Translation of Satellite Tobacco Necrosis Virus Ribonucleic Acid by an in Vitro System from Wheat Germ†

David W. Leung, Carl W. Gilbert, Robert E. Smith, Nancy L. Sasavage, and John M. Clark, Jr.*

ABSTRACT: The RNA of satellite tobacco necrosis virus (STNV) is an effective messenger RNA when translated in an in vitro system from wheat germ. This RNA codes for only STNV coat protein, as indicated (1) by coincidence of the tryptic fingerprints of the translation product and of STNV coat protein, (2) by equivalent size of the translation product and STNV coat protein, and (3) by isolation of an initial peptide of the in vitro product containing the amino acid sequence of the N terminus of STNV coat protein. STNV RNA does not contain a 5′-terminal $m^7G(5')ppp(5')Np\cdots$ group and translation of STNV RNA by the wheat germ system does not involve prior formation of 5′-terminal $m^7G(5')ppp(5')Np\cdots$ groups on STNV RNA. STNV RNA and ^{125}I-labeled STNV RNA form a specific initiation complex when incubated with initiator tRNA, GTP, initiation factors, and wheat germ ribosomes. Treatment of this specific initiation complex with ribonuclease A allows isolation of an ^{125}I-labeled oligonucleotide protected from ribonuclease A by the initiation complex. This specific oligonucleotide contains approximately 38 nucleotides, including nucleotide sequences that coincide with the codons of the N-terminal amino acids of STNV coat protein.

What features of a messenger RNA define certain AUG sequences as sites for initiation of protein synthesis or "initiator AUGs" remains a basic question in protein synthesis. Three proposed mechanisms attempt to answer this question. First, analysis of the nucleotide sequences about initiator AUGs of several procaryotic messenger RNAs suggests that certain initiator AUGs occur at the ends of loops created by the secondary structure of the messenger RNAs (Steitz, 1969; Hindley and Staples, 1969; Min Jou et al., 1972; Barrell et al., 1975). Extension of these observations predicts that initiation of translation occurs only at AUG sequences uniquely exposed by the secondary structure of the messenger RNA. Second, these and related nucleotide sequence data reveal messenger RNAs to contain one or more unique nucleotide sequence immediately to the 5′ side of the initiator AUGs (Robertson et al., 1973; Shine and Dalgarno, 1974). An initiator AUG region of a messenger RNA containing a purine-rich nucleotide sequence on the 5′ side of an initiator AUG forms specific base pairs with nucleotides at the 3′ ends of 16S ribosomal RNA (Steitz and Jakes, 1975). Thus, it is assumed that interactions between the 16S or 18S ribosomal RNA and specific messenger RNA nucleotides 5′ adjacent to AUGs define initiator AUGs. Thirdly, the 5′ ends of a variety of eucaryotic messenger RNAs contain a $m^7G(5')ppp(5')Np\cdots$ "capping" group, which is essential for the translation of these messenger RNAs (Both et al., 1975a; Muthukrishnan et al., 1975). Such capping groups are required for formation of translation initiation complexes with ribosomes (Both et al., 1975b). Competitive analogues of the $m^7G(5')ppp(5')Np\cdots$ structure inhibit the formation of translation initiation complexes by messenger RNAs containing the 5′-terminal capping group (Hickey et al., 1976). Thus, it is assumed that this capping group functions in the initiation of translation of certain eucaryotic messenger RNAs.

It is likely that all of these theories define, in part, phenomena associated with the selection of initiator AUGs of messenger RNAs. For example, the recent characterization of a nucleotide sequence about an initiator AUG of a brome mosaic virus RNA (BMV[1]) reveals the initiator AUG to be (1) in a region of the RNA apparently lacking extensive secondary structure, (2) within a region of the messenger RNA having affinity for 18S ribosomal RNA, and (3) close to a 5′ terminal $m^7G(5')ppp(5')Np\cdots$ group (Dasgupta et al., 1975). Further understanding of the role of each of these proposed mechanisms requires more data concerning the sites of initiation of translation of various messenger RNAs.

Satellite tobacco necrosis virus RNA provides an ideal system for study of features that define initiator AUGs within messenger RNAs. STNV RNA is a monocistronic messenger RNA coding for only STNV coat protein in in vitro systems obtained from procaryotes and eucaryotes (Klein et al., 1972; Lundquist et al., 1972; Klein and Clark, 1973). Thus, STNV RNA must contain the basic features used by both procaryotes and eucaryotes to govern the initiation of translation of messenger RNA. The in vitro system from wheat embryo of Marcus et al. (1968), previously used to demonstrate correct translation of STNV RNA by eucaryotes, is inefficient in that it requires large quantities of cell-free extract to measure protein synthesis. The in vitro system from wheat germ of Roberts and Paterson (1973) would appear to offer a more efficient in vitro system for study of the translation of STNV RNA by eucaryotes.

This paper reports the characteristics of the translation of STNV RNA by the in vitro system from wheat germ and evaluates the initiation of STNV RNA translation in terms of the above theories of initiator AUG selection. This paper further reports the isolation of a unique 38 nucleotide-long

† From the Biochemistry Department, University of Illinois, Urbana Illinois 61801. *Received April 14, 1976.* This work was supported by United States Public Health Service Grant GM-08647.

[1] Abbreviations used are: BMV, brome mosaic virus; STNV, satellite tobacco necrosis virus; TNV, tobacco necrosis virus; TMV, tobacco mosaic virus; $m^7G(5')ppp(5')Np\cdots$, 7-methylguanosine linked by its 5′ position through three phosphate anhydrides to the 5′ position of a nucleoside 3′-phosphate or 2′-*O*-methylnucleoside 3′-phosphate; $tRNA^{Met}_i$, methionine-specific initiator tRNA; Met-$tRNA^{Met}_i$, $tRNA^{Met}_i$ aminoacylated with methionine; DBAE-cellulose, dihydroxyboryl-substituted aminoethylcellulose; DEAE, diethylaminoethyl; ATP, GTP, adenosine and guanosine 5′-triphosphates.

fragment of STNV RNA containing the initiator AUG region of STNV RNA. Characterization of the nucleotide sequence within this RNA fragment should help to further define features of a messenger RNA that define specific AUGs as initiator codons.

