# THE CONTROL OF RNA TRANSCRIPTION IN BACTERIOPHAGE gh-1-INFECTED PSEUDOMONAS PUTIDA

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ABSTRACT

THE CONTROL OF RNA TRANSCRIPTION IN BACTERIOPHAGE gh-1-INFECTED *PSEUDOMONAS PUTIDA*

By

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The process of RNA transcription was examined in bacteriophage gh-1-infected *Pseudomonas putida*. The purpose was to define the mechanisms of transcriptional control in gh-1-infected cells. The approach to this problem was to determine the specificity of gh-1 RNA transcription at different time intervals of the infectious cycle and to define the conditions necessary to duplicate the specificity of gh-1 RNA transcription *in vitro* by utilizing highly purified RNA polymerase and gh-1-DNA.

The specificity of RNA transcription during three different time intervals of gh-1 phage development was determined. During the early time interval (0-5 minutes post infection) the host RNA polymerase transcribes early RNA sequences from the L strand of gh-1 DNA. The host RNA polymerase continues to transcribe early RNA sequences

from the L strand of gh-1 DNA during the intermediate time interval (5-12 minutes post infection) but about 90% of the gh-1 RNA sequences synthesized during this period of infection are late RNA sequences transcribed by the gh-1 phage-induced RNA polymerase[1] from the L strand of gh-1 DNA. Host RNA synthesis is completely inhibited during the intermediate time interval of gh-1 phage development.

The host RNA polymerase continues to transcribe early RNA sequences from the L strand of gh-1 DNA and the gh-1 RNA polymerase continues to transcribe late RNA sequences from the L strand of gh-1 DNA during the late time interval (12 minutes post infection to lysis), but the relative amount of early RNA synthesis is much greater (50-60%) than during the intermediate time interval. In addition, during the late time interval complementary RNA is transcribed by host RNA polymerase from the H strand of gh-1 DNA and represents about 20% of the gh-1 RNA synthesized in gh-1-infected cells during the late time interval.

The transcriptional specificity of highly purified RNA polymerase was examined *in vitro* to determine if the *in vivo* transcriptional specificity could be duplicated. It was determined that the host RNA polymerase could be limited essentially to transcription

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[1]Howard C. Towle; James F. Jolly; and J. A. Boezi (1975), J. Biol. Chem., 250, 1723.

of early RNA sequences from the L strand of gh-1 DNA *in vitro* if the molar ratio of host RNA polymerase to gh-1 DNA was below 1. The sigma factor was also required for the limitation of transcription to early RNA sequences. At higher molar ratios of enzyme to gh-1 DNA the host RNA polymerase was no longer limited to transcription of early RNA sequences but was capable of transcribing the entire gh-1 genome.

Highly purified gh-1 RNA polymerase was limited to transcription of the L strand of gh-1 DNA *in vitro* as it is during gh-1 phage development but the enzyme was not limited to transcription of late RNA sequences *in vitro*. This result was not affected by the molar ratio of gh-1 RNA polymerase to gh-1 DNA.

The following model is suggested for the control of gh-1 RNA transcription during gh-1 phage development. During the early time interval of gh-1 phage development the host RNA polymerase is limited to transcription of the early region from the L strand of gh-1 DNA. This limitation is probably due to a low molar ratio of host RNA polymerase to gh-1 DNA *in vivo* since host DNA is in large excess and most host RNA polymerase molecules are probably associated with the host genome. The gh-1 RNA polymerase is produced and begins to transcribe late RNA sequences from the L strand of gh-1 DNA during the intermediate time interval of phage development while the host

RNA polymerase is still limited to the early region. During the late time interval of gh-1 phage development host DNA is completely degraded. Thus, all host RNA polymerase molecules should be available for gh-1 RNA synthesis. If all host RNA polymerase molecules in an infected cell are available for gh-1 RNA synthesis the molar ratio of host RNA polymerase to gh-1 DNA would be about 50:1. Under these conditions host RNA polymerase transcribes complementary RNA *in vitro*.

# THE CONTROL OF RNA TRANSCRIPTION IN BACTERIOPHAGE gh-1-INFECTED PSEUDOMONAS PUTIDA

By

James Frederick Jolly

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DEDICATION

To Setsuko

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BSA - bovine serum albumin

CAM - chloramphenicol

DEAE - diethylaminoethyl

DTT - dithiothreitol

EDTA - ethylenedinitrilo-tetraacetic acid

GSC - glucose-salts-casamino acid media

NTP - nucleoside triphosphate

PFU - plaque forming unit

SDS - sodium dodecyl sulfate

SSC - standard saline citrate (0.15M NaCl, 0.015M sodium citrate)

TCA - trichloroacetic acid

Tris - tris-(hydroxymethyl)-methylamine

# LITERATURE REVIEW

## Introduction

How genetic expression is controlled in the cell has been a fundamental question of molecular biology since the development of molecular biology as a science. Since the science of molecular biology developed historically from the study of bacteriophages (1), it is not surprising that the study of bacteriophages has provided much of our current knowledge of how control of genetic expression occurs. It was clear from the beginning of such studies that a fundamental shift in genetic expression occurs during phage development. The control of host biosynthetic mechanisms and the utilization of host molecules after phage infection resulting in the production of hundreds of phage particles within a period of twenty minutes was dramatic evidence of a shift of genetic expression in infected cells.

The study of DNA phage development during the past thirty years has indicated that the control of gene expression in phage systems is primarily at the level of transcription. Transcription of a phage genome is carried out initially by the host RNA polymerase, but transcription of later classes of phage genes is carried

out by either a modified host RNA polymerase or a new, phage-specific RNA polymerase encoded by the phage genome. In both cases transcription of the host genome is inhibited by phage infection.

The second case, that involving the synthesis of a new, phage-encoded RNA polymerase, will be reviewed here in detail because of its relevance to the research presented in this dissertation. As will be described below, the Pseudomonas putida bacteriophage gh-1 induces the appearance of a new RNA polymerase in gh-1-infected cells. The most extensively studied phages which encode for their own specific RNA polymerases are the Escherichia coli bacteriophage T7 and the closely related T3 phage. The process of T7 phage infection will first be discussed in detail and then the transcriptional control of T3 phage development will be compared to the T7 phage system. Several reviews on transcription in T7-infected cells are available (2,3).

Before the process of T7 phage infection is described, a short discussion of the structure and function of bacterial RNA polymerases will be presented.

## Structure and Function of Bacterial RNA Polymerase

The following is a short discussion of the structure and function of bacterial RNA polymerases. This discussion is based on the reviews of bacterial

RNA polymerase literature by Chamberlin (4) and Burgess (5).

RNA polymerase from bacterial sources is composed of four types of subunits: $\alpha$, $\beta$, $\beta'$ and $\sigma$. The molecular weights of the subunits vary somewhat for each source of RNA polymerase. The subunit molecular weights for the P. putida RNA polymerase subunits are: $\alpha$, 44,000; $\beta$, 155,000; $\beta'$, 165,000 and $\sigma$, 98,000.

Bacterial RNA polymerases exist in two forms: holoenzyme and core enzyme. The subunit stoichiometry of the holoenzyme form of RNA polymerase is $\alpha_2\beta\beta'\sigma$. The subunit stoichiometry of the core enzyme form of RNA polymerase is $\alpha_2\beta\beta'$. Chromatography of E. coli holoenzyme on phosphocellulose dissociates the enzyme into core enzyme and the $\sigma$ subunit while both holoenzyme and core enzyme can be obtained from phosphocellulose chromatography of P. putida holoenzyme.

Both forms of bacterial RNA polymerase are capable of transcribing DNA. Both holoenzyme and core enzyme utilize poly [d(A-T)] and calf thymus DNA efficiently. The holoenzyme, however, is much more efficient with most phage DNA templates, such as T4 and T7 DNA. The holoenzyme also transcribes phage DNA asymmetrically; only the strand that is transcribed in vivo is transcribed by holoenzyme in vitro. The core enzyme transcribes both

strands of phage DNA. For this reason σ factor is believed to be necessary for correct initiation of RNA synthesis.

The σ factor acts catalytically during RNA synthesis. After initiation of RNA synthesis by holoenzyme, the σ factor is released from the DNA-RNA polymerase complex and the core polymerase continues the process of RNA chain elongation. The σ subunit can bind to another molecule of core enzyme to allow correct initiation.

The antibiotic rifampicin inhibits bacterial RNA polymerase activity by binding to the β subunit of RNA polymerase. Inhibition of RNA polymerase by rifampicin occurs prior to RNA chain initiation. The process of RNA chain elongation is not inhibited by rifampicin.

## Organization of the T7 Genome

The advantage of using the T7 phage system for experimentation is that it is genetically well defined. The size of the T7 genome is small enough ($25X10^6$ daltons [6]) that many gene functions are known, as well as the size of each RNA transcript and the size of the corresponding polypeptide product.

Twenty-five genes have been identified on the T7 genome: 19 essential genes have been identified by the isolation and characterization of amber mutants essential for T7 growth (7-9) and 6 non-essential genes have been

identified by studying deletion mutants (7,10). The genes have been ordered on a linear genetic map and are numbered from left to right (7,8). The essential genes are numbered by integral numbers, from 1 to 19, while the non-essential genes are numbered by fractional numbers depending on their map position (eg, gene 1.3 is located three-tenths of the distance between gene 1 and gene 2).

Approximately thirty T7-specific polypeptides with molecular weights ranging from 7,000 to 150,000 daltons have been identified in T7-infected cells by SDS-gel electrophoresis. These proteins account for virtually all the coding capacity of T7 DNA (7).

Three temporal classes of proteins appear in a T7 time course of infection (7): (a) class I proteins are synthesized between 4 and 8 minutes after infection and are synthesized at a normal rate during infection with T7 gene 1 mutants grown under non-permissive conditions. Class I proteins are the products of T7 genes 0.3 to 1.3; these genes are referred to as early genes and comprise the early region of T7 DNA. Early genes are transcribed in vivo and in vitro by the host RNA polymerase. Ligase (gene 1.3) is classified as a class I protein because it is synthesized at a normal rate during infection with gene 1 mutants even though it usually continues to be synthesized until class II protein synthesis ends. (b) Class II proteins are synthesized between 6 and 15 minutes post infection

and are specified by genes 1.7 to 6. (c) Class III proteins are specified by genes 7 to 19 and are synthesized from about 6 minutes after infection until lysis (about 25 to 30 minutes at 30°). The genes specifying Class II and III proteins are referred to as late genes and are expressed only if functional T7 gene 1 product is present.

As with other phage genomes, genes with related functions tend to cluster along the genetic map of T7 DNA: genes 2 to 6 affect DNA synthesis (9), genes 7 to 17 specify proteins that are found in mature T7 phage particles (8), and genes 18 and 19 are involved in maturation of T7 DNA from DNA replicating intermediates (11,12).

## Protein Synthesis in T7-Infected Cells

The order of appearance of T7 proteins in infected cells is the same as the order of the corresponding genes on the T7 genetic map (7); T7 genes are expressed according to their genetic map position.

It has been found that the stop point for the transcription of the early region by host RNA polymerase (to the right of the ligase gene) is not 100% effective--small amounts of class II and class III proteins are made after infection with T7 gene 1 mutant phage in which the stop signal is present. The reason for this

may be that a small amount of gene 1 product is present, which regulates late genes, or host RNA polymerase post termination signals are present to prevent widespread transcription of late genes by host RNA polymerase. The latter appears more likely because a double mutation in gene 1 produces the same amount of late proteins.

It is not known why the synthesis of class II proteins stop midway during phage infection; the synthesis of class III proteins slows at the same time. Regulation of class II and III protein synthesis could be controlled at either the level of transcription or translation, or possibly both. The mechanism responsible for determining the relative rates of synthesis of different phage proteins is also unknown.

At least 20 major T7 polypeptides have been synthesized *in vitro* in a protein synthesizing system derived from uninfected *E. coli* and programed by RNA extracted from T7-infected cells (13). Identification of polypeptides as T7 gene-specific products was made in experiments using T7 amber and deletion mutants. The pattern of polypeptide synthesis *in vitro* programed with RNA extracted from infected cells was compared to the pattern observed *in vivo* throughout the time course of T7 infection. The results indicated that nearly all polypeptides displayed a similar time course of synthesis *in vitro* and *in vivo*--the fidelity of the *in vitro* pattern extended

even to relative differences in the levels of synthesis among most of the various T7 polypeptides. These results make it unlikely that alterations in ribosomes, or initiation factors play a major role in the regulation of T7 gene expression.

Not only is phage protein synthesis controlled during T7 infection, but host protein synthesis is inhibited. This inhibition may be required to insure that a sufficient supply of amino acid precursors is available for phage protein synthesis and that the host protein synthesis machinery is available for the synthesis of phage proteins. T7 phage infection induces two negative control mechanisms of protein synthesis (14): host protein synthesis is "repressed" by a "T7 repressor" and early T7 phage protein synthesis is inhibited by a late phage protein. The "repressor" for host protein synthesis is an early T7 protein, but is not one of the known early genes. The "repressor" must be an early T7 gene product because mutations in gene 1 are capable of inhibiting host protein synthesis. These mechanisms do not distinguish between a translational or transcriptional control mechanism for synthesis of host and early phage proteins. It has been suggested that the expression of early phage genes is inhibited by a late protein (14).

## Pleiotropic Effect of the T7 Gene 1 Product

T7 amber mutants in genes 2 to 19 delete only one of the 25 to 30 proteins normally found in wild-type T7-infected cells. In contrast, gene 1 has a highly significant effect on T7 protein synthesis. During infection with gene 1 amber mutants only two or three of the T7 early proteins are synthesized while none of the T7 late genes are expressed (15). Thus, it is clear that unlike the other T7 genes, gene 1 is capable of exerting a pleiotropic effect on the expression of T7 late genes. The mechanism by which gene 1 exerts its pleiotropic effect on late gene functions was determined by Chamberlin and co-workers who found that the gene 1 product is a T7-specific RNA polymerase (16). It was found that the T7 RNA polymerase transcribes only the late region of T7 DNA in vitro which explains why the gene 1 product has a pleiotropic effect on the expression of late phage genes. The T7 RNA polymerase was found to be insensitive to the antibiotics rifampicin and streptolydigin. Two derivatives of rifamycin were found to be effective inhibitors of T7 RNA polymerase when present at high concentrations (100 ug per ml); these derivatives inhibit bacterial RNA polymerase when present at a much lower concentration (10 ug per ml).

The results of Chamberlin and co-workers on T7 RNA polymerase were extended by Gelfand and Hayashi (17) using a coupled transcription-translation system from uninfected E. coli cells dependent on T7 DNA and host RNA polymerase. When rifampicin was added to this system after 20 minutes of RNA synthesis, RNA synthesis became rifampicin resistant which is consistent with the presence of a new rifampicin-resistant RNA polymerase activity. However, when T7 DNA with a gene 1 mutation was used as template, RNA synthesis stopped immediately when rifampicin was added, indicating that the rifampicin-resistant RNA polymerase activity was not present.

RNA Synthesis in T7-Infected Cells

The pattern of RNA synthesis in T7-infected cells was found to closely resemble the pattern of T7-specific protein synthesis (8,15). After infection of E. coli with phage T7, 12 or 13 phage-specific RNA species are synthesized while only 5 of the normal T7 RNA species are synthesized if protein synthesis is blocked by chloramphenicol. If cells are infected with T7 phage containing an amber mutant in gene 1 then only four T7-specific RNA species are detected. These results indicate that gene 1 is transcribed in the absence of protein synthesis and is required for transcription of

the remainder of the T7 genome (late region). The control of T7 late protein synthesis is therefore mediated at the level of transcription by means of positive regulation--the appearance of the T7 RNA polymerase controls the transcription of T7 late genes and therefore the synthesis of late proteins.

The RNA transcripts and proteins specified by the 5 early T7 genes have been identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (18, 19). The five early transcripts are from genes 0.3, 0.7, 1, 1.1, and 1.3. Several small RNA species of unknown function were found which probably originate from the left of the 0.3 gene.

The early T7 RNA species can be mapped by heteroduplex mapping of deletion mutants in the early region, which together with genetic and electrophoretic analysis of T7 RNA transcripts and proteins has produced a detailed physical map of T7 DNA (20). The position of genes on T7 DNA is given as a percentage of the total length of wild type T7 DNA measured from the left end of the T7 genome. The 5 early genes occupy the region between 1.8 and 20.2, while the host RNA polymerase stop signal is mapped at 20.2. The size of the five early RNA species found in vivo are, from the left of the genome, 0.24, 0.70, 1.15, 0.24 and $0.47 \times 10^6$ daltons corresponding to genes 0.3, 0.7, 1, 1.1 and 1.3. The

molar amount of each RNA is the same except for the gene 0.3 transcript which is present in a 10-fold excess (20).

Using the electron microscope to observe DNA-RNA hybrids constructed *in vitro* with *in vivo* RNA synthesized in T7-infected cells and the H strand of T7 DNA, Hyman (21) found that after infection essentially the entire T7 genome is expressed as RNA. The early RNA species mapped contiguously on the genome starting at map position 0.010 and ending at 0.198 (the repetitive ends on T7 DNA are terminally redundant and extend to 0.007 [22]). The size of the early region measured in this way is in good agreement with the size of the early region determined by heteroduplex mapping (20).

Unlike bacterial messenger RNA T7 RNA is metabolically stable. Actinomycin D and rifampicin decay experiments show that T7 RNA is long lived, with a half life of more than 20 minutes (23,24). Marrs and Yanofsky (25) have shown that messenger RNA transcribed from the *tryp*-operon of the host genome is degraded normally in T7-infected cells with a half-life of 2.5 minutes. Marrs and Yanofsky also confirm that T7 RNA is stable when measured by hybridization to T7 DNA.

With the use of T7 amber mutants in gene, which produce only early RNA *in vivo*, Yamada *et al* (26,27) has found that the functional activity of T7 early RNA--tested in an *in vitro* protein synthesizing system--decayed

at a rapid rate (half-life of 6.6 minutes). The ability of the RNA to direct f-met tRNA binding also decayed rapidly. This functional decay was related to a loss of structural integrity of the RNA, as detected by polyacrylamide-agarose gel electrophoresis, which indicated that the size of the RNA became smaller with loss of functional activity. Thus, although T7 RNA is metabolically stable, the ability of the RNA to direct in vitro protein synthesis decayed at a rapid rate. Why T7 RNA is stable and E. coli RNA is not stable is not clear. Perhaps T7 RNA has some structural features that make it resistant to degradation.

Summers and Szybalski (28,29) were the first to find that poly (G) binds to only one of the two strands of T7 DNA. This allowed separation of the two T7 DNA strands by CsCl gradient centrifugation in the presence of poly (G). The DNA strand that bound poly (G) banded at a region of high density in CsCl equilibrium gradients and was referred to as the "heavy" or H strand, while the DNA strand that did not bind poly (G) banded at a lower density and was referred to as the "light" or L strand of T7 DNA.

The poly (G) binding sites on T7 DNA consist of dC-rich clusters of 15 to 40 nucleotides, as determined by the melting temperature and sedimentation rates of $^{3}$H-labeled poly (G) fragments recovered from RNase $T_1$

treated T7 DNA-poly (G) hybrids (28,29). Summers and Szybalski determined that 30 to 75 poly (G) binding sites exist evenly distributed on each T7 DNA molecule. It was further determined (30) that only the poly (G) binding strand (H strand) of T7 DNA was transcribed during all phases of T7 phage development. Pulse-labeled phage RNA isolated from T7-infected cells during different phases of phage development hybridized only to the H strand of T7 DNA.

## The Shutoff of Host RNA Synthesis in T7 Phage-Infected Cells

Summers and Siegel have found (31) that after infection with wild type T7 phage, host RNA synthesis gradually ceases until 5 to 7 minutes post infection when it is undetectable by hybridization. The shutoff of host RNA synthesis may be due to the degradation of host DNA or to a more direct mechanism such as the modification of host RNA polymerase. The breakdown of host DNA is a late gene function, while host RNA shut-off occurs in infection with gene 1 mutants. Therefore, an early phage protein is probably responsible for the shutoff of host RNA synthesis, not the degradation of host DNA. In support of this idea, a T7 mutant which fails to synthesize one of the early T7 proteins of 40,000 daltons (gene 0.7) has been shown by Brunovskis

and Summers to be deficient in the ability to shutoff host RNA synthesis (32,33).

A possible mechanism of host RNA synthesis shutoff has been recently elucidated by Hesselback et al (34,35) who find that a phage-induced protein is associated with host RNA polymerase and is responsible for inactivation of host RNA polymerase activity in infected cell extracts. This inhibitor protein can be removed from host RNA polymerase by phosphocellulose column chromatography; removal of the inhibitor protein resulted in the reactivation of host RNA polymerase. The inhibitor protein has been purified to homogeneity and consists of a 14,000 dalton polypeptide. The purified protein specifically inhibits the holoenzyme form of RNA polymerase. It does not inhibit core enzyme or the T7 RNA polymerase. The inhibitor protein was found to inhibit RNA synthesis at the level of initiation by binding to the holoenzyme and preventing it from binding to DNA. The inhibition was not specific for T7 DNA but occurred when other DNA templates were used. These results have been confirmed by Ponta et al (36).

Although it is reported that gene 0.7 is the host RNA shutoff gene (32,33), extracts of gene 0.7 mutant T7-infected cells have completely inactivated RNA polymerase activity (35); it is more likely that the host RNA shutoff gene is gene 0.3 which has been shown by

Ratner (37) to be the only DNA binding protein among the T7 early phage proteins and to have a molecular weight of 14,000 daltons. This possibility, however, has not yet been tested directly.

Bacteriophage T7 codes for a protein kinase (38). From a study of RNA and protein synthesis after T7 infection it has been suggested that the phage specific kinase may be responsible for the early transcriptional control observed during T7 infection, including the shutoff of host RNA synthesis. It was shown that in kinase-deficient mutants early RNA species are over-produced and the shutoff of host RNA synthesis is delayed (39). Zillig and co-workers (40) have recently proposed a mechanism for the action of the T7 protein kinase in early transcriptional control. Their work indicates that the β' subunit, and to a lesser extent the β subunit, of E. coli RNA polymerase are phosphorylated by the T7 protein kinase along with about 40 other host polypeptides during T7 infection. Phosphorylation of the β' and β subunits of E. coli RNA polymerase may modify the transcriptional specificity of E. coli RNA polymerase in T7-infected cells resulting in the shutoff of host RNA and early phage RNA synthesis. It is also possible that some of the other phosphorylated proteins may function in turning off genes. It has yet to be

shown that phosphorylated E. coli RNA polymerase has altered transcriptional specificity in vitro.

## Binding of Host RNA Polymerase to T7 DNA

Hinkle and Chamberlin (41-43) have utilized the fact that RNA polymerase is quantitatively retained on nitrocellulose filters to study the binding of RNA polymerase to T7 DNA. Complexes between RNA polymerase and labeled T7 DNA are retained on nitrocellulose filters with an efficiency of 70% while T7 DNA itself is not retained. The attachment of a single RNA polymerase molecule to a molecule of T7 DNA is sufficient to cause retention of the complex.

Both RNA polymerase holoenzyme and core enzyme bind to T7 DNA. Holoenzyme binds to two classes of binding sites on T7 DNA: class A sites which result in the formation of a stable complex and class B sites which result in formation of an unstable complex. Eight RNA polymerase (holoenzyme) molecules can bind to a T7 DNA molecule to form highly stable complexes. These stable complexes are thought to be located at specific promotor regions (44). There are many sites on the T7 DNA molecule for unstable complex formation, where the holoenzyme binds weakly and reversibly. It was found by Chamberlin and co-workers that holoenzyme can locate and bind tightly to class A sites with a half-time of

20 seconds after being added to T7 DNA. The rate limiting step in site selection by holoenzyme is the binding and release from the many class B sites on T7 DNA which occurs before the enzyme forms a stable complex at a class A site. The relatively low affinity of holoenzyme for class B sites is essential for rapid location of class A sites. In contrast, core enzyme binds to only one class of binding site but there are many such sites on T7 DNA. It is apparent that the sigma subunit of RNA polymerase is not required for binding of RNA polymerase to DNA--both enzymes bind with high affinity to T7 DNA. It is possible to suggest that RNA polymerase may exist in two conformational states--one designed for promotor binding and initiation (holoenzyme) and the other for RNA chain elongation (core enzyme). The sigma subunit may function in site selection by switching the enzyme between the two conformational states.

Single-stranded breaks in T7 DNA also serve as binding sites for RNA polymerase. Single-stranded breaks in T7 DNA enhance transcription by core enzyme by providing sites for RNA chain initiation (45). Single-stranded breaks in T7 DNA inhibit holoenzyme activity. Inhibition of holoenzyme activity is due to a decrease in the amount of enzyme that can initiate RNA synthesis when DNA containing single-stranded breaks serves as

template (single-stranded breaks are tight binding sites for holoenzyme but few such sites can serve as RNA chain initiation sites). Thus, the structure requirements for a tight binding site for RNA polymerase on T7 DNA are less stringent than the requirements for an RNA chain initiation site; fidelity of initiation of RNA synthesis depends not only on recognition of binding sites by RNA polymerase but also on rigid structural requirements for RNA chain initiation once the enzyme is bound.

## Transcription of T7 Phage Early RNA

The exact mechanism by which early RNA is transcribed by host RNA polymerase has not been completely defined. A number of models have been proposed, including:

(a) post-transcriptional cleavage of a precursor RNA transcript of the entire early region by a sizing factor, RNase III, into five early RNA species.

(b) punctuation of transcription of the early region by the transcription termination factor rho.

(c) existence of independent promotor and terminator sequences for each early phage gene. The experimental basis for these different models will be discussed below.

(a) Transcription of T7 DNA *in vitro* by purified *E. coli* RNA polymerase without added factors produces

large RNA molecules which correspond to the entire early region of T7 DNA (46-48). The early region is transcribed starting at three closely spaced independent initiators located near the left end of T7 DNA and ending at a terminator at 20.2; RNA chains started from two of the initiators begin with ATP, those from the third promotor begins with GTP. Endonuclease activity from uninfected cells can cleave the large RNA molecules at specific sites to generate RNA species essentially the same size as the early T7 RNA species found *in vivo*. The large precursor RNA species can also be cut by purified RNase III to yield similar results: 5 large and 3 small RNA molecules; the small 3 RNA species come from the left of gene 0.3.

In agreement with this (49), the early region of T7 DNA is transcribed as a single, large RNA molecule in a RNase III-deficient mutant. This RNA precursor is essentially identical to the RNA molecules produced *in vitro* by host RNA polymerase. These RNA molecules are cleaved by RNase III in the same manner as are the *in vitro* precursor RNA molecules.

It should be noted that the RNase III mechanism predicts equal amounts of each early RNA, but, as described above, this is not seen *in vivo*.

(b) The action of rho termination factor during transcription as originally described by Roberts (50)

can be summarized as follows: rho factor depresses overall synthesis of RNA by decreasing the length of RNA chains and restricts transcription of host RNA polymerase to particular regions of DNA. Rho factor interacts with RNA polymerase at specific DNA sites leading to formation of RNA species of well-defined length. Rho factor acts catalytically affecting many enzyme molecules.

The following model was suggested by Dunn et al (51) in order to explain early T7 RNA transcription in vitro: all host RNA polymerase molecules with sigma factor start transcription on T7 DNA at a single unique initiation site at the left end of the T7 genome and propagate towards the right. There are several rho-dependent RNA termination signals, one for each early RNA, where rho-mediated release of RNA occurs. The RNA polymerase is not released and continues to propagate along the H strand of T7 DNA. After transcription of the early region, a second kind of termination site releases the polymerase.

Goldberg and Hurwitz (52) have found that rho factor can interact with DNA to form a protein-DNA complex, but it is doubtful that this is the mechanism of action. The binding of rho to DNA is not stoichiometrically related to its effects on transcription, nor is the effect of rho factor altered by a 10-fold excess of DNA. The ratio of rho factor to RNA polymerase

is the most critical factor. Rho factor appears to interact with host RNA polymerase at specific DNA sites leading to formation of RNA chains of well defined length. Even though rho factor can terminate RNA synthesis at specific sites it does not bind tightly to RNA polymerase, in fact its presence is not needed until RNA polymerase approaches the termination site.

The mechanism of rho termination is not yet known. Richardson et al (53) have recently found that rho factor has RNA-dependent ATPase activity and may be the product of the host SuA gene. Poly (C) is particularly effective in stimulating the ATPase activity of rho factor (54). If this is true then rho factor may play a significant role in causing polar effects (55). Richardson et al propose that rho factor recognizes some structural features in nascent RNA as it emerges from the RNA polymerase molecule. It is proposed that the binding of rho factor to this nascent RNA site affects RNA polymerase in such a way that RNA polymerase is released from DNA template resulting in termination of RNA synthesis. If, however, the rho binding site on the nascent RNA is translated by a ribosome before rho factor binds then termination of RNA synthesis would be prevented. If translation is prematurely terminated at a nonsense codon, then the ribosome would leave the nascent RNA and expose the emerging nascent RNA to rho

factor. Thus, a mutation in one gene of an operon could reduce the level of expression of the genes in the operon that lie on the operator-distal side of the mutant gene producing the polar effect.

In summary, in the absence of rho factor one large RNA transcript is synthesized, while transcription in the presence of rho factor produces five different RNA species. In addition, transcription in the presence of rho factor is totally asymmetrical and restricted to the early region of the T7 genome (56). The role of rho factor *in vivo*, however, is not clear since only two of the RNA species transcribed in the presence of rho factor *in vitro* appear to be identical to *in vivo* early RNA species.

An alternative explanation for the function of rho factor has been proposed (56). Since it has been shown that only some of the RNA products of *in vitro* RNA transcription in the presence of rho factor match RNA species present in T7-infected cells *in vivo*, rho factor may be a defense mechanism of the host cell to disrupt the transcription of the early region by terminating transcription at incorrect positions; this would be particularly fatal if transcription of gene 1 were interrupted. However, there is no evidence that rho factor acts *in vitro* in the same manner that it does

in vivo; it may in fact recognize only correct termination points in vivo.

(c) Another model for in vivo early RNA synthesis has been proposed by Minkley (57,58). He suggests that each early gene is controlled by its own promotor and terminator.

He has identified 12-specific RNA species synthesized at early times during T7 infection in ultraviolet-irradiated host cells. Ultraviolet-irradiation of host cells prevents host protein and RNA synthesis but not phage macromolecular synthesis (59). The molecular weights of these RNA species range from $0.02\text{-}1.05 \times 10^6$ daltons. The sum of the molecular weights can fit within the early region on the T7 genome without overlaps.

The early RNA species identified by Minkley were not present in equimolar amounts. Thus, they could not originate by post-transcriptional cleavage of a single precursor molecule of the entire early region as suggested by the RNase III model of transcription. The ability to correlate a specific T7 protein with a monocistronic transcript of its gene by gel electrophoresis indicated to Minkley that the rate at which proteins are synthesized during phage T7 infection may be dependent on the number of copies of each RNA transcript present; in otherwords, on the efficiency of

initiation sites for each RNA transcript. The location of the four largest transcripts have been mapped by Minkley within the early region of T7 DNA.

Three ATP initiation sites, one GTP initiation site and four termination sites within the early region were found in vitro by labeling RNA molecules at their 5' termini with $\gamma$ -$^{32}$P-labeled nucleoside triphosphates (58); an extremely short GTP initiation transcript from the incorrect L strand of T7 DNA was also found. A possible reason for the failure of other investigators (46-48) to obtain these results is that highly specific initiation of in vitro RNA synthesis occurs only at low molar ratios of RNA polymerase to DNA template. RNA polymerase is capable of initiating RNA synthesis at random nonspecific sites at high molar ratios of enzyme to DNA template.

Minkley and Pribnow have found that dinucleotides can be used to stimulate transcription in vitro from selected initiation sites on T7 DNA (60). Using this method seven initiation sites (three very close together in the early promotor region) and five termination sites were mapped relative to known deletions in the early region of the T7 genome. Over twenty H strand-specific RNA products were formed; most resulted, however, from readthrough of termination signals. Despite this there was a strong correlation between the size of the products

synthesized in vitro by this method and the size of in vivo early RNA species. The ratio of products initiated with ATP to GTP by this method varied from about one to four, showing only a modest preference for ATP. ATP was used exclusively at three initiation sites, GTP was used at two sites, and either seem to be used at one site. In addition, Minkley and Pribnow found that holoenzyme was capable of transcribing in vitro a product initiated with GTP with a molecular weight of 80,000 daltons from the L strand of T7 DNA.

Initiation sites may consist of a strong RNA polymerase binding sequence adjacent to or including the DNA sequence of the 5' end of the corresponding RNA (61-63). Thus, the dinucleotide-DNA complex may be stabilized by the presence of RNA polymerase. RNA polymerase should be capable of binding to the RNA polymerase binding sequence on DNA and also bind the dinucleotide, if the dinucleotide contains the sequence of the 5' end of the corresponding RNA. Minkley and Pribnow found that several different dinucleotides could stimulate synthesis from the same initiation site (60). Thus, there may be a short region (5-6 nucleotides) where RNA polymerase can initiate with a paired dinucleotide.

CpA was ten times more efficient in priming RNA synthesis than was ATP, probably because of the extra

stability provided by the dinucleotide. The most effective dinucleotide at most initiation sites contained a purine nucleoside in the 3' position of the dinucleotide which corresponds to the 5' triphosphate of the RNA chain initiated at that site. Less efficient dinucleotides contained the purine nucleoside triphosphates in the 5' position and presumably have the RNA initiation base as the 3' end group. CpC was very efficient in initiating RNA synthesis, but it is not known if it is contained in the sequence before or after the 5' end of the corresponding RNA. One possible fault of this procedure is that it may increase the relative efficiency of weak initiation sites (60).

In summary, three models for early RNA transcription have been proposed:

1. The host RNA polymerase synthesizes one continuous transcript from the entire early region. The precursor RNA transcript is cleaved by RNase III into the five RNA species observed *in vivo*.

2. Only one major RNA polymerase binding site exists and transcripts are synthesized by a rho mediated termination-reinitiation mechanism without release of the polymerase at several rho termination sites throughout the early region.

3. Autonomous RNA initiation and termination sites exist throughout the early region, each of which

controls the synthesis of RNA from an independent transcriptional unit.

