TRANSLATION OF BACTERIOPHAGE T4 mRNA INTO ACTIVE LYSOZYME BY A WHEAT GERM CELL-FREE SYSTEM

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Received October 20, 1976

SUMMARY: T4 bacteriophage mRNA for lysozyme was extracted from T4 phage infected E. coli cells, partially purified by column chromatography, and translated in a heterologous cell-free protein synthesizing system prepared from wheat germ. The translation product was confirmed by SDS polyacrylamide gel electrophoresis and enzymatic activity - bacteriolysis as tested with Micrococcus luteus. The specific activity of the enzyme prepared was 660 U/mg.

INTRODUCTION: We wanted to develop a system which would allow a comparison of the functional activity of isolated components of the protein biosynthetic machinery from normal and transformed cells. We were seeking a heterologous, easy to handle system which translates an isolated mRNA into an active enzyme. An enzyme as a translational product would be advantageous because not only the amount but the biophysical and biochemical activity of the product could be examined. Thus, for example, reduced fidelity of translation caused by altered ribosomes could be detected and studied.

The T4 lysozyme mRNA and wheat germ S30 system were selected for several reasons: The T4 lysozyme mRNA is relatively easy to isolate in an enriched form and was successfully translated in the E. coli in vitro system by Salser et al. (1). The S30 wheat germ fraction is easily prepared and compares favorably to the E. coli system. Low nuclease activity has made it possible to translate several messengers (2,3,4,5,8,14,15).

Thang et al. (6) reported obtaining active mouse interferon by translating its specific mRNA in the wheat germ system. Sarkar and Griffith (7) isolated and partially purified the mRNA for glutamine synthetase from polysomes of embryonic chick retina cells. They reported obtaining enzyme activity but no experimental evidence was included in their report.

In this communication we present evidence that the T4 lysozyme mRNA can be translated into the active enzyme in a heterologous system. The T4 lysozyme had a specific activity of 660 U/mg.

MATERIALS: T4 bacteriophage (wild type) was provided by Dr. H. Martin, Microbiology, TH Darmstadt. Escherichia coli MRE 600 RNase I^- and Micrococcus luteus were obtained from Merck, Darmstadt. Wheat germ was milled fresh by H. Matthes & Söhne, Ober-Ramstadt. Triphosphates were purchased from Pharma Waldhof, Düsseldorf. Unlabeled amino acids and spermine-HCl were from Serva, Heidelberg. Radioactive ^{3}H-Leu and ^{14}C-Phe was from Amersham-Buchler, Braunschweig. Creatine phosphate, creatine kinase, egg white lysozyme and Qβ RNA were from Boehringer, Mannheim. All other chemicals were from Merck, Darmstadt.

METHODS: E. coli MRE 600 RNase I^- were grown in minimal suspension medium to A_{630} = 0.8 and infected with wild type T4 phages at a multiplicity of infection of 4 as previously described by Salser et al. (1), modified by T. Eikhom (a personal communication) and altered by us in that the incubation temperature was lowered from 30° to 20°C during the 25 min infection cycle in order to prevent early lysis. Plaque counts were determined at 3 and 25 min after infection. 25 min after infection the culture flask was immersed in an ice bath for 10-15 min, centrifuged, and the cell pellet resuspended in 100 ml of 10 mM Tris-HCl buffer, pH 9.0, 10 mM NaCl, 5 mM $MgCl_2$. The lysis and phenol extraction procedure followed that of Salser et al. (1) except that the phenol extraction was carried out at 4°C for 30 min. Care was taken not to lose the mRNA in the interphase during the phenol extraction. T. Eikhom suggested that the lysozyme mRNA is trapped at the interphase complexed with protein. The phenol extractions were repeated until no protein remained at the interphase barrier. The total T4 RNA was dissolved in sterile water at a concentration of 6-7 mg/ml.

Sepharose 4B Column Chromatography: Total T4 RNA was applied to a Sepharose 4B column (1.0 cm x 100 cm) as described by Woo et al. (9). 15-20 mg of the RNA were suspended in 10 ml of 1 M LiCl, heated for 1.5 min at 70°C and quickly cooled in an ice bath before applying to the column which was developed at 4°C with 0.1 M NaOAc, pH 5.0, containing 1 mM EDTA at a flow rate of 4-5 ml/h. Fractions of 5 ml were collected and $A_{260/280}$ determined. Those peaks $A_{260/280} > 1.4$ were made 0.2 M NaCl and precipitated with 2 vol.

of cold absolute ethanol, dialyzed against double destilled water and assayed in the wheat germ system. 0.1 A_{260} of mRNA stimulated the incorporation of 35.5 pmoles of ^{3}H-Leu into protein.

