Fidelity of in Vitro Transcription of T3 Deoxyribonucleic Acid by Bacteriophage T3-induced Ribonucleic Acid Polymerase and by Escherichia coli Ribonucleic Acid Polymerase*

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SUMMARY

The fidelity of in vitro transcription of bacteriophage T3 DNA by T3 RNA polymerase and Escherichia coli RNA polymerase have been studied by DNA-RNA hybridization-competition studies with regard to (a) asymmetry of transcription and (b) the region of the T3 DNA transcribed by either polymerase.

RNA transcribed by T3 RNA polymerase hybridized exclusively with the H strand of T3 DNA—the only strand that is copied in vivo at all times following T3 phage infection. In contrast, RNA transcribed by E. coli RNA polymerase hybridized with both the H and the L strands of T3 DNA, although a high proportion of RNA chains (>70%) hybridized with the H strand. Approximately 15 to 20% of RNA chains hybridized with the L strand, the strand that does not appear to be copied in vivo. The ratio of H to L strand copying by E. coli polymerase was unaltered by the presence of either excess σ or ρ factor or by changes in the ratio of polymerase to DNA in the reaction mixture.

Competition-hybridization studies between RNA synthesized in vitro and RNA isolated after T3 phage infection indicated that RNA transcribed in vitro by T3 RNA polymerase contained all of the sequences present in late in vivo RNA. In addition, all sequences present in early in vivo RNA were also present in in vitro T3 RNA polymerase products. Thus, in vitro, T3 RNA polymerase transcribed the entire early and late regions of the T3 genome.

RNA transcribed by E. coli RNA polymerase contained all sequences present in early in vivo RNA. In addition, such in vitro RNA also contained nearly 50% of the sequences present in late in vivo RNA. In contrast, RNA transcribed by E. coli RNA polymerase in the presence of termination factor ρ contained all of the sequences present in early in vivo RNA, but contained very few late RNA sequences. These results demonstrate that E. coli RNA polymerase, in the absence of ρ factor, can read through early termination signals to transcribe part of the late regions. The presence of ρ factor restricts E. coli RNA polymerase to copying primarily early regions.

In agreement with the above results, it was observed that RNA synthesized either by T3 RNA polymerase or by E. coli RNA polymerase could direct in vitro synthesis of both early and late T3 phage-specific enzymes—S-adenosylmethionine-cleaving enzyme and lysozyme, respectively—in an in vitro protein-synthesizing system. When RNA transcribed by E. coli RNA polymerase in the presence of ρ factor was used as messenger, lysozyme synthesis was markedly depressed.

It is now well established that expression of genetic information in bacteriophages is subject to temporal control; not all genes are expressed simultaneously. Early and late functioning cistrons have been distinguished in λ, T-even, and other bacteriophages like T3 and T7. In these viruses, temporal regulation of gene expression seems to occur at the transcriptional level. At a certain time after infection, mRNA synthesis, originally specific for early phage proteins, is altered to produce RNA that directs the synthesis of late phage proteins. Evidence for this control mechanism has been reviewed (6, 7). Two broad classes of phage-specific mRNAs have thus been distinguished in these bacteriophage-infected cells, “early” and “late” mRNA, so termed because of their order of appearance during the infectious cycle.

In T3- or T7-infected cells, it has been demonstrated that only early RNA is synthesized during the first 2 to 3 min after infection, followed by a switch to the synthesis of late mRNA. It is also known that in T7-infected cells, the synthesis of late RNA is under the pleiotropic control of T7 gene 1. A mutation in gene 1 or the addition of chloramphenicol—a drug that blocks
new protein synthesis—to the bacterial culture prior to infection with phage prevents expression of late genes. Under these conditions, only early mRNA is synthesized in infected cells. This phenomenon has been explained by Chamberlin et al. (8), who elegantly demonstrated that T3 gene 1 codes for the synthesis of a new DNA-dependent RNA polymerase; this de novo synthesis of T7 RNA polymerase coincides with the appearance of late mRNA in infected cells. Summers and Siegel later demonstrated that T7 RNA polymerase produces T7 phage-specific late mRNA in vitro (9).

Since the original discovery by Chamberlin et al. (8), reports from this laboratory (1) and that of Dunn et al. (10) have described the isolation of a new DNA-dependent RNA polymerase in Escherichia coli infected with bacteriophage T3. The phage-induced RNA polymerase was shown to be physically and biochemically distinct from E. coli RNA polymerase. The phage polymerase uses only T3 DNA as template and is inactive with a variety of other native DNA preparations tested so far. More recently, the T3 phage-induced RNA polymerase has been purified to homogeneity and shown to be free of detectable RNase and DNase activities (3). The availability of highly purified polymerase preparations has permitted a detailed biochemical characterization of various aspects of the transcription process catalyzed by T3 RNA polymerase. Some of these studies have already been reported (2-5).

The purpose of the present communication is to describe studies on the role of both the phage polymerase and the host E. coli RNA polymerase in the synthesis of early and late mRNA in vitro. By the use of specific DNA RNA hybridization competition studies with separated strands of T3 DNA, we have determined the extent of sequence homology between RNA products transcribed in vitro by each polymerase and RNA transcribed in vivo at various times following T3 phage infection. In addition, we have measured the synthesis of early and late phase-specific enzymes in an in vitro protein-synthesizing system directed by RNA polymerase products made in vitro both by the phage polymerase and by E. coli RNA polymerase. These studies indicate that transcription of T3 DNA by T3 RNA polymerase produces RNA chains that contain all of the sequences present both in early and in late in vivo RNA. Thus, T3 RNA polymerase in vitro can copy the entire early and the entire late regions of the T3 genome. RNA transcribed in vitro by E. coli RNA polymerase, in addition to containing the entire early in vivo RNA sequences, also contains some of the sequences present in late in vivo RNA. The presence of E. coli RNA chain termination factor, σ, restricts E. coli RNA polymerase to copying mainly the early regions.

