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The Selective Inhibition of Protein Initiation by T4 Phage-Induced Factors

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Abstract. The phenomenon of selective translation of T4 template RNA by ribosomes from T4-infected cells, or factors derived therefrom, has been extended to studies on the initiation of protein synthesis. A high-salt extract derived from T4-infected ribosomes inhibits the formation of initiation complexes of MS2 and *Escherichia coli* template RNA with uninfected ribosomes while efficiently supporting the formation of initiation complexes with T4 template RNA. T4 factors also permit T5 template RNA to bind to *E. coli* ribosomes, which indicates that the T4 selective effect is not exclusive for T4 templates. Other evidence indicates that T4 factors do not alter the process of polypeptide chain elongation.

Various reports have now described specific changes in the translational machinery of *E. coli* when infected by T-even bacteriophage. These changes include alterations in host tRNAs[1–3] and the enzymes which modify them,[4,5] changes in aminoacyl-tRNA synthetases,[6] and the synthesis of phage-coded tRNAs.[7–9] Another induced alteration was found by Hsu and Weiss,[10] who observed that ribosomes from T4-infected cells efficiently translate T4 template RNA but are restricted in their translation of host and MS2 RNA's. This phenomenon is an early phage function and is not manifested when infection occurs in the presence of chloramphenicol[10] or with T4 ghosts (unpublished results of the authors).

In the same report, Hsu and Weiss found that a high-salt extract of T4-infected ribosomes (T4 factors) imparts a similar property of "selective" T4 RNA translation when added to uninfected ribosomes. These findings were interpreted by the authors as a form of translational control exerted by the invading phage on its host. Subsequent reports by Schedl *et al.*,[11] Dube and Rudland,[12] and Steitz *et al.*[13] confirm the above observations. Refs. 12 and 13 also show that T4 factors restrict the binding of host, f2, and R17 RNA to ribosomes but permit the binding of T4 RNA. In this communication, we provide independent evidence that T4 factors exert their restrictive effect at the level of the formation of initiation complexes, and that the apparent selective translation of T4 template RNA, *in vitro*, is not exclusive. In addition, evidence is presented which indicates that T4 factors exert no effect on the elongation of polypeptide chains.

Materials and Methods. Ribosomes, factors, and template RNAs: The preparation of ribosomes and ribosomal factors from uninfected and T4-infected *E. coli* and template RNAs from *E. coli*, T4-infected cells, and MS2 phage were as previously reported.[10] Washed ribosomes from normal *E. coli* were prepared by extraction of unwashed ribosomes with 1 M NH_4Cl and then treated with DEAE-cellulose by the method of Iwasaki *et al.*[14] T5 template RNA was isolated from *E. coli* F infected with T5 phage at 37°C for 20 min.

Radioactive template RNAs and fMet-tRNA: *E. coli* was grown in a salts–glucose medium supplemented with 0.04 vol of 3 × D medium[15] as reported elsewhere.[5] MS2 [^{3}H]RNA (1×10^4 cpm/μg) was isolated from MS2 phage grown on *E. coli* K12W1485 in the presence of [^{3}H]uracil (1 mCi/40 ml of culture). *E. coli* [^{3}H]RNA (1×10^4 cpm/μg) was isolated from log phase *E. coli* B which had been pulsed for 2 min at 37°C with [^{3}H]uracil (2 mCi/40 ml of culture). T4 and T5 [^{3}H]RNAs (0.97×10^4 and 61×10^4 cpm/μg, respectively) were isolated from *E. coli* B which was pulsed from 5 to 15 min after infection with [^{3}H]uracil (1 mCi/40 ml of culture). *E. coli* [^{3}H-fMet]$tRNA_f$ was prepared by charging *E. coli* $tRNA_f^{Met}$ (97% pure and a gift of Dr. G. D. Novelli, Oak Ridge National Laboratory) with [^{3}H]methionine in the presence of leucovorin, with a dialyzed supernate from *E. coli* S165 as enzyme. The [^{3}H-fMet]$tRNA_f$ was isolated by phenol extraction, alcohol precipitation, and dialysis, and had a specific activity of 57,000 cpm/μg.