Preparation of Cell-Free Extracts: The freshly milled wheat germ was ground using quartz sand following procedures published by Marcu and Dudock (10). The pre-incubation step reported by Roberts and Paterson (8) was found to increase activity. Also, tRNA's from wheat germ prepared according to Zubay (11) increased translational efficiency of the system by 30%.

Wheat Germ in Vitro Reaction Mixture: Contained in a final volume of 300 µl: 90 µl of pre-incubated S30 wheat germ, 20 mM HEPES, pH 7.6, 1 mM ATP, 1 mM Dithioerythritol, 0.1 mM spermine-HCl, 0.375 mM GTP, 8 mM creatine phosphate, 40 µg/ml of creatine phosphokinase, 4 mM $Mg(OAc)_2$, 100 mM KCl, 0.25 mM of 19 unlabeled amino acids, either 4 µCi ^{14}C-Phe (spec. act. 412) or 4 µCi ^{3}H-Leu (spec. act. 1,000), and 150 µg of wheat germ tRNA. The mixture was incubated at 28-30°C for 90 min.

The preparative reaction mixture used for the enzyme assay was increased to 1 ml maintaining the appropriate concentrations and incubation time to produce maximum protein synthesis.

SDS Polyacrylamide Gel Electrophoresis of Translation Product: Amino acid incorporation was monitored during incubation by removing 10 µl samples from the reaction mixture, pipetting on GF/A filters, drying, and precipitating the protein with 10% TCA at 90°C with sequential washing in 5% TCA at 20°C, ethanol, ethanol-ether (3:1 v/v), and ether. Radioactivity was determined by counting in 2 ml toluene scintillant. The efficiency of counting was 83% for ^{14}C and 25% for ^{3}H.

The mixture was adjusted to 10 mM EDTA at the end of the incubation period, immediately frozen, and lyophilized overnight. The freeze-dried reaction mixtures with and without mRNA were dissolved in 50 µl H_2O, adjusted to 2% SDS and heated at 60°C for 15 min. Following the addition of 1% 2-mercaptoethanol and 5 µl 0.05% bromphenol blue it was applied to 10% polyacrylamide gels prepared according to Weber and Osborn (12). 10 µg egg white lysozyme was used as an internal and external standard.

The gels were fractionated into 3 mm slices, dehydrated overnight at 65°C and dissolved in 1 ml 30% H_2O_2 at 65°C. Sucrose grains were added to neutralize the H_2O_2 and 5 ml Bray's solution

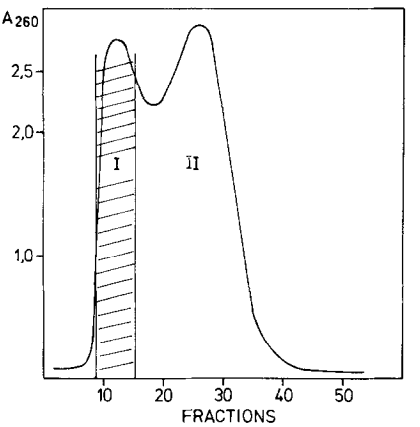

Fig. 1 Purification of crude lysozyme mRNA on a Sepharose 4B column. 18-20 mg of crude T4 lysozyme mRNA in 10 ml 1 M LiCl solution were applied to a Sepharose 4B column (1 cm x 100 cm) equilibrated 0.1 M NaOAc, pH 5.1. The column was eluted with the same solvent, flow rate 3 ml/h. The shaded area from peak I was pooled, precipitated with ethanol and tested in the wheat germ system.

