IN VITRO SYNTHESIS OF T4 PROTEINS

Janwillem Coolsma* and Robert Haselkorn

Department of Biophysics

University of Chicago, Chicago, Illinois 60637

Received November 27, 1968

SUMMARY

Biologically active bacteriophage T4 lysozyme is synthesized *in vitro* using RNA from T4-infected cells to program a protein-synthesizing system from uninfected cells (Salser et al.,1967). We have confirmed this finding, and have extended it to a number of T4 proteins characterized by their migration on acrylamide gel. Two widely used protein-synthesizing systems differ considerably in their suitability for such experiments.

The synthesis of T4 lysozyme *in vitro* by Salser, Gesteland and Bolle (1967) suggests a potentially powerful way of studying the regulation of protein synthesis in T4 infections. Since hybridization-competition studies characterize whole classes of RNA species, and 5-FU rescue experiments cannot be interpreted unambiguously (Haselkorn, Baldi and Doskocil, 1968), we have attempted to characterize preparations of T4 messenger RNA in terms of the proteins whose synthesis is directed by them *in vitro*. The proteins synthesized *in vitro* are in turn characterized by their position as bands after electrophoresis on acrylamide gels, after Hosoda and Levinthal (1968).

Before examining the proteins synthesized *in vitro* on gels, we wanted to check the reliability of the amino acid incorporation system used by repeating the synthesis of T4 lysozyme. In our first attempts, we used a system patterned after Nirenberg (1961), which incorporates amino acids actively when programmed with RNA isolated from cells 18 min after T4 infection at 30° C. These failed to make

---

Present address: Biochemisch Laboratorium, The University, Groningen, The Netherlands

detectable amounts of lysozyme. We then followed a suggestion from W. Salser and switched to the system of Capecchi, with the results shown in Table 1. The princi finding of Salser et al. (1967) is confirmed: late T4 m-RNA directs the synthesis active lysozyme, in an extract from uninfected cells, whereas early T4 m-RNA does not. We found that ribosomes inhibit the lysozyme assay slightly; entries 5 and 6 in the Table show that removal of ribosomes from the system directed by late RNA increases the measured lysozyme activity, whereas removal of ribosomes from the

TABLE I

T4 LYSOZYME SYNTHESIS IN CELL-FREE EXTRACTS OF UNINFECTED E. COLI

	RNA addition	c.p.m. C^{14}-Proline incorporated per 0.2 ml	% Decrease of turbidity in 2 min
1)	none	230	2
2)	TYMV-RNA (60 μg)	12,640	2
3)	5 min RNA from T4-infected cells (860 μg)	3,980	3
4)	18 min RNA (314 μg)	4,850	12
5)	5 min RNA (S-100)	---	2
6)	18 min RNA (S-100)	---	20
7)	Same as (1), but lysate of infected cells equivalent to 2.5 x 10^6 cells added after incubation		20

Protein synthesis after Capecchi (1966). Each tube contains 0.1 ml dialyzed S-30 and 0.1 ml preincubation mix containing 10 μmoles Tris-Cl, pH 7.6, 12 μmoles NH_4Cl, 1 μmole $MgSO_4$, 0.1 μmole dithiothreitol, 2 nmoles each of 19 amino acids, and 0.4 nmole proline. After incubation at 37°C for 30 min, an additional 0.1 ml containing 20 μmoles Tris, 16 μmoles NH_4Cl, 1.4 μmoles $MgSO_4$, 0.5 μmoles dithiothreitol, 2 μmoles phosphoenol pyruvate, 8 μg pyruvate kinase, 1.2 μmoles ATP, 0.08 μmoles GTP are added, followed by 0.04 ml containing approximately 10^5 cpm C^{14}-proline (200 mC/mmole) and 4 nmoles of 19 other amino acids; 0.05 ml of RNA; and finally 0.01 ml containing 30 μg leucovorin. Incubation was for 20 min at 37°C. This system differs from that of Salser et al.(1967) in the omission of polyethylene glycol and the inclusion of leucovorin; S-30 from E. coli B, MRE 600, and CR 63 have all produced active lysozyme. RNA for the experiments reported here was prepared by the hot phenol method of Bolle et al.(1968); subsequent preparations using room temperature phenol extractions and DNase are as active as those made with hot phenol. Lysozyme was assayed by measuring the rate of decrease of turbidity at 450 mμ of a suspension of $CHCl_3$-treated E. coli, after Sekiguchi and Cohen (1964).

system directed by early RNA leaves no activity above the background due to spontaneous degradation of the substrate. Entry 6 also shows that active lysozyme is released from the ribosomes during the synthetic reaction.