Fremilimentary reports of this work have already appeared (11, 12).

**EXPERIMENTAL PROCEDURE**

**Materials**

**Enzymes and Factors**—T3 RNA polymerase was isolated and characterized as described previously (3). E. coli RNA polymerase holoenzyme (containing both the "core" enzyme and σ factor) was purified by the procedure of Maitra and Hurwitz (13). Enzymatic units of each polymerase were defined as described in those communications. E. coli RNA polymerase "core enzyme" and σ subunit were prepared by the procedure of Burgess (14) and were kind gifts of Dr. L. Yarbrough of this Institution. Protein factor σ was isolated and purified from E. coli MRE 600 as described by Roberte (15). All enzyme and factor preparations described above are free of detectable RNase, DNase, and nucleoside triphosphatase activities by the criteria described previously (3, 13).

**Isolation of Phage and DNA**—Crude bacteriophage T3 (wild type) lysate and T3 phage-infected cells were prepared as described previously (3). T3 phages were further purified from the crude lysate by precipitation with polyethylene glycol followed by extraction of the precipitated material with 1 M KCl solution as described by Yamamoto et al. (16). Subsequently, bacteriophage was purified by isopyknic banding in CsCl. T3 DNA was isolated from phage preparations purified through the CsCl step by extraction described by Siedowki and Hurwitz (17).

**Separation of T3 DNA Strands**—Strands of T3 DNA were isolated from purified phage suspensions by adaptation of the procedure described by Guha and Szybalski (18). The procedure utilizes alterations in buoyant density resulting from differential interaction of certain ribonucleoside homo- and copolymers with DNA complementary strands to enable physical separation of the DNA strands in CsCl density gradient solutions. In the present experiments, separation of T3 DNA strands was accomplished with the aid of ribonucleopolymer, poly(U, G) (base ratio, 0.5:1), as follows: a suspension of purified T3 phage was dialyzed for 4 hours against sterile water containing 1 mM EDTA, pH 8.9. The dialyzed phage suspension (0.1 ml) containing 250 to 300 μg of DNA was added to 1.0 ml of a mixture containing 40 μg of poly(U, G), 0.15% of sodium dodecyl sarcosinate, and 5 mM NaOH. The mixture was adjusted to 5 min at 98° and then rapidly cooled to 0°. Approximately 4 ml of a saturated solution of CsCl were added to the reaction mixture and the refractive index of the solution was adjusted to 1.402 at 25°. The mixture was divided into two 2.5-ml aliquots and centrifuged in an SW 50.1 rotor for 60 hours at 30,000 rpm in a polyethylene tube. Following centrifugation, 5-drop fractions were collected through a hole pierced in the bottom of the tube. To each fraction 0.2 ml of 2 X SSC (a buffer solution containing 0.3 M NaCl and 0.05 M trisodium citrate, pH 7.4) was added and the absorbance at 260 nm was determined. Two major DNA peaks were evident (Fig. 1). In addition, a small peak banding at the same density as poly(U, G) was detected, presumably representing unreacted poly(U, G). Of the two major peak fractions, the fraction showing the greater density change on interaction with poly(U, G) (51.4 = 1.4044) was designated as the H (heavy) strand, while the other fraction (51.4 = 1.4022) was designated as the L (light) strand.

Each of these bands representing complexes of the separated strands of T3 DNA and poly(U, G) were heated for 5 min at 97° to separate the ribonucleopolymer from the DNA strand. Each of the separated T3 DNA strands was self-annealed in 2 X SSC for 3 hours at 65° in order to form duplex DNA molecules with any contaminating complementary strands. Since double-stranded DNA is not retained by nitrocellulose membranes (19), this procedure was employed to achieve such conditions for hybridized DNA-DNA strands unavailable for hybridization with complementary RNA.

**Isolation of in Vivo RNA**—Early or late in vivo unlabeled RNA was isolated from T3 phage-infected cells as follows: E. coli B (strain SY106) was grown in glucose-Gasamin acid medium (Medium A (3)) at 37° as described before (3). When the cell density reached 5 X 10⁶ cells per ml, the culture was infected with phage T3 at a multiplicity of infection of 10. Three types of in vivo RNA were prepared; early RNA (0 to 2 min), late RNA (0 to 11 min), and "chloramphenicol"-early RNA. For the preparation of early RNA (0 to 2 min), infected cells were harvested after 2 min following phage infection, while for the preparation of late RNA (0 to 11 min), cells were harvested at 11 min after infection with phage T3. Under the conditions of phage infection employed, cell lysis was complete in 16 min. For preparation of "chloramphenicol"-early RNA, chloramphenicol (200 μg per ml) was added to bacterial cultures 5 min before infection with T3 and infection was then carried out for 5 min. Infected cells were harvested by pouring cultures onto 0.5 volume of crushed frozen "acidic buffer" containing 20 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, and 10 mM NaCl, followed by centrifugation at 7000 rpm for 5 min. In vivo late RNA was prepared as follows: [3H]uridine (25 μCi per ml) was added to infected bacterial cultures 9 min after infection with phage T3; infection was allowed to proceed for an additional 2 min and cells were harvested as described above. "Chloramphenicol"-early [3H]RNA was prepared by pulse-labeling...
The procedures used are identical with those described above for the synthesis of nonradioactive RNA product for hybridization-competition studies except that following infection, each reaction was extracted twice with phenol equilibrated against 50 mM Tris-
Cl buffer, pH 7.8. Carrier E. coli tRNA (5 A260 units per ml) was
added to the aqueous layer, which was then adjusted to 0.2 M
with potassium acetate followed by the addition of 2 volumes of
ethanol. The mixture was stored at -20°C overnight and the pre-
cipitated RNA was isolated by centrifugation at 14,000 x g for
15 min, dried in vacuo, and dissolved in water. DNA-RNA hy-
brids were treated with pancreatic DNase (10 μg per ml) for 10
min at 37°C.

