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Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid. II. Initiation of *in Vitro* Translation in Procaryotic and Eucaryotic Systems†

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ABSTRACT: This paper reports the N-terminal amino acid of the product of *in vitro* translation of satellite tobacco necrosis virus ribonucleic acid (STNV-RNA) by both a procaryotic (*Escherichia coli*) and eucaryotic (wheat embryo) system. *In vitro* translation of satellite tobacco necrosis virus RNA by the procaryotic (*Escherichia coli*) system initiates with fMet-tRNA$_f^{Met}$. Specifically, deformylation of the *in vitro* product protein followed by end-group analysis with fluorodinitrobenzene reveals DNP-Met. At 6–7 mM Mg^{2+} levels, extracts from *Escherichia coli* deprived of formyl donors by the action of trimethoprim require formyltetrahydrofolic acid for translation of the RNA. The viral-RNA-dependent incorporation of [^{3}H]formate from [^{3}H]formyltetrahydrofolic acid into protein results in the preferential labeling of one tryptic fingerprint peptide. Digestion of the *in vitro* product protein with specific proteases followed by ion-exchange procedures yields *N*-formylmethionine. Similar ion-exchange analyses of the product of *in vitro* translation of STNV-RNA by the eucaryotic (wheat embryo) system fail to detect fMet in the product. In contrast, end-group analyses of the *in vitro* eucaryotic product reveal Ala as the most prevalent N-terminal amino acid. These data support the theory that the original, eucaryotic, STNV-RNA translation product has specifically lost an N-terminal methionine to yield an alanine-terminated protein.

Protein chain initiation in procaryotes (Marcker and Sanger, 1965; Adams and Cappechi, 1966; Eisenstadt and Lengyel, 1966; Horikoshi and Doi, 1968), and presumedly mitochondria and chloroplasts of higher organisms (Smith and Marcker, 1968; Galper and Darnell, 1969), utilizes the codon AUG (Thach *et al.*, 1966) as a chain-initiation signal to code for fMet-tRNA$_f^{Met}$.[1] The resultant initial product proteins of such systems, containing formylmethionine in their N termini, are then modified, if necessary, by deformylation and/or exopeptidase action, to yield the final product proteins (Takeda and Webster, 1968).

The mechanism of protein chain initiation in the cytoplasmic fraction of cells of higher organisms (eucaryotes) is not as well characterized. Current evidence indicates that a nonformylated, yet methionine-specific tRNA is uniquely involved in protein chain initiation in the cytoplasm of eucaryotic systems (Smith and Marcker, 1970; Marcus *et al.*, 1970; Housman *et al.*, 1970; Wigle and Dixon, 1970). Other evidence suggests that a deacylated, possibly methionine-specific, tRNA is involved in this initiation process (Culp *et al.*, 1970).

A role for some form of methionine-specific tRNA is central to all of these initiation schemes. Thus, the methionine codon AUG is presumedly involved. Yet protein chain initiation must require a mechanism more complex than the existence of the methionine codon sequence, AUG. Specifically, protein chain initiation must avoid "missense" initiations

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[1] Abbreviations used are: STNV, satellite tobacco necrosis virus; TYMV, turnip yellow mosaic virus; fMet, *N*-formylmethionine; fH$_4$Fol, N^{10}-formyltetrahydrofolic acid; tRNA$_f^{Met}$, methionine-specific-tRNA capable of accepting a formyl group on its methionine; Met-tRNA$_f^{Met}$, methionyl ester containing tRNA$_f^{Met}$; fMet-tRNA$_f^{Met}$, formylmethionyl containing tRNA$_f^{Met}$; 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$_i^{Met}$; FDNB, fluorodinitrobenzene.

spanning portions of two consecutive codons. Further, protein synthesis must distinguish between AUG sequences that code for protein chain-initiation positions and AUG sequences that code for internal methionine residues.

