Construction and Properties of a Cell-free System for Bacteriophage T4 Late RNA Synthesis*

Dietmar Rabussay and E. Peter Geiduschek

From the Department of Biology, University of California, San Diego, La Jolla, California 92037

A cell-free system for synthesizing bacteriophage T4 late RNA is described. The system, which is based on the "cellophane disc" technique introduced by Schaller and co-workers (Schaller, H., Otto, B., Naselain, V., Huf, J., Hermann, R., and Bonnhoeffer, F. (1972) J. Mol. Biol. 63, 183-200), provides favorable conditions for T4 DNA and RNA synthesis in vitro. Total RNA synthesis can be sustained for more than 1 h at 25°C and initiation of early and late RNA chains occurs in vitro. The capacity to yield cell-free systems which make T4 late RNA in vitro is acquired by virus-infected cells as they make late RNA in vivo. The in vitro synthesized RNA is highly asymmetric. The conditions which optimize the in vitro system with respect to several parameters (total extent of T4 transcription, rate of transcription, asymmetry, fraction of T4 late RNA, and sensitivity to inhibition by rifampicin and streptolydigin) are described.

The regulatory mechanisms for the transcription of the late genes of bacteriophage T4 are likely to differ from those that are already known to function in procaryotes. 1) Late genes, as opposed to T4 early and middle genes, normally require T4 DNA replication for their transcription. This "coupling" of T4 DNA replication and late transcription occurs at two levels, via the common use of at least one T4 protein, gene product (gp) and via the provision of a "competent" template structure. 2) T4 DNA contains \( \text{hmC} \) instead of C. The substitution of \( \text{hmC} \) by C results in severely defective late transcription which abolishes the synthesis of T4 late proteins. The effect of the nucleotide substitution on early and middle transcription has not been thoroughly studied. 3) The RNA polymerase which functions in T4-infected cells during the late period of infection is an extensively modified host enzyme; its \( \alpha \) subunits are ADP-ribosylated and it contains four T4-specific subunits (gp 33, 55, and two proteins coded by as yet unidentified genes). That these modifications are so much more numerous than any others that are now known is compatible with the speculation that the structure of T4 late promoters differs fundamentally from the structures of known promoters (for a review of all the above, see Ref. 1).

A suitable in vitro system for the study of T4 late transcription has not previously been available. Several attempts to transcribe T4 late genes in vitro using purified T4 DNA and RNA polymerase, including templates and enzymes isolated from T4-infected cells, have failed. The search for "factors" which would stimulate T4 late transcription in these systems has not been rewarded with sustained success. Crude lysate systems prepared from T4-infected cells have shown varying amounts of T4 late transcription in vitro. In all cases, the transcription efficiency, the ratio of late to early transcription, or the duration of late transcription in vitro were low and no initiation of late transcription in vitro was demonstrated (1).

We have developed a cell-free system based on the "cellophane disc" technique introduced by Schaller and co-workers (2). Since T4 DNA replication and late transcription are connected in vivo, it was our intention to provide favorable conditions in vitro for T4 DNA replication as well as transcription. In addition, we wanted a system that would allow us to add or remove, activate or inactivate, relevant components. In this paper, we describe the properties of the system and present proof that this in vitro system resembles very closely the transcription pattern of T4-infected cells in vivo. We also show that the synthesis of late T4 RNA is initiated in vitro. We have recently shown that added, T4-modified RNA polymerase specifically stimulates transcription of T4 late genes in vitro (3). The relation between T4 DNA replication and late transcription in vitro is the subject of a subsequent paper.2

**MATERIALS AND METHODS**

*Escherichia coli* B\(^ {\text{su}} \) (stl\(^ {\text{su}} \)) was the standard host strain. A streptolydigin-sensitive (stl\(^ {\text{su}} \)) mutant of B\(^ {\text{su}} \) (DPR 4) and a DPR 4 mutant with a streptolydigin-resistant RNA polymerase (DPR 4-10, stl\(^ {\text{su}} \)) were isolated as described elsewhere.\(^ ^{\text{1}} \) *E. coli* AB105 (RNAase I-, RNAase III-, met\(^ {\text{su}} \), (h), Hfr) was from P. H. Hofschneider (Max Planck Institut für Biochemie, Munich, West Germany). CR63 (su\(^ {\text{su}} \)) was the permissive host for T4am mutants.

T4am M41 (gene e, lysozyme) was used as the standard phage; it shows a normal development except that it does not lyse the infected host cell. T4am 292 (gene 55) is deficient in late transcription.

**Section b: Chemicals**

Streptolydigin was a gift of the Upjohn Co. (Kalamazoo). Rifampicin was a gift of Lepeittp s.p.a. (Milano). The following substances were purchased from sources noted in parentheses: actinomycin, NAD, thymidine, and spermidine (Calbiochem, La Jolla); dithiothreitol, Brij 58, and Mops (4-morpholinopropanesulfonic acid) (Sigma, St. Louis); deoxyribonucleotide and ribonucleotide triphosphates (P-L Biochemicals, Milwaukee, or Schwarz Bio Research, Orangeburg); phosphoenolpyruvate (Boehringer, Mannheim); egg white lysozyme, DNase I, RNAase I and RNAase T1 ( Worthington, Freehold); various bacterial culture media, Bacto Agar and Noble

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* This work was supported by grants of the Cancer Research Coordinating Committee, University of California (to D. R.) and the National Institute of General Medical Sciences (to E. P. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The abbreviations used are: stl, streptolydigin; rif, rifampicin; CM RNA, chloramphenicol RNA; SDS, sodium dodecyl sulfate; c.e., cell equivalent; \( \text{hmC} \), 5-hydroxymethyl cytosine; C, cytosine; Mops, 4-morpholinopropanesulfonic acid; gp, gene product; PA RNA, preannealed and late RNA; SSC, 0.15 M NaCl, 0.015 M sodium citrate.

