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"Host Shutoff" Function of Bacteriophage T7: Involvement of T7 Gene 2 and Gene 0.7 in the Inactivation of *Escherichia coli* RNA Polymerase

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The "host shutoff" function of bacteriophage T7 involves an inactivation of the host *Escherichia coli* RNA polymerase by an inhibitor protein bound to the enzyme. When this inhibitor protein, termed I protein, was removed from the inactive RNA polymerase complex prepared from T7-infected cells by glycerol gradient centrifugation in the presence of 1 M KCl, the enzyme recovered its activity equivalent to about 70 to 80% of the activity of the enzyme from uninfected cells. Analysis of the activity of *E. coli* RNA polymerase from *E. coli* cells infected with various T7 mutant phages indicated that the T7 gene 2 codes for the inhibitor I protein. The activity of *E. coli* RNA polymerase from gene 2 mutant phage-infected cells, which was about 70% of that from uninfected cells, did not increase after glycerol gradient centrifugation in the presence of 1 M KCl, indicating that the salt-removable inhibitor was not present with the enzyme. It was found that the reduction in *E. coli* RNA polymerase activity in cells infected with $T7^+$ or gene 2 mutant phage, i.e., about 70% of the activity of the enzyme compared to that from uninfected cells after glycerol gradient centrifugation in the presence of 1 M KCl, results from the function of T7 gene 0.7. *E. coli* RNA polymerase from gene 0.7 mutant phage-infected cells was inactive but recovered a full activity equivalent to that from uninfected cells after removal of the inhibitor I protein with 1 M KCl. *E. coli* RNA polymerase from the cells infected with newly constructed mutant phages having mutations in both gene 2 and gene 0.7 retained the full activity equivalent to that from uninfected cells with or without treatment of the enzyme with 1 M KCl. From these results, we conclude that both gene 2 and gene 0.7 of T7 are involved in accomplishing complete shutoff of the host *E. coli* RNA polymerase activity in T7 infection.

The "host shutoff" function of bacteriophage T7 has been thought to result from an inactivation of the host *Escherichia coli* RNA polymerase which transcribes host RNA and T7 early mRNA (2, 3, 14). When T7 infection proceeds, the synthesis of host RNA and T7 early mRNA is shut off and the T7-specific RNA polymerase (6), the product of gene 1 of T7, transcribes T7 late mRNA. Thus, host shutoff is another control element involved in the "early to late" switch in T7 gene expression in addition to the switch from the host to the phage-coded RNA polymerase transcribing early mRNA and late mRNA, respectively, at different times of infection and from different regions of T7 DNA.

We have previously reported that the host shutoff function of phage T7 involves an inactivation of the host RNA polymerase by an inhibitor protein bound to the enzyme (8, 10). We have shown that when this inhibitor protein is removed from the inactive host RNA polymerase prepared from T7-infected cells, the RNA polymerase recovers its activity (8, 9). The inhibitor protein, removed from the inactive RNA polymerase from T7-infected cells, has been purified and termed I protein (8, 9). I protein inhibits initiation of RNA synthesis by binding directly to the *E. coli* RNA polymerase holoenzyme and preventing the holoenzyme from binding to the T7 template DNA (9). On the other hand, I protein does not inhibit the activity of T7-specific RNA polymerase or the *E. coli* RNA polymerase core enzyme (8, 10).

In this paper, we describe our genetic analysis which indicated that gene 2 of T7 codes for the I protein. We also present evidence that, in addition to gene 2, T7 gene 0.7 which codes for a protein kinase (12, 22) is also required in accomplishing complete shutoff of the host *E. coli* RNA polymerase activity in T7 infection.

MATERIALS AND METHODS

Chemicals. [5-^{3}H]UTP (22.7 Ci/mmol) and [*methyl*-^{3}H]thymidine (6.7 Ci/mmol) were purchased from

New England Nuclear Corp.; ATP, CTP, GTP, and UTP were from Sigma Chemical Co.; poly[d(A-T)] was from P-L Biochemical Co.

Bacteria and T7 phages. *E. coli* D10 F^-(*met*$^-$, $B1^-$, RNase I^-), from our laboratory stock, was derived from D10 F^+ (20). This strain was commonly used as the host for T7 phages. An amber-suppressor strain *E. coli* 011′ (17) and a host-restriction minus strain *E. coli* C (19) were from F. W. Studier's collection.

Wild-type phage T7 ($T7^+$) and amber and deletion mutant phages were from the collection of F. W. Studier. Mutant phages are described in Results (see Table 1). Double mutant phages, BH3 and BH4, having mutations in both gene 0.7 and gene 2, were constructed in this laboratory by a cross between mutant phages H3 (deletion in gene 0.7) and 2-64(amber mutation in gene 2).

Growth of bacteria and phage T7 infection. *E. coli* D10 F^- was grown in M9-glucose medium supplemented with 50 μg of L-methionine per ml and 2 μg of thiamine per ml at 30°C to approximately 7×10^8 cells/ml and infected with phage T7 at a multiplicity of infection of 5 to 7 as described previously (8). $T7^+$ and mutant phages with deletions in non-essential genes were grown on *E. coli* D10 F^-, T7 amber mutant phages were grown on *E. coli* 011′, and T7 mutant phages with mutations in gene 0.3 were grown on *E. coli* C.

Standard buffer solutions. The following buffer solutions were frequently used in this work: (i) buffer A, consisting of 20 mM Tris-hydrochloride (pH 7.9), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 5% glycerol; and (ii) buffer G, consisting of 50 mM Tris-hydrochloride (pH 7.6), 150 mM KCl, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 5% glycerol.