Satellite tobacco necrosis virus RNA provides an ideal probe to investigate these subtleties of protein chain initiation. First, STNV-RNA is a small, possibly monocistronic message for the viral coat protein (Reichmann, 1964). Second, STNV-RNA serves as a functional message in cell-free systems from both procaryotic (*Escherichia coli*) and eucaryotic (wheat embryo) sources (Clark *et al.*, 1965; Klein *et al.*, 1972). Further, typtic digestion of the products produced from these two *in vitro* systems, followed by fingerprint analysis, reveals coincidence between the detectable radioactive peptides and a majority of the tryptic peptides derived from STNV coat protein (Klein *et al.*, 1972). Thus, within the limits of detection by fingerprint analysis, translation of STNV-RNA by both the procaryotic and eucaryotic systems is correct and essentially complete.

These convenient features emphasize the need for a closer examination of the initiation of translation of STNV-RNA. This paper reports on the initial amino acids of the STNV-RNA translation product made by *in vitro* procaryotic and eucaryotic systems.

Experimental Procedures

Materials. Cell-free extracts of *E. coli* A-19 were prepared from cells grown and extracted as previously described (Clark *et al.*, 1965). Extracts of *E. coli* A-19 deficient in formyl components were prepared by trimethoprim treatment (Eisenstadt and Lengyel, 1966). The cell-free eucaryotic system employed S-23 extracts of wheat embryo (Marcus *et al.*, 1968).

STNV-RNA was obtained from STNV by phenol extraction (Kirby, 1965) of virus grown on tobacco (Reichmann, 1964) or mung beans (Liu *et al.*, 1969). The STNV-RNAs obtained from virus isolated from these varied sources are identical in physical properties and yield identical translation products upon fingerprint analysis. TYMV-RNA was obtained by phenol extraction (Kirby, 1965) of TYMV grown on Chinese cabbage.

[^{3}H]Formyltetrahydrofolic acid of specific activity of 1.27 Ci/mmole was prepared enzymatically from tetrahydrofolic acid (Goldthwait and Greenberg, 1955). Sodium [^{3}H]formate was purchased from Tracerlab, Co., Waltham, Mass. [^{14}C]-Amino acids and amino acid mixtures were as sold by New England Nuclear Co. Trimethoprim was the generous gift of Dr. G. H. Hitchings of Burroughs Wellcome Co., Tuckahoe, N. Y. Specific enzymes were purchased from Worthington Biochemical Corp., Freehold, N. J. fMet was purchased from Mann Research Laboratories, New York, N. Y. fMet-Ala-Lys was the generous gift of Dr. Phillip Leder, NIH, Bethesda, Md.

Enzyme Incubations. Unless stated otherwise, reaction conditions for the cell-free *E. coli* assays were as previously reported (Klein *et al.*, 1972) with the following exceptions. Where listed, certain reactions involved Mg^{2+} concentrations other than 0.01 M Mg^{2+}. As listed, certain reactions include 50 μM L-[^{14}C]Met (specific activity of 100–150 mCi/mmole) supplemented with 40 μM concentrations of the 19 other L-[^{12}C]amino acids required for protein synthesis. As indicated, certain reactions contained 40 μM [^{3}H]fH_4Fol (specific activity 1.27 Ci/mmole) supplemented with 40 μM levels of the 20 L-[^{12}C]amino acids found in proteins. The 20-min, 37° incubations employed are sufficient to allow all reactions to go to completion.

The incubation conditions for assays with the cell-free wheat embryo system were as reported (Klein *et al.*, 1972) except that the incorporation utilized single [^{3}H]- or [^{14}C]-amino acids supplemented with 3×10^{-5} M concentrations of the other 19 L-[^{12}C]amino acids commonly found in proteins. The 30-min, 30° incubations employed are sufficient to allow all reactions to go to completion.

