloroacetic acid at 0°. The precipitates were centrifuged at 12,000 x g for 10 min (2°) and the pellets washed once with cold 7% trichloroacetic acid. The washed pellets were resuspended in 2 ml of 7% trichloroacetic acid and heated for 20-min intervals at 95°. Particulate material was removed by centrifugation and DNA in 1.0 ml of the supernatants was determined by the diphenylamine reaction (22). The data plotted were corrected for the contribution of E. coli DNA (5.2 µg/ml of culture), which was determined from a sample taken before phage addition.

extracts prepared by the more gentle freeze-thaw technique without employing ionic detergents or elevated temperatures. This approach was used since it was thought that the harsher extraction procedures could inactivate non-DNA components in the extract which might otherwise influence the expression of the DNA in vitro.

Sucrose Gradient Analysis of Vegetative Templates—As stated above, initial experiments with the preparations from gently lysed cells suggested that the DNA template was either very large or in a condensed structure, or was attached to cellular components that caused the material to be pelleted by relatively low speed centrifugation. The finding that only about 20% of the total cellular DNA is in the S-5 fraction

\textsuperscript{1}Unpublished observations.
prompted experiments to determine the template properties of the remaining DNA components and to clarify their relationship to the S-5 fraction.

Consequently, lysates of [3H]thymidine-labeled T4-infected cells (20 min past infection) were prepared by the lysozyme-freeze-thaw technique (without detergents) and analyzed by centrifugation through sucrose density gradients. Each gradient was assayed for acid-precipitable [3H]thymidine material absorbing at 260 nm, and for DNA template activity in the cell-free amino acid-incorporating system. Fig. 2 reveals that the component that sediments to the 70% sucrose shelf, which is shown at about fraction 0.9 in Fig. 2. However, since whole cells and unlysed spheroplasts would populate the sedimented fraction (31), it was not studied further.

A similar lysate was prepared from unlabeled T4-infected cells. One-half was centrifuged at 5000 x g for 5 min to prepare an S-5 fraction. The unfraccionated half of the lysate and the S-5 fraction were then layered onto sucrose gradients. Fig. 3 shows that the component that sediments to the 70% sucrose shelf, called the “pad” fraction, is absent in the 5000 x g supernatant indicating that the slower sedimenting material contains the S-5 DNA template. Assuming that these two major fractions contain all of the T4-specific intracellular DNA present at 20 min past infection (30”), one can estimate that approximately 65 to 80% of the DNA sediments as the pad fraction, and 20 to 35% is present in the slower sedimenting form.

Approximate sedimentation coefficients were calculated for the two species relative to a T4 phage marker from these and similar gradients which were centrifuged for only 10 min so that both species remained above the 70% sucrose pad. These are 150 S and 2000 S for the slow and fast sedimenting components, respectively. The effect of the DNA concentration on the sedimentation rate was not investigated, but in one analysis where the DNA concentration was lowered 10-fold, the sedimentation was about 5% faster. The approximate nature of the sedimentation constants determined in this study should be emphasized. It is difficult to compare the sedimentation rate of linear DNA to that of whole phage due to obvious differences in the frictional coefficients of such particles. However, if the replicative DNA is in a compact rather than linear form, as suggested by Huberman (32), this comparison may be legitimate.

Stimulation of Protein Synthesis by S-5 and Pad Components—Because of the crude state of the preparation containing the template activity, it was thought that it might contain all of the components necessary for DNA-dependent protein synthesis. However, Table IV shows that it is dependent for its activity on added fractions from uninfected cells. It is noted that both ribosomes and a soluble high speed supernatant fraction (S-145) are required and that maximal incorporation is obtained with the S-30 extract. Since any ribosomes present in the S-5 fraction might have been destroyed during dialysis in the absence of magnesium (see “Experimental Procedure”), the experiment was repeated with an S-5 that had been dialyzed against standard buffer, which contains 10 mM MgCl₂. The same results were obtained.