The first two models are not capable of completely describing early RNA synthesis as it exists in vivo. The first model cannot adequately explain the 10 fold excess amount of gene 0.3 RNA observed in vivo. The second model is inadequate because rho factor fails to produce RNA molecules in vitro that are the same size as those found in vivo. The third model predicts that proteins are synthesized at a rate dependent on the efficiency of the initiation sites for each RNA transcript. If this is true, there should be a correlation between the molar amount of a given monocistronic transcript and the molar amount of the corresponding protein. This is found to be true: the gene 0.3 product is the most plentiful early protein in T7 infected cells and the gene 0.3 RNA transcript is the most prominent RNA transcript. The other early proteins are present in more nearly equal molar amounts as are their RNA transcripts. However, the specificity of in vitro transcription observed by Minkley is not in total agreement with the in vivo specificity of transcription.

## Characterization of the T7-Specific RNA Polymerase

The T7 RNA polymerase has been extensively studied by Chamberlin and co-workers (64-68). The T7

RNA polymerase is distinguishable from host RNA polymerase by its stringent template specificity and resistance to the bacterial RNA polymerase inhibitors rifampicin and steptolydigin.

Only T7, T3 and salmon sperm DNA have appreciable template activity. The activity of T7 RNA polymerase with T3 DNA as template is about one-half that with T7 DNA as template. The enzyme shows some activity with salmon sperm DNA but large concentrations of salmon sperm DNA are needed for maximum activity.

Denaturation of T7 DNA decreases its template activity and also abolishes the absolute requirement of T7 RNA polymerase for its specific promotor. Denaturation of T7 DNA results in a loss of strand specificity during transcription. Thus, poly (dT) is an active template for T7 RNA polymerase while poly (dA) · poly (dT) is not; but the loss of specificity is not complete since T7 RNA polymerase will utilize only poly (dT) and poly (dC) as templates. The DNA templates poly (dC) · poly (dG) and poly (dI) · poly (dC) are active templates with T7 RNA polymerase, but only the dC-containing strand is transcribed.

Promotors recognized by holoenzyme are called class I promotors while those recognized by T7 RNA polymerase are called class II promotors. All phage and bacterial DNA templates tested contain class I promotors,

while only T7, T3, and possibly M13 DNA contain class II promotors. Class II promotors may also be present on salmon sperm DNA, but at a much lower concentration than on T7 DNA. The specificity of host RNA polymerase for class I sites is lost when single-stranded DNA templates are transcribed or when DNA templates containing single-stranded breaks are transcribed. The T7 RNA polymerase also loses specificity when some single-stranded DNA templates are transcribed but does not lose specificity when DNA templates containing single-stranded breaks are transcribed.

Chamberlin and co-workers have also studied the inhibition of T7 RNA polymerase activity. Synthesis of RNA by T7 RNA polymerase is inhibited by actinomycin D, heparin, high salt concentrations and poly r(U); all these inhibitors block transcription immediately, even after initiation of RNA synthesis, indicating that RNA chain growth is specifically inhibited. RNA chain elongation by holoenzyme is not sensitive to these inhibitors (except actinomycin D). RNA synthesis catalyzed by T7 RNA polymerase is not affected by the bacterial RNA polymerase inhibitors rifampicin and streptolydigin (except those derivatives noted above) or by preincubation of the enzyme with poly r(C), poly r(I) or a variety of heterologous DNA molecules. Host RNA polymerase is stimulated by KCl up to a concentration of 0.15M, then

activity is inhibited sharply between 0.15-0.3M KCl while the T7 RNA polymerase is inhibited between 0-0.15M KCl with no activity observed above 0.2M KCl.

When poly r(U), or heparin are added before RNA synthesis is initiated the host RNA polymerase is inhibited; however, when added 15 seconds after initiation of RNA synthesis they have no effect on RNA synthesis. In contrast, poly r(U) and heparin inhibit T7 RNA polymerase activity equally well if added before or after RNA synthesis has begun. Ultraviolet-irradiated T7 DNA inhibits initiation of RNA synthesis by T7 RNA polymerase (ultraviolet-irradiation of T7 DNA blocks its template activity).

The following mechanisms of inhibition by heparin and ultraviolet-irradiated T7 DNA have been suggested by Chamberlin and co-workers (65). Heparin inhibits chain elongation catalyzed by T7 RNA polymerase presumably because the DNA binding site of T7 RNA polymerase is partly exposed during RNA synthesis and heparin interacts with it. It is also possible that it interacts with another site on the enzyme. Ultraviolet-irradiated T7 DNA acts as an inhibitor of T7 RNA polymerase activity by providing a promotor for the binding of T7 RNA polymerase but inhibiting RNA chain elongation by the bound T7 RNA polymerase molecules. When ultraviolet-irradiated T7 DNA is added after RNA synthesis has begun with native

T7 DNA as template it has little effect on the reaction (for a short reaction period). Ultraviolet-irradiated DNA is therefore an inhibitor of free T7 RNA polymerase and does not affect RNA chain growth catalyzed by T7 RNA polymerase once the enzyme has already initiated RNA synthesis.

## Late Phage RNA Synthesis in Infected Cells

Golomb and Chamberlin (67-68) have found that *in vitro* transcription of T7 DNA by T7 RNA polymerase produces six discrete size classes of T7 RNA species with a broad range of molecular weights (from $2 \times 10^5$-$5 \times 10^6$ daltons). The six major T7 RNA species are synthesized in approximately equimolar amounts, with the exception of species III, which is present in twice the amount of the other species and may actually consist of two RNA spices transcribed from separate regions of the T7 genome. The RNA species are initiated independently with GTP at the 5' end and elongated at a rate of 230 nucleotides per second under standard *in vitro* conditions. When artificially shortened T7 DNA templates are transcribed, four of the seven RNA spices are truncated or deleted. This indicates that the four RNA spices are terminated near the right end of T7 DNA, probably at a common termination site near 98.5%. Since the approximate lengths of the transcripts are known, the promotor sites

for the T7 RNA spices can be mapped at 56, 64, 83, and 97% on T7 DNA. Only one major T3 RNA species is transcribed by T7 RNA polymerase with a promotor at 83% and a terminator at 98.5%.

Niles et al (69) found that the RNA products synthesized in vitro by the T7 RNA polymerase are larger than the late RNA transcripts found in infected cells. It has been concluded therefore by Dunn and Studier (47) that late RNA transcripts may also be cleaved by RNase III.

Niles et al (70,71) have recently used fractionated and unfractionated late T7 RNA species synthesized in vitro by the T7 RNA polymerase to program an in vitro protein synthesizing system derived from uninfected E. coli. They conclude from observing polyacrylamide gels of both the RNA transcripts and the translation products that virtually the entire late region of T7 DNA is transcribed in vitro by T7 RNA polymerase. The initiation sites found agree with those described by Golomb and Chamberlin, but in addition they found an initiation site between gene 1 and 1.3 for T7 RNA polymerase, which, if recognized in vivo, explains why gene 1.3 continues to be transcribed after the other early genes are shutoff. They have also found a termination site to the right of gene 10 not described by Chamberlin. Band VI, as described by Chanberlin,

does not appear to stimulate the synthesis of any T7 protein *in vitro*. In addition, they found that the majority of the RNA transcripts transcribed by T7 RNA polymerase *in vitro* translate into class III proteins *in vitro*.

The individual T7 late RNA spices are transcribed by a pattern of overlapping transcription and are translated with different efficiencies (70,71). Genes 17 and 19 are transcribed from three initiation sites, but both proteins are translated in small amounts *in vivo* and *in vitro*. However, gene 10 is also transcribed from two or three initiation sites but is very effectively translated both *in vitro* and *in vivo*. More dramatically, gene 11 is transcribed from only one initiation site but is in greater molar yield than either gene 17 or 19.

## Control of RNA Synthesis in T3-Infected Cells

The control of RNA synthesis in T3-infected cells is similar to that described above for T7-infected cells. Dunn *et al* (51) have found that five early T3-specific RNA species are synthesized from the H strand of T3 DNA *in vitro*. In addition, he found that *in vitro* transcription of T3 DNA by *E. coli* RNA polymerase can closely approximate the *in vivo* pattern of RNA products only if the rho termination factor is present under optimal ionic conditions. In the absence of rho factor host RNA

polymerase transcribes both strands of T3 DNA but synthesizes only anti-late RNA species. In vitro transcription from the H strand of T3 DNA is limited to the early region of the T3 genome in the presence of rho factor at low ionic strength (0.05M KCl). The RNA products were similar in size to those found in vivo. Transcription from the L strand of T3 DNA is strongly depressed in the presence of rho factor. L strand termination occurs even at high ionic strength (0.2M KCl) indicating that there may be strong preferential termination of L strand transcripts.

It was concluded (51) that the termination site between the early and late regions of T3 DNA operates at approximately 50% efficiency in vitro in the absence of rho factor, while the same site operates at 95% efficiency on T7 DNA. Thus, rho factor is needed to prevent readthrough to the late region of T3 DNA but not T7 DNA. On T7 DNA there is incorrect L strand initiation but also rho-independent termination. With T3 DNA the corresponding termination site has a low efficiency and rho factor is needed to improve asymmetry of transcription.

The T3 specific RNA polymerase transcribes only the H strand of T3 DNA in vitro (51). The products are discrete RNA species similar in size to those isolated late in infection from T3-infected cells. Rho factor

has no effect on transcription by T3 RNA polymerase. Unlike the T7 RNA polymerase, the T3 RNA polymerase transcribes both early and late regions of the T3 genome *in vitro*.

Dunn *et al* have confirmed these results using another experimental technique (72). The size distribution of the RNA species coding for three T3-specific enzymes--the SAM cleaving enzyme, T3 RNA polymerase and T3 lysozyme--were determined by sedimenting the RNA isolated from T3-infected cells in a sucrose gradient. A cell-free protein synthesizing system was utilized, followed by enzymatic analysis of the translation products, to determine the position of the corresponding RNA transcript in the sucrose gradient. This procedure was followed with *in vivo* RNA and with RNA synthesized *in vitro* by host RNA polymerase from T3 DNA. In the presence of rho factor, synthesis of early RNA *in vitro* (coding for the SAM cleaving enzyme and the T3 RNA polymerase) was identical in size and coding capacity to early RNA isolated from infected cells. It was also found that RNA transcribed by T3 RNA polymerase consisted mainly of late RNA (coding for T3 lysozyme) but also contained functional early RNA.

The T3-specific RNA polymerase has been characterized by Maitra and by Bautz and their co-workers (73-77) and is similar to the T7-specific RNA polymerase

described by Chamberlin et al (16). It consists of a single polypeptide of 105,000 daltons and requires the presence of $Mg^{+2}$, nucleoside triphosphates and T3 DNA for optimal activity. T7 DNA is utilized as a template, but only at 2-5% of the optimal activity with T3 DNA as template. It was found that rho factor has no effect on the rate, yield or size of RNA formed in vitro with T3 DNA as template. T3 RNA polymerase was found to be highly sensitive to salt concentrations above 0.03M KCl and to sulfhydryl group reagents. The enzyme catalyzes a T3 DNA dependent $^{32}$P-labeled PPi exchange into nucleoside triphosphate, but the reaction is limited to GTP alone. No pyrophosphorolysis of free RNA was found.

Maitra and co-workers have also studied the transcription of denatured DNA templates with the T3 RNA polymerase (78). The T3 RNA polymerase transcribes a variety of denatured DNA templates including denatured T3, T7, calf thymus, ØX174 and fd DNA templates, but at markedly reduced rates. Thus, as with the T7 RNA polymerase, it is evident that the high degree of template specificity of the T3 RNA polymerase resides both in the enzyme and in the secondary structure of T3 DNA template.

The kinetics of in vitro RNA synthesis catalyzed by T3 RNA polymerase show that activity is critically dependent on GTP concentration (79). The nature of the

data obtained indicates that T3 RNA polymerase initiates chains with pppGpG, while with T7 DNA as template T3 RNA polymerase initiates RNA chains with pppGpA. A high level of pyrophosphate exchange is obtained in the presence of GTP alone as substrate which confirms this interpretation. T7 RNA polymerase, unlike T3 RNA polymerase, shows a high rate of PPi exchange with GTP alone as substrate on both T3 and T7 DNA. This suggests that there are differences in initiation regions for T3 and T7 RNA polymerases--T3 RNA polymerase may be more stringent in its site specificity. A further indication that the two phage polymerases may differ in structure is the fact that RNA synthesis by T3 RNA polymerase is not inhibited by heparin, unless it is added at the same time as substrate, when it inactivates both unbound RNA polymerase and RNA polymerase bound to DNA. Heparin will inhibit the T7 RNA polymerase regardless of when it is added.

Maitra and co-workers have recently studied *in vitro* transcription by both the T3 and host RNA polymerase (80). RNA synthesized by T3 RNA polymerase *in vitro* hybridizes only to the H strand of T3 DNA, as does all *in vivo* phage RNA. Host RNA polymerase transcribes RNA that hybridizes with both the H and L strands of T3 DNA, although greater than 70% hybridizes with the H strand. In contrast to other studies summarized above, the ratio of RNA hybridization to the H or L strand was

unaltered by the presence of excess sigma factor, or the presence of rho factor, or by the molar ratio of RNA polymerase to DNA present in the reaction mixture.

It was shown by Maitra et al that the in vitro products of T3 RNA polymerase contain all late RNA sequences and all early RNA sequences. Thus, T3 RNA polymerase transcribes the entire early and late region of T3 DNA in vitro. The RNA products of host RNA polymerase transcription of T3 DNA in vitro contain all the sequences present in early in vivo RNA, but also contain 50% of the sequences present in late in vivo RNA. In the presence of rho factor all early sequences were present, but few late RNA sequences were present. Thus, rho factor restricts host RNA polymerase transcription to the early region of T3 DNA in vitro.

Dunn and Bautz have found different template specificities with the T3 and T7 RNA polymerases (81). The two RNA polymerases transcribe T3 and T7 DNA with greatly different efficiencies. T7 RNA polymerase transcribes T7 DNA about twice as effectively as T3 DNA, while T3 RNA polymerase has very low activity on T7 DNA but is highly active with T3 DNA.

Hercules et al have found (82) that the processing of early T3 and T7 RNA by RNase III is necessary for their efficient translation both in vivo and in vitro. Uncleaved T3 and T7 RNA species are poor templates for in vitro

synthesis of the phage enzymes SAMase, DNA ligase and lysozyme. Only small amounts of phage enzymes are synthesized after T3 or T7 infection of E. coli RNase III-deficient strains (83). This low rate of phage-specific enzyme synthesis in vivo is not due to a lack of phage-specific RNA, since equal amounts of phage RNA are found in wild-type and RNase III-deficient hosts. There is a low rate of translation of phage RNA from RNase III-deficient host cells in an in vitro RNase III-deficient protein synthesizing system; this activity is probably a result of residual RNase III activity (83). If sizing of RNA were required for translation of bacterial mRNA, then the RNase III-deficient strain should be inviable. However, the strain used retains some RNase III activity, which may be enough to maintain the slow growth rate observed.

Srinivasan and co-workers have presented evidence that the host RNA polymerase is modified after T3 phage infection (84). The modified host RNA polymerase behaves differently in its elution properties from DEAE-cellulose, phosphocellulose and DNA-cellulose columns. The specific activity of the modified enzyme is about one-fourth that of the unmodified host RNA polymerase which Srinivasen indicates may be due to an alteration in the $\beta'$ subunit of host RNA polymerase. Modification of the host RNA polymerase also occurs in infection with T3 gene 1 mutant

phage. However, an analogous modification was not found by Srinivasan in E. coli infected with T7 phage. Thus, it appears that a T3 early gene controls the modification of host RNA polymerase but a similar gene does not exist on the T7 genome.

Srinivasen et al also found evidence for an inhibitor protein capable of inhibiting E. coli RNA polymerase (85,86). This protein has no effect on core polymerase, indicating a requirement for sigma factor. The protein did not bind directly to DNA or RNA polymerase or to DNA-RNA polymerase complex, instead the presence of ATP and GTP are required for the binding of the inhibitor protein to RNA polymerase-DNA complex. The inhibitor protein is not an early protein, thus it must be a late gene under the control of the T3 RNA polymerase. This is in contrast to the T7 inhibitor protein which appears to be an early T7 protein capable of directly binding to host RNA polymerase (34,35).

# INTRODUCTION

The studies in this dissertation were initiated largely with the discovery by Chamberlin and co-workers (16) that bacteriophage T7 codes for a new RNA polymerase upon infection of E. coli. It was hoped that the bacteriophage gh-1 which was previously characterized by Lee and Boezi (87) would also induce a novel RNA polymerase upon infection of Pseudomonas putida. The search for a new RNA polymerase in gh-1-infected P. putida cells was then undertaken. The results of the purification and characterization of the gh-1-induced RNA polymerase have been recently published and a reprint is presented in the appendix.

The gh-1-induced RNA polymerase proved to be similar to the T7 RNA polymerase. Despite this it was hoped that the control of transcription in gh-1-infected cells could be defined on a molecular level and that the control mechanisms found in gh-1-infected cells would be significantly different from the mechanism of transcriptional control in T7-infected E. coli which was being elucidated at the same time as work was progressing on the gh-1 phage system. It was also hoped that the mechanisms of transcriptional control operating

in gh-1-infected cells could be duplicated in vitro with highly purified RNA polymerase and gh-1 DNA.

The results described in this dissertation clearly show that the control of transcription in gh-1-infected cells is lost during late periods of infection. A model describing how this loss of control may take place at the molecular level is presented in the Discussion. The loss of control during late time intervals of gh-1 infection is indicated by the appearance of complementary RNA. Complementary RNA is not synthesized during T7 phage infection and is known to be synthesized in very few systems either procaryotic or eucaryotic.

This dissertation provides evidence that the gh-1 phage system is unlike other phage systems described in that transcription of gh-1 phage RNA is not stringently controlled during infection. This dissertation also indicates that the molar ratio of RNA polymerase to phage DNA may be important for the control of RNA transcription in vivo. It has been shown by other investigators (57, 58) that the molar ratio of RNA polymerase to DNA is important in limiting transcription to certain regions of DNA in vitro but no evidence has been provided that it is important to the control of RNA transcription in vivo. Finally, the gh-1 phage system is the only phage system so far described in which large amounts of

complementary RNA are synthesized *in vivo*. While the function of complementary RNA, if any, is not known the gh-1 phage system provides an excellent opportunity to study the significance of complementary RNA.

Before the results of this dissertation are discussed previous work on the gh-1 phage system will be quickly reviewed. The gh-1 phage was originally isolated by Lee and Boezi (87). The size and shape of the gh-1 phage particle is similar to the T7 phage particle. In addition, the size of the gh-1 genome ($23X10^6$ daltons) is also similar to the size of the T7 genome. The latent period of the gh-1 phage lytic cycle is about 21 minutes with a burst size of 100 plaque forming units (PFU) per infected cell.

The RNA polymerase from uninfected *Pseudomonas putida* has been purified to near homogeneity and characterized by Johnson *et al* (42). The enzyme has a subunit composition analogous to the *E. coli* RNA polymerase and like the *E. coli* enzyme transcribes a large variety of DNA templates *in vitro* and is sensitive to the drug rifampicin.

The gh-1-induced RNA polymerase has also been purified to near homogeneity and characterized as described in the appendix. The reader should read the appendix first since these results are not discussed in the Results section.

## MATERIALS AND METHODS

### Materials

Whatman DEAE-cellulose (DE 52) and phosphocellulose (PCl and PC 11) were purchased from Reeve Angel. Lysozyme, chloramphenicol, bovine serum albumin and β-mercaptoethanol were obtained from Sigma. Calf thymus DNA, unlabeled ribonucleoside triphosphates and dithiothreitol were from P-L Biochemicals. $^{3}$H-labeled thymidine (6.7 Ci/mmol), $^{3}$H-labeled CTP (26.2 Ci/mmol), $^{3}$H-labeled uridine (26.2 Ci/mmol), and Aquasol were purchased from New England Nuclear. Bio-Gel P-150, Bio-Gel A-1.5m, and agarose were obtained from Bio-Rad Laboratories. Poly (U,G), poly d(A-T) and poly d(C)· poly d(G) were from Miles Laboratories. E. coli RNA polymerase was purchased from Boehringer Mannheim, ribonuclease from Cal Biochem and deoxyribonuclease I from Worthington Biochemical Corporation. Nitrocellulose membrane filters were obtained from Schleicher and Schuell. "Stains-all" dye was from the Eastman Kodak Company. The rifamycin derivatives were gifts from Dr. Luigi Silvestri, Gruppo Lepetit, Inc., Milan, Italy. Cordycepin triphosphate and E. coli ribosomal RNA were the kind gifts of Dr. Fritz Rottman of this department. T3, T4 and T7 phage were generous

gifts of Dr. Loren Snyder, Department of Microbiology and Public Health, Michigan State University.

## Growth of Pseudomonas Putida and Infection with Bacteriophage gh-1

Pseudomonas putida (ATCC 12633) was grown at 33°C in a medium containing, in grams per liter: yeast extract, 5; tryptone, 5; glucose, 5; NaCl 8; $Na_2HPO_4$, 6; $KH_2PO_4$, 3. After cell growth had reached the mid-logarithmic phase, gh-1 phage was added to a multiplicity of 5-10 plaque forming units (PFU) per bacterial cell. The infected cells were allowed to incubate for 10 minutes and were then poured onto a half volume of crushed, cooled (-20°C) ice and centrifuged. The infected cell pellets were quick-frozen in an acetone-dry ice bath and stored at -20°C. Cells infected by this procedure were used for the preparation of gh-1 RNA polymerase and competitor RNA. In all other cases, where complete and synchronous infection was required, another procedure was used. To insure complete and synchronous infection, after P. putida cells had reached the mid-logarithmic stage of growth they were concentrated ten-fold by centrifugation and chilled to 4°C. Gh-1 phage was then added at a multiplicity of 5-10 PFU per cell. At 4°C adsorption of gh-1 phage to P. putida cells takes place. After 10 minutes at 4°C, the culture was diluted into

pre-warmed media (33°C). The time of dilution into pre-warmed media was taken as the 0 time of infection.

## Plaque Assay of Bacteriophage and One Step Growth Experiment

Bacteriophage gh-1 was assayed by the plaque assay technique described by Lee and Boezi (87). The one step growth experiments were as described by Lee (90) with the exception that rifampicin (100 ug per ml) was added to the media at the indicated times.

The one step growth experiments with T3 phage were done in a similar way except that T3 antiserum was not available. The multiplicity of infection was 1 PFU per *E. coli* cell.

Plaque assays of the infected cell culture during the one step growth experiments were done at 10 different time intervals during a 60 minute incubation period. The dilution of the infected culture was such that at least 100 plaques were formed for each plaque assay. The one step growth experiments were repeated at least twice with identical results.

## Purification of Bacteriophage and Isolation of DNA

Bacteriophage gh-1 was purified from *P. putida* lysate by the method of Lee and Boezi (87). The phage was purified from cell debris by differential centrifugation and further purified by chromatography on

DEAE-cellulose. Bacteriophage T3 was purified from E. coli lysate by differential centrifugation and by banding in a preformed CsCl density gradient. Bactriophage DNA was isolated by the method of Thomas and Abelson (88) while P. putida DNA was isolated by the procedure of Thomas et al (89). Commercial calf thymus DNA was extracted twice with phenol (in the presence of 0.1% SDS) and extensively dialyzed before use.

RNA Polymerase Assay

The RNA polymerase assay of P. putida and gh-1 RNA polymerase measured the incorporation of $^{3}$H-labeled CMP into a TCA-insoluble product. The volume of the standard reaction mixture for host RNA polymerase was 0.125 ml and contained: 50mM Tris-HCl (pH 8), 10mM $MgCl_2$, 2.5mM $MnCl_2$, 50mM KCl, 1mM DTT, 0.4 mM in each of the four NTP's, 500 ug per ml BSA, 50uCi per ml $^{3}$H-labeled CTP and 100 ug per ml calf thymus DNA. The final specific activity of the $^{3}$H-labeled CTP was $2X10^4$ CPM per nmol. The assay was started by the addition of enzyme. The reaction was incubated at 30°C for 10 minutes and stopped by the addition of 0.2ml of a 0.1% SDS solution followed by 5ml of cold 10% TCA containing 1% sodium pyrophosphate. A few drops of 2.5mg per ml herring sperm DNA as carrier was added. The mixture was allowed to stand for about five minutes at 4°C and then

the precipitate was collected on nitrocellulose membrane filters. After washing the filters with 15ml of cold 10% TCA, they were dried at 80°C for ten minutes and counted by liquid scintillation spectroscopy using a toluene-based fluor (5ml).

The procedure was the same for the assay of gh-1 RNA polymerase except the standard reaction mixture contained 100ug per ml gh-1 DNA and the $MnCl_2$ and KCl were omitted. Various components were either added or omitted from the standard reaction mixtures as indicated in the legends to the appropriate tables and figures.

Purification of RNA Polymerase

The gh-1-induced RNA polymerase was purified by the method described by Towle, Jolly and Boezi (91). The P. putida RNA polymerase holoenzyme and core enzyme were purified by the method of Johnson et al (92) with the following modifications: (a) all buffers contained 15% glycerol (V/V) with the exception of the final storage buffer which contained 50% glycerol (V/V). (b) Cell disruption was accomplished by use of a French pressure call at 7000 lb per in. (c) The 30% ammonium sulfate fractionation step and the second DEAE-cellulose column chromatography step were omitted; instead, the final step in the enzyme purification was gel filtration using a Bio-Gel A-1.5m column. The final concentration of

enzyme was 22 mg per ml and the specific activity was 900 units per mg.

By the procedures outlined above all RNA polymerase preparations were greater than 90% pure as determined by SDS-polyacrylamide gel electrophoresis. Both enzymes were judged to be free of RNase activity by the failure of the enzymes to alter the profile of $^{3}$H-labeled ribosomal RNA as analyzed by polyacrylamide-agarose gel electrophoresis after incubation of 0.1mg per ml enzyme with $^{3}$H-labeled ribosomal RNA (150,000 CPM per ml) for 30 minutes at 30°C. Similarly, the enzymes were judged to be free of DNase activity by their failure to change the sucrose density gradient sedimentation profile of $^{32}$P-labeled gh-1 DNA. RNase III activity was also found to be absent when assayed by the procedure of Robertson et al (93).

Preparation of $^{3}$H-labeled RNA in vitro

$^{3}$H-labeled RNA synthesis was carried out in vitro by the P. putida holoenzyme and core enzyme and the E. coli RNA polymerase in a reaction mixture containing 50mM Tris-HCl (pH 8), 10mM $MgCl_2$, 2.5 mM $MnCl_2$, 50mM KCl, 1mM DTT, 0.4mM in each of the four NTPs, 500 ug per ml BSA, and 0.2 mCi per ml $^{3}$H-labeled CTP. The final specific activity of CTP was about $8X10^4$ CPM per nmol. The amount of DNA and enzyme in

each reaction mixture was varied as indicated for each figure. The reaction was begun by the addition of enzyme. The reaction was incubated for 45 minutes at 30°C and then chilled to 4°C. A small amount of solid DNase was added and the mixture was incubated at 37°C for 15 minutes. The $^{3}$H-labeled RNA was extracted twice with an equal volume of phenol and the aqueous phase was dialyzed extensively against 2XSSC. The procedure for $^{3}$H-labeled RNA synthesis in vitro by the gh-1 RNA polymerase was the same except the reaction mixture contained gh-1 DNA and the $MnCl_2$ and KCl were omitted. The conditions of in vitro $^{3}$H-labeled RNA synthesis were such that the rate of reaction was proportional to enzyme concentration.

The procedure for $^{3}$H-labeled RNA synthesis using infected cell extracts was similar to that above except various RNA polymerase inhibitors were added to inhibit either the host RNA polymerase or the gh-1 RNA polymerase. To inhibit the host RNA polymerase rifampicin was added to a final concentration of 10 ug per ml and streptolydigin to 100 ug per ml. The gh-1 RNA polymerase was inhibited in the crude extract mixture by the addition of cordycepin triphosphate (3'-dATP) to a final concentration of 50uM (91). All components of the standard reaction mixture for RNA synthesis were present except DNA. The inhibitors were added 5 minutes prior to the addition of $^{3}$H-labeled

CTP. The crude extract of infected cells was prepared by lysing infected cells with lysozyme buffer as described. The final protein concentration in the reaction mixture was about 0.5 mg per ml. The $^{3}$H-labeled RNA was extracted by the procedure of Bøvre and Szybalski (94).

Preparation of Pulse-Labeled RNA

P. putida cells were infected as described with the exception that the cells were grown in GSC media. The RNA was pulse-labeled with $^{3}$H-labeled uridine (25 uCi per ml) at appropriate time intervals. The pulses were terminated by rapidly chilling the cells in an ice-water bath and collecting by centrifugation. Following centrifugation, the cell pellets were frozen in an acetone-dry ice bath and stored at -20°C until use. $^{3}$H-labeled RNA was extracted by the procedure outlined below for preparation of competitor RNA. Pulse-labeled chloramphenicol RNA was prepared in the same manner except chloramphenicol (400 ug per ml) was added to the media 5 minutes before infection and the RNA was pulse-labeled from 0-5 minutes post infection.

Preparation of Competitor RNA

P. putida cells were grown and infected as described. At appropriate times the infection was terminated by pouring the culture onto a half volume

of crushed, cooled (-20°C) ice. The infected cells were collected by centrifugation and the infected cell pellets were quick-frozen in an acetone-dry ice bath and stored at -20°C. Chloramphenicol competitor RNA (CAM RNA) was prepared from cells infected for 5 minutes in the presence of 400 ug per ml chloramphenicol (added five minutes before infection). Total RNA was extracted according to the hot phenol method of Bøvre and Szybalski (94). Infected cells were lysed with lysozyme (200 ug per ml in a buffer containing 50mM tris-HCl (pH 8), 10mM $MgCl_2$ and 0.1M KCl) and alternately freezing and thawing the infected cell suspension in the presence of 0.1% SDS. After lysis, an equal volume of 0.1M sodium acetate buffer (pH 5.4) was added, followed by two volumes of freshly distilled phenol. The mixture was then vigorously swirled in a 65°C water bath for three minutes followed by rapid chilling in an ice-water bath. The liquid phases were separated by centrifugation and the aqueous phase was extracted with phenol two more times. The RNA in the final aqueous phase was precipitated with 2 volumes of cold ethanol (-20°C), and then collected by centrifugation at 12,000xg for 30 minutes. The RNA pellet was dissolved in a minimal amount of 2 SSC and dialyzed overnight against 2xSSC at 4°C. The final concentration of RNA was at least 10 mg per ml.

E. coli competitor RNA was prepared as described by Bøvre and Szybalski (94).

DNA-RNA Hybridization

$^{3}$H-labeled RNA was hybridized to DNA by two methods. The procedure of Gillespie and Spiegelman (95) involved the hybridization of labeled RNA to denatured DNA immobilized on nitrocellulose membrane filters. Gh-1 DNA or host DNA (100 ug) in buffer containing 10mM Tris-HCl (pH 8) and 10mM NaCl was denatured by heating for ten minutes in a boiling water bath, followed by quick chilling in an ice-water bath. Three ml of cold 10XSSC was added and the denatured DNA was slowly filtered onto nitrocellulose membrane filters that had been pre-soaked for 6 hours in 6xSSC. Each filter was then washed extensively with 6xSSC (100ml per filter) and allowed to air-dry overnight. The DNA containing filters were then baked at 80°C for 3 hours. Labeled RNA was incubated with DNA filters in 2xSSC (enough volume to cover the surface of the filter, about 2ml) for 24 hours at 65°C. The filters were then treated with RNase, dried and assayed for radioactivity as described below.

The presence of gh-1 DNA sequences in DNA extracted from infected cells was determined by hybridization of $^{3}$H-labeled gh-1 RNA synthesized in vitro by P. putida holoenzyme to extracted DNA. DNA

filters were prepared from DNA extracted from infected cells at different time intervals post infection (100 ug per filter) and $^{3}$H-labeled gh-1 RNA was hybridized to the DNA. The time course of gh-1 DNA synthesis was followed by the amount of $^{3}$H-labeled gh-1 RNA that hybridized to the DNA extracted from infected cells at different time intervals.

Labeled RNA was hybridized to the separated strands of gh-1 DNA by the method of Nygaard and Hall (96). $^{3}$H-labeled RNA was hybridized to 2ug of either of the separated strands of gh-1 DNA in a volume of 0.5 ml and a final salt concentration of 2xSSC. The hybridization mixtures were incubated at 65°C for 6 hours, chilled to 4°C, and then treated with RNase (5ug per ml in 2xSSC) for 15 minutes at 37°C. The RNase solution was pre-heated at 98°C for 10 minutes to destroy contaminating DNase activity. The hybridization mixtures were then diluted with 2xSSC to a total volume of 10ml and filtered slowly through nitrocellulose membrane filters which had been soaked in 2xSSC for at least 6 hours. Each filter was washed with 100ml of 2xSSC and dried and counted as described. Since only denatured DNA and DNA-RNA hybrids but not RNA, duplex RNA or duplex DNA bind to nitrocellulose membrane filters under these conditions, the radioactivity of the filter reflects the amount of hybrid formed in each hybridization mixture.

To determine if DNA was in excess during hybridization increasing amounts of input $^{3}$H-labeled RNA were hybridized to a fixed amount of DNA. If DNA is in excess, the relationship between input $^{3}$H-labeled RNA and amount of hybrid formed should be linear. This was found to be true for hybridization involving DNA immobilized on a nitrocellulose filter. Under the conditions of hybridization employed for this method $^{3}$H-labeled gh-1 or host RNA synthesized *in vitro* hybridized with nearly 100% efficiency. The hybridization efficiency (to both gh-1 and host DNA) of $^{3}$H-labeled *in vivo* RNA under conditions of excess DNA by this method of hybridization was about 50%.

Hybridization with the separated strands of gh-1 DNA was not done under conditions of excess DNA due to the difficulty in preparing appreciable amounts of separated strands. Hybridization of *in vitro* $^{3}$H-labeled RNA to the separated strands of gh-1 DNA was accomplished with a 40% hybridization efficiency. The hybridization efficiency of *in vivo* $^{3}$H-labeled early RNA isolated 0-5 minutes post infection to the separated strands of gh-1 DNA was less than 1% because of the presence of excess $^{3}$H-labeled host RNA. The hybridization efficiency of *in vivo* $^{3}$H-labeled late RNA (isolated at 8 minutes or later post infection) to the separated strands of gh-1 DNA was about 20%.

