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N-Terminal Sequence of the Eucaryotic *in Vitro* Product Made upon Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid†

William H. Klein and John M. Clark, Jr.*

ABSTRACT: This paper presents a thorough analysis of the N-terminal amino acid sequence of the satellite tobacco necrosis virus (STNV) coat protein produced by translation of STNV ribonucleic acid in an *in vitro* wheat embryo system. Short term incubations detect a limited incorporation of [^{35}S]methionine from [^{35}S]Met-tRNA$_i^{Met}$. This incorporated [^{35}S]Met is largely lost on prolonged incorporation. Sucrose gradient analysis of protein synthesis initiation complexes of [^{3}H]Met-tRNA$_i^{Met}$, STNV-RNA, and wheat embryo ribosomes demonstrate that formation of the complexes requires the methionine-specific initiator tRNA. Electrophoretic resolution of labeled products found in these complexes detects the STNV-RNA dependent production of the peptide Met-Ala-Lys and its precursors. Comparison of this apparent N-terminal sequence with the Ala-Lys · · · N-terminal sequence of *in vivo* STNV coat protein dictates that STNV-RNA translation by an *in vitro* procaryotic system in that correct translation begins on a Met codon immediately prior to the codons governing the final *in vivo* sequence.

Many reports describe the *in vitro* translation of a messenger or viral RNA in heterologous cell-free extracts (Heywood, 1969; Housman *et al.*, 1971; Lane *et al.*, 1971; Laycock and Hunt, 1969; Stavnezer and Huang, 1971). Proof of the accuracy of such heterologous *in vitro* translation requires characterization of the *in vitro* products. In particular, proof of common initiation signals between mRNAs of various origins requires N-terminal amino acid sequence analysis of the *in vitro* products.

We have previously reported the *in vitro* translation of STNV-RNA[1] in both procaryotic (*Escherichia coli*) and eucaryotic (wheat embryo) extracts produces protein containing the amino acid sequence of STNV coat protein (Klein *et al.*, 1972). STNV coat protein (*in vivo*) has the N-terminal sequence I

$$\text{Ala-Lys-}\begin{matrix}\text{Glu}\\\text{Gln}\end{matrix}\cdots \qquad \text{I}$$

(Klein *et al.*, 1972). The major *in vitro* product of procaryotic translation of STNV-RNA has the N-terminal sequence fMet-Ala-Lys · · · (Lundquist and Clark, 1971; Lundquist *et al.*, 1972). This suggests that procaryotic translation of STNV-RNA initiates with an fMet immediately prior to the eventual *in vivo* N terminus. The *in vitro* eucaryotic translation of STNV-RNA yields a product containing alanine as the predominant N-terminal amino acid (Lundquist *et al.*, 1972). This paper reports that this N-terminal alanine arises from a eucaryotic initiation of STNV-RNA translation with the sequence Met-Ala-Lys · · · followed by specific removal of the N-terminal methionine. Thus both the procaryotic and eucaryotic *in vitro* systems initiate STNV-RNA translation at the same site.

Experimental Procedures

Materials. STNV and STNV-RNA were prepared as described previously (Liu *et al.*, 1969; Clark and Klein, 1973). The cell-free wheat embryo system used was an S-23 extract (Marcus *et al.*, 1968) or ribosomes and S-100 derived from such S-23 extracts. Wheat embryo ribosomes were washed two times (Marcus, 1970). S-100 extracts were further freed of endogenous tRNA by passage over DEAE-cellulose preequilibrated with 0.1 M Tris-Cl, pH 7.4, and 8 mM β-mercaptoethanol. The resultant DEAE S-100 was then equilibrated with 1 mM Tris-Cl, pH 7.4, 2 mM $Mg(OAc)_2$, 50 mM KCl, and 5 mM β-mercaptoethanol, by brief (2 hr) dialysis.

Crude tRNA was prepared from wheat germ (Glitz and Dekker, 1963). The two methionine-accepting tRNAs (tRNA$_i^{Met}$ and RNA$_M^{Met}$) of this preparation were resolved from each other on benzoylated DEAE-cellulose (Leis and Keller, 1970) and then separately aminoacylated with [^{35}S]Met according to the method of Loehr and Keller (1968).

