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Negative Control of Protein Synthesis after Infection with Bacteriophage T7

(*E. coli*/*S*-adenosyl methionine cleavage/T3 phage/RNA polymerase)

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ABSTRACT T7 phage induces two negative control mechanisms of protein synthesis: (*a*) Host-gene expression is repressed by a "T7 repressor," and (*b*) early T7 protein synthesis is inhibited by a late phage protein.

(*a*) The repressor for host enzyme synthesis is an early T7 protein. Its gene is none of the known early genes; it is located promotor-proximal to gene 1. The repressor function of this protein can be demonstrated by DNA-dependent enzyme synthesis *in vitro*.

(*b*) Expression of early phage gene is depressed by a late phage protein or by T7 RNA polymerase. Control takes place on the level of transcription.

The mechanisms by which bacteriophage T7 regulates protein synthesis are only partially known. Soon after infection, a group of phage proteins (early proteins) is synthesized temporarily in a sequential manner. This time sequence is caused by a sequential arrangement of the early genes in an early transcription unit (1). The early transcription unit is transcribed by host RNA polymerase, and corresponds to a total RNA of 2.4×10^6 daltons (2). Within the early region, which is located at one end of the T7 genome, with the promotor close to the end (3), the genes for T7 RNA polymerase (4), ligase, the unknown gene-2 product, endonuclease, and lysozyme are clustered (1). Phage-specific polymerase transcribes the late genes (5). Parallel to these positive controls of phage protein synthesis, host RNA and protein synthesis ceases during phage T7 development. Protein synthesis is necessary for inhibition of host-enzyme synthesis (6). Formation of phage polymerase does not seem to be required for interference with protein synthesis of the host. Production of T7 RNA polymerase, however, is necessary for another negative control: the depression of T7 ligase synthesis (1). The mechanisms of both negative regulations are unknown.

Here, we report that for the repression of host-protein synthesis an early protein of T7 is essential. This "repressor" protein is not the product of one of the known early genes. Its gene is located promotor-proximal to the RNA polymerase gene. The "T7 repressor" probably acts on the level of initiation of protein synthesis. The formation of early T7 proteins is depressed by a mechanism that needs newly synthesized T7 RNA polymerase.

METHODS

Protein Synthesis In Vitro. "Brij-extracts" (7) and "DEAE-system (8) were described (see Fig. 2).

Crude Extracts for Enzyme Assays. For the determination of most enzyme activities *in vivo*, 5 to 8×10^8 cells were harvested on ice containing 50 μg/ml of chloramphenicol, and lysed by the Brij-lysozyme method for low concentrations of cells (7). For determination of lysozyme activity, the cells were either sonicated or lysed by freeze-thawing (5×10^8 cells/0.1 ml Tris-Mg-Acetate (10 mM Tris·HCl pH 7.5–10 mM $MgCl_2$–22 mM NH_4Cl with 100 μg/ml of albumin). For β-galactosidase assays, aliquots of the culture were treated with toluene directly.

Enzyme Assays. *S*-Adenosyl methionine-cleaving enzyme (9), lysozyme (8), ligase (1), and endonuclease (1) were measured as described. β-galactosidase tests were as described (10). *Escherichia coli* RNA polymerase assay during the