Fig. 4 shows the effect of increasing the DNA concentration

FIG. 2. Sucrose gradient analysis of two template components in lysates from T4-infected cells. Escherichia coli B cells were grown, infected with T4 phage, and labeled with [CH₃-3H]thymidine as described under “Experimental Procedure.” The lysates were prepared according to Procedure B (freeze-thaw only); final cell concentration was 1.6 x 10⁹ cells/ml. Then 1 ml of the lysate was centrifuged through a 25-m1 to 40% sucrose gradient over a 5-m1 70% sucrose pad and fractionated as described under “Experimental Procedure.” Buffer for the sucrose solutions contained 1 M NH₄Cl [CH₃-3H]thymidine (□—□) was determined by pipetting 50 μl of each fraction onto 2.4-cm circles of Whatman No. 3MM paper supported on steel pins. After drying, the discs were washed three times in 7% trichloroacetic acid, dried, and counted in toluene scintillation fluid. The absorbance at 260 nm (□—□) was determined on 50-μl aliquots diluted in 3.0 ml of distilled water. Amino acid incorporation (△—△) was determined in reaction mixtures containing 4.6 nmol of [³⁵S]arginine (50 μCi/μmol), 1.0 mg of S-30 protein, and 0.015 ml of each gradient fraction in a final volume of 0.150 ml. Incubation was at 37° for 20 min. Recovery of tritium and A₂₆₀ material were both 90 to 100%.

FIG. 3. Sedimentation analysis of DNA templates in S-5 fractions and unfractionated lysates from T4-infected Escherichia coli B. Infected cells (3.1 x 10⁹ cells/ml) were lysed according to Procedure B (freeze-thaw only). One-half of the lysate was centrifuged at 5000 x g for 5 min. Then, 1 ml of the S-5 fraction and 1 ml of the unfractionated lysate were separately analyzed on sucrose gradients (see “Experimental Procedure” and Fig. 2). The gradients contained 0.1 M KCl. Absorbance at 260 nm (□—□) was meaured as in Fig. 2. Assay for template activity (△—△) was carried out in reaction mixtures containing 1.6 nmol of [³⁵S]lysine (310 μCi/μmol), 0.95 mg of S-30 protein, and 0.015 ml of each gradient fraction in a final volume of 0.150 ml. Incubation was at 37° for 20 min.
TABLE IV
Stimulation of vegetative DNA-dependent amino acid incorporation by fractions from uninfected cells

The S-5 fraction was prepared according to Procedure A. Reaction mixtures for protein synthesis contained 14.0 nmol of [\(^{14}C\)]lysine (20 \(\muCi/\mumol\)), 10.0 \(\mug\) of mature T4 DNA, or 5.75 \(\mug\) of S-5 DNA. These were supplemented with 1.24 mg of S-30 protein, 0.70 mg of S-145 protein, or 0.35 mg of ribosomes as indicated, all in a final volume of 0.150 ml. The ribosomes and S-145 fraction were prepared from uninfected cells as described under “Experimental Procedure.” Incubation was at 37\(^\circ\) for 30 min.

<table>
<thead>
<tr>
<th>Source of template</th>
<th>Fraction from uninfected cells</th>
<th>([^{14}C)]lysine incorporated cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mature T4 DNA</td>
<td>None</td>
<td>198</td>
</tr>
<tr>
<td>Mature T4 DNA</td>
<td>S-30</td>
<td>9695</td>
</tr>
<tr>
<td>S-5</td>
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<td>188</td>
</tr>
<tr>
<td>S-5</td>
<td>S-30</td>
<td>4341</td>
</tr>
<tr>
<td>Mature T4 DNA</td>
<td>S-145 + ribosomes</td>
<td>1236</td>
</tr>
<tr>
<td>S-5</td>
<td>S-145</td>
<td>128</td>
</tr>
<tr>
<td>S-5</td>
<td>Ribosomes</td>
<td>107</td>
</tr>
<tr>
<td>S-5</td>
<td>S-145 + ribosomes</td>
<td>518</td>
</tr>
</tbody>
</table>