The *in vitro* synthesis is not very efficient. The 314 μg of total RNA (entries 4 and 6) correspond to the RNA from approximately 3×10^9 cells. During the linear phase of lysozyme synthesis, the pool of functional lysozyme messenger RNA corresponds to about 10 minutes' worth of lysozyme at 30°C (Bose and Warren, 1967; M. Vogel and R. Haselkorn, unpublished results). The 30 minute lysate represented in entry 7 contains 18 minutes' worth of lysozyme, since synthesis begins around the twelfth minute. Allowing for this factor of 1.8, comparison of entries 6 and 7 indicates that the *in vitro* system translates at best 0.2% of the lysozyme-synthetic capacity of the cells from which the mRNA is prepared. More recent experiments of J. Wilhelm suggest that even this estimate is high; with better recovery of lysozyme activity from 30 min lysates, the ratio of *in vivo* to *in vitro* synthetic capacity, with respect to messenger RNA, is of the order of 1000.

Gesteland and Salser (personal communications) have been unable to detect translational controls for lysozyme synthesis in preincubated S-30 prepared from cells infected with T4 for 5 or 20 min. We wished to see if such control elements could be detected in the messenger RNA preparations themselves. These should be revealed by mixing experiments, which ask for inhibitors of lysozyme messenger translation in 5 min RNA, or potentiators of lysozyme messenger translation in 18 min RNA. Neither control element was detected as shown by the data in Table 2. The first series of entries shows that with up to 315 μg of 18 min RNA the extent of amino acid incorporation and of lysozyme synthesized is proportional to the amount of 18 min RNA added. The second and third series show that, when ribosomes are limiting, 5 min RNA inhibits lysozyme synthesis roughly in proportion to the extent of competition for ribosomes. The fourth series shows that, when ribosomes are in excess, 5 min RNA does not inhibit lysozyme synthesis, and 18 min RNA does not unmask a potential for lysozyme synthesis in the 5 min RNA.

TABLE II

LACK OF SPECIFIC INHIBITION OF CELL-FREE LYSOZYME SYNTHESIS

BY RNA FROM CELLS INFECTED WITH T4 FOR 5 MINUTES

RNA, μg		c.p.m. C^{14}-aminoacid incorporated per 0.2 ml	% decrease of turbidity in 2 min
5 min	18 min		
865	----	8700	1
---	63	1000	5
---	126	2000	7
---	252	4300	14
---	315	5700	23
---	63	1000	5
692	63	7300	2
---	126	2000	7
519	126	6700	3
---	252	4300	14
173	252	6900	12
	30 min lysate from 5×10^6 infected cells		13
	30 min lysate from 10^7 infected cells		26

Protein synthesis and lysozyme assay as in Table I

We then returned to the failure of our early attempts to make lysozyme. We found that freshly prepared Nirenberg S-30 could indeed make a small amount of lysozyme, and then asked if the defect is in the ribosomes or the supernatant fraction. Nirenberg and Capecchi S-30's were centrifuged and the four possible combinations of ribosomes and supernatant were compared for amino acid incorporatic activity, release of nascent protein, and lysozyme activity, with the results shown in Table 3. The Nirenberg supernatant is more efficient for incorporation

TABLE III

CELL-FREE SYNTHESIS OF T4 LYSOZYME IN RECONSTITUTED AMINO ACID INCORPORATION SYSTEMS

iRNA	Ribosomes	Supernatant	cpm C^{14}-proline incorporated per 0.2 ml	Fraction of cpm released from ribosomes	Lysozyme % decrease of turbidity in 2 min.
.8 min.	Nirenberg	Nirenberg	13,700	0.35	4
" "	"	Capecchi	7,380	.37	15
" "	Capecchi	Nirenberg	13,550	.46	5
" "	"	Capecchi	7,430	.48	17
5 min.	"	"	5,850	.40	1
		Lysate equivalent to 10^7 infected cells			16
		Lysate equivalent to 2×10^6 infected cells			3

Portions of Nirenberg and Capecchi S-30 (after preincubation 30 min at 37°) ere spun 2 hours at 40,000 cpm and the supernatants carefully removed. Individual ellets were suspended in the appropriate supernatant, concentrated mix (Table I) dded, the Mg^{++} level adjusted to 8.5 mM, then 1.5 mg RNA added, and finally eucovorin. The final volume was 0.8 ml; after incubation at 37° for 20 min, 0.05 ml as removed to measure TCA precipitable radioactivity and the remainder centrifuged hours at 40,000 cpm. Aliquots of the supernatants were counted and assayed for ysozyme.

of radioactive amino acids, but defective in lysozyme synthesis. The Capecchi ribosomes are slightly more efficient in release of nascent protein, but this difference is only marginally reflected in the lysozyme activity. The major differences in the two preparative procedures are the following: the Nirenberg S-30 is preincubated first and then dialyzed overnight; the Capecchi S-30 is dialyzed for six hours, and preincubated just before use. Presumably the higher amino acid incorporation activity of the Nirenberg supernatant is due to the longer dialysis, which lowers the level of endogenous amino acids; unfortunately a factor necessary for lysozyme synthesis is also lost. Since propagation and release are as efficient in the Nirenberg system, we believe the factor lost is concerned with correct initiation.