Ribosome binding assay for template [^{3}H]RNA and [^{3}H-fMet]$tRNA_f$: The components of the reaction mixtures used are indicated in the footnotes of the experiments in the text. Experiments using [^{3}H]RNAs were assayed by centrifugation on a 5 ml 5–20% linear sucrose gradient [containing 5 mM $Mg(OAc)_2$, 50 mM Tris·HCl (pH 7.8) and 80 mM NH_4Cl] for 65 min at 50,000 rpm in a Spinco SW 50 rotor. Fractions were collected and their radioactive content was determined by scintillation counting. Binding of [^{3}H-fMet]$tRNA_f$ to ribosomes was determined by trapping on nitrocellulose filters.[16] The filters were pretreated with 0.5 N NaOH for 20 min and washed prior to their use as described by Smolarsky and Tal.[17]

Incorporation of [^{3}H]fMet into polypeptides: The reaction mixtures are indicated in the footnotes of the experiments in the text. Incorporation of [^{3}H]fMet into a hot-acid-precipitable fraction was as described elsewhere.[10]

Isotopic compounds: The [^{3}H]uracil and [^{3}H]methionine used in this work had a specific activity of 25.4 and 3.3 Ci/mmol, respectively, and were purchased from Schwarz BioResearch, Orangeburg, N.Y.

Results. Since the T4 "selective" translational factor is associated with ribosomes and behaves similarly to initiation factors with respect to certain properties (i.e., extraction with high salt concentration and chromatographic distribution on DEAE-cellulose), it seemed likely that the T4 factors might exert their effect on the initiation steps of protein synthesis. Studies on ribosomal complex formation showed that in the presence of fMet-tRNA, GTP, and magnesium acetate, binding of T4[^{3}H]RNA to washed *E. coli* ribosomes required the addition of factors extracted with high salt from either uninfected or infected (T4) *E. coli* ribosomes (Fig. 1). However, with MS2 and *E. coli* (EC) [^{3}H]-RNAs, effective complex formation occurs only with EC factors while T4 factors exhibit a marked inhibition. On the other hand, T4 factors do not restrict the binding of T5 [^{3}H]RNA to washed ribosomes and are as effective as EC factors for promoting complex formation.

Using the nitrocellulose trapping technique of Nirenberg and Leder,[16] we obtained results on the binding of [^{3}H-fMet]$tRNA_f$ to ribosomes that were in agreement with the above findings. Table 1 shows that in the presence of

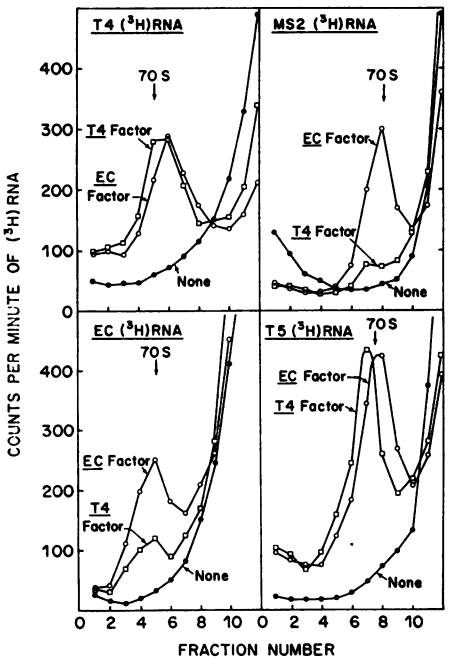

FIG. 1. Sucrose gradient analysis of the binding of template [³H]RNA to ribosomes. The reaction system (0.125 ml) contained 5 mM $Mg(OAc)_2$; 50 mM Tris (pH 7.8); 80 mM NH_4Cl; 14 mM mercaptoethanol; 40 μg of *E. coli* tRNA charged with fMet; 100 μg of washed *E. coli* ribosomes; one ³H-labeled RNA (2×10^4 cpm for *E. coli*, T4, and T5 RNAs, and 1.5×10^4 cpm for MS2 RNA); nonradioactive RNA from the same source as carrier (80 μg of *E. coli* and T4 RNAs, 20 μg of T5 RNA, and 26 μg of MS2 RNA); and 60 μg of either *E. coli* or T4 factors where indicated above. After 15 min at 37°C, the mixtures were subjected to sucrose density centrifugation as indicated under *Methods*. Only the bottom half of the gradients (70S region) are shown. EC, uninfected *E. coli*.