2 D. Rabussay and E. P. Geiduschek, manuscript in preparation.
Agar (Difco, Detroit); EGTA (ethylenebis(oxyethylenenitrilo)tetraacetic acid and various other reagent grade chemicals (J. T. Baker, Phillipsburg); Triton X-100 (Atlas Chemical, San Diego); [14C]thymidine and [3H]UTP (Schwarz-Mann, Orangeburg).

5-Hydroxymethyl-DCF (hmDCMP) was prepared in two ways which yielded final products with virtually identical properties: (a) unglucosylated T4 DNA was hydrolyzed and the mononucleotides were separated by ion exchange chromatography on Dowex 1 (4); (b) hydroxymethylation of DCF (5). In each case, hmDCMP was reacted and purified on QAE-Sephadex (quaternary aminoethyl Sephadex) acetate and checked for purity by thin layer chromatography (6).

The following filters and membranes were used: nitrocellulose filters, 24-mm diameter, type HA, 0.45-μm pore size (Millipore, Bedford); glass microfiber filters, 24-mm diameter, type GF/C (Whatman, England); cellophane membrane discs, 12-mm diameter, 22-μm thick (Kalle, Wiesbaden Biebrich, West Germany).

Section c: Media, Buffers, and Incorporation Mixtures
M9S medium, which is M9 medium supplemented with 1% casamino acids (7), was used for growing phage stocks. Phage were plated on tryptone bottom agar using tryptone top layer agar.

Agar plates A and B were as described (2) except that Noble Agar (Difco, Detroit); EGTA [ethylenebis(oxyethylenenitrilo)tetraacetic acid and various other reagent grade chemicals (J. T. Baker, Phillipsburg); Triton X-100 (Atlas Chemical, San Diego); [14C]thymidine and [3H]UTP (Schwarz-Mann, Orangeburg).

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Table I: Composition of incorporation mixtures

<table>
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<tr>
<th>Mixture</th>
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<th>B</th>
<th>C</th>
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a Concentrations of hmDCTP are given in parentheses.

first formed on an isotonic agar plate. The discs supporting the spheroplasts is then transferred to a hypotonic agar plate. B. The resulting osmotic shock lyses the spheroplasts. In detail, the basic protocol (Method A) is as follows. E. coli B were grown in 31 ml of Hershey broth at 37°C in a rotary shaker bath (generation time, 25 min) to A570 = 0.3 (1.2 × 10^7 cells/ml), infected with T4am M41 (1 plaque forming units/cell), and 17 mCi/ml of [3H]thymidine (5Ci/mol) was added. Survivors were determined 1 min later (usually less than 0.1%). Eighteen minutes after infection, cells were chilled in ice water, centrifuged, and resuspended in 1 ml of TNGO buffer; the suspension was transferred to an Eppendorf conical tube and centrifuged in an SS 34 Sorvall rotor accelerated to 10,000 rpm, maintained at that speed for ½ min, and then turned off. The supernatant was completely removed and the pellet was resuspended in 200 μl of TNGO/Brij 58 (about 1.5 × 10^7 cells/ml).

One microliter of lysosome solution (1 mg/ml in 0.1 M potassium Mops, pH 6.5) was spread on a cellophane disc on agar plate A at 5°C and 1 μl of the cell suspension was added. After 20 min at 5°C, the disc was transferred to agar plate B and air (at about 20°C) was blown over the disc to evaporate all "excess" liquid. This was judged visually.

The "drying" time was 6 to 8 min.

Method A has been modified according to the needs of specific experiments with respect to the following parameters: volume of Hershey or Penassay broth, amount of [3H]thymidine (5 to 30 nCi/ml), cell density at time of infection (0.6 to 1.2 × 10^7 cells/ml) and density of the final cell suspension, and drying procedure. Whenever the specific values of these parameters are considered to be important, they are given in the legends to the tables and figures.

Section e: RNA Synthesis in Vitro
Cellophane discs were removed from agar plate B and placed on a 90-μl drop of incorporation mixture. Drops of incorporation mixture were placed in a closed plastic petri dish which was kept on a temperature-controlled brass plate at 25°C. The temperature inside the drops was 23-24°C. Standard incorporation time was 30 min. RNA synthesis was terminated by transferring a disc into 1.5 ml of stop solution and heating in boiling water for 2 min. The sample, including the cellophane disc, was cooled, precipitated with 1.5 ml of 10% CCl₄COOH, filtered through a nitrocellulose membrane, washed with 2.5% CCl₄COOH and 70% ethanol, dried, and counted in a toluene-based scintillation liquid. Radioactivity incorporated into RNA was normalized to 10^7 cells equivalents, using the internal standardization provided by the [14C]-labeled DNA. The internal standardization factor was determined by precipitating a sample of the infected bacterial culture of known optical density immediately after harvesting. The background (which is subtracted in the presented data unless otherwise specified) was determined from discs which had been incubated on a drop of incorporation mixture for 2 min in the presence of 40 mM EDTA.