All hybridization experiments were repeated several times. Hybridization blanks (minus DNA) were less than 100 CPM.

Hybridization-Competition

Hybridization-competition was carried out by the method of Nygaard and Hall (96) as described by Bøvre and Szybalski (94). The reagents were mixed at 4°C and contained in a total volume of 0.5 ml 10ug of denatured gh-1 DNA (or 2 ug of either separated strand of gh-1 DNA), varying amounts of competitor RNA, $^{3}$H-labeled RNA (at least 10,000 CPM per hybridization mixture), and a final salt concentration of 2xSSC. Hybridization was carried out at 65°C for 6 hours. Under these conditions the hybridization efficiency was about 65% for in vitro $^{3}$H-labeled RNA, 35% for in vivo RNA pulsed at late times post infection and less than 1% for in vivo RNA pulsed at early times of infection. Hybridization efficiency with separated strands of gh-1 DNA was the same as that described above. After hybridization the hybridization mixtures were rapidly chilled to 4°C and then treated as described for the Nygaard and Hall method. Each point on the hybridization-competition curves represents an average of 2-4 determinations. Blanks (minus DNA) were less than 100 CPM.

Degradation of Host DNA During Infection

A 10ml culture of P. putida was grown in glucose-salts-casamino acid (GSC) media, which contained in grams per liter: N $HPO_4$, 6; $KH_2PO_4$, 3; NaCl, 8; $NH_4Cl$, 2; casamino acids, 5; glucose, 20; and trace amounts of $FeCl_3$, manganese acetate, and $Na_2SO_4$. The GSC media contained in addition 2.5 mg per 10ml adenine and $^3H$-labeled thymidine (750uCi per 10ml). The cells were allowed to grow for three generations at which time unlabeled thymidine (0.6 mg per 10ml of growth medium) was added. After incubation for an additional 10 minutes the cells were chilled and collected by centrifugation. The cells were then suspended in 1ml of GSC media containing 0.6mg per 10ml thymidine and 2.5 mg per 10ml adenine, chilled and infected. Infection was begun by adding the cells to 9ml of pre-warmed GSC media (33°C) containing 0.6mg per 10ml thymidine and 2.5mg per 10ml adenine. Aliquots (1 ml) of the infected-cell culture were precipitated in 5ml of cold 10% TCA at the indicated time intervals and filtered onto nitrocellulose filters. The filters were dried and counted by liquid scintillation spectroscopy. The radioactivity of each filter was taken to be a measure of the amount of $^3H$-labeled DNA present at each time interval.

## Separation of gh-1 DNA Strands

Samples (1 ml) of a solution containing 160 ug gh-1 DNA, 300 ug poly (U,G), 0.1% SDS and 0.35mM EDTA were heated in a boiling water bath for 10 minutes and quickly cooled in an ice-water bath. CsCl solution (2ml, $\rho$=1.4080 [25°C]) was added to silicone-treated nitrocellulose centrifuge tubes. Sample (0.4ml) was layered into each tube and the tubes were filled with mineral oil to within 5mm of the top of the centrifuge tube. The tubes were centrifuged for 60 hours at 114,000xg in a SW 50.1 rotor at 10°C. After puncturing the bottom of the centrifuge tube 5-drop fractions were collected. The absorbance at 260nm and refractive index (25°C) were determined for alternate fractions. The fractions containing the separated strands were pooled and dialyzed against 2xSSC for 12 hours (at 4°C). The pooled fractions were then dialyzed against 0.1N NaOH for 8 hours at 37°C followed by extensive dialysis against 2xSSC at 4°C. The separated strands were self-hybridized for 3 hours at 65°C and then chilled in an ice-water bath.

## Detection of RNA Duplex Formation by S1 Nuclease Digestion

Samples of $^{3}$H-labeled RNA were heated in a boiling water bath for 10 minutes and then rapidly chilled in an ice-water bath. Aliquots of the RNA were

incubated under annealing conditions at 65°C for 6 hours and then chilled to 4°C. The "self-hybridized" RNA and aliquots of unhybridized, denatured RNA (to serve as controls) were digested with S1 nuclease at 45°C in an assay mixture (0.200 ml) containing: 30mM sodium acetate (pH 4.5), 1mM $ZnSO_4$, 5% glycerol and 50 ug S1 nuclease. S1 nuclease was purified by the procedure of Vogt (97) through the DEAE-cellulose chromatography step. Samples of the digestion mixture were precipitated in cold 10% TCA at the indicated time intervals and filtered onto nitrocellulose filters which were assayed for radioactivity. Digestion was continued until the control digestion mixtures no longer contained TCA insoluble radioactivity and there was no further digestion by S1 nuclease in the experimental mixture. Because the S1 nuclease digests only single-stranded nucleic acid the radioactivity found in the "self-hybridized" RNA digestion mixture which is not digested by S1 nuclease is a result of RNA duplex formation during annealing of the RNA sample. The amount of radioactivity resistant to S1 nuclease digestion is therefore a measure of the amount of complementary RNA present in the $^3$H-labeled RNA sample. The amount of $^3$H-labeled RNA self-hybridized was at least 100,000 CPM. The amount of radioactivity remaining after digestion of the unhybridized, $^3$H-labeled

RNA controls was less than 500 CPM. Digestion was usually complete in 2 hours.

Polyacrylamide Gel Electrophoresis

$^{3}$H-labeled RNA was synthesized *in vitro* for electrophoresis as described except BSA was not added to the reaction mixtures (because of possible RNase contamination). The reaction mixtures were incubated for 30 minutes at 30°C. Rifampicin (10ug per ml) or the rifamycin derivative DNPI (97,98) (100 ug per ml) were then added to the reaction mixtures to inhibit initiation of RNA synthesis by the *P. putida* or gh-1 RNA polymerase respecively. After addition of the inhibitors reaction mixtures were incubated for an additional 10 minutes to insure that synthesis of all RNA molecules was complete. The reactions were terminated by addition of SDS to a final concentration of 0.1% and an additional 5 minutes incubation at 30°C to dissociate the RNA polymerase and RNA product from DNA template. The reaction mixtures were then chilled to 4°C and centrifuged to remove precipitated SDS and used directly for gel electrophoresis.

RNA was pulse labeled 16-18 minutes post infection as described above except the pulse was terminated by addition of rifampicin DNPI (100ug per ml). The culture was incubated for an additional 2 minutes and cells were

collected as described above. $^{3}$H-labeled RNA was extracted by rapid freezing and thawing in lysozyme buffer followed by phenol extraction as described above. The $^{3}$H-labeled RNA was used directly for polyacrylamide gel electrophoresis.

Electrophoresis was carried out on 1.75% polyacrylamide-0.5% agarose cylindrical gels according to the procedure of Peacock and Dingman (99). Electrophoresis was done at 5mA per gel for about 2.5 hours (30 minutes after the bromophenol blue marker had completely run off the end of the gels). The gels were sliced into 2mm slices using a gel slicer and each gel slice was placed in a scintillation vial and incubated with 0.5ml of 0.5N NaOH for 1 hour at 92-98°C. The vials were then cooled and 5ml of Aquasol containing 5ml of glacial acetic acid per liter was added. The vials were then vigorously shaken and radioactivity was determined by liquid scintillation spectroscopy.

Polyacrylamide gel electrophoresis of each $^{3}$H-labeled RNA sample described in Results was repeated at least twice with essentially the same results in all cases. The yield of radioactivity from the gels was greater than 75%.

E. coli ribosomal RNA provided standard RNA markers for estimating the molecular weights of the labeled RNA species. The gel containing E. coli

ribosomal RNA (40ug) was stained in a 100ml solution of 0.005% stains-all dye in 50% formamide for at least 24 hours. The gel was destained with distilled water in the dark and the positions of the ribosomal RNA species were measured directly. The molecular weight of RNA was estimated from a plot of log molecular weight vs fraction number. It was assumed that the molecular weight of E. coli 23s ribosomal RNA was $1.1 \times 10^6$ and 16s ribosomal RNA was $0.55 \times 10^6$.

## Other Procedures

Protein concentration was determined by the method of Lowry (100), using BSA as a standard protein. The concentration of nucleic acid was determined by assuming that the extinction coefficient of pure nucleic acid is absorbance at 260nm (1% solution) = 200.

# RESULTS

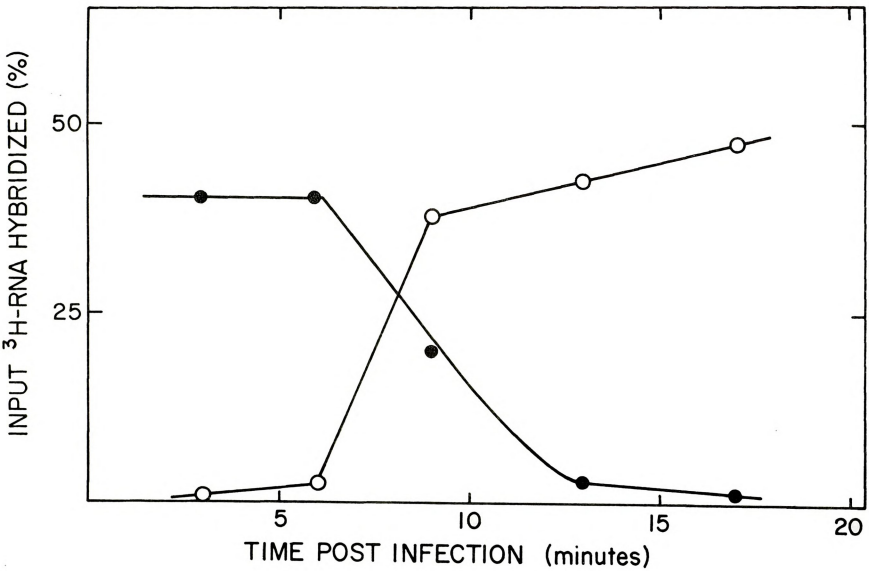
## Time Course of Nucleic Acid Synthesis In gh-1-Infected Cells

The time course of RNA and DNA synthesis in gh-1-infected cells was examined to determine when phage-specific RNA and DNA are synthesized during the gh-1 infectious cycle.

As seen in Figure 1, gh-1 RNA synthesis for the first 6 minutes of the infectious cycle is very limited, less than 1% of the total RNA synthesized during this period hybridized to gh-1 DNA. After this initial period, however, the relative amount of RNA hybridizing to gh-1 DNA increases greatly. The RNA pulse-labeled 12-14 minutes post infection hybridized almost exclusively to gh-1 DNA.

Figure 1 also indicates a corresponding decrease in the relative amount of host RNA synthesis during the infectious cycle. RNA synthesized during the initial 6 minutes of the infectious cycle hybridized almost exclusively to host DNA. However, after 6 minutes of the infectious cycle the relative amount of RNA hybridizing to host DNA decreases drastically. Little or none of the RNA pulse-labeled 12-14 minutes post infection hybridized to host DNA.

Figure 1.--Time course of RNA synthesis in gh-1-infected cells. $^{3}H$-labeled RNA synthesized in gh-1-infected cells during 2 minute pulse labeling periods was hybridized to both gh-1 and P. putida DNA filters under conditions of excess DNA. The percent input $^{3}H$-labeled RNA hybridized to either the gh-1 DNA filter (o) or the P. putida DNA filter (•) was determined for each pulse (designated by the midpoint of the 2 minute pulse interval). The amount of input $^{3}H$-labeled RNA varied from 10,000 CPM to 100,000 CPM. The procedure for pulse-labeling infected cells and the conditions for hybridization are described in Materials and Methods.


INPUT $^{3}$H-RNA HYBRIDIZED (%)
50
25
5
10
15
20
TIME POST INFECTION (minutes)

The hybridization data in Figure 1 provides information about the relative amount of RNA synthesis that is gh-1-specific or host-specific. It does not yield quantitative information about the absolute amount or rate of RNA synthesis in infected cells. Thus, the conclusion from Figure 1 is that the relative amount of gh-1 RNA synthesis increases greatly and host RNA synthesis is shutoff after 6 minutes of the infectious cycle.

The large increase in the relative amount of gh-1-specific RNA can be explained by assuming that the appearance of the gh-1 RNA polymerase results in a large increase in the transcription of gh-1 DNA in infected cells. This appears likely because the gh-1 RNA polymerase is first detected by 5 minutes post infection (98) and the large increase in the relative amount of gh-1-specific RNA synthesis starts shortly afterward at 6 minutes post infection (Figure 1).

The appearance of the gh-1 RNA polymerase, however, cannot account for the shutoff in host RNA synthesis observed in Figure 1. The two most plausible reasons for the shutoff of host RNA synthesis are that the host RNA polymerase is modified or inhibited so that it no longer is capable of transcribing host DNA, or host DNA is degraded during infection and is no longer available to support RNA synthesis.

To determine if host DNA is degraded during gh-1 infection host DNA was labeled by incorporation of $^{3}$H-labeled thymidine before infection. The labeled cells were then infected with gh-1 phage and the degradation of host DNA, determined by the amount of $^{3}$H-labeled thymidine remaining TCA insoluble, was followed throughout the infectious cycle. Figure 2 indicates that host DNA was degraded but not until 8-10 minutes post infection. By 12 minutes post infection 65% of the labeled host DNA was degraded. The complete degradation of host DNA is not observed in Figure 2. Instead, the amount of TCA insoluble $^{3}$H-labeled DNA increases after 12 minutes post infection and reaches 75% of the amount of TCA insoluble $^{3}$H-labeled DNA found in uninfected cells by 16 minutes post infection.

$^{3}$H-labeled gh-1 RNA synthesized in vitro was hybridized to DNA extracted from infected cells at the indicated times in Figure 2. The $^{3}$H-labeled gh-1 RNA served as a qualitative probe for the appearance of gh-1 DNA in the DNA extracted from infected cells. The results in Figure 2 indicate that gh-1 DNA synthesis begins after 12 minutes post infection and thus undoubtedly accounts for the increase in TCA insoluble $^{3}$H-labeled DNA in infected cells after 12 minutes post infection.

Figure 2.--Degradation of host DNA during infection. DNA in uninfected cells was labeled by incorporation of $^{3}H$-labeled thymidine and the cells were infected with gh-1 phage as described in Materials and Methods. At different time intervals after infection the radioactivity of 1 ml aliquots of the infected culture was determined by TCA precipitation (O) as described in Materials and Methods. The ability of DNA isolated from infected cells at different times post infection to hybridize $^{3}H$-labeled gh-1 RNA (●) was also determined. The amount of $^{3}H$-labeled thymidine incorporated into DNA in a 1 ml aliquot of culture before infection (control) was 21,000 CPM. The amount of $^{3}H$-labeled gh-1 RNA in each hybridization mixture was 50,000 CPM.

$^3$H-DNA TCA INSOLUBLE (%)
100
50
4
8
12
16
20
TIME POST INFECTION (minutes)
100
50
RELATIVE HYBRIDIZATION (%)

It is apparent that host DNA degradation does occur in gh-1-infected cells, and that the process begins between 8 and 10 minutes post infection. The nucleotides resulting from the degradation of host DNA are then used for the synthesis of gh-1 DNA beginning at 12 minutes post infection, with the result that about 75% of the nucleotides originally present in host DNA become incorporated into gh-1 DNA.

It is concluded from Figures 1 and 2 that shut-off of host RNA synthesis is probably a result of the degradation of host DNA in infected cells. This conclusion is reasonable because the time course of the shutoff of host RNA synthesis and the degradation of host DNA are similar.

## Time Course of Early and Late gh-1-Specific RNA Synthesis

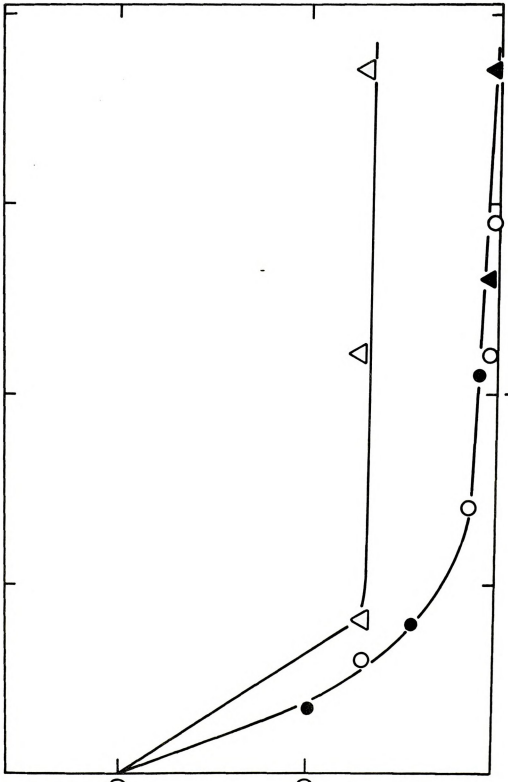
The gh-1-specific RNA that is synthesized before the gh-1 RNA polymerase is present in infected cells will be defined as "early" gh-1-specific RNA, or simply early RNA. Early RNA can be labeled with $^{3}$H-labeled uridine during the first 5 minutes of the infectious cycle in the presence of chloramphenicol. Since protein synthesis is inhibited by chloramphenicol, the synthesis of the gh-1-induced RNA polymerase will be inhibited. Thus, under these conditions, the transcription of gh-1-specific RNA will be limited to transcription of early

RNA by the host RNA polymerase. RNA synthesized by this procedure will be referred to as chloramphenicol RNA or CAM RNA. CAM RNA is equivalent to early RNA. Early RNA sequences by definition are those gh-1 sequences synthesized by host RNA polymerase during the initial period of gh-1 infection. This does not mean, however, that early RNA sequences are not synthesized at other periods of infection nor does it mean that they must be transcribed by the host RNA polymerase.

The time course of early RNA synthesis was followed by determining the ability of RNA isolated from infected cells at different times during infection to compete with $^{3}$H-labeled CAM RNA in hybridization-competition experiments. It is clear that if all early RNA sequences or their complements are present then full competition of $^{3}$H-labeled CAM RNA will be observed. $^{3}$H-labeled early RNA can be prevented from hybridizing to gh-1 DNA by competition from an excess amount of unlabeled early RNA for the same DNA site or by formation of RNA duplexes with an excess of RNA complementary to early RNA. RNA duplexes form under the same conditions that DNA-RNA hybrids form, but RNA duplexes are not retained by nitrocellulose filters.

The results in Figure 3 indicate that by 2 minutes post infection the majority of early RNA sequences or their complements are present. By 4 minutes post

Figure 3.--Hybridization-competition of *in vivo* $^{3}H$-labeled RNA synthesized 0-5 minutes post infection in the presence of chloramphenicol. $^{3}H$-labeled gh-1 RNA isolated from cells infected for 5 minutes in the presence of $^{3}H$-labeled uridine and chloramphenicol (400 ug/ml) was competed with the following unlabeled RNA competitors: 2 minute RNA (▲), 4 minute RNA (▲), CAM RNA (O), and 18 minute RNA (●). The procedure for pulse labeling infected cells and the conditions for hybridization-competition are described in Materials and Methods.


RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)

infection all early RNA sequences or their complements are present. As a control it was found that unlabeled CAM RNA completely competed out $^{3}$H-labeled CAM RNA.

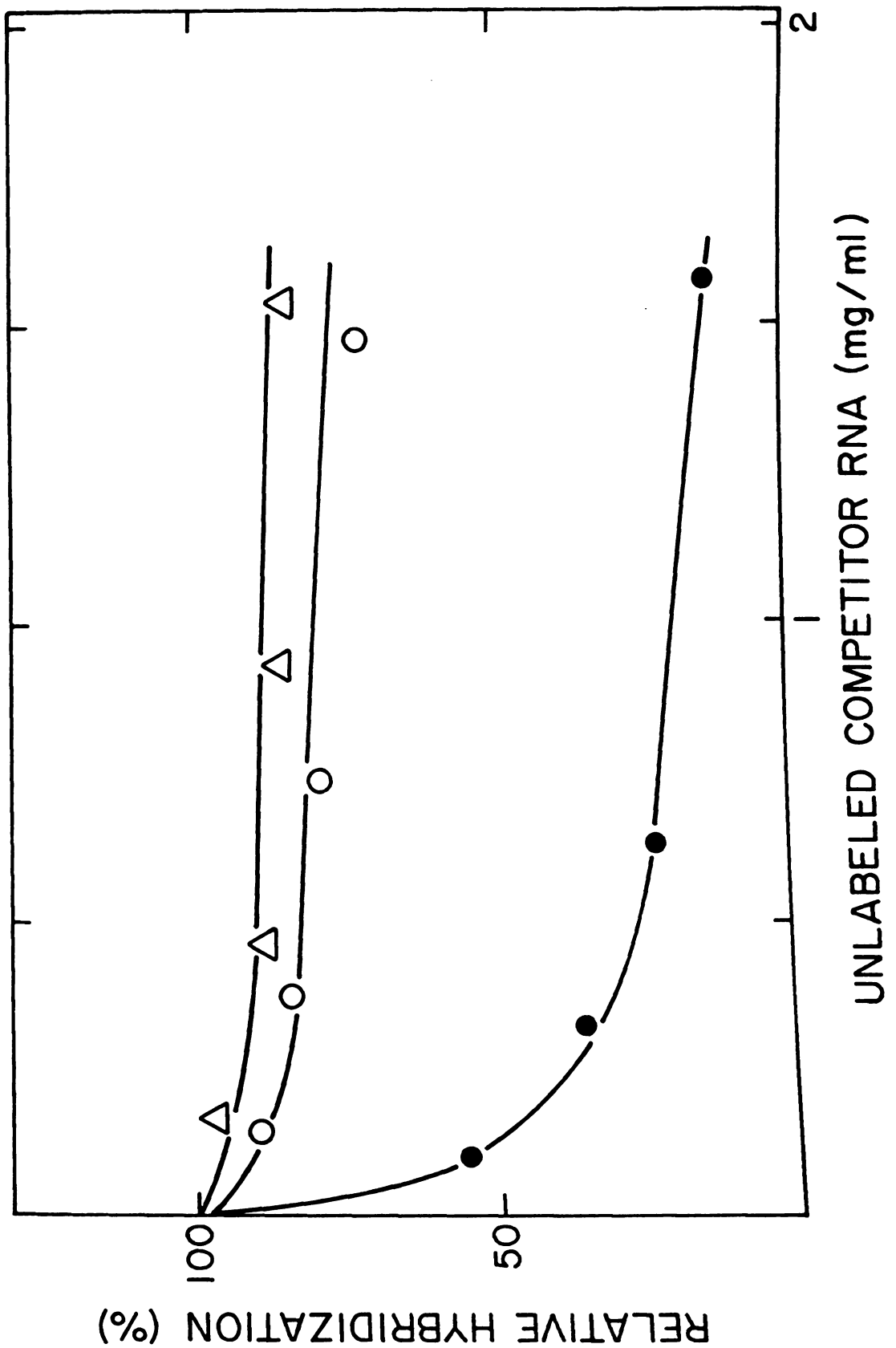
When unlabeled RNA from cells infected for 18 minutes was tested for the presence of early RNA sequences it was found that all early RNA sequences or their complements were present. The presence of early RNA sequences at 18 minutes post infection indicates that either early RNA is metabolically stable or early RNA is transcribed at late periods of the infectious cycle as well as during the initial period of infection.

"Late" gh-1-specific RNA, or simply late RNA, is defined as that RNA which is competed by 18 minute competitor RNA but not by CAM RNA. This definition of late RNA does not specify which RNA polymerase is responsible for transcribing late RNA sequences in infected cells. The presence of late RNA, like early RNA, is detected by hybridization-competition studies. The presence of RNA sequences complementary to late RNA sequences cannot be detected by hybridization-competition studies alone because both late RNA sequences and their complements are competed by 18 minute competitor RNA. Sequences complementary to late RNA sequences can be detected only by RNA duplex formation as described later.

Only early RNA was detected when cells were infected in the presence of chloramphenicol. To determine the relative amount of early and late RNA synthesized in gh-1-infected cells when gh-1 RNA polymerase is present, cells were pulse labeled with $^{3}$H-labeled uridine 8-10 minutes post infection. During this time period the specific activity of gh-1 RNA polymerase in infected-cell extracts is at a maximum level (98). The $^{3}$H-labeled RNA was extracted and the relative amount of early and late RNA that hybridized to gh-1 DNA was determined by hybridization-competition. The results indicate (Figure 4) that about 90% of the $^{3}$H-labeled RNA that hybridized to gh-1 DNA was late RNA or sequences complementary to late RNA. Thus, it is evident that once the gh-1 RNA polymerase is produced in the infected cell there is a shift in the specificity of transcription from early RNA to mostly late RNA.

RNA synthesis during a later period of infection was also examined. When $^{3}$H-labeled RNA synthesized from 16-18 minutes post infection was examined by hybridization-competition (Figure 5) it was found that a significant amount of $^{3}$H-labeled early RNA hybridized to gh-1 DNA. About 60% of the $^{3}$H-labeled RNA that hybridized to gh-1 DNA was competed by CAM RNA. Thus, a large amount of early RNA synthesis is occurring at 16-18 minutes post infection. This is in contrast to $^{3}$H-labeled RNA

Figure 4.--Hybridization-competition of *in vivo* $^{3}H$-labeled RNA synthesized 8-10 minutes post infection. $^{3}H$-labeled RNA pulse-labeled 8-10 minutes post infection was competed with the following unlabeled RNA competitors: CAM RNA (O), *E. coli* RNA (▲), and 18 minute RNA (●). The procedure for pulse-labeling infected cells and the conditions of hybridization-competition are described in Materials and Methods.


RELATIVE HYBRIDIZATION (%)
100
50
UNLABELED COMPETITOR RNA (mg/ml)
2
1

synthesized 8-10 minutes post infection since only about 10% of the $^{3}$H-labeled RNA that hybridized to gh-1 DNA was found to be early RNA (Figure 4).

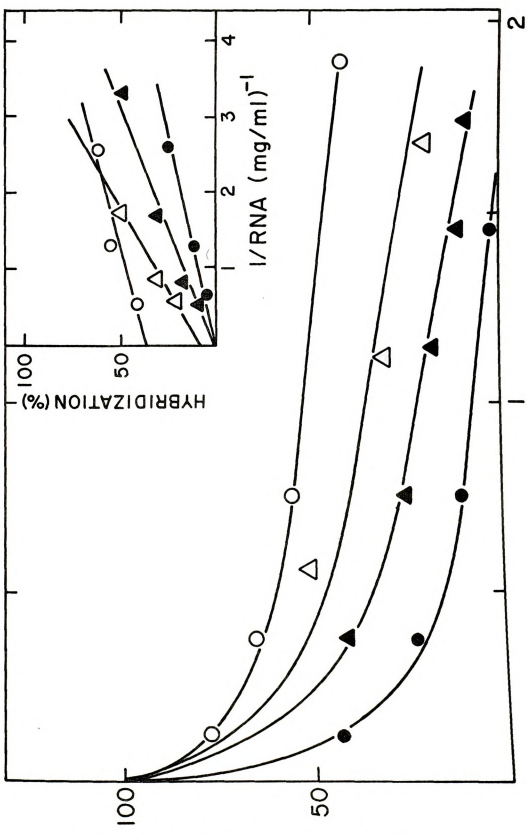
It appears that there are at least 3 different time intervals of RNA synthesis in gh-1-infected cells. During the early time interval of infection, before the gh-1 RNA polymerase is present, only early RNA is synthesized. During the intermediate time interval of RNA synthesis the gh-1 RNA polymerase is present and mostly late RNA is synthesized. During the late time interval of RNA synthesis early RNA is again synthesized in relatively large amounts.

The results from Figure 5 also indicate that nearly all RNA sequences or their complements synthesized during the 16-18 minute pulse are present by 8 minutes post infection, while by 12 minutes post infection all late RNA sequences or their complements are present. As a control it was found that unlabeled 18 minute competitor competed fully with the 16-18 minute pulse-labeled RNA for sites on gh-1 DNA.

## RNA Polymerase Activity in Late Infected Cell Extracts

If early RNA sequences or their complements are synthesized during late periods of infection, as indicated in Figure 5, they may be the products of transcription by either the gh-1 RNA polymerase, which is

Figure 5.--Hybridization-competition of *in vivo* $^{3}H$-labeled RNA synthesized 16-18 minutes post infection. $^{3}H$-labeled RNA pulse-labeled from 16 to 18 minutes post infection was competed with the following unlabeled RNA competitors: CAM RNA (○), 8 minute RNA (△), 12 minute RNA (▲) and 18 minute RNA (●). The procedure for pulse-labeling infected cells and the conditions of hybridization-competition are described in Materials and Methods. The data is replotted in the inset to yield straight lines. The intercept of each line is the value of the relative hybridization at infinite RNA competitor concentration.

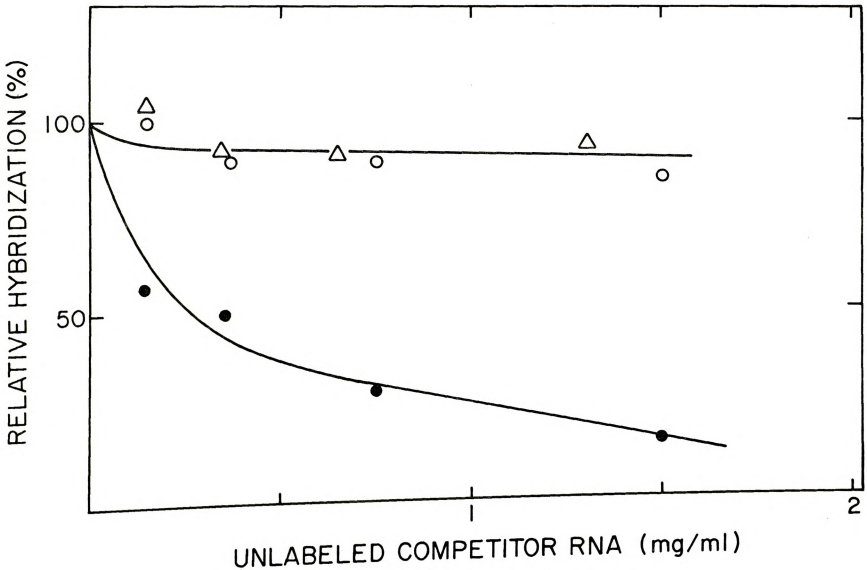

RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)
HYBRIDIZATION (%)
100
50
1
2
3
4
1/RNA (mg/ml)$^{-1}$

present after 5 minutes of infection (98), or host RNA polymerase, which can still be detected during late periods of infection (98).

In an attempt to resolve this question crude extracts of cells infected for 18 minutes were used to synthesize $^{3}$H-labeled RNA *in vitro* with endogenous DNA serving as template. RNA synthesis was limited to synthesis by the gh-1 RNA polymerase or the host RNA polymerase by either adding rifampicin and streptolydigin to inhibit host RNA polymerase in the extract or by adding the proper concentration of 3'-dATP to inhibit specifically the gh-1 RNA polymerase. After the RNA synthesis inhibitors were added $^{3}$H-labeled CTP was added to label the RNA synthesized by either RNA polymerase. The labeled RNA was then extracted and tested for the presence of early and late RNA sequences.

When labeled RNA synthesized by the gh-1 RNA polymerase *in vitro* was examined (Figure 6) it was found that only late RNA sequences or their complements were present since 18 minute competitor RNA fully competed the labeled RNA while CAM RNA competitor was no more effective than an *E. coli* RNA control in competing the labeled RNA. It is evident then that the gh-1 RNA polymerase present in late infected-cell extracts does not transcribe early RNA sequences or their complements.

Figure 6.--Hybridization-competition of $^{3}$H-labeled RNA synthesized *in vitro* by gh-1 RNA polymerase activity in infected-cell extracts. $^{3}$H-labeled RNA synthesized *in vitro* with gh-1 RNA polymerase in a crude extract of infected cells (18 minutes post infection) is competed with the following unlabeled RNA competitors: CAM RNA (○), *E. coli* RNA (△), and 18 minute RNA (●). The conditions of hybridization-competition and the procedure for preparing $^{3}$H-labeled RNA *in vitro* with infected-cell extracts is described in the Materials and Methods.


RELATIVE HYBRIDIZATION (%)
100
50
2
UNLABELED COMPETITOR RNA (mg/ml)

When labeled RNA synthesized *in vitro* by the host RNA polymerase was examined (Figure 7) it was found that early RNA sequences or their complements were present, as indicated by competition with unlabeled CAM RNA; however, the labeled RNA was not fully competed indicating that the host RNA polymerase in late infected-cell extracts also transcribes late RNA sequences or their complements. It was also shown that unlabeled 18 minute RNA fully competed the $^{3}$H-labeled RNA.

It should be realized that these two experiments depend on complete inhibition of either RNA polymerase by the added inhibitors and reflect RNA polymerase activity in infected-cell extracts and not under *in vivo* conditions.

Figure 7 indicates that host RNA polymerase transcribes early RNA sequences or their complements during the late time interval of infection. Cells were infected with gh-1 phage and RNA was pulse-labeled from 16-18 minutes post infection in the presence of rifampicin, which was added 5 minutes prior to the addition of $^{3}$H-labeled uridine. The $^{3}$H-labeled RNA was tested for the presence of early RNA sequences (Figure 8). It was found that unlabeled CAM RNA competitor did not compete with the $^{3}$H-labeled RNA more effectively than did the control *E. coli* competitor RNA, while unlabeled 18 minute competitor RNA fully

Figure 7.--Hybridization-competition of $^{3}$H-labeled RNA synthesized *in vitro* by host RNA polymerase activity in infected-cell extracts. $^{3}$H-labeled RNA synthesized *in vitro* with host RNA polymerase in an infected-cell extract (18 minutes post infection) is competed with the following unlabeled RNA competitors; CAM RNA (○), and 18 minute RNA (●). $^{3}$H-labeled RNA synthesized *in vitro* by host RNA polymerase in an infected-cell extract (5 minutes post infection) was competed with unlabeled CAM RNA (△). The conditions for hybridization-competition and the procedure for RNA synthesis with infected-cell extracts is described in Materials and Methods.

