Translation of tobacco necrosis virus and its satellite in a cell-free wheat germ system

(Maria S. Salvato and Heinz Fraenkel-Conrat)

ABSTRACT Tobacco necrosis virus (TNV) and its satellite virus (STNV) each contain a single-stranded RNA genome, of about 1.4 × 10^6 and 0.4 × 10^6 daltons, respectively, which is very active in stimulating amino acid incorporation in a wheat germ cell-free system. With STNV RNA the predominant incorporation product of 22,000 daltons coelectrophoreses with viral coat protein and crossreacts with antibody to viral coat protein. A similar result is obtained with TNV RNA, the only major translation product being a 30,000-dalton protein which corresponds to the coat protein by gel sizing, serological tests, and tryptic peptide analysis. Other products appearing in smaller amounts are about 83,000, 43,000, and 26,000 daltons and smaller. The possible nature of these products is discussed, as well as the unusual feature of a large, presumably multigenic, viral RNA yielding the coat protein as the predominant translation product in a eukaryotic system.

Much less STNV RNA than TNV RNA produces maximal translation. Cotranslation of both RNAs in vitro indicates that STNV RNA has a translational advantage over TNV RNA. The fact that these RNAs lack 3'-terminal capping and 3'-terminal poly(A) is discussed.

Early attempts to study the translation products of mRNAs in vitro utilized the only pure RNA species then available, namely, those of viruses, and the results were disappointing. Only when the small RNAs of multicomponent viruses became available was it possible to obtain clear translation results, in that these RNAs proved to yield only the respective viral coat proteins upon translation. This was the case also for the RNA of the satellite of tobacco necrosis virus (STNV), a similarly small RNA, although the translation product of this RNA did not correspond exactly in size and charge to the viral coat protein, regardless of whether Escherichia coli or the then new wheat germ system was used (1, 2). In the case of multigenic mRNAs, recent evidence indicates that multiple translation initiations are avoided in various ways by eukaryotic cells. For two "classical" plant viruses [tobacco mosaic virus (TMV) and turnip yellow mosaic virus (TYMV)] in particular, it was recently shown that the coat protein gene was separately produced or carried (3–6).

As part of our current efforts to elucidate the nature of the dependence of STNV replication on that of its helper, tobacco necrosis virus (TNV), we have studied the translation of the respective RNAs in the in vitro wheat germ system. The finding that not only STNV RNA but also the much larger TNV RNA yields mainly the respective coat protein was surprising in view of the findings with TMV and TYMV. The finding that much less STNV RNA than TNV RNA is required for maximal translation, and that STNV RNA is preferentially translated even in the presence of much more TNV RNA, may supply an explanation for the frequently encountered difficulty in obtaining and maintaining certain strains of TNV free of STNV.

MATERIALS AND METHODS

A strain of TNV originally isolated by C. E. Yarwood, was used in the present study because it has been used in parallel studies of the RNA replicase in this laboratory (7, 8), and its replication in protoplasts is also under investigation. The original inocula yielded predominantly STNV, but after 2 years of passage through tobacco, almost only TNV was produced under all conditions (7). This trend was somewhat reversed by twice passaging the virus through cowpea.

Wheat germ was supplied by General Mills, Vallejo, CA. Hepes (N-2-hydroxypiperazine-N'-2-ethanesulfonic acid), ATP (disodium salt), GTP (sodium salt), creatine phosphate (disodium salt), spermine tetrachlorohydrochloride, and creatine phosphokinase (EC 2.7.3.2) were purchased from Sigma Biochemicals. Trypsin (EC 3.4.21.4) treated with tosylphenylalanyl chloromethyl ketone (TPCK) was from Worthington. Aminex cation-exchange resin was from Bio-Rad Laboratories. Tritiated algal hydrolysate was from Schwarz/Mann and ^14C-labeled algal hydrolysate was from New England Nuclear.

