Translation of the Initial Codons of Satellite Tobacco Necrosis Virus Ribonucleic Acid in a Cell-free System from Wheat Embryo*

SAMARENDRA N. SEAL AND ABRAHAM MARCUS
From The Institute for Cancer Research, Philadelphia, Pennsylvania 19111

SUMMARY

Satellite tobacco necrosis virus RNA directs the ribosomal binding of methionyl-, alanyl-, and lysyl-tRNA when incubated under the appropriate conditions in the in vitro wheat embryo amino acid incorporating system. The products of the various binding reactions have been identified as methionyl-tRNA, methionyl-alanyl-tRNA, and methionyl-alamyl-lysyl-tRNA. This sequence corresponds to the known NH₂-terminal sequence of the satellite tobacco necrosis virus coat protein thereby establishing the accurate initiation and translation of at least the three initial codons of the translated portion of the viral RNA.

Methionyl-tRNA binding requires, in addition to ATP and GTP, the presence of the two initiation factors C and D, and occurs readily at 1.3 mM Mg²⁺. Alanyl-tRNA binding is obtained only when the system is supplemented with phosphoenolpyruvate, pyruvate kinase, and elongation factor 1 and when the Mg²⁺ concentration is raised to 3.6 mM. These latter conditions also allow an additional increment of methionyl-tRNA to be bound to ribosomes. The amount of extra methionyl-tRNA bound is equal to the alanyl-tRNA bound under these conditions and to the methionyl-tRNA bound in the original initiation reaction. Chlortetracycline completely inhibits binding of alanyl-tRNA as well as the extra increment of methionyl-tRNA. It is, however, without effect on the initial binding of methionyl-tRNA. Binding of lysyl-tRNA to ribosomes occurs only upon further supplementation with elongation factor 2. These observations provide further evidence that factors C and D are initiation factors: that elongation factor 1 functions in dipeptide synthesis and that elongation factor 2 is required only for tripeptide formation. The ability to bind the extra increment of methionyl-tRNA under conditions of dipeptidyl tRNA synthesis suggests the existence of a third ribosomal binding site specific for methionyl-tRNA.

An in vitro wheat embryo system catalyzes the incorporation of radioactive amino acids into protein in response to exogenous plant messenger RNAs (1–4). The reaction requires ATP (2), GTP (5), and four soluble factors (6). Two of the factors (C and D) are considered to function in chain initiation. They are required for the formation of a ribosome-mRNA complex whose capacity for amino acid incorporation is insensitive to aurintricarboxylic acid, and they catalyze the mRNA-dependent binding of methionyl-tRNA to ribosomes (6). The other two factors (EF-1 and EF-2) are assumed to be elongation factors on the basis of their absolute requirement for the mRNA-dependent incorporation reaction, and their ability to catalyze the poly(U)-dependent polymerization of phenylalanine.

In order to characterize further the specific functions of the factors, as well as to probe the roles of ATP and GTP, it appeared desirable to utilize an mRNA whose translation would yield a single chemically defined peptide product. Klein et al. (4) have recently reported that incubation of STNV-RNA, the RNA of satellite tobacco necrosis virus, in the wheat embryo system resulted in the formation of only one product, the satellite virus coat protein. Translation of this mRNA should therefore provide a particularly suitable system for detailed study. A primary prerequisite, however, is a high degree of accuracy both in initiation and in the translation of at least the initial three codons of the mRNA. In the present study we inquire as to whether such accuracy of STNV-RNA translation is obtained in the resolved wheat embryo system. The NH₂-terminal dipeptide of STNV coat is Ala-Lys (4, 7). Assuming that initiation with STNV-RNA utilizes Met-tRNA as has been shown for other eucaryotic mRNAs (8–10), the initial di- and tripeptide that would be expected as a consequence of the translation of STNV-RNA are Met-Ala and Met-Ala-Lys, respectively. We show herein, that indeed only these products are formed in the controlled translation of STNV-RNA. The delineation of the requirements for the specific synthesis of these products supports the categorizing of the translation process into separate phases of initiation and chain elongation, and allows the functional definition of the two elongation factors.