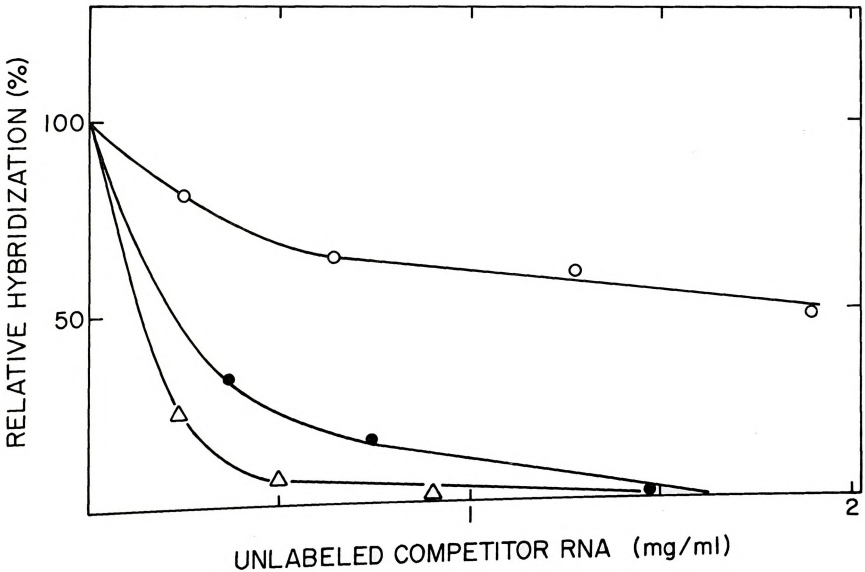
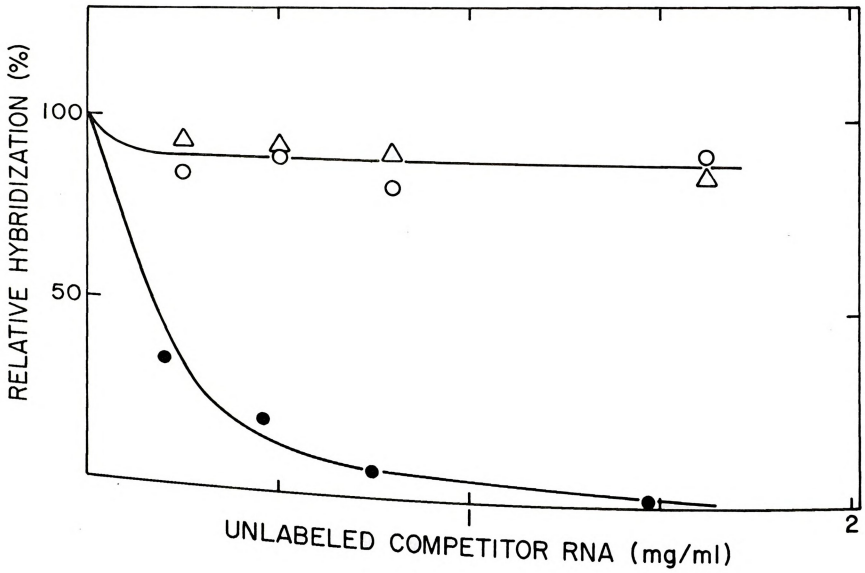

RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)

Figure 8.--Hybridization-competition of *in vivo* $^{3}H$-labeled RNA synthesized 16-18 minutes post infection in the presence of rifampicin. RNA was pulse-labeled 16-18 minutes post infection in the presence of rifampicin (100 ug/ml) added 5 minutes before addition of $^{3}H$-labeled uridine. The $^{3}H$-labeled RNA was extracted and competed with the following unlabeled RNA competitors: CAM RNA (o), *E. coli* RNA (Δ) and 18 minute RNA (●). The conditions of hybridization-competition and the procedure for pulse-labeling infected cells is described in Materials and Methods.


RELATIVE HYBRIDIZATION (%)
100
50
UNLABELED COMPETITOR RNA (mg/ml)
2

competed the $^{3}$H-labeled RNA. Thus, in the presence of rifampicin which inhibits host RNA polymerase, no early RNA sequences or their complements were transcribed. The late RNA sequences or their complements present during the late time interval of infection must be transcribed by the gh-1 RNA polymerase which is not inhibited by rifampicin. It can therefore be concluded that early RNA sequences or their complements are transcribed by the host RNA polymerase during the entire infectious cycle while the gh-1 RNA polymerase transcribes only late RNA sequences or their complements *in vivo*.

Asymmetry of gh-1-Specific RNA Transcription

Gh-1-specific RNA can be characterized not only as early or late RNA--the region of the gh-1 genome from which it is transcribed--but also from which strand of gh-1 DNA it is transcribed. The strand specificity of transcription can be determined by hybridizing labeled gh-1-specific RNA to either of the two separated strands of gh-1 DNA.

The two strands of DNA can be separated by CsCl equilibrium gradient centrifugation in the presence of poly (U,G) if the DNA strands bind poly (U,G) to different extents (101). This has been accomplished with gh-1 DNA (Figure 9). One strand of gh-1 DNA binds more poly (U,G) than the other strand and therefore bands at a relatively

Figure 9.--CsCl density gradient centrifugation of denatured gh-1 DNA complexed with poly (U,G) gh-1 DNA complexed with poly (U,G) was prepared as described in Materials and Methods and centrifuged in a CsCl equilibrium density gradient as outlined in Materials and Methods. The resulting fractions were analyzed for absorbance at 260nm ( ● ) and refractive index of CsCl (25°C) which was used to determine the density of CsCl in alternate fractions ( o ). The positions of the H strand (H) and the L strand (L) of gh-1 DNA are indicated. The position of denatured gh-1 DNA is indicated by an arrow.

ABSORBANCE AT 260 nm
0.2
0.1
H
L
10
20
30
40
50
FRACTION NUMBER
1.8
1.7
1.6
DENSITY OF CsCl (g/cm³, 25°C)

heavier density in the CsCl equilibrium gradient--this strand is referred to as the "heavy" or H strand of gh-1 DNA. The other strand binds less poly (U,G) and bands at a less dense position of the CsCl gradient--this strand is referred to as the "light" or L strand of gh-1 DNA. Unbound poly (U,G) can be detected at the bottom of the CsCl gradient. The position of denatured gh-1 DNA indicates that both strands of gh-1 DNA bind poly (U,G); the H strand binds relatively more as indicated by its banding at a higher density and also by its slightly greater absorbancy at 260nm. Fractions containing the H and L strands were pooled and the poly (U,G) was selectively degraded by incubation with NaOH. The separated strands of gh-1 DNA were self-annealed so that contamination with the opposite DNA strand was removed by formation of DNA duplexes.

To show conclusively that the strands of gh-1 DNA were separated, it is necessary to show that both strands of gh-1 DNA contain DNA molecules with one half the molecular weight of duplex gh-1 DNA. This was not done; however, since both T7 and T3 DNA can be separated into heavy and light strands (101) it is probable that gh-1 DNA was also separated into heavy and light strands. When additional data concerning the asymmetry of transcription is considered (see below) it is almost certain that the two peaks in Figure 9 represent separated strands of gh-1 DNA.

The separated strands of gh-1 DNA were used to determine which strand is transcribed at different periods of the gh-1 infectious cycle (Table 1). $^{3}H$-labeled early RNA isolated from cells infected in the presence of chloramphenicol (0-5 minutes) hybridized almost exclusively to the L strand of gh-1 DNA. $^{3}H$-labeled RNA pulse-labeled from 8-10 minutes post infection also hybridized almost exclusively to the L strand of gh-1 DNA. However, $^{3}H$-labeled RNA extracted from cells pulse-labeled from 12-14 minutes and 16-18 minutes post infection was capable of hybridizing to the H strand of gh-1 DNA. In contrast, when infected cells were pulse-labeled from 16-18 minutes post infection in the presence of rifampicin the $^{3}H$-labeled RNA hybridized only to the L strand of gh-1 DNA.

The hybridization studies presented in Table 1 were done under conditions of excess RNA because of an inability to obtain separated strands of gh-1 DNA in large quantity. However, enough DNA was present in the hybridization mixtures to hybridize an estimated 50% of the gh-1-specific RNA. Thus, while the asymmetry data in Table 1 is strictly valid for only that proportion of the gh-1 RNA that hybridized, enough RNA is hybridized so that the data can be extended, with caution, to include all the gh-1 RNA present in the hybridization mixture. The validity of this assumption is enhanced by the results

TABLE 1.--Asymmetry of gh-1-specific RNA Transcription *in vivo*.

| Time of Labeling | Hybridization to H Strand (CPM) | Hybridization to L Strand (CPM) |
|---|---|---|
| 5 minutes (+CAM) | 64 (5%) | 1168 (95%) |
| 8-10 minutes | 379 (4.8%) | 7569 (95.2%) |
| 12-14 minutes | 2016 (18.9%) | 8639 (81.1%) |
| 16-18 minutes | 1403 (22.7%) | 4792 (77.3%) |
| 16-18 minutes (+100 ug/ml rifampicin) | 134 (4%) | 3088 (96%) |

$^{3}$H-labeled RNA was pulse-labeled with $^{3}$H-labeled uridine, extracted and hybridized to either the H or L strand of gh-1 DNA. The procedure for pulse-labeling infected cells and the conditions for hybridization to separated gh-1 DNA strands are described in Materials and Methods. The percentages refer to relative hybridization only and not to the percent input RNA hybridized.

described below (Figure 11) which indicate that the amount of complementary RNA in in vivo RNA is essentially equal to the amount of H strand-specific RNA present.

It is concluded from these results and the results described above that during the first 5 minutes of infection the host RNA polymerase transcribes early RNA from the L strand of gh-1 DNA. The host RNA polymerase continues to transcribe early RNA during later periods of infection, and in addition appears to transcribe RNA from the H strand of gh-1 DNA beginning about 12 minutes post infection. The gh-1 RNA polymerase transcribes late RNA from the L strand of gh-1 DNA during the gh-1 infectious cycle.

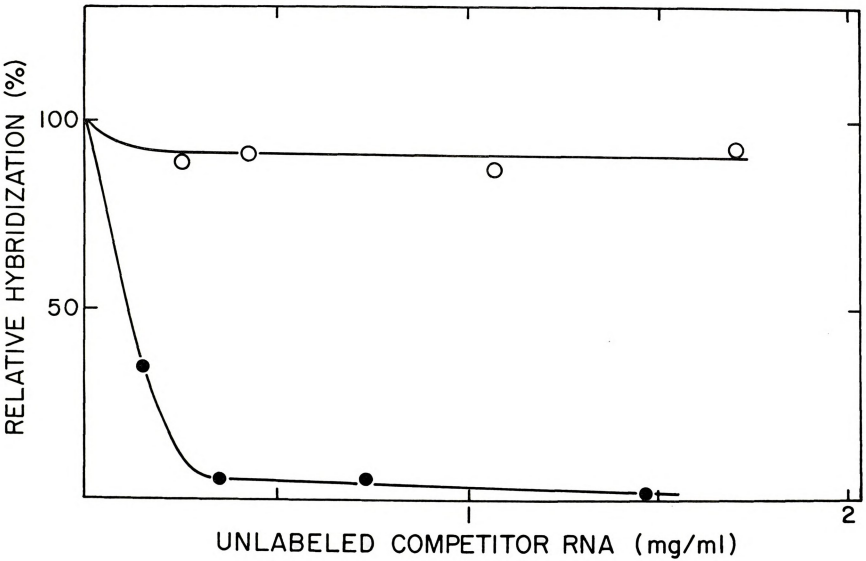
For purposes of clarity H strand-specific RNA that is complementary to L strand-specific RNA will be defined as the complementary RNA. RNA transcribed from the H strand of gh-1 DNA that is complementary to late RNA will be called complementary late RNA while RNA transcribed from the H strand of gh-1 DNA that is complementary to early RNA will be called complementary early RNA. This definition, however, does not assume that complementary RNA is anti-messenger RNA since it is not known from which strand of gh-1 DNA the true messenger RNA is transcribed.

## Characterization of the H Strand-Specific RNA Transcribed by the Host RNA Polymerase

It was indicated above (Figure 7) that host RNA polymerase is capable of transcribing early RNA sequences or their complements in extracts of cells infected for 18 minutes. It was also suggested that the host RNA polymerase transcribes another class of gh-1-specific RNA in infected cells, which is presumably late RNA, because the RNA is present at 18 minutes post infection. If this is true it may be hypothesized that the H strand-specific RNA transcribed by the host RNA polymerase (Table 1) is late RNA.

To test this hypothesis, RNA pulse-labeled from 16-18 minutes post infection was hybridized to the H strand of gh-1 DNA in the presence of unlabeled CAM RNA competitor and unlabeled 18 minute RNA competitor (Figure 10). CAM RNA failed to compete with the labeled RNA indicating that no early RNA sequences or their complements were synthesized while the 18 minute competitor RNA competed fully with labeled RNA. Thus, all sequences capable of hybridizing to the H strand of gh-1 DNA are late gh-1-specific RNA sequences or their complements. It is concluded that the host RNA polymerase transcribes early RNA sequences or their complements from the L strand of gh-1 DNA throughout the infections cycle and also transcribes late RNA sequences or their

Figure 10.--Hybridization-competition of *in vivo* $^{3}H$-labeled RNA synthesized 16-18 minutes post infection to the H strand of gh-1 DNA. RNA pulse-labeled from 16-18 minutes post infection was competed with unlabeled CAM RNA (o) and 18 minute competitor RNA (●) for sequences on the H strand of gh-1 DNA. The procedure for pulse-labeling infected cells and the conditions for hybridization-competition are described in Materials and Methods.

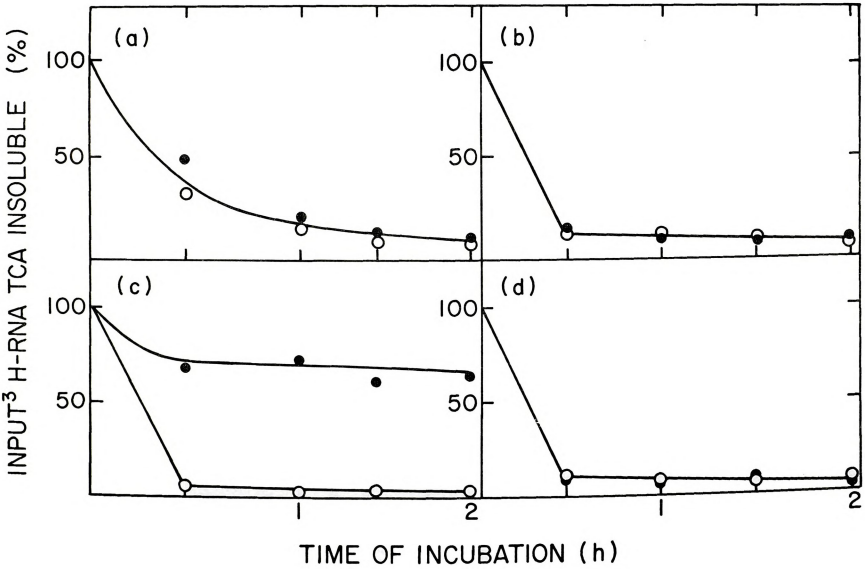

RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)

complements from the H strand of gh-1 DNA beginning approximately 12 minutes post infection.

To determine if host RNA polymerase transcribes complementary late RNA sequences from the H strand of gh-1 DNA *in vivo*, pulse-labeled RNA from infected cells was extracted and self-hybridized. If complementary RNA is present RNA-RNA duplex structures will form which can be detected by their resistance to degradation by S1 nuclease. RNA-RNA duplexes are not present in *in vivo* RNA before the RNA is self-hybridized (data not shown).

When this was done it was found (Figure 11) that $^{3}$H-labeled CAM RNA does not form RNA-RNA duplexes when self-annealed which is consistent with the fact that $^{3}$H-labeled CAM RNA hybridizes to only the L strand of gh-1 DNA. RNA pulse-labeled from 8-10 minutes post infection also does not form RNA-RNA duplexes indicating an absence of complementary RNA. When RNA pulse-labeled from 16-18 minutes post infection was tested for the presence of complementary RNA it was found that about 65% of the $^{3}$H-labeled RNA became resistant to S1 nuclease after self-hybridization. Thus, about 30% of the labeled RNA must be complementary RNA. It is possible that all the H strand-specific RNA sequences detected by hybridization to the H strand of gh-1 DNA are complementary RNA, indicating that the host RNA polymerase may transcribe complementary RNA sequences *in vivo* beginning 12 minutes

Figure 11.--Detection of RNA duplex formation by S1 nuclease digestion of *in vivo* $^{3}$H-labeled RNA $^{3}$H-labeled RNA was tested for the presence of complementary RNA by self-hybridization and S1 nuclease digestion of the hybridized RNA (●). A sample of $^{3}$H-labeled RNA that was not self-hybridized was also treated with S1 nuclease to serve as a control (○). The following RNA samples were tested for the presence of complementary RNA: (a) $^{3}$H-labeled CAM RNA, (b) $^{3}$H-labeled 8-10 minute RNA (c) $^{3}$H-labeled 16-18 minute RNA and (d) $^{3}$H-labeled 16-18 minute RNA synthesized in the presence of rifampicin (100 ug/ml). Rifampicin was added 5 minutes prior to the addition of $^{3}$H-labeled uridine. The procedure for pulse-labeling infected cells and for self-hybridization and S1 nuclease digestion are described in Materials and Methods. The amount of radioactivity TCA insoluble at 0 time of incubation was about 10,000 CPM for all experiments.


(a)
(b)
(c)
(d)
100
50
1
2
INPUT $^{3}$H-RNA TCA INSOLUBLE (%)
TIME OF INCUBATION (h)

post infection and continuing until at least 18 minutes post infection. This conclusion is strengthened by testing RNA pulse-labeled from 16-18 minutes post infection in the presence of rifampicin for the presence of complementary RNA sequences. As seen in Figure 11(d) no complementary RNA sequences were detected, indicating that all complementary RNA sequences must by synthesized by the host RNA polymerase.

An attempt was made to determine if transcription by host RNA polymerase is required throughout the infectious cycle. This was done by observing the effect of rifampicin addition at different time intervals of the infectious cycle on phage production. Phage production was measured by burst size in one step growth experiments (Table 2). When rifampicin was added at the time of infection (0 minutes) no phage production was detected. This result was expected since the host RNA polymerase was inhibited before gh-1-specific RNA was transcribed. When rifampicin was added at 5 minutes post infection phage production was decreased by 75%. By 5 minutes post infection some message for the gh-1 RNA polymerase is probably transcribed and in the process of translation as judged by the fact that gh-1 RNA polymerase activity can first be detected by 5 minutes post infection (98).

Thus, if host RNA polymerase activity is inhibited at the time gh-1 RNA polymerase is first detectable

TABLE 2.--Effect of Rifampicin on Burst Size.

| Phage | Time of Rifampicin Addition (Min) | Burst Size (PFU/infected cell) |
|---|---|---|
| gh-1 | -rifampicin | 100 |
| | 0 | 0 |
| | 5 | 24 |
| | 12.5 | 72 |
| T3 | -rifampicin | 200 |
| | 0 | 0 |
| | 5 | 150 |
| | 12.5 | 150 |

Rifampicin (100 ug/ml) was added to either gh-1-infected P. putida cells or T3-infected E. coli B cells at the indicated times post infection. The effect of rifampicin addition on burst size was determined by one step growth experiments. The procedure for infecting cells and for the one step growth experiment are described in Materials and Methods.

then phage production is drastically reduced. It appears that the continued synthesis of early RNA sequences and possibly the synthesis of complementary late RNA sequences is necessary for maximal phage production.

When rifampicin was added at 12.5 minutes post infection phage production was decreased by only 25%. By 12.5 minutes post infection the gh-1 RNA polymerase is at its maximal amount in infected cells (98) while synthesis of complementary late RNA is just begun (Table 1).

Thus, once the gh-1 RNA polymerase is present at its maximal level transcription by the host RNA polymerase is no longer needed to produce phage effectively. The synthesis of complementary late RNA by host RNA polymerase from 12 minutes post infection appears to be unnecessary, or at least has little effect on phage production. This does not, however, prove that complementary late RNA is anti-messenger RNA since it is possible that it may contain messages for non-essential proteins.

The effect of rifampicin on T3 phage production in E. coli was also observed to determine if rifampicin has some additional effects on phage production other than inhibition of transcription (Table 2). When rifampicin was added at the time of infection T3 phage production was completely inhibited as expected since rifampicin inhibits the E. coli RNA polymerase and therefore prevents late T3 RNA transcription. When rifampicin was added at 5 minutes post infection T3 phage production was decreased by 25%. The same result was obtained when rifampicin was added at 12.5 minutes post infection. It is known that early RNA synthesis is shutoff in the T3 and T7 phage systems at about 5 minutes post infection (7), so the addition of rifampicin should have no effect on T3 phage production if added at either 5 or 12.5 minutes post infection. The 25% decrease in T3 phage production may be due to some additional

effect of the drug on phage production. Similar results have been reported for the effect of rifampicin on T7 phage infection (119). Thus, the 25% decrease in phage production when rifampicin is added at 12.5 minutes after gh-1 infection of P. putida may also be due to additional effects of the drug.

It seems certain that transcription of early gh-1 RNA sequences by host RNA polymerase is necessary, at least until the gh-1 RNA polymerase is synthesized. If the host RNA polymerase is inhibited before phage RNA synthesis is begun no phage production is observed. The continued synthesis of early RNA sequences directed by the host RNA polymerase is necessary for maximal phage production until the gh-1 RNA polymerase is present at its maximal level (about 12 minutes post infection [98]). If host RNA polymerase is inhibited before 12 minutes post infection, gh-1 phage production is decreased but not prevented. Transcription of complementary late RNA sequences by host RNA polymerase is not necessary for phage production since the inhibition of host RNA polymerase at the time of complementary late RNA transcription (12.5 minutes post infection) has little if any effect on phage production.

## Size of the in vivo gh-1-Specific RNA

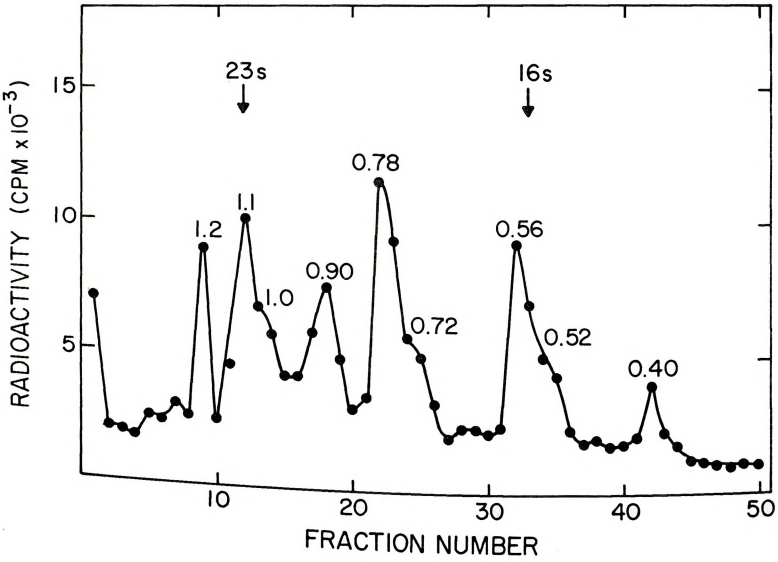
The molecular weight of gh-1-specific RNA was estimated by polyacrylamide-agarose gel electrophoresis

with the mobility of E. coli ribosomal RNA as molecular weight standards. RNA was pulse-labeled from 16-18 minutes post infection with $^{3}$H-labeled uridine. The extracted labeled RNA, which is all or almost all gh-1-specific RNA since host RNA synthesis is shutoff by 16 minutes post infection (Figure 1), was subjected to electrophoresis as described in Materials and Methods (Figure 12). Nine RNA peaks were observed with sizes ranging from 0.40 to $1.2 \times 10^{6}$ daltons. Since both early and late RNA should be present in the sample (Figure 5), either the entire geneome is not expressed or the RNA peaks are comprised of more than one sequence of RNA of the same size. The largest RNA species ($1.2 \times 10^{6}$ daltons) could be the gh-1 RNA polymerase messenger RNA, since it is large enough to code for a protein of about 100,000 daltons. It could also be a polycistronic messenger coding for 3 30,000 dalton proteins.

## Sequence Homology Between gh-1 DNA and T3 and T7 DNA

Because the size of the gh-1 genome is very close to that of the T3 and T7 genomes and because the gh-1 RNA polymerase appears to be similar to the T3 and T7 RNA polymerases it was decided to determine if gh-1 DNA contains sequences common to either the T3 or T7 genomes. It is known that the T3 and T7 genomes contain homologous sequences (102).

Figure 12.--Polyacrylamide gel electrophoresis of *in vivo* $^{3}H$-labeled RNA synthesized 16-18 minutes post infection. RNA was pulse-labeled 16-18 minutes post infection by the procedure described under polyacrylamide gel electrophoresis in Materials and Methods. The $^{3}H$-labeled RNA was subjected to polyacrylamide gel electrophoresis on 1.75% polyacrylamide-0.5% agarose gels at 5mA per gel for 2.5 hours. The gel was then sliced into 2mm slices and the radioactivity in each slice (●) determined as described in Materials and Methods. *E. coli* ribosomal RNA (40ug) was subjected to electrophoresis and run parallel to the $^{3}H$-labeled RNA sample. The position of 23s and 16s ribosomal RNA was determined as described in Materials and Methods and are indicated by arrows. The position of the *E. coli* ribosomal RNA markers was used to estimate the molecular weight of each RNA peak as described in Materials and Methods. The estimated molecular weight of each peak is indicated in daltons x $10^{-6}$.


23s
16s
1.2
1.1
1.0
0.90
0.78
0.72
0.56
0.52
0.40
RADIOACTIVITY (CPM x10$^{-3}$)
FRACTION NUMBER
15
10
5
10
20
30
40
50

Gh-1 DNA was transcribed *in vitro* utilizing either the gh-1 or host RNA polymerase. The $^{3}$H-labeled RNA product was then hybridized to gh-1, T3 and T7 DNA. If any sequence homology exists between gh-1 DNA and either T3 or T7 DNA the $^{3}$H-labeled gh-1 would hybridize to either T3 or T7 DNA. As seen in Table 3 this is not the case. The gh-1 $^{3}$H-labeled RNA synthesized *in vitro* by either the host or gh-1 RNA polymerase hybridized efficiently to gh-1 DNA but little or no hybridization was detected with T3 or T7 DNA. It appears that little or no sequence homology exists between gh-1 DNA and either T3 or T7 DNA, assuming that all the gh-1 genome is transcribed *in vitro* by the host and gh-1 RNA polymerase.

## Region Specificity of gh-1 RNA Synthesis in vitro

Once the specificity of RNA transcription in gh-1-infected cells became defined, it was hoped that purified *in vitro* systems could be developed that would be capable of duplicating the *in vivo* specificity of RNA transcription. Such systems could help define the necessary conditions for the control of RNA synthesis *in vivo*.

Since the host RNA polymerase transcribes early RNA sequences from the L strand of gh-1 DNA *in vivo*, the possibility that highly purified host RNA polymerase

TABLE 3.--Hybridization of gh-1-Specific RNA to T3 and T7 DNA.

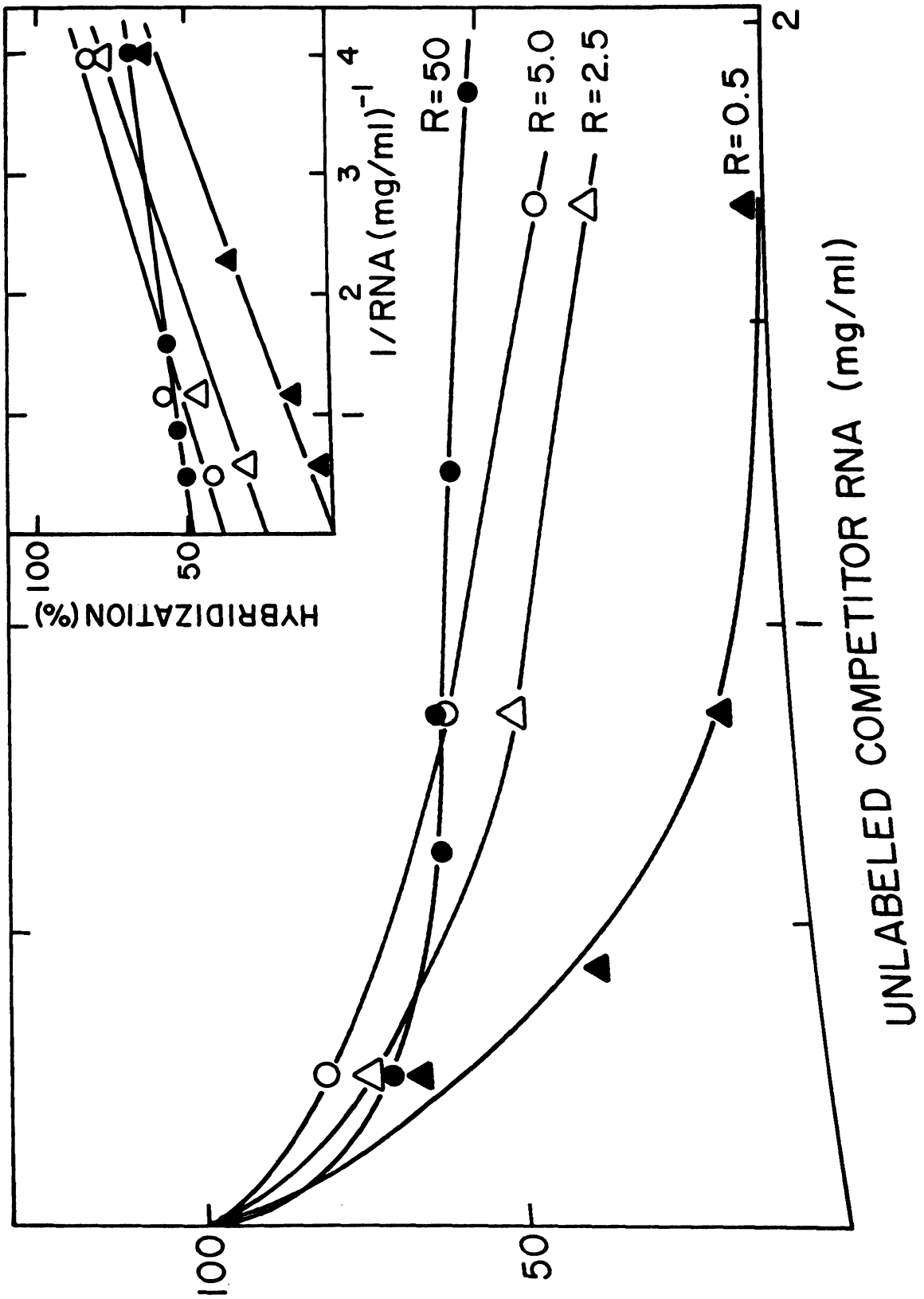
| RNA Polymerase | DNA Used In Hybridization | Hybrid Formed (CPM) | Rel. Hyb. (%) |
|---|---|---|---|
| gh-1 | None | 63 | -- |
| gh-1 | gh-1 | 6087 | 100 |
| gh-1 | T3 | 308 | 4.1 |
| gh-1 | T7 | 192 | 2.1 |
| *P. pugida* | None | 24 | -- |
| *P. putida* | gh-1 | 10369 | 100 |
| *P. putida* | T3 | 62 | -- |
| *P. putida* | T7 | 49 | -- |

$^{3}$H-labeled gh-1 RNA was synthesized *in vitro* with either the gh-1 RNA polymerase (2.5ug/ml) or the *P. putida* RNA polymerase (14.5ug/ml) and gh-1 DNA as template as described in Materials and Methods. The $^{3}$H-labeled RNA was then hybridized to gh-1 DNA (control), T3 or T7 DNA as indicated. The extent of hybridization to T3 and T7 DNA was compared to hybridization to gh-1 DNA (100%). The conditions for hybridization are described in Materials and Methods.

(holoenzyme) may transcribe only early RNA sequences *in vitro* was explored (Figure 13). The extent of early RNA synthesis was determined by hybridization-competition experiments in which $^{3}$H-labeled RNA synthesized *in vitro* was competed with unlabeled CAM RNA. If competition is complete, then only early RNA sequences or their complements are synthesized *in vitro*. It was found that transcription by holoenzyme could be limited to the early region of gh-1 DNA *in vitro* if the molar ratio of RNA polymerase to gh-1 DNA was below 1.0. Apparently an excess of holoenzyme allows initiation at other promotors (late region) which are not normally transcribed *in vivo*. This experiment however does not distinguish between early RNA transcripts from the L strand of gh-1 DNA and complementary RNA transcripts from the H strand of gh-1 DNA since both species are competed by CAM RNA competitor.

The host core enzyme transcribed predominately early RNA sequences or their complements *in vitro* (Figure 14), but was not limited to transcription of early RNA sequences by decreasing the molar ratio of core enzyme to gh-1 DNA template. Apparently the presence of sigma factor is necessary to limit transcription to the early region of gh-1 DNA.

Figure 13.--Hybridization-competition of $^{3}$H-labeled RNA synthesized in vitro by host RNA polymerase holoenzyme. $^{3}$H-labeled RNA synthesized in vitro by host RNA polymerase holoenzyme at different molar ratios (R) of enzyme to gh-1 DNA template was competed with unlabeled CAM RNA. The procedure for in vitro RNA synthesis and the conditions for hybridization-competition are described in Materials and Methods. In the following cases the concentration of gh-1 DNA was 250 ug/ml and the concentration of enzyme was: 2.7 ug/ml (R=0.5) (▲), 13.7 ug/ml (R=2.5) (△), and 27.5 ug/ml (R=5.0) (○). In one case the concentration of gh-1 DNA was 100 ug/ml and the concentration of enzyme was 120 ug/ml (R=50) (●). The data is replotted in the inset to yield straight lines. The intercept of each line is the value of the relative hybridization at infinite RNA competitor concentration.


RELATIVE HYBRIDIZATION (%)
100
50
UNLABELED COMPETITOR RNA (mg/ml)
1
2
R=0.5
R=2.5
R=5.0
R=50
1/RNA (mg/ml)$^{-1}$
1
2
3
4
HYBRIDIZATION (%)
50
100

A commercial preparation of E. coli RNA polymerase was found to be limited to transcription of gh-1 early RNA sequences or their complements in vitro (Figure 15). This enzyme fraction contained both holo- and core enzyme. The molar ratio of RNA polymerase to gh-1 DNA template was estimated to be about 3. Thus, E. coli RNA polymerase, which transcribes only early RNA sequences from T3 and T7 DNA (8,51), surprisingly transcribed only early RNA sequences or their complements from gh-1 DNA as well.