Preparation of cell-free extracts from T7-infected and uninfected cells. *E. coli* D10 F^- cells were infected with phage T7 at 30°C as described. At indicated times before and after infection, aliquots of the culture were withdrawn and chilled on ice, and the cells were collected by centrifugation. Each cell pellet was suspended in 1 ml of buffer G in a 12-ml Sorvall conical centrifuge tube and disrupted by sonic treatment with a Sonifier cell disruptor. Sonic extraction was for a total of 3 min with alternating 15-s periods of sonic disruption (setting 4 to 6) and chilling in an ice bath. Sonic extracts were centrifuged at $13,000 \times g$ for 10 min, and cell debris and unbroken cells were removed.

Purification of *E. coli* RNA polymerase holoenzyme from T7-infected and uninfected cells. *E. coli* RNA polymerase holoenzyme was purified from *E. coli* D10 F^- cells harvested 9 min after $T7^+$ infection and also from uninfected cells by the method of Burgess (4) as modified by Hesselbach and Nakada (9).

Purification of *E. coli* RNA polymerase core enzyme and sigma factor from T7-infected and uninfected cells. *E. coli* RNA polymerase core enzyme and sigma factor were separated from the purified RNA polymerase holoenzyme by subjecting the enzyme to phosphocellulose column chromatography as described by Berg et al. (1).

Assay for *E. coli* RNA polymerase. The standard assay system contained the following in a total volume of 0.25 ml: 10 mM Tris-hydrochloride (pH 7.9), 150 mM KCl, 10 mM $MgCl_2$, 12 mM β-mercaptoethanol, 50 nmol each of ATP, CTP, and GTP, 20 nmol of [^{3}H]UTP (1 μCi/20 nmol), and 50 μg of bovine serum albumin. Different DNA templates were used, usually 5 μg of T4 DNA, 10 μg of T7 DNA, or 5 μg of poly[d(A-T)] per reaction mixture and RNA polymerase or cell-free extract as indicated. The reaction mixture was incubated at 37°C for 10 min unless otherwise specified. The reaction was terminated by the addition of 0.3 ml of 10% trichloroacetic acid, and the radioactivity in acid-insoluble material retained on a glass fiber filter (Reeve Angel 934AH, 24-mm diameter) was measured. The activity of an enzyme preparation was given as nanomoles of UMP incorporated into RNA in 10 min at 37°C. One activity unit equals 1 nanomole of UMP incorporated into acid-insoluble material in 10 min at 37°C. Specific activity was given as units per milligram of protein.

Assay for the inhibition of *E. coli* RNA polymerase by the inhibitor protein (I protein). The standard RNA-synthesizing system described above was used with the addition of 3 μg of purified *E. coli* RNA polymerase holoenzyme and 10 μg of T7 DNA. Inhibitor material was added as indicated in each experiment. The inhibitor activity was presented as the reduction in nanomoles of UMP incorporated into RNA compared to the control reaction.

Assay for T7-specific RNA polymerase. T7-specific RNA polymerase was assayed following the procedure of Chamberlin et al. (6). The reaction mixture contained the following in a total volume of 0.25 ml: 40 mM Tris-hydrochloride (pH 7.9), 20 mM $MgCl_2$, 12 mM β-mercaptoethanol, 50 nmol each of ATP, CTP, and GTP, 20 nmol of [^{3}H]UTP(1 μCi/20 nmol), 10 μg of T7 DNA, 250 μg of bovine serum albumin, and 5 μg of rifampin. Cell-free extracts from T7-infected cells were added as indicated in each experiment. After a 10-min incubation at 37°C, the reaction was terminated with 0.3 ml of cold 10% trichloroacetic acid, and the acid-insoluble radioactivity was measured as described for the assay of *E. coli* RNA polymerase.

RESULTS

Time course of the host shutoff measured by the inactivation of *E. coli* RNA polymerase after T7 infection. Time course of the switch from the host *E. coli* RNA polymerase to the T7-specific RNA polymerase in normal phage $T7^+$ infection is shown in Fig. 1. Cell-free extracts were prepared from T7-infected cells withdrawn from the culture at different times after infection and assayed for the two enzymes. A rapid decline in host RNA polymerase activity coincided with a rapid appearance of T7-specific RNA polymerase activity. Since it was shown that the addition of rifampin 5 min or later after T7 infection did not change the kinetics and the final activity of T7-specific RNA polymerase (21), we assumed that no new initiation of T7 early mRNA synthesis occurred after 5 min of infection, probably in parallel with the shutoff of new host RNA synthesis. When the cells were

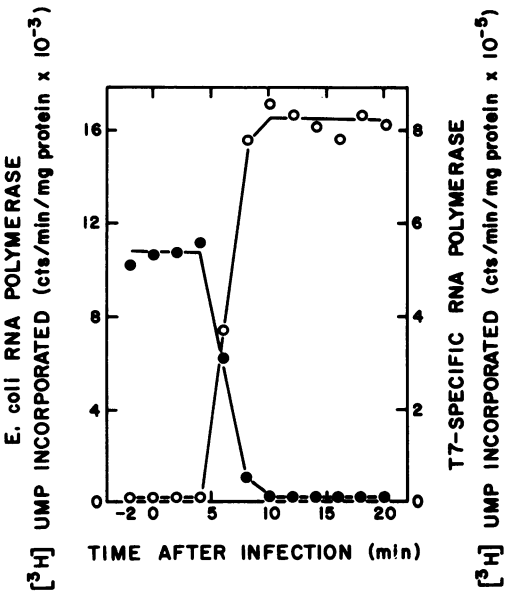

FIG. 1. *Activities of E. coli RNA polymerase and T7-specific RNA polymerase during T7⁺ infection. E. coli D10 F⁻ cells were infected with T7⁺ phage at 30°C as described in the text. Aliquots of the culture (70 ml) were removed at indicated times and sonically disrupted cell-free extracts were prepared. E. coli RNA polymerase was assayed by using a portion of cell-free extract (about 300 μg of protein) with 5 μg T4 DNA as described in the text. T7-specific RNA polymerase was assayed by using a portion of cell-free extract (about 50 μg of protein) with 10 μg of T7 DNA as described in the text. Enzyme activities were expressed as counts of [³H]UMP incorporated per minute into RNA per milligram of protein in 10 min of incubation at 37°C. Symbols: ●, E. coli RNA polymerase activity; ○, T7-specific RNA polymerase activity.*