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received November 17, 1972.* Supported in part by National Institutes of Health Research Grant No. GM-08647. Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid. III. See Lundquist *et al.* (1972) for the preceding paper in the series.

[1] Abbreviations used are: STNV, satellite tobacco necrosis virus; tRNA$_i^{Met}$, methionine-specific initiator tRNA not capable of accepting a formyl group on its methionine; Met-tRNA$_i^{Met}$, methionyl ester containing tRNA$_i^{Met}$; tRNA$_M^{Met}$, noninitiator methionine-specific tRNA; Met-tRNA$_M^{Met}$, methionyl ester containing tRNA$_M^{Met}$.

fMet-Ala-Lys, a gift from Dr. Philip Leder, National Institutes of Health, Bethesda, Md., was converted to Met-Ala-Lys by treatment with methanolic HCl and subsequent NH_4OH treatment (Sheehan and Yang, 1958). Met-Ala was purchased from Cyclo Chemical Co. ^3H, ^{14}C, or ^{35}S amino acids were purchased from New England Nuclear.

Assays. Protein synthesis assays were as described previously (Klein *et al.*, 1972) with the exception that amino acids were provided as 0.3 pmol (0.05 μCi) of [^{35}S]Met-tRNA$_i^{Met}$ or [^{35}S]Met-tRNA$_M^{Met}$ plus 3.0 $\times$ 10^{-6} M of the 20 ^{12}C amino acids common to proteins.

Binding assays of Met-tRNA–STNV-RNA–ribosome complex formation were carried out in 0.36-ml volumes containing 0.03 M Tris-Cl, pH 7.4, 0.04 M KCl, 1.2 mM $Mg(OAc)_2$, 8.3 mM β-mercaptoethanol, 1.5 mM ATP, 0.2 mM GTP, 40 μg of STNV-RNA, 20 μg of tRNA$_i^{Met}$ or 40 μg of tRNA$_M^{Met}$, 0.05 ml of ribosomes (5.5 A_{260} units), 0.2 ml of DEAE S-100, and 1.1 $\times$ 10^{-5} M [^{3}H]methionine (5 Ci/mmol) or 2.5 $\times$ 10^{-5} M [^{14}C]methionine (220 mCi/mmol). Binding assays employing a double label utilized 1.1–2.2 $\times$ 10^{-5} M [^{3}H]methionine (5 Ci/mmol) and 1.6–3.5 $\times$ 10^{-5} M of a [^{14}C]amino acid (alanine, 158 mCi/mmol; lysine, 342 mCi/mmol; isoleucine, 338 mCi/mmol, or arginine, 251 mCi/mmol). Binding assays were incubated for 30 min at 30°, before centrifugation (2 hr, 201,000g) on an 11-ml, 5–20% linear sucrose gradient containing 10 mM Tris-OAc (pH 7.6)–70 mM KCl–5 mM $Mg(OAc)_2$. Fractions (0.3 ml) from these gradients were then assayed for A_{260}.

When just gradient resolution data were desired all fractions were analyzed for counts per minute. When specific ribosome bound materials were to be further analyzed, the A_{260} peak representing the 80S ribosomes was pooled. The resultant aminoacyl-tRNA–STNV-RNA–ribosome complexes were then bound to Millipore filters (Seal and Marcus, 1972) and washed two times with 10 mM Tris-OAc (pH 7.6), 70 mM KCl, and 5 mM $Mg(OAc)_2$. The filter-bound complexes were then decomposed with three, 1-ml washes of water-saturated triethylamine. The released labeled amino acids or peptides in these triethylamine washings were then lyophilized to dryness, taken up in 0.05 ml of pH 3.5 pyridine–acetate buffer (pyridine–acetic acid–H_2O, 1:6.6:192.5), spotted on Whatman 3MM paper, and finally resolved by 3.5 hr of electrophoresis (92.5 V/cm) in pH 3.5 pyridine–acetate buffer. Paper segments (1 cm) from this electrophoretic separation were counted by liquid scintillation counting and compared against ninhydrin-detectable standards electrophoresed in an identical manner.