The gh-1 RNA polymerase transcribes only late RNA sequences in vivo, but is capable of transcribing both early and late RNA sequences or their complements in vitro (Figure 16). The enzyme synthesized about equal amounts of early and late RNA in vitro. This result was not dependent on the molar ratio of enzyme to DNA template. Perhaps an additional factor is necessary to limit the transcription of gh-1 RNA polymerase to the late region of gh-1 DNA.

## Strand Specificity of gh-1 RNA Synthesis in vitro

To demonstrate that an in vitro transcriptional system can duplicate RNA transcription in vivo, the strand specificity of transcription must also be duplicated. It was determined that early RNA is transcribed only from the L strand of gh-1 DNA by host RNA

Figure 14.--Hybridization-competition of $^{3}$H-labeled RNA synthesized in vitro by host RNA polymerase core enzyme at different molar ratios of enzyme to DNA template. $^{3}$H-labeled gh-1 RNA was synthesized in vitro by the P. putida core enzyme at two different molar ratios (R) of enzyme to gh-1 DNA template. $^{3}$H-labeled RNA synthesized at a molar ratio of R=50 (45 ug/ml enzyme and 100 ug/ml gh-1 DNA) is completed by CAM RNA competitor (o) and 18 minute RNA (●). RNA synthesized at a molar ratio of R=0.5 (2.7 ug/ml enzyme and 250 ug/ml gh-1 DNA) is competed with CAM RNA (Δ) and 18 minute competitor RNA (▲). The procedure for in vitro RNA synthesis and the conditions for hybridization-competition are described in Materials and Methods.

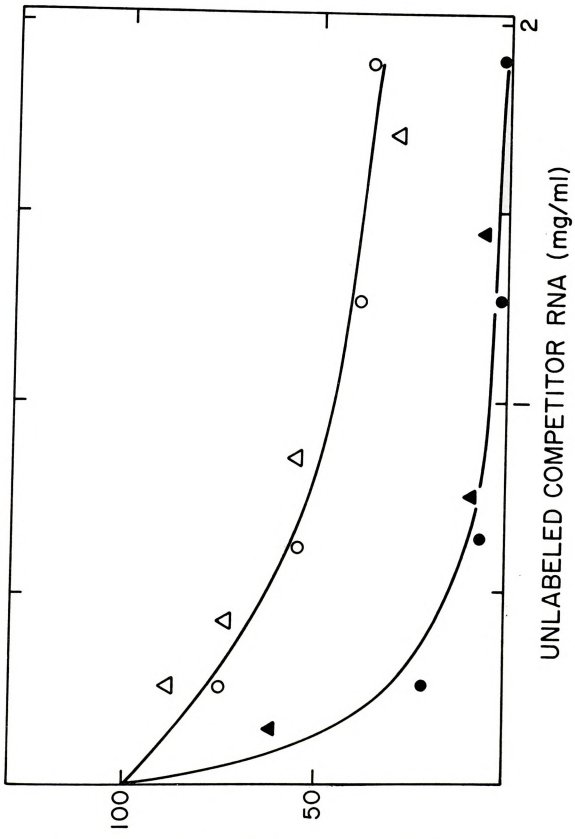
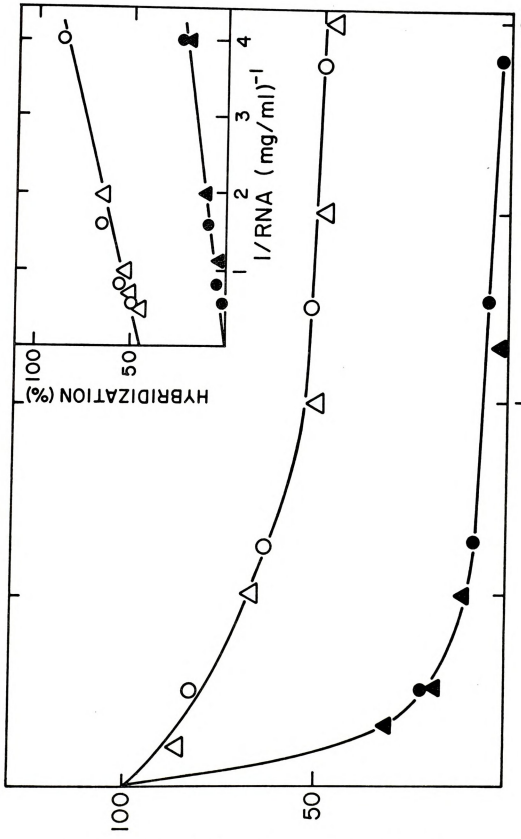

RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)

Figure 15.--Hybridization-competition of $^{3}$H-labeled RNA synthesized in vitro with E. coli RNA polymerase. $^{3}$H-labeled RNA was synthesized in vitro with E. coli RNA polymerase (67 ug/ml) and gh-1 DNA (100 ug/ml) as template. The molar ratio was estimated to be R=3 assuming that the enzyme preparation is 10% pure. The $^{3}$H-labeled RNA was competed with unlabeled CAM RNA ( o ) and 18 minute RNA ( ● ). The procedure for in vitro RNA synthesis and the conditions for hybridization-competition are described in Materials and Methods.

RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)

Figure 16.--Hybridization-competition of $^{3}$H-labeled RNA synthesized *in vitro* by gh-1 RNA polymerase. $^{3}$H-labeled RNA was synthesized *in vitro* with gh-1 RNA polymerase and gh-1 DNA as template to two different molar ratios (R) of enzyme to DNA. $^{3}$H-labeled RNA synthesized at R=50 (22.5 ug/ml gh-1 RNA polymerase and 100 ug/ml gh-1 DNA) was competed with unlabeled CAM RNA (o) and 18 minute competitor RNA (Δ). $^{3}$H-labeled RNA synthesized at R=2.5 (7.7 ug/ml gh-1 RNA polymerase and 680 ug/ml gh-1 DNA) was competed with unlabeled CAM RNA (●) and 18 minute competitor RNA (▲). The data is replotted in the inset to yield straight lines. The intercept of each line is the value of the relative hybridization at infinite RNA competitor concentration.


RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)
HYBRIDIZATION (%)
100
50
1
2
3
4
1/RNA (mg/ml)⁻¹

polymrease in vivo, while late RNA is transcribed in vivo by gh-1 RNA polymerase also from only the L strand of gh-1 DNA. The host RNA polymerase transcribes, in addition, late complementary RNA from the H strand of gh-1 DNA in vivo.

As seen in Table 4 the asymmetry of in vitro transcription by holoenzyme is increased with a decrease in the molar ratio of enzyme to gh-1 DNA template. When the ratio is 0.5 transcription is nearly limited to the L strand of gh-1 DNA. It is possible then to nearly duplicate early RNA synthesis in vivo by utilizing highly purified holoenzyme at a low molar ratio of enzyme to gh-1 DNA. Under these conditions transcription is limited to the early region of gh-1 DNA and mostly to the biologically correct L strand of gh-1 DNA.

In contrast, transcription by P. putida core enzyme and E. coli RNA polymerase is not limited to the L strand of gh-1 DNA (Table 4). In both cases the H and L strand appear to be transcribed with equal efficiency. The asymmetry of transcription by P. putida core enzyme is not affected by altering the ratio of enzyme to DNA template. Thus, while the P. putida core enzyme is limited to mainly early RNA synthesis and the E. coli RNA polymerase is totally limited to early RNA synthesis in vitro with gh-1 DNA as template, neither

TABLE 4.--Asymmetry of gh-1-Specific RNA Transcription *in vitro*.

| RNA Polymerase | Molar Ratio of Enzyme to Template (R) | Hybridization to H Strand (CPM) | Hybridization to L Strand (CPM) |
|---|---|---|---|
| *P. putida* (holoenzyme) | 50 | 1731 (40%) | 2616 (60%) |
| *P. putida* (holoenzyme) | 0.5 | 1468 (20%) | 5872 (80%) |
| *P. putida* (core enzyme) | 50 | 807 (42%) | 1113 (48%) |
| *P. putida* (core enzyme) | 0.5 | 2079 (54.6%) | 1723 (45.4%) |
| *E. coli* (holoenzyme and core enzyme) | 3[a] | 5239 (52%) | 4785 (48%) |
| gh-1 | 50 | 5 | 2463 (100%) |
| gh-1 | 2.5 | 98 (3.6%) | 2584 (96.4%) |

[a]The value of R is an estimation based upon the assumption that the enzyme preparation is about 10% pure as calculated from the specific activity of the preparation.

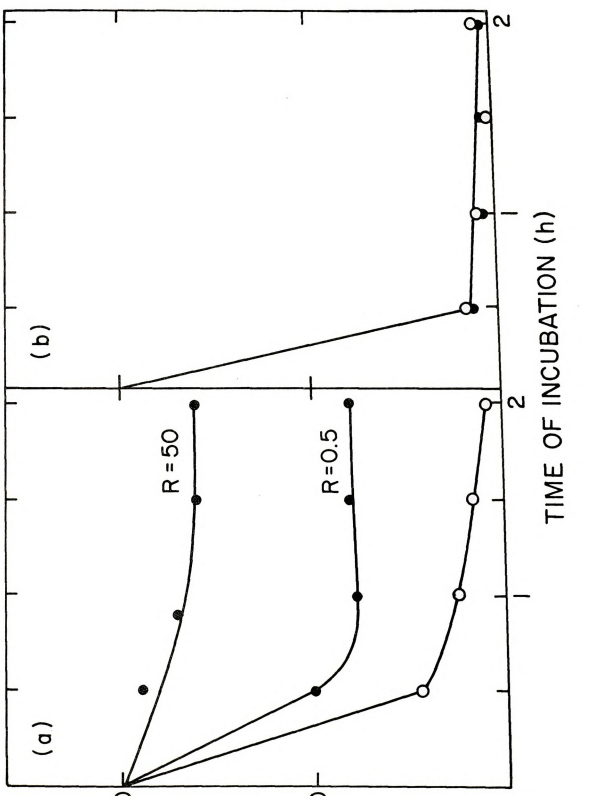
$^3$H-labeled RNA is synthesized *in vitro* by the procedures outlined in the legends to figures 13, 14, 15, and 16. The $^3$H-labeled RNA was then hybridized to the separated strands of gh-1 DNA. The conditions for hybridization to the separated strands of gh-1 DNA are described in Materials and Methods. The percentages refer to relative hybridization only and not to the percent input RNA hybridized.

enzyme is capable of transcribing only the biologically correct L strand of gh-1 DNA in vitro.

The gh-1 RNA polymerase is found to be capable of transcribing only the L strand of gh-1 DNA in vitro (Table 4), even though it is not limited to late RNA transcription in vitro (Figure 16).

When RNA synthesized by holoenzyme in vitro was examined for the presence of complementary RNA by the S1 nuclease assay it was found that nearly all H strand-specific RNA sequences were complementary sequences (Figure 17). For example, when the molar ratio of enzyme to DNA template was 50, about 40% of the RNA transcribed in vitro was H strand-specific (Table 4). If all the H strand-specific RNA were complementary RNA then after self-hybridization 80% of the RNA should be in the form of S1 nuclease-resistant RNA-RNA duplexes. This was found to be the case as seen in Figure 17 (a). When the molar ratio of enzyme to template was lowered to 0.5, about 20% of the RNA transcribed in vitro by holoenzyme hybridized to the H strand of gh-1 DNA (Table 4). If all H strand-specific RNA were complementary RNA then 40% of the RNA following self-hybridization should be S1 nuclease-resistant. This also was found to be true as indicated in Figure 17(a). In contrast, RNA synthesized by the gh-1 RNA polymerase in vitro is all L strand-specific (Table 4) and therefore should not

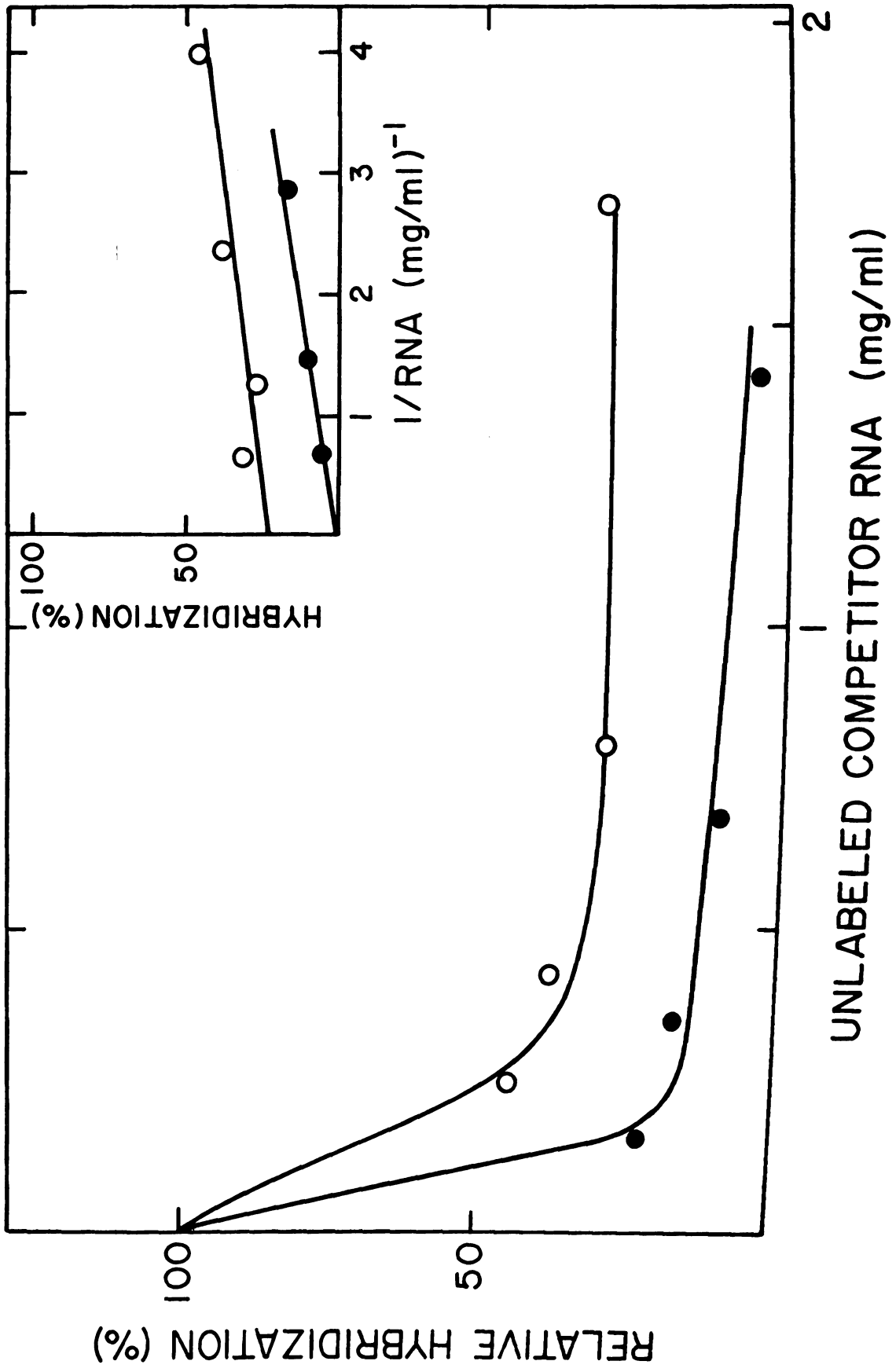
Figure 17.--Detection of RNA duplex formation by S1 nuclease digestion of $^{3}$H-labeled RNA synthesized *in vitro*. $^{3}$H-labeled RNA synthesized *in vitro* by *P. putida* holoenzyme (a) is tested for the presence of complementary RNA by self-hybridization and S1 nuclease digestion (●) at molar ratios of enzyme to gh-1 DNA template of R=50 and R=0.5 as indicated in the figure. An unhybridized RNA control was also digested with S1 nuclease (o). $^{3}$H-labeled RNA synthesized *in vitro* by the gh-1 RNA polymerase (R=50) (b) was also tested for the presence of complementary RNA as described in (a). The procedure for RNA synthesis *in vitro* by *P. putida* RNA polymerase is described in the legend to Figure 14. The procedure for RNA synthesis *in vitro* by gh-1 RNA polymerase is described in the legend to Figure 17. The conditions of self-hybridization and S1 nuclease digestion are described in Materials and Methods.


(a)
(b)
R=50
R=0.5
100
50
2
1
2
INPUT $^3$ H-RNA TCA INSOLUBLE (%)
TIME OF INCUBATION (h)

form S1 nuclease-resistant RNA-RNA duplexes after self-hybridization. Figure 17(b) shows that this was observed to be true.

It is apparent that nearly all RNA transcribed by holoenzyme from the H strand of gh-1 DNA is complementary RNA. The amount of the H strand transcribed was determined by hybridization-competition of labeled *in vitro* RNA (synthesized by holoenzyme) by CAM RNA and 18 minute late RNA for sequences on the H strand of gh-1 DNA (Figure 18). This experiment was complicated by the fact that competition of labeled *in vitro* RNA can occur in two ways: (a) direct competition for H strand sequences by unlabeled complementary RNA present in *in vivo* competitor RNA (due to transcription of complementary late RNA by host RNA polymerase *in vivo*) and (b) indirect competition due to RNA-RNA duplex formation between labeled *in vitro* RNA and complementary *in vivo* competitor RNA. Formation of such duplexes would prevent labeled RNA from hybridizing to the H strand of gh-1 DNA. The data in Figure 18 indicates that CAM RNA competitor competed 75-80% of the $^{3}$H-labeled *in vitro* RNA. Since there is no complementary RNA present in CAM RNA (Figure 11) the competition observed must have been indirect, indicating that 75-80% of the RNA synthesized *in vitro* by holoenzyme from the H strand of gh-1 DNA was complementary early RNA. The remaining

Figure 18.--Hybridization-competition of $^{3}$H-labeled RNA synthesized in vitro by holoenzyme to the H strand of gh-1 DNA. $^{3}$H-labeled RNA was synthesized by P. putida holoenzyme in vitro at a molar ratio of R=50 as described in the legend to Figure 14. The $^{3}$H-labeled RNA was competed by unlabeled CAM RNA (o) and 18 minute competitor RNA (●) for sequences on the H strand of gh-1 DNA. The H strand was self-hybridized before use in hybridization-competition as described in Materials and Methods. The conditions of hybridization-competition are described in Materials and Methods. The data is replotted in the inset to yield straight lines. The intercept of each line is the value of the relative hybridization at infinite RNA competitor concentration.


RELATIVE HYBRIDIZATION (%)
100
50
1
2
UNLABELED COMPETITOR RNA (mg/ml)
HYBRIDIZATION (%)
100
50
1
2
3
4
1/RNA (mg/ml)$^{-1}$

20-25% must have been complementary late RNA as indicated by the fact that 18 minute late RNA completely competed the $^{3}$H-labeled RNA, although competition must have been both direct and indirect. It is not possible to conclude that the complementary late messenger RNA synthesized in vitro by holoenzyme is the same as the complementary late RNA synthesized in vivo by host RNA polymerase.

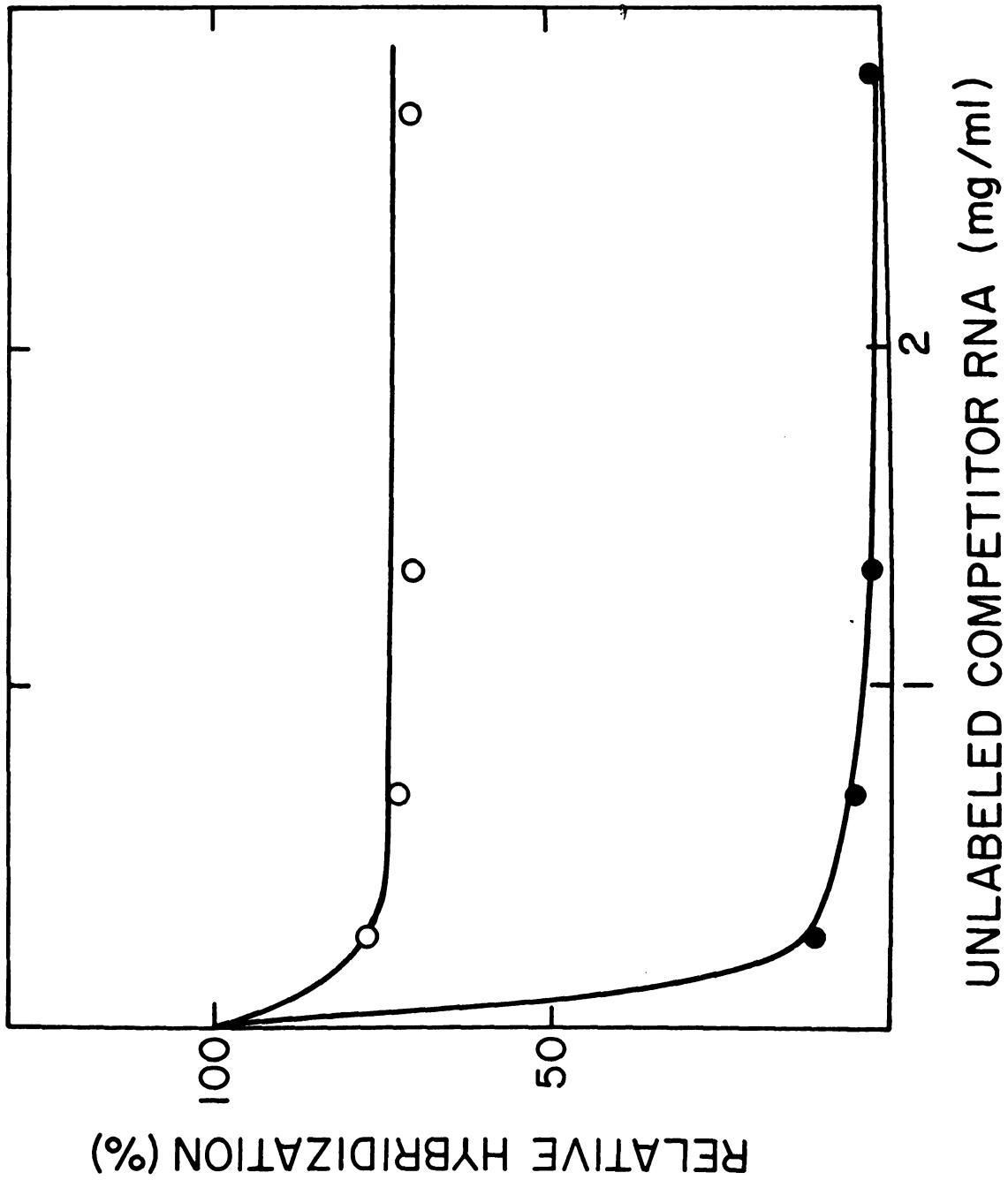
The same experiment was also done with the L strand of gh-1 DNA (Figure 19). This data indicates that 30% of the L strand-specific RNA transcribed by holoenzyme in vitro is early RNA while the remaining 70% must be late RNA.

## Size of gh-1-Specific RNA Transcribed in vitro

The size of RNA transcribed by holoenzyme in vitro was determined by polyacrylamide gel electrophoresis. The conditions of RNA synthesis were such (as outlined in Materials and Methods) that only complete transcripts were synthesized. The RNA product was determined to be entirely from the early region of gh-1 DNA and at least 75% L strand-specific.

The results (Figure 20) indicate that five major RNA transcripts are synthesized in vitro with molecular weights of approximately 1.2, 1.5, 1.8, 2.2 and $2.5 \times 10^{6}$ daltons. In addition, a heterogenous group of smaller

Figure 19.--Hybridization-competition of $^{3}$H-labeled RNA synthesized *in vitro* by holoenzyme to the L strand of gh-1 DNA. $^{3}$H-labeled RNA synthesized *in vitro* by the *P. putida* holoenzyme (as described in the legend to Figure 18) was competed by unlabeled CAM RNA (o) and 18 minute RNA competitor RNA (●) for sequences on the L strand of gh-1 DNA. The conditions for hybridization-competition are described in Materials and Methods.


RELATIVE HYBRIDIZATION (%)
100
50
UNLABELED COMPETITOR RNA (mg/ml)
2
1

RNA species are present on the gel. The total molecular weight of the five major RNA species, assuming each species is unique in sequence, is $9.2 \times 10^6$ daltons, which corresponds to about 80% of the gh-1 genome.

If the largest RNA species ($2.5 \times 10^6$ daltons) is assumed to be a precursor molecule of the entire early region then the early region would comprise 21.7% of the gh-1 genome, which is in agreement with the size of the early region of T7 DNA. The smallest major RNA peak observed ($1.2 \times 10^6$ daltons) is the correct size to code for the gh-1 RNA polymerase, which almost certainly is the product of a gh-1 phage early gene.

An attempt was made to elute $^3$H-labeled RNA from the polyacrylamide gel and characterize the RNA as early or late by hybridization-competition and as H or L strand-specific by hybridization to the separated strands of gh-1 DNA. This, with the exception of one case, was not possible because the eluted $^3$H-labeled RNA did not hybridize efficiently to gh-1 DNA. The inability to hybridize RNA eluted from polyacrylamide-agarose gels with high efficiency has been observed by other investigators (57) and is probably due to an inhibitory effect of agarose in the hybridization mixture. It was possible, however, to show that the pooled, eluted group of small heterogenious RNA species seen in Figure 20 hybridized to the H strand of gh-1 DNA. These

Figure 20.--Polyacrylamide gel electrophoresis of $^{3}H$-labeled RNA synthesized in vitro by holoenzyme. $^{3}H$-labeled RNA was synthesized in vitro by P. putida holoenzyme for gel electrophoresis as described in Materials and Methods. The molar ratio of enzyme to DNA was R=0.5 (250 ug/ml gh-1 DNA and 2.7 ug/ml enzyme). The procedure for gel electrophoresis is described in Materials and Methods. The estimated molecular weight of each peak is indicated in daltons $x10^{-6}$.

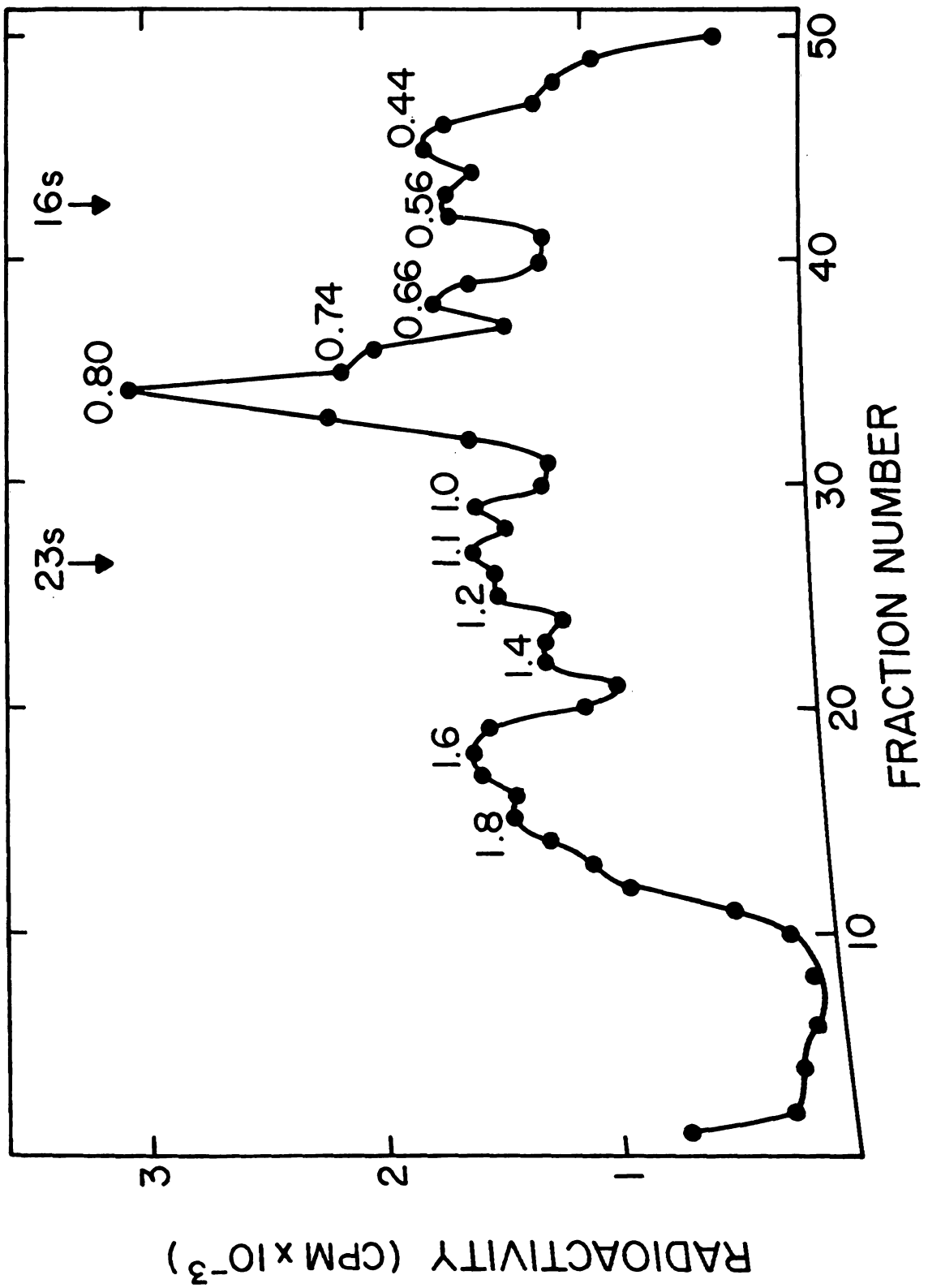
RADIOACTIVITY (CPM x 10⁻³)
15
10
5
10
20
30
40
50
FRACTION NUMBER
23s
16s
2.5
2.2
1.8
1.5
1.2

species may represent all the H strand-specific RNA in the sample since they comprise about 20% of the RNA on the gel. If this is true it indicates that only small RNA species are transcribed from the H strand of gh-1 DNA *in vitro*.

It is seen in Figure 12 that *in vivo* RNA contains a RNA species of $1.2 \times 10^6$ daltons, which may be the same RNA species as that detected *in vitro*. It is also noted that no RNA species larger than $1.2 \times 10^6$ daltons is present *in vivo*. This indicates that the large RNA species synthesized *in vitro* are probably large precursor molecules of early region of the gh-1 genome. It is not known if such precursor molecules are synthesized *in vivo*.

The size of RNA synthesized *in vitro* by the gh-1 RNA polymerase was also determined by polyacrylamide gel electrophoresis (Figure 21). The RNA species present, if unique in sequence, would represent about 100% of the gh-1 genome. The 1.8, 1.6 and $1.4 \times 10^6$ dalton RNA species are probably large precursor molecules since RNA species of this size are not detected *in vivo* (Figure 12).

Figure 21.--Polyacrylamide gel electrophoresis of $^{3}H$-labeled RNA synthesized *in vitro* by the gh-1 RNA polymerase. $^{3}H$-labeled RNA was synthesized *in vitro* by gh-1 RNA polymerase at a molar ratio of R=30 (12.5 ug/ml enzyme and 100 ug/ml gh-1 DNA) for gel electrophoresis as described in Materials and Methods. The procedure for gel electrophoresis is described in Materials and Methods. The estimated molecular weight of each peak is indicated in daltons $x10^{-6}$.


RADIOACTIVITY (CPM x 10⁻³)
FRACTION NUMBER
16s
23s
0.80
0.74
0.66
0.56
0.44
1.0
1.1
1.2
1.4
1.6
1.8

## DISCUSSION

### Model for the Control of gh-1 RNA Synthesis in Infected Cells

A model for the control of gh-1 RNA synthesis in infected cells is presented in Figure 22.

The gh-1 DNA is shown in the model to contain a L strand and a complementary H strand. The evidence that gh-1 DNA contains H and L strands is that the strands of gh-1 DNA can be separated by CsCl density gradient centrifugation when complexed with poly (U,G) (Figure 9). The gh-1 genome is also shown to contain an early region and a late region. For simplicity the early region is placed at the left end of the gh-1 genome. The early region is shown to be 20% of the gh-1 genome because the size of the early region was estimated to be about 20% of the gh-1 genome by determining the size of early RNA species transcribed *in vitro* (Figure 20). The evidence for the size of the early region of gh-1 DNA is not strong evidence but it is consistent with the size of the early region of T7 DNA (20,21).

In the model the time course of gh-1 infection is divided into three time intervals. The RNA species synthesized during each time interval and the RNA

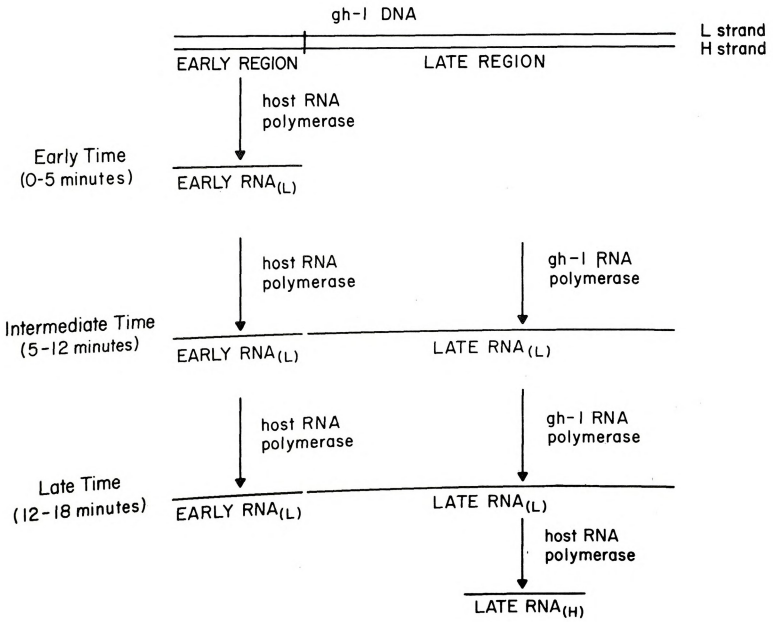
polymerase responsible for the transcription of the RNA species is illustrated in the model.