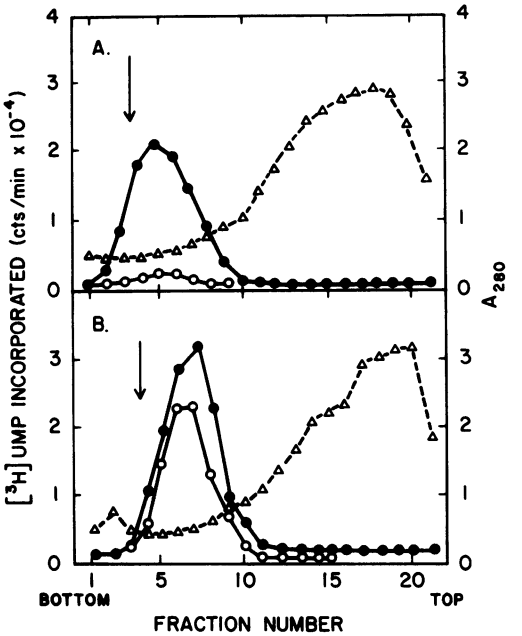

FIG. 2. *E. coli RNA polymerase activity in cell-free extracts prepared from uninfected and T7⁺-infected cells after glycerol gradient centrifugation in the presence of 0.15 M KCl (A) and 1 M KCl (B). Sonically disrupted cell-free extracts were prepared from uninfected E. coli D10 F⁻ cells and from T7⁺-phage infected cells harvested 10 min after infection. Cell-free extracts were treated with 50 μg of DNase per ml for 1 h at 4°C and centrifuged at 13,000 × g for 10 min. A portion of the cell-free extracts containing 12 mg of protein (0.6 ml) was applied to 12-ml gradients containing 10 to 30% glycerol in buffer A with 0.15 M KCl (A) and 10 to 30% glycerol in buffer A with 1 M KCl (B). Gradients were centrifuged in an SW41 rotor at 40,000 rpm for 24 h (gradients A) or for 30 h (gradients B). E. coli RNA polymerase activity was assayed by using a 40-μl volume from each gradient fraction with 2.4 μg of T4 DNA as described in the text. The final KCl concentration in the RNA polymerase assay system was adjusted to 0.16 M for all the samples from gradients A and gradients B. (A) 10 to 30% glycerol gradient with 0.15 M KCl; (B) 10 to 30% glycerol gradient with 1 M KCl. Symbols: ●, E. coli RNA polymerase activity from uninfected cells; ○, E. coli RNA polymerase activity from T7⁺-infected cells; △, protein content (A_{280}). Arrows in the figure indicate the position of β-galactosidase added to the gradients as a sedimentation marker.*

infected with T7 in the presence of chloramphenicol, no reduction in the activity of the host RNA polymerase was observed (8), indicating that protein synthesis after T7 infection is required to manifest the shutoff function.

Recovery of enzyme activity from the inactive *E. coli* RNA polymerase by glycerol gradient centrifugation in the presence of 1 M KCl. A partial purification of *E. coli* RNA polymerase was achieved by glycerol gradient centrifugation of sonically disrupted cell-free extracts after treatment with DNase to remove endogenous DNA. As shown in Fig. 2A, *E. coli* RNA polymerase sedimented faster than most other proteins of the cell and resulted in about a fivefold purification of the enzyme. DNase sedimented in gradient fractions (Fig. 2A, fractions 14 to 20) apart from the fractions containing RNA polymerase activity. Pelleted material contained less than a few percent of the total RNA polymerase activity.

A comparison of the activities of *E. coli* RNA polymerase in cell-free extracts from T7-infected cells harvested 10 min after infection and from uninfected cells after glycerol gradient centrifugation in the presence of 0.15 M KCl showed that T7 infection caused a marked reduction in the enzyme activity (Fig. 2A). The *E. coli* RNA polymerase activity was assayed by using T4

DNA as a template to eliminate the activity of T7-specific RNA polymerase. However, when T7 DNA was used as a template in the presence and absence of rifampin to differentiate the activities of the two RNA polymerases (T7-specific RNA polymerase is insensitive to rifampin), the same activity for *E. coli* RNA polymerase was obtained as assayed on T4 DNA. When the gradient fractions containing the *E. coli* RNA polymerase activity were pooled and again assayed, the gradient fractions from the cell-free extract of T7-infected cells contained about 15% of the activity observed in uninfected cells.

The inactivation of the host RNA polymerase in T7-infected cells involves an association of inhibitor material with the enzyme (Fig. 2B). A portion of the same DNase-treated cell-free extract from T7-infected cells or uninfected cells shown in Fig. 2A was subjected to glycerol gradient centrifugation in the presence of 1 M KCl. When the activity of *E. coli* RNA polymerase from T7-infected cells was assayed in the standard RNA-synthesizing system containing a normal concentration of KCl at 0.15 M, about 70% of the uninfected cell activity was detected (Fig. 2B). The result indicated that the inhibitory material associated with the enzyme was removed by exposing the inactive enzyme to a high concentration of KCl.