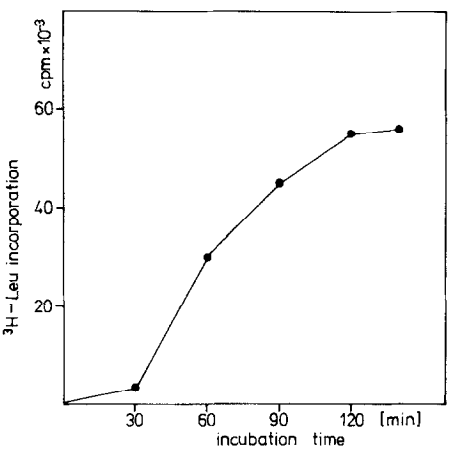

Fig. 2 Kinetics of protein synthesis as followed by ^{3}H-Leu incorporation. The kinetics were followed in a 1 ml preparative reaction mixture by removing 10 µl samples at the times indicated.

added to each sample which was allowed to stand overnight in the dark. Counting followed the next day.

The molecular weight was determined on the basis of mobility according to Weber and Osborn (12).

Lysozyme Activity Assay: A 1 ml reaction mixture, as stated above, was incubated with 250 µl of T4 RNA (6.8 mg/ml) for 90 min at

Table 1: Comparison of amino acid incorporation directed by Poly(U), Qβ RNA, crude Lysozyme mRNA, and Lysozyme mRNA purified by Sepharose 4B.

mRNA	CPM/100 µl	Stimulation (fold)
Poly(U)	188,388	42.8
Qβ RNA	14,520	3.3
Lysozyme mRNA	14,080	3.2
Lysozyme mRNA after purification - peak I	22,880	5.2
Control	4,400	-

300 µl incubation reaction mixtures were used and ^{14}C-Phe (spec. act. 412) as well as the corresponding concentrations of all other components.

28-30°C. The in vitro reaction was made 10 mM EDTA and immediately freeze-dried.

The freeze-dried reaction mixture was assayed according to Shugar (13). Micrococcus luteus was suspended in a balanced phosphate buffer (KH_2PO_4/K_2HPO_4), pH 7.1, to A_{450} = 0.8, the lyophilisate was dissolved in 100 µl of the same buffer, incubated at 37°C for 15 min, and added to a cuvette containing 400 µl of the adjusted M. luteus suspension. The decrease in absorbance was monitored on a Beckman Spectrophotometer with recorder at 1A, 100 nm/min, 1 inch/min. Activity is expressed as a measure of decreasing absorbance due to bacteriolysis measured against a reference of balanced phosphate buffer. As a control 10 µg egg white lysozyme were added to the assay mixture 5 min after the initial addition of the T4 mRNA synthesized lysozyme extract (See Fig. 4).

RESULTS: A five liter culture of T4 infected E. coli MRE 600 RNase I$^-$ normally yields 35-49 mg of T4 RNA of which only a small percentage is active lysozyme mRNA. The temperature change from 30° to 20°C during the infection cycle increased the yield to 55-60 mg RNA.

The RNA isolated by this procedure was further purified by Sepharose 4B column chromatography as per Woo et al. (9) with the difference that LiCl was increased to 1 M. Figure 1 shows the RNA elution profile and Figure 2 the kinetics of in vitro synthesis directed by lysozyme mRNA. The degree of stimulation as compared to other mRNA's is summarized in Table 1.

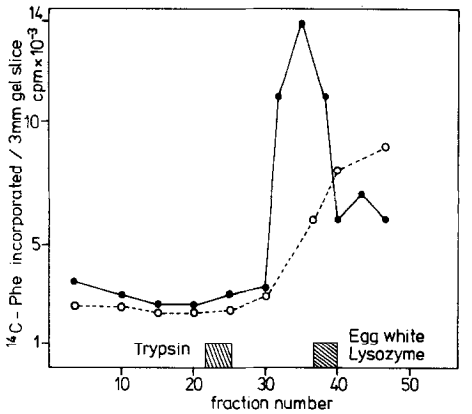

Fig. 3 SDS polyacrylamide gel electrophoresis profile of the proteins synthesized. The lyophylisate of a 300 µl reaction mixture containing 11.3 pmol protein was applied to the gel. 10 mg of egg white lysozyme and trypsin each were applied as internal standards. The filled circles show the separation pattern obtained with mRNA the open circles the minus mRNA as a control.

As reported by Davies and Kaesberg (2) an optimal concentration of 0.375 mM GTP was required to translate the Qβ RNA and the lysozyme mRNA. Below 0.100 mM no stimulation occurred. This holds true even in the presence of a triphosphate regenerating system.