Results

Few proteins isolated from eucaryotic cells contain methionine as the N-terminal amino acid, yet several reports demonstrate eucaryotic initiation of protein synthesis, with N-terminal methionine (Smith and Marcker, 1970; Marcus *et al.*, 1970). These observations suggest specific removal of the N-terminal methionine by eucaryotic systems. To date, only a few reports exist which describe such a specific removal of initiating methionine (Housman *et al.*, 1970; Wigle and Dixon, 1970). In the light of these observations, it is essential that we further examine our initial observation that translation of STNV-RNA in an *in vitro* eucaryotic (wheat embryo) system yields an STNV coat protein containing an N-terminal alanine. We report here several experiments designed to detect initiation of STNV coat protein synthesis with an N-terminal

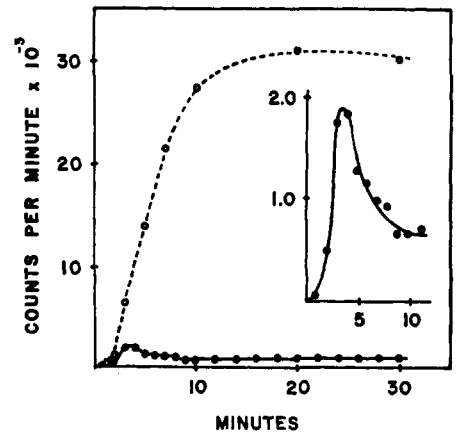

FIGURE 1: [^{35}S]Met incorporation from [^{35}S]Met-tRNA$_i^{Met}$ (●) or [^{35}S]Met-tRNA$_M^{Met}$ (○) into STNV coat protein as a function of time. A protein synthesis assay 10 $\times$ normal for [^{35}S]Met-tRNA$_M^{Met}$ or 20$\times$ normal for [^{35}S]Met tRNA$_i^{Met}$ was incubated at 30°. Samples (0.36 ml) were removed at indicated times and assayed for hot trichloroacetic acid insoluble counts. Counts reported reflect subtraction of counts derived from a minus STNV-RNA control.

methionine followed by specific removal of the initiating methionine.

One proof of the involvement of N-terminal methionine in STNV coat protein synthesis by the wheat embryo system is detection of the transient incorporation of [^{35}S]methionine from [^{35}S]Met-tRNA$_i^{Met}$ into the partially synthesized STNV coat protein. As seen in Figure 1, incubation of the wheat embryo system with 20 amino acids plus [^{35}S]Met-tRNA$_i^{Met}$ leads to an early and limited "pulse" of [^{35}S]Met incorporation into acid-insoluble material. Continued incubation of this system results in the loss of much of this label. Incubation of this same system with 20 amino acids plus [^{35}S]Met-tRNA$_M^{Met}$ (instead of [^{35}S]Met-tRNA$_i^{Met}$) leads to an incorporation of [^{35}S]Met that mimics the incorporation of other labeled amino acids into STNV coat protein.

Qualitatively, these data of Figure 1 suggest utilization of the eucaryotic initiator tRNA, Met-tRNA$_i^{Met}$, during STNV-RNA translation by the wheat embryo system. Quantitative correlation of this observation from these data is more difficult. Specifically, the experiment requires a high level of unlabeled Met to minimize migration of [^{35}S]Met label from the added [^{35}S]Met containing methionine-specific tRNA form to the other methionine-specific tRNA form present in the S-23 extract. This added [^{12}C]Met is also taken up by the system and results in an approximately 40-fold isotopic dilution of [^{35}S]Met incorporation as compared with direct incorporation of labeled Met by this system. Similar, yet unquantitated, isotopic dilution takes place during the transient incorporation of [^{35}S]Met from the initiator tRNA, [^{35}S]Met-tRNA$_i^{Met}$. Stoichiometric analysis of this transient incorporation is no doubt further complicated by the simultaneous incorporation of [^{35}S]Met into some initiating chains of STNV coat protein and removal of [^{35}S]Met from other partially synthesized chains of STNV coat protein. Regardless of the quantitative interpretation of these results, the only explanation for the transient incorporation of [^{35}S]Met from [^{35}S]Met-tRNA$_i^{Met}$ by this system is initiation with an N-terminal Met followed by subsequent specific removal of this Met.