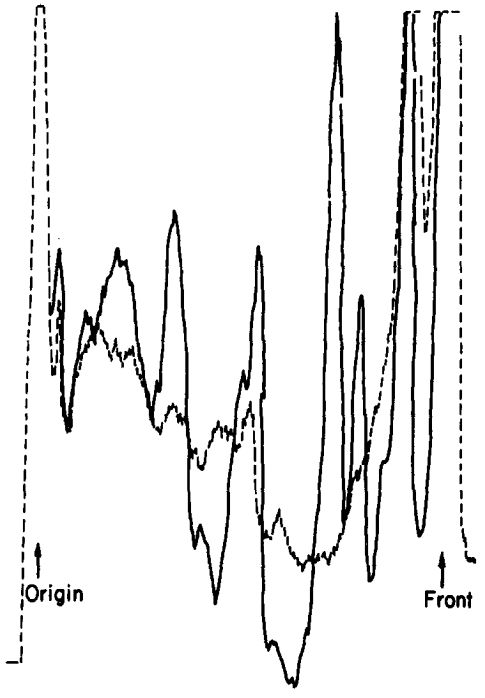

Fig. 1. Densitometer scans of autoradiograms obtained from acrylamide gel electropherograms of *in vitro* synthesized T4 proteins. The electrophoresis procedure is that of Hosoda and Levinthal (1968), using 7% gels at pH 8.7. 120 µl of released protein (spun 2 hr at 40,000 rev/min) from Capecchi (——) or Nirenberg (---) incorporation systems were applied to each of two gels. The applied sample contained 20,000 cpm from the Capecchi system, 10,000 cpm from Nirenberg. Electrophoresi was at room temperature, continued until the tracking dye had migrated 5.5 cm from the origin. Autoradiography was for 30 days. In both cases 0.4 ml systems had been programmed with 570 µg 18 min T4 mRNA; lysozyme synthesis was five-fold higher in the Capecchi system than in the Nirenberg system.

Finally we compared the two S-30's with respect to the gel patterns of protei synthesized *in vitro*. Figure 1 shows densitometer traces of two autoradiograms, displaying the protein bands found after electrophoresis of the released proteins directed by 18 min T4 RNA in Nirenberg and Capecchi S-30 from uninfected cells. The gel of proteins from the Capecchi S-30 shows at least nine discrete bands; that from the Nirenberg S-30 contains several of the same bands, but with lower contrast, and several important bands are missing. Another qualitative result not apparent in the traces is that a smaller proportion of the total radioactive mater

enters the gel from the Nirenberg system. Again we conclude that although some specific proteins are made in the Nirenberg S-30, the proportion of specific synthesis is higher in the Capecchi S-30.

At present we are characterizing the proteins made in the Capecchi S-30 by RNA preparations from cells infected with T4 and various amber mutants, using several gel systems. It has been possible to identify several in vitro bands with their in vivo counterparts on the basis of amber mutations (J. Wilhelm and R. Haselkorn, to be published).

This work was supported by grant GM-12344 from the U.S. Public Health Service. J.C. was supported by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) R.H. holds a Research Career Development Award from the U.S.P.H.S.

REFERENCES

Bolle, A., Epstein, R.H., Salser, W. and Geiduschek, E.P. (1968). J. Mol. Biol. 31, 325.
Bose, S.K. and Warren, R.J. (1967). Proc. Natl. Acad. Sci. U.S. 58, 2408.
Capecchi, M. (1966). J. Mol. Biol. 21, 173.
Haselkorn, R., Baldi, M.I. and Doskocil, J. (1968). The Biochemisty of Virus Replication, Universitetsforlaget, Oslo. p.79.
Hosoda, J. and Levinthal, C. (1968). Virology 34, 709.
Nirenberg, M. and Matthaei, J.H. (1961). Proc. Natl. Acad. Sci., U.S. 47, 1588.
Salser, W., Gesteland, R.F. and Bolle, A. (1967). Nature 215, 588.
Sekiguchi, M. and Cohen, S.S. (1964). J. Mol. Biol. 8, 638.