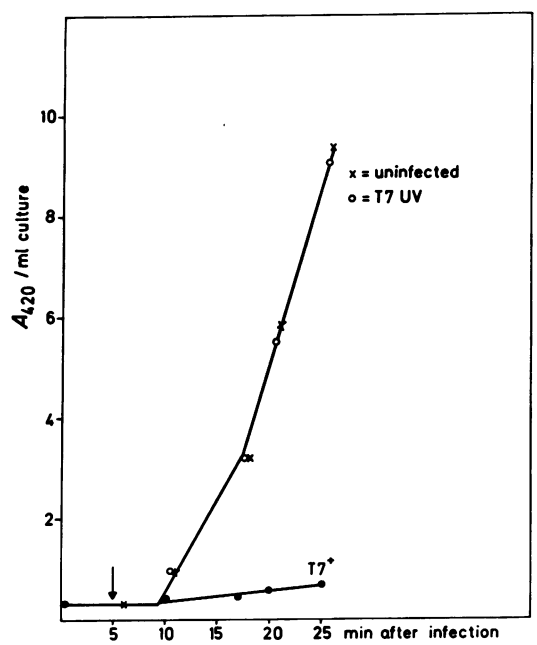

FIG. 1. β-Galactosidase induction after infection with various mutants of bacteriophage T7. *E. coli* B_{s-1} was grown in Mg medium and concentrated to 5×10^9 cells/ml in Mg-free M9 buffer at 30°. Aliquots of 0.5 ml were mixed with phage (MOI = 10). This time is time zero of the *abscissa:* After 5 min, the cells were transferred to 10 ml of M9–glycerol medium containing 1 mM isopropyl-*l*-thio-β-D-galactoside (IPTG) (*arrow*). UV-irradiated phage received a dosage that would suffice to inactivate *S*-adenosyl methionine-cleaving enzyme synthesis in T3 50%. The infection was performed in dim yellow light. Shut-off of β-galactosidase synthesis caused by $T7^+$ was also seen in cells infected with T7 H13 (gene 1^-) and with double mutants in gene 1 and the following genes: 1.5 (ligase), 2, 3 (endonuclease), 3.5 (lysozyme).

TABLE 1. *Activity of partially purified RNA polymerase after T7 infection*

Source	DEAE-cellulose fraction*	Total volume (ml)	Protein (mg/ml)	Total activity (units) with various DNA templates: T7 DNA	T4 DNA	poly d(A–T)	Calf-thymus DNA
Uninfected *E. coli* B	0.13	4	0.74	— (<2)	—	—	<2
	0.26	3	0.22	80 (<2)	131	318 (<2)	39
E. coli B Infected with T7 H13	0.13	4	1.83	— (<2)	—	—	<2
	0.26	3	0.40	71 (<2)	151	338 (<2)	43
E. coli B Infected with T7+	0.13	4	1.70	— (110)	—	—	<2
	0.26	3	0.48	37 (13)	92	175 (<2)	28

Partial purification of host RNA polymerase from infected cells: *E. coli* were grown to a cell density of 8×10^8/ml in 1 liter of M9 (18) medium supplemented with 0.5% casamino acids and 0.4% glucose. The cells were harvested and resuspended in 30 ml of M9 buffer (18). The suspension was divided. 10 ml remained uninfected, 10 ml received T7+, and 10 ml received T7 H13, both at a multiplicity of infection of 12. The phage were allowed to adsorb at 10° for 4 min. The cells were then transferred to 400 ml of warmed M9 medium and shaken with optimal aeration at 33° for 10 min. The cells were collected on ice containing 20 μg/ml of chloramphenicol. RNA polymerase was then isolated (11) from all three samples. 2 ml of the $(NH_4)_2SO_4$ fraction (Fraction 3) were applied to a DEAE-cellulose column (pasteur pipette, column volume 1 ml) that had been equilibrated with buffer A (11). The column was washed with buffer A and enzyme fractions were eluted stepwise with 4 ml of 0.13 M KCl, 3 ml of 0.26 M KCl, and 2 ml of 0.5 M KCl (all in buffer A). The 0.13 M KCl fraction contained most of the T7-specific RNA polymerase, while the 0.26 M KCl fraction contained most of the host polymerase.

