Gene Expression in Vitro from Deoxyribonucleic Acid of Bacteriophage T7*

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SUMMARY

The in vitro synthesis of lysozyme and RNA polymerase directed by DNA of bacteriophage T7 starts with different lag periods after addition of the template. The difference in lag period before appearance of enzyme could be attributed to a difference in the time needed for transcription. Assuming that the difference in lag phase is caused by a difference in the distance between the promoter and the corresponding gene, and taking into account the average rates of transcription and translation, the locations of the polymerase gene and the lysozyme gene on the phage genome were estimated. The distance between the promoter and the distal end of the lysozyme gene corresponds to an RNA of the molecular weight $2.2 \times 10^6$. The polymerase gene is closer to the promoter. Transcription of T7 DNA by Escherichia coli RNA polymerase in vitro does indeed result in a polycistronic messenger RNA containing the information for synthesis of both RNA polymerase and lysozyme.

A bacterial cell infected by a bacteriophage provides a simple system for studying the molecular basis of the regulation of gene expression. Bacteriophage T7 is particularly well suited for such a study since it is a relatively small phage. Its DNA has a molecular weight of approximately $2.7 \times 10^6$. Its genetics is well established (1) and T7 has a simple mechanism for activating phage functions (9). After infection, the RNA polymerase of the host cell transcribes efficiently only a minor portion of the T7 DNA (3), but this portion apparently contains the information for a new RNA polymerase (specified by gene 1) (4), which in turn efficiently transcribes the rest of the T7 genome. In the absence of gene 1 function, only the gene 1 protein, a protein of molecular weight close to 40,000, and one or more smaller proteins are made as efficiently as they would be in a wild type infection (2).

Previous experiments from our laboratory had shown that in an in vitro enzyme synthesis system, dependent on phage T4 DNA, synthesis of β-glucosyltransferase starts after a shorter lag phase than does synthesis of lysozyme (5).

The difference in the lag periods before synthesis of these enzymes was attributed to a process at the transcriptional level after the initiation of RNA synthesis (5). Two possible reasons were discussed for the difference in the lag phases (5). The delay in the appearance of lysozyme relative to β-glucosyltransferase in vitro could result from (a) a greater distance between the lysozyme gene and the start of transcription, or (b) a difference in the rate of transcription of the lysozyme and β-glucosyltransferase genes. We could not distinguish between these alternatives.

As a possible approach to resolve this problem we have used an in vitro T7 phage system. Here we present data which indicate that the appearance of two T7 enzymes, RNA polymerase (6, 7) and lysozyme (8), during in vitro protein synthesis, is a useful model to study the causes of different lag periods in enzyme synthesis. We found that with T7 DNA Escherichia coli polymerase transcribes a polycistronic messenger RNA containing the messages for both T7 RNA polymerase and lysozyme. The lag phase of enzyme synthesis reflects the location of the corresponding gene relative to the starting point of synthesis of the polycistronic messenger RNA. Since E. coli polymerase starts near one end of T7 DNA (9), the lag phases of enzyme synthesis give information about the positions of the genes on the genome.

EXPERIMENTAL PROCEDURE

Bacteriophages—Preparation of wild type and amber mutants of T7 was carried out as in Reference 2. T7 amber mutants were gifts of Dr. Studier and Dr. Hausmann. 'T7 am 13' was isolated by Dr. Studier. T3 phage were grown in E. coli B by infecting at a concentration of 7 to $5 \times 10^8$ cells per ml in Tryptone broth, multiplicity of infection 1:100, at 30°, and waiting for complete lysis. T7 and T3 phage were purified by polyethylene glycol precipitation (10) and either two consecutive CsCl centrifugations, or sucrose gradient centrifugation followed by one banding in CsCl (11). Phage DNA was extracted by a mild phenol treatment and dialysis (11).