During the early time interval of infection (0-5 minutes post infection) early RNA is transcribed from the early region of the gh-1 genome by the host RNA polymerase. As indicated in the model, the early RNA ($early_{[L]}$) is complementary to the L strand of gh-1 DNA. The gh-1 RNA synthesized before the gh-1 RNA polymerase is present is early RNA by definition. The duration of the early time interval of infection is not known precisely, but it was shown (Figure 3) that all early DNA sequences are not transcribed in infected cells until about 4 minutes post infection. The gh-1 RNA polymerase is first detectable in infected-cell extracts at about 5 minutes post infection (98). Thus, the early time interval of infection probably lasts until about 5 minutes post infection when all early DNA sequences are transcribed and the gh-1 RNA polymerase is present. The early RNA must be transcribed by host RNA polymerase because early RNA is transcribed in the presence of chloramphenicol, which prevents the synthesis of the gh-1 RNA polymerase in infected cells (98). The evidence that early RNA is complementary to the L strand of gh-1 DNA is that early RNA synthesized in infected cells hybridized to only the L strand of gh-1 DNA (Table 1).

During the intermediate time interval of infection (5-12 minutes post infection) early RNA complementary to the L strand of gh-1 DNA (early $RNA_{[L]}$) continues to be transcribed by the host RNA polymerase. In addition, as indicated in the model, late RNA is transcribed from the L strand of gh-1 DNA (late $RNA_{[L]}$) by the gh-1 RNA polymerase. The evidence that early RNA continues to be synthesized during the intermediate time interval of infection is that about 10% of the RNA pulse-labeled 8-10 minutes post infection that hybridized to gh-1 DNA was competed by CAM RNA (Figure 4). The remaining RNA that hybridized to gh-1 DNA was competed by 18 minute competitor RNA indicating that most of the RNA synthesized during the intermediate time interval of infection is late RNA. The fact that the RNA pulse-labeled from 8-10 minutes post infection hybridized almost exclusively to the L strand of gh-1 DNA (Table 1) indicates that both the early RNA and late RNA synthesized during this time interval are complementary to the L strand of gh-1 DNA.

It was not proven that host RNA polymerase transcribes early RNA complementary to the L strand of gh-1 DNA during the intermediate time interval of infection as indicated in the model. However, it was shown that host RNA polymerase transcribes early RNA complementary to the L strand of gh-1 DNA during the early time interval

Figure 22.--Model for the control of gh-1 RNA synthesis in infected cells. Details of this model are presented in the text. Early RNA and late RNA are represented as single lines for simplicity. It is not suggested that early and late RNA are transcribed as large precursor molecules. The length of the line representing late RNA complementary to the H strand of gh-1 DNA (late $RNA_{(H)}$) indicates that about 20% of the RNA synthesized during the late time interval of infection capable of hybridizing to gh-1 DNA is late $RNA_{(H)}$. It does not indicate that only a limited amount of the H strand late region is transcribed. The extent to which the late region of the H strand of gh-1 DNA is transcribed is not known.


gh-1 DNA
L strand
H strand
EARLY REGION
LATE REGION
host RNA polymerase
Early Time (0-5 minutes)
EARLY RNA(L)
host RNA polymerase
gh-1 RNA polymerase
Intermediate Time (5-12 minutes)
EARLY RNA(L)
LATE RNA(L)
host RNA polymerase
gh-1 RNA polymerase
Late Time (12-18 minutes)
EARLY RNA(L)
LATE RNA(L)
host RNA polymerase
LATE RNA(H)

of infection and also during the late time interval of infection. It is therefore assumed that host RNA polymerase also transcribes early RNA complementary to the L strand of gh-1 DNA during the intermediate time interval of infection. Likewise, it was not proven that the gh-1 RNA polymerase transcribes late RNA from the L strand of gh-1 DNA during the intermediate time interval of infection but it was shown that the gh-1 RNA polymerase transcribes late RNA from the L strand of gh-1 DNA during the late time interval of infection (Table 1 and Figure 8). It is therefore assumed that the gh-1 RNA polymerase also transcribes late RNA from the L strand of gh-1 DNA during the intermediate time interval of infection.

The duration of the intermediate time interval of infection as presented in the model is an estimation only. It was shown that all late DNA sequences are transcribed in infected cells by about 10 minutes post infection (Figure 5). Thus, the intermediate time interval probably lasts until about 10 minutes post infection. However, a small amount of RNA complementary to the H strand of gh-1 DNA may be present in RNA pulse-labeled 8-10 minutes post infection (Table 1), while large amounts of RNA complementary to the H strand of gh-1 DNA are present in RNA pulse-labeled 12-14 minutes post infection and 16-18 minutes post infection (Table 1). The synthesis of complementary RNA is a characteristic

of the late time interval of infection (see model). It is not possible to determine exactly when the intermediate time interval of infection begins or ends. For simplicity, the intermediate time interval is extended in the model until 12 minutes post infection, when large amounts of H strand complementary RNA are first detected.

As indicated in the model, host RNA polymerase continues to transcribe early RNA complementary to the L strand of gh-1 DNA during the late time interval of infection. In addition, host RNA polymerase transcribes late RNA complementary to the H strand of gh-1 DNA (late $RNA_{[H]}$). The gh-1 RNA polymerase continues to transcribe late RNA complementary to the L strand of gh-1 DNA during the late time interval of infection.

Evidence that host RNA polymerase transcribes early RNA complementary to the L strand of gh-1 DNA during the late time interval of infection was obtained in the following way. Rifampicin was added to inhibit host RNA polymerase during a late pulse-labeling period (16-18 minutes post infection). The labeled RNA was not competed by CAM RNA in hybridization-competition studies indicating that host RNA polymerase and not the gh-1 RNA polymerase transcribes early RNA sequences (or their complements) during the late time interval of infection (Figure 8). The early RNA must be complementary to the L strand of gh-1 DNA because all the RNA complementary

to the H strand of gh-1 DNA was shown by hybridization-competition to be late RNA (Figure 10). The late RNA complementary to the H strand of gh-1 DNA must be transcribed by the host RNA polymerase because the labeled RNA synthesized in the presence of rifampicin did not hybridize to the H strand of gh-1 DNA (Table 1). Late RNA complementary to the L strand of gh-1 DNA must be transcribed by the gh-1 RNA polymerase because its transcription is not affected by the addition of rifampicin during a late RNA pulse (16-18 minutes post infection, Figure 8).

Most if not all, of the late RNA transcribed by the host RNA polymerase from the H strand of gh-1 DNA is complementary to the late RNA transcribed by the gh-1 RNA polymerase from the L strand of gh-1 DNA. The evidence for this is that about 60% of the RNA pulse-labeled 16-18 minutes post infection is capable of forming RNA duplexes (Figure 11[c]); half of the RNA capable of forming RNA duplexes (i.e., 30% of the total RNA) must be complementary to the H strand of gh-1 DNA. About 25% of the RNA pulse-labeled 16-18 minutes post infection hybridized to the H strand of gh-1 DNA (Table 1). This RNA was shown to be late RNA as already indicated. Thus, most if not all the late RNA transcribed from the H strand of gh-1 DNA must be complementary to the late RNA transcribed from the L strand of gh-1 DNA.

The novel features of this model of gh-1 transcription, when compared to the pattern of T7 and T3 transcription, is the continued synthesis of early RNA during all time periods of infection and the synthesis of complementary RNA during the late time interval of infection. The appearance of complementary RNA during gh-1 infection is particularly significant because it has been found in very few systems. These systems will be described later as will speculation about the function of complementary gh-1 RNA.

The RNA species transcribed *in vitro* by purified host RNA polymerase were examined to determine if the enzyme contains the specificity to transcribe *in vitro* the RNA species predicted by the model of gh-1 RNA transcription. It was found (Figure 13, Table 4) that the host RNA polymerase holoenzyme could transcribe almost exclusively early RNA from the L strand of gh-1 DNA (early $RNA_{[L]}$) if the molar ratio of enzyme to DNA was low (R less than 1). Under these conditions the enzyme also transcribed a small amount of early RNA from the H strand of gh-1 DNA. This RNA was transcribed as a heterogeneous class of small RNA molecules (Figure 20). It was found that the core enzyme could not be limited to the transcription of early RNA or to transcription of the L strand of gh-1 DNA at either high or low molar ratios of enzyme to DNA template (Figure 14,

Table 4). It is concluded that the host RNA polymerase can be essentially limited to the transcription of early RNA from the L strand of gh-1 DNA *in vitro* if the sigma subunit is present and if the molar ratio of enzyme to DNA template is low. This is the specificity of RNA transcription expected of the host RNA polymerase during the early time interval of infection as described in the model.

It was found that at a high molar ratio of holoenzyme to gh-1 DNA template the enzyme could transcribe late RNA from the H strand of gh-1 DNA *in vitro* as well as early RNA from the L strand of gh-1 DNA (Figure 18). This is the situation postulated in the model of gh-1 transcription during the late time interval of infection. However, the enzyme was not limited to transcription of these classes of RNA. It also transcribed a large amount of early RNA from the H strand of gh-1 DNA and late RNA from the L strand of gh-1 DNA. It is not possible to determine if the host RNA polymerase transcribes late RNA from the L strand of gh-1 DNA during the late interval of infection, but it is clear that the enzyme does not transcribe early RNA from the H strand of gh-1 DNA during the late time interval of infection. Thus, at a high molar ratio of holoenzyme to DNA template the enzyme can transcribe late RNA from the H strand of gh-1 DNA as presented in the model, but

it also transcribes early RNA from the H strand of gh-1 DNA which is not consistent with the model of gh-1 transcription.

The specificity of RNA transcription by the gh-1 RNA polymerase was also examined *in vitro*. It was found that the enzyme transcribed only the L strand of gh-1 DNA, but transcription was not limited to the late region of gh-1 DNA as suggested by the model of gh-1 transcription (Figure 16). This result was not affected by the molar ratio of enzyme to gh-1 DNA template. The evidence that gh-1 RNA polymerase transcribes only late RNA *in vivo* rests in the interpretation of the hybridization-competition data in Figure 8. While it may not be correct to conclude that *no* early RNA is synthesized, the data clearly shows that if early RNA is synthesized it amounts to very little of the total RNA synthesized by the gh-1 RNA polymerase. The gh-1 RNA polymerase transcribes about 50% early RNA *in vitro* (Figure 16), so it is obvious that early RNA synthesis by gh-1 RNA polymerase is mostly, if not totally, suppressed *in vivo*. It is possible that a transcriptional factor not present in the purified gh-1 RNA polymerase preparation is capable of limiting transcription by the enzyme to the late region of gh-1 DNA. This could be done by interacting with the gh-1 RNA polymerase directly to modify its transcriptional specificity, or it could be

bound to gh-1 DNA so that promotors recognized by the gh-1 RNA polymerase in the early region of gh-1 DNA are not accessible to the enzyme in vivo. It is also possible that the gh-1 RNA polymerase and host RNA polymerase compete for the same early RNA promotor sites and host RNA polymerase has a greater affinity for such sites.

## Synthesis of Complementary RNA in Other Systems

It has been shown that complementary RNA transcripts are made during T4 phage development (103,104). These RNA species have been characterized as anti-late RNA transcribed from a large region of the l strand of T4 DNA. Extensive anti-late RNA synthesis, like delayed early T4 RNA synthesis is dependent on protein synthesis. Anti-late RNA appears at 3 minutes post infection at the same time as delayed early RNA and the synthesis of anti-late RNA is shutoff or decreased at later stages of T4 development as is pre-replicative early RNA. Anti-late RNA reaches a steady-state concentration at 4 minutes post infection when it constitutes about 2% of the total RNA. It has been postulated that the synthesis of anti-late RNA is due to either defective termination or initiation of RNA synthesis (103,104).

Complementary RNA has also been observed in λ phage development (105). It was found that early RNA

transcription from the l strand of λ DNA and late RNA transcription from the r strand of λ DNA slightly overlap in the b2 region of the λ genome. This "buffer zone" apparently does not code for a functional protein.

Double-stranded RNA has been observed in vaccinia virus-infected cells (106). It was found that 3% of the RNA in chick cells infected with vaccinia virus is RNase resistant. The RNA in the duplexes is complementary to vaccinia virus DNA but a small amount is also complementary to chicken DNA (1%). Complementary RNA (0.4% of the total RNA) is also found in uninfected chick cells. The duplex RNA isolated from vaccinia-infected cells was capable of inducing the production of interferon.

Attardi and co-workers (107,108) have found that complementary RNA is synthesized in HeLa cell mitochondria. Both the heavy and light strands of mitochondrial DNA are completely transcribed. Isolated mitochondrial RNA contains double-stranded RNA molecules which appear as long as the entire mitochondrial genome. The proportion of double-stranded RNA present in the mitochondrial RNA can be enhanced by self-annealing. The amount of complementary RNA after self-annealing was about 20-35% of the total RNA. The L strand RNA appears to be either rapidly degraded or exported from the mitochondria.

## Speculation Concerning the Function and Appearance of Complementary RNA in gh-1-Infected Cells

The complementary RNA detected in gh-1-infected cells (late $RNA_{[H]}$) is either anti-messenger RNA or messenger RNA coding for a non-essential protein for gh-1 phage production. The evidence for this is that when host RNA polymerase is inhibited by the addition of rifampicin at 12.5 minutes post infection phage production is not significantly affected (Table 2). Since complementary RNA is synthesized by the host RNA polymerase beginning about 12 minutes post infection, this indicates that synthesis of complementary RNA is not required for phage production.

If the complementary RNA synthesized in gh-1-infected cells is messenger RNA then the synthesis of complementary RNA should be regulated by some means of transcriptional control. This may involve the modification of host RNA polymerase at about 12 minutes post infection resulting in a change in the specificity of the enzyme. The modified host RNA polymerase may then be capable of transcribing complementary RNA. It is known that the B. subtilus host RNA polymerase is modified during SP01 phage infection so that new classes of phage RNA are transcribed (109-113). The modified B. subtilus RNA polymerase has been purified from infected cells and shown to have an altered transcriptional

specificity in in vitro RNA synthesis (111-113). It has also been shown that the E. coli RNA polymerase is modified during T4 phage infection (114,115).

It is also possible that the transcription of complementary RNA is coupled to the replication of gh-1 DNA which also occurs about 12 minutes post infection. It is known that late phage RNA synthesis in T4 infected E. coli and SP01-infected B. subtilus requires phage DNA replication (117,118). It is not known why transcription of a new class of phage RNA is coupled to DNA replication but it has been suggested that a change in the structure of phage DNA during replication may be responsible (118).

The experiment in which rifampicin was added to gh-1-infected cells at 12.5 minutes post infection indicated that complementary RNA synthesis is not required for phage production. It also indicates that the continuation of early RNA synthesis is not required after 12.5 minutes post infection since early RNA is also transcribed by host RNA polymerase. Early RNA is messenger RNA and synthesis of early RNA is required until at least 5 minutes post infection because the addition of rifampicin at 5 minutes post infection severely limits phage production (Table 3). If early RNA synthesis is not required for phage production after 12.5 minutes post infection, why is up to 50% of the RNA synthesized between 16-18 minutes post infection

early RNA? It is possible that there is a breakdown in transcriptional control during the late interval of infection and the synthesis of complementary RNA is one manifestation of this breakdown. It is therefore speculated that complementary RNA synthesized by the host RNA polymerase during the late interval of infection (late $RNA_{[H]}$) is anti-messenger RNA and that the synthesis of anti-messenger is a result of a breakdown of transcriptional control during the late interval of gh-1 infection.

The hypothesized breakdown in transcriptional control during the late time interval of gh-1 infection may occur because of a change in the molar ratio of host RNA polymerase to gh-1 DNA during infection. During the early time interval of infection the ratio of host RNA polymerase molecules to gh-1 DNA molecules is probably low because the host genome is much larger than the gh-1 genome and most host RNA polymerase molecules are probably associated with host DNA at the time of infection. As evidence for this, less than 1% of the RNA synthesized in gh-1-infected cells during a 2-4 minute pulse hybridized to gh-1 DNA (Figure 1). In addition, the specificity of transcription during this period of infection (early $RNA_{[L]}$) closely resembles the specificity of transcription in vitro at low molar ratios of holoenzyme to gh-1 DNA template.

During the intermediate time interval of infection the molar ratio of host RNA polymerase molecules to gh-1 DNA molecules probably does not change significantly. The rapid increase in the relative proportion of gh-1 RNA synthesis during this period (Figure 1) is probably due to the presence of gh-1 RNA polymerase which transcribes gh-1 DNA exclusively. As an indication of this about 90% of the RNA synthesized 8-10 minutes post infection capable of hybridizing to gh-1 DNA is late RNA (Figure 4).

However, between 10 and 12 minutes post infection host DNA is rapidly degraded (Figure 2) with the result that host RNA polymerase molecules may be free for transcription of gh-1 DNA. The replication of gh-1 DNA also begins about this time post infection (Figure 2). It has been estimated that about 5000 host RNA polymerase molecules exist in a P. putida cell (116). If all are available for transcription of gh-1 DNA and if there are 100 gh-1 DNA molecules per cell after gh-1 DNA replication (Table 3) then the ratio of host RNA polymerase molecules to gh-1 DNA molecules would be about 50:1. It has been shown that host RNA polymerase holoenzyme is no longer restricted to transcription of the early region of gh-1 DNA in vitro at this molar ratio of enzyme to gh-1 DNA and is capable of synthesizing complementary RNA in vitro. Thus, it is

suggested that the increase in the ratio of host RNA polymerase molecules to gh-1 DNA molecules during the late time interval of infection is responsible for the synthesis of complementary RNA during this period.

The increase in the molar ratio of host RNA polymerase to gh-1 DNA could also be responsible for the increase in the relative amount of early RNA synthesis during the late time interval of infection since more host RNA polymerase molecules would be available for synthesis of early RNA.

The suggestion that an increase in the molar ratio of host RNA polymerase to gh-1 DNA during the late time interval of infection is responsible for the synthesis of complementary RNA in vivo is based on the observation that late RNA complementary to the H strand of gh-1 DNA is transcribed in vitro under conditions of a high molar ratio of holoenzyme to gh-1 DNA. However, under these conditions the holoenzyme transcribes early RNA from the H strand of gh-1 DNA and late RNA from the L strand of gh-1 DNA. There is no evidence that host RNA polymerase transcribes these two RNA classes during the late time interval of infection.

As stated before, there is no evidence indicating that host RNA polymerase does or does not transcribe late RNA from the L strand of gh-1 DNA during the late time interval of infection. The difficulty in obtaining such

evidence is that if the host RNA polymerase is inhibited by rifampicin during the late time interval of infection late RNA is still transcribed by the gh-1 RNA polymerase. Since it is not possible to selectively inhibit the gh-1 RNA polymerase *in vivo* no conclusion about the ability of host RNA polymerase to transcribe late RNA from the L strand of gh-1 DNA can be made.

It is clear that early RNA is not transcribed from the H strand of gh-1 DNA because all H strand-specific RNA transcribed *in vivo* is late RNA. It is possible that if early RNA is transcribed from the H strand *in vivo* it is rapidly degraded in the infected cell. It is also possible that early RNA synthesis from the H strand of gh-1 DNA may be preferentially depressed *in vivo* by a termination factor analogous to the rho termination factor found in *E. coli*. Evidence has been presented that rho termination factor can eliminate RNA transcription from the biologically incorrect strand of T3 DNA when present during *in vitro* transcription of T3 DNA by *E. coli* RNA polymerase holoenzyme (51).

Another possible explanation for the synthesis of complementary RNA during the late time interval of gh-1 infection is that host RNA polymerase may lose its complement of sigma factor during infection. It has been shown that host RNA polymerase core enzyme is not limited to transcription of early RNA *in vitro*. This

result is independent of the molar ratio of enzyme to gh-1 DNA (Figure 14). While it was shown that core enzyme can transcribe the H strand of gh-1 DNA *in vitro* (Table 4), it is not known if the H strand-specific RNA is late RNA. *E. coli* RNA polymerase is known to lose its associated sigma factor during T4 infection, but it is apparently only one of several changes in *E. coli* RNA polymerase after infection (114).

Finally, even though transcriptional control may breakdown during the late time interval of infection, control of genetic expression may then be regulated at the level of translation. Thus, the early $RNA_{(L)}$ and late $RNA_{(H)}$ transcribed by the host RNA polymerase during this period of infection may not be translated. It would be to the advantage of the phage if only late RNA coding for late proteins needed for phage production were translated during late stages of phage development. The inhibition of host protein and early phage protein synthesis is known to occur in phage systems (14). Early phage protein synthesis may be inhibited in gh-1-infected cells during the late time interval of infection because the specific activity of gh-1 RNA polymerase (which is presumed to be an early gene product) increases rapidly from 5 to 10 minutes post infection and levels off after 12 minutes post infection (data not shown) despite the fact that early RNA synthesis continues

throughout the gh-1 infectious cycle and accounts for about 50% of the gh-1 RNA synthesized in infected cells during a 16-18 minute pulse that hybridizes to gh-1 DNA (Figure 5).

# BIBLIOGRAPHY

BIBLIOGRAPHY


1. Cairns, J.; Stent, G. S.; and Watson, J. D., Eds. (1966) Phage and the Origins of Molecular Biology. Cold Spring, N.Y.: The Cold Spring Harbor Laboritory.

2. Studier, F. W. (1972) Science, 176, 367.

3. Chamberlin, M. J. (1974) Ann. Rev. Biochem, 43, 721.

4. Chamberlin, M. J. (1970) Cold Spring Harbor Symp. Quant. Biol, 35, 851.

5. Burgess, R. R. (1971) Ann. Rev. Biochem., 41, 711.

6. Dubin, S. D.; Benedele, G. B.; Bancroft, D.; and Freifelder, D. (1970) J. Mol. Biol., 54, 547.

7. Studier, F. W. (1972) Science, 176, 367.

8. Studier, F. W. and Maizel, J. V. (1969) Virology, 39, 575.

9. Studier, F. W. (1969) Virology, 39, 562.

10. Studier, F. W. (1973) J. Mol. Biol., 79, 227.

11. Hausmann, R., and LaRue, K. (1969) J. Virol, 3, 278.

12. Kelly, T. J., Jr. and Thomas, C. A. (1969) J. Mol. Biol., 44, 459.

13. Hopper, J. E.; Grant, K.; and Young, E. T. (1975) J. Mol. Biol., 94, 539.

14. Schweiger, M.; Herrlich, P.; Scherzinger, E.; and Rahmsdorf, H. J. (1972) Proc. Nat. Acad. Sci. USA, 69, 2203

15. Siegel, R. B. and Summers, W. C. (1970) J. Mol. Biol., 49, 115.

16. Chamberlin, M.; McGrath, J.; and Waskell, L. (1970) Nature, 228, 227.

17. Gelfand, D. H. and Hayashi, M. (1970) Nature, 228, 1162.

18. Studier, F. W. (1973) J. Mol. Biol., 79, 237.

19. Summers, W. C.; Brunovskis, I.; and Hyman, R. W. (1973) J. Mol. Biol., 74, 291.

20. Simon, M. N. and Studier, F. W. (1973) J. Mol. Biol., 79, 249.

21. Hyman, R. W. (1971) J. Mol. Biol., 61, 367.

22. Ritchie, D. A.; Thomas, C. A.; MacHattie, L. A.; and Wensink, P. C. (1967) J. Mol. Biol., 23, 365.

23. Summers, W. C. (1970) J. Mol. Biol., 51, 671.

24. Herrlick, P.; Rahmsdorf, A. J.; Pai, S. H.; and Schweiger, M. (1974) Proc. Nat. Acad. Sci. USA, 71, 1088.

25. Marrs, B. L. and Yanofsky, C. (1971) Nature, New Biol., 234, 168.

26. Yamada, Y.; Whitaker, P. A.; and Nakada, D. (1974) Nature, 248, 335.

27. Yamada, Y.; Whitaker, P. A.; and Nakada, D. (1974) J. Mol. Biol., 89, 293.

28. Summers, W. C. and Szybalski, W. (1968) Biochem. Biophys. Acta., 166, 371.

29. Mushynski, W. E. and Spencer, J. H. (1970) J. Mol. Biol., 52, 91.

30. Summers, W. C. and Szybalski, W. (1968) Virology, 34, 9

31. Summers, W. C. and Siegel, R. B. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 255.

32. Brunovskis, I. and Summers W. C. (1971) Virology, 45, 224.

33. Brunovskis, I. and Summers, W. C. (1972) Virology, 50, 322.

34. Hesselback, B. A. and Nakada, D. (1975) Nature, 258, 354.

35. Hesselback, B. A.; Yamada, Y.; and Nakada, D. (1974) Nature, 252, 71.

36. Ponta, H.; Rahmsdorf, H. J.; Pai, S. H.; Herrlich, P.; and Schweiger, M. (1974) Molec. gen. Genet., 134, 29.

37. Ratner, D. (1974) J. Mol. Biol., 88, 373.

38. Rahmsdorf, H. J.; Pai, S. H.; Ponta, H.; Herrlich, P.; Roskaski, R., Jr.; Schweiger, M.; and Studier, F. W. (1974) Proc. Nat. Acad. Sci. USA, 71, 586.

39. Ponta, H.; Rahmsdorf, H. J.; Pai, S. H.; Hirsch-Kauffmann, M.; Herrlich, P.; and Schweiger, M. (1974) Mol. Gen. Genet., 134, 281.

40. Zillig, W.; Fiyiki, H.; Blum, W.; Janekovic, D.; Schweiger, M.; Rahmsdork, H. J.; Ponta, H.; and Hirsch-Kauffmann, M. (1975) Proc. Nat. Acad. Sci. USA, 72, 2506.

41. Hinkle, D. C.; Mangel, W. F.; and Chamberlin, M. J. (1972) J. Mol. Bio., 70, 209.

42. Hinkle, D. C. and Chamberlin, M. J. (1972) J. Mol. Biol., 70, 157.

43. Hinkle, D. C. and Chamberlin, M. J. (1972) J. Mol. Biol., 70, 187.

44. Bautz, E. K. F. and Bautz, F. A. (1970) Nature, 226, 1219.

45. Hinkle, D. C.; Ring, J.; and Chamberlin, M. J. (1972) J. Mol. Biol., 70, 197.

46. Davis, R. W. and Hyman, R. W. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 269.

47. Dunn, J. J. and Studier, F. W. (1973) Proc. Nat. Acad. Sci. USA, 70, 1559.

48. Millette, R. L.; Trotter, C. D.; Herrlich, P.; and Schweiger, M. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 135.

49. Dunn, J. J. and Studier, F. W. (1973) Proc. Nat. Acad. Sci. USA, 70, 3296.

50. Roberts, J. W. (1969) Nature, 224, 1168.

51. Dunn, J. J.; McAllister, W. T.; and Bautz, E. K. F. (1972) Virology, 48, 112.

52. Goldberg, A. R. and Hurwitz, J. (1972) J. Biol. Chem., 247, 5637.

53. Richardson, J. P.; Grimley, C.; and Lowery, C. (1975) Proc. Nat. Acad. Sci. USA, 72, 1725.

54. Lowery-Goldhammer, C. and Richardson, J. P. (1974) Proc. Nat. Acad. Sci. USA, 71, 2003.

55. Zipser, D. (1969) Nature, 221, 21.

56. Darlix, J. (1973) Eur. J. Biochem., 35, 517.

57. Minkley, E. G., Jr. (1974) J. Mol. Biol., 83, 289.

58. Minkley, E. G., Jr. (1974) J. Mol. Biol., 83, 305.

59. Ptashne, M. (1967) Proc. Nat. Acad. Sci. USA, 57, 306.

60. Minkley, E. G., Jr. and Pribnow, D. (1973) J. Mol. Biol., 77, 255.

61. Pribnow, D. (1975) Proc. Nat. Acad. Sci. USA, 72, 784.

62. Heyden, M. Nusslein, J. and Schaller (1975) Eur. J. Biochem, 55, 147.

63. Hinkle, D. C. and Chamberlin, M. J. (1970) Cold Spring Harbor Symp. Quant. Biol., 35, 65.

64. Chamberlin, M. J. and Ring, J. (1973) J. Biol. Chem., 248, 2235.

65. Chamberlin, M. J. and Ring, J. (1973) J. Biol. Chem., 248, 2245.

66. Chamberlin, M. J. and Ring, J. (1972) Biochim. Biophys. Res. Commun., 49, 1129.

67. Golomb, M. and Chamberlin, M. J. (1974) J. Biol. Chem., 249, 2858.

68. Golomb, M. and Chamberlin, M. J. (1974) Proc. Nat. Acad. Sci. USA, 74, 760.

69. Niles, E. G.; Conlon, W. W.; and Summers, W. C. (1974) Biochemistry, 13, 3904.

70. Skare, J.; Niles, E. G.; and Summers, W. C. (1974) Biochemistry, 13, 3912.

71. Niles, E. G. and Condit, R. C. (1975) J. Mol. Biol., 98, 57.

72. Dunn, J. J.; McAllister, W. T.; and Bautz, E. K. F. (1972) Eur. J. Biochem., 29, 500.

73. Chakroborty, P. R.; Sarkar, P.; Huang, H. H.; and Maitra, U. (1973) J. Biol. Chem., 248, 6637.

74. Maitra, U. (1971) Biochem. Biophys. Res. Commun., 43, 443.

75. Maitra, U. and Huang, H. H. (1972) Proc. Nat. Acad. Sci. USA, 69, 55.

76. Kupper, H. A.; McAllister, W. T.; and Bautz, E. K. F. (1973) Eur. J. Biochem., 38, 581.

77. Bautz, E. K. F. (1973) FEBS (Fed. Eur. Biochem. Soc.) Lett., 36, 123.

78. Salvo, Ringo A.; Chakrobority, P. R.; and Maitra, U. (1973) J. Biol. Chem., 248, 6647.

79. McAllister, W. T.; Kupper, H.; and Bautz, E. K. F. (1973) Eur. J. Biochem., 34, 489.

80. Chakroborty, P. R.; Bandyopadhyay, P.; Huang, H. H.; and Maitra, U. (1974) J. Biol. Chem., 249, 6901.

81. Dunn, J. J.; Bautz, F. A.; and Bautz, E. K. F. (1971) Nature, New Biol., 230, 94.

82. Hercules, K.; Schweiger, M.; and Sauerbier, W. (1974) Proc. Nat. Acad. Sci. USA, 71, 840.

83. Kindler, P.; Keil, T.; and Hofschneider, P. H. (1973) Molec. Gen. Genet., 126, 53.

84. Dharmgrongartama, B.; Mahadik, S. P.; and Srinivasan, P. R. (1973) Proc. Nat. Acad. Sci. USA, 70, 2845.

85. Makadik, S. P.; Dharmgrongartama, B.; and Srinivasan, P. R. (1974) J. Biol. Chem., 249, 1787.

86. Mahadik, S. P.; Dhamgrangartama, B.; and Srinivasan, P. R. (1972) Proc. Nat. Acad. Aci. USA, 69, 162.

87. Lee, L. F. and Boezi, J. A. (1966) J. Bacteriol., 92, 1821.

88. Thomas, C. A. and Abelson, J. (1966) In Procedures in Nucleic Acid Research. Edited by G. L. Contani and D. R. Davies. New York, New York: Harper and Row, Publishers, p. 553.

89. Thomas, C. A.; Berns, K. I.; and Kelly, T. J. (1966) In Procedures in Nucleic Acid Research. Edited by G. L. Contoni and D. R. Davies. New York, New York: Harper and Row, Publishers, p. 535.

90. Lee, L. F. (1966) Ph.D. Thesis, Michigan State University.

91. Towle, H. C.; Jolly, J. F.; and Boezi, J. A. (1975) J. Biol. Chem., 250, 1723.

92. Johnson, J. C.; DeBacker, M.; and Boezi, J. A. (1971) J. Biol. Chem., 246, 1723.

93. Robertson, H. D.; Webster, R. E.; and Zinder, N. D. (1968) J. Biol. Chem., 243, 82.

94. Bøvre, K. and Szybalski, W. In Methods in Enzymology, Vol. XXI, Edited by L. Grossman and K. Moldave. New York and London: Academic Press, p. 350.

95. Gillespie, D. and Spiegelman, S. (1965) J. Mol. Biol., 12, 829.

96. Nygaard, A. P. and Hall, B. D. (1964) J. Mol. Biol., 9, 125.

97. Riva, S. and Silvestri, L. G. (1972) Ann. Rev. Microbiol, 26, 199.

98. Towle, H. C. (1974) Ph.D. Thesis, Michigan State University.

99. Peacock, A. C. and Dingman, C. W. (1967) Biochemistry, 6, 1818.

100. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; and Randall, R. J. (1951) J. Mol. Biol., 193, 265.

101. Szybalski, W.; Kubinski, H.; Hradecna, Z.; and Summers, W. C. In Methods of Enzymology, Vol. XXI, Edited by L. Grossman and K. Moldave. New York and London: Academic Press, p. 383.

102. Hausmann, R. and Gomez, B. (1967) J. Virol., 1, 779.

103. Geiduschek, E. P. and Grau, O. Lepetit, Colloq. on RNA polymerase and Transcription, Edited by L. Silvestri (North-Holland Publ. Co., Amsterdam, 1970), p. 190.