All glassware and stock solutions used for isolation of in vitro
and in vivo RNA were sterilized and stored at -20°C as precautions
against RNAse contamination.

Methods

DNA-RNA Competition-Hybridizations were performed in
liquid at saturating DNA concentrations using the method of
Nygaard and Hall (22) as modified by Bøvre and Szybalski (23).
Each reaction mixture (0.5 ml) contained 0.5 μg of
either strand of T3 DNA, 2 to 5 pmoles of [3H]RNA (1 x 10⁶ cpm)
and, where indicated, increasing concentrations of unlabeled com-
petitor RNA. Each reaction mixture as template in the protein-
synthesizing system, all RNA samples were treated with pancreatic
DNase (10 μg per ml) for 10 min at 37°C.

In vitro Synthesis of Protein—The reaction was carried out in
two stages. In the first stage, reaction mixtures (0.3 ml) contain-
ing 50 mM Tris-Cl buffer, pH 7.8, 10 mM MgCl₂, 50 mM potassium
acetate, 100 mM NH₄Cl, 2 mM dithiothreitol, 24 nmoles of each
of the 20 amino acids, and an S-30 extract (containing 4.2 mg of
protein) were incubated for 15 min at 37°C. Following incubation,
reactions were chilled and 0.1 ml of the chilled mixture was added
to a second reaction mixture (Stage II reaction) that contained
50 mM Tris-Cl buffer, pH 7.8, 1 mM dithiothreitol, 10 mM mag-
nesium acetate, 50 mM NH₄Cl, 2 mM ATP, 0.5 mM GTP 10 mM
phosphoenylpyruvate, 7.5 A₂₆₀ units of E. coli total tRNA, 0.2
mm of all 20 amino acids, 150 nmoles of [¹⁴C]valine to label transcrip-
tion during protein synthesis. At Stage II, reaction mixtures were incubated for 20 min at 37°C
and subsequently chilled. Aliquots of the chilled reaction mix-
tures were then assayed for de novo synthesis of the enzymes—
lysozyme and S-adenosylmethionine-cleaving enzyme as de-
scribed in the following section. To quantitate the amount of
total protein formed under these conditions, ['⁴C]valine (1 x 10⁶
cpm per μmol) was added to a reaction mixture as template in the
protein-synthesizing system, and the radioactivity was counted in
a liquid scintillation counter to determine the amount of hybrid formed. The absolute hybridiza-
tion efficiency, in the absence of added competitor RNA, was
greater than 90% for in vitro [³H]RNA product while with in vitro
early and late [³H]RNA, the efficiency was 20% and 60%, respec-
tively. This lower efficiency of hybridization of early in vivo
RNA was expected since, at this time after infection, substantial
amounts of bacterial specific RNA were still being synthesized.
Thus, a large proportion of the labeled material corresponds
to bacterial RNA.

Isolation of RNA Product for in Vitro Protein Synthesis—The
sources of all other chemicals were described in previous com-
munications from this laboratory (2–4).
onine (2 \times 10^3 \text{ cpm per pmole}) and a 20-\mu l aliquot of an incubated Stage II reaction mixture. After incubation at 37\(^\circ\) for 30 min, the reaction was stopped by placing the assay tubes in a Dry Ice-acetone bath for 10 s. The solutions were thawed and immedi-
ately placed on columns of Amberlite CG 50 (0.3 \times 1 \text{ cm}), pre-
viously equilibrated with 20 mM potassium phosphate buffer, pH 7.0. The column was washed with 10 ml of the same buffer and the ef-
luence, containing \(^3H\)-labeled thiaminyladenosine, were col-
collected and aliquots counted in Bray’s solution (25). Under these conditions, unreacted substrate remained adsorbed in the column and could be eluted with 4 N acetic acid. Control values (those obtained from mixtures lacking enzyme) usually ranged between 3 and 5% of the total input radioactivity and have been subtracted from the experimental values. A unit of enzyme activity was defined as that amount that catalyzed the formation of 1 pmole of thiaminyladenosine in 20 min under the above assay conditions.