This notion was supported by the experiment shown in Fig. 3, which employed *E. coli* RNA polymerase preparations from T7-infected and uninfected cells that had been processed by using the standard RNA polymerase purification procedure through glycerol gradient centrifugation in the absence of KCl (4, 9). At this stage of purification, without exposing the enzyme to a high concentration of KCl, the enzyme preparation from T7-infected cells contained only marginal activity when assayed on T4 or T7 DNA templates. When this enzyme preparation from T7-infected cells was sedimented in a glycerol gradient containing 0.15 M KCl, little activity was found in the gradient fractions (Fig. 3A). However, when the same enzyme preparation was sedimented in a glycerol gradient containing 1 M KCl, more than a 10-fold increase in the activity of *E. coli* RNA polymerase assayed on T4 DNA was observed (Fig. 3B). KCl concentrations from 0.7 to 1.3 M achieved the same effect in recovering the activity of the inactive enzyme from T7-infected cells.

The result shown in Fig. 3B also demonstrated the appearance of an inhibitor material of *E. coli* RNA polymerase near the top of the gradient which was not observed in the parallel gradient with 0.15 M KCl (Fig. 3A). A comparison of the results in Fig. 3A and B indicated that the increase in the inhibitor activity in the presence of 1 M KCl equaled the increase in

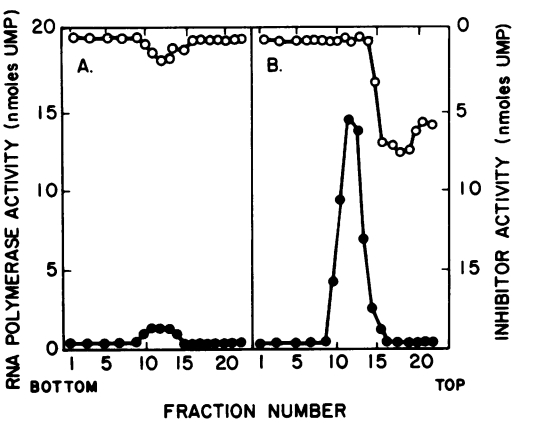

FIG. 3. *Recovery of the activity and release of the inhibitor from partially purified E. coli RNA polymerase from T7⁺-infected cells by glycerol gradient centrifugation in the presence of 1 M KCl. E. coli RNA polymerase was partially purified from the cells infected with T7⁺ and harvested after 10 min of infection as described previously (8). This enzyme preparation, which has a very low activity on T4 DNA, was layered on a 12-ml, 10 to 30% glycerol gradient in buffer A containing 0.15 M KCl (A) or 1 M KCl (B) and centrifuged in an SW41 rotor at 40,000 rpm for 16 h. Each gradient fraction was assayed for the E. coli RNA polymerase activity in the standard assay system with 10 μg of T7 DNA. The inhibitor activity of each gradient fraction was assayed in the standard assay system with 10 μg of T7 DNA and 3 μg of purified E. coli RNA polymerase holoenzyme as described in the text. The final KCl concentration in the assay system was adjusted to 0.15 M. (A) Glycerol gradient with 0.15 M KCl; (B) glycerol gradient with 1 M KCl. Symbols: ●, E. coli RNA polymerase activity in nanomoles of UMP per fraction; ○, the inhibitor activity as reduction in nanomoles of UMP into RNA per fraction.*

the *E. coli* RNA polymerase activity in terms of nanomoles of UMP incorporated into RNA in the assay system. Glycerol gradient centrifugation of the inactive *E. coli* RNA polymerase preparation from T7-infected cells in the presence of 0.3 M and 0.5 M KCl resulted in a recovery of about 25 and 40% of the control enzyme activity, respectively, accompanied by a corresponding appearance of the inhibitor activity near the top of the gradient.

These observations suggested a stoichiometric association between the *E. coli* RNA polymerase and the inhibitor in T7-infected cells resulting in an inactive enzyme complex formation which is reversible in vitro by a high concentration of salt. The fact that an inhibitor from T7-infected cells which is responsible for the inactivation of the host RNA polymerase can be separated from the enzyme prompted us to proceed with the purification of the inhibitor. As described in a separate paper (9), we purified

the inhibitor (I protein) from T7-infected cells and presented evidence that the I protein directly binds to *E. coli* RNA polymerase holoenzyme and prevents the binding of the enzyme to the promoter site of T7 DNA, thus inhibiting the initiation of RNA synthesis by the enzyme.

Identification of the T7 gene which codes for the inhibitor I protein. To identify the T7 gene which codes for the inhibitor I protein, our first attempt was to screen possible candidates by analyzing the activity of *E. coli* RNA polymerase in cell-free extracts from various mutant phage-infected cells after subjecting the extracts to glycerol gradient centrifugation in the presence of 0.15 M KCl. Under these conditions, the cell-free extract from cells infected with mutant phage having a mutation in the gene responsible for the inactivation of the *E. coli* RNA polymerase was expected to show an uninhibited activity of the enzyme. The second step to identify the mutation depended on the reactivation of the inactive *E. coli* RNA polymerase by removing the inhibitor I protein with a high concentration of KCl as observed in the results shown in Fig. 2 and 3. According to this screening, it was predicted that if the cells infected with a particular mutant phage showed an uninhibited *E. coli* RNA polymerase activity in its cell-free extract after glycerol gradient centrifugation with 0.15 M KCl, then glycerol gradient centrifugation of the same cell-free extract in the presence of 1 M KCl should not show any increase in the activity of the enzyme because there should not be a salt-removable inhibitor associated with the enzyme.

Since the inactivation of *E. coli* RNA polymerase starts at about 5 min and reaches a maximum inactivation at 9 to 10 min after T7 infection (Fig. 1), *E. coli* D10 F^- cells infected with $T7^+$ and various mutant phages were harvested 10 and 16 min after infection, and sonically disrupted cell-free extracts were prepared. After DNase treatment, these cell-free extracts were subjected to glycerol gradient centrifugation in the presence of 0.15 M KCl, and the fractions containing *E. coli* RNA polymerase activity were pooled and assayed for activity relative to that of the cell-free extract from uninfected cells. Results obtained with mutant phages having mutations in class I genes ("early" genes, genes 0.3 to 1.3) and class II genes ("early-late" genes, genes 1.7 to 3.5) are shown in Table 1. Results obtained with mutant phages with mutations in class III genes ("late" genes, genes 4 to 19) were not included in Table 1 because the results were all negative.