Experimental Procedures

Materials. Cell-free S-30 extracts were prepared from wheat germ (General Mills Corp.) by the method of Roberts and Paterson (1973) and stored at −80 °C (without incubation) for later assay. Ribosomes were sedimented (1 h, 1 °C, 160 000*g*) from 1 volume of S-30 extracts and washed one time by resuspension in, and sedimentation from (1 h, 1 °C, 160 000*g*), 1 volume of 0.1 M KCl, 3 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 20 mM Hepes-K salt, pH 7.2 (KMH buffer), prior to resuspension in KMH buffer (400 A_{260} units/ml) and storage at −80 °C. DEAE S-160 was prepared by passage of the initial 160 000*g* supernatant (above) through a column of DEAE-cellulose equilibrated with KMH buffer. Fractions containing three-fourths of the eluted A_{280} absorbing materials were pooled and stored at −80 °C. Initiator tRNA ($tRNA_i^{Met}$) was prepared from wheat germ by the method of Leis and Keller (1970).

STNV RNA was prepared from STNV isolated from germinating mung beans infected with the B strain of TNV and the SV1 strain of STNV (Clark and Klein, 1974). STNV [^{32}P]RNA was prepared by incubation of 500 g of germination mung beans, previously infected for 2 days with TNV and STNV, with 30 mCi of, pH 7, carrier free ^{32}P, followed by isolation of STNV [^{32}P]RNA (Clark and Klein, 1974). STNV [^{125}I]RNA was prepared in vitro by the method of Commerford (1971) except that the resultant STNV [^{125}I]RNA was purified by alcohol precipitation and two subsequent resuspensions in, and sedimentations from (10 min, 1 °C, 10 000*g*), 3 M NaOAc, pH 6. The resultant washed STNV [^{125}I]RNA containing less than 1% of the cytidine residues derivitized as [5-^{125}I]iodocytidine (sp act. 5×10^6 γ cpm/μg) was finally sedimented (10 min, 1 °C, 10 000*g*) and dissolved in H_2O to a concentration of 2 mg/ml. Hemoglobin mRNA of rabbit reticulocytes was the generous gift of Dr. Byron Kemper.

[methyl-^{3}H]Methionine, [8-^{3}H]GTP, [α-^{32}P]GTP, a mixture of 15 L-[U-^{14}C]amino acids, and carrier free Na^{125}I were obtained from Amersham/Searle. $tRNA^{Phe}$ was purified from yeast tRNA (Gillam and Tener, 1971). Sparsomycin was obtained from the Upjohn Co. Poly(ethyleneimine)cellulose films for thin-layer chromatography were obtained from Brinkmann Instruments, Inc. Various m^{7}G(5′)ppp(5′)Np standards were obtained from P-L Biochemicals, Inc. Aurintricarboxylic acid was purchased from Kodak. Purified enzymes were obtained from Worthington Biochemical Corp. Other reagents were supplied by Sigma Chemical Co., or as specified.

Assays. In vitro protein synthesis was assayed at 25 °C for indicated times in 50-μl reactions containing KMH buffer, 30 μM L-asparagine, L-cysteine, L-glutamine, L-methionine, and L-tryptophan, 0.5 μCi of a mixture of 15 L-[U-^{14}C]amino acids (55 Ci/atom), 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 2 μg of creatine kinase, 20 μl of wheat germ S-30 extract, and 1–10 μg of mRNA. [^{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). [^{14}C]Protein synthesized by the wheat germ system in 60 min, tenfold (i.e., 500 μl), reactions was mixed with 4 mg of STNV coat protein and then subjected to tryptic fingerprint analysis as previously described (Clark et al., 1965). The resultant fingerprints represent incomplete, but reproducible, tryptic digestion, yielding more tryptic peptides than expected from the known arginine and lysine content of STNV coat protein (Rees et al., 1970; Reichmann, 1964). Gel electrophoresis analyses employed [^{14}C]protein samples previously treated with 5% trichloroacetic acid for 10 min at 90 °C and then washed four times by suspension in, and sedimentation from (5 min, 2000*g*), 5% trichloroacetic acid before neutralization and analysis (Weber and Osborn, 1969).

Initiation complexes containing Met-$tRNA_i^{Met}$, STNV RNA, and ribosomes were formed in 50-μl reactions containing KMH buffer, 30 μM L-methionine, 2 μM sparsomycin, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 2 μg of creatine kinase, 15 μl of DEAE S-160, 5 μl of ribosomes (400 A_{260} units/ml), 10 μg of $tRNA_i^{Met}$, and 4 μg of STNV RNA. As indicated, these incubations featured [methyl-^{3}H]methionine (5.3 Ci/mmol), [8-^{3}H]GTP (5.6 Ci/mmol), or STNV [^{125}I]RNA (5×10^6 γ cpm/μg) at the above concentrations. After 25 min at 25 °C, reactions were layered onto cold 11.6 ml, 15–30%, linear sucrose gradients containing 0.1 M KCl, 5 mM $Mg(OAc)_2$, 10 mM Tris–OAc, pH 7.5 (KMT buffer), and centrifuged (4 h, 1 °C, 35 000 rpm, i.e., 148 000*g*) in a Beckman SW-41 rotor. Fractions (0.5 ml) collected by draining punctured tubes were assayed for absorbance and radioactivity with a spectrophotometer and a scintillation counter, respectively.

Limited synthesis of protein with resultant synthesis of the initial peptides translated from STNV RNA employed initiation complexes prepared in 250-μl reactions as described above, except that sparsomycin was omitted, 0.1 mM L-alanine and 0.1 mM L-lysine were added, and the concentrations of L-methionine, STNV RNA, and $tRNA_i^{Met}$ were altered to 13 μM [methyl-^{3}H]methionine (7.5 Ci/mmol), 10 μg of STNV RNA/50 μl of reaction, and 75 μg of $tRNA_i^{Met}$/50 μl of reaction, respectively. After 25 min at 25 °C, ribosomes and associated materials were resolved and analyzed on sucrose gradients as above. Ribosome-bound materials in the pooled gradient fractions were further analyzed for [^{3}H]Met-containing compounds by the triethylamine-dependent hydrolysis and paper electrophoresis assay of Klein and Clark (1973).