Antiserum to TNV-AC36 and STNV-AC36 were generously provided by R. G. Grogan of the University of California, Davis. They were found to crossreact with our TNV and STNV strains, respectively, by double gel-diffusion precipitation in 1% agarose in 0.15 M NaCl/0.01 M phosphate buffer, pH 7.2/0.1% NaN₃. The antiserum to TNV did not crossreact with STNV and vice versa. Coat anti-rabbit serum was from Wellcome Laboratories.

Preparation of Viruses and Viral RNAs. TNV and STNV were prepared by sedimenting for 2–3 hr at 105,000 × g (39,000 rpm in the SW40 rotor) virus-containing plant extracts that had previously been clarified at 8,000–16,000 rpm (10–20 min). The extracts had been prepared either in 0.1 M phosphate (pH 7.0) or polymerase extraction buffer (0.01 M Tris, pH 8.1/0.01 M KCl/0.025 M NaH₂PO₄/0.1% mercaptoethanol) (7). The pellet, redissolved in water, was clarified at 8000 rpm and the viruses were again sedimented as above. The crude virus solution was again dissolved and clarified and was subjected to sucrose gradient fractionation (5–30%, 3 hr at 33,000 rpm in the SW41 rotor), with or without prior fractionation with ammonium sulfate, to obtain purified TNV and STNV, as described (7). If the ammonium sulfate fractionation was omitted, there was often a peak of intermediate sedimentation rate which seemed to represent a complex of TNV with a 55,000-dalton protein, as determined by gel electrophoresis. RNA was prepared from the viruses and purified further by sucrose gradient fractionation either as reported (7) or by spinning the RNA in a buffer containing 0.1% sodium dodecyl sulfate (0.1M NaCl/0.01 M Tris, pH 7.4/10⁻⁴ M EDTA) for 3 hr at 25° at 33,000 rpm (SW41 rotor). To separate RNA complexes, the

Abbreviations: TNV, tobacco necrosis virus; STNV, satellite virus of TNV; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus.
latter technique was used also after the RNA was heated to 65° for 3 min and quickly cooled. 

To obtain 14C-labeled virus preparations, plants were cut off 2–3 days after inoculation. Each was immersed in and allowed to imbibe 50 µCi of the 14C-labeled amino acid mixture, in about 0.2 ml of water, followed by several additions of water. Then the plants were kept in water under 16 hr of illumination for an additional 1 or 2 days. The viruses were prepared as above.

**Standard Protein Synthesis Assays.** These contained 20 mM Hepes (pH 7.6), 2 mM dithiothreitol, 80 mM KCl, 3.5 mM Mg acetate, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 30 µM each amino acid, 23 µg of wheat trRNA per ml, 16 µg of creatine phosphokinase per ml, 30 µg of spermine tetrahydrochloride per ml, varying amounts of TNV RNA or STNV RNA, 10 µCi of 3H-labeled algal hydrolysate per ml, and 10 µl of S-23 wheat extract per 25-µl assay. Wheat extracts were made according to Marcu and Dudock (9).

After 90 min at 28°, reactions were stopped by addition of 1 ml of 10% trichloroacetic acid. The mixture was heated for 10 min at 90° and then centrifuged. The precipitate was re-suspended in water or 0.01 M NaOH and washed successively with ethanol and ether.

**Immunoprecipitation.** All sera and cell-free extracts were clarified by a low-speed centrifugation prior to immunoprecipitation, as described (10). Twenty microliters of the cell-free incubation mixture was added to 40 µl of immunoprecipitation buffer (2 mg of bovine serum albumin per ml/1% Triton X-100) and reacted 1 hr at 37° with 5 µl of the first antibody (rabbit antivirus). A 50-µl portion of the second antibody (goat anti-rabbit) was then added and the incubation was continued at 37° for 1 hr and overnight at 4°. The precipitate was pelleted at 2000 rpm for 10 min, washed four times, and mixed on a Vortex with buffer (20 mM Tris-HCl, pH 7.5/50 mM NaCl/0.5% Nonidet P-40). The pellet was then mixed on a Vortex in gel sample buffer and prepared for gel electrophoresis (11).