If the *in vitro* translation of STNV-RNA by the eucaryotic system does initiate with an N-terminal methionine, then one should be able to detect protein synthesis initiation complexes

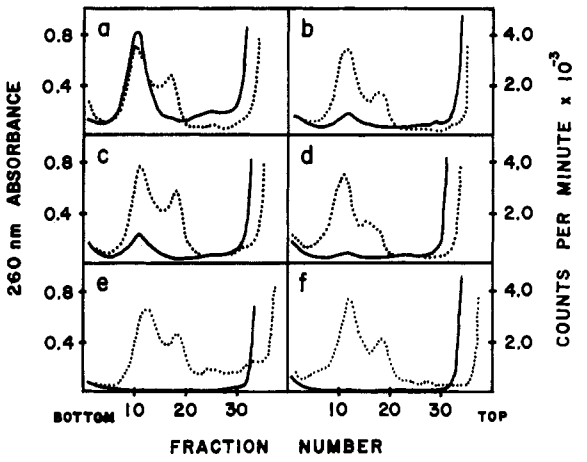

FIGURE 2: Sucrose gradient analysis of [^{3}H]Met-tRNA^{Met}–STNV-RNA–wheat embryo ribosome complexes: solid lines represent ^{3}H counts from [^{3}H]Met; dotted lines represent A_{260} absorbing material; (a) complete system; (b) omission of STNV-RNA; (c) substitution of $tRNA_M^{Met}$ for $tRNA_i^{Met}$; (d) substitution of $tRNA_M^{Met}$ for $tRNA_i^{Met}$ and omission of STNV-RNA; (e) omission of DEAE S-100; (f) omission of DEAE S-100 and STNV-RNA.

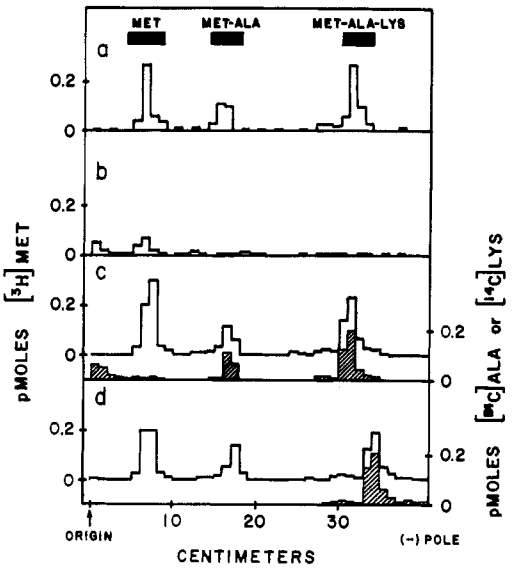

FIGURE 3: Electrophoretic analysis of peptides isolated from aminoacyl-tRNA–STNV-RNA–wheat embryo ribosome complexes: enclosed areas represent picomoles from [^{3}H]Met; hash marked areas represent picomoles from [^{14}C]Ala or [^{14}C]Lys; (a) [^{3}H]Met label from a complex of a single sucrose gradient was isolated as described in Experimental Procedures; electrophoresis was performed along with the indicated standards; (b) same as in (a) except STNV-RNA was omitted; (c) [^{3}H]Met and [^{14}C]Ala label from complexes of four sucrose gradients; (d) [^{3}H]Met and [^{14}C]Lys label from complexes of three sucrose gradients.