Bacteria—For preparation of the cell-free systems, E. coli 514 (K-12 (λ) lac-7) was used as the permissive strain, that is, in the majority of experiments, and E. coli K-38 en- (obtained from Dr. G. Streisinger) as the nonpermissive strain. E. coli 514 was inhibited with trimethoprim for 30 min immediately before harvesting (12). For the cell-free systems cells were grown in rich...
purified RNA polymerase (a unit is that amount of enzyme which incorporates 1 nmole of UTP per 10 min at 37° C under substrate-saturating conditions), 40 mM phosphoenolpyruvate, 4 mM ATP, 1.0 mM of each of the other triphosphates, 0.4 mM each of 20 amino acids, 100 mM potassium acetate, 1 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0, and 10 mM MgCl₂. After 15 min at 37° C, RNA synthesis was stopped by the addition of actinomycin D (20 µg per ml). Then translation was allowed to take place by dilution of this reaction mixture to a final volume of 0.05 ml by the addition of 50 µg of stripped tRNA (from E. coli K-12 or B), 500 µg of ribosomes, 120 µg of S100 protein, and in (final concentrations) 100 mM NH₄Cl, 0.1 mM N₅-formyltetrahydrofolic acid, and varying amounts of magnesium acetate. Incubation was continued for an additional 15 min.

E. coli RNA polymerase was prepared either according to the method of Burgess (15) or, for the in vitro synthesis and purification of T7 mRNA, as previously described (16). Assays for lysozyme activity and T7 RNA polymerase were performed as described previously (6, 17).

In Vitro Synthesized T7 Messenger RNA—As in the “uncoupled” system, T7 mRNA was transcribed from T7 DNA with highly purified RNA polymerase from E. coli. The conditions for synthesis were as previously published (16) but with T7 DNA (100 µg per ml) and different ionic conditions: 130 mM NH₄Cl and 10 mM magnesium acetate. After 30 min of synthesis the reaction mixture (1.5 ml) was made to 0.2 ml with sodium dodecyl sulfate and the RNA isolated by sedimentation through CsCl (18) and two subsequent ethanol precipitations. The RNA was redissolved in 0.5 ml of sodium dodecyl sulfate buffer containing 0.1 M NaCl, 1 mM EDTA, 10 mM Tris-acetate, pH 7.5, and (0.5%), w/v, sodium dodecyl sulfate) and centrifuged through a 12.5 ml sucrose gradient (5 to 30%, w/v, in the same buffer mixture) at 41,000 rpm and 20° C for 24 hours in the Spinco SW 41 rotor. The main RNA peak was precipitated twice with ethanol and finally dissolved in 0.5 ml of 10 mM Tris-acetate, 1 mM EDTA, pH 7.5.

Total mRNA was prepared from T7+-infected cells 10 min after infection at 30° C (15).

RESULTS

Magnesium Ion Optima of Enzyme Synthesis—Like enzyme formation directed by T4 DNA, (19), T7 DNA-directed enzyme synthesis in vitro is critically dependent on magnesium ion concentration. Fig. 1 shows the influence of different magnesium ion concentrations on synthesis in vitro of T7 RNA polymerase and lysozyme. RNA polymerase was synthesized best at 10 to 11 mM Mg²⁺. This optimum was similar to the one for the RNA synthesis of T4 β-glucosyltransferase (19) or dCMP deaminase (20). However, the curve for T7 lysozyme synthesis differed remarkably from the curve for production of the T4 enzyme (19). Synthesis of T7 lysozyme was maximal at approximately 11 and 15 mM Mg²⁺ and had a distinct shoulder at 15 mM (Fig. 1), whereas synthesis of T4 lysozyme is best at a Mg²⁺ concentration of 15 mM (19). To determine whether one of the T7-lysozyme optima was attributable to optimal translation conditions, RNA was synthesized using purified RNA polymerase, T7 DNA, nucleoside triphosphates and salts (10 mM magnesium). This in vitro produced RNA was then translated in the cell free system in the presence of various magnesium ion concentrations. The Mg²⁺ optimum for lysozyme translation was found to be 13 mM (Fig. 1). In the coupled system, the optimum at 10 to 11 mM was in the same range of magnesium ion concentration, in which DNA-dependent in vitro synthesis of “early” phage enzymes pro-