Section f: Analysis of RNA
(i) RNA Size Determination on Sucrose Gradients—RNA synthesis was stopped by placing the disc in 0.3 to 0.5 ml of 1% SDS, 1 mM EDTA, pH 7. The sample was heated in boiling water for 2 min, cooled, and a 0.1-ml aliquot was layered on top of a sucrose gradient. The 5 to 20% (w/v) linear sucrose gradients were prepared in nitrilotriacetic acid and various other reagent grade chemicals (J. T. Baker, Phillipsburg). The 5 to 20% (w/v) linear sucrose gradients were prepared in nitrilotriacetic acid, 0.1 M NaCl, and 0.1% SDS. After centrifugation, solutions containing 10 mM Tris-HCl, pH 8, 10 mM NaCl, 1 mM EDTA, and 0.1% SDS. After centrifugation, 4% h, 48,000 rpm, 18°C, fractions were collected from the tube bottom. To each fraction (approximately 0.2 ml), 0.5 ml of 10% CCl₄COOH was added and 25 samples were simultaneously filtered through a sheet of GF/C glass fiber paper in an apparatus designed by D. Freifelder (8). (The complete processing time for 25 samples with this device was about 10 min.) Precipitates were washed extensively with 2.5% CCl₄COOH, 0.1 M Na₄P₂O₇, then with 70% (v/v) ethanol in water. Finally, the whole sheet of GF/C paper was rinsed with ethanol/water after the lid of the filtering apparatus had been removed. The paper was dried and cut to separate the individual samples which were counted in toluene-based scintillation fluid. The amount of radioactive material from the gradient was determined by precipitation and counting a 0.1-ml aliquot of each fraction. We usually recovered greater than 90% of input material in the gradient fractions.

(ii) DNA-RNA Hybridizations—In vitro RNA synthesis was stopped by placing the cellophane disc in 0.5 ml of 0.1 M EDTA, 0.25% SDS, pH 7.0, and boiling for 2 min. The cellophane disc was removed, the volume of 1 mM sodium acetate, pH 5.2, was added and the sample
was repeatedly extended with equal volumes of water-saturated phenol at 70°C. After dialysis against 0.1 SSC, the [3H]RNA was hybridized to the separated strands of T4 DNA (10, 11). The input radioactivity was determined by mixing an aliquot of [3H]RNA sample (usually 50 μl) with 0.2 ml stop solution, precipitating with 0.2 ml of 10% CCl₄:COOH, filtering onto a nitrocellulose membrane filter, washing with 2.5% CCl₄:COOH and 70% ethanol, drying, and counting in a toluene-based scintillation fluid. The concentration of [3H]RNA never exceeded 10 ng/ml and it was accompanied by RNA from less than 3 × 10⁶ cells (this would contribute ~18 μg/ml of unlabeled RNA if extracted directly from cells rather than from the discs after preparation and incubation). T4 DNA l and r strands were each used at 10 μg/ml, except for the experiment shown in Fig. 8. In the absence of DNA, l to 2% of the radioactivity of the [3H]RNA samples bound to filter and this was subtracted as background. The percentage of r-strand-specific [3H]RNA was calculated as (counts per min hybridized to the r strand) × 100/(sum of counts per min hybridized to the l and r strands).

Percentage of hybridization efficiency was calculated as 100 × (sum of counts per min hybridized to r and l strands)/(input counts per min) × 1.13. The factor 1.13 corrects for the different counting efficiencies of the hybridized and acid-precipitated RNA samples on nitrocellulose membrane filters. This factor was determined by hybridizing T4 early in vitro [3H]RNA, which contains only labeled T4 RNA, to a saturating excess of T4 DNA l strand and dividing the number of counts per min hybridized (y) by the number of input acid-precipitable counts per min (z) (1/1.13). Comparable experiments with T4 in vivo late RNA, T4 DNA l and r strands, yielded the same correction factor.

In routine assays, with single concentrations of different batches of DNA strands, the hybridization efficiency of in vivo RNA varied between 80 and 95%. We therefore normalized the hybridization efficiency obtained in individual sets of experiments with in vitro RNA to the hybridization efficiency obtained with in vivo T4 late [3H]RNA which was used as a standard. We refer to this normalized quantity, i.e. (percent hybridization efficiency with [3H]RNA sample) × 100/(percent hybridization efficiency with the in vivo T4 probe) as the relative T4 hybridization.

RESULTS

Section a: General Properties of the System; Time Course

The cellophane system's unconventional constitution is associated with certain peculiarities, which it is well to consider at the outset. 1) The cellophane disc which serves as a mechanical support for the concentrated cell lysate, is a semipermeable membrane. This allows rapid changes of composition of substrates and other low molecular weight components to be made by simply transferring the disc from one drop of incorporation mixture to another. On the other hand, even relatively low molecular weight substances added to the incorporation mixture do not act as promptly or as efficiently as would be anticipated for homogeneous systems. High molecular weight substances, such as heparin or RNA polymerase must be added directly to the cell lysate, that is, on top of the cellophane disc. To distribute material uniformly on top of a disc requires care.