**Tryptic Peptide Analysis.** Both 3H- and 14C-labeled in vitro products were dissolved in 67% formic acid and incubated at 37° for 24 hr. Samples were then lyophilized, suspended in water, and again lyophilized several times before they were dissolved in 2% ammonium bicarbonate, pH 8.2/1% trypsin (2). This mixture was incubated for 6 hr at 37°, treated with an additional 1/10 volume of 1% trypsin, and incubated for 6 hr longer. Tryptic digestion was stopped with 1/10 volume acetic acid, the buffer was removed by repeated lyophilization with addition of water, and the residue was finally suspended in 100 µl of 0.5% pyridine-acetate (pH 2.5). Peptide samples, double-labeled with 3H and 14C, were run on an Aminex cation-exchange resin using a pyridine-acetate gradient of pH 2.5–4.6. Two-milliliter fractions were collected and dried down onto filters in scintillation vials. Radioactivity was measured in 5 ml of toluene/Omniflour.

**RESULTS**

**Translation Products of STNV RNA and TNV RNA.** Maximal incorporation of 3H-labeled amino acids was obtained with 32 µg of STNV RNA per ml and about 100 µg of TNV RNA per ml, as contrasted with TMV RNA, of which 200 µg/ml was required for maximal incorporation (Fig. 1). The use of radioactive amino acid mixtures in all experiments has facilitated a quantitative analysis of tryptic peptides and minor translation products, which is not possible if [35S]methionine or any other single-labeled amino acid is used.

Gel electrophoretic analysis of the sodium dodecyl sulfate-treated products made with STNV RNA as messenger showed one very predominant product of about 22,000 daltons (Fig. 2) which coelectrophoresed with unlabeled STNV coat protein detected by staining, as well as with 14C-labeled viral coat protein (Fig. 2). The significance, if any, of the 18,000-dalton peak and the very much smaller amounts of other products will be discussed below.
Table 1. Products of protein synthesis in vitro

<table>
<thead>
<tr>
<th>RNA (per 25-μl assay)</th>
<th>Amino acid incorporation (cpm)</th>
<th>% of total incorporation into protein peaks (daltons × 10⁻⁵)</th>
</tr>
</thead>
<tbody>
<tr>
<td>μg</td>
<td>mol × 10⁻¹²</td>
<td></td>
</tr>
<tr>
<td>TNV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.9</td>
<td>0.65</td>
<td>57,300</td>
</tr>
<tr>
<td>1.8</td>
<td>1.3</td>
<td>82,600</td>
</tr>
<tr>
<td>2.7</td>
<td>2.0</td>
<td>120,020</td>
</tr>
<tr>
<td>8</td>
<td>3.7</td>
<td>105,100</td>
</tr>
<tr>
<td>TNV + STNV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.6 + 0.6</td>
<td>2.7 + 1.5</td>
<td>40,700</td>
</tr>
<tr>
<td>1.8 + 0.6</td>
<td>1.3 + 1.5</td>
<td>59,800</td>
</tr>
<tr>
<td>0.9 + 0.6</td>
<td>0.65 + 1.5</td>
<td>57,400</td>
</tr>
<tr>
<td>STNV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.6</td>
<td>1.5</td>
<td>108,600</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>4,200</td>
</tr>
</tbody>
</table>

* This component is more evident and distinct in this than some other experiments.
† n.d. means not detected.

Similar patterns were obtained with TNV RNA as messenger, with the main peak being of 30,000 daltons and coinciding with TNV coat protein (Fig. 3). However, besides the main peak there occur consistently minor products, partly or at times not forming peaks but shoulders, which have been roughly quantitated, as illustrated by a typical experiment in Table 1. The amounts of these lesser components cannot be clearly differentiated from the background of nonspecific degradation products, and thus the percents listed in Table 1 are probably falsely high. From these it was calculated that for every 100 TNV coat protein-sized molecules (30,000 daltons), there are maximally four molecules of 63,000 daltons, four of 43,000 daltons, 14 of a 35,000-dalton peak that was not always seen, 22 of 26,000 daltons, and 32 of 22,000 daltons. There may also be traces of lower-molecular-weight peptide chains barely above the background of presumed degradation products towards the end of the gel. Less than optimal concentrations of viral RNA give essentially the same products, except for the 35,000-dalton product, which is decreased compared to the other products as RNA is increased. Upon simultaneous use of both TNV and STNV RNAs, total incorporation is decreased and the STNV coat protein, as well as the smaller product, prevails greatly over the TNV-specific products, as shown in Table 1.