Enzyme units reflect the activity of the enzyme at 0.15 M KCl, the units given in parentheses were obtained by assay at 0.02 M KCl with 20 μg/ml of rifampicin present. Polymerase assays: The incubation mixtures contained in 0.1 ml; 0.04 M Tris·HCl (pH 7.9), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.15 M KCl, 0.4 mM potassium phosphate, 0.5 mM (each) of ATP, CTP, GTP, and [^{3}H]UTP (with poly d(A–T) as template, GTP and CTP were omitted), 0.5 mg/ml of bovine-serum albumin, 2.5 μg of T7 DNA, 2.5 μg of poly d(A–T), 5 μg of T4 DNA, or 10 μg of calf-thymus DNA, and a 10-μl aliquot of the fraction to be tested. Incubation at 37° for 10 min.

* Expressed as molarity of KCl used to elute the fraction.

purification procedure was described (11). Units were defined as the amount of enzyme incorporating 1 nmol of UMP at 37° in 10 min (12).

Extraction of RNA from E. coli Infected with T7 Phage. The procedure for mRNA isolation was modified from one described (13). We used an SuA strain as the host, although we have not observed polarity in T7.

RESULTS

Regulation of host enzymes

Induction of β-galactosidase and derepression of alkaline phosphatase are prevented by infection with T7+ or with T7 gene-1 mutants. However, ultraviolet irradiation of T7+ before infection prevented the phage from manifesting this repression (Fig. 1). This experiment indicates that phage DNA-directed protein synthesis is essential for the repression of host-enzyme synthesis. The repressor is an early phage protein, since gene-1 mutants inhibit host-enzyme induction. Within the "early" region of the T7 genome, 5 genes have been characterized. None of these known early genes is the gene for the T7 repressor (Fig. 1): double mutants of gene 1 with the genes 1.5 (DNA ligase), 2 (unidentified protein), 3 (endonuclease), or 3.5 (lysozyme) all interfered with host enzyme formation.

Induction of β-galactosidase is inhibited after the third minute of infection (Fig. 2). To elaborate the mechanism of repression of enzyme induction, cell-free extracts from T7-infected cells were prepared, and the capacity for T3 DNA-directed synthesis of *S*-adenosyl methionine-cleaving enzyme *in vitro*, mediated by *E. coli* RNA polymerase, was used as an indicator of T7 repressor activities. Extracts prepared from cells later than 3 min after infection had increased repressor activities (Fig. 2). Rates of protein synthesis in these extracts were about three times lower than in intact cells. Therefore, it is unlikely that the repressor was synthesized in the cell-free extracts during the incubation for enzyme synthesis. The repressor activities of the extracts reflect the amounts of repressor present in the cells at the time of harvest.

Detailed analysis of the time course of enzyme synthesis *in vitro* and its inhibition by the T7 protein indicates that initiation of transcription or translation was prevented (Fig. 3). In the *in vitro* synthesis reaction, 5 min was needed for the appearance of the first enzyme activity. However, inhibition decreased immediately without any distinct lag period after start of the reaction (Fig. 3). In case the repressor affected a step other than initiation, a period of complete inhibition, corresponding to the time needed for completion of the first mRNA for *S*-adenosyl methionine-cleavage enzyme or of enzyme itself, should be expected.

Transcription on the host genome seems to be reduced soon after T7 infection (6). However, the host RNA polymerase itself is not altered by the T7 repressor. The polymerase could be purified from T7 cells, and there was only little decrease of activity (Table 1). This decrease could be caused by T7-specific nucleases, which are eluted from DEAE-cellulose at the same salt concentration. In addition, it can not be excluded that some T7 repressor might still be present in the partly-purified RNA polymerase.

Regulation of T7 early proteins

As was shown previously, T7 mutants in gene 1 (polymerase) induce higher amounts of ligase than does T7+ (1). After appearance of T7 RNA polymerase the rate of ligase formation is reduced. The reduction of ligase synthesis is caused by inhibition of transcription later than 7.5 min after infection (Fig. 4). In consequence, cells harvested later than 7.5 min after infection with mutants defective in gene 1 contained more messenger RNA for ligase than did cells infected with T7+. This was found by use of the extracted RNAs as templates for cell-free enzyme synthesis. In parallel, the T7+ *in vivo* RNA had much less *in vitro* template activity for polymerase synthesis than did H13 RNA (not shown). Since the gene-1 mutant was an amber mutant, it could not induce polymerase formation in nonpermissive cells. However, in the *in vitro* system, the amber mutation could be suppressed by addition of suppressor tRNA and the messenger activity could be measured.