Isolations of a ribosome-protected fragment of STNV [^{125}I]RNA were patterned after those of Steitz (1969). Initiation complexes migrating in the 80S ribosome region of 5–10 of the above sucrose gradient assays of initiation complexes were pooled, sedimented (90 min, 1 °C, 160 000*g*), and resuspended in 1 ml of KMT buffer. This preparation was treated with 0.2 μg of ribonuclease A for 12 min at 25 °C prior to isolation of the ribonuclease-treated initiation complexes by pelleting (90 min, 1 °C, 160 000*g*) through 7 ml of 15% sucrose in KMT buffer. [^{125}I]RNA, protected from ribonuclease action by the ribosomes of initiation complexes, i.e., "initiator region [^{125}I]oligonucleotide", was isolated from the pelleted ribosomes by extraction with sodium dodecyl sulfate–phenol–chloroform (Palmiter, 1974). This initiator region [^{125}I]oligonucleotide was analyzed by gel electrophoresis on 20% polyacrylamide gels (DeWachter and Fiers, 1971) in the presence of 7 M urea using the two half molecules derived from $tRNA^{Phe}$ (Theibe and Zachau, 1971) as standards. The initiator region [^{125}I]oligonucleotide was also analyzed by high-pressure (250 psi) liquid chromatography on a 2 × 500 mm column of RPC-5 (Miles Labs) in the presence of a 40-ml linear gradient of 0.25–2.0 M NaCl in 10 mM Tris-OAc, pH 7.5, 10 mM $Mg(OAc)_2$. ^{125}I-containing 1-mm thick gel slices

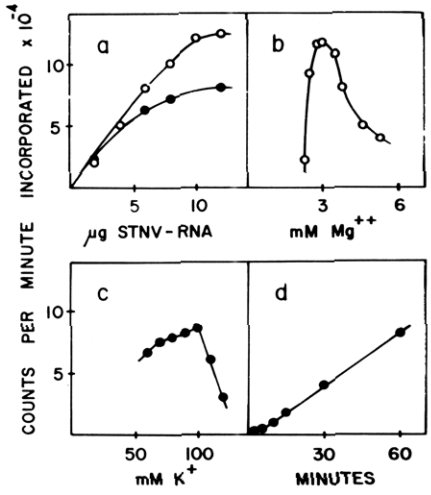

FIGURE 1: Characteristics of translation of STNV RNA in wheat germ extracts. Assays of incorporation of 15 [^{14}C]amino acids in two different wheat germ extracts, designated by ○ and ●, were performed with 10 μg of STNV RNA, 3 mM Mg^+, and 100 mM K^+ for 60 min, unless varied as indicated.

and 1-ml eluate fractions from the RPC-5 column were analyzed for ^{125}I by scintillation counting of γ radiation detectable in NaI sample vials (Bicron Co., Newbury, Ohio). The initiator region [^{125}I]oligonucleotide and STNV [^{125}I]RNA were both characterized by exhaustive digestion with ribonuclease A (30 min, 37 °C, 1 μg of ribonuclease A/20 μg of RNA) and subsequent two-dimensional fingerprint analysis (Sanger et al., 1965) and radioautography of the ^{125}I-labeled compounds.

Two milligrams of STNV [^{32}P]RNA was hydrolyzed with alkali, neutralized with $HClO_4$, and the resultant ^{32}P-labeled products were then separated by electrophoresis on DEAE paper by the method of Horst et al. (1971). Slower moving, nonnucleotide components, identified by radioautography, were eluted with 2 M triethylamine-HCO_3^-, pH 9.0, and retreated with alkali and again resolved on DEAE paper in order to confirm their nonmononucleotide character. Materials characterized as spots 1 and 2 by Horst et al. (1971) were eluted with 2 M triethylamine-HCO_3^-, pH 9.0, divided into two portions, and dried. One portion was dissolved in 10 μl of H_2O and incubated (37 °C, 10 min) with 0.5 unit of bacterial alkaline phosphatase and reanalyzed by electrophoresis on DEAE paper by the method of Horst et al. (1971). The other portion was dissolved in 1 ml of 0.2 M NaCl, 0.01 M $MgCl_2$, 0.05 M 4-methylmorpholine buffer, pH 7.7, and 20% ethanol (NMM buffer), and loaded onto a 0.7 × 15 cm column of acetylated DBAE-cellulose (Collaborative Research Inc., Waltham, Mass.) equilibrated in NMM buffer. The column was washed with five column volumes of NMM buffer and then eluted by 0.2 M NaCl, 0.05 M sodium acetate, pH 5.0, a reagent known to elute *cis*-glycols from acetylated DBAE-cellulose (McCutchan et al., 1975). ^{32}P-labeled materials on DEAE paper or in eluted fractions of these phosphatase and acetylated DBAE-cellulose assays were detected by Cerenkov counting in a scintillation counter.

When specified, STNV RNA was treated with IO_4^- and aniline according to the method of Steinschneider and Fraenkel-Conrat (1966), as modified by Mayer et al. (1975).

Results

STNV RNA-Dependent Amino Acid Incorporation by the Wheat Germ System. STNV RNA greatly enhances the incorporation of a mixture of 15 [^{14}C]amino acids into protein exhibited by in vitro S-30 extracts of wheat germ prepared and assayed by the method of Roberts and Paterson (1973). The extent of this amino acid incorporation varies somewhat among individual S-30 extracts but demonstrates maximum incorporation with approximately 10 μg of added STNV RNA, 3 mM Mg^+, and 100 mM K^+ (Figure 1a–c). These ion optima differ slightly from the Mg^+ and K^+ concentration optima reported for the translation of other messenger RNAs by this system (Roberts and Paterson, 1973; Davies and Kaesberg, 1974). Thus, this wheat germ system demonstrates slightly different properties for different messenger RNAs. STNV RNA-dependent amino acid incorporation proceeds, after an initial lag, in a linear manner for at least 1 h (Figure 1d). Such STNV RNA-dependent amino acid incorporation is totally inhibited by 2 μM sparsomycin and 70% inhibited by 50 μM aurintricarboxylic acid.

FIGURE 2: Fingerprint analysis of radioautogram-positive tryptic peptides from [^{14}C]protein labeled with 15 [^{14}C]amino acids during translation of STNV RNA by a cell-free extract from wheat germ.

Characterization of the STNV RNA-Dependent Product. Three lines of evidence dictate that amino acid incorporation at the ion optima of Figure 1 represents correct translation of STNV RNA with resultant production of only STNV coat protein. First, tryptic fingerprint analysis of the STNV RNA-dependent product obtained in the presence of 15 [^{14}C]amino acids (Figure 2) yields a pattern coincident with tryptic fingerprints of STNV coat protein and of products of STNV RNA translation in in vitro systems from *Escherichia coli* and wheat embryo (Klein et al., 1972). Our analysis does not detect significant quantities of labeled tryptic peptides that are not coincident with tryptic peptides of STNV coat protein. Thus, within the limits of fingerprint analysis, this amino acid incorporation only yields material with the amino acid sequence of STNV coat protein.

Second, sodium dodecyl sulfate gel electrophoretic analysis of the amino acid incorporation product reveals a single product with a molecular weight of 22 000. The size of this product coincides well with the 22 000 known molecular weight of STNV coat protein (Rees et al., 1970).