104. Notani, G. W. (1973) J. Mol. Biol., 73, 231.

105. Bøvre, K. and Szybalski, W. (1969) Virology, 38, 614.

106. Colby, C. and Duesberg, P. H. (1969) Nature, 222, 940.

107. Aloni, Y. and Attardi, G. (1971) Proc. Nat. Acad. Sci. USA, 68, 1757.

108. Young, P. G. and Attardi, G. (1975) Biochem. Biophys. Res. Commun., 65, 1201.

109. Spiegelman, G. B. and Whiteley, H. R. (1974) J. Biol. Chem., 249, 1476.

110. Spiegelman, G. B. and Whiteley, H. R. (1974) J. Biol. Chem., 249, 1483.

111. Pero, J.; Nelson, J.; and Fox, T. D. (1975) Proc. Nat. Acad. Sci. USA, 72, 1589.

112. Duffy, J. J. and Geiduschek, E. P. (1975) J. Biol. Chem., 250, 4530.

113. Duffy, J. J.; Petrusek, R. L.; and Geiduschek, E. P. (1975) Proc. Nat. Acad. Sci. USA, 72, 2366.

114. Stevens, A. (1972) Proc. Nat. Acad. Sci. USA, 69, 603.

115. Horvitz, H. R. (1973) Nature, New Biol., 244, 137.

116. Johnson, J. C. (1971) Ph.D. Thesis, Michigan State University.

117. Riva, S.; Cascino, A.; and Geiduschek, E. P. (1970) J. Mol. Biol., 54, 103.

118. Riva, S.; Cascino, A.; and Geiduschek, E. P. (1970) J. Mol. Biol., 54, 110.

119. Summers, W. C. and Siegel, R. B. (1969) Nature, 223, 1111.

# APPENDIX

# APPENDIX

The following publication is the result of a two year collaboration of Howard Towle and I on the purification and characterization of the gh-1-induced RNA polymerase. We were both involved in the purification of the enzyme and the determination of the template specificity and the effect of RNA synthesis inhibitors on gh-1 RNA polymerase. Howard was then involved with the effect of varying the concentration of substrates and the effect of cordycepin triphosphate on gh-1 RNA polymerase activity (Figures 4-7), while I began to examine the control of gh-1 RNA transcription *in vivo* and the transcriptional specificity of the host and gh-1 RNA polymerases *in vitro* described in this dissertation.

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# Purification and Characterization of Bacteriophage gh-1-induced Deoxyribonucleic Acid-dependent Ribonucleic Acid Polymerase from *Pseudomonas putida**

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## SUMMARY

**Infection of *Pseudomonas putida* by the bacteriophage gh-1 induced the synthesis of a novel DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase was purified to near homogeneity. It was shown to be distinct from the host RNA polymerase ($\alpha_2\beta\beta'\sigma$) physically and in respect to many of its catalytic properties. The gh-1-induced RNA polymerase was composed of a single polypeptide of approximately 98,000 molecular weight. The divalent metal ion requirement for *in vitro* RNA synthesis by the gh-1 polymerase could be satisfied with $Mg^{2+}$, but not with $Mn^{2+}$. RNA synthesis by the gh-1 polymerase was highly resistant to inhibition by rifampicin and streptolydigin but could be inhibited by relatively low concentrations of KCl or the rifamycin derivative AF/013. The structural analog of ATP, 3'-deoxyadenosine 5'-triphosphate, inhibited both the gh-1-induced and the host RNA polymerases by competing for a single binding site with ATP. The phage polymerase was extremely sensitive to this inhibitor, exhibiting an apparent $K_i$ value ($2 \times 10^{-8}$ M) approximately 100 times lower than that for the host RNA polymerase. The gh-1 polymerase had a highly specific template requirement for DNA from the homologous gh-1 phage. It would not efficiently utilize denatured DNA templates and had only low levels of activity with pyrimidine-containing polydeoxyribonucleotide homopolymers.**

When a bacterial cell becomes infected with a virulent bacteriophage, a shift in RNA synthesis occurs from entirely host-specific (transcription from the host DNA) to largely phage-specific (transcription from the viral DNA). There are two general types of mechanisms by which this shift in transcription can occur. In one mechanism, the host DNA-dependent RNA polymerase is utilized throughout the infectious cycle for the transcription of all classes of viral genes. Modifications of the host RNA polymerase in the viral infected cell, however, alter the specificity of the enzyme to program changes in transcription during the infectious cycle. This mechanism most likely occurs in T4 and λ bacteriophage infections of *Escherichia coli* (1, 2), SPO1 and SP82 infections of *Bacillus subtilis* (3, 4), and $\phi$29 bacteriophage infection of *Bacillus amyloliquefaciens* (5). The exact nature of the modification causing altered specificity of the host RNA polymerase is unknown. Several chemical alterations of the subunits of the host RNA polymerase have been demonstrated after T4 infection of *E. coli* (6–8). Furthermore, polypeptides, some of which have been shown to be the products of T4 regulatory genes, have been found to be associated with the host RNA polymerase in T4-infected cells (9, 10). It has not been demonstrated, however, which, if any, of these modifications confer altered transcriptional specificity to the host RNA polymerase.

The second mechanism to account for the shift in DNA transcription after bacteriophage infection involves the synthesis of a new, viral coded DNA-dependent RNA polymerase. This mechanism has been shown to occur in both T3 and T7 infection of *E. coli* (11–14). The new RNA polymerases synthesized after infection by these coliphages are quite different from the host RNA polymerase in both structure and catalytic properties. These phage-induced RNA polymerases are composed of single polypeptides of approximately 108,000 to 110,000 molecular weight (11, 14). The *E. coli* RNA polymerase is composed of five subunits, $\alpha_2\beta\beta'\sigma$, with a combined molecular weight of 470,000 (15). The phage-induced RNA polymerases show highly stringent template specificities *in vitro* for their homologous phage DNA, whereas the host RNA polymerase can utilize DNA from a variety of sources (11, 13, 14). A comparison of other properties of these two types of RNA polymerases has been presented recently (16).

We have examined the regulation of RNA synthesis after the infection of *Pseudomonas putida* by the bacteriophage gh-1. gh-1 is a small, virulent bacteriophage isolated in this laboratory (17). The nucleic acid component of phage gh-1 is a linear, double-stranded DNA having a molecular weight of $23 \times 10^6$ (18). In this paper, we report that gh-1 infection of *P. putida* induces the synthesis of a new DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase has been purified and its structure and catalytic properties studied. A preliminary report on some of this work has been presented previously (19).

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## EXPERIMENTAL PROCEDURE

*Materials*—Whatman DEAE-cellulose (DE52) and phosphocellulose (P-11) were purchased from Reeve Angel. Dithiothreitol, calf thymus DNA, yeast glucose-6-phosphate dehydrogenase, and unlabeled nucleoside triphosphates were obtained from P-L Biochemicals. $^{3}$H-labeled ribonucleoside triphosphates were from Schwarz-Mann and $\gamma$-$^{32}$P-labeled ATP and GTP were from New England Nuclear. Poly(dC)·poly(dG) and poly[d(A-T)] were purchased from Miles Laboratories. *E. coli* alkaline phosphatase, beef heart lactate dehydrogenase, bovine hemoglobin, rabbit muscle phosphorylase *a*, bovine serum albumin, and chloramphenicol were obtained from Sigma. Beef liver catalase was from Worthington Biochemicals. Blue dextran 2000 was purchased from Pharmacia Fine Chemicals and Bio-Gel P-200 from Bio-Rad Laboratories. T4 DNA and T7 DNA were the kind gifts of Dr. Loren Synder, Department of Microbiology and Public Health, Michigan State University. Rifamycin derivatives were the gifts of Dr. Luigi Silvestri, Gruppo Lepetit, Inc., Milan, Italy. $^{3}$H-labeled ribosomal RNA, 3′-deoxyadenosine, 3′-deoxyadenosine 5′-diphosphate, 3′-deoxyadenosine 5′-triphosphate, and 3′-*O*-methyladenosine 5′-triphosphate were the very generous gifts of Ron Desrosiers and Dr. Fritz Rottman of this department. All other materials were obtained from sources described previously (20, 21).

*Growth of gh-1-infected P. putida—P. putida* (ATCC 12633) was grown at 33° in a medium containing, in grams per liter: yeast extract, 5; tryptone, 5; glucose, 5; NaCl, 8; $Na_2HPO_4$, 6; and $KH_2PO_4$, 3. After cell growth had reached mid-logarithmic phase, gh-1 phage was added to a multiplicity of 5 plaque-forming units per cell. After 10 min of incubation, the culture was poured onto a half-volume of crushed ice (−20°) and was collected immediately by centrifugation at 0°. Infected cells were quick-frozen in an acetone-Dry Ice bath and stored at −20°. The yield of infected cells was approximately 4 g of wet weight per liter of medium.

*Purification of gh-1-induced RNA Polymerase*—All of the procedures were performed at 0–4°. Frozen gh-1-infected *P. putida* (90 g of wet weight) were ground in a mortar and pestle in 2 volumes of acid-washed glass beads until cell breakage occurred. The cell homogenate was extracted in 4 to 6 volumes of buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol (Buffer A; initial extract fraction). This fraction was centrifuged at 105,000 × *g* for 2 hours. The pellet, which contained 75 to 90% of the RNA polymerase activity, was extracted with 1 M $NH_4Cl$ in Buffer A and was centrifuged at 105,000 × *g* for 1½ hours. The supernatant solution was dialyzed for 12 hours against Buffer A minus $MgCl_2$ ($NH_4Cl$ wash fraction) and was applied to a DEAE-cellulose column (4 × 16 cm) equilibrated with the same buffer. The RNA polymerase activity was eluted with a linear gradient from 0 to 0.4 M KCl in Buffer A minus $MgCl_2$ (total volume of 1 liter). A single peak of RNA polymerase activity eluted at approximately 0.17 M KCl. The concentration of KCl in various fractions was determined by conductivity measurements. The fractions containing the majority of the RNA polymerase activity were pooled and dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.5, 5 mM 2-mercaptoethanol, and 15% (v/v) glycerol (Buffer B) for 12 hours (DEAE fraction). The dialyzed fraction was applied to a phosphocellulose column (2.5 × 12 cm) equilibrated with Buffer B. The column was eluted with a linear gradient from 0 to 0.6 M KCl in Buffer B (total volume of 400 ml). RNA polymerase activity appeared as a single peak at approximately 0.35 M KCl. The fractions with the majority of RNA polymerase activity were pooled and concentrated to a volume of approximately 2 ml using an Amicon Micro-Ultrafiltration System, model 8MC, with a PM-30 Diaflo membrane. This fraction (phosphocellulose fraction) was dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, and 7.5% (v/v) glycerol for 8 hours before being layered on the top of a column (1.5 × 85 cm) Bio-Gel P-200 (50 to 100-mesh). The Bio-Gel column was equilibrated and developed in a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, and 5% (v/v) glycerol. The fractions with RNA polymerase activity were pooled again, were concentrated by ultrafiltration, and were dialyzed against a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 50% (v/v) glycerol (Bio-Gel fraction). This fraction was stored at −20°.

For purposes of further purification, the Bio-Gel fraction was dialyzed against a buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol, 0.2 M KCl, and 5% (v/v) glycerol for 12 hours. Samples of 0.10 to 0.15 ml were layered onto the top of 4.8 ml of 10 to 30% glycerol gradients made in 20 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol, and 0.2 M KCl. Centrifugation was performed at 44,000 rpm in a Spinco SW 50.1 rotor at 2° for 13 hours. Fractions of 0.16 ml were collected and small aliquots were assayed for activity. Fractions with the majority of RNA polymerase activity were pooled and dialyzed against a buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 50% (v/v) glycerol (glycerol gradient fraction).

*Assay for RNA Polymerase Activity*—The assay of RNA polymerase activity measured the incorporation of CMP into a form insoluble in trichloroacetic acid. The standard reaction mixture contained, in a final volume of 0.125 ml: 40 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 10 mM $MgCl_2$, 400 μg per ml of bovine serum albumin, 0.4 mM each of ATP, [$^{3}$H]CTP, $GTP_\gamma$ and UTP, and 50 μg per ml of gh-1 DNA and RNA polymerase, as indicated. The specific activity of [$^{3}$H]CTP was $1 \times 10^{4}$ cpm per nmol. In experiments in which the apparent $K_m$ value of CTP was measured, [$^{3}$H]UTP was used as the labeled nucleoside triphosphate at the same specific activity. Reactions were initiated by the addition of enzyme and were incubated for 10 min at 30°. Termination of the reaction, filtration onto nitrocellulose membrane filters, and analysis of the filters for radioactivity were as described previously (20). One unit of enzyme activity was equal to the incorporation of 1 nmol of CMP in 1 hour. The specific enzyme activity was the number of units per mg of protein as determined by the method of Lowry *et al.* (22) using bovine serum albumin as the standard.

*Assay of Other Enzyme Activities—E. coli* alkaline phosphatase was assayed by following the rate of release of *p*-nitrophenol from *p*-nitrophenyl phosphate, as determined spectrophotometrically at 410 nm. The sedimentation coefficient of alkaline phosphatase was taken as 6.1 S (23) and the molecular Stokes radius as 29.3 Å (24). Glucose-6-phosphate dehydrogenase was assayed by following the reduction of $NAD^{+}$ in the presence of glucose 6-phosphate, as measured by the increase in absorbance at 340 nm. The diffusion coefficient of glucose-6-phosphate dehydrogenase was taken as $5.77 \times 10^{-7}$ cm$^{2}$ s$^{-1}$ (25). Lactate dehydrogenase was assayed by following the oxidation of NADH in the presence of pyruvate, as determined spectrophotometrically at 340 nm. The sedimentation coefficient of lactate dehydrogenase used was 7.4 S and the diffusion coefficient was taken as $5.05 \times 10^{-7}$ cm$^{2}$ s$^{-1}$ (26). The molecular Stokes radii of lactate dehydrogenase and glucose-6-phosphate dehydrogenase were determined from their respective diffusion coefficients as described by Siegel and Monty (27).

RNase activity was assayed by determining whether any change occurred in the sucrose density gradient sedimentation profile of $^{3}$H-labeled ribosomal RNA after incubation at 30° for 20 min with 6 μg per ml or 60 μg per ml of gh-1 polymerase (Bio-Gel fraction). RNase was assayed similarly using native $^{3}$H-labeled gh-1 DNA. RNase III activity was assayed by the procedure of Robertson *et al.* (28) using $^{3}$H-labeled poly[r(A,U)] as substrate.

*Preparation of Bacteriophages and DNA—P. putida* bacteriophage gh-1 was purified from cell lysates by two rounds of differential centrifugation, followed by DEAE-cellulose chromatography (17). *E. coli* bacteriophage T3 was purified from cell lysates by differential centrifugation, followed by banding in a preformed CsCl density gradient. All bacteriophage DNA preparations were purified by the method of Thomas and Abelson (29). *P. putida* DNA was prepared by the procedure of Thomas *et al.* (30). Commercially obtained calf thymus DNA was further purified by two SDS[1]-phenol extractions, followed by extensive dialysis.

*SDS-Polyacrylamide Gel Electrophoresis*—SDS-polyacrylamide gel electrophoresis was performed using a modification of the procedure of Shapiro *et al.* (31), as described by Johnson *et al.* (20). Samples of 2 to 15 μg of protein were layered on 5% (w/v) polyacrylamide gels (0.5 × 10 cm). Electrophoresis was performed for 6 to 8 hours at 4 volts per cm of gel length. Gels were stained for protein with 0.5% (w/v) Coomassie brilliant blue in 10% (w/v) trichloroacetic acid and 33% (v/v) methanol for 8 to

[1] The abbreviations used are: SDS, sodium dodecyl sulfate; 3′-dATP, 3′-deoxyadenosine 5′-triphosphate; 3′-AmTP, 3′-*O*-methyladenosine 5′-triphosphate.

12 hours. Destaining was performed on a diffusion destainer in 10% trichloroacetic acid and 33% methanol for 6 hours. Gels were removed from the diffusion destainer and were incubated in 10% trichloroacetic acid at 30° until the background was clear (approximately 4 hours). Gels were stored at 4° in 10% trichloroacetic acid.

*Other Methods*—Extracts of either gh-1-infected or uninfected *P. putida*, which were used to assay RNA polymerase activity directly, were made by suspending cells in 2 volumes of Buffer A. These suspensions were sonicated for 11½ min (in 30-s bursts) at a setting of 70 on a Biosonik sonicator and then were centrifuged at 16,000 × *g* for 20 min to remove cellular debris. RNA polymerase assays were performed with varying amounts of extract to ensure the enzyme activity was linearly proportional to the protein concentration.

The purification of *P. putida* RNA polymerase was performed by the method of Johnson *et al.* (20). The preparation of RNA polymerase used in these studies was more than 95% pure, as determined by SDS-polyacrylamide gel electrophoresis.

## RESULTS

*A Novel RNA Polymerase Activity in Bacteriophage gh-1-infected P. putida*—The first evidence that a novel RNA polymerase is synthesized after gh-1 infection of *P. putida* was obtained from measurements of the RNA polymerase activity in extracts of uninfected and gh-1-infected cells. In extracts from uninfected cells, RNA polymerase activity was inhibited 97% by the addition to the reaction mixture of the antibiotics, rifampicin and streptolydigin (Table I). This activity is largely, if not entirely, due to the *P. putida* RNA polymerase, which is known to be sensitive to these antibiotics (20). In extracts from gh-1-infected cells, the specific activity of RNA polymerase was 11 times greater than the specific activity in extracts from uninfected cells. Furthermore, this activity from infected cells was inhibited only 4% by the addition to the reaction mixture of the two bacterial RNA polymerase inhibitors. Addition to the reaction mixture of actinomycin D and nogalamycin, which inhibit RNA synthesis by binding to DNA, almost completely inhibited the activity from extracts of both uninfected and gh-1-infected cells (32, 33). These activities were, therefore, due to DNA-directed processes. In extracts from cells infected with gh-1 in the presence of chloramphenicol, the specific activity of RNA polymerase was essentially the same as that in uninfected cells. This activity also was sensitive to rifampicin and streptolydigin. Thus, protein synthesis was necessary for the appearance of the rifampicin- and streptolydigin-resistant RNA polymerase activity. Although other interpretations are possible, these results can be explained most readily by the synthesis of a novel DNA-dependent RNA polymerase after gh-1 infection of *P. putida*. This explanation was verified by the purification of the gh-1-induced RNA polymerase and by a study of its structure and catalytic properties.

### TABLE I

*Specific activity of RNA polymerase in extracts of uninfected and bacteriophage gh-1-infected Pseudomonas putida*

Components of the standard reaction mixture and preparation of cell extracts were as described under "Experimental Procedure." Rifampicin and streptolydigin, when added to the reaction mixture, were at concentrations of 5 μg per ml and 100 μg per ml, respectively. Actinomycin and nogalamycin, when present, were both at a concentration of 10 μg per ml. Reactions were initiated by the addition of extract to a final protein concentration between 50 and 400 μg per ml.

| Components of the reaction mixture | Specific activity of RNA polymerase | | |
|---|---|---|---|
| | Extract of uninfected *P. putida* | Extract of *P. putida* infected with gh-1 | Extract of *P. putida* infected with gh-1 in the presence of chloramphenicol[a] |
| | *units/mg* | | |
| Standard | 17 | 193 | 15 |
| Standard plus rifampicin and streptolydigin | 0.6 | 186 | 0.5 |
| Standard plus actinomycin and nogalamycin | 0.3 | 1.7 | 0.4 |

[a] Chloramphenicol was added to the growth medium to a final concentration of 100 μg per ml 1 min before the addition of gh-1 phage.

*Purification of the gh-1-induced RNA Polymerase*—The results of the purification of the gh-1-induced RNA polymerase, performed as described under "Experimental Procedure," are shown in Table II. The Bio-Gel fraction which was used for many of the catalytic studies reported below had a specific enzyme activity of 42,000 units per mg. This represents a 280-fold purification from the initial extract fraction. An accurate determination of the specific enzyme activity of the glycerol gradient fraction could not be made due to the difficulty of determining protein concentration at the relatively low level present in this fraction. An estimate of the protein concentration of the glycerol gradient fraction, however, could be made from the SDS-polyacrylamide gel electrophoresis of this fraction (Fig. 1*C*). By measuring the area under the peaks of the scan at 550 nm of the SDS-polyacrylamide gel and comparing with the area under the peaks of known amounts of the reference proteins, the amount of protein present in the gel could be determined. This determination is dependent on the demonstration that the amount of stain absorbed by the SDS-polyacrylamide gel is linearly related to the amount of protein present (20). From this estimate of protein concentration, a specific enzyme activity of 86,000 units per mg was calculated for the glycerol gradient fraction.

Analysis of the Bio-Gel fraction for RNase and DNase activities, contaminants of RNA polymerase preparations which can alter the observed RNA polymerase activity, were negative. The Bio-Gel fraction also did not contain any RNase III activity, the enzyme involved in the "sizing" of T7 early mRNA in *E. coli* (34, 35). The Bio-Gel fraction also is free of any host RNA polymerase activity. The slowest migrating polypeptides on SDS-polyacrylamide gel electrophoresis of the Bio-Gel fraction

### TABLE II

*Summary of purification*

Summary of purification of gh-1-induced RNA polymerase from 30 g (wet weight) of gh-1-infected *Pseudomonas putida* as described under "Experimental Procedure."

| Fraction | Total protein | Total enzyme activity | Recovery of enzyme activity | Specific activity |
|---|---|---|---|---|
| | *mg* | *$10^{-4}$ units* | % | *units/mg* |
| Initial extract fraction | 2,900 | 44 | 100 | 150 |
| $NH_4Cl$ wash fraction | 1,300 | 39 | 89 | 300 |
| DEAE fraction | 330 | 21 | 48 | 640 |
| Phosphocellulose fraction | 7.2 | 6.3 | 14 | 8,700 |
| Bio-Gel fraction | 0.62 | 2.6 | 6 | 42,000 |
| Glycerol gradient fraction | (0.23)[a] | 2.0 | 5 | (86,000)[a] |

[a] Based on the protein concentration determination made from SDS-polyacrylamide gel electrophoresis of the sample as described under "Results."

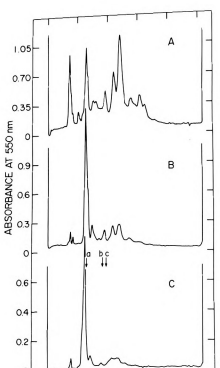


FIG. 1. SDS-polyacrylamide gel scans of fractions from the purification of gh-1-induced RNA polymerase. Samples of the phosphocellulose fraction (*A*, 12.7 μg), Bio-Gel fraction (*B*, 9 μg), and glycerol gradient fraction (*C*, approximately 5 μg) of gh-1 polymerase were subjected to SDS-polyacrylamide gel electrophoresis as described under "Experimental Procedure." Electrophoresis was performed at 4 volts per cm of gel length for 6.25 hours at 25°. After staining and destaining, the gels were scanned at 550 nm on a Gilford linear transport. The direction of migration was from left to right. The *arrows* indicate the peak positions of the reference proteins: phosphorylase *a* (*a*), bovine serum albumin (*b*), and catalase (*c*).

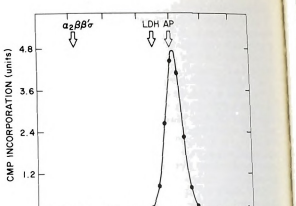


FIG. 2. Glycerol gradient centrifugation of gh-1-induced RNA polymerase. gh-1 polymerase (phosphocellulose fraction, 18 μg) was mixed with 150 μg of bovine serum albumin and diluted to 0.15 ml in a buffer containing 20 mM Tris-HCl, pH 8.0, 0.5 mM dithiothreitol, and 0.2 M KCl. After dialysis for 6 hours against the same buffer, a 0.1-ml sample was layered on a 4.8-ml 10 to 30% linear glycerol gradient prepared in the above buffer and containing 0.5 mg per ml of bovine serum albumin. On three parallel gradients, 0.1-ml samples of the reference proteins (*Pseudomonas putida* RNA polymerase holoenzyme (80 μg), *Escherichia coli* alkaline phosphatase (70 μg), and beef heart lactate dehydrogenase (7 μg)) were layered. All gradients were centrifuged for 15 hours at 4° in a Spinco SW 50.1 rotor at 45,000 rpm. After centrifugation, 32 fractions of 0.16 ml were collected from each gradient and enzyme assays were performed on the fractions as described under "Experimental Procedure." The *arrows* indicate the peak positions of the reference proteins, *P. putida* RNA polymerase ($\alpha_2\beta\beta'\sigma$), alkaline phosphatase (*AP*), and lactate dehydrogenase (*LDH*). The recovery of gh-1 polymerase activity was approximately 90%.

migrated significantly ahead of the β and β′ subunits of purified *P. putida* RNA polymerase. The phosphocellulose and Bio-Gel fractions could be stored at −20° in buffer containing 50% glycerol for several months with little loss of activity, if the protein concentration was equal to or greater than 0.5 mg per ml.

Analysis of the phosphocellulose fraction, the Bio-Gel fraction, and the glycerol gradient fraction was performed by SDS-polyacrylamide gel electrophoresis (Fig. 1). The glycerol gradient fraction contained one major polypeptide which comprised approximately 80%, by weight, of the total protein present (Fig. 1*C*). No other polypeptide present comprised more than 8% of the total protein. The molecular weight of the major polypeptide was determined by comparison of its mobility to the mobility of the reference proteins, phosphorylase *a* (subunit molecular weight of 94,000), catalase (68,000), and bovine serum albumin (60,000). Using a standard curve of the logarithms of the molecular weights of the reference proteins to the distances of migration, a molecular weight of 97,000 was estimated for the major polypeptide of the glycerol gradient fraction. This major polypeptide is thought to be the only polypeptide comprising the gh-1 polymerase. It is the only polypeptide which increased in relative purity in the last two steps of the purification procedure. Its increase in purity parallels the increase in specific enzyme activity of gh-1 polymerase in these last two steps. Finally, the molecular weight of the gh-1 polymerase, as determined by SDS-polyacrylamide gel electrophoresis, is consistent with a determination of 98,000 made by glycerol gradient centrifugation and gel filtration (see below). The gh-1 polymerase polypeptide was 10 to 15%, by weight, of the total protein in the phosphocellulose fraction (Fig. 1*A*) and 50 to 55% of the Bio-Gel fraction (Fig. 1*B*).

*Molecular Weight and Structure of the gh-1-induced RNA Polymerase*—The molecular weight of the gh-1 polymerase was calculated using experimentally obtained values for its sedimentation coefficient and molecular Stokes radius. A molecular weight value calculated in this manner is not dependent on assumptions concerning the shape of the macromolecule (27).

The sedimentation coefficient of gh-1 polymerase was determined by sedimentation velocity centrifugation in a 10 to 30% glycerol gradient (Fig. 2). The reference proteins (alkaline phosphatase, lactate dehydrogenase, and *P. putida* RNA polymerase) were centrifuged under identical conditions. Based on the sedimentation coefficients of the reference proteins, the gh-1 polymerase exhibited a sedimentation coefficient of 6.1 ± 0.2 S.

The molecular Stokes radius of the gh-1-induced RNA polymerase was obtained by gel filtration on a Bio-Gel P-200 column (Fig. 3). The reference proteins (alkaline phosphatase, lactate dehydrogenase, glucose-6-phosphate dehydrogenase, and hemoglobin) were chromatographed under identical conditions to standardize the column. Using the relationship, derived by Ackers (36), between molecular radius and distribution coefficient, the molecular radius of gh-1 polymerase was calculated to be 38 A. By combining the molecular radius determined by gel filtration and a sedimentation coefficient from sedimentation

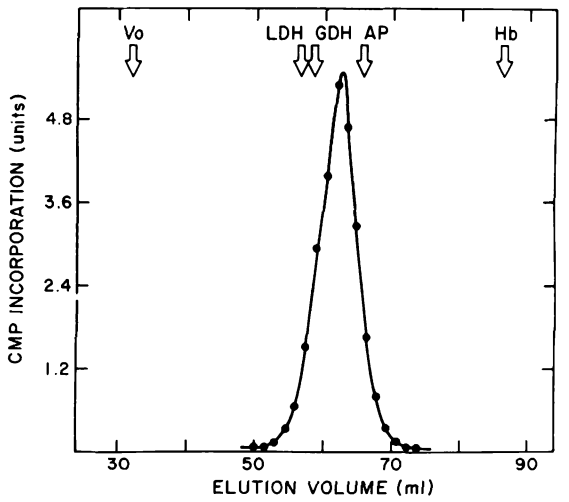


FIG. 3. Gel filtration of gh-1-induced RNA polymerase on Bio-Gel P-200. gh-1 polymerase (phosphocellulose fraction, 60 $\mu$g) was diluted to 1 ml in a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, and 7.5% (v/v) glycerol and was dialyzed against the same buffer for 6 hours. The 1-ml sample was layered on the top of a Bio-Gel P-200 (100- to 200-mesh) column (1.5 × 77 cm) which had been equilibrated previously with a buffer containing 20 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.2 M KCl, 5% (v/v) glycerol, and 0.5 mg per ml of bovine serum albumin at 4°. The column was developed in the same buffer at a flow rate of 2.1 ml per hour and fractions of 1.5 ml were collected. To standardize the column, two samples containing markers were chromatographed under exactly the same conditions in subsequent runs. One sample contained the markers 0.2% (w/v) blue dextran 2000, *Escherichia coli* alkaline phosphatase (1 mg), and beef heart lactate dehydrogenase (100 $\mu$g). The markers in the second sample were 0.2% (w/v) blue dextran 2000, yeast glucose-6-phosphate dehydrogenase (500 $\mu$g), and bovine hemoglobin (1.5 mg). Fractions from the column were analyzed for various enzyme activities as described under "Experimental Procedure." Blue dextran 2000 and hemoglobin were assayed spectrophotometrically at 650 nm and 410 nm, respectively. The peak position of the markers are shown by the *arrows*: blue dextran 2000, $V_0$; lactate dehydrogenase, *LDH;* glucose-6-phosphate dehydrogenase, *GDH*; alkaline phosphatase, *AP*; and hemoglobin, *Hb*.

velocity centrifugation and assuming a $\bar{v}$ of 0.73 ml per g, a molecular weight estimate of 98,000 can be calculated for the gh-1 polymerase. This value for the molecular weight is in good agreement with the value of 97,000 obtained using SDS-polyacrylamide gel electrophoresis. Together these results indicate that the gh-1-induced RNA polymerase is composed of a single polypeptide with a molecular weight of 95,000 to 100,000.

*Characterization of RNA Synthesis by gh-1-induced RNA Polymerase Using gh-1 DNA as Template*—The general requirements for *in vitro* RNA synthesis by the purified gh-1 polymerase were examined by varying the components of the standard reaction mixture (Table III). When the enzyme, the gh-1 DNA, one of the four ribonucleoside triphosphates, or the $Mg^{2+}$ was removed from the reaction mixture, little or no RNA synthesis occurred. Near maximal enzyme activity was maintained over a broad concentration range of 5 to 20 mM $Mg^{2+}$ with the optimal activity occurring at approximately 10 mM (data not shown). No detectable RNA synthesis occurred when the $Mg^{2+}$ was replaced in the standard reaction mixture by the divalent metal ions ($Mn^{2+}$, $Zn^{2+}$, or $Ca^{2+}$) at concentrations between 0.5 and 8 mM (Table III). In fact, the addition of any of these divalent metal ions at 2 mM to the reaction mixture containing $Mg^{2+}$ inhibited the enzyme activity 93 to 100%. The activity of the gh-1 polymerase was also inhibited quite markedly by relatively low

TABLE III

*Characteristics of RNA synthesis by gh-1-induced RNA polymerase*

The components of the standard reaction mixture were as described under "Experimental Procedure." Where indicated, the appropriate component was removed from or added to the standard reaction mixture. Reactions were initiated by the addition of 2.4 $\mu$g per ml of gh-1 polymerase (Bio-Gel fraction).

| Components of the reaction mixture | CMP incorporated |
|---|---|
| | *nmol/hr* |
| Standard | 9.30 |
| Minus enzyme | 0 |
| Minus gh-1 DNA | 0 |
| Minus ATP, GTP, or UTP | 0–0.04 |
| Minus $MgCl_2$ | 0 |
| Minus $MgCl_2$; Plus $MnCl_2$, $CaCl_2$, or $ZnCl_2$ (0.5–8 mM) | 0 |
| Plus 2 mM $MnCl_2$ or $CaCl_2$ | 0.84 |
| Plus 2 mM $ZnCl_2$ | 0 |
| Plus 85 mM KCl | 4.45 |
| Plus 200 mM KCl | 0.18 |

concentrations of monovalent ions. At a concentration of 85 mM KCl, the gh-1 polymerase activity was inhibited 50%, whereas at 200 mM, the reaction was essentially completely inhibited. An almost identical inhibition of enzyme activity was observed with either NaCl or $NH_4Cl$ (data not shown).

Apparent $K_m$ values for each of the four ribonucleoside triphosphates which are substrates for RNA synthesis were determined. For these studies, the concentration of three of the ribonucleoside triphosphates was fixed at a high level, greater than 5 times the $K_m$ value for any ribonucleoside triphosphate. The concentration of the fourth ribonucleoside triphosphate was varied and the initial reaction rates were measured at each concentration. To analyze the results, Michaelis-Menten kinetics was assumed applicable to this complex reaction, and the results were plotted in Lineweaver-Burk double reciprocal plots ($1/v$ *versus* $1/[S]$). All data were analyzed by a computer program to determine the highest correlation to a least squares straight line for the equation:

$$v = V_{max} - K_m{}^n(v/[NTP]^n)$$

as $n$ was varied in increments of 0.05 unit (37). An $n$ value so determined is equivalent to the Hill coefficient, $n$, and should equal 1.0 if the double reciprocal plot is linear.

For the purine ribonucleoside triphosphate ATP, the double reciprocal plot was linear (Fig. 4*A*). The apparent $K_m$ value for ATP was $3.5 \times 10^{-5}$ M. Likewise, the pyrimidine ribonucleoside triphosphates, CTP and UTP, yielded linear double reciprocal plots (data not shown). The apparent $K_m$ value for both of these substrates in the RNA polymerase reaction was $4.0 \times 10^{-5}$ M. For the purine ribonucleoside triphosphate GTP, however, the double reciprocal plot was curvilinear (Fig. 4*A*). An $n$ value of 1.2 for GTP was determined by the computer analysis. Thus, the best fit to a straight line was obtained when $1/v$ was plotted *versus* $1/[GTP]^{1.2}$ (Fig. 4*B*). The kinetics of RNA synthesis at the lowest GTP concentration used in the $K_m$ study was linear for at least 5 min and showed no appreciable lag in initiation (data not shown). Thus, the higher order $n$ value is not due to nonlinear reaction rates at the lower substrate concentrations. The apparent $K_m$ value for GTP, using the higher order value of substrate concentration in the Michaelis-Menten equation, was $8.0 \times 10^{-5}$ M or twice that seen for the other three ribonucleoside triphosphates.