Assay for Lysozyme Activity—Lysozyme activity was measured by the method of Gold and Schweiger (26). The measurement was based on the release of radioactivity from filter paper discs containing adsorbed \(^3H\)dihamino-
pinic acid. The method of preparation of each filter paper disc was described by Gold and Schweiger (26) except that \(E.\ coli\) M145, a diaminopimelate-requiring mutant, kindly sup-
plied by Dr. Charles Gilvarg of Princeton University, was used. Each filter paper disc contained 70,000 to 100,000 cpm of \(^3H\)-radio-
activity. Only 50% of the radioactivity on each filter paper disc could be released by the addition of excess egg white lysozyme; the remaining radioactivity on the disc could be released by tryp-
in or chymotrypsin. Thus, lysozyme-sensitive substrate on each disc was between 35,000 and 50,000 cpm. The assay method was as follows. An aliquot (0.02 ml) of incubated Stage II reaction mixture was diluted to 1 ml with 0.1 M ammionium acetate. The mixture was incubated with a filter paper disc containing \(^3H\)-labeled cell wall for 4 hours at 37\(^\circ\). The amount of radioactivity released was measured by removing 0.5 ml of the fluid above the disc and counting it with 10 ml of Bray’s solution in a liquid scintillation counter. A unit of lysozyme activity was defined as that amount that catalyzed the release of 1000 cpm of \(^3H\)-radio-
activity from the disc under the above conditions.

RESULTS

Strand Selection during in Vitro RNA Synthesis Catalyzed by \(E.\ coli\) and T3 RNA Polymerases

The nature of the RNA produced in \(in vitro\) from native T3 DNA templates by both the \(E.\ coli\) and T3 RNA polymerases was investigated by DNA-RNA hybridization studies with separated H and L strands of T3 DNA (Table I). For com-
parison, the hybridization of \(in vivo\) early (0 to 2 min) and late (8 to 11 min) \(^3H\)RNA to T3 DNA strands was also determined. In addition, \(^3H\)RNA isolated from cells infected with plaque T3 in the presence of chloramphenicol (called “chlorampheni-
col”-early RNA) was also used in these studies (Table I). It was observed that both the \(in vitro\) T3 RNA polymerase products and \(in vivo\) early and late RNAs hybridized exclusively with the H strand of T3 DNA. Thus, \(in vitro\), T3 RNA polymerase copies the same strand that is copied \(in vivo\) at all times following T3 phage infection. When \(^3H\)RNA products synthesized by \(E.\ coli\) RNA polymerase in either the presence or absence of KCl (\(\mu = 0.2 \text{ M}\)) were used for hybridization studies, it was ob-
served that about 70 to 75% of RNA chains, as expected, hybrid-
ized with the H strand. However, approximately 15% of the RNA synthesized \(in vitro\) by \(E.\ coli\) RNA polymerase hybridized with the L strand—the strand that appears not to be copied at all \(in vivo\).

It has previously been reported from a number of laboratories that deficiency of \(\sigma\) subunit in the \(E.\ coli\) RNA polymerase holo-
enzyme causes the polymerase to lose its strand specificity (27). However, in the present experiments, transcription of L strand

<table>
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<tr>
<th>PHIRNA samples</th>
<th>Input RNA hybridized with saturating amounts of (\text{H} + \text{L}) strand</th>
<th>Assymetry (%=\left(\frac{\text{H}}{\text{H} + \text{L}}\right))</th>
</tr>
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<tbody>
<tr>
<td>Experiment A ((in vivo) RNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early-“chloramphenicol”</td>
<td>12</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Late (8-11 min)</td>
<td>56</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Late (11-13 min)</td>
<td>60</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Experiment B ((in vitro) RNA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(E.\ coli) RNA polymerase product made in low ionic conditions</td>
<td>70</td>
<td>15</td>
</tr>
<tr>
<td>(E.\ coli) RNA polymerase product made in presence of 0.2 M KCl</td>
<td>10</td>
<td>12</td>
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<tr>
<th>Additions to reaction with (E.\ coli) RNA polymerase</th>
<th>Input (^3H)RNA hybridized with saturating amounts of (\text{H} + \text{L}) strand</th>
<th>(\sigma) factor (15 pmoles)</th>
<th>(\rho) factor (10 pmoles)</th>
<th>Additional T3 DNA (100 nmols of nucleotide residues) (low enzyme/DNA ratio)</th>
<th>RNA polymerase (10 pmoles) (\times) T3 DNA (5 nmols) (high enzyme/DNA ratio)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H strand</td>
<td>L strand</td>
<td>%</td>
<td>%</td>
<td>75</td>
<td>22</td>
</tr>
<tr>
<td>Additional T3 DNA (100 nmols of nucleotide residues) (low enzyme/DNA ratio)</td>
<td>72</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNA polymerase (10 pmoles) (\times) T3 DNA (5 nmols) (high enzyme/DNA ratio)</td>
<td>80</td>
<td>18</td>
<td></td>
<td></td>
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</tr>
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</table>

was not due to \(\sigma\) deficiency of the polymerase preparation; addition of a molar excess of \(\sigma\) subunit to the reaction mixture did not affect the relative ratio of copying of the T3 DNA strands by \(E.\ coli\) RNA polymerase (Table II). It should be noted that
the polymerase preparations used in the present experiments already appear to contain a full complement of σ factor since the net incorporation of [3H]UMP on native T4 DNA templates was not further stimulated by adding excess σ factor. In addition, sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of the polymerase preparation revealed intense protein bands corresponding to all subunits—ασβγ. Similarly, [3H]-RNA synthesized by E. coli RNA polymerase in the presence of saturating amounts of E. coli RNA chain termination factor ρ, hybridized with both the H and the L strands in the same ratio as that observed with [3H]RNA synthesized in the absence of ρ factor (Table II). The ratio of H strand to L strand transcription was not influenced by the alteration of the ratio of RNA polymerase to DNA template used during the in vitro RNA synthesis. Identical transcriptional patterns were obtained with a large excess of polymerase as well as with limiting amounts of polymerase and excess template (Table II). Thus, the small extent of L strand copying by E. coli RNA polymerase seems to be a reproducible in vitro reaction. Since in T3-infected cells all isolated RNA transcripts hybridized exclusively with the H strand, all competition-hybridization studies with the E. coli RNA polymerase products were carried out with only the H strand.