Analysis Methods. [^{14}C]Amino acid incorporation into protein was assayed by means of scintillation counting of acid-precipitable material collected on Millipore-type HAWP filters after treatment with hot 5% trichloroacetic acid (Conway and Lipmann, 1964). Assays involving [^{3}H]formyl incorporation from [^{3}H]formyl-tetrahydrofolic acid into protein were terminated by 10-min, 37° incubations in the presence of 100 μg of pancreatic RNase followed by four repetitive washings onto Millipore filters with cold (0–2°) 5% trichloroacetic acid. Precipitated labeled protein retained on Millipore filters after drying at 27° for 8–12 hr was counted by liquid scintillation counting in either a Packard Model EX-314 or a Beckman Model LS-133 scintillation counter.

Reactions destined for derivatization with FDNB were terminated with pacreatic RNase, as above, adjusted to pH 11 with KOH, dialyzed against 1% $(NH_4)_2CO_3$ and then water until no label was detected in the dialysate, and finally freeze-dried prior to FDNB derivatization and subsequent protein hydrolysis (Fraenkel-Conrat *et al.*, 1955). Where indicated, the existence of individual labeled amino acids in the N-terminal position of the protein product was detected by ether extraction (6 extractions of 3 ml each) of the diluted (1 M HCl) acid hydrolysate of the derivatized protein and subsequent scintillation counting of the ether extract. As indicated for other experiments, the ether extract of the acid hydrolysis mixture was analyzed by two-dimensional paper chromatography (Fraenkel-Conrat *et al.*, 1955) along with DNP-amino acid standards. Paper sectors containing the appropriate DNP-amino acid standards were counted directly by scintillation counting without correction for localized quenching on the paper by the yellow standards. Label coincident with DNP-Met, DNP-Met sulfoxide, and DNP-Met sulfone was summed and defined as DNP-Met. Where indicated, deformylation of the protein (prior to derivatization with FDNB) was carried out by 12-hr, 30° incubation of dialyzed and freeze-dried samples in 0.5 M HCl in anhydrous CH_3OH.

Reaction mixtures destined for digestion with trypsin and carboxypeptidases A and B were terminated, titrated, dialyzed, and lyophilized as the samples prepared for FDNB derivatization. The samples were then each suspended in 1.5 ml of 0.1 M Tris-Cl–0.02 M $CaCl_2$ (pH 8.3) and incubated (37°) with 0.1 mg of trypsin for 8 hr before addition of another 0.1 mg of trypsin and an additional 10–12 hr of 37° incubation. After 5 min of 100° treatment to denature residual trypsin, 0.5 ml of 0.4 M NaCl–0.1 M Tris-Cl–0.02 M $CaCl_2$ (pH 8.3)–100 μg of solubilized carboxypeptidase A–40 μg of carboxypeptidase B were added to each sample and incubations was continued for 8 hr at 37°.

The samples were then stored at −15° prior to passage over a 1.2 × 15 cm Dowex 50-X2 (H^+ form) column with water elution (flow rate 1 ml/min). Material adhering to the column is subsequently eluted (1 ml/min) with 0.3 M NH_4OH.

The deformylation of fMet was measured by ninhydrin reaction before and after varying 30° treatments with 0.5 M HCl in anhydrous CH_3OH or 1.0 M HCl in H_2O. Tryptic

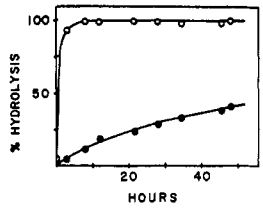

FIGURE 1: Relative ratio of deformylation of fMet at 30°: (○) 0.5 M HCl in anhydrous CH_3OH; (●) 1.0 M aqueous HCl.

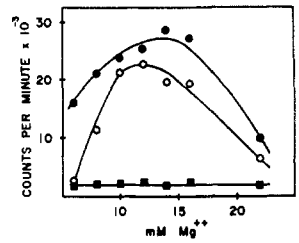

FIGURE 2: Formyl donor dependency of STNV-RNA translation by extracts from trimethoprim-treated *E. coli* cells: (●) STNV-RNA-dependent [^{14}C]amino acid incorporation in the presence of 30 μg of fH_4Fol; (○) STNV-RNA-dependent [^{14}C]amino acid incorporation in the absence of formyl donor; (■) STNV-RNA-independent amino acid incorporation in the presence of 30 μg of fH_4Fol.

fingerprints were prepared as previously described (Clark *et al.*, 1965). When a ^{3}H counts distribution was desired, the fingerprint paper was cut into 1 × 0.5 in. segments, counted by scintillation counting, washed in toluene, and then treated with ninhydrin in order to locate the tryptic peptides derived from added STNV coat protein.