Third, initiation of the STNV RNA-dependent amino acid incorporation under conditions of limited protein synthesis yields initial (N terminal) peptides with amino acid sequences corresponding to the N terminus of STNV coat protein. Specifically, incubation of STNV RNA, ribosomes, DEAE S-160 GTP, ATP, Ala, Lys, [^{3}H]Met, and partially purified $tRNA^{Met}_i$ yields the formation of STNV RNA and DEAE S-160 dependent ribosome complexes containing [^{3}H]Met (Figure 3a). Similar experiments in an in vitro wheat embryo system reveal the partially purified $tRNA^{Met}_i$ to contain $tRNA^{Ala}$ and $tRNA^{Lys}$ and, therefore, facilitate the synthesis of initial aminoacyl-tRNA and peptidyl-tRNA products of

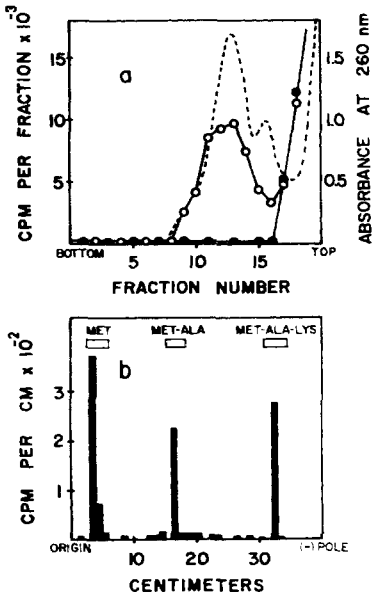

FIGURE 3: Analysis of initial peptides produced during translation of STNV RNA. (a) Sucrose density gradient resolution of [^{3}H]Met-RNA$^{Met}_i$ in initiation complexes with complete system (○) and systems lacking either STNV RNA or DEAE S-160 (●). Dashed line indicates A_{260}. (b) Paper electrophoretic analysis of amino acid and peptides released from tRNA forms in the ribosome complexes of Figure 3a.

STNV RNA translation. Mild alkaline hydrolysis of these products yields the expected Met, Met-Ala, and Met-Ala-Lys (Klein and Clark, 1973). Analysis of the [^{3}H]Met-containing forms present in the STNV RNA-dependent ribosome complexes of Figure 3a also yields only these products expected from initial partial translation of STNV RNA (Figure 3b). Thus, the in vitro system of wheat germ both initiates the translation of STNV RNA correctly and then carries out complete translation of the STNV RNA cistron to form STNV coat protein.

No 5′-Terminal m^{7}G(5′)ppp(5′)Np· · · Group Present on in Vivo STNV RNA. A number of messenger RNAs from eucaryotes, namely certain animal (Furuichi et al., 1975b; Desrosiers et al., 1975), animal viral (Furuichi et al., 1975a; Abraham et al., 1975; Lavi and Shatkin, 1975; Furuichi et al., 1975c; Martin and Moss, 1975) and plant viral (Zimmern, 1975) messenger RNAs, contain 5′-terminal m^{7}G(5′)ppp(5′)Np· · · "capping" groups. Experiments designed to test the role of such groups in the translation of messenger RNAs show these 5′-terminal capping groups to be essential for efficient translation of the messenger RNA (Both et al., 1975a,b; Muthukrishnan et al., 1975; Hickey et al., 1976). It follows that many or most messenger RNAs of eucaryotic protein synthesis may contain a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group that functions in the translation of messenger RNAs. STNV is a virus of eucaryotic cells. It is therefore necessary to determine whether a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group is present on in vivo STNV RNA.

Four lines of evidence establish that in vivo STNV RNA does not contain a m^{7}G(5′)ppp(5′)Np· · · group. First, a previously reported stoichiometric analysis of the 5′ end of STNV RNA recovers 85% of the 5′ termini of STNV RNA molecules as [^{32}P]pAp residues as a result of limited hydrolysis with alkaline phosphatase followed by 5′ phosphorylation of the resultant STNV RNA with polynucleotide 5′-kinase in the presence of [γ-^{32}P]ATP (Wimmer et al., 1968). This phosphorylation of STNV RNA requires an initial treatment with alkaline phosphatase; thus, a majority of in vivo STNV RNA molecules contain a 5′-phosphate or polyphosphate anhydride on 5′-terminal adenosine.

Table I: Effect of IO_4^- Oxidation and β Elimination upon Messenger RNA Potential of RNAs.

Messenger RNA	μg of RNA	Counts min^{-1} Assay^{-1} No treatment	Counts min^{-1} Assay^{-1} IO_4^-, β Elimination
STNV RNA	1	11 400	14 800
STNV RNA	2	17 500	17 200
STNV RNA	10	49 600	50 000
Hemoglobin mRNA	1	83 300	12 300
Hemoglobin mRNA	2	68 000	19 200
None		10 600	

Second, alkaline hydrolysis-mediated characterization of the 5′ end of uniformly labeled STNV [^{32}P]RNA detects [^{32}P]pppAp· · · and [^{32}P]ppAp· · · as the 5′ termini of STNV RNA (Horst et al., 1971). These data plus the stoichiometric recovery of approximately one [^{32}P]pppAp· · · or [^{32}P]ppAp· · · group per one STNV [^{32}P]RNA allowed Horst et al. (1971) to characterize the 5′ end of STNV RNA as pppAp· · · and ppAp· · · .

Third, we have repeated the above analysis of STNV [^{32}P]RNA and obtained the results of Horst et al. (1971). We have further confirmed the pppAp and ppAp character of the presumed [^{32}P]pppAp and [^{32}P]ppAp generated from STNV [^{32}P]RNA by alkaline hydrolysis. Specifically, the presumed [^{32}P]pppAp and [^{32}P]ppAp both release all of their label as ^{32}P upon treatment with alkaline phosphatase. Alkaline hydrolysis of STNV [^{32}P]RNA also does not release any ^{32}P material that adheres to the *cis*-glycol specific reagent, acetylated DBAE-cellulose (McCutchan et al., 1975) known to take up m^{7}G(5′)ppp(5′)Np groups released from 5′ capped mRNAs by alkali (Furuichi et al., 1975c). Thus, we further prove that STNV RNA contains a 5′-terminal pppAp· · · or ppAp· · · group and that STNV RNA does not contain a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group.