Region of the T3 Genome Copied by T3 RNA Polymerase and by E. coli RNA Polymerase

**Competition of Hybridization of in Vitro [3H]RNA Products by in Vivo RNA**—We have used competition-hybridization to determine the regions of the T3 genome transcribed in vitro by T3 RNA polymerase and by E. coli RNA polymerase. Fig. 2 shows the results of competition-hybridization studies between the in vitro [3H]RNA products synthesized by either polymerase and unlabeled early and late in vivo RNA isolated from T3-infected cells. The hybridization of in vitro [3H]RNA products transcribed by T3 RNA polymerase to the H strand of T3 DNA was completely prevented by in vivo late RNA isolated 11 min after T3 phage infection (Fig. 2A). Thus, all RNA sequences present in T3 RNA polymerase products were also present in late in vivo RNA. The experiment presented in Fig. 2A also shows that an excess of early (0 to 2 min) or “chloramphenicol”-early RNA could also compete with a small fraction (15 to 20%) of the in vitro T3 polymerase products for complementary sites on the T3 DNA. To prove that this small inhibition of hybridization observed was really due to competition for hybridization, we used unlabeled RNA isolated from T4 phage-infected cells as competitor. In this case, no inhibition of hybridization of [3H]-labeled T3 RNA polymerase products was observed (data not shown). These results indicate that T3 RNA polymerase in vitro, in addition to copying the late regions, transcribes early regions of the T3 genome. When similar competition-hybridization studies were carried out with [3H]RNA transcribed by E. coli RNA polymerase, it was observed that the hybridization of [3H]RNA samples was decreased nearly 50% in the presence of unlabeled early in vivo RNA, while essentially 100% inhibition of hybridization was achieved in the presence of excess late in vivo RNA (Fig. 2B). In contrast, hybridization of [3H]RNA products synthesized by E. coli RNA polymerase in the presence of ρ factor, was almost completely prevented (90 to 95% inhibition) by both early and late (0 to 11 min) in vivo RNA (Fig. 2C). These results indicate that nearly 60% of the RNA sequences transcribed by E. coli RNA polymerase in the absence of ρ factor correspond to those present in early in vivo RNA, the remaining 40% correspond to RNA sequences present only in late in vivo RNA. In contrast, the RNA product synthesized by E. coli polymerase in the presence of ρ factor contains almost only early in vivo RNA sequences. It should be noted that late in vivo RNA used in these studies was isolated 11 min after infection with phage T3. Because of the stability of mRNA in T3- and T7-infected cells (28), this RNA, in addition to containing late RNA, also contained all of the early RNA. Thus, a complete inhibition of hybridization of [3H]-labeled E. coli RNA polymerase products by in vivo (0 to 11 min) late RNA was obtained.

The experiment presented in Fig. 2A represents competition of [3H]RNA synthesized in vitro by T3 RNA polymerase by unlabeled in vivo RNA isolated 2 min and 11 min following T3 phage infection. When the same competition-hybridization experiments were carried out with unlabeled in vivo RNA isolated 4 and 6 min following T3 infection as competitor (Fig. 3A), it was observed that (0 to 4 min) in vivo RNA inhibited hybridization of [3H]-labeled T3 RNA polymerase products nearly 80% while essentially 100% inhibition of hybridization was obtained in the presence of excess unlabeled (0 to 6 min) in vivo RNA. Thus, all RNA sequences transcribed by T3 RNA polymerase in vitro are synthesized in vivo within 6 min after T3 phage in-
decreased hybridization of late in vivo RNA by only 20%.

The amount of in vivo [3H]RNA used in each experiment was as follows. In Experiment A, approximately 10,000 cpm of in vivo late [3H]RNA; Experiment B, approximately 50,000 cpm of in vivo RNA (Fig. 3B). These results indicate that in vivo early [3H]RNA and unlabeled in vivo RNA as competitor; A—A, RNA product synthesized by E. coli RNA polymerase in the presence of saturating amounts of ρ factor; O—O, RNA product synthesized by E. coli RNA polymerase alone; A—A, RNA transcribed by T3 RNA polymerase.

When similar competition-hybridization tests were performed between in vivo early [3H]RNA and unlabeled in vitro RNA polymerase products (Fig. 4B), it was observed, as expected, that an excess of unlabeled RNA synthesized by E. coli RNA polymerase, in either the presence or absence of ρ factor, completely prevented the hybridization of early [3H]RNA. These results indicate that in vivo E. coli RNA polymerase products contained all RNA sequences present in in vivo early RNA.

Surprisingly, in vitro T3 RNA polymerase products were also equally effective in preventing hybridization of in vivo early [3H]RNA, indicating the presence of all early in vivo RNA sequences in in vitro T3 RNA polymerase products (Fig. 4B).

These experiments demonstrate that T3 RNA polymerase can transcribe the entire early and the entire late regions of the T3 genome. Similar conclusions have also been reported by Dunn et al. (29). However, E. coli RNA polymerase can copy only the early regions provided that ρ factor is present. In the absence of ρ factor, E. coli RNA polymerase, in addition to copying the entire early region, can read through the early termination.
signals to transcribe part of the late region. The presence of ρ factor prevents this readthrough to a great extent and restricts transcription by E. coli RNA polymerase to primarily the early regions.