between [^{3}H]Met-$tRNA_i^{Met}$, STNV-RNA, and wheat embryo ribosomes. Accordingly, we incubated [^{3}H]Met with STNV-RNA, wheat embryo ribosomes, DEAE S-100, ATP, GTP, and one of the two forms of methionine-specific tRNA ($tRNA_i^{Met}$ or $tRNA_M^{Met}$) under conditions that lead to both aminoacyl-tRNA synthesis and formation of specific protein synthesis initiation complexes. As seen in Figure 2, sucrose gradient analysis of such reactions reveals the formation of [^{3}H]Met containing complexes on ribosomes that are dependent upon STNV-RNA, enzymes, and added $tRNA_i^{Met}$. As reported earlier (Seal and Marcus, 1972) wheat embryo ribosomes also form a low level of message-specific Met-$tRNA_M^{Met}$–ribosome–mRNA complexes under these conditions.

Full characterization of the above results as "initiation complexes" requires proof of the aminoacyl-tRNA character of the bound isotopic label. We therefore pooled the ribosome bound [^{3}H]Met containing gradient fractions from a complete system (as in Figure 2a), adsorbed the ribosome bound ^{3}H material(s) on Millipore filters, decomposed the labeled tRNA forms of the ribosome complexes with aqueous triethylamine washes, and then analyzed the character of the ^{3}H label by paper electrophoresis against standards. As seen in Figure 3a, much of the label migrates as [^{3}H]Met, but significant portions of the label migrate at two other locations. This suggests the existence of a limited degree of specific peptide synthesis analogous to the initial amino acid sequence of STNV coat protein. STNV coat protein (*in vivo*) has the N-terminal sequence I shown previously (Klein *et al.*, 1972). Assuming the above theorized limited degree of correct synthesis of the N-terminal end of STNV coat protein, and assuming an N-terminal methionine is removed to yield the final (*in vivo*) STNV coat protein, one must predict that the three labeled peaks of Figure 3a are [^{3}H]Met, [^{3}H]Met-Ala, and [^{3}H]Met-Ala-Lys.

Three lines of experimentation prove that the labeled components of Figure 3a are, in fact, [^{3}H]Met, [^{3}H]Met-Ala, and [^{3}H]Met-Ala-Lys. First, Figure 3a shows that the three ^{3}H-labeled peaks co-run with the three known standards Met, Met-Ala, and Met-Ala-Lys. Secondly, if the peptide synthesis is specific one would expect to find alanine and lysine, but not other amino acids, in the ribosomal complex. Figure 4 shows sucrose gradient analysis of reactions containing [^{3}H]methionine and [^{14}C]alanine or [^{14}C]lysine. Both [^{14}C]-alanine and [^{14}C]lysine are present in the ribosomal complex. Separate control experiments utilizing [^{14}C]arginine or [^{14}C]-isoleucine show no ^{14}C counts in this 80S region.

Final proof of the N-terminal sequence arises from the data of Figures 3b and 3c. In these experiments ribosome complexes containing [^{3}H]methionine and [^{14}C]alanine or [^{3}H]-methionine and [^{14}C]lysine were isolated from sucrose gradients, and decomposed with aqueous triethylamine. The released materials (peptides) were then resolved by paper electrophoresis as before and quantitatively analyzed for ^{3}H and ^{14}C content. This stoichiometric analysis reveals that the peak co-running with the Met-Ala standard contains methionine and alanine in a 1 : 1 ratio while the peak co-running with the Met-Ala-Lys standard contains methionine, alanine, and lysine in a 1 : 1 : 1 ratio (Table I).

Thus, the *in vitro* translation of STNV-RNA by the eucaryotic wheat embryo system initiates with the sequence Met-Ala-Lys · · · .[2] Our previous observation (Lundquist *et al.*, 1972) that the extended translation of STNV-RNA by this system results in STNV coat protein containing N-terminal alanine strongly suggests that the initiating methionine is specifically removed to yield the observed alanine-terminated, STNV coat protein.

[2] An independent and similar isolation of Met-Ala-Lys· · · as the initial peptide obtained upon limited translation of STNV-RNA has recently been made (S. N. Seal and A. Marcus, manuscript in preparation).