Fourth, chemical treatments that destroy capping groups, and thereby destroy the coding functions of messenger RNAs, do not effect the coding potential of STNV RNA. Specifically, periodate oxidation and subsequent treatment with aniline facilitate a β-elimination reaction that removes terminal, *cis*-glycols containing nucleosides from RNAs (Whitfeld, 1954; Brown et al., 1955). This treatment decomposes m^{7}G(5′)ppp(5′)Np· · · groups of mRNAs (Abraham et al., 1975) and destroys the message potential of mRNAs that require 5′-terminal m^{7}G(5′)ppp(5′)Np· · · groups for their translation (Muthukrishnan et al., 1975). As seen in Table I, such periodate oxidation and aniline treatment of STNV RNA do not alter the mRNA potential for STNV RNA in the wheat germ system.[2] One can see that the β-elimination reaction conditions of this assay are adequate for the same treatments and greatly reduce the functional character of optimum levels of hemoglobin mRNA, a mRNA known to contain a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group (Muthukrishnan et al., 1975).

No in Vitro Introduction of a 5′-Terminal m^{7}G(5′)ppp-

[2] Kemper (1976) recently and independently reported a similar observation that IO_4^- and aniline-facilitated β-elimination procedures do not decrease the mRNA potential of STNV RNA in the wheat germ system.

(5′)Np· · · Group by the Wheat Germ System Prior to Translation of STNV RNA. The above data establish that the STNV RNA introduced into the wheat germ systems does not contain a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group. However, the wheat germ system can introduce a 5′-terminal m^{7}G-(5′)ppp(5′)Np· · · group onto known mRNAs (Both et al., 1975b). Thus, although the original STNV RNA lacks a 5′ capping group, such a 5′-terminal group could be introduced by the wheat germ system as an obligate prerequisite to translation of STNV RNA into STNV coat protein by the system.

Three approaches can be used to demonstrate that the wheat germ system does not introduce a 5′-terminal m^{7}G(5′)-ppp(5′)Np· · · group onto STNV RNA. Both et al. (1975a, b) report that high concentrations of *S*-adenosyl-L-homocysteine in wheat germ extracts inhibit the *S*-adenosyl-L-methionine dependent methylation steps involved in the synthesis of 5′-terminal m^7(5′)ppp(5′)Np· · · groups on mRNAs. This inhibition precludes the translation of mRNAs that lack a 5′-terminal capping group and require such a 5′-terminal capping group for their translation. As shown above, STNV RNA lacks a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group. If such a 5′-terminal group is required for translation of STNV RNA by the wheat germ system, then high concentrations of *S*-adenosyl-L-homocysteine should inhibit the translation of STNV RNA by the wheat germ system. We observe that neither high concentrations of *S*-adenosyl-L-homocysteine, nor high concentrations of the methl donor, *S*-adenosyl-L-methionine, influence the translation of STNV RNA by the wheat germ system.[3]

The above experiment with *S*-adenosyl-L-homocysteine provides indirect proof that the wheat germ system does not employ a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group on STNV RNA during the translation of STNV RNA. However, characterization of the 5′ terminus of STNV RNA during and after its translation by the wheat germ system provides a more direct proof of use of such a 5′-terminal capping group during the translation of STNV RNA in the wheat germ system. All available evidence suggests that m^{7}G(5′)ppp(5′)Np· · · groups on mRNAs enhance the ability of such capped mRNAs to form "initiation complexes" containing Met-tRNA$^{Met}_i$ and ribosomes complexed at an initiator AUG site on the capped mRNA (Both et al., 1975b; Hickey et al., 1976). If STNV RNA is capped by the wheat germ system as an obligate step prior to its translation, initiation complexes on STNV RNA should contain one m^{7}G(5′)ppp (5′)Np· · · group for each Met-tRNA$^{Met}_i$ found in the complexes. A resolved wheat germ system can form specific initiation complexes in the presence of sparsomycin (so as to inhibit limited protein synthesis, such as seen in Figure 3) and [^{3}H]methionine or [8-^{3}H]-GTP. [^{3}H]Methionine found in these initiation complexes will be [^{3}H]Met-tRNA$^{Met}_i$. The ^{3}H label from [8-^{3}H]GTP found in these complexes will represent synthesis of m^7-G(5′)ppp(5′)Np· · · groups on STNV RNA (Martin and Moss, 1975). As seen in Figure 4, such initiation complexes contain the expected [^{3}H]Met-tRNA$^{Met}_i$ when incubated with [^{3}H]methionine, yet such complexes do not contain significant ^{3}H label when formed in the presence of [8-^{3}H]GTP with specific activity equivalent to the [^{3}H]Met detected in [^{3}H]-Met-tRNA$^{Met}_i$. Thus, formation of initiation complexes does not require or involve prior formation of m^{7}G(5′)ppp(5′)Np· · · groups on STNV RNA.

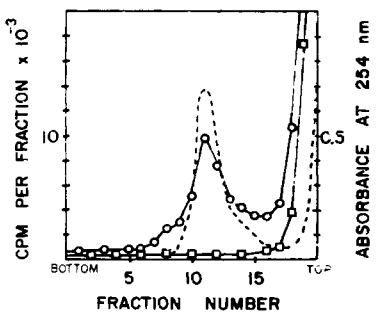

FIGURE 4: Sucrose gradient analysis of the uptake of [methyl-^{3}H]methionine (○) and [8-^{3}H]GTP (□) into initiation complexes. Dashed line indicates A_{254}.

The wheat germ system readily translates STNV RNA in the presence of [α-^{32}P]GTP. The ^{32}P label from such [α-^{32}P]GTP should be incorporated into any 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group formed on STNV RNA prior to, or during, translation (Martin and Moss, 1975). Yet, extensive translation of STNV RNA in the presence of high specific activity (9250 Ci/mol) [α-^{32}P]GTP does not cause STNV RNA-dependent uptake of ^{32}P label into STNV RNA. Further alkaline hydrolysis of this posttranslational STNV RNA, followed by characterization of alkaline hydrolysate by chromatography on poly(ethyleneimine)cellulose (Rapaport and Zamecnik, 1975) with appropriate standards, fails to detect significant levels of ^{32}P label in m^{7}G(5′)ppp(5′)Np. Thus, a m^{7}G(5′)ppp(5′)Np· · · group is not formed on the 5′ terminus of STNV RNA prior to in vitro translation of STNV RNA by wheat germ extracts.

Isolation of an Initiator Region Oligonucleotide of STNV RNA. Characterization of the nucleotide sequence about the initiator AUG site of STNV RNA offers a direct approach to predict features of STNV RNA that define the single translation initiation site of this message. Ribosome protection experiments (Steitz, 1969) offer a potential route for such a characterization. In such experiments, ribosomes present in initiation complexes on the mRNA allow treatment of the initiation complexes with endonucleases and subsequent isolation of mRNA fragments protected from endonucleases actions by the ribosomes. If the original mRNA is radioactive, one can use this radioactive label for purification or characterization of the protected fragments of mRNA.