2) The lysate on top of the cellophane disc is a nonuniform gel of variable thickness. This implies different accessibility of different parts of the gel to substances diffusing in from the incorporation drop and variably restricts lateral and vertical diffusion of small and large molecules within the gel.

Despite these peculiarities of the cellophane system and its laborious preparation, it has been found more suitable for the transcription of late T4 genes in vitro than other systems that were tested. These included a crude lysate system based on the procedure of Wickner and co-workers (12), a system employing toluenized cells based on the procedure of Peterson and co-workers (13), and a system consisting of highly concentrated "nucleoid" structures prepared according to a modification of the method of Stonington and Pettijohn (14). Such systems synthesized RNA less efficiently and for only approximately 10 min.

Before turning to the details of the experiments on the effects of substrate composition, we want to outline their rationale. The main goal was to generate in vitro RNA synthesis which resembled T4 RNA synthesis in vivo in the late period of infection as closely as possible. Accordingly, we had to optimize RNA synthesis for T4 specificity, high proportion of late RNA, low symmetry, high rate, and duration. T4 late transcription in vivo is related to T4 DNA replication. Therefore, we also maintained favorable conditions for DNA synthesis. The conditions of the latter and the relation between T4 DNA replication and transcription in vitro are the subject of a forthcoming publication.

Our present understanding of the system evolved from numerous experiments, only a fraction of which are presented here. Optimization of the system was done in many steps, over an extensive period of time. Each of the optimizations that is summarized below was ultimately established by confirmatory experiments in which the relevant parameter alone was varied. In developing the system, we used five different incorporation mixtures (Table I), some of which have similar properties. There are two reasons for this variety: (1) the system can be, and has been, separately optimized for different properties; and (2) optimization of the different components was done in several steps. A judgment of the suitability of the different mixes for different purposes will be given at the end of this section.

The time course of RNA synthesis with two different incorporation mixtures is shown in Fig. 1. The approximately 1-min lag in the onset of RNA synthesis probably represents the time required for the diffusion of substrates to sites of transcription. A linear phase of RNA synthesis follows for 40 min in incorporation mixture A (Fig. 1a) or for 20 min in incorporation mixture B (Fig. 1b). (The differences are due to the substrate composition and not to the different rates of drying the discs in the two experiments; longer drying times have been observed for mixture A.)

4 Portions of this paper (including some of the results, Figs. 2, 3, 4, and 5, and Tables II, III, and IV) are presented in mimeo at the end of this paper. Mimeo is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9565 Rockville Pike, Bethesda, Md. 20014. Request Document No. 735M-577, cite author(s), and include a check or money order for $2.10 per set of photocopies. Number key for reference to major headings: 1, Introduction; 2, Materials and Methods; 3, Results; 4, Discussion.

R. Wu, unpublished results.
shorten the duration of the linear phase of RNA synthesis, data not shown). The subsequent slowing down is not avoided by transferring a disc to a fresh drop of incorporation mixture (inset of Fig. 1a). We have observed variations of the degree of subsequent slowdown, depending partly on particular batches of components of the incorporation mixture but also depending on steps in the preparation which we cannot yet identify. With an incorporation mixture that has been optimized for extensive T4 RNA synthesis (incorporation mixture E), the initial rate of RNA synthesis of Fig. 1b can be sustained for more than 60 min (data not shown). The initial rate for all three mixes is about the same. For Mix E it is 4.0 pmol of UMP or about 12 pmol of nucleotide incorporated/min/10^7 cells. The inset shows the effect of transferring discs at 60 min (arrow) to a drop of fresh incorporation mixture at 25°C (C). Control discs remained on the original drop of incorporation mixture (D). System preparation by Method A (9.4 x 10^4 c.e./disc, on the average) except that drying of the discs was with 5°C air (drying time, approximately 20 min). Incorporation mixture B was used. The relative T4 hybridization efficiency was 97 ± 4% for all RNA samples. *, total RNA synthesis; δ, t-strand-specific T4 RNA; ω, r-strand-specific T4 RNA.

**Fig. 1.** Time course of RNA synthesis with two different incorporation mixtures. a, cell-free systems were prepared from T4am M41-infected E. coli B by Method A but drying on agar plate B was for 45 min at 5°C (2.1 x 10^4 c.e./disc, on the average). Incorporation mixture A, but with hmdCTP, 0.1 mM [3H]UTP, no uridine, and 0.2 mM dithiothreitol, was used. Relative T4 hybridization increases with increasing in vitro incubation time from 80% at 10 min to 100% at 60 min. The inset shows the effect of transferring discs at 60 min (arrow) to a drop of fresh incorporation mixture at 25°C (C). Control discs remained on the original drop of incorporation mixture (D). System preparation by Method A (9.4 x 10^4 c.e./disc, on the average) except that drying of the discs was with 5°C air (drying time, approximately 20 min). Incorporation mixture B was used. The relative T4 hybridization efficiency was 97 ± 4% for all RNA samples. *, total RNA synthesis; δ, t-strand-specific T4 RNA; ω, r-strand-specific T4 RNA.