Among the cell-free extracts tested, only those from the cells infected with mutant phage having a mutation in gene 1 (*am*193) or in gene 2 (2-64) showed a relatively high activity of *E. coli* RNA polymerase. Since gene 1 codes for T7-specific RNA polymerase (6), infection with

TABLE 1. *Relative activity of E. coli RNA polymerase in cell-free extracts from various T7 mutant phage-infected cells after glycerol gradient centrifugation in the presence of 0.15 M KCl*[a]

T7 phage	Type of mutation	T7 gene affected by mutation	*E. coli* RNA polymerase activity (% of uninfected control value)	
			10 min postinfection	16 min postinfection
		Uninfected control	100	100
$T7^+$			17	10
$T7^+$		Chloramphenicol[b]	98	102
CR3b	Amber	0.3	12	16
CR10b	Point	0.3	16	10
D111	Deletion	0.3	21	20
H1	Deletion	0.3–0.7	20	14
H3	Deletion	0.7	20	9
A57	Amber	0.7	15	12
*am*193	Amber	1	72	71
LG3	Deletion	1.1–1.3	15	10
LG26	Deletion	1.3-terminator-1.7	21	10
2-64	Amber	2	65	67
3-29	Amber	3	16	13
Ha13a	Amber	3.5	18	11

[a] Sonically disrupted cell-free extracts were prepared from *E. coli* D10 F^- cells infected with various T7 mutant phages and treated with DNase as described in the text. These extracts were layered on 10 to 30% glycerol gradients in buffer A containing 0.15 M KCl and centrifuged in an SW41 rotor at 40,000 rpm for 24 h. The gradient fractions containing *E. coli* RNA polymerase activity (fractions 1 to 9, see Fig. 2A) were pooled and a 40-μl volume of the pooled fractions from each gradient was assayed in the standard *E. coli* RNA polymerase assay system with 2.4 μg of T4 DNA.

[b] Chloramphenicol (150 μg/ml) was added 5 min before infection.

gene 1 mutant phage does not produce class II and class III RNAs and proteins. Therefore, the gene 2 product will not be produced in gene 1 mutant phage-infected cells. Gene 2 is one of the class II genes and has been implicated in the synthesis of T7 DNA (5, 15); the exact mechanism by which mutation in gene 2 causes reduced DNA synthesis has not been determined, and the protein product of gene 2 is unidentified (7,16). The molecular weight of purified I protein was estimated at about 7,000 to 9,000 (9), and the coding capacity of gene 2 (nucleotide length) is sufficient to specify a protein of this size (16). Therefore, it is likely that gene 2 codes for the I protein.

In the cells infected with gene 1 mutant phage, the only T7 genes that can produce the product proteins are the early genes 0.3, 0.7, 1.1, and 1.3. The results (Table 1) with gene 1 mutant-infected cells indicated that these early genes are not involved in the inactivation of the *E. coli* RNA polymerase. This agrees with the results shown in Table 1 that the cells infected with phages having mutation in genes 0.3, 0.7, 1.1, and 1.3, individually or in combination, all showed a greatly inhibited level of *E. coli* RNA polymerase activity.

Ratner (13) has shown that the gene 0.3 protein, with a molecular weight of 14,000, is the only T7 protein which binds to *E. coli* RNA polymerase. However, no evidence has been presented that gene 0.3 protein inhibits *E. coli* RNA polymerase. From the results presented in Table 1, gene 0.3 does not seem to be involved in the inactivation of *E. coli* RNA polymerase. In addition, the molecular weight of the inhibitor I protein is now estimated to be 7,000 to 9,000 (9), smaller than the molecular weight of gene 0.3 protein. Therefore, gene 0.3 apparently does not code for the inhibitor I protein.

T7 gene 0.7 has been implicated as the gene responsible for the host shutoff function of T7 (2, 3, 14). However, the results presented in Table 1 indicate that this gene is not primarily involved in the inactivation of the host *E. coli* RNA polymerase activity 10 min after infection when assayed in vitro by the method used in this screening.

Table 2 shows that when cell-free extract from gene 2 mutant phage-infected cells was analyzed, *E. coli* RNA polymerase activity from the glycerol gradient containing 1 M KCl was about the same as that from the gradient containing 0.15 M KCl. In a parallel glycerol gradient analysis, a cell-free extract from $T7^+$-infected cells showed greatly increased activity of *E. coli* RNA polymerase when subjected to centrifugation in the presence of 1 M KCl compared to 0.15 M KCl (Table 2). The results shown in Table 2 satisfied our criterion that the uninhibited *E. coli* RNA polymerase should not increase its activity after an exposure to 1 M KCl because the salt-removable inhibitor should not be present with the enzyme.

TABLE 2. *Effect of $T7^+$ phage and T7 gene 2 mutant phage 2-64 infection on the activity of E. coli RNA polymerase analyzed after glycerol gradient centrifugation of cell-free extracts from phage-infected cells in the presence of 0.15 M KCl and 1 M KCl*[a]

Time after infection (min)	*E. coli* RNA polymerase activity (% of uninfected control value)			
	$T7^+$		2-64	
	0.15 M KCl	1.0 M KCl	0.15 KCl	1.0 M KCl
Uninfected control	100	100	100	100
10	17	74	65	69
16	10	71	67	70

[a] Preparation of cell-free extracts, glycerol gradient centrifugation, and *E. coli* RNA polymerase assay were the same as described in legends for Fig. 2 and 3.