TABLE I: Stoichiometric Analysis of Electrophoretically Resolved Peptides Eluted from Ribosomal Complexes.[a]

Radioact. Co-migrating with	pmol [^{3}H]Met	pmol [^{14}C]Ala
Expt 1: Reaction Labeled with [^{3}H]Met and [^{14}C]Ala[b]		
Met	0.57	0.05
Met-Ala	0.23	0.20
Met-Ala-Lys	0.47	0.40
Expt 2: Reaction Labeled with [^{3}H]Met and [^{14}C]Lys[b]		
Met	0.45	0.02
Met-Ala	0.22	0.01
Met-Ala-Lys	0.40	0.45

[a] Data obtained from Figures 3c and 3d. [b] Quantities of individual radioactive amino acids present and derived from comparative scintillation counting analysis of paper-bound ^{3}H and ^{14}C standards and paper-bound labeled materials co-migrating with the indicated marker peptides. The labeled components were eluted (triethylanine) from Millipore filter bound materials obtained from sucrose gradient resolved ribosome complexes as described in Experimental Procedures.

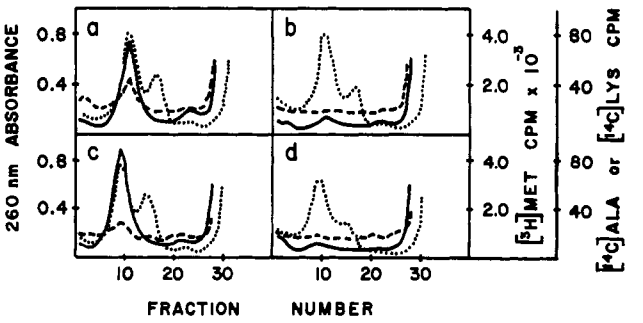

FIGURE 4: Sucrose gradient analysis of [^{3}H]Met tRNA$_i^{Met}$–STNV-RNA–wheat embryo ribosome complexes with added [^{14}C]Ala or [^{14}C]Lys: solid lines represent ^{3}H counts from [^{3}H]Met; dotted lines represent A_{260} absorbing material; dashed lines represent ^{14}C counts from [^{14}C]Ala or [^{14}C]Lys; (a) complete system with [^{14}C]-Ala; (b) same as (a) except STNV-RNA was omitted; (c) complete system with [^{14}C]lysine; (d) same as (c) except STNV-RNA was omitted.

Discussion

The relative amounts of Met, Met-Ala, and Met-Ala-Lys detected in Figure 3 possibly provide some insight into the workings of the limited translation of STNV-RNA by this ribosome complex system. If one subtracts the limited [^{3}H]-Met binding that occurs in the absence of STNV-RNA from the STNV-RNA-dependent [^{3}H]Met binding, one observes that the system produces more bound Met-Ala-Lys than bound Met-Ala or bound Met. This observation plus our failure to detect significant quantities of other ^{3}H components suggests the system contains limited quantities of the fourth amino acid and/or its tRNA. The limited protein synthesis by this ribosome binding system of Figure 3 also suggests that the specific removal of an initiating, N-terminal Met takes place on developing peptide chains larger than three amino acids long.

This article suggests that the *in vitro* product of eucaryotic translation of STNV-RNA initiates only with the sequence Met-Ala-Lys$\cdots$. Our data do not, however, rule out the existence of other points of initiation of STNV-RNA translation by the wheat embryo system. Specifically, our failure to isolate other initial peptides from the initiation complexes may reflect the limited tRNA and amino acid populations present during the formation of these complexes on the ribosomes. In any event, the Met-Ala-Lys sequence detected closely resembles fMet-Ala-Lys, the major N-terminal sequence found from the *in vitro* procaryotic (*E. coli*) translation of STNV-RNA (Lundquist and Clark, 1971; Lundquist *et al.*, 1972), and overlaps the known *in vivo* N-terminal sequence I of STNV coat protein (Klein *et al.*, 1972). Thus, translation of STNV-RNA by these diverse systems is both similar and correct. It follows that the initiation factors and ribosomes from these diverse systems must both recognize a common sequence and/or structural feature of STNV-RNA that constitutes the correct site of initiation for STNV-RNA translation.

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