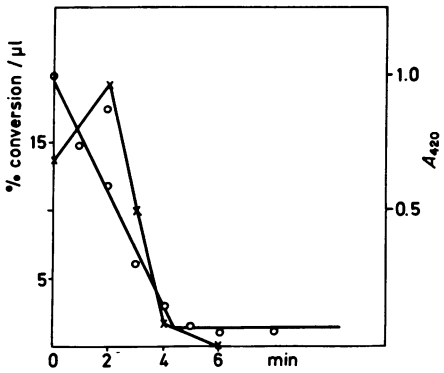

FIG. 2. Rate of formation of "T7 repressor," measured by enzyme synthesizing capacity of crude extracts at various times after infection with T7 phage and the inducibility of β-galactosidase. *E. coli* K12 514 was grown in rich medium at 30°, harvested at 4 × 10^8 cells/ml, and resuspended in cold magnesium-free M9 buffer to give a density of 1 × 10^{10}/ml. T7+ were allowed to adsorb at a MOI of 10 at 10° for 3 min. The infected cells were transferred to rich medium of 30° to give a density of 4 × 10^8/ml. Time of transfer is time zero of the *abscissa*. The surviving cell count was below 1% at 1 min. Aliquots of about 1 × 10^{10} cells were harvested on frozen TMA buffer at various times after infection; the cells were pelleted, washed twice with TMA buffer, and lysed by the Brij-lysozyme method (for concentrated cells) (7). The lysates were mixed vigorously on a Vortex mixer. T3 DNA-dependent synthesis was in 0.05-ml incubation mixtures containing 20 µl of cell extract, with the ion concentrations derived from the cell content plus the ingredients (0.01 M $MgCl_2$–3.6 mM EDTA) of the lysis method; in addition to these: 30 mM NH_4Cl, 13 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM potassium acetate, 50 mM Tris·HCl (pH 8.0), 2 mM ATP, 0.5 mM (each) of CTP, GTP, and UTP, 20 mM phosphoenolpyruvate, 0.2 mM of each of 20 amino acids, 50 µg of stripped tRNA from *E. coli* B, 6 µg of Ca-leucovorin, and 5 µg of T3 DNA. After incubation for 40 min, enzyme activity was determined. ×—× *S*-Adenosyl methionine-cleaving enzyme synthesis of extracts at various times after infection. The ability to induce β-galactosidase was tested by induction of cells in M9 (0.5% glycerol) medium with (IPTG) (see legend of Fig. 4) at various times after infection with T7 H13. In this case, the infection was done at 300 at a density of 4 × 10^8/ml. Aliquots of 0.5 ml were collected at 10 min after induction, and the time of induction was plotted against the resulting β-galactosidase activity (A_{420}/0.5 ml culture, O——O).

DISCUSSION

Inhibition of host-protein synthesis requires synthesis of a phage protein, as demonstrated by the inability of UV-irradiated T7 phage to disturb the host functions. The inhibiting protein is the product of an early T7 gene, since mutants in gene 1 interfered with host-enzyme synthesis, although these mutants did not induce late phage proteins. None of the known early T7 genes corresponds to the repressor. From the rate of *in vivo* induction of the repressor, which was measured by the capacity to synthesize enzymes in cell-free extracts from cells at various times after infection, it can be concluded that the corresponding gene is located promotor-proximal to the phage polymerase. The first repressor appears after a lag period of 2.5 min, whereas phage polymerase does not appear before 4.5 min after infection. If one takes the time needed for synthesis of T7 polymerase into account, the repressor gene should be located just in front of gene 1.