**Competition-Hybridization Studies between RNA Synthesized in Vitro by T3 and E. coli RNA Polymerases—**The competition-hybridization experiments between labeled and unlabeled in vitro RNA polymerase products (Fig. 5) are also consistent with the above conclusion. The experimental observations can be summarized as follows. (a) The hybridization of [3H]RNA transcribed in vitro by E. coli RNA polymerase in the presence of ρ factor was completely prevented by the addition of excess unlabeled RNA synthesized either by E. coli RNA polymerase in the absence of ρ factor or by T3 RNA polymerase (Fig. 5A). (b) The hybridization of [3H]RNA synthesized in vitro by E. coli RNA polymerase in the absence of ρ factor was also completely inhibited by an excess of unlabeled T3 RNA polymerase products. However, unlabeled RNA, synthesized by E. coli RNA polymerase plus ρ factor, maximally reduced such hybridization by 70% (Fig. 5B). (c) In contrast, increasing concentrations of unlabeled E. coli RNA polymerase products decreased the hybridization of [3H]RNA polymerase products maximally 60% when the unlabeled RNA was isolated from polymerase reactions carried out in the absence of ρ factor. When reactions were carried out with ρ factor, hybridization was reduced by only 30% (Fig. 5C). These results demonstrate that E. coli and T3 RNA polymerases copy overlapping regions of T3 DNA.

In vitro, T3 RNA polymerase can copy the same regions of T3 DNA that are copied by E. coli RNA polymerase in the presence or absence of ρ factor. The phage polymerase copies additional regions of the T3 DNA not copied by E. coli RNA polymerase. In the presence of ρ factor, E. coli RNA polymerase copies a restricted region of the T3 DNA. In the absence of ρ factor E. coli RNA polymerase, in addition to copying this region, also transcribes additional regions of the T3 DNA also copied by T3 RNA polymerase.

**Synthesis of Biologically Active Early and Late T3 Phage-Specific Enzymes by in Vitro RNA Polymerase Products—**The results obtained in hybridization-competition studies have been confirmed by measuring the ability of in vitro transcription products to direct the synthesis of early and late T3 phage-specific enzymes in an in vitro protein-synthesizing system derived from E. coli. We found that RNA products synthesized from T3 DNA by either E. coli or T3 RNA polymerase were efficient in directing amino acid incorporation into proteins. This encouraged a direct attempt to show that these RNA species direct the synthesis of functional early and/or late enzymes. S-Adenosylmethionine-cleaving enzyme has been reported to be an early T3 phage enzyme and the synthesis of this enzyme in vivo shuts off at about 8 min (30). In addition, mRNA specific for S-adenosylmethionine-cleaving enzyme appears to be synthesized in vivo in chloramphenicol-treated cells infected with phage T3. In contrast, lysozyme is a typical late enzyme, since the enzyme activity does not appear in T3-infected cells until 6 min after infection at 30° (28). Moreover, lysozyme-specific mRNA is not detected in chloramphenicol-treated T3-infected cells. As shown in Table III, RNA products synthesized either by T3 RNA polymerase or by E. coli RNA polymerase in the absence of ρ factor directed the synthesis of both S-adenosylmethionine-cleaving enzyme and lysozyme. RNA synthesized by E. coli RNA polymerase in the presence of ρ factor, while being active in directing the synthesis of S-adenosylmethionine-cleaving enzyme, was inactive in the synthesis of lysozyme. As expected, early in vivo RNA directed the synthesis of only S-adenosylmethionine-cleaving enzyme, while (0 to 8 min) in vivo RNA, which contained a mixture of early and late mRNAs, directed the synthesis of both enzymes. These results therefore demonstrate that RNA synthesized either by T3 RNA polymerase or by E. coli RNA polymerase in the absence of ρ factor contained information for the synthesis of both the early enzyme, S-adenosylmethionine-cleaving enzyme, and the late enzyme, lysozyme. In contrast, RNA products synthesized by E. coli RNA polymerase in the presence of ρ factor contained sequences coding for early enzyme but lacked mRNA activity for the late enzyme, lysozyme. In agreement with the above results, it was observed that RNA synthesized from DNA of a T3 mutant, defective in the production of S-adenosylmethionine-cleaving enzyme, did not direct in vitro synthesis of this enzyme activity. Such RNA preparations, however, were fully active in directing amino acid incorporation into proteins as well as lysozyme synthesis (Experiment 2, Table III). It is to be noted that DNase, actinomycin D, or rifampicin—compounds which inhibit DNA-dependent RNA synthesis—were each without effect on RNA-dependent incorporation of amino acids into proteins and concomitant synthesis of both enzymes (data not shown). For this reason, these compounds were routinely included in reaction mixtures to prevent transcription during protein synthesis. RNase or inhibitors of protein synthesis such as chloramphenicol, fusidic acid, or puromycin eliminated both amino acid incorporation and synthesis of both enzymes (only the data with puromycin are shown in Table III).