SDS polyacrylamide gel electrophoresis of the in vitro product repeatedly rendered one definitive peak. Figure 3 represents the gel profile.

Our results compare favorably with those of Davies and Kaesberg (2) using the Qβ RNA in the wheat germ system. On the basis of mobility - ratio of fronts - the molecular weight of the in vitro product on the gel compared to egg white lysozyme, mol. wt. 14,300 and trypsin, mol. wt. 23,300 was shown to be 17,800. This, also, is in the reported range of 18,000 for T4 lysozyme.

Lysozyme lyses Gram positive bacteria by hydrolyzing the (α1-4) glycosidic bond of the glucosamines of peptidoglycan in the cell wall. Due to a decrease in absorbance because of this lysis, the activity of the enzyme can be expressed accordingly. Shugar (13) characterized the activity of egg white lysozyme. His results were used as a standard of comparison for our studies. Refering to Figure 4 it can be observed that 5.2 pmoles in 400 µl of Micrococcus l. at A_{450} = 0.8 produces a spectral change of

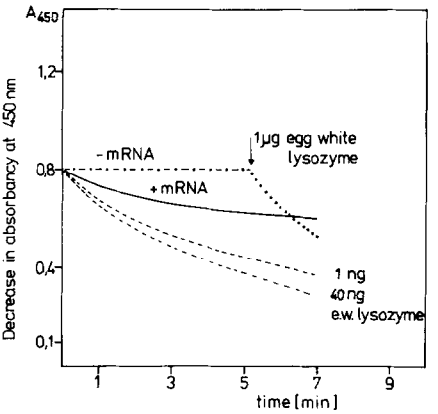

Fig. 4 Lysozyme activity assay of in vitro synthesized product directed by lysozyme mRNA. The enzyme assay was performed as described under methods. 1 unit of lysozyme activity is defined as a decrease in absorbancy of 1 at 450 nm per min. Specific activity is calculated per mg protein. Under the conditions listed the specific activity of egg white lysozyme was 5200 compared to 660 of the synthesized product. As a control 1 µg of egg white lysozyme was added after 5 min to the minus mRNA during the assay.

0.07 A_{450} units per min. From these data a specific activity of 660 U/mg can be calculated (See legend to Figure 4).

Reaction mixtures without mRNA and mRNA alone produced absolutely no lysozyme activity. The assay was extended for a period up to 30 min to check for any delayed enzyme activity.

DISCUSSION: In the wheat germ system a procaryotic mRNA from T4 infected E. coli cells can be translated into an enzymatically active protein, namely lysozyme. The system used by us shows the advantage, that minute amounts i. e. in the range of pmoles can be detected by a very sensitive assay as shown in Fig. 4.

The activity was determined from the initial velocity of the decrease in absorbancy at 450 nm. Using this method a specific activity of 660 U/mg protein was found for the protein synthesized compared to 5200 U/mg for egg white lysozyme tested in the same system. If one assumes that the activity for both pure proteins is similar, one may calculate that about 15% of the protein synthesized is actually T4 lysozyme.

Since addition of preisolated tRNA from wheat germ increases the amount of protein synthesized by 30% it seems that the availability of sufficient amounts of charged tRNA is the rate limiting process in translation. Supplementing the incubation mixture

with preisolated 80S ribosomes did not increase protein synthesis (data not shown). Polyamines as well as higher concentrations of GTP both stimulate the degree of translation as has been found by others (10,16).

The T4 lysozyme mRNA contains neither a cap sequence at the 5'end nor a poly(A) segment at the 3'end. Capped and noncapped mRNA's have been successfully translated in a eukaryotic translational system (17). The effect of these mRNA modifications may be examined by comparing it with lysozyme mRNA isolated from hen oviducts.

In further work we intend to use the cell free synthesis of active lysozyme as a screening system, to examine the functional integrity of ribosomes from both normal and transformed cells.

Acknowledgements: O.E.B. wishes to express his sincere thanks to Dr. T.S. Eikhom of Bergen, Norway for his unselfish assistance in improving the extraction methods for T4 RNA. Dr. F. Zimmermann has our gratitude for allowing us to use his facilities for culturing purposes. This work was supported from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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