Furthermore, our preliminary experiment showed that *E. coli* RNA polymerase from an amber suppressor host 011′ strain infected with the T7 gene 2 amber mutant phage 2-64 exhibited an inhibited level of activity comparable to the enzyme activity of the same host infected with $T7^+$ after glycerol gradient centrifugation of the cell-free extracts in the presence of 0.15 M KCl. Both cell-free extracts showed an activity of *E. coli* RNA polymerase equivalent to about 70% of that of the uninfected control after glycerol gradient centrifugation in the presence of 1 M KCl. The result suggests that the T7 amber mutant phage 2-64 produces I protein in the amber suppressor host 011′ cells, and the suppressed I protein inhibits the activity of *E. coli* RNA polymerase unless it is removed by 1 M KCl. We are currently attempting to isolate and identify the suppressed I protein from the suppressor host cells infected with amber mutant phage 2-64, assuming that the suppressed I protein should be found with the inactive *E. coli* RNA polymerase.

From the results presented here, we have tentatively assigned coding of the T7 inhibitor I protein to gene 2.

Involvement of T7 gene 0.7 in the inactivation of *E. coli* RNA polymerase. As noticed from the results in Table 2, the activity of *E. coli* RNA polymerase, which was recovered by removal of the inhibitor with 1 M KCl from the cells infected with either $T7^+$ or gene 2 mutant phage, was not 100% of the uninfected control value; usually about 70% of the control value

was obtained. In fact, purified *E. coli* RNA polymerase from T7$^+$-infected cells always showed a reduced activity on the T4 DNA template when compared to a similarly purified *E. coli* RNA polymerase from uninfected cells (9). The reduction in the *E. coli* RNA polymerase activity, about 30% from that of the uninfected control, is comparable to the similar reduction observed in the assay which employed a direct glycerol gradient centrifugation of the sonically disrupted cell-free extract from T7$^+$-infected cells (Fig. 2 and Table 2). Therefore, another possible inhibitory mechanism which affects the activity of *E. coli* RNA polymerase in T7-infected cells in addition to the gene 2-mediated inhibition of the enzyme by I protein was investigated.

For this purpose, cell-free extracts prepared from *E. coli* D10 F$^-$ cells infected with various mutant phages and harvested 10 min after infection were subjected to centrifugation in two glycerol gradients containing 0.15 M KCl and 1 M KCl in parallel, and the activities of *E. coli* RNA polymerase in two gradients assayed on T4 DNA template were compared. In this screening, it was expected that a full activity in *E. coli* RNA polymerase should be found in the cell-free extract from cells infected with a particular mutant phage having a mutation in the gene which is responsible for the partial inhibition of the enzyme after removal of the I protein by glycerol gradient centrifugation with 1 M KCl.

It was found that only the cell-free extracts prepared from the cells infected with gene 0.7 mutant phages showed a full activity of *E. coli* RNA polymerase comparable to the activity of that from uninfected control after glycerol gradient centrifugation in the presence of 1 M KCl. As shown in Table 1, *E. coli* RNA polymerase in cell-free extracts from gene 0.7 mutant phage (for an example, H3)-infected cells showed about 15 to 20% of the uninfected control level activity when centrifuged in a glycerol gradient containing 0.15 M KCl. However, when the same cell-free extract was centrifuged in a gradient in the presence of 1 M KCl, the enzyme activity was recovered to 100% of the uninfected control level as shown in Table 3.

The results indicated that gene 0.7 product, a protein kinase which phosphorylates many host proteins including the β' subunit of *E. coli* RNA polymerase (12, 22), is responsible for the partial inhibition (usually by 30%) of *E. coli* RNA polymerase activity in addition to the stronger inhibition by the gene 2 product I protein. Therefore, it appears that gene 2 and gene 0.7 are both involved in the inactivation of *E. coli* RNA polymerase.

TABLE 3. *E. coli RNA polymerase activity in cell-free extracts from T7$^+$- and T7 mutant phage-infected cells after glycerol gradient centrifugation in the presence of 0.15 M KCl and 1 M KCl*[a]

T7 phage	Mutation in T7 gene	*E. coli* RNA polymerase activity, (% of uninfected control value)	
		0.15 M KCl	1.0 M KCl
	Uninfected control	100	100
T7$^+$		12	67
H3	Gene 0.7 deletion	21	102
2-64	Gene 2 amber	64	68
BH3	Gene 0.7 deletion and gene 2 amber	93	100
BH4	Gene 0.7 deletion and gene 2 amber	105	101

[a] All phage-infected cells were harvested at 10 min after infection. Preparation of cell-free extracts, glycerol gradient centrifugation, and *E. coli* RNA polymerase assay were the same as described in legends for Fig. 2 and 3.

In our preliminary experiments, *E. coli* RNA polymerase holoenzyme preparations purified from uninfected and T7$^+$-infected cells were separated into respective core polymerase and sigma factor moieties. When holoenzyme was constituted by using core RNA polymerase from T7$^+$-infected cells and sigma factor from either uninfected or T7$^+$-infected cells, the activity of the reconstituted holoenzyme was 20 to 25% less than that of reconstituted holoenzyme formed between core enzyme from uninfected cells and sigma factor from either source. Therefore, the partial inactivation of *E. coli* RNA polymerase by the gene 0.7 product appears to result from phosphorylation of the core enzyme moiety, presumably β' subunit (22), rather than the inactivation of the sigma factor.