Previously reported ribosome protection experiments employ in vivo labeled [^{32}P]mRNA containing at least 10^6 dpm/μg of RNA. STNV [^{32}P]RNA prepared from STNV- and TNV-infected mung beans and STNV [^{32}P]RNA prepared in cowpea leaves (Horst et al., 1971) contain ^{32}P levels far below this 10^6 dpm/μg of RNA needed for convenient ribosome protection experiments. Accordingly, all of the ribosome protection experiments reported here employ STNV [^{125}I]RNA (sp act. 5×10^6 γ cpm/μg of RNA) prepared by the in vitro procedure of Commerford (1971). Such STNV [^{125}I]RNA is judged to be specific in that STNV [^{127}I]RNA, prepared in a manner identical to STNV [^{125}I]RNA, is equally effective a messenger RNA as in vivo STNV RNA.

Wheat germ ribosomes readily form complexes with STNV [^{125}I]RNA (Figure 5). These complexes are true and specific initiation complexes in that their formation requires added tRNA$^{Met}_i$, nucleoside 5′-triphosphate, and DEAE S-160, and they are formed in the presence of the peptide bond synthesis inhibitor, sparsomycin. Fifty micromolar aurintricarboxylic

[3] Roman et al. (1976) recently and independently reported a similar lack of inhibition by *S*-adenosyl-L-homocysteine of STNV RNA translation in an in vitro wheat germ system.

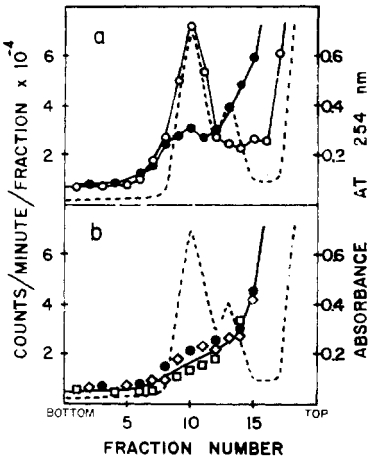

FIGURE 5: Sucrose gradient analysis of initiation complexes containing STNV [^{125}I]RNA. (a) Initiation complexes formed in a complete system (○) and a complete system containing 50 μM aurintricarboxylic acid (●). (b) Initiation complexes formed by complete system lacking tRNA$^{Met}_{i}$ (◇), complete system lacking DEAE S-160 (●), and complete system lacking ATP and GTP (□). Dashed lines represent A_{254}.

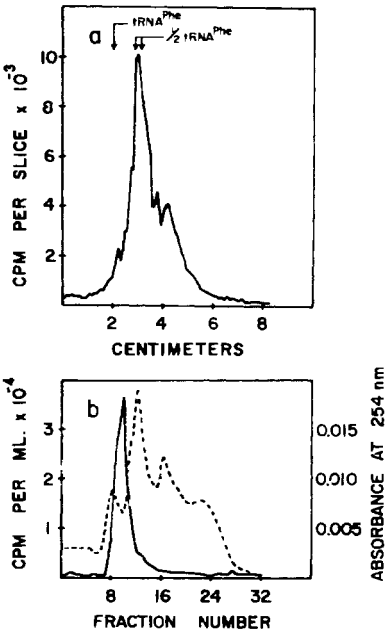

FIGURE 6: Analyses of ribosome protected STNV [^{125}I]RNA fragment. (a) 20% acrylamide gel electrophoretic analysis in the presence of 7 M urea and designated tRNA standards. (b) High-pressure liquid chromatographic analysis on RPC-5. Dashed line represents A_{254}.

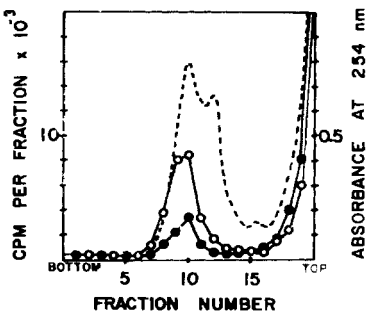

FIGURE 7: Sucrose density gradient analysis of initiation complexes formed with the STNV [^{125}I]RNA oligonucleotide material of Figure 6 in a complete system (○) and a complete system lacking tRNA$^{Met}_{i}$, DEAE S-160, ATP, and GTP (●). Dashed line represents A_{254}.

Table II: Codons for Translation of the N Terminus of STNV Coat Protein.[a]

In vivo amino acid sequence	Ala - Lys - Gln - · · · ·
Initial biosynthetic product	Met - Ala - Lys - Gln - · · · ·
Potential codon assignments	· · · · -AUG - GC$\begin{matrix}A\\G\\U\\C\end{matrix}$ - AA$\begin{matrix}A\\G\end{matrix}$ - CA$\begin{matrix}A\\G\end{matrix}$ - · · · ·
Sites cleaved by ribonuclease A	· · · · -AU | GGC | $\begin{matrix}A\\G\\U\\C\end{matrix}$ | AA$\begin{matrix}A\\G\end{matrix}$C | A$\begin{matrix}A\\G\end{matrix}$ - · · · ·
Oligonucleotides found in "initiator region [^{125}I] oligonucleotide"	| GGC | | AAAC |

[a] Amino acid assignments are from known N-terminal sequence of STNV coat protein (Klein et al., 1972; Tashian, 1975) and the expected initiation of STNV RNA translation by eucaryotes with N-terminal Met (Klein and Clark, 1973). Codon assignments are from the genetic code.

acid markedly inhibits the formation of these initiation complexes. Treatment of these initiation complexes with ribonuclease A, followed by sedimentation of the treated ribosomes, disruption of the sedimented ribosomes with sodium dodecyl sulfate-phenol-chloroform, and final alcohol precipitation of materials in the aqueous phase of the sodium dodecyl sulfate-phenol-chloroform extracts, yields ^{125}I-labeled material coprecipitated with a population of ribosomal-RNA fragments. This ^{125}I-labeled material contains approximately 3% of the total ^{125}I label in the initiation complexes, suggesting that approximately 40 of the original 1200 nucleotides of STNV RNA are retained as a ribosome-protected RNA fragment.

Several lines of evidence dictate that this ^{125}I-labeled material, precipitated by alcohol, is predominantly a unique initiator region oligonucleotide-containing nucleotides at and around the initiator AUG of STNV RNA. First, the ^{125}I-labeled material is RNA in that it adheres to DEAE-cellulose at neutral pH and is made alcohol soluble by treatment with ribonuclease A, takadiastase T_1, or strong alkali. Second, size analysis of this ^{125}I-labeled material on acrylamide gel electrophoresis and by high-pressure liquid chromatography both reveal the ^{125}I-labeled material to be a rather homogeneous preparation with an average equivalent to 38 nucleotides (Figure 6).