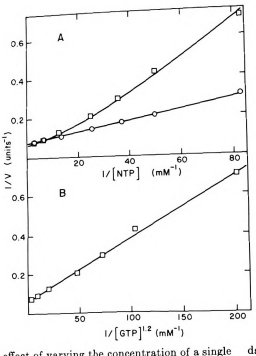


FIG. 4 (*left*). The effect of varying the concentration of a single nucleoside triphosphate on the activity of gh-1-induced RNA polymerase. Reaction mixtures for gh-1 polymerase were prepared as described under "Experimental Procedure," except that the concentration of one nucleoside triphosphate was varied whereas the concentrations of the other three nucleoside triphosphates were kept constant at 0.4 mM. The reaction mixtures were prewarmed to 30° and RNA synthesis initiated by the addition of gh-1 polymerase (Bio-Gel fraction) to a final concentration of 2.4 $\mu$g per ml. After 5 min of incubation, the reactions were terminated and the incorporation of [$^3$H]CTP into acid-insoluble material was determined as described under "Experimental Procedure." *A*, double reciprocal plot of 1/*v* *versus* 1/concentration of nucleoside triphosphate for ATP (○) and GTP (□). *B*, the data of *A* for GTP replotted as 1/*v* *versus* 1/concentration of GTP raised to the 1.2 power.

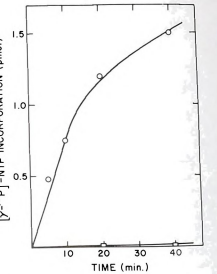


FIG. 5 (*right*). The kinetics of incorporation of [$\gamma$-$^{32}$P]GTP and [$\gamma$-$^{32}$P]ATP into RNA by the gh-1-induced RNA polymerase. Reaction mixtures for gh-1 polymerase were prepared as described under "Experimental Procedure," except that the final concentrations of ATP and GTP were lowered to 0.2 mM. Either the ATP (□) or GTP (○) was labeled with $\gamma$-$^{32}$P to a final specific activity of 2100 to 2500 cpm per pmol. In all of the assays in which [$\gamma$-$^{32}$P]-ATP was the labeled substrate, 0.1 mM ADP was included in the reaction mixture to inhibit any trace amounts of polyphosphate kinase which might be present (38). Reactions were initiated by the addition of 0.3 $\mu$g of gh-1 polymerase (Bio-Gel fraction) and were incubated at 30° for the times indicated. The reactions were terminated and processed as described by Maitra *et al.* (39).

The initiation process of RNA synthesis by gh-1-induced RNA polymerase with gh-1 DNA as template was measured using $\gamma$-$^{32}$P-labeled purine ribonucleoside triphosphates. As shown in Fig. 5, gh-1 polymerase incorporated [$\gamma$-$^{32}$P]GTP into acid-insoluble material and this incorporation continued during the entire period of incubation. On the other hand, [$\gamma$-$^{32}$P]ATP was not incorporated significantly under the same conditions. The incorporation of [$\gamma$-$^{32}$P]GTP into acid-insoluble material did not occur in the absence of either enzyme or gh-1 DNA. The $^{32}$P-labeled product, isolated from the reaction mixture after 20 min of incorporation, was rendered completely acid-soluble by treatment with either pancreatic RNase (1 $\mu$g per ml at 37° for ½ hour) or alkali (1 N NaOH at 37° for 6 hours). Thus, the [$\gamma$-$^{32}$P]GTP is being incorporated into RNA by the gh-1 polymerase in a DNA-dependent process. For the purine ribonucleoside triphosphates, gh-1 polymerase initiates RNA synthesis on gh-1 DNA exclusively with GTP.

Several antibiotics and antibiotic derivatives, which are inhibitors of host RNA polymerase activity, were added to the standard reaction mixture for gh-1 polymerase to test their effect on *in vitro* RNA synthesis catalyzed by the phage enzyme (Table IV). The antibiotics, actinomycin D and nogalamycin, inhibit RNA synthesis by intercalating into the DNA structure at G-C-rich and A-T-rich regions, respectively (32, 33). These two antibiotics are effective inhibitors of the host and phage polymerases, as expected, because both catalyze DNA-dependent proc-

TABLE IV

*Effect of various RNA synthesis inhibitors on Pseudomonas putida and gh-1-induced RNA polymerase activities*

The components of the standard reaction mixture were as described under "Experimental Procedure." All inhibitors except streptolydigin were added from stock solutions in 10% (v/v) dimethyl sulfoxide. The final concentrations of dimethyl sulfoxide in the reaction mixtures (0.4 to 0.8%) did not alter the over-all incorporation of [$^3$H]CTP by either enzyme. Reactions were initiated by the addition of 2.4 $\mu$g per ml of gh-1 polymerase (Bio-Gel fraction) or 12.8 $\mu$g per ml of *P. putida* RNA polymerase. In the standard reaction mixture with no additions, 1.85 nmol of CMP were incorporated by the gh-1 polymerase in 10 min and 0.95 nmol by the *P. putida* RNA polymerase.

| Addition to the standard reaction mixture | Concentration | Relative activity | |
|---|---|---|---|
| | | gh-1 polymerase | *P. putida* polymerase |
| | $\mu$g/ml | % | |
| None | | 100 | 100 |
| Actinomycin D | 4 | 10 | 7 |
| Nogalamycin | 4 | 5 | 13 |
| Streptolydigin | 100 | 96 | 5 |
| Rifampicin | 10 | 95 | 0.5 |
| Rifamycin AF/013 or AF/DNFI | 35 | 50 | 0.5 |

esses. The activity of the gh-1 polymerase is highly resistant to the antibiotics, rifampicin and streptolydigin, present at concentrations which markedly inhibit the host RNA polymerase. The effect of 13 other derivatives of rifamycin on gh-1 polymerase activity was tested. The derivatives examined, using the nomenclature of Gruppo Lepetit, were: AF/AOP, Rifamycin AG, AF/APR, AF/DEI, AF/DA-AMP, Rifamide, 4-Dessosi SV, PR/14, AF/013, AF/ABDP-cis, AF/AP, AF/BO, AF/DNFI, and PR/19 (for review of structures see Ref. 40). All 13 of these derivatives were effective inhibitors (>95%) of RNA synthesis by the host RNA polymerase when present at a concentration of 10 μg per ml. When added to the gh-1 polymerase reaction mixture at 100 μg per ml, seven of the derivatives (AF/013, AF/DNFI, AF/BO, AF/AOP, AF/ABDP, PR/19, and AF/DEI) were found to inhibit polymerase activity to a significant degree (>20%) (data not shown). The most effective inhibitors were AF/013 and AF/DNFI, which inhibited RNA synthesis by 50% at concentrations of 35 μg per ml and almost completely at concentrations of 80 μg per ml or more. The relative order of effectiveness of the rifamycin derivatives in inhibiting gh-1 polymerase activity was virtually the same as that observed for T7-induced RNA polymerase (41). Inhibitors of the phage polymerase activities, however, were far more effective. Even those rifamycin derivatives which were more effective against the activity of the host RNA polymerase.

3′-Deoxyadenosine 5′-triphosphate, the triphosphate derivative of the antibiotic cordycepin, has been shown to be an *in vitro* inhibitor of RNA synthesis by certain bacterial RNA polymerases (42, 43). This ATP analog presumably inhibits RNA synthesis by being enzymatically incorporated into an RNA chain at a position normally occupied by an AMP residue. If incorporated, the 3′-dAMP would act as a chain terminator in RNA synthesis, because it does not contain a 3′-hydroxyl group necessary for the formation of the next phosphodiester bond. As shown in Fig. 6, 3′-dATP, when added to the standard reaction mixture, inhibited RNA synthesis by both the gh-1-induced and *P. putida* RNA polymerases. It was a much more effective inhibitor, however, of the gh-1 polymerase. The 3′-dATP concentration required to produce a given level of inhibition with the host RNA polymerase was approximately 80 times greater than that required to inhibit the gh-1 polymerase to the same extent. Thus, at the concentration of ATP present in the standard reaction mixture, 0.4 mM, 50% inhibition of the host polymerase occurred at an ATP:3′-dATP molar ratio of 20, whereas the same degree of inhibition of the phage enzyme occurred at an ATP:3′-dATP molar ratio of 1600. By selecting the appropriate concentration of 3′-dATP, the gh-1 polymerase activity can be essentially completely inhibited, whereas the host polymerase activity is almost completely unaffected. Neither the nucleoside, 3′-deoxyadenosine (cordycepin), nor the diphosphate derivative, 3′-deoxyadenosine 5′-diphosphate, had any effect on either enzyme activity at concentrations up to 1 mM (data not shown).

Double reciprocal plots of $1/v$ *versus* 1/[ATP] in the absence and presence of 3′-dATP were experimentally determined to study further this interesting inhibitory effect (Fig. 7). Within experimental error, 3′-dATP acted as a competitive inhibitor of ATP for both enzymes. The apparent $K_m$ values for ATP for both enzymes were similar: $6 \times 10^{-5}$ M for the host enzyme

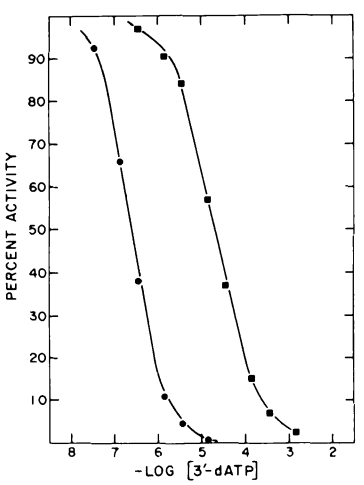


FIG. 6 (*left*). The effect of 3′-deoxyadenosine 5′-triphosphate on *in vitro* RNA synthesis by *Pseudomonas putida* and gh-1-induced RNA polymerases. Standard reaction mixtures were prepared as described under "Experimental Procedure," except that 3′-dATP was added to some reactions as indicated. Reactions were initiated by the addition of either 0.3 μg of gh-1 polymerase (Bio-Gel fraction) (●) or 1.6 μg of *P. putida* RNA polymerase (■). After 10 min of incubation, the reactions were terminated and the incorporation of [$^3$H]CTP into acid-insoluble material determined as described under "Experimental Procedure." Incorporation in reactions containing various concentrations of 3′-dATP were compared to control reactions containing no 3′-dATP. For gh-1 polymerase, 100% activity (no 3′-dATP) was equal to 1.95 nmol of CMP incorporated in 10 min and for *P. putida* RNA polymerase, 0.95 nmol.

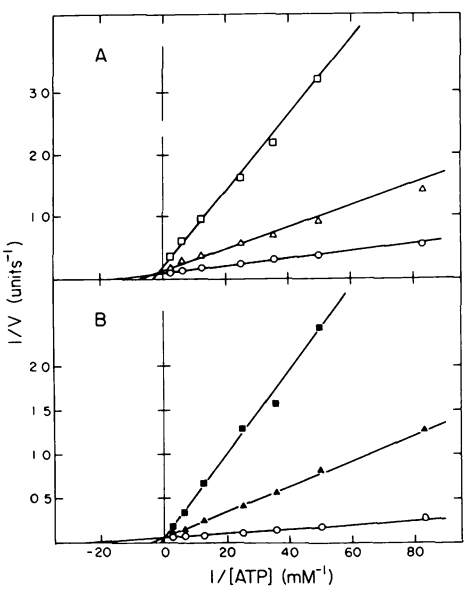


FIG. 7 (*right*). The effect of varying the concentration of ATP in the absence and presence of 3′-deoxyadenosine 5′-triphosphate on *in vitro* RNA synthesis by gh-1-induced and *Pseudomonas putida* RNA polymerases. Reaction mixtures were prepared as described in the legend to Fig. 4, except that some reaction mixtures included 3′-dATP at the concentrations listed below. Reactions were initiated by the addition of either 1.6 μg of *P. putida* RNA polymerase (*A*) or 0.3 μg of gh-1 polymerase (*B*). After 10 min of incubation, the reactions were terminated and the incorporation of [$^3$H]CTP into acid-insoluble material was determined as described under "Experimental Procedure." Final concentrations of 3′-dATP in the reaction mixtures were: 0, (○); 0.12 μM, (▲); 0.4 μM, (■); 8 μM, (△); or 40 μM, (□).

and $3.5 \times 10^{-5}$ M for the gh-1 polymerase. The apparent $K_i$ values for 3′-dATP were, however, quite different: $2 \times 10^{-6}$ M for the host enzyme and $2 \times 10^{-8}$ M for the phage enzyme. Thus, the difference in sensitivity of the two enzymes toward 3′-dATP, as seen in Fig. 7, was reflected in the relative difference of the apparent $K_i$ values. These results indicate that 3′-dATP inhibited the polymerase by competing for a common binding site with ATP. This conclusion was substantiated by the finding that the poly(dC)·poly(dG)-primed polymerization of GTP by the gh-1 polymerase (see below) was not affected by the presence of 3′-dATP at levels which completely inhibit the gh-1 DNA-primed reaction (data not shown).

Another structural analog of ATP is 3′-*O*-methyladenosine 5′-triphosphate. 3′-AmTP is similar to 3′-dATP in that it differs from ATP only at the 3′ position of the ribose moiety. 3′-AmTP was an inhibitor of RNA synthesis by both the *P. putida* and the gh-1-induced RNA polymerases (data not shown). The large differential inhibitory effect seen for these two RNA polymerases with 3′-dATP was not observed for 3′-AmTP. The apparent $K_i$ value for 3′-AmTP calculated for the *P. putida* RNA polymerase was $4.1 \times 10^{-5}$ M, or approximately 20 times higher than the apparent $K_i$ value for 3′-dATP. For the gh-1 polymerase, the apparent $K_i$ value of 3′-AmTP was $1.3 \times 10^{-4}$ M; over 3 orders of magnitude greater than that of 3′-dATP. Thus, the 3′-*O*-methylated analog of ATP is not as efficient as an inhibitor of *in vitro* RNA synthesis as the 3′-H analog for these RNA polymerases.

*Template Specificity of gh-1-induced RNA Polymerase*—One of the most striking characteristics of the gh-1-induced RNA polymerase-catalyzed reaction is the stringent template specificity. When DNA from many sources was tested, only the homologous phage gh-1 DNA was found to be an efficient template for *in vitro* RNA synthesis (Table V). The gh-1 polymerase would not utilize DNA from coliphages T3, T4, or T7, nor would it utilize calf thymus or *P. putida* DNA. When the gh-1 DNA was denatured, it became an inefficient template for the gh-1 polymerase. Thus, some feature inherent in the double-stranded structure of the gh-1 phage DNA is necessary for its function as an efficient template. Likewise, denatured DNA from either coliphage T3 or calf thymus supported little or no RNA synthesis. By contrast, the host RNA polymerase can utilize all of the above templates, although at varying efficiencies.

Several synthetic polydeoxyribonucleotides were tested as templates for RNA synthesis by the gh-1 polymerase (Table VI). The alternating copolymer, poly[d(A-T)], which was an efficient template for the host RNA polymerase, was not utilized effectively by the gh-1 polymerase. The gh-1 polymerase utilized the homopolymer duplex, poly(dC)·poly(dG), to direct the polymerization of GTP at a rate 7 times higher than the polymerization of CTP from this template. Several single-stranded polydeoxyribonucleotide homopolymers also were tested as templates for gh-1 polymerase. Either of the pyrimidine-containing polymers, poly(dT) or poly(dC), would support the synthesis of the corresponding ribohomopolymers. Little or no template activity, however, could be detected with the purine-containing homopolymers, poly(dA) or poly(dI). Thus, with either single-stranded or double-stranded deoxyribonucleotide homopolymers, the gh-1 polymerase markedly prefers to utilize the pyrimidine-containing templates as compared to the purine-containing ones. It should be noted that the highest enzyme activity on any template other than native gh-1 DNA, namely that for poly(dT), was less than 5% of the enzyme activity on native gh-1 DNA, in terms of total nanomoles of nucleotide incorporated per hour per mg of protein.

TABLE V

*Template specificity of gh-1-induced and Pseudomonas putida RNA polymerase toward DNA from various sources*

RNA polymerase reactions were prepared and run as described under "Experimental Procedure," except that the gh-1 DNA was replaced by DNA from various sources as indicated. The final concentration of DNA in all cases was 50 μg per ml. The assays contained 4.3 μg per ml of gh-1 polymerase (Bio-Gel fraction) or 12.8 μg per ml of *P. putida* RNA polymerase. With gh-1 DNA as template, the gh-1 polymerase incorporated 3.3 nmol of CMP in 10 min, and the *P. putida* RNA polymerase incorporated 1.4 nmol. Where indicated, DNA solutions were denatured immediately before use by heating for 10 min at 100°, followed by rapid chilling at 0°.

| DNA template | Relative activity | |
|---|---|---|
| | gh-1 polymerase | *P. putida* polymerase |
| | % | |
| gh-1 | 100 | 100 |
| T3 | 0.8 | 99 |
| T7 | <0.5 | 137 |
| T4 | <0.5 | 28 |
| Calf thymus | 0.6 | 61 |
| *P. putida* | <0.5 | 29 |
| Denatured gh-1 | 1.8 | 33 |
| Denatured T3 | 1.2 | 22 |
| Denatured calf thymus | <0.5 | 25 |

## DISCUSSION

The infection of *P. putida* by the bacteriophage gh-1 induces the synthesis of a novel DNA-dependent RNA polymerase. This gh-1-induced RNA polymerase has been purified to near homogeneity. It is composed of a single polypeptide chain with a molecular weight of approximately 98,000. The structure of

TABLE VI

*Template specificity of gh-1-induced and Pseudomonas putida RNA polymerase toward synthetic polydeoxyribonucleotides*

RNA polymerase reactions were prepared and run as described under "Experimental Procedure," except that the template and nucleoside triphosphates were changed as indicated. Each nucleoside triphosphate was present at a final concentration of 0.4 mM. The following concentrations of template were employed: poly[d(A-T)], 3 $A_{260}$ units per ml; Poly(dC)·poly(dG), 2.5 $A_{260}$ units per ml; poly(dA), poly(dC), poly(dI), and poly(dT), 50 μM (expressed in terms of nucleotide phosphate). The assays contained either 4.3 μg per ml of gh-1 polymerase (Bio-Gel fraction) or 12.8 μg per ml of *P. putida* RNA polymerase.

| Template | Nucleoside triphosphate substrates | gh-1 polymerase | *P. putida* polymerase |
|---|---|---|---|
| | | *nmol [³H]NMP incorporated/hour* | |
| gh-1 | [³H]CTP, ATP, GTP, UTP | 19.8 | 8.72 |
| Poly[d(A-T)] | [³H]ATP, UTP | 0.22 | 21.3 |
| Poly(dC)·poly(dG) | [³H]CTP, GTP | 0.23 | 0.83 |
| Poly(dC)·poly(dG) | [³H]GTP, CTP | 1.66 | 7.57 |
| Poly(dA) | [³H]UTP | <0.01 | 2.30 |
| Poly(dC) | [³H]GTP | 1.87 | 1.58 |
| Poly(dI) | [³H]CTP | 0.54 | 1.65 |
| Poly(dT) | [³H]ATP | 3.39 | 11.5 |

the gh-1 polymerase is, therefore, relatively simple compared to the structure of the host *P. putida* RNA polymerase, which is composed of five subunits, $\alpha_2\beta\beta'\sigma$, with a combined molecular weight of approximately 506,000 (20).

Although the gh-1-induced and host *P. putida* RNA polymerases both catalyze the template-directed incorporation of ribonucleoside triphosphates into RNA, they differ in their response to several factors affecting RNA synthesis. Whereas the host polymerase can utilize either $Mg^{2+}$ or $Mn^{2+}$ to satisfy the divalent metal ion requirement, the phage polymerase can only utilize $Mg^{2+}$. Low concentrations of monovalent ions, which do not appreciably affect the activity of the host polymerase, inhibit the gh-1 polymerase markedly. The antibiotics, rifampicin and streptolydigin, inhibit the activity of the host enzyme at concentrations which do not affect the activity of the phage enzyme. The host RNA polymerase will utilize as an *in vitro* template every DNA with which it has been tested. The ability of the host polymerase to utilize a wide range of templates may be due to the diversity of sites it must recognize to perform its role in the transcription of the bacterial chromosome. On the other hand, the gh-1 polymerase is highly specific in its template requirement for DNA from the homologous gh-1 phage.

The infection of *E. coli* by the coliphages T3 or T7 has been shown to induce the synthesis of viral specific RNA polymerases (11, 13, 14). These coliphage-induced RNA polymerases are similar in structure to the gh-1 polymerase; both are single polypeptides of 108,000 to 110,000 molecular weight (11, 14). The induction of a novel RNA polymerase activity also has been demonstrated after infection of *E. coli* by the helper-dependent bacteriophage P4 (44). The P4-induced RNA polymerase could synthesize polyriboguanylic acid from the duplex homopolymer, poly(dC)·poly(dG); however, no naturally occurring DNA has been found yet to serve as an *in vitro* template for this enzyme. Its actual function, therefore, is still a matter of conjecture. These three phage-induced RNA polymerases of *E. coli* are the only bacteriophage-specific RNA polymerases which have been described previously.

A comparison of the catalytic properties of the gh-1-induced RNA polymerase with those of the T3 and T7 RNA polymerases shows that these three phage polymerases are quite similar (11, 13, 14). All three phage polymerases cannot utilize $Mn^{2+}$ as divalent metal ion in place of $Mg^{2+}$. The activities of the phage polymerases were highly resistant to inhibition by rifampicin and streptolydigin but could be inhibited by the rifamycin derivative AF/013 at concentrations higher than 10 $\mu$g per ml (40, 45). Low concentrations of monovalent ions inhibited the activities of the three phage enzymes. Finally, all three phage-induced RNA polymerases showed highly stringent specificities for DNA from the homologous bacteriophage as *in vitro* templates.

The stringent template specificities of the gh-1, T3, and T7 RNA polymerases are quite interesting. All three polymerases can utilize pyrimidine-containing homopolymers, either single-stranded or as part of duplex pairs, as templates, but are far less efficient with the purine-containing homopolymers (13, 46). The ability of the pyrimidine-containing polymers to serve as efficient templates may result from the preferential initiation by these enzymes with purine ribonucleoside triphosphates (47). T7 polymerase can utilize T3 DNA approximately 50% as efficiently as T7 DNA, whereas T3 polymerase is approximately 10% as active on T7 DNA as its homologous T3 DNA (13, 14, 46). The gh-1 polymerase, however, will not utilize either T3 or T7 DNA as templates to any detectable degree. Thus, the exact nucleotide sequences of DNA necessary for either binding or initiation of RNA synthesis must be different between the coliphage-induced and the gh-1-induced RNA polymerases. The coliphage-induced RNA polymerases can utilize denatured or single-stranded DNA from many sources as templates for RNA synthesis at rates from 4 to 35% of the rates on native homologous phage DNA (46, 48). With the gh-1 polymerase, very little RNA synthesis is detected when any denatured templates are used.

The gh-1-induced RNA polymerase can initiate RNA synthesis on gh-1 DNA with the ribonucleoside triphosphate, GTP. This nucleotide has an apparent $K_m$ value approximately twice as high as the other three ribonucleoside triphosphates. The Hill coefficient of GTP is 1.2, as opposed to 1.0 for ATP, CTP, and UTP. The higher apparent $K_m$ value for GTP and its curvilinear double reciprocal plot may result from the role of GTP in the initiation process.

The process of RNA synthesis by bacterial and phage-induced RNA polymerases has been postulated to involve two binding sites for ribonucleoside triphosphates: an initiation site, which binds the 5′-terminal ribonucleoside triphosphate during the initiation process and the 3′-terminal nucleotide of the growing RNA chain during elongation, and an elongation site, which binds the ribonucleoside triphosphate which is to be incorporated into the 3′ terminus of the growing RNA chain (49, 50). These two sites may have very different $K_m$ values. The apparent $K_m$ value of any ribonucleoside triphosphate involved only in the elongation process will be the $K_m$ value of the elongation binding site. For gh-1 polymerase, this value is apparently 35 to 40 $\mu$M for the ribonucleoside triphosphates. The over-all apparent $K_m$ value for any ribonucleoside triphosphate involved in both initiation and elongation will depend on the relative $K_m$ values for the two individual binding processes. If the relative $K_m$ value of one of the two binding sites is substantially higher than that of the other site, the over-all apparent $K_m$ value for that ribonucleoside triphosphate will reflect primarily the higher $K_m$ binding site. This is evidently the case for *E. coli* and T3 RNA polymerases, for which the apparent $K_m$ values of the ribonucleoside triphosphates involved in initiation are 10 times and 5 times higher, respectively, than the apparent $K_m$ values of nucleoside triphosphates involved only in elongation (49, 50). If the $K_m$ values for the initiation and elongation binding processes are similar for any ribonucleoside triphosphate, the over-all apparent $K_m$ value should contain contributions from both binding processes. This may be the case with gh-1 polymerase for GTP, where the apparent $K_m$ value of the initiating ribonucleoside triphosphate GTP is only twice as high as that seen for the nucleoside triphosphates involved only in elongation. Therefore, varying the concentration of GTP may affect both the initiation and the elongation binding processes simultaneously, causing a curvilinear double reciprocal plot with respect to GTP.

A second explanation for the curvilinear double reciprocal plot with respect to GTP for gh-1 polymerase is based on the requirement for 2 substrate molecules for the formation of the first phosphodiester bond, whereas subsequent polymerization only involves the addition of single nucleotides. If both of the first two ribonucleoside triphosphates used in the initiation process are the same nucleotide, then the double reciprocal plot with respect to that nucleoside triphosphate should be second order (50). For the gh-1 polymerase, it is conceivable that a portion of the RNA chains synthesized using gh-1 DNA are initiated with the dinucleotide pppGpG-, giving rise to a Hill coefficient between 1.0 and 2.0 with respect to GTP. Although it is not possible to distinguish between the two explanations presented for the curvilinear double reciprocal plots of GTP, it should be noted that both ex-

planations involve the role of GTP as the initiating nucleoside triphosphate in RNA synthesis.

The inhibitor 3′-dATP was shown to compete with ATP for a common binding site on the gh-1 polymerase and host RNA polymerase molecules. The exact mechanism by which 3′-dATP inhibited *in vitro* RNA synthesis of the host and gh-1 RNA polymerases is unknown. The inhibition of *in vitro* RNA synthesis by 3′-dATP could be due to a simple competition with ATP for a single binding site. On the other hand, the inhibition could be due to the enzymatic incorporation of 3′-dATP into the growing RNA chain, thus causing chain termination. Once RNA synthesis has terminated, the RNA polymerase molecule would have to be released from the enzyme-DNA-nascent RNA complex and then bind to a proper initiation sequence in the DNA before it could once again participate in normal RNA synthesis. It is also possible that RNA polymerase molecules terminated by incorporation of 3′-dATP could be released less rapidly than RNA polymerase molecules terminated at natural termination sites. The determination of whether 3′-dATP is incorporated into RNA would be greatly facilitated by the use of radioactively labeled 3′-dATP. Experiments on the size of RNA transcribed *in vitro* by the gh-1 polymerase after incubation periods long enough to ensure several rounds of transcription revealed that the RNA synthesized in the presence of 3′-dATP was significantly shorter than that synthesized in its absence (data not shown). These experiments indicated that the 3′-dATP can cause premature termination of RNA synthesis by the phage enzyme. Although these experiments do not directly demonstrate that 3′-dATP is incorporated into RNA by the gh-1 polymerase, they are presumptive evidence of this point.

The apparent $K_i$ value for 3′-dATP for the gh-1 polymerase ($2 \times 10^{-8}$ M) is strikingly low compared to that for the host *P. putida* RNA polymerase ($2 \times 10^{-6}$ M) or for the eukaryotic RNA polymerase I and II isolated from Novikoff hepatoma tissue culture cells ($1.4 \times 10^{-5}$ M and $7 \times 10^{-6}$ M, respectively).[2] This higher sensitivity of the gh-1 polymerase to 3′-dATP could indicate that it is not as competent at discriminating between the substrate analog (3′-dATP) and the natural substrate (ATP) for binding to the active site as the other RNA polymerases. 3′-dATP provides a tool for selectively inhibiting gh-1 polymerase activity in the presence of host RNA polymerase activity in *in vitro* RNA synthesis.

For T7 infection of *E. coli*, development of the bacteriophage requires both the host and phage-induced RNA polymerases (11, 12). The host polymerase transcribes approximately 20% of the length of the T7 DNA, giving rise to the early RNA species (51). One of the products of this transcription is the mRNA for the T7 polymerase, which is then responsible for the transcription of the late region of coliphage T7 DNA (12). In gh-1 infection of *P. putida*, two temporally appearing classes of RNA have been identified.[2] The gh-1 polymerase transcribes from only one strand of gh-1 DNA, the biologically correct strand, and synthesizes both early and late gh-1 RNA.[3] It is likely, therefore, that the gh-1 polymerase acts similarly to the T7 and T3 RNA polymerase to provide a positive control in turning on transcription of viral genes.

## REFERENCES


1. Haselkorn, R., Vogel, M., and Brown, R. D. (1969) *Nature* **221**, 836–838
2. Takeda, Y., Oyama, Y., Nakajima, K., and Yura, T. (1969) *Biochem. Biophys. Res. Commun.* **36**, 533–538
3. Geiduschek, E. P., and Sklar, J. (1969) *Nature* **221**, 833–836
4. Spiegelman, G. B., and Whiteley, H. R. (1974) *J. Biol. Chem.* **249**, 1476–1482
5. Holland, M., and Whiteley, H. R. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 2234–2237
6. Seifert, W., Rabussay, D., and Zillig, W. (1971) *Fed. Eur. Biochem. Soc. Lett.* **16**, 175–179
7. Goff, C. G., and Weber, K. (1970) *Cold Spring Harbor Symp. Quant. Biol.* **35**, 101–108
8. Travers, A. A. (1969) *Nature* **223**, 1107–1110
9. Stevens, A. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 603–607
10. Horvitz, H. R. (1973) *Nature New Biol.* **244**, 137–140
11. Chamberlin, M., McGrath, J., and Waskell, L. (1970) *Nature* **228**, 227–231
12. Summers, W. C., and Siegel, R. B. (1971) *Nature* **228**, 1160–1162
13. Maitra, U. (1971) *Biochem. Biophys. Res. Commun.* **43**, 443–450
14. Dunn, J. J., Bautz, F. A., and Bautz, E. K. F. (1971) *Nature New Biol.* **230**, 94–96
15. Burgess, R. R. (1969) *J. Biol. Chem.* **244**, 6168–6176
16. Bautz, E. K. F. (1973) *Fed. Eur. Biochem. Soc. Lett.* 36, 123–129
17. Lee, L. F., and Boezi, J. A. (1966) *J. Bacteriol.* 92, 1821–1827
18. Lee, L. F., and Boezi, J. A. (1967) *J. Virol.* **1**, 1274–1276
19. Towle, H. C., Jolly, J. F., and Boezi, J. A. (1973) *Fed. Proc.* **32**, 645
20. Johnson, J. C., DeBacker, M., and Boezi, J. A. (1971) *J. Biol. Chem.* **246**, 1222–1232
21. Gerard, G. F., Johnson, J. C., and Boezi, J. A. (1972) *Biochemistry* **11**, 989–997
22. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
23. Schlesinger, M. J., and Barrett, K. (1965) *J. Biol. Chem.* **240**, 4284–4292
24. Laurant, T. C., and Killander, J. (1965) *J. Chromatogr.* 14, 317–330
25. Yue, R. H., Noltmann, E. A., and Kuby, S. A. (1967) *Biochemistry* **6**, 1174–1183
26. Jaenicke, R., and Knof, S. (1968) *Eur. J. Biochem.* **4**, 157–163
27. Siegel, L. M., and Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346–362
28. Robertson, H. D., Webster, R. E., and Zinder, N. D. (1968) *J. Biol. Chem.* **243**, 82–91
29. Thomas, C. A., and Abelson, J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., and Davies, D. R., eds) pp. 553–561, Harper and Row, New York
30. Thomas, C. A., Berns, K. I., and Kelly, T. J. (1966) in *Procedures in Nucleic Acid Research* (Cantoni, G. L., and Davies, D. R., eds) pp. 535–540, Harper and Row, New York
31. Shapiro, A. L., Vinuela, E., and Maizel, J. V. (1967) *Biochem. Biophys. Res. Commun.* **28**, 815–820
32. Bhuyan, B. K., and Smith, C. P. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **54**, 566–572
33. Goldberg, I. H., Rabinowitz, M., and Reich, E. (1962) *Proc. Natl. Acad. Sci. U. S. A.* **48**, 2094–2101
34. Dunn, J. J., and Studier, W. F. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 1559–1563
35. Dunn, J. J., and Studier, W. F. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70**, 3296–3300
36. Ackers, G. K. (1964) *Biochemistry* **3**, 723–730
37. Dunne, C. P., Gerlt, J. A., Rabinowitz, K. W., and Wood, W. A. (1973) *J. Biol. Chem.* **248**, 8189–8199
38. Kornberg, A., Kornberg, S. R., and Simms, E. S. (1956) *Biochim. Biophys. Acta* **20**, 215–227
39. Maitra, U., Nakata, Y., and Hurwitz, J. (1967) *J. Biol. Chem.* **242**, 4908–4918
40. Riva, S., and Silvestri, L. G. (1972) *Annu. Rev. Microbiol.* 26, 199–224
41. Chamberlin, M. J., and Ring, J. (1972) *Biochem. Biophys. Res. Commun.* **49**, 1129–1136
42. Shigeura, H. T., and Boxer, G. E. (1964) *Biochem. Biophys. Res. Commun.* **17**, 758–763
43. Sentenac, A., Simon, E. J., and Fromageot, P. (1968) *Biochim. Biophys. Acta* **161**, 299–308


[2] H. Towle and R. Desrosiers, unpublished data.

[3] J. F. Jolly, personal communication.

44. Barrett, K. J., Gibbs, W., and Calendar, R. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 2986–2990
45. Kupper, H. A., McAllister, W. T., and Bautz, E. K. F. (1973) *Eur. J. Biochem.* **38**, 581–586
46. Chamberlin, M., and Ring, J. (1973) *J. Biol. Chem.* **248**, 2235–2244
47. Maitra, U., and Huang, H. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 55–59
48. Salvo, R. A., Chakraborty, P. R., and Maitra, U. (1973) *J. Biol. Chem.* **248**, 6647–6654
49. Anthony, D. D., Wu, C. W., and Goldthwait, D. A. (1969) *Biochemistry* **8**, 246–256
50. McAllister, W. T., Kupper, H., and Bautz, E. K. F. (1973) *Eur. J. Biochem.* **34**, 489–501
51. Hyman, R. W. (1971) *J. Mol. Biol.* **61**, 369–376