Results

Several plant viral RNAs have been translated in *in vitro* procaryotic systems. These systems either demonstrate a requirement for initiation with fH_4Fol or fMet-$tRNA_f^{Met}$ and/or yield product protein(s) containing *N*-formylmethionine in their N-terminal positions (Kolakofsky and Nakamoto, 1966; Schwartz, 1967; Reinecke *et al.*, 1968; Hoogendam *et al.*, 1968). Our preliminary study (Reichmann *et al.*, 1966) of the N-terminal amino acid of the *in vitro* procaryotic (*E. coli*) product of STNV-RNA translation failed to detect fMet at the N terminus. Our assay for fMet was dependent upon an acid (1 N HCl, 8 hr, 27°) deformylation of the *in vitro* product followed by derivatization of the freed N-terminal amino group with FDNB and eventual chromatographic resolution (Fraenkel-Conrat *et al.*, 1955) of the DNP-[^{14}C]methionine released by acid hydrolysis. We have subsequently determined (Figure 1) that our acid hydrolysis conditions employed for deformylation are not adequate to deformylate fMet and expose methionine for derivatization with FDNB. Use of more rigorous HCl–methanol deformylation conditions (Sheehan and Yang, 1958) followed by derivatization with FDNB, acid hydrolysis of the derivatized product, and analysis as previously described, leads to the ready detection of an exposed, N-terminal methionine and and the apparent initiation of STNV-RNA translation with fMet-tRNA (Table I).

Additional proof that translation of STNV-RNA by an *in vitro* procaryotic system involves fMet-$tRNA_f^{Met}$ comes from use of extracts derived from *E. coli* grown in the presence of trimethoprim. Trimethoprim inhibits dihydrofolate reductase activity (Burchall and Hitchings, 1966). As a result, trimethoprim-treated cells are unable to synthesize fMet-$tRNA_f^{Met}$ and rapidly exhaust their endogenous fMet $tRNA_f^{Met}$. At low Mg^{2+} concentrations (5–7 mM), extracts from such trimethoprim-treated cells are totally dependent upon fH_4Fol or fMet-$tRNA_f^{Met}$ for the translation of fMet-$tRNA_f^{Met}$ requiring messenger RNAs (Eisenstadt and Lengyel, 1966).

As seen in Figure 2, the translation of STNV-RNA at low Mg^{2+} concentrations by such trimethoprim extracts is also totally dependent upon added fH_4Fol. At higher Mg^{2+} concentrations, this dependency disappears. Yet, the same reaction product is made over the Mg^{2+} range of Figure 2, for radioautographs of tryptic fingerprints of the ^{14}C product made at the various Mg^{2+} concentrations are essentially identical and all mimic (within the limits of the assays) the STNV coat protein fingerprint.

The fH_4Fol dependence of the translation of STNV-RNA by extracts of *E. coli* treated with trimethoprim facilitates experiments involving the uptake of [^{3}H]formyl from [^{3}H]-formyltetrahydrofolic acid into the STNV-RNA translation product. As seen in Table II, an extract from trimethoprim-treated *E. coli* cells readily takes up [^{3}H]formate from [^{3}H]-formyl-tetrahydrofolic acid. This ^{3}H-labeled product is a large protein as indicated by its exclusion on Sephadex G-25 gel filtration and its subsequent inclusion on Sephadex G-25 after tyrpsin digestion. Further, this [^{3}H]formate-labeled protein contains its ^{3}H in an acid-labile linkage with the acid lability characteristics analogous to [^{3}H]formyl label incorporated from [^{3}H]formyltetrahydrofolic acid during translation of TYMV-RNA. Such formyl incorporation in *E. coli* extracts directed by TYMV-RNA is known to represent [^{3}H]fMet-$tRNA_f^{Met}$ mediated synthesis of [^{3}H]formylmethionine-containing protein(s) (Kolakofsky and Nakamoto, 1966).