FIG. 4. Effect of increasing amounts of mature T4 and S-5 DNA on amino acid incorporation. The S-5 extract was prepared according to Procedure A (freeze-thaw only). Reaction mixtures for protein synthesis contained 1.6 nmol of [\(^{14}C\)]lysine (50 \(\muCi/\mumol\)), 0.93 mg of S-30 protein, and DNA as indicated. Incubation was at 37\(^\circ\) for 30 min. Note the change in scales for the quantities of DNA added; \(\bullet\), mature T4 DNA; \(\Delta\), S-5 DNA.

on [\(^{14}C\)]lysine incorporation for both mature and S-5 DNA (note the change in scales). In both cases the amount of amino acid incorporated was proportional to the amount of DNA added until saturation was reached at around 10 to 12 \(\mug\) of mature DNA and 2 \(\mug\) of S-5 DNA/reaction mixture. Under conditions where DNA is limiting, the S-5 template is generally about 50% as active based on DNA content as mature DNA for stimulating amino acid incorporation in S-30 extracts from uninfected cells.

To check whether components of the S-5 other than the DNA itself might have caused the abrupt saturation at such low DNA concentrations, an S-5 extract was sonicated to shear the DNA and eliminate its template activity. The sheared extract was added to reaction mixtures which contained mature DNA or unsheared S-5 and compared to control mixtures which contained mature or S-5 DNA but no sonicate. There was no apparent inhibition under these conditions. However, it should also be mentioned that some of the extracts prepared with Na dodecyl-SO\(_4\) were maximally active at concentrations comparable to those for mature DNA.

The stimulation of amino acid incorporation in response to the pad fraction was also examined. As seen in Fig. 5, this fast sedimenting fraction is less active than mature DNA, but the response was linear with respect to the amount of DNA added, even at 16 \(\mug\) of DNA/reaction mixture. This is in sharp contrast to the response produced by mature DNA and S-5 DNA where saturation is reached at lower concentrations (Fig. 4). Incorporation is completely sensitive to DNase under the assay conditions.

The kinetics of protein synthesis in response to S-5 and mature DNA are presented in Fig. 6. As seen, reaction mixtures supplemented with either DNA incorporate lysine linearly for about 20 min. The rate of incorporation with mature DNA, however, is about four times greater than that with the S-5 extract and the extent of incorporation is about 2-fold higher.

Because amino acid incorporation in this system is dependent on the synthesis of messenger RNA, the rate of RNA synthesis using the different DNA templates was compared. The data in Fig. 7 shows that the rate and extent of GTP incorporation is lower in reaction mixtures primed with DNA present in the pad fraction than in those primed with mature DNA.

RNA Polymerase Associated with Vegetative DNA—The unusually large sedimentation coefficient of the pad fraction (1700 to 2300 S) suggested a resemblance to other fast sedimenting, DNA-containing particles such as those observed by Altman and Lerman (33) from T4-infected cells and by Stonington and Pettijohn (34) from uninfected E. coli. Al-
FIG. 6. Rate of protein synthesis in response to mature and S-5 DNA. The S-5 fraction was prepared by Procedure A (freeze-thaw only). In vitro reaction mixtures contained 1.6 nmol of [14C]lysine (100 μCi/μmol), 0.88 mg of S-30 protein, and either 3.4 μg of mature T4 DNA (○-○), 3.2 μg of S-5 DNA (O--O), or no DNA (A--A) as indicated. The final reaction volume was 0.150 ml and incubation was at 37° for the times indicated.