washed ribosomes, GTP, and magnesium ions, the trapping of labeled fMet-$tRNA_f$ on filters requires the participation of both template RNA and ribosomal factors. With RNAs from T4- and T5-infected cells, [³H-fMet]tRNA is trapped equally well with either *E. coli* or T4 factors, but with MS2 and *E. coli* template RNAs, T4 factors significantly inhibit the accumulation of label on the filters. When similar experiments with [³H-fMet]$tRNA_f$ were carried out under conditions of active polypeptide synthesis, incorporation of radioactivity into hot-acid-precipitable material was also dependent upon the presence of ribosomal factors (Table 2). Both *E. coli* and T4 factors support good incorporation of [³H]fMet into protein with RNA templates from T4 and T5 infected cells, but with MS2 and *E. coli* RNAs, T4 factors are inhibitory.

Although the experimental data shown above indicate that T4 ribosomal factors restrict protein synthesis at the level of initiation with certain templates, they do not exclude the possibility that these same factors might exert an addi-

TABLE 1. *Effect of T4 factors on [³H-fMet]$tRNA_f$ binding to ribosomes.*

Source of ribosomal factors	[³H-fMet]$tRNA_f$ bound with different templates (cpm)				
	No RNA	T4 RNA	T5 RNA	MS2 RNA	*E. coli* RNA
None	108	212	76	100	125
E. coli	118	3450	3510	2150	2260
T4	136	3520	3400	448	1200

The reaction mixtures (0.125 ml each) were the same as in Fig. 1 except as follows. No labeled template RNAs were used, but nonradioactive template RNAs were present (as indicated) in the following amounts: T4 (160 μg), MS2 (40 μg), T5 (66 μg), and *E. coli* (250 μg). Purified [³H-fMet]$tRNA_f$ (0.35 μg, 20,000 cpm) was added to each reaction. After incubation at 37°C for 15 min, the mixtures were assayed for ribosome-bound radioactivity by trapping on nitrocellulose filters.[16] The *E. coli* ribosomes used were washed with 1 M NH_4Cl.

TABLE 2. *Effect of T4 factors on incorporation of [^{3}H]fMet into protein.*

Source of ribosomal factors	[^{3}H]fMet incorporated into acid-insoluble form (cpm)			
	T4 RNA	T5 RNA	MS2 RNA	*E. coli* RNA
None	86	100	112	47
E. coli	5411	4445	2182	3929
T4	6145	5163	765	2810

The reaction mixture (0.125 ml) contained 40 mM Tris·HCl (pH 7.8), 10 mM $Mg(OAc)_2$, 80 mM NH_4Cl, 4 mM mercaptoethanol, 2 mM ATP, 0.2 mM GTP, 2 mM PEP, 3 μg of PEP kinase, 8×10^{-3} mM each of 20 amino acids, 50 μg of *E. coli* tRNA, 0.35 μg of [^{3}H-fMet]tRNA (20,000 cpm), 100 μg of washed *E. coli* ribosomes, 75 μg of soluble S165 protein, and where indicated, 160 μg of T4 RNA, 40 μg of MS2 RNA, 66 μg of T5 RNA, and 250 μg of *E. coli* RNA as template, and 60 μg of either *E. coli* or T4 factors. Incorporation of radioactivity into a hot-acid-insoluble fraction was determined as described elsewhere.[10]

Abbreviation: PEP, phosphoenolpyruvate.

tional effect on the elongation of the protein chain. Experiment 1 of Table 3 shows that with MS2 RNA as template, [^{3}H-fMet]$tRNA_f$ is bound to *unwashed* ribosomes in the absence of added ribosomal factors and is completely blocked by aurintricarboxylic acid, a chemical agent previously shown to inhibit protein chain initiation.[18,19] Although the addition of *E. coli* factors has no effect on binding of labeled fMet-$tRNA_f$, T4 factors reduce the amount of [^{3}H]fMet-$tRNA_f$ complexed by nearly 40% as compared to *E. coli* factors, which suggests a partial reversal of the binding process. On the other hand, once chain elongation has begun, T4 factors have no significant effect on [^{3}H]fMet incorporation into a polypeptide form (Table 3, Experiment 2). In this last experiment,