Purified *E. coli* RNA polymerase from T7$^+$-infected cells, which had about 70% of the activity of normal holoenzyme after removal of I protein, was inhibited by purified I protein to a similar extent as the normal holoenzyme when tested in a standard RNA-synthesizing system using T4 and T7 DNA (9). This suggests that although the RNA polymerase from T7$^+$-infected cells was less active, the ability to bind with I protein (thus to be inhibited) was not altered. The reduction in the activity of *E. coli* RNA polymerase from T7$^+$-infected cells does not appear to alter the template specificity or to cause more frequent read-through at transcriptional termination sites. This was indicated by analyzing the size of RNA synthesized by the less active RNA polymerase on T7 DNA using agarose-acrylamide gel electrophoresis. Therefore, about 30% reduction in the activity of *E. coli* RNA polymerase which resulted from the function of T7 gene 0.7 seems to be a nonspecific, partial inactivation of the enzyme.

Involvement of both T7 gene 2 and gene 0.7 in the inactivation of *E. coli* RNA polymerase. To confirm the notion that T7 gene 2 and gene 0.7 are both involved in the inactivation of the host *E. coli* RNA polymerase, double mutant phages having mutations in both gene 2 and gene 0.7 were constructed by a cross between gene 2 mutant phage 2-64 (amber mutation in gene 2) and gene 0.7 mutant phage H3 (deletion in gene 0.7). Double mutant phages, BH3 and BH4, were used to examine the activity of *E. coli* RNA polymerase after infection.

Cell-free extracts from *E. coli* D10 F^- cells infected with BH3 and BH4 phages were centrifuged in glycerol gradients containing 0.15 M KCl or 1 M KCl, and the activity of *E. coli* RNA polymerase in each gradient was assayed on T4 DNA template. As shown in Table 3, *E. coli* RNA polymerase in these extracts retained about 100% of the uninfected control level activity after centrifugation in the presence of both 0.15 M and 1 M KCl. The result supports the hypothesis that gene 2 and gene 0.7 are both involved in the inactivation of *E. coli* RNA polymerase in T7 infection.

DISCUSSION

Previous work on the host shutoff function of T7 phage. T7 gene 0.7 has been thought to be responsible for the host shutoff. This assumption was based on the observations that gene 0.7 mutant phage-infected cells continued to synthesize host RNAs (or host proteins such as β-galactosidase) and T7 early mRNA for a longer period of time compared to $T7^+$-infected cells (2, 3, 14).

Recently, it was found that the product of T7 gene 0.7, a protein with a molecular weight of approximately 40,000 (18), is a protein kinase which phosphorylates many host proteins including the β′ subunit of *E. coli* RNA polymerase (12, 22). Based on this finding, combined with an additional observation that gene 0.7 mutant infection results in an overproduction of T7 early mRNA, Ponta et al. (11) proposed that modification of *E. coli* RNA polymerase by the gene 0.7-coded protein kinase is responsible for the host shutoff. However, they were unable to demonstrate a reduced synthesis of RNA in cell-free extracts from T7-infected cells in which *E. coli* RNA polymerase should have been phosphorylated by the protein kinase. Therefore, they speculated that an endogenous phosphatase in the extract might have removed the newly attached phosphate groups from the RNA polymerase thus restoring the activity to the enzyme.

The results presented in this and previous papers (8, 9) were not in accord with their observation. Our results showed a greatly reduced *E. coli* RNA polymerase activity in cell-free extracts from $T7^+$-infected cells.

Inhibitor of *E. coli* RNA polymerase: T7 gene 2 codes for the inhibitor I protein. Based on our preliminary finding that *E. coli* RNA polymerase in a cell-free extract from $T7^+$-infected cells is inactive when assayed in vitro, we took a different approach to the problem of T7 phage-mediated host shutoff. We initiated our studies by attempting to isolate and identify an inhibitor protein bound to the *E. coli* RNA polymerase that causes inactivation of the enzyme. As described in this and in previous papers (8, 9), we were able to partially purify the "inactive" *E. coli* RNA polymerase from $T7^+$-infected cells and found that the inactive enzyme is associated with an inhibitor protein which is removable with 1 M KCl. After centrifugation in a glycerol gradient containing 1 M KCl, the inactive enzyme recovered its activity and the inhibitor was separated from the enzyme. As described in the accompanying paper (9), the inhibitor protein, termed I protein, was purified and its molecular weight was estimated to be about 7,000 to 9,000. Since the protein product of T7 gene 0.7 (protein kinase) has a molecular weight of approximately 40,000 (18), it is unlikely that the I protein is coded by gene 0.7.

As shown in Tables 1 and 2, our search for the T7 gene which codes for the I protein depended on a screening to detect an uninhibited activity in *E. coli* RNA polymerase in cell-free extracts from the cells infected with various T7 mutant phages. By this screening, we were able to assign T7 gene 2 as the gene which codes for the I protein. It is of special interest that by this screening *E. coli* RNA polymerase from gene 0.7 mutant phage-infected cells harvested 10 min after infection showed a greatly reduced activity which was not different from that of the $T7^+$-infected cells. Therefore, T7 gene 0.7 was ruled out as the gene coding for the inhibitor of *E. coli* RNA polymerase.

However, it was found that removal of the I protein from the inactive *E. coli* RNA polymerase from $T7^+$-infected cells did not restore 100% of the control activity to the enzyme; usually about 70% of uninfected control value was obtained. The activity of *E. coli* RNA polymerase from T7 gene 2 mutant phage-infected cells was also about 70% of the uninfected control activity.

Reevaluation of the involvement of T7 gene 0.7 in the host shutoff. It was found that gene 0.7 is involved in the host shutoff function of T7, although it was eliminated as the gene which codes for I protein as described above.