Since during this investigation it became known to us that TYMV carries a complex of the large RNA with a smaller RNA molecule, and that the latter represents the coat protein gene (5, 6), we felt it necessary to ascertain whether in TNV also a smaller coat protein gene could be separated from the viral RNA. Thus, TNV RNA was heated to 65° for 3 min before it was repurified on a sucrose gradient in the presence of sodium dodecyl sulfate, and it was tested for messenger activity. The pattern of products obtained with the large RNA (1.4 × 10⁶ daltons) was the same as usual, with the only significant peak coinciding with TNV coat protein. The lower-molecular-weight RNAs failed to yield any significant products upon translation and, hence, are probably degraded RNA rather than a noncovalently bound RNA components of TNV.

**Immunoprecipitation of In Vitro Products.** Antiserum to TNV formed a stable precipitate with 24% of the total TNV RNA-directed in vitro products (Table 2). Disruption of this precipitate and subsequent electrophoresis (data not shown) revealed sharp peaks corresponding to the coat protein (30,000 daltons) and the 26,000-dalton product, as well as smaller products. The crossreacting peptides smaller than 30,000 dalton

![Fig. 3. Polyacrylamide gel electrophoreses of TNV protein (14C]leucine-labeled) and products of 1H-labeled amino acid incorporation in the wheat system stimulated by TNV RNA (100 μg/ml). The numbers refer to molecular weights as derived from marker proteins (E. coli RNA polymerase, phosphorylase A, bovine serum albumin, TNV, and TMV). (○ --- ○) 14C-labeled (in vivo) virus; (● --- ●) 1H-labeled (in vitro) products.](image-url)
tons, including the 26,000-dalton product, are presumably degradation products of coat protein. A shoulder of 35–43,000 daltons was also among the precipitation products and may represent a readthrough of TNV coat protein.

Antiserum to STNV precipitated 26% of the total in vitro products. These also showed two sharp peaks upon electrophoresis, corresponding to STNV coat protein and the 18,000-dalton peptide, as well as smaller peptides, all presumably from coat protein degradation.

The fact that antiserum to TNV precipitated less than 2% of the STNV products and vice versa (Table 2) indicates that there was negligible nonspecific precipitation and that TNV and STNV in vitro coat proteins are antigenically distinct.

**Tryptic Analysis of TNV Proteins.** The tryptic pattern of 14C-labeled in vitro TNV coat protein, as illustrated by one example on Fig. 4, shows approximately 12 high peaks and several less well-defined ones. Only one of the high peaks has no corresponding 3H-labeled in vitro peptide; another peak has a disproportionately small amount of corresponding peptide made in vitro; and three or four of the less well-defined peaks have undetectable correspondence to in vitro peptides. On the whole, however, considering that the in vitro products were not in any way fractionated, there is good similarity between in vitro and in vivo peptides, the total noncorrespondence amounting to no more than 5%.

**DISCUSSION**

In contrast to in vitro translation of TMV RNA, which yields a broad spectrum of products the largest of which approaches the entire coding capacity of the RNA (3), TNV and STNV RNAs each yield a greatly predominant product of relatively low molecular weight and corresponding to the respective coat protein.

The first reports on the use of the wheat system for translation of a viral RNA dealt with STNV RNA, a molecule of 0.4 X 10^6 daltons believed to carry but one gene (1). Tryptic patterns indicated that proper phase translation occurred; however, the main product did not exactly correspond to complete STNV coat protein. The results with the wheat system were quite similar to those obtained in parallel with the E. coli system (1), as well as in our laboratory (2). From that same laboratory there has now come a study of the mechanism of translation of STNV RNA by the wheat system (12), in which complete translation is postulated, but unfortunately not documented.