Extracts from normal *E. coli* cultures (no trimethoprim) will also utilize [^{3}H]fH_4Fol during translation of STNV-RNA. Such extracts are known to produce STNV coat protein when utilizing STNV-RNA as mRNA (Clark *et al.*, 1965). [^{3}H]Formyl incorporation into this product should, therefore, yield synthesis of a single, fMet-containing protein. Tryptic digestion of such a [^{3}H]formyl-labeled protein followed by fingerprint analysis should result in a single,

TABLE I: Counts per Minute Recoverable in DNP-Methionine Relative to Prior Deformylation of STNV-RNA-Dependent Translation Product.[a]

Mg^{2+} Level during Protein Synthesis (mM)	Treatment of Protein Product	Cpm Recovd in DNP-Met	Total Cpm Incorporated
7	CH_3OH, HCl deformylation	5500	99,500
7	No deformylation	1801	99,500
10	CH_3OH, HCl deformylation	5801	117,500
10	No deformylation	2949	117,500

[a] ^{14}C-labeled DNP-Met was isolated by paper chromatography and counted as specified in Experimental Procedures.

TABLE II: Incorporation of [^{3}H]Formyl Label from [^{3}H]-Formyltetrahydrofolic Acid into Proteins by a Cell-Free Extract from Trimethoprim-Treated *E. coli* Cells.[a]

	Counts per Minute			
	6 mM Mg^{2+}		10 mM Mg^{2+}	
mRNA	Cold Acid Washed	Hot Acid Treatment	Cold Acid Washed	Hot Acid Treatment
STNV-RNA	1060	265	2055	1032
TYMV-RNA	1195	458	3725	1840
None			545	185

[a] The mRNA levels employed are saturating. The 20-min incubations are sufficient to allow all reactions to go to completion. Cold acid washed samples are assayed as described in Experimental Procedures. Hot acid treated samples were handled like the cold acid samples except that they were subjected to 10 min at 90° prior to the cold acid washes.

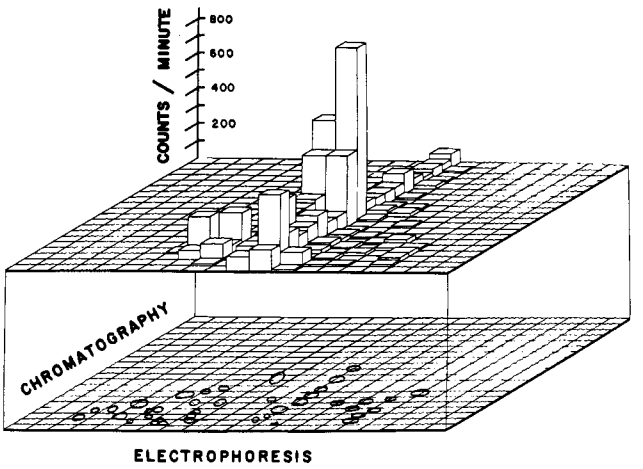

FIGURE 3: Counts distribution on a tryptic fingerprint of STNV-RNA-dependent counts incorporated from [^{3}H]fH_4Fol. The tryptic fingerprint was prepared as in Experimental Procedures; 25% of the ^{3}H counts remained as insoluble, "trypsin-resistant" material removed by centrifugation (2000*g*, 5 min) prior to fingerprint analysis.

predominant ^{3}H peptide containing the ^{3}H label. As seen in Figure 3, over 50% of the ^{3}H label detected on such a fingerprint of the *in vitro* product of STNV-RNA translation by our system does indeed reside in one location on the fingerprint. The remaining, diversified, ^{3}H label most likely reflects side products or artifacts such as ^{3}H peptides representing incomplete tryptic digestion, ^{3}H peptides generated by "missence" starts in the system, and undigested ^{3}H protein remaining at the origin.