When the same experiment was repeated with E. coli AB 105 (RNase I, III), essentially the same result was obtained. Despite the considerable RNase activity of the system, it is possible to demonstrate the growth of RNA chains in vitro, although it is not possible to determine their growth rate accurately. A pulse-chase experiment yielded the results shown in Fig. 6. A 3 min pulse with [3H]UTP in vitro at 37°C was followed by different periods of RNA synthesis in the presence of excess unlabeled UTP, which shut off further incorporation of label (data not shown). Chase periods of 1 and 3 min show a shift of the labeled RNA to larger size. At 0 min, degradation of that larger RNA is clearly seen.

In the next experiment, we examine the size distribution of the transcripts created during the course of RNA synthesis in vitro. RNA synthesized with Mix A (but containing 0.2 M KCl instead of 0.1 M KCl) shows the following sedimentation patterns (Fig. 7a): The “background” sample, obtained by a

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**Section b: Substrate Requirements**—This section is presented in the miniprint supplement which follows this paper.

**Section c: Preparation of the Cell-free System**—This section is presented in miniprint at the end of the paper.

**Section d: Sedimentation Analysis of the in Vitro RNA Product**—The cellophane system, which contains all macromolecular components of the cells from which it has been derived, has a relatively high level of nucleic acid activity. In order to observe the rate of T4 RNA decay during the preparation of the system and during in vitro RNA synthesis, we labeled E. coli B with [3H]uridine and harvested the cells at 18 min. The system was prepared and in vitro RNA synthesis with unlabeled precursors was allowed for 0, 1, 3, 6, 10, 20, or 40 min, respectively. Samples were collected at these times, heat-denatured, layered on top of sucrose gradients, and centrifuged. Total ribosomal RNA was used as a sedimentation marker in a separate gradient run under identical conditions. The sedimentation profile obtained from an aliquot of cells taken just before preparation of the in vitro system was comparable with previously reported patterns (18) or showed slightly smaller RNA than some previous estimates (19). At zero time, that is, when the cell-free system had been prepared but there had been no in vitro RNA synthesis, the sedimentation profile showed only a slight drift to smaller RNA size. Increasing periods of in vitro RNA synthesis shifted the sedimentation pattern more and more to smaller chain length. We calculated that there is, on the average, approximately one break per 10^6 nucleotides of RNA per min.5

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5 This rough calculation was done as follows: weight average sedimentation constants, s_w, were calculated for each gradient (not shown). These were then normalized to the sedimentation constant of the control sample (s_w). Since s is proportional to M_w, in this medium (20), the ratio of weight average chain length of the control sample to all other RNA samples is given as (s_w/s)_{E} or (s_w/s)_{E} for s_w. For s_w, we calculated a value of 12.2 S, giving a weight average chain length of 1020 nucleotides. The size distribution of all samples is broad. We, therefore, assumed a ratio of weight to number average chain lengths of 2 for all samples and took 510 to be the initial number average chain length, n. The best line through the time points of s_w/s gave n = 2.5 at 30 min, corresponding to the introduction of 1.5 breaks/510 nucleotides in 30 min or 1 break/min/10^6 nucleotides, assuming zero order kinetics of phosphodiester bond cleavages.
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1-min incubation in the presence of 40 mM EDTA, shows a small amount of radioactivity (presumably bound to protein and nucleic acids) sedimenting between 0 and 7 S. The pattern remains the same for in vitro incubations up to 40 s in the absence of EDTA (data not shown). This is consistent with a lag of about 1 min in the incorporation of [³H]UTP into RNA (Fig. 1). One-minute in vitro RNA contains RNA chains with sedimentation constant up to 16 S (i.e., 1500 nucleotides long). This supports the notion that a considerable portion of all RNA chains which grow during the first minutes in vitro have already been initiated in vivo since, in 20 s, one would expect polymerization of no more than 200 to 300 nucleotides corresponding to a rate of 10 to 15 nucleotides/s for T4 RNA synthesis at 25°C. The same argument can be made for the sedimentation profile for 3-min in vitro RNA (i.e., for 2 min and 20 s of incorporation of [³H]UTP into RNA). The largest components of 9 min in vitro RNA are about 23 S and the median size is about 9 S. Thirty-minute in vitro RNA clearly contains a high proportion of degradation products as indicated by the high proportion of RNA that is smaller than 7 S. RNA synthesis with 0.1 M instead of 0.2 M KCl generates essentially the same size distribution of products (data not shown).

The size distribution of RNA that has been pulse-labeled after a 30-min synthesis period in vitro is shown in Fig. 7b. The average sedimentation rate, which is greater than that of the 30-min sample in Panel a, reflects the size distribution of growing RNA chains at 30 min in vitro (with, doubtless, some contribution from degradation). The average chain length increases in the 2- and 3-min samples but decreases again in the 9-min sample of Fig. 7b. The average and maximum size of in vitro RNA is larger when synthesis takes place at high ionic strength (0.4 to 1 M KCl). The sedimentation patterns in Panels a, b, c, and d. Each disc was then processed as described under "Materials and Methods." The arrows locate the positions of 23, 16, and 5 S RNA.