MATERIALS AND METHODS

Ribosome-binding assays were carried out by the Millipore filter assay as previously described (6). The preparation of

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1 The abbreviations used are: STNV-RNA, the RNA of the satellite virus; STNV, satellite tobacco necrosis virus.
The ribosome pellets obtained from the first wash are suspended in 2 ml of 2 mM Tris acetate pH 7.4, 100 mM KCl, 2 mM MgAc₂, 3 mM 2-mercaptoethanol; kept in ice for 30 min, layered over 7 ml of 10% sucrose containing the same solution and centrifuged for 135 min at 150,000 x g. These ribosomes are referred to as "K₂₀M₂" ribosomes (11). In some experiments, ribosomes washed by the earlier procedure (i.e. 20 mM KCl, 1 mM MgAc₂) were used. These ribosomes are referred to as "K₅₀M₅" ribosomes. Procedures for the various aminoacyl-tRNAs and the separation of the soluble embryo factors have been previously described (6, 9, 11). The aminoacyl tRNAs were prepared by charging unfractionated tRNA with one radioactive amino acid and 19 unlabeled amino acids. Factors C and D are the resolved initiation factors purified through Steps 2a and 3 (Reference 6). The aminoacyl tRNAs were analyzed (Fig. 1), only one radioactive component corresponding to free methionine was observed. The incubation mixture contains greater than an equivalent amount of the other 19 aminoacyl-tRNAs so that substrates for di- and tripeptide synthesis (see Table III). When the product bound to the ribosomes was analyzed (Fig. 1), 0.08 pmoles (binding in the absence of mRNA) is subtracted.

**RESULTS**

**Ribosome Binding of Methionyl-tRNA (Step 1)—**In earlier studies (14, 15) we observed that STNV-RNA catalyzed the binding of Met-tRNA to ribosomes under initiating conditions. The results reported in Tables I and II examine this reaction in greater detail. The STNV-RNA-dependent binding reaction is specific for Met-tRNA both at 1.3 and 3.6 mM Mg⁺² and requires factors C and D, ATP and GTP. Factor C can be partially replaced by factor EF-1. The Met-tRNA bound in this latter reaction is, however, nonreactive either with puromycin (data not shown) or with Ala-tRNA in dipeptide synthesis (see Table III). When the product bound to the ribosomes was analyzed (Fig. 1), only one radioactive component corresponding to free methionine was observed. The incubation mixture contains greater than an equivalent amount of the other 19 aminoacyl-tRNAs so that substrates for di- and tripeptide synthesis (see Table III). When the product bound to the ribosomes was analyzed (Fig. 1), only one radioactive component corresponding to free methionine was observed. The incubation mixture contains greater than an equivalent amount of the other 19 aminoacyl-tRNAs so that substrates for di- and tripeptide synthesis (see Table III).

### Table I

| Substrate | Aminoacyl-tRNA Bound |
|-----------|----------------------|
| Mg⁺²      | +STNV-RNA | -STNV-RNA |
|           | pmoles    |
| [¹⁴C]Met-tRNA | 1.3 | 1.46 | 0.22 | 1.24 |
| [³⁵S]Ala-tRNA | 3.6 | 1.47 | 0.22 | 1.25 |
| [¹⁴C]Lys-tRNA | 1.3 | 0.39 | 0.25 | 0.14 |
|            | 3.6 | 0.28 | 0.22 | 0.06 |

### Table II

**Requirements for STNV-RNA-catalyzed ribosomal binding of Met-tRNA**

The incubation conditions were those of Table I with MgAc₂ at 1.3 mM and [³⁵S]Met-tRNA added to all vessels. EF-1, 30 μg, and EF-2, 21 μg, were added where indicated. A blank value of 0.21 pmoles (binding in the absence of mRNA) is subtracted.

| Conditions | Met-tRNA bound |
|------------|----------------|
|            | pmoles         |
| Complete   | 1.25           |
| Omit C     | 0.00           |
| Omit D     | 0.00           |
| EF-1 + EF-2 in place of D | 0.00 |
| EF-1 in place of C | 0.18 |
| EF-2 in place of C | 0.00 |
| Omit GTP   | 0.51           |
| Omit ATP   | 0.20           |
tRNA was added to a volume of 0.34 ml and the incubation was for 10 min at 20°. The components of the second incubation (5.6 mM phosphoenolpyruvate, 25 μg of pyruvate kinase (PEP), 30 μg of EF-1, 21 μg of EF-2 where indicated, MgAc2 and KCl to provide final concentrations of 3.6 mM and 51 mM, respectively) were added to a volume of 0.43 ml and the incubation was continued for 20 min at 20°. In the experiment where ATP was omitted, the phosphoenolpyruvate and the pyruvate kinase were added at the start of the first incubation. The data are corrected for a blank value of 0.29 pmole (binding in the absence of mRNA).

The reaction (see legend to Table I) with the addition of Met-tRNA added at the start of the first incubation. A blank value of 0.21 pmole has been subtracted. Essentially identical results are obtained when STNV-RNA is replaced with 10 μg of TMV-RNA.

The increment in Met-tRNA binding that is obtained by the antibiotic at a concentration as high as 5.5 × 10⁻⁴ M. This type of observation, i.e. higher activity for ribosomes washed in low salt, has been noted in a number of assays measuring capacity for protein synthesis (see Reference 11). Attempts to restore activity with ribosomal wash fractions have been unsuccessful.