Final proof of a role for fMet-$tRNA_f^{Met}$ in the translation of STNV-RNA by procaryotic (*E. coli*) extracts requires identification of fMet in the *in vitro* product. The blocked α-amino group of an fMet present in a protein provides a simplified method for the detection and characterization of such fMet. Trypsin and carboxypeptidases A and B catalyzed digestion of an fMet-containing protein followed by cation-exchange chromatography at neutral pH will result in retention of all the amino acids generated by such an enzymatic total digestion except for the N terminally blocked fMet.

As seen in Table III, such as anionic, ^{3}H-containing material is readily obtained from a trypsin and carboxypeptidase A and B catalyzed digest of [^{3}H]Met-labeled STNV-RNA translation product made by the *in vitro E. coli* system. This noncationic ^{3}H material is [^{3}H]fMet in that it runs coincident with fMet upon electrophoresis (3500 V, 2 hr, pyridine–acetate, pH 6.5 or 3.5) and chromatography (butanol–acetic acid–H_2O, 4:1:5). Further, methanolic HCl deformylation (Sheehan and Yang, 1958) of this labeled material yields an ^{3}H compound that migrates as [^{3}H]Met upon electrophoresis and chromatography. Methanolic HCl deformylation and subsequent drying of similar material, as produced from [^{3}H]fH_4Fol, results in complete volatilization of the ^{3}H label. Thus, the *in vitro* translation of STNV-RNA by a procaryotic (*E. coli*) system yields a protein containing fMet.

The cell-free protein-synthesizing system from wheat embryo (Marcus *et al.*, 1968) provides a useful system for comparative study of the initation of STNV-RNA translation. This *in vitro* system translates STNV-RNA to yield STNV coat protein (Klein *et al.*, 1972), thereby providing a useful eucaryotic system for comparison to the *in vitro* procaryotic translation of STNV-RNA of *E. coli*.

The specific enzymatic digestion and cation-exchange analysis procedures of Table III provides a direct assay for the existence of fMet in the product of wheat embryo translation of STNV-RNA. As seen in Table III, trypsin and carboxypeptidases A and B catalyzed digestion and subsequent cation-exchange analysis of [^{3}H]methionine-labeld protein produced by the *in vitro* wheat embryo system fails to yield any significant quantity of the nonabsorbed material characterizable as fMet. Further, this lack of fMet within the product does not reflect enzymatic deformylation of the protein product for incubation of fMet-containing procaryotic product of STNV-RNA translation with the wheat embryo system

TABLE III: Per Cent of Noncationic ^{3}H Label Derived from Protease-Treated, [^{3}H]Met-Containing STNV-RNA Translation Product.[a]

Source of Protein Synthesis System	% of Total Incorporated ^{3}H[c]
E. coli (3 experiments)	8.4, 11.5, 13.0
Wheat Embryo	0.2
E. coli followed by incubation of protein product with wheat embryo system[b]	9.4

[a] Protein products are digested with trypsin and carboxypeptidases A and B and then passed over Dowex 50-X2 (H^+ form) as described in Materials and Methods. Tryptic digestion alone (no carboxypeptidase) of the translation products results in passage of only insignificant amounts of ^{3}H material through Dowex 50-X2. [b] A pancreatic RNase-treated, dialyzed, and lyophilized [^{3}H]Met-labeled translation product made during 30-min incubation with the *E. coli* system was incubated with the standard wheat embryo system (minus STNV-RNA and isotopic amino acid) for 30 min prior to subsequent treatment with trypsin and carboxypeptidases A and B and analysis as in Experimental Procedures. [c] That passes through cation exchanger after exhaustive proteolytic digestion.