![Fig. 6](image)

**Fig. 6.** Growth and degradation of in vitro labeled RNA. Cellophane discs were prepared from T4am M41-infected E. coli B according to Method A. Discs were incubated 3 min on incorporation mixture A containing 0.2 M KCl and 50 μM [³H]UTP (10,000 Ci/mol). Then, the underside of each disc was blotted on filter paper and it was transferred to a fresh drop of incorporation mixture which contained 200 μM unlabeled UTP instead of [³H]UTP. Incubation on unlabeled mix was for 0 (direct transfer to stop mix), 1, 3, and 9 min.

![Fraction number Fraction number Fraction number](image)

![Fraction number Fraction number Fraction number](image)

![Fraction number Fraction number Fraction number](image)

**Fig. 7.** Size of RNA chains growing in vitro under different conditions. Cellophane systems were prepared from T4am M41-infected E. coli B by Method A, except that no [³H]thymidine was added and the final resuspension of cells was in 300 μl of TCNO/Brij 58. Discs were transferred from agar plate B to 50-μl drops of incorporation mixture A containing 0.2 mM UTP. Incubation was at 25°C for 1, 3, 9, or 30 min. One disc was incubated on incorporation mixture A containing 40 mM EDTA for 1 min ("0 min" sample). Each disc was put into 0.3 ml of 0.1% SDS, 1 mM EDTA (pH 7.5), heated to 100°C for 2 min, and kept on ice until a 100-μl aliquot was layered on a sucrose gradient (run and processed as described under "Materials and Methods"). a, incorporation mixture A with 0.2 mM KCl and [³H]UTP (10,000 Ci/mol). b, RNA synthesis was allowed for 30 min on incorporation mixture A with 0.2 M KCl and unlabeled UTP. Then, discs were transferred to a drop of the same incorporation mixture but containing [³H]UTP (10,000 Ci/mol). The underside of each disc was blotted on filter paper before transfer to the new drop. Incorporation of [³H]UTP was allowed for 1, 2, 3, or 9 min; c, same as a but with 1 M KCl. Symbols indicate the time period of in vitro RNA synthesis: ○, 0 min (EDTA); ●, 1 min; △, 2 min; ▲, 3 min; □, 9 min; ■, 30 min. The solid line without data points is the sedimentation profile of E. coli total ribosomal [³H]RNA (23, 16, and 5 S).
for RNA synthesized in 1 x KCl are shown in Fig. 7c. The appearance of distinct peaks, including one of very low molecular weight material, is characteristic for this RNA. The observed differences between high and low ionic strength RNA could reflect effects on synthesis or degradation of RNA, or both.

Section e: Hybridization Analysis of the in Vitro Synthesized T4 RNA—We now examine fidelity of T4 transcription in the cellophane system in detail, using three hybridization methods: 1) hybridization of the in vitro synthesized RNA to the separated strands of T4 DNA; 2) hybridization-competition with various T4 in vitro RNAs, and 3) RNA-RNA duplex formation between labeled in vitro and excess unlabeled in vivo RNA.

For this analysis, the in vitro RNA was synthesized with incorporation mixture E for 30 min at 25°C. The composition of Mix E is derived from the optimization experiments of Section b and the preparation of the cell-free system is derived from the optimization experiments of Section c (see “Results” in miniprint). Two in vivo labeled T4 RNA samples serve as reference. One (Sample 2, Table V) has been labeled from 17 to 19 min after T4 infection at 37°C and represents RNA synthesis at the time of harvesting cells for preparation of the in vitro system. The other (Sample 3, Table V) has been labeled 3 to 5 min after infection and represents T4 early RNA synthesis. The result of hybridizing this RNA to an excess of the separated strands of T4 DNA is shown in Table V. The high hybridization efficiency of the in vitro [3H]RNA indicates that it is almost exclusively T4-specific and the proportion of r-strand-specific RNA is high in both Samples 1 and 2. In view of the low and comparable degree of symmetry of all three RNA samples (see below and Table VI), the r transcripts that are made in vitro must come from late genes; in this turn means that the proportion of late RNA synthesis is only 20 to 25% lower in vitro than in vivo.

Further analysis of this in vitro RNA by hybridization-competition employs four unlabeled competitor RNA probes (“Materials and Methods, Section f”): (a) “chloramphenicol” (CM) RNA mainly contains RNA sequences proximal to T4 early promoters and contains essentially no anti-message RNA; (b) “early” RNA contains essentially all the sequences which are transcribed from the l strand in the early region of the T4 genome, including low concentrations of l-strand-specific anti-late RNA; (c) “late” RNA contains essentially all the late gene messages, many of the early RNA sequences, and low concentrations of anti-late as well as anti-early RNA; (d) “preannealed early and late” (PA) RNA contains essentially all early “sense” sequences in single-stranded form but sequesters anti-message in double-stranded RNA. The results of hybridization-competition experiments with limiting amounts of T4 l strand DNA are shown in Fig. 8. The competition patterns are very similar (Panels a and c). It is significant that CM RNA competes with labeled in vitro late RNA and with the in vitro RNA less completely than with labeled in vivo early RNA (for the last named RNA, we observe a competition plateau at about 15% residual hybridization: data not shown). This difference in competition ability reflects differences in the synthesis of early-promoter-proximal RNA: the l-strand-specific RNA which is made late in infection, probably is initiated at other than early promoters, i.e. at middle promoters and possibly at a small number of late promoters. This result suggests that the cellophane system may also be useful for studying T4 middle transcription.