FIG. 7. Rate of RNA synthesis in response to mature and pad DNA. The pad fraction was isolated in sucrose gradients as described in Fig. 3. Reaction mixtures for RNA synthesis contained 2.6 nmol of [3H]GTP (38.2 nCi/μmol), 0.20 mg of bovine serum albumin, 0.025 ml of each gradient fraction, and 2.5 μg of rifampin where indicated. Incubation was for 10 min at 37° in a total volume of 0.150 ml. Symbols indicate the absence (O) or presence (O) of rifampin in the reaction mixtures.

though there may be several explanations for the fast sedimenting behavior of these particles in sucrose gradients, it is possible that the DNA is attached to cellular components. Thus, the “bottom” component of Altman and Lerman (33) probably consists of a DNA-protein complex since it equilibrates in CsCl gradients in a broad band with a density spectrum between 1.25 and 1.40 g/ml, a value far lower than that expected for pure T4 DNA (ρ = 1.70 g/ml).

The compact structure isolated from E. coli D10 by Stonington and Pettijohn (34), which contained nascent RNA chains and core RNA polymerase in addition to DNA, prompted the experiment illustrated in Fig. 8. A lysate from infected cells was centrifuged through a sucrose gradient and fractionated as described under “Experimental Procedure.” Then an aliquot of each fraction was assayed for its ability to incorporate [3H]GTP into acid-insoluble material in the absence of either added DNA or RNA polymerase. A duplicate assay was carried out in the presence of 2.5 μg of rifampin, a drug which inhibits initiation of RNA synthesis by the E. coli DNA-dependent RNA polymerase but not elongation of RNA chains started before its addition (35). The results show RNA polymerase activity in the absence of the drug at both the S-5 and pad positions in the gradient (compare to Fig. 3). However, the RNA polymerase activity associated with the pad fraction is about 85% resistant to the action of rifampin whereas that associated with the top fractions is completely inhibited by the drug. This result is in agreement with data presented earlier showing that S-5 DNA is rifampin-sensitive (Table III). When the salt concentration of the gradient was raised from 0.1 to 1.0 M KCl, the RNA polymerase activity in the pad fraction was still 50% resistant to the action of rifampin. This resistance at a high salt concentration strongly suggests that the polymerase is not only bound to the DNA in these fractions but has initiated RNA chains (36, 37). It is suspected that the polymerase lacks the E. coli sigma subunit because this factor dissociates from the core polymerase upon initiation (38) and appears to be lost as a result of T4 infection (39, 40).

The kinetics of RNA synthesis by DNA-dependent RNA polymerase in the pad fraction is shown in Fig. 7. Incorporation is substantial with the pad fraction alone, but can be stimulated 3- to 4-fold by the addition of S-145 protein.

Protein Products Synthesized in Vitro in Response to Vegetative DNA—The nature of the polypeptide products synthesized in response to the vegetative DNA templates was of considerable interest since Brody et al. (11) have shown that the E. coli DNA-dependent RNA polymerase will transcribe...
only early cistrons on both mature and highly purified vegetative T4 DNA. However, since the crude Snyder-Geiduschek (15) extracts had been shown to be competent for late messenger RNA transcription, it seemed possible that the vegetative templates might direct the synthesis of late proteins in the coupled cell-free system.

The in vitro labeled proteins synthesized in response to mature or S-5 T4 DNA were analyzed by polyacrylamide gel electrophoresis. Fig. 9A shows that several labeled polypeptides from the two reaction mixtures migrate with the same mobilities but that at least four differences are apparent. Two polypeptides (Peaks 24 and 33) specified by the mature DNA (solid line) are missing from the profile elicited by the S-5 template. On the other hand, two proteins (Peaks 18 and 38) synthesized in response to S-5 DNA (dashed line) are absent in the reaction mixture programmed with mature T4 DNA. The polypeptides synthesized in response to mature DNA, S-5 template, and the pad fraction were also compared by electrophoresis in Na dodecyl sulfate-polyacrylamide gels. Autoradiograms of these gels are presented in Fig. 9B. The S-5 and mature DNA patterns have differences that are readily apparent, whereas proteins labeled in response to DNA contained in the pad fraction resemble the pattern obtained using mature T4 DNA.