**DISCUSSION**

As in the case of phage T7, the growth of phage T3 depends on the participation of two different DNA-dependent RNA polymerases. These are the E. coli RNA polymerase and a T3 phage-specific RNA polymerase; the latter is the product of T3 gene 1. In the present communication, we have attempted to determine the fidelity of in vitro transcription of T3 DNA by these two polymerases with regard to the synthesis of early and late T3 phage-specific mRNAs. For this purpose, we have compared by specific DNA-RNA hybridization-competition studies the sequence homologies of RNA transcribed in vitro by either polymerase with those synthesized in vivo in T3-infected cells with regard to (a) asymmetry of strand transcription and (b) the region of the T3 genome copied by each polymerase. The results indicate that T3 RNA polymerase in vitro maintains the asymmetry of strand transcription. The same strand (strand H) that is copied in vivo at all times following T3 phage infection was exclusively copied in vitro by T3 RNA polymerase. No RNA transcript corresponding to the L strand was found among in vitro T3 RNA polymerase products. In contrast, E. coli RNA polymerase in vitro copies both the H and the L strands, although a high proportion of RNA chains (>70%) were copied from the biologically correct H strand; the remaining 15 to 20% of the product hybridized with the L strand. It is to be noted that no stable RNA product transcribed from the L strand can be found at any time in T3-infected cells.

It has previously been reported from a number of laboratories (27) that deficiency of the σ subunit in E. coli RNA polymerase holoenzyme causes the polymerase to lose asymmetric transcription. In addition, in the case of in vitro transcription of T7 DNA by E. coli RNA polymerase, it has been demonstrated (21) that asymmetry of in vitro transcription is dependent on the ratio of RNA polymerase to DNA. At E. coli RNA polymerase to DNA ratios of greater than 4, the L strand was also transcribed.
In vitro synthesis of lysozyme and S-adenosylmethionine-cleaving enzyme directed by RNA polymerase products from T3 DNA template

Reaction mixtures for RNA-directed protein synthesis were prepared with all 20 unlabeled amino acids as described under "Methods" except that various additions of RNA product as mRNA and of other compounds were made as indicated in the table during the Stage II reaction. Following RNA-directed protein synthesis, aliquots of reaction mixtures (25 μl) were assayed for lysozyme activity, while 50- to 100-μl aliquots were assayed for S-adenosylmethionine-cleaving enzyme activity as described under "Methods." Synthesis of each of these enzymes is expressed as total units of each enzyme produced in 0.3 ml of total Stage II reaction. Units of each enzyme are defined under "Methods." T3 RNA-dependent enzyme synthesis resulted in sufficient lysozyme production to cleave 20 to 100% of lysozyme-sensitive [3H]substrate. Control reaction mixtures (i.e., without added mRNA) released about 3% of the total radioactivity. In the assays for S-adenosylmethionine-cleaving enzyme, aliquots of enzyme activity assayed cleaved between 25 and 100% of the added substrate. Control values (those obtained from mixtures lacking S-30 extract) usually ranged between 4 and 6% of the total input radioactivity and were subtracted from the experimental values reported above. Total protein synthesis in Stage II reaction was also measured in parallel incubations carried out in the presence of [3H]-valine (100 cpm per μmole) and measuring the incorporation of radioactivity into hot CCl₃COOH-insoluble material as described under "Methods."

The results presented in this communication show that T3 RNA polymerase in vitro copies not only the entire late but also the entire early regions of the T3 DNA. This conclusion is based on competition-hybridization studies showing that hybridization of both early and late in vitro [3H]RNA to the H strand of T3 DNA was effectively and completely prevented by unlabeled in vitro T3 RNA polymerase products. In agreement with these results we found that RNA synthesized in vitro by T3 RNA polymerase can direct the in vitro synthesis of both late and early T3 phage-specific enzymes, lysozyme and S-adenosylmethionine-cleaving enzyme, respectively. The extent of competition of hybridization of [3H]RNA synthesized by T3 RNA polymerase by in vitro early RNA was approximately 20%, indicating that nearly 20% of the RNA synthesized in vitro by T3 RNA polymerase is the early class while the remainder (80%) is the late class. This result is in agreement with the fractional length of the T3 genome corresponding to early and late regions (33). Similar conclusions regarding the in vitro transcription of T3 DNA by T3 RNA polymerase have also been reached by Dunn et al. (29). The observation that T3 RNA polymerase in vitro transcribes both the early and late regions of the T3 genome is in contrast to the reports of Summers and Siegel (9) on transcription by T7 RNA polymerase. These workers reported that T7 RNA polymerase in vitro transcribes only the late regions of 17 DNA. The reason for this difference in transcription specificity of these two apparently similar phage polymerases is unclear. The observation that, in vitro, T3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Source of mRNA</th>
<th>Amount added</th>
<th>[3H]-Valine incorporated</th>
<th>Lysozyme activity</th>
<th>S-Adenosylmethionine-cleaving enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>T3 RNA polymerase product</td>
<td>None, 56, 112, 56</td>
<td>&lt;5, 60, 312, 558</td>
<td>&lt;3, 60, 90</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>T3 RNA polymerase product synthesized from DNA of T3 SAMase</td>
<td>+ puromycin (0.5 mM) 56</td>
<td>832, 289</td>
<td>&lt;3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>E. coli RNA polymerase product</td>
<td>29, 58, 38</td>
<td>706, 1562, 324</td>
<td>160, 333</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>E. coli RNA polymerase + p factor product</td>
<td>+ puromycin (0.5 mM) 10, 20</td>
<td>219, 500</td>
<td>8, 19</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Early-&quot;chloramphenicol&quot; in vitro RNA</td>
<td>18,000, 30,000</td>
<td>625, 575</td>
<td>&lt;5, 112</td>
<td></td>
</tr>
</tbody>
</table>

a SAMase, S-adenosylmethionine-cleaving enzyme. (Phage T3 SAMase was the kind gift of Dr. R. Hausman.)
b Most of the RNA nucleotides in in vitro RNA represent tRNA and rRNA; mRNA constitutes only a small fraction of the total RNA nucleotides added here.