The competition patterns with T4 r strand DNA are also comparable for labeled late in vivo and in vitro RNA (Fig. 8, Panels b and d). However, about 4% of the in vitro RNA is not competed out and must therefore be rarely present in, or altogether absent from, in vivo RNA. In summary, by the criteria of hybridization-competition analysis, our in vitro RNA resembles the corresponding in vivo RNA very closely.

T4 transcription in vivo is not strictly asymmetric. At about 2 min after infection, when middle transcription starts, anti-messenger RNA also first appears. It is this anti-messenger content of T4 transcription in vivo which complicates the use of asymmetry of transcription as an absolute criterion for transcription fidelity. However, by comparing labeled in vivo and in vitro RNA and by using the PA and CM RNA probes in RNA-RNA duplex formation, it is possible to characterize the quality and quantity of anti-message synthesis. CM RNA is almost completely asymmetric but its usefulness as a probe for anti-message synthesis is limited because CM RNA is transcribed from only a part of the early region of the T4 genome. PA RNA is the most sensitive probe for anti-message, since it should contain a complete set of sense sequences but little or no anti-sense sequences in the form of single-stranded RNA.

The results of the RNA-RNA duplex formation analysis are shown in Table VI. A large fraction of the label in Sample 2, the in vivo late RNA, is driven into lRNA-lRNA duplexes by the anti-late RNA in unlabeled in vitro early RNA (Column 3). In PA RNA, this anti-late RNA has been predriven into RNA-RNA duplexes and does not react with the labeled RNA (Column 4). The residual RNA-RNA duplex formation may be due to late transcription on the anti-sense r strand of early genes (Columns 2 and 4). The in vivo early RNA (Sample 3) shows very low symmetry with all three of these probes. RNA made with E. coli holoenzyme and intact double-helical T4 DNA in vitro (Sample 4) is, of course, highly asymmetric (Column 5) but contains a significant, though low proportion of anti-late sequences (Column 4) due to anti-late RNA synthesis along the l strand (11, 21, 22). RNA made with yeast RNA polymerase II and denatured T4 DNA (Sample 5) or with E. coli core polymerase and “nicked” T4 DNA (Sample 6) is almost, if not completely, symmetric: the 40 to 48% r transcription (Column 5) is matched by a very high degree of complementarity to PA RNA (Column 4) and by considerable complementarity to CM RNA (Column 2). Examining the in vitro RNA (Sample 1) in comparison with these other mate-

<table>
<thead>
<tr>
<th>Sample</th>
<th>Period of labeling</th>
<th>Specific activity</th>
<th>Input</th>
<th>Relative T4 hybridization</th>
<th>% r transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>30 min in vitro</td>
<td>8.4 x 10^4</td>
<td>2330</td>
<td>96</td>
<td>50</td>
</tr>
<tr>
<td>2</td>
<td>17 to 19 min in vivo (37°C)</td>
<td>8.8 x 10^4</td>
<td>1750</td>
<td>(100)&quot;</td>
<td>65</td>
</tr>
<tr>
<td>3</td>
<td>3 to 5 min in vivo (30°C)</td>
<td>4.2 x 10^4</td>
<td>790</td>
<td>(100)&quot;</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

"Value for Sample 1 normalized to Samples 2 and 3 as described under “Materials and Methods, Section f.”
Section f: Initiation of Late T4 Transcription Occurs in the Cellophane System—Initiation of late transcription is crucial for the usefulness of this in vitro system. We therefore provide evidence for such initiation in two independent ways. First, the time course of RNA synthesis in the absence and presence of the transcription-initiation inhibitor, rifampicin, is examined. We have already noted that the in vitro system is not completely inhibited by rifampicin. Fig. 9 shows that RNA synthesis levels off prematurely if rifampicin is present in the incorporation mixture from the beginning (Curve b, ○); while RNA synthesis continues in the control lacking rifampicin (Curve a, ■). The shape of Curve b and the height of its plateau suggests that the system contains many initiated RNA chains when the in vitro incubation starts. This is to be expected since the distribution of nascent RNA chain lengths must be, to a first approximation, random upon harvest of the cells and they should be completed in vitro. Some chains may also be initiated upon transfer of the discs onto the incorporation mixture before the drug efficiently blocks further initiation. When discs are transferred after 10 min of $[^3H]UTP$ incorporation to a second rifampicin-containing, but otherwise identical, drop of incorporation mixture, an intermediate result is obtained (Curve c, ●). Analysis of the RNAs synthesized in 60 min under the conditions described for Curves a, b, and c all show the same proportion of $r$- to $l$-strand transcription ($\sim0.4:0.6$). This result indicates that the difference between the plateaux of Curves $b$ and $c$ is a measure of both early and late RNA chain initiation during the first 10 min of incubation.

Another experiment which provides evidence for initiation of late transcription in vitro is based on the kinetics of T4 RNA synthesis at different ionic strengths (Fig. 4). In the presence of 800 mM KCl, RNA synthesis levels off approximately as it does in the presence of rifampicin (compare Figs. 4 and 9, Curve c). We interpret this to mean that 800 mM KCl, like rifampicin, allows elongation but not initiation of RNA chains. However, the blocking effect of KCl is completely and rapidly reversible (Fig. 10). RNA synthesis resumes upon transfer of a disc from an incorporation mixture containing 800 mM KCl (○) to one with 200 mM KCl (■). The initial rates of RNA synthesis at 800 mM KCl and after transfer to 200 mM KCl are comparable. RNA labeled from the time of transfer to low ionic strength until 60 min later had a $r:l$ transcription ratio of 0.35:0.65. The corresponding ratio for RNA labeled from 0 to 30 min in 800 mM KCl in the same experiment was 0.46:0.54.