TABLE IV: Extraction of DNP-Amino Acids Derived from Acid Hydrolysis of Protein Synthesized during Eucaryotic Translation of STNV-RNA.

Amino Acid	Extraction Phase	Cpm	% Cpm in[a] Ether Phase	Expected %[b] if N Terminal
Methionine	Ether	176	1.5	16.7
	Water	11,802		
Alanine	Ether	358	6.3	6.3
	Water	5,308		
Lysine	Ether	72	0.4	12.5
	Water	17,087		
Leucine	Ether	21	0.4	6.3
	Water	4,806		
Phenylalanine	Ether	0	0.0	14.3
	Water	1,368		
Tyrosine	Ether	11	0.6	25.0
	Water	1,735		
Valine	Ether	6	0.1	7.1
	Water	5,062		

[a] Per cent cpm in the ether phase represents cpm in the ether phase divided by the total cpm (ether and water phases) times 100. [b] Expected per cent if N terminal is derived from the amino acid composition of STNV coat protein (Rees *et al.*, 1970).

does not decrease the yield of fMet obtainable subsequently upon enzymatic digestion and ion-exchange analysis. Thus, this eucaryotic translation of STNV-RNA does not lead to an fMet-containing product.

Failure to detect an N-blocked methionine derivative (specifically fMet) in the product produced upon translation of STNV-RNA by the cell-free wheat embryo system necessitates other attempts to characterize the initial or N-terminal amino acid utilized by this eucaryotic system. Protein synthesis initiation studies with other *in vitro* eucaryotic systems (Smith and Marcker, 1970; Marcus *et al.*, 1970; Housman *et al.*, 1970; Wigle and Dixon, 1970) suggest that eucaryotic systems produce protein carrying N-terminal methionine with a free amino group. Such free N-terminal amino groups can be detected in isotopically labeled proteins through use of FDNB derivatization, acid hydrolysis, and subsequent ether extraction. The original N-terminal amino acid with the free amino group is converted to DNP amino acid by this procedure and can be detected in the ether phase of the final acidic extraction (Marcus *et al.*, 1970).

Use of this procedure with various [^{14}C]amino acids (Table IV) reveals that of the amino acids tested, only [^{14}C]-methionine and [^{14}C]alanine yield significant labeled DNP derivatives. Assuming that eucaryotic systems initiate protein biosynthesis with Met-tRNA$_i^{Met}$ (Smith and Marcker, 1970; Marcus *et al.*, 1970; Housman *et al.*, 1970; Wigle and Dixon, 1970) and knowing that STNV-RNA codes for STNV coat protein *in vivo* which eventually has the N-terminal sequence

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

(Klein *et al.*, 1972), the data of Table IV, with their limited isolation of DNP-Met relative to DNP-Ala, suggest that this eucaryotic system contains an active system to remove N-terminal methionine from the ends of proteins. Preliminary attempts to detect such a specific exopeptidase have not been very rewarding. Although the wheat embryo extract will not hydrolyze dipeptides containing N-terminal fMet, the extract does hydrolyze dipeptides containing a wide variety of N-terminal amino acids. Thus, the apparent exopeptidase specificity that causes this eucaryotic system to yield a STNV-RNA-dependent protein containing (predominantly) N-terminal alanine may reflect three-dimensional features of the biosynthesized protein. Lacking more complete data on this, one must assume that the *in vitro* translation of STNV-RNA by this eucaryotic system yields a protein-containing N-terminal methionine. This N-terminal methionine is then presumedly removed to yield a protein-containing N-terminal alanine.