Further delineation of the reactions occurring in the two sequential steps was obtained with the antibiotic chlorotetracycline (16-18) (Table V). Binding of Met-tRNA in Step 1 is unaffected by the antibiotic at a concentration as high as 5.5 × 10⁻⁴ M. In contrast, Ala-tRNA binding in the two-step sequence is completely prevented with chlorotetracycline at 5.5 × 10⁻⁴ M. The increment in Met-tRNA binding that is obtained by the two-step sequence is also affected in a manner similar to Ala-tripeptide synthesis are available. Clearly, no such compounds are formed.

**Formation of Met-Ala-tRNA (Step 2)—** A series of experiments were performed to ascertain the conditions necessary for the binding of Ala-tRNA (Table III). The total requirements were found to include the components of the initiation reaction (Step 1), a phosphoenolpyruvate-pyruvate kinase, nucleotide triphosphate generating system ("PEP"), factor EF-1, and a shift in the Mg²⁺ concentration to 3.6 mM Mg²⁺. The reaction could be carried out either in a single 30-min incubation at 3.6 mM Mg²⁺ or as a sequence of two incubations, the first for 10 min at 1.3 mM Mg²⁺ and the second for 20 min at 3.6 mM Mg²⁺. Factor EF-2 failed to replace factor EF-1 and when added as a supplement resulted in only a slight increment in Ala-tRNA binding. Carrying out the reaction with K₂₀M₁ ribosomes produced a considerable increase of bound Ala-tRNA. These latter ribosomes are, however, slightly contaminated with EF-2 so that the time and temperature of incubation must be carefully controlled to prevent tripeptide formation.

**Fig. 1. Product analysis of the Step 1 reaction.** A 3-fold incubation was carried out with components as in Table II. The product was isolated and chromatographed as described under "Materials and Methods." Strips, 1 cm, were cut from the dried chromatogram and counted in a toluene-based scintillator.

**TABLE III**

**Requirements for ribosomal binding of Ala-tRNA**

The reaction was carried out in two sequential incubations. The first incubation contained the components of the binding reaction (see legend to Table I) with Mg²⁺ at 1.3 mM. [¹⁴C]Alanyl-tRNA was added to a volume of 0.34 ml and the incubation was for 10 min at 20°. The components of the second incubation (5.6 mM phosphoenolpyruvate, 25 μg of pyruvate kinase (PEP), 30 μg of EF-1, 21 μg of EF-2 where indicated, MgAc₂ and KCl to provide final concentrations of 3.6 mM and 51 mM, respectively) were added to a volume of 0.43 ml and the incubation was continued for 20 min at 20°. In the experiment where ATP was omitted, the phosphoenolpyruvate and the pyruvate kinase were added at the start of the first incubation. The data are corrected for a blank value of 0.29 pmole (binding in the absence of mRNA).

**TABLE IV**

**Requirements for extra Met-tRNA binding**

The incubation conditions were those of Tables II and III with [¹⁴C]Met-tRNA added at the start of the first incubation. A blank value of 0.21 pmole has been subtracted. Essentially identical results are obtained when STNV-RNA is replaced with 10 μg of TMV-RNA.

**Fig. 1. Product analysis of the Step 1 reaction.** A 3-fold incubation was carried out with components as in Table II. The product was isolated and chromatographed as described under "Materials and Methods." Strips, 1 cm, were cut from the dried chromatogram and counted in a toluene-based scintillator.
TABLE VI
Requirements for ribosomal binding of lysyl-tRNA

The reaction was carried out in three sequential incubations. The first two incubations were identical to those of Table III except that 14C]lysyl-tRNA was added at the start of the first incubation. At the completion of the second incubation, 21 µg of EF-2 were added to a volume of 0.46 ml and the vessels were kept for 10 min at 20°C. A blank value of 0.22 pmole (binding in the absence of mRNA) is subtracted.

| Conditions          | Lys-tRNA bound (pmoles) |
|---------------------|-------------------------|
| Complete            | 1.33                    |
| Omit EF-1           | 0.06                    |
| Omit EF-2           | 0.09                    |
| Omit C              | 0.00                    |
| Omit D              | 0.00                    |

Fig. 2. Product analysis of the Step 2 reaction. A, 3-fold incubations were carried out with either [14C]Met-tRNA (■—■) or [14C]Ala-tRNA (○—○) and all other components as in Table III. The remainder of the procedure was as in Fig. 1. B, the [14C]Ala product from the chromatogram of A was eluted and cleaved with cyanogen bromide as described under “Materials and Methods.” The product was then rechromatographed.