In the present experiments, however, the ratio of H to L strand transcription was unaltered by adding either excess σ factor or by altering the ratio of enzyme to DNA in the polymerase reaction. Similarly, we have found the same transcriptional pattern in the presence of saturating amounts of σ factor as in its absence. These results are quantitatively different from those of Dunn et al. (29), who reported that σ factor induces asymmetry of transcription of T3 DNA by E. coli RNA polymerase. These workers reported that, in the absence of σ factor, E. coli RNA polymerase transcribed both strands of 17 DNA with equal facility. This resulted in the production of antisense from late regions of the T3 DNA. Addition of σ resulted in the preferential termination of transcription of L strand, lowering the amount of L strand-specific RNA formed (29). In contrast, we have found no effect of σ on strand selection of T3 DNA by E. coli RNA polymerase. In addition, in our system the extent of asymmetry is much higher (H to L strand transcription between 3:1 and 4:1) compared to the results of Dunn et al. (29), who found a nearly 1:1 ratio of copying of each strand. The discrepancy between these two results remains to be resolved.

The small extent of L strand copying by E. coli RNA polymerase may be an in vitro artifact; alternatively, such L strand-specific RNA may also be synthesized in vivo. The absence of such RNA in in vivo RNA isolated from T3-infected cells may be a consequence of rapid turnover of this class of RNA. Such L strand-specific RNA may be involved as primers in DNA replication (32) and thus be rapidly degraded.
RNA polymerases copy the entire early and the entire late regions of the T3 genome warrants a careful study of in vitro transcription.

Studies on the specificity of transcription by E. coli RNA polymerase indicate that this polymerase, as expected, copies the entire early regions of the T3 genome. Similar results have been observed in transcription studies with DNA from phages T4 and T7 (28, 31, 34). However, in the absence of ρ factor, the enzyme reads through early termination signals into significant portions of the late regions. The extent of competition of both in vivo late [3H]RNA and [3H]RNA synthesized by T3 RNA polymerase by unlabeled E. coli RNA polymerase products indicates that nearly 50 to 60% of the late mRNA sequences are copied in vitro by E. coli RNA polymerase. The addition of ρ factor to E. coli RNA polymerase reaction markedly suppresses this readthrough and restricts E. coli polymerase to copying mainly the early regions. However, even under these conditions, a small amount of readthrough occurs. For example, RNA products synthesized by E. coli RNA polymerase in the presence of ρ factor competed with late in vivo [3H]RNA hybridization to the extent of approximately 20% as against 50 to 60% competition by RNA synthesized in the absence of ρ factor (Fig. 4). Since late in vivo [3H]RNA does not contain any early RNA sequences (Fig. 3B), the above results suggest that efficiency of in vitro termination by ρ factor is not absolute.

The results on in vitro transcription by E. coli polymerases are consistent with the available in vivo data on transcription pattern by E. coli RNA polymerase in related phage T7 (28). In this phage system, it has been demonstrated that E. coli RNA polymerase recognizes an initiation site at a position located about 15% from the left end of T7 DNA. This is the only site at which the enzyme can bind to T7 DNA. At about 20% from the left end of T7 DNA there exists a termination site (T1) which is recognized by E. coli RNA polymerase. The termination at this site (T1 site) is not absolute and as a result a small but significant amount of RNA synthesis proceeds through this site. There exists a second termination site (T2) located about 30% from the left end of T7 DNA molecule at which all E. coli RNA transcription halts. Termination at T1 in vivo occurs with about 80% efficiency as judged by gel electrophoretic analysis of early RNA (28).

The results presented in this paper are thus consistent with the following view of regulation of gene expression in T3-infected cells. As in the case of phage T7, the growth of T3 phage requires the participation of two different DNA-dependent RNA polymerases. The bacterial RNA polymerase, punctuated by termination signals, is required in the initial stages of infection to transcribe the early regions of the T3 genome. This early region comprises approximately 20% of the H strand of T3 DNA. Transcription of the remainder of the T3 genome requires the synthesis of a protein product of T3 gene 1 which is a T3-specific RNA polymerase. The phage RNA polymerase then transcribes the late T3 genes comprising approximately 80% of the H strand, which are inaccessible to E. coli RNA polymerase. In vitro, at least, the phage polymerase also recopies early regions.

At present, the number of discrete size classes of late mRNA made in T3-infected cells is unknown; thus, it is not possible to determine whether T3 RNA polymerase in vitro is able to synthesize all discrete size classes of late mRNA as those produced in vivo in T3-infected cells. The question also remains as to whether initiation, termination, and release of RNA chains by T3 RNA polymerase in vitro proceeds by the same mechanism as in T3-infected cells in vivo. Comparative studies between the different sizes of RNA chains formed in vitro by T3 RNA polymerase and those produced in vivo in T3-infected cells by polynucleotide gel electrophoretic analysis, together with the oligonucleotide sequence analysis at the 3'-triphosphate and 3'-OH ends of the in vivo and in vitro RNA chains are necessary to answer this question.

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Fidelity of in Vitro Transcription of T3 Deoxyribonucleic Acid by Bacteriophage T3-induced Ribonucleic Acid Polymerase and by Escherichia coli Ribonucleic Acid Polymerase

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