Since many of the phage late proteins are phage structural components, antiserum against phage particles should react specifically with any late proteins synthesized in vitro. Table V shows the results of an experiment in which mature T4 DNA and S-5 template were used to stimulate $[^{14}C]$lysine incorporation by S-30 fractions from uninfected cells. After incubation, the fraction of the total radioactivity which could be precipitated with T4 antiserum was determined. The labeled extracts from uninfected and T4-infected cells served as controls. Both the in vitro reaction primed with mature T4 DNA and the labeled uninfected extract contained some cross-reacting material, although a 2-fold increase in radioactivity in the immune precipitate was seen with the reaction primed with S-5 DNA. The radioactive proteins from the extract labeled in vivo late after infection reacted almost completely with the T4 antiserum in this experiment. Since it has been shown that mature T4 DNA does not direct the synthesis of late proteins in vitro (13, 26), it is assumed that the cross-reacting material observed in these tubes is due to nonspecific binding. Other experiments have shown smaller percentages of antibody-precipitable material (11 to 19%) synthesized in response to S-5 preparations, but in all cases S-5 template yielded 2-fold more radioactive protein capable of reacting with T4 antiserum than proteins whose synthesis was stimulated by mature DNA (5 to 10%). In these experiments only 30 to 45% of the extract labeled in vivo late after infection reacted with antibody to form a precipitate.

**DISCUSSION**

A large body of evidence suggests that the early events in phage infection are directed by the expression of genes of the infecting parental DNA while the late functions are the result of the expression of genes in the newly synthesized progeny DNA. In particular, the experiments of Sechaud and Streisinger (41) showed that infection with two different tail fiber T4 mutants allowed tail fiber protein to be formed as a result of recombination, which was presumed to occur entirely in a pool of replicating DNA.

Bolle et al. (42) and Riva et al. (43) showed by DNA-RNA hybridization-competition experiments that late mRNA species were not synthesized in cells infected with T4 mutants unable to synthesize DNA. This requirement of DNA replication for late messenger RNA synthesis could reflect a coupling of late transcription with DNA synthesis or the necessity to
form a modified DNA template, which is an initial product of replication. This last possibility is strengthened by the observation of Riva et al. (44) who showed that late transcription could be uncoupled from DNA replication in cells infected with T4 ligase mutants (gene 30) when they carried a second mutation in gene 46 or 47.

Geiduschek et al. (14) have shown that the DNA-dependent RNA polymerase from uninfected cells will transcribe in vitro only those regions transcribed in vivo early after infection on mature T4 or T2 DNA. Brody et al. (11) extended these observations to show that the Escherichia coli enzyme could also transcribe early regions of purified T4 vegetative DNA, that is, the replicative form of T4 DNA isolated from infected cells, and that the polymerase binds more tightly to single-stranded and denatured DNA than to the native duplex DNA. The saturation constant \( K_a \) of E. coli RNA polymerase for DNA varies with the DNA preparation. They found that the \( K_a \) determined for single-stranded DNA was about 10-times lower than that for mature T4 DNA. Maruschige and Bonner (48) showed an apparent \( K_a \) for S-5 DNA about 2- to 10-times lower than that for mature T4 DNA. Hurwitz et al. (49) have shown that the affinity for DNA of T4 polymerase associated proteins (45).