TABLE V
Effect of chlortetracycline on ribosomal binding of Met-tRNA and Ala-tRNA

The incubation conditions for assay of Met-tRNA binding were those of Table II with the exception of the two incubations noted with an asterisk. These latter assays as well as those measuring the binding of Ala-tRNA were carried out under the conditions of Table III. Corrections of 0.21 and 0.29 pmole, are subtracted from the Met-tRNA and Ala-tRNA data, respectively.

| Chlortetracycline | Aminocarlyl-tRNA bound (pmoles) |
|-------------------|--------------------------------|
|                   | Met-tRNA | Ala-tRNA        |
| x × 10⁻⁴         |          |                 |
| 2.7 × 10⁻⁴        | 1.15     | 1.44            |
| 5.5 × 10⁻⁴        | 1.14     | 1.21            |
| 1.2 × 10⁻⁴        | 1.16     | 0.22            |
| 5.5 × 10⁻⁴        | 1.15     | 0.10            |
| 1.2 × 10⁻⁴        | 1.09     | 0.09            |
| 5.5 × 10⁻⁴        | 2.65*    |                 |
| 1.2 × 10⁻⁴        | 1.29*    |                 |

Fig. 3. Product analysis of the Step 3 reaction. A, 3-fold incubations were carried out with either Ala-tRNA (■—■) or Lys-tRNA (○—○) with all other components as in Table VI. The remainder of the procedure was as in Fig. 1. B, the radioactive products of A were eluted, cleaved with cyanogen bromide, and rechromatographed.

binding of Lys-tRNA. The reaction could be carried out either as a single 30-min incubation or as a sequence of three reactions. Table VI documents the factor requirements for Lys-tRNA binding. In addition to EF-2 there is a complete requirement for factors C, D, and EF-1. Presumably, these latter components are needed for the prior formation of the products of the Step 1 and Step 2 reactions.

The products of the three-step reaction are examined in Fig. 3.
With Lys-tRNA only one radioactive product is found. A standard of Met-Ala-Lys was unavailable for direct comparison. Cleavage with cyanogen bromide, however, caused complete disappearance of the initial product with the formation of a new compound that cochromatographed with Ala-Lys. Further confirmation of the structure of the product of the three-step sequence was obtained by carrying out the incubation with radioactive Ala-tRNA. Again, only one product was obtained identical to that obtained from the incubation with radioactive Lys-tRNA, both in its original migration and in its migration after cyanogen bromide cleavage.

**DISCUSSION**

*In vitro* translation of the initial codons of STNV-RNA results in ribosomally bound Met-, Met-Ala-, and Met-Ala-Lys-tRNA. The specific synthesis of these products and the lack of formation of other di- or tripeptides indicates that both the initiation of STNV-RNA and the subsequent translation of at least the initial two codons are accurate. In the earlier study (19), some uncertainty was indicated with regard to the function of Met-tRNA in initiating STNV coat protein because of the inability to detect appreciable NH$_2$-terminal methionine in the in *vivo* product. Our results demonstrate unambiguously that methionine does initiate the reaction. It would therefore appear that the lack of NH$_2$-terminal methionine obtained with the unfractionated extract is due to the rapid removal of the initiating methionine from the nascent peptidyl-tRNA.

The requirements for factors C and D in the STNV-RNA dependent Met-tRNA binding reaction, as well as the aminocacyl-tRNA specificity of this latter reaction, are consistent with previous studies utilizing TMV-tRNA (6) and strongly indicate that these factors function in chain initiation. A similar argument can be made for ATP and GTP. It should be noted, however, that the experimental system that we describe does not preclude the function of these components (C, D or the nucleotide triphosphates) in some part(s) of the elongation reaction, in addition to their role in chain initiation. Experiments testing this possibility, in which the specific steps are studied as separate reactions, are in progress.

The function of EF-1, in the biosynthesis of Met-Ala-tRNA, and its specific inhibition by chlortetracycline are consistent with the earlier designation of this factor as the aminocacyl-tRNA binding enzyme (6). The ability to bind an extra increment of Met-tRNA under conditions of dipeptidyl-tRNA synthesis (Table IV) appears to be due to the “clearing” of the Met-tRNA initially bound (during the initiation reaction) by the synthesis of dipeptidyl-tRNA. Thus, the increment of bound Met-tRNA is essentially equal to the Met-tRNA initially bound (Table IV, and to the quantity of Met-Ala-tRNA formed in dipeptide synthesis (Fig. 2). In addition, the conditions required for obtaining the increment in bound Met-tRNA and the sensitivity of this reaction to chlortetracycline are identical with that which is observed for dipeptidyl tRNA synthesis. The occurrence of the “extra Met-tRNA” reaction per se is unexpected. Although several schemes might account for this observation, the most likely explanation would modify the accepted model of ribosomal function (20, 21), utilizing a third ribosomal site where Met-tRNA could bind without participating or interfering in the subsequent reactions of chain elongation.  

We have recently observed that the “extra” Met-tRNA remains ribosome-bound as Met-tRNA during the three-step reaction. At the same time, the initially bound Met-tRNA is converted to Met-Ala-Lys-tRNA.

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