Most likely, the repressor affects initiation, since the kinetics of repressor action on enzyme synthesis *in vitro* does not show any distinct period of total prevention of *S*-adenosyl methionine-cleavage enzyme synthesis. Inhibition of other steps, such as elongation or termination of RNA or elongation of polypeptide chains, would result in a period of total inhibition. This period would be about as long as the lag period of enzyme synthesis. Inhibition of ribosome–mRNA complex formation, however, would also result in kinetics similar to those found, if we assume that this complex is formed exclusively

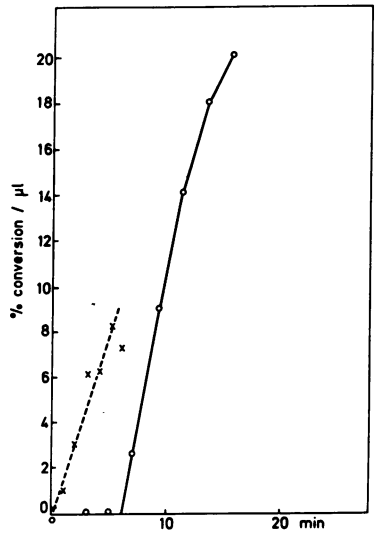

FIG. 3. Kinetics of *S*-adenosyl methionine-cleaving enzyme synthesis *in vitro* by crude extracts, and its inhibition by "T7 repressor." Extracts from uninfected *E. coli* 514 were obtained as described in Fig. 2, and incubated under protein synthesis conditions—as in Fig. 2—at 37° with T3 DNA as template. After various periods of time, aliquots of 20 µl were taken and either harvested on ice and chloramphenicol (O——O) (*a*) or mixed with 5 µl of extract from cells 6 min after infection with T7+ (plus 5 µl of $MgCl_2$ and NH_4Cl solution to keep the ion concentrations constant), and incubated further for 15 min (×----×) (*b*). The enzyme activity finally measured, corresponds to (*a*) the amount of enzyme completed at the time of chloramphenicol addition, and (*b*) to the amount of enzyme that had been completed or still could be completed at the time of T7+ extract addition, respectively. Activity is given in % conversion to methyladenosine.