<table>
<thead>
<tr>
<th>Source of radioactive antigen</th>
<th>Precipitated by anti-T4-T7 poly. cm</th>
<th>Precipitated by anti-T4-T7 poly. cm</th>
<th>Labeled proteins in immune precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro reaction mixture +</td>
<td>33,712</td>
<td>6,016</td>
<td></td>
</tr>
<tr>
<td>mature T4 DNA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro reaction mixture +</td>
<td>19,757</td>
<td>8,515</td>
<td>43</td>
</tr>
<tr>
<td>S-5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Extract from uninfected cells</td>
<td>16,188</td>
<td>2,293</td>
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<tr>
<td>Extract from infected cells</td>
<td>5,025</td>
<td>5,066</td>
<td>97</td>
</tr>
<tr>
<td>labeled in vivo</td>
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The S-5 DNA template was found to saturate the amino acid incorporation activity of the S-30 fraction at a lower concentration than mature T4 DNA (Fig. 4). Double reciprocal plots showed an apparent \( K_a \) for S-5 DNA about 2- to 10-times lower than that for mature T4 DNA. Maruschige and Bonner (48) suggest that the amount of DNA template required to achieve half-maximal velocity is dependent on the concentration of RNA polymerase and that template response curves are essentially titrations of the amount of polymerase present. On the other hand, Hurwitz et al. (49) have shown that the affinity constant \( K_a \) of E. coli RNA polymerase for DNA varies with the DNA preparation. They found that the \( K_a \) determined for single-stranded DNA was about 10-times lower than that for double-stranded DNA and that for heat-denatured DNA was about 6-times lower than that for native DNA. Thus, the polymerase binds more tightly to single-stranded and denatured DNA than to the native duplex DNA. The saturation curves observed with fixed concentrations of the RNA polymerase in the S-30 fraction are probably also influenced by single-stranded regions or nicks in the S-5 DNA. In this regard, analysis of data presented in the following paper (50) shows that the S-5 DNA from cells infected with a T4 DNA ligase mutant (gene 30, am H39X) saturates the S-30 extract at a lower DNA concentration than that from the wild-type infection.

Shah and Berger (51) and Shalitin and Naot (46) have proposed the existence of membrane-bound phage DNA in infected cells. It is possible that the activity of the pad fraction is a complex of phage replicating DNA and the bacterial cell membrane. In experiments not shown here, it was found that chloramphenicol (50 \( \mu \)g/ml) added at 10 min after infection inhibited the release of DNA from this fraction and incubation of a lysate with Pronase (1 mg/ml for 20 min at 30°C) caused a 40% reduction in DNA sedimenting with the pad material.

Others have observed that the infecting parental T4 DNA enters into a fast sedimenting complex, containing both host and phage-induced proteins, shortly after infection (52-55). Also, the experiments by Earhart et al. (56, 57) suggest that the spectrum of proteins synthesized in response to the S-5 DNA since the pad fraction, which also has polymerase associated with it, stimulates the synthesis of a set of proteins like those programmed by mature DNA (Fig. 9B). It is possible that the RNA polymerase itself is different, perhaps because of a change in the binding properties of one or more of the T4-polymerase associated proteins (45).

Although we did not specifically investigate the possibility, the presence of an endonuclease in the S-5 extracts might also account for the differences in its template properties. The fact that DNA may be removed from a membrane replication complex by endonucleolytic breakage (46) and that formation of a late transcription-competent template probably results from endonucleolytic action on newly replicated DNA (44) are in accord with this possibility. In this regard, we have observed that extracts prepared from infected cells that had been stored overnight at 0°C contained higher DNA concentrations in the S-5 fraction and that this DNA saturated the RNA polymerase in S-30 extracts at lower template concentrations than S-5 fractions prepared immediately from infected cells, a result possibly reflecting nuclease activity (see below). Although a nuclease with this specificity has not been reported in cell-free preparations, endonuclease activity associated with the product of gene 49 appears to be responsible for the formation of 200 S vegetative DNA (47).
both parental and newly synthesized phage DNA are associated with the cell membrane throughout the eclipse period and that detachment appears to be due to a late function as it can be inhibited by chloramphenicol treatment 9 min after infection. However, association with proteinaceous material other than membrane is suggested by the presence of active RNA polymerase initiation complexes (Fig. 6), as are condensed than membrane is suggested by the presence of active RNA 27.

REFERENCES

Template properties of bacteriophage T4 vegetative DNA. I. Isolation and characterization of two template fractions from gently lysed T4-infected bacteria.
G S Cox and T W Conway


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