TABLE 3. *Effect of T4 factors on polypeptide initiation and elongation.*

Expt. no.	Binding reaction	Additions	[^{3}H-fMet]$tRNA_f$ binding (cpm)	Incorporation of [^{3}H]fMet into protein (cpm)
1.	Complete	None	2310	
	Omit MS2 RNA	None	184	
	Complete	ATA	193	
	Complete	EC factors	2440	
	Complete	T4 factors	1560	
2.	Complete	Elongation mixture + ATA		3000
	Omit MS2 RNA	Elongation mixture + ATA		42
	Complete	Elongation mixture + ATA + *E. coli* factors		3560
	Complete	Elongation mixture + ATA + T4 factors		3140

Expt. 1. The binding mixtures (0.065 ml) contained the same components as indicated in Fig. 1, except that 30 μg of MS2 RNA, 230 μg of *unwashed E. coli* ribosomes and 37,000 cpm of [^{3}H-fMet]-$tRNA_f$ ([^{3}H]methionine, 3.3 Ci/mmol) were used. The binding mixtures were incubated for 10 min at 37°C before the "additions" shown above were made. After incubation for another 10 min, each reaction was assayed for [^{3}H-fMet]$tRNA_f$ trapping on filters as described under *Methods*. The concentration of aurintricarboxylic acid (ATA) was 0.15 mM and 30 μg of either *E. coli* or T4 factors was used.

Expt. 2. The binding mixture (0.065 ml) was the same as described for Expt. 1. After reaction for 15 min at 37°C, the additions were made in the following order: elongation mixture and ATA simultaneously, 30 sec incubation, and then the ribosomal factors. Incubation was continued for another 10 min, the reaction was terminated, and the incorporation of [^{3}H]fMet into an acid-insoluble form was assayed.[10] The concentration of ATA was 0.15 mM and, in addition to the binding mixture, the elongation mixture (0.065 ml) contained all the components required for protein synthesis, as shown in Table 2. [^{3}H]fMet]tRNA, *E. coli*, and T4 factors were the same as described above. At the time of factor addition, no significant incorporation of [^{3}H]fMet was detectable.

aurintricarboxylic acid was included to prevent initiation of new chains during elongation, and at the time when factors were added (30 sec after the addition of the elongation components), the amount of [^{3}H]fMet incorporated into protein was negligible; maximum incorporation was achieved by 2.5 min under the conditions used. Similar results were obtained with other labeled amino acids.

Discussion. The present study shows that factors derived from ribosomes of T4-infected cells permit initiation complexes to form with T4 and T5 template RNAs but inhibit similar complex formation with *E. coli* and MS2 RNAs. These results support and explain our previous findings that T4-infected ribosomes, or factors derived therefrom, allow efficient translation of T4 RNA but not of host and MS2 RNA. An explanation is also provided for the earlier observations[20-22] that RNA-phage replication is blocked by infection with T-even phage. Similar defects in the translation of host and RNA-phage templates have now been observed with ribosomes from T5-infected cells (unpublished results from this laboratory) and with ribosomes from T7-infected cells (P. Leder, personal communication).

In view of the fact that T4 factors support ribosomal binding and fMet incorporation into polypeptides with T5 RNA, the translational selectivity of T4 infected ribosomes can no longer be considered exclusive for T4 RNA templates. Since T4 ribosomal factors recognize some, but not all template RNAs, this suggests that nucleotide sequences which specify sites for polypeptide chain initiation may be similar for certain templates but, most probably, are different for others.

Besides blocking the formation of MS2 RNA initiation complexes, T4 factors also seem capable of partially reversing these complexes, once formed, which suggests some type of reversible competition between *E. coli* factors and the T4 inhibitory component. Similar observations have been made by Dube and Rudland.[12] However, once the process of polypeptide chain elongation begins, T4 factors have little or no effect on the incorporation of amino acids into peptide linkage when MS2 RNA is used as template. This information reinforces the idea that the inhibitory factor for T4 translation exerts its action solely at the level of initiation and not on peptide chain extension.

The mechanism by which ribosomal factors from T4-infected cells prevent the binding of certain template RNAs to ribosomes and their function in the phage replicative process is still not known. We have previously suggested that the T4-induced ribosomal phenomenon could play a role in the shut-off of host protein synthesis and provide an effective regulatory mechanism for translational control. It is possible that such a mechanism might be operative in phage-infected cells as well as in other biological systems, but experimental proof for this idea remains unconfirmed.

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