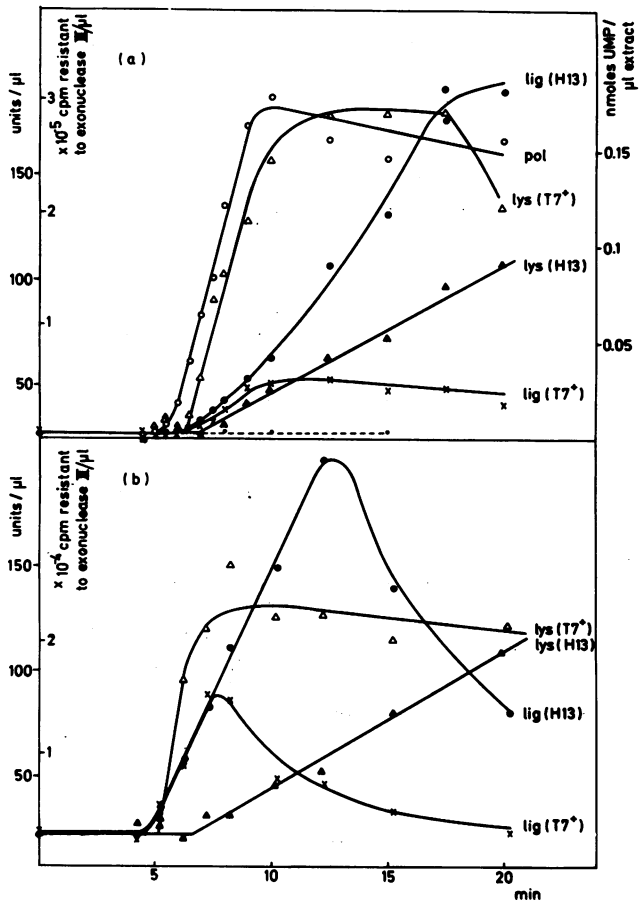

FIG. 4. Enzyme and messenger RNA synthesis in cells infected with T7 H13$^+$ and T7 H13. XA 7007 was grown in 0.5% tryptone–0.5% yeast extract–0.5% NaCl to a density of 4×10^8/ml. The cells were resuspended in M9 buffer to a concentration of 1×10^{10} cells/ml. Phage was added at a MOI of 7, and allowed to adsorb at 10° for 3 min. The cells were transferred to warmed rich medium at 4×10^8 cells/ml. Aliquots of 2 ml were harvested for the preparation of crude Brij-extracts and the determination of enzyme activities. Aliquots of 20 ml were collected on 10 ml of frozen buffer [0.01 M Tris (pH 7.6)–0.01 M KCl]. These aliquots were pelleted and the cells were resuspended in 0.8 ml of cold buffer (as above). Egg-white lysozyme (200 μg/ml) and EDTA (to give 1.0 mM) were added. The mixture was allowed to stand at 4° for 10 min, then treated twice by freeze–thawing. Pancreatic DNase I (RNase free, Worthington) and $MgCl_2$ were added to a final concentration of 20 μg/ml and 20 mM, respectively. After 5 min at 21°, clear nonviscous lysates were obtained by treatment with 1% sodium dodecyl sulfate, and 0.2 M sodium acetate (pH 5.2) was added. After two extractions with redistilled water-saturated phenol, the aqueous phase received another 0.1 ml of 2 M sodium acetate (pH 5.2) and the nucleic acids were precipitated by ethanol. The precipitates were washed, dried, and dissolved in 0.2 ml H_2O, yielding a solution of about 80 A_{260}/ml. Enzyme assays as in *Methods*. Activity of T7 phage RNA polymerase was tested in Brij-extracts of cells after infection. The incubation mixtures contained in 0.05 ml: 20 μl Brij-extract, 30 mM $MgCl_2$ (additionally to compensate for EDTA), 40 mM NH_4Cl, 5 μg of T7 DNA or T3 DNA, 2 mM (each) of ATP, CTP, GTP, and [^{3}H]UTP, 400 μg/ml of bovine-serum albumin, 1 mM dithiothreitol, 80 mM phosphoenol-pyruvate, 20 mM Tris·HCl (pH 8.0). The extracts were preincubated with 100 μg/ml of rifampicin at 37° for 5 min, and rifampicin was present during subsequent incorporation. Incubation time was 30 min at 30°. (*a*) Enzyme activities of extracts at various times after infection: O——O, phage RNA polymerase in cells infected with T7 H13$^+$; ●– – –●, rifampicin-resistant RNA polymerase in cells infected with T7 H13; ●——●, DNA ligase activity (T7 H13), ×——× DNA ligase (T7 H13$^+$); △——△, T7 lysozyme (T7 H13$^+$), ▲——▲ Lysozyme (T7 H13). (*b*) mRNA content was determined by use of the isolated RNA as template in the purified cell-free system. The resulting enzyme activity corresponds to the amount of specific mRNA that was present at the time of harvest of the cells. ●——●, ligase mRNA in T7 H13 cells; ×——×, ligase mRNA in T7 H13$^+$ cells; ▲——▲, lysozyme mRNA in T7 H13 cells; △——△, lysozyme mRNA in T7 H13$^+$ cells.

at the 5′ end of RNA in DNA-dependent *in vitro* synthesis, an hypothesis that has indeed been shown to be likely (2, 14). The conclusion, that the T7 repressor affects the initiation of transcription or translation, can also be drawn from indirect results; the time course of prevention of β-galactosidase induction and the lag period of induction of repressor *in vivo*. The lag period of β-galactosidase induction at 30° is less than 5 min. Complete prevention of induction takes place when the inducer is added later than 3.5–4 min after infection. Induction at 2 min after infection—as an example—still allows some enzyme to be synthesized at 7–8 min after infection. The repressor, however, appears at 3–4 min after infection. The repressor cannot affect enzyme synthesis after initiation.