Third, if the ^{125}I-labeled oligonucleotide material contains the nucleotide sequence of the translation initiation site of STNV RNA, then it should demonstrate a low efficiency, yet specific, rebinding into ribosome-containing initiation complexes. As seen in Figure 7, the ^{125}I-labeled oligonucleotide material does form complexes with wheat germ ribosomes. This rebinding, like other reported rebinding of initiator region fragments of mRNAs (Steitz, 1973; Dasgupta et al., 1975), is inefficient in terms of percent rebinding of radioactive label. However, this rebinding is largely specific, depending upon added tRNA$^{Met}_{i}$, nucleoside 5′-triphosphate, and DEAE S-160.

Fourth, the ^{125}I-labeled oligonucleotide contains a nucleotide sequence which agrees with the amino acid sequence of the N terminus of STNV coat protein. As seen in Table II, if the ^{125}I-labeled material protected by ribosomes is the initiator fragment of STNV RNA, it must yield, upon digestion with ribonuclease A, two rather distinctive oligonucleotides, both

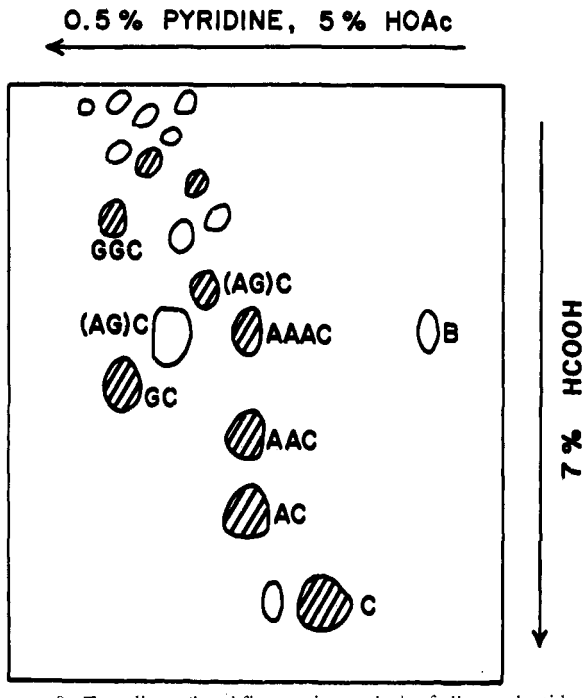

FIGURE 8: Two-dimensional fingerprint analysis of oligonucleotides released from STNV [^{125}I]RNA and ribosome-protected STNV [^{125}I]RNA oligonucleotide by ribonuclease A. All spots represent ^{125}I-containing compounds detected upon radioautographic analysis of ribonuclease A treated STNV [^{125}I]RNA. Cross-hatched spots represent ^{125}I-containing compounds detected upon radioautographic analysis of ribonuclease A treated STNV [^{125}I]RNA oligonucleotide material of Figure 6. Nucleotide and oligonucleotide characterizations, and the designated spot B (xylene cyanol F. F., blue marker dye), are based upon the analyses of Horst et al. (1971) and Robertson et al. (1973b).

containing a 3′-terminal Cp. Both of these specific oligonucleotides would contain ^{125}I due to the cytidine-specific iodination procedure used to make the original STNV [^{125}I]RNA (Robertson et al., 1973b). Thus, these two ^{125}I-labeled oligonucleotides should be among the few ^{125}I-labeled oligonucleotides detected in a ribonuclease A digest of ^{125}I-labeled, previously ribosome protected, mRNA fragment. Figure 8 contrasts the oligonucleotides and nucleotides obtained upon digestion of STNV [^{125}I]RNA with those obtained from digestion of the ^{125}I-labeled, previously ribosome protected, apparent initiator region fragment of STNV RNA (i.e., the ^{125}I-labeled material of Figure 6). STNV [^{125}I]RNA yields the expected wide variety of ^{125}I-labeled degradation products, while the apparent initiation fragment of STNV RNA yields only nine ^{125}I-labeled degradation products. The small number of oligonucleotide and nucleotide products derived from the apparent initiation fragment of STNV RNA supports the unique character of the isolated RNA fragment. Further, these nine ^{125}I-labeled degradation products are in good agreement with the observed 38 nucleotides in the apparent initiator region fragment and the known (Reichmann, 1964) 22% cytidylic acid content of STNV RNA. Most importantly, treatment of the apparent initiator region fragment of STNV RNA with ribonuclease A yields ^{125}I-labeled GGC and AAAC, two oligonucleotides specifically predicted from the known N-terminal amino acid sequence of STNV coat protein (Table II). Further, the failure to detect AAAAC or GAAAC suggests that the nucleotide on the 3′ end of the alanine codon adjacent to the initiator AUG (Table II) is a site for cleavage by ribonuclease A, namely, a pyrimidine. Thus, the nucleotide sequence for the first 12 nucleotides translated on STNV RNA is most likely AUGGC$^{U}_{C}$UAAACA$^{A}_{G}$.

Discussion

The results presented here establish that the in vitro system from wheat germ of Roberts and Paterson (1973) is an effective system for the in vitro translation of STNV RNA. Specifically, the system is efficient in that small quantities of cell-free wheat germ extract support significant levels of translation of STNV RNA (Figure 1). Further, this translation is correct, starting at the single site for initiation of translation of STNV RNA (Figure 3) and yielding only STNV coat protein (Figure 2). Thus, experiments that employ this in vitro wheat germ system can provide meaningful assays of the initiation of STNV RNA translation.

The experiments reported here also provide some insight into features of messenger RNAs that define specific AUG sequences as sites for the initiation of protein synthesis. STNV RNA does not contain a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · capping group (Table I) and such a 5′-terminal capping group is not added prior to or during initiation and translation of STNV RNA by the wheat germ system (Figure 4). Thus, STNV RNA is like poliovirus RNA (Nomoto et al., 1976) in that it is a functional messenger RNA that is not capped prior to or during translation by a eucaryotic system. These findings therefore establish that a preexisting or subsequently formed 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group is not an obligate prerequisite for translation of messenger RNAs in eucaryotic systems. These findings also support the theory (Steitz and Jakes, 1975) that recognition of initiator AUG sites on mRNAs involves a combination of effects generated by factors and structural relationships of the ribosomes, mRNA, and tRNA$^{Met}_{i}$. Thus, one can predict that the selection of specific AUG sequences on mRNAs as sites for initiation of protein synthesis by eucaryotes is favored by, but may not necessarily require, (1) the presence of a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group on the mRNA, (2) a mRNA structure that exposes the initiator AUG region of the mRNA, (3) specific interaction of an exposed portion of the mRNA with a similarly exposed and unique portion of the 18S ribosomal-RNA. In this connection, it is of interest to note (Table I) that hemoglobin mRNA, an mRNA containing a 5′-terminal m^{7}G(5′)ppp(5′)Np· · · group (Muthukrishnan et al., 1975), saturates the wheat germ system with 1 μg of hemoglobin mRNA, while this same system requires 10 μg of STNV RNA for saturation.