E. coli RNA polymerase from gene 0.7 mutant phage-infected cells showed a greatly inhibited activity similar to that from T7$^+$ phage-infected cells when analyzed in the presence of 0.15 M KCl. However, when I protein was removed from the inactive enzyme from gene 0.7 mutant phage-infected cells by glycerol gradient centrifugation in the presence of 1 M KCl, the enzyme recovered a full activity equivalent to the uninfected control level. This indicated that gene 0.7 (protein kinase) plays a role in the inactivation of *E. coli* RNA polymerase in T7 infection. However, the inactivation of *E. coli* RNA polymerase by gene 0.7-coded protein kinase is only a partial reduction of the enzyme activity (about 30%) and is usually masked by a stronger inactivation (about 80% or more) by gene 2-coded I protein.

On the other hand, in in vivo situations one can easily envision that gene 0.7 still plays a significant role in the T7-mediated host shutoff. Kinetically, it is known that gene 0.7-coded protein kinase is produced earlier than the gene 2-coded I protein. Therefore, a partial inhibition of *E. coli* RNA polymerase by the T7-coded protein kinase can result in a significant decrease in the amount of both host RNA and T7 early mRNA synthesized by *E. coli* RNA polymerase during an early period in T7 infection. This can also explain why gene 0.7 mutant phage-infected cells overproduce T7 early mRNA (11). Since previous in vivo studies on the inactivation of the host RNA polymerase were performed using T7 gene 1 mutant phages to eliminate expression of all late genes (class II and III) including gene 2, the lack of gene 0.7 product may have resulted in a profound effect in reducing the in vivo activity of the host RNA polymerase (2, 3, 14).

E. coli RNA polymerase from the cells infected with newly constructed mutant phages having mutations in both gene 2 and gene 0.7 (mutant phages BH3 and BH4) retained a full control level activity through 20 min after infection when the enzyme activity was assayed in vitro. This confirmed the involvement of both gene 2 and gene 0.7 in the host shutoff by T7.

Dual function of T7 gene 2. *E. coli* RNA polymerase in cell-free extracts from gene 2 mutant phage-infected cells harvested at various times after infection maintained an uninhibited level of activity when assayed on T4 DNA (Tables 1 and 2). Therefore, it is expected that the synthesis of host RNA and T7 early mRNA will continue during gene 2 mutant phage infection because *E. coli* RNA polymerase will not be inactivated. Since T7-specific RNA polymerase is the product of one of the early genes, the synthesis of T7-specific RNA polymerase, which is dependent on the synthesis of T7 early mRNA, is expected to continue in gene 2 mutant phage infection.

However, that was not the case. As can be seen in Fig. 4, the increase in T7-specific RNA polymerase activity in gene 2 mutant phage-infected cells sharply leveled off at 9 to 10 min after infection, whereas the host *E. coli* RNA polymerase maintained an uninhibited level of activity. An abrupt termination of T7-specific RNA polymerase synthesis in this case is very similar to the situation observed in T7$^+$ phage infection as shown in Fig. 1. However, this abrupt termination of T7-specific RNA polymerase synthesis in gene 2 mutant phage-infected cells is unlikely to be due to the inactivation of *E. coli* RNA polymerase because *E. coli* RNA polymerase actually retained its activity when assayed in vitro using an exogenous template T4 DNA as shown in Fig. 4. The result suggests that T7 DNA is impaired by the lack of the gene 2 product and is not functioning as the template for *E. coli* RNA polymerase.

T7 gene 2 has been implicated in the synthesis of T7 progeny DNA. A mutation in gene 2 results in a reduction in the rate of T7 DNA synthesis (5, 15). We have confirmed this notion by comparing the rate of DNA synthesis in T7$^+$ phage-infected and gene 2 mutant phage-infected cells. Recently, Center (5) has shown that the reduced synthesis of T7 progeny DNA in gene 2 mutant phage-infected cells is accompanied by the formation of DNA species which are smaller in size than the unit length of mature T7 phage DNA; no concatemer T7 progeny DNA molecule

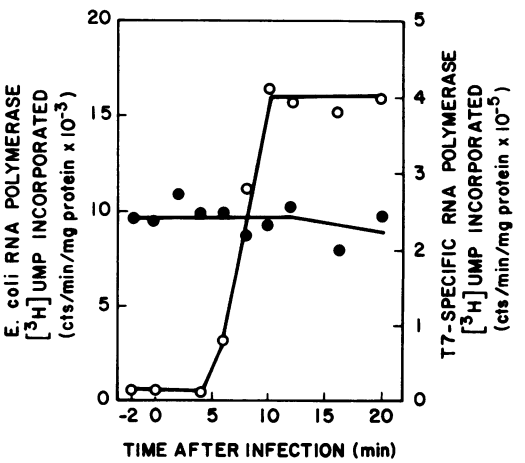

FIG. 4. *Activities of E. coli RNA polymerase and T7-specific RNA polymerase during T7 gene 2 mutant phage 2-64 infection. This experiment was performed by using the same procedure described for the T7$^+$ phage infection shown in Fig. 1. Symbols: ●, E. coli RNA polymerase activity; ○, T7-specific RNA polymerase activity.*

was found in gene 2 mutant phage-infected cells. Therefore, it is very likely that the termination of the synthesis of the T7-specific RNA polymerase at about 9 min after gene 2 mutant phage infection is due to the loss of template activity in T7 DNA, probably because of the loss of the promoter sites which are located near the left end of the T7 DNA. In this aspect, the product of gene 2 appears to be required either for the formation of T7 progeny DNA concatemers or for the protection of T7 DNA from nucleolytic cleavage by a yet unknown function of the host.

Due to the difficulty in identifying the product of T7 gene 2, mainly because of its small size, the role played by the gene 2 product in T7 progeny DNA synthesis has not been well understood. We now propose a dual function of the gene 2 product, one role as the inhibitor I protein of *E. coli* RNA polymerase and another as a protective agent for the production of T7 progeny DNA. Since we have already isolated gene 2 protein as a pure protein, i.e., the I protein (9), we are currently investigating the second function of gene 2 in T7 DNA synthesis.

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