From the present data, transcription and translation as possible targets of the "T7 repressor" cannot be distinguished. Decreased UMP incorporation after T7 infection (6) could also be caused by fast digestion of unprotected mRNA after inhibition of translation.

Repression of gene expression is specific. Whereas the T7 repressor, which is formed 2.5–3.5 min after infection, efficiently inhibited the induction of β-galactosidase *in vivo* and T3 DNA-dependent enzyme synthesis *in vitro*, it did not depress T7 protein synthesis. Ligase mRNA and ligase, as well as lysozyme mRNA and lysozyme, were synthesized in cells infected with gene-1 mutants with constant rates for more than 12 min. The action of the inhibitor should be apparent earlier, at 7–8 min. The specific repression by the T7 repressor may explain mutual exclusion (15). This mechanism enables T7 to exclude coinfecting phages, like T3.

Recently, an inhibitor of transcription in T3-infected cells was described (16). Since T3 is related to T7, one could imagine that this T3 inhibitor might be closely related to the T7 repressor. However, this does not seem to be the case. In contrast to the T7 repressor, the T3 inhibitor appears to be a late protein, as indicated by the time of induction *in vivo* (16). It was shown that prior infection with either UV-irradiated T3 or with T3 gene-1 mutants—which still could induce the early proteins, except for the gene-1 product—did not reduce significantly phage T4 development. This observation indicated that no early T3 protein inhibits the action of the host RNA polymerase, which is essential for T4 development (17). In addition, the T3 inhibiting protein interferes more effectively with transcription on T3 DNA than with transcription on *E. coli* DNA (16). In contrast, the T7 repressor inhibits the expression of the *E. coli* genome efficiently, but not expression of the phage genome. For these reasons, it is unlikely that the T7 repressor is related to the inhibiting protein from T3-infected cells.

Expression of the early T7 proteins is inhibited after formation of T7 RNA polymerase. Phage polymerase could be

directly involved. However, participation of a late protein in depression of ligase synthesis is very likely, since the ligase mRNA formation is stopped as soon as late proteins appear.

Since the synthesis of ligase messenger RNA was shut-off immediately after late T7 proteins had been formed, it can be concluded that this "late inhibitor" blocks elongation of RNA rather than initiation. Blockage of the initiation would not stop the production of ligase mRNA before 4.5 min after appearance of late proteins. Late proteins appear at 7–7.5 min; inhibition should be effective only after 11.5–12 min after infection. Therefore, the "late inhibitor" should affect elongation of RNA synthesis.

The question remains, what stops ligase mRNA synthesis in gene-1 mutants? A possibility would be that the same inhibitor that switches off ligase mRNA formation in cells infected with $T7^+$ is responsible. The delay in establishing the transcriptional block would be the result of the longer period needed for *E. coli* RNA polymerase to reach the gene of the inhibitor than the period needed for transcription of this gene by T7 polymerase. Indeed, the lysozyme gene, for instance, is transcribed later when *E. coli* polymerase has to read all the way to this gene than when it is transcribed by T7 phage polymerase, since the total stretch of DNA that must be copied in the latter case is smaller (1). Another possibility would be a third inhibition mechanism. The gene for this inhibitor should be located near the ligase gene, and its synthesis, therefore, should be mediated by *E. coli* polymerase 7–8 min after infection. Inhibition of initiation of transcription would then cause a switch-off at 11.5–12 min since the readout of started mRNA would take another 4.5 min. We cannot decide between these possible mechanisms of inhibition of early gene expression.

In conclusion: T7 induces at least two independent control mechanisms to repress protein synthesis. A T7 repressor specific for the shut-off of host protein synthesis, and a late phage protein that inhibits early T7 protein synthesis.

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