Discussion

The translation of STNV-RNA by both cell-free procaryotic and eucaryotic systems yields STNV coat protein (Clark *et al.*, 1965; Klein *et al.*, 1972). Yet the results presented here establish that these two systems initiate STNV coat protein synthesis in different manners. Specifically, the procaryotic (*E. coli*) system utilizes the well-known fMet-tRNA$_f^{Met}$ mechanism yielding a product initiated with fMet, while the eucaryotic (wheat embryo) system yields a product carrying N-terminal alanine, presumedly as a result of chain initiation with Met-tRNA$_i^{Met}$ and subsequent removal of the N-terminal methionine. Specifically, the observation (Table III) that the wheat embryo system does not deformylate the fMet-containing *E. coli* product of STNV-RNA translation supports the observation (Hunter and Jackson, 1971) that eucaryotes do not utilize an fMet-containing tRNA. Marcus *et al.* (1970) report that translation of tobacco mosaic virus RNA by the same wheat embryo system followed by FDNB derivatization, acid hydrolysis, and ether extraction yields large quantities of DNP-Met derived from the N-terminal amino acid of the newly synthesized protein(s). Their failure to detect appreciable removal of N-terminal methionine from their protein(s), and our ability to detect such methionine removal, may reflect differences in the sequence, structure, and general characteristics of the protein products.

The [^{3}H]formyl incorporation and fingerprint analysis data of Figure 3 provide additional evidence to support the monocistronic character of STNV-RNA. Specifically, only one region of the tryptic fingerprint contains significant [^{3}H]-formyl label incorporated from [^{3}H]fH_4Fol during STNV-RNA translation by the *E. coli* system. In separate experiments, this ^{3}H label moves coincident with the compound fMet-Ala-Lys. This fact and the known

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

sequence of the N terminus of STNV coat protein (Klein *et al.*, 1972) suggests that this ^{3}H label represents the correct initiation of STNV coat protein by this procaryotic system. Similarly, the indication of a Met-Ala-$\cdots$ N-terminal sequence on the product of eucaryotic (wheat embryo) translation of STNV-RNA (Table IV) suggests that both the procaryotic and eucaryotic *in vitro* systems initiate protein synthesis at the same site upon STNV-RNA.

A stoichiometric analysis of the data of Table III further

supports the concept that STNV-RNA translation on the procaryotic (*E. coli*) system leads to STNV coat protein containing fMet. There are 5 methionine residues in STNV coat protein (Rees *et al.*, 1970). Assuming that *in vitro* procaryotic translation adds an initial fMet containing a sixth methionine residue, then one-sixth or 16.66% of the incorporated [^{3}H]Met should be isolatable as [^{3}H]fMet. The isolated 8–13% [^{3}H]fMet (Table III) compares favorably with the 16.66% expected for fMet-initiated STNV coat protein.

The failure to isolate the full 16.66% [^{3}H]fMet expected from the *in vitro* procaryotic STNV-RNA translation product can be due to several causes. Varying degrees of a specific deformylase action (Takeda and Webster, 1968) can reduce the yield of [^{3}H]fMet and lead to the variations we observe in isolatable [^{3}H]fMet. Alternately, a limited amount of correct initiation may take place with a deformylated, yet methionine-containing tRNA (Met-tRNA$_f^{Met}$). Our study of the available N-terminal amino acids in the procaryotic translation product (Table I) does not rule out a limited amount of initiation with Met-tRNA$_f^{Met}$. Further, data from the artificial situation in which an *E. coli* extract is formyl deficient (Figure 2) support this alternate hypothesis. Specifically, Figure 2 shows that at 10 mM and higher Mg^{2+} concentrations, formyl-deficient *E. coli* extracts can initiate translation of STNV-RNA by a mechanism not requiring a formyl donor. Yet the products of this higher Mg^{2+} level translation by these formyl-deprived extracts yield fingerprints analogous to STNV coat protein. Thus, this high Mg^{2+} level translation of STNV-RNA mimics the lower Mg^{2+} level, formyl-dependent procaryotic system. The observation (Zamir *et al.*, 1966) that deformylated, yet methionine-containing tRNA (Met-tRNA$_f^{Met}$) replaces the formylated fMet-tRNA$_f^{Met}$ in protein chain initiation at 10 mM and higher Mg^{2+} concentrations also supports the concept that a limited amount of protein chain initiation may be taking place through use of Met-tRNA$_f^{Met}$.

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