Further analysis of features of STNV RNA that influence the selection of a specific AUG sequence as an initiator AUG requires determination and analysis of the nucleotide sequence at and around the site of initiation of STNV RNA translation. Formation of specific initiation complexes between tRNA$^{Met}_{i}$, wheat germ ribosomes, and STNV [^{125}I]RNA facilitates ribosome protection experiments and allows the isolation of an initiation region oligonucleotide of STNV RNA (Figures 5 and 6). This [^{125}I]oligonucleotide contains nucleotide sequences that coincide with the codons of the N-terminal amino acids of STNV coat protein (Figure 8). Thus in vitro labeling of messenger RNAs with ^{125}I by the method of Commerford (1971) offers a meaningful method for mRNA nucleotide sequence studies that employ the ribosome protection approach.

Acknowledgment

The authors acknowledge the invaluable technical assistance of Ms. Ronnie Barker.

References

Abraham, G., Rhodes, D. P., and Banerjee, A. K. (1975), *Cell 5,* 51.

Barrell, B. G., Weith, H. L., Donelson, J. E., and Robertson, H. D. (1975), *J. Mol. Biol. 92,* 377.

Both, G. W., Banerjee, A. K., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1189.

Both, G. W., Furuichi, Y., Muthukrishnan, S., and Shatkin, A. J. (1975b), *Cell 6,* 185.

Brown, D. M., Fried, M., and Todd, Sir, A. (1955), *J. Chem. Soc.,* 2206.

Clark, J. M., Jr., Chang, A. Y., Spiegelman, S., and Reichmann, M. E. (1965), *Proc. Natl. Acad. Sci. U.S.A. 54,* 1193.

Clark, J. M., Jr., and Klein, W. H. (1974), *Methods Enzymol. 30,* 754.

Commerford, S. L. (1971), *Biochemistry 10,* 1993.

Conway, T. W., and Lipmann, F. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1462.

Dasgupta, R., Shih, D. S., Saris, C., and Kaesberg, P. (1975), *Nature (London) 256,* 624.

Davies, J. W., and Kaesberg, P. (1974), *J. Gen. Virol. 25,* 11.

Desrosiers, R. C., Friderici, K. H., and Rottman, F. M. (1975), *Biochemistry 14,* 4367.

DeWachter, R., and Fiers, W. (1971), *Methods Enzymol. 21,* 167.

Furuichi, Y., Morgan, M., Muthukrishnan, S., and Shatkin, A. J. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 362.

Furuichi, Y., Morgan, M., Shatkin, A. J., Jelinek, W., Salditt-Georgieff, M., and Darnell, J. E. (1975b), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1904.

Furuichi, Y., Shatkin, A. J., Stavnezer, E., and Bishop, J. M. (1975c), *Nature (London) 257,* 618.

Gillam, I. C., and Tener, G. M. (1971), *Methods Enzymol. 20,* 55.

Hickey, E. C., Weber, L. A., And Baglioni, C. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 19.

Hindley, J., and Staples, D. H. (1969), *Nature (London) 224,* 964.

Horst, J., Fraenkel-Conrat, H., and Mandeles, S. (1971), *Biochemistry 10,* 4748.

Kemper, B. (1976), *Nature (London) 262,* 321.

Klein, W. H., and Clark, J. M., Jr. (1973), *Biochemistry 12,* 1528.

Klein, W. H., Nolan, C., Lazar, J. M., and Clark, J. M., Jr. (1972), *Biochemistry 11,* 2009.

Lavi, S., and Shatkin, A. J. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2012.

Leis, J. P., and Keller, E. B. (1970), *Biochem. Biophys. Res. Commun. 40,* 416.

Lundquist, R. E., Lazar, J. M., Klein, W. H., and Clark, J. M., Jr. (1972), *Biochemistry 11,* 2014.

Marcus, A., Luginbill, B., and Feeley, J. (1968), *Proc. Natl. Acad. Sci. U.S.A. 59,* 1243.

Martin, S. A., and Moss, B. (1975), *J. Biol. Chem. 250,* 9330.

Mayer, S. A., Abraham, G., Alder, R., and Banerjee, A. K. (1975), *Cell 5,* 59.

McCutchan, T. F., Gilham, P. T., and Söll, D. (1975), *Nucleic Acids Res. 2,* 853.

Min Jou, W., Haegeman, G., Yaebaert, M., and Fiers, W. (1972), *Nature (London) 237,* 82.

Muthukrishnan, S., Both, G. W., Furuichi, Y., and Shatkin, A. J. (1975), *Nature (London) 255,* 33.

Nomoto, A., Lee, Y. F., and Wimmer, E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 375.

Palmiter, R. D. (1974), *Biochemistry 13,* 3606.

Rapaport, E., and Zamecnik, P. C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 314.

Rees, M. W., Short, M. N., and Kassanis, B. (1970), *Virology 40,* 448.

Reichmann, M. E. (1964), *Proc. Natl. Acad. Sci. U.S.A. 52,* 1009.

Roberts, B. E., and Paterson, B. M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2330.

Robertson, H. D., Barrell, B. G., Weith, H. L., and Donelson, J. E. (1973a), *Nature (London), New Biol. 241,* 38.

Robertson, H. D., Dickson, E., Model, P., and Prensky, W. (1973b), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3260.

Roman, R., Brooker, J. D., Seal, S. N., and Marcus, A. (1976), *Nature (London) 260,* 359.

Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13,* 373.

Shine, J., and Dalgarno, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 1342.

Steinschneider, A., and Fraenkel-Conrat, H. (1966), *Biochemistry 5,* 2735.

Steitz, J. A. (1969), *Nature (London) 224,* 957.

Steitz, J. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2605.

Steitz, J. A., and Jakes, K. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4734.

Tashian, R. (1975) (unpublished results).

Thiebe, R., and Zachau, H. G. (1971), *Methods Enzymol. 20,* 179.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.

Whitfeld, P. (1954), *Biochem. J. 58,* 390.

Wimmer, E., Chang, A. Y., Clark, J. M., Jr., and Reichmann, M. E. (1968), *J. Mol. Biol. 38,* 59.

Zimmern, D. (1975), *Nucleic Acids Res. 2,* 1189.