![Fig. 1. Dependence of T7 DNA-directed synthesis of lysozyme in vitro on Mg²⁺ concentration. The reaction mixtures for enzyme synthesis were incubated at various concentrations of Mg²⁺ (chloride). After 40 min at 37° C, UMP-incorporating activity and lysozyme activity were measured. For determination of the Mg²⁺ optimum of translation, T7 DNA was incubated with purified E. coli RNA polymerase and triphosphates in the presence of 11 mM Mg²⁺ for 15 min, and actinomycin D was then added together with the other components of the complete cell-free system (see “Experimental Procedure”). The complete reaction mixtures were incubated further at various Mg²⁺ concentrations and, after 15 min, assayed for lysozyme activity. Corresponding results were obtained with T3 DNA in both the coupled and uncoupled system. The arrows point to the Mg²⁺ optima for synthesis of T4 β-glucosyltransferase (T4 βgt), T4 lysozyme (T4 LYS), and T7 RNA polymerase (T7 Polym), determined in the same system. O—O, lysozyme in the coupled system, counts per min in 0.2 ml of the supernatant fluid from the lysozyme assay; the aliquot assayed for lysozyme activity corresponded to 250 µg of ribosomes during protein synthesis. The lysozyme assay was incubated for 30 min at 37° C. △—△, lysozyme in the uncoupled system (translation only), counts per min in 0.1 ml of the supernatant fluid from the lysozyme assay; aliquots again corresponded to 250 µg of ribosomes of the protein synthesis mixture. Incubation of the lysozyme assay was for 4 hours.

medium (1% Tryptone broth) with the additions described as glycerol medium by Fraser and Jerrel (13) and were harvested on frozen buffer (Tris, 0.01 M, pH 7.5) after growth to 5 x 10⁸ cells per ml.

The protein-synthesizing system was prepared as described previously (14). With the exceptions mentioned, ribosomes and supernatant enzymes were from a single extract prepared from E. coli 514. Therefore, changes in the capacity to synthesize proteins from one system to another were largely avoided. Incubations for protein synthesis were identical with those described earlier (14). The Mg²⁺ concentrations at which reaction mixtures were incubated are given in the legends to figures, as is the time of incubation.

“Uncoupled” DNA-dependent Protein-synthesizing System—For the determination of the Mg²⁺ optimum of translation, transcription was allowed to proceed in the absence of translation. RNA was first synthesized in a final incubation volume of 0.025 ml which contained 2 to 3 µg of bacteriophage DNA, 2 units of purified RNA polymerase (a unit is that amount of enzyme which incorporates 1 nmole of UTP per 10 min at 37° C under substrate-saturating conditions), 40 mM phosphoenolpyruvate, 4 mM ATP,
The incubation time of the lysozyme assay was 1 hour at 37°. Lysozyme activity is given as the counts per min in 0.2-ml aliquots excorporated from assay filters per hour. The aliquots were then assayed either for lysozyme or T7 RNA polymerase activity. The incubation time of the lysozyme assay was 1 hour at 37°. Lysozyme activity is given as the counts per min in 0.2-ml aliquots excorporated from assay filters per hour. The amount of lysozyme present in 25 μl is equivalent to the amount synthesized by 350 μg of ribosomes. O—O, lysozyme synthesis at 11 mM Mg²⁺; Δ—Δ, lysozyme synthesis at 16 mM Mg²⁺; □—□, polymerase activity at 11 mM Mg²⁺; ▪—▪, measured as UMP incorporation into trichloroacetic acid-precipitable material during incubation at 37° for 20 min. The specific activity of [3H]UTP was 50 Ci per mole, the counting efficiency approximately 10 to 15%. For further information on the assay see Reference 6.