That resumption of RNA synthesis after transfer to low ionic strength due to initiation of new RNA chains is demonstrated by its complete inhibition by rifampicin (Fig. 10, ○). Streptolydigin has essentially the same effect as rifampicin (Fig. 10, ▲). We conclude from the experiments represented in Figs. 8 and 9 that initiation of early as well as late RNA chains does occur with high efficiency in the cellophane system.

For some time, we were not consistently able to observe rifampicin- and streptolydigin-dependent inhibition of RNA synthesis. Upon further analysis, we found three reasons for this irreproducibility. 1) Systems prepared with Brij 58 are less sensitive to drug action. This may be due to the formation of mixed micelles between the detergent and the drugs. 2) Incorporation mixture A usually requires higher drug concen-
TABLE VI

RNA-RNA duplex formation between different preparations of T4 in vitro and in vivo RNA

<table>
<thead>
<tr>
<th>Sample</th>
<th>%r-transcription</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>none</td>
</tr>
<tr>
<td>1</td>
<td>30 min in vitro</td>
</tr>
<tr>
<td>2</td>
<td>17 to 19 min in vivo</td>
</tr>
<tr>
<td>3</td>
<td>3 to 5 min in vivo</td>
</tr>
<tr>
<td>4</td>
<td>In vitro RNA: E. coli holopolymerase and double helical T4 DNA</td>
</tr>
<tr>
<td>5</td>
<td>In vitro symmetric RNA: yeast RNA polymerase II and denatured T4 DNA</td>
</tr>
<tr>
<td>6</td>
<td>In vitro symmetric RNA: E. coli core polymerase and nicked T4 DNA</td>
</tr>
</tbody>
</table>

The capacity for T4 late RNA synthesis increases immediately after infection, rises to a maximum about 5 min after infection and continues thereafter. The sharp decrease of the in vitro [3H]TTP incorporation capacity after 10 min does not reflect the in vivo situation and we do not know the reason for this discrepancy. However, the increase in the capacity for [3H]TTP incorporation in vitro precedes the appearance of late transcription capacity in vitro just as T4 DNA replication precedes late transcription in vivo.
DNA synthesis during T4 infection. Transcription is only 20 to 30% less than it is in vivo and when from the T4 DNA r strand. The rate of total RNA synthesis in vitro, roughly one-half of which are transcribed from the T4 DNA r strand, may make it difficult to construct a coupled system for DNA-dependent protein synthesis without extensive modification. However, the system is adaptable to certain kinds of complementation experiments. Those which we have found most successful thus far have involved DNA replication proteins and RNA polymerases containing the T4-specific regulatory subunits.

As far as we are able to determine, the cell-free system is strikingly better for T4 late transcription than any of the conventional alternative systems that have previously been tried. The advantages of the present system may have to do with the high concentrations of all components or with the mechanical requirements of T4 late gene expression (27), or both. A requirement for components at high concentration might reflect participation of assemblies of weakly bound components in T4 late transcription. The E. coli cytoplasm is a densely packed gel of protein and nucleic acid (29) and the cellophane disc comes much closer to achieving such concentrations of reacting components than does any other in vitro system. If there are mechanical requirements of T4 late transcription, they might be due to constraints on the tertiary structure of DNA. The analysis of such requirements could lead us to a better understanding of DNA competence for T4 late gene expression.

ACKNOWLEDGMENTS—We thank M. Filip for technical assistance, C. and K. G. Lark for their introduction to the cellophane system, and S. Fuhrman and K. Jacobs for critically reading the manuscript.

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The effect of t3l concentration on dsDNA synthesis is presented in Fig. 5. The concentration of T4 DNA polymerase is held constant at 1 mg/ml throughout the reaction. The highest t3l concentration, 5,000 units/ml, is significantly lower than the optimal concentration of 75,000 units/ml, obtained with 500 units/ml. However, the optimal t3l concentration is reached with a t3l concentration of 10 units/ml, which is 5 times lower than the optimal concentration. The optimal t3l concentration increases with increasing levels of T4 DNA polymerase.

Table 1: Table of Enzyme Activity

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Concentration (units/ml)</th>
<th>Activity (nmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4 DNA polymerase</td>
<td>100</td>
<td>500</td>
</tr>
<tr>
<td>T3 DNA polymerase</td>
<td>500</td>
<td>2500</td>
</tr>
</tbody>
</table>

Table 2: Table of Kinetic Parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m</td>
<td>0.5</td>
</tr>
<tr>
<td>V_max</td>
<td>100</td>
</tr>
</tbody>
</table>

Fig. 1: Time course of dsDNA synthesis activity at different t3l concentrations. The t3l concentration was varied from 0 to 1000 units/ml, and the enzyme activity was measured at 30 units/ml intervals. The optimal t3l concentration is reached at 100 units/ml, and the activity increases with increasing t3l concentration up to 100 units/ml. The enzyme activity decreases with further increases in t3l concentration.