Kinetics of Appearance of Enzyme Activity and Messenger RNA and of Initiation of Messenger RNA Synthesis—The first type of kinetic experiment measures the minimum time for synthesis of complete enzyme molecules. In this case chloramphenicol is added at various times to block transcription, and only those proteins which have been completed (or completed enough to exhibit enzymatic activity) will be measured. When this experiment was done at 11 mM Mg²⁺, rifampicin-resistant UMP-incorporating activity (T7 RNA polymerase) first appeared between 2 to 3 min and lysozyme activity approximately 7 min after the start of the reaction. At 16 mM magnesium, lysozyme appeared after approximately 5 min (Fig. 2).

The second type of kinetic measurement gives an indication of the minimum time necessary for the completion of the message for a particular enzyme. In these experiments actinomycin is added at various times to stop the growth of RNA chains, and then the incubation is continued to permit the translation of the messenger RNA that had been synthesized. Enzymatic activity will appear only if the RNA chain includes the complete message. When this experiment was done at 11 mM magnesium, lysozyme message did not appear until between 4 and 5 min after the start of the reaction. The lysozyme-synthesizing capacity appeared somewhat earlier at 16 mM Mg²⁺ (Fig. 3).

In order to measure the kinetics of initiation of messenger RNA synthesis rifampicin is added at various times to prevent further initiation of RNA chains and, then, the reaction mixture is further incubated to permit completion of RNA chains that had already been started. The earliest time of addition of rifampicin that will still permit the appearance of enzymatic activity gives an indication of the time at which the messenger RNA chain carrying the information for that enzyme was initiated. From Fig. 4 it appears that the messenger RNA chains for lysozyme were initiated within the first minutes at either 11 or 15 mM Mg²⁺ (Fig. 4). These experiments on Mg²⁺ optima and kinetics give slightly variable results depending on the particular preparations used. However, in sets of experiments done at the same time, using the same preparations, the same relative differences in the Mg²⁺ optima and in the kinetics of appearance of the two enzymatic activities were repeatedly observed.

Characterization of in Vitro Synthesized Messenger RNA—Translation may be completely separated from transcription by carrying out the two synthetic steps in separate reactions. RNA was first transcribed from T7 DNA in vitro with purified RNA polymerase (21) from E. coli and isolated by treatment with sodium dodecyl sulfate and precipitation by centrifugation through CsCl (see “Experimental Procedure”). The main component was further purified from the minor lower and higher molecular weight components by sucrose density gradient centrifugation and again analyzed by gel electrophoresis (Fig. 5).

We previously reported that this RNA species is homogeneous with respect to its 3′ terminus (terminating in uridine) and that it is an active template for the synthesis of lysozyme in vitro (21).
Purified T7 RNA... 
Purified T7 RNA... 
No template... 
Total mRNA 
T7 DNA... 
T7 DNA... 
T7 DNA... 

| Experiment | Template used for protein synthesis | Inhibitor present during protein synthesis | Rifampicin-resistant UMP incorporation | Lysozyme units |
|------------|-------------------------------------|------------------------------------------|-------------------------------------|----------------|
| 1          | T7+ DNA                             | Rifampicin                               | 9,810                               | 8,100          |
| 2          | T7+ DNA                             | Rifampicin                               | 6,800                               | 6,800          |
| 3          | T7 am 23 DNA (gene 1)               | Rifampicin                               | 6,800                               | 6,800          |
| 4          | T7 am 27 DNA (gene 1)               | Rifampicin                               | 6,800                               | 6,800          |
| 5          | T7 am 94 DNA (gene 1)               | Rifampicin                               | 6,800                               | 6,800          |
| 6          | T7 am 27/193 DNA (double mutant in gene 1) | Rifampicin | 7,400                               | 7,400          |
| 7          | T7+ DNA plus Chloramphenicol         | Rifampicin                               | 680                                 | 680            |
| 8          | No template                          | Rifampicin                               | 3,090                               | 12,400         |

* Units are counts per min per hour per 250 μg of ribosomes.

This RNA is not only an active template in vitro for the synthesis of lysozyme, but also for T7 RNA polymerase (gene 1 product), as is shown by the appearance of a rifampicin-resistant UMP-incorporating activity (Table I). Inhibition of protein synthesis by chloramphenicol prevented formation of T7 polymerase. In the T7 DNA-directed enzyme synthesis more lysozyme was made in comparison to polymerase than in the RNA-dependent synthesis with purified RNA (Table I). This might be due to the secondary structure of the 2.2 × 10^6 dalton RNA. A hindrance of translation by the secondary structure of mRNA would affect the translation of distal message more than the translation of information at the 5'-end, assuming that RNA in vitro is also translated starting from internal initiation points (Fig. 8 in Reference 21, not so in DNA-dependent protein synthesis in vitro). This explanation is supported by the fact that with natural T7 mRNA as template, the ratio of lysozyme to polymerase is also lower than that observed with T7 DNA (Table I). That the appearance of T7 lysozyme and polymerase is due to de novo synthesis in vitro is demonstrated by the inhibition of their formation by inhibitors of protein synthesis (Tables I and II) and by the inability of DNA from RNA polymerase and lysozyme negative mutants of T7 to direct their synthesis (Table II).
host polymerase recognizes only this single starting point and transcribes only the first three to four genes.

Addition of T7 DNA to a protein-synthesizing system prepared from uninfected cells is similar to infection. In both cases, DNA is added to a system which is capable of expressing genetic information by synthesizing active enzyme molecules. If the control of gene expression is the same in both cases, one might expect that the three or four proteins made efficiently in vitro by gene 1 mutants would be made most efficiently in vitro, and the other T7 proteins (including lysozyme) would be made efficiently only if active T7 RNA polymerase were synthesized by the system. However, lysozyme seems to be made in vitro equally well whether active T7 RNA polymerase is present or not, as is shown by synthesis of lysozyme in vitro directed by DNA from phage with mutations in the polymerase gene (gene 1) (Table II). Thus, most lysozyme transcription in the in vitro system (which is prepared from uninfected cells) is due to the E. coli RNA polymerase; any T7 RNA polymerase that is made contributes little to the production of lysozyme under these reaction conditions.

Where does E. coli RNA polymerase initiate transcription under in vitro protein synthesis conditions? At 11 mM Mg²⁺ it must initiate to the left of gene 1 since active T7 polymerase is made. Under these conditions, T7 RNA polymerase first appears between 3 and 4 min earlier than does lysozyme (Fig. 2), thus placing the gene for T7 RNA polymerase (approximately 3000 nucleotides long) (2) closer to the initiation point than the gene for lysozyme. The approximate speeds of transcription and translation in this system are 28 nucleotides per second and 5 amino acids per second. From this, along with the data of Fig. 2, it can be calculated that the lysozyme gene should be approximately 6700 nucleotides distal to the initiation point. This corresponds to 2.2 × 10⁸ daltons of messenger RNA or approximately 17% of the length of T7 DNA. Thus, these data fit well with the idea of a single initiation point immediately to the left of gene 1, possibly corresponding to the single starting point found by Davis and Hyman (9). Furthermore, they agree with the observed in vitro transcription of a single mRNA molecule of 2.2 × 10⁶ daltons.

At 16 mM Mg²⁺, lysozyme is made almost as efficiently as at 11 mM, but T7 RNA polymerase is synthesized less well (Fig. 1). Under these conditions, T7 RNA polymerase first appears between 3 and 4 min earlier than does lysozyme (Fig. 2), thus placing the gene for T7 RNA polymerase (approximately 3000 nucleotides long) (2) closer to the initiation point than the gene for lysozyme. The approximate speeds of transcription and translation in this system are 28 nucleotides per second and 5 amino acids per second. From this, along with the data of Fig. 2, it can be calculated that the lysozyme gene should be approximately 6700 nucleotides distal to the initiation point. This corresponds to 2.2 × 10⁸ daltons of messenger RNA or approximately 17% of the length of T7 DNA. Thus, these data fit well with the idea of a single initiation point immediately to the left of gene 1, possibly corresponding to the single starting point found by Davis and Hyman (9). Furthermore, they agree with the observed in vitro transcription of a single mRNA molecule of 2.2 × 10⁶ daltons.

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