Our results support the belief of these authors that, with proper optimization of all component concentrations, what appears to be authentic STNV protein is being made in vitro. However, the results reported recently on small differences between the in vitro coat protein and the in vitro products of the short RNA of TMV-B (4) should caution one against definitive conclusions until the peptide pattern and both ends of the in vitro product have been shown rigorously to be identical with the viral coat protein.

STNV RNA translation yields two previously unreported minor products, which have also been shown but not discussed by Roman et al. (13). One, larger than coat protein, our 35,000-dalton protein, is sometimes observed as a clean peak (Table 1) and sometimes not at all (Fig. 3). It could be a result of readthrough of STNV RNA, but it is more likely a product of endogenous mRNA since its appearance is independent of STNV RNA concentration. Since both the 22,000-dalton and 18,000-dalton proteins can be precipitated with antiserum to STNV, the smaller is probably a cleavage product of the larger.

The finding of a very predominant major product when TNV RNA is used as messenger is surprising. The possibility that this TNV product may be attributed to the presence and preferred translation of a small RNA, as is the case for TYMV (5, 6), seems to be ruled out by our finding that heating and then fractionating TNV RNA under conditions that dissociate the RNA complexes of TYMV (5, 6) and Rous sarcoma virus (14) did not cause TNV RNA to lose its coat protein mRNA function. The possibility of pretranslational cleavage within the wheat extract has not been precluded, and could be tested by gel analysis of radioactive RNA that has been incubated in the wheat system. If TNV RNA is not cleaved, the preferential translation of one gene may be assumed to be due to a particularly strong ribosomal initiation site or a particular availability of that initiation site due to RNA configuration. Minor products would then be translated from less available genes or genes with weaker initiation sites. The in vitro translation of the small component of TRV RNA (15) is analogous in that the major translation product is a coat protein representing only one-fourth of the coding capacity of RNA.

The major protein is by size, antigenicity, and tryptic pattern clearly TNV coat protein. Of the minor translation products, the 63,000- and 43,000-dalton proteins are present in such low amounts that they are probably not structural proteins, but may, if they are genuine products, serve regulatory or catalytic
functions. The 63,000-dalton protein is a good candidate for a component of a viral RNA polymerase since a 60,000-dalton protein was observed in fractions of TNV-infected tobacco that had high polymerase activity (7). The 35,000-dalton protein corresponds in size to an endogenous protein observed in incorporation experiments lacking viral mRNA, as well as being irregularly detectable, when either TNV RNA or STNV RNA is used as messenger. However, there can be at least 10 times more of this protein made when TNV (or STNV) RNA is used, seemingly decreasing in proportion to the other products as the concentration of TNV RNA is increased (see Table 1). This protein thus may represent the product of an endogenous wheat messenger the production of which is stimulated in the presence of added RNAs, or it may result from a low-preference initiation site on both viral RNAs which is used less as the abundance of others increases. The 22,000-dalton product may well represent the STNV coat protein, since contamination of TNV and its RNA by 1% of STNV and its RNA cannot be ruled out, and thus could, in view of its ready translatability, easily account for that much STNV protein. All of the minor products mentioned correspond to minor products found in vivo by Jones and Reichmann (16), except for the 35,000- and 26,000-dalton proteins. The larger of these may be, as stated, wheat-specific, and both could have remained undetected because of their proximity to the greater amount of 30,000-dalton coat protein-sized product. Immunoprecipitation indicates that the 26,000-dalton protein and some smaller products have coat protein antigenicity and are therefore probably cleavage products. It may be noted that TNV RNA could carry three genes coding for the 63,000- and 43,000-dalton proteins, besides the 30,000-dalton coat protein, the others representing degradation products.

Attention should be drawn to the fact that neither STNV RNA nor TNV RNA is capped at the 5' end (12, 14, 17, 18), thus differing from most mRNAs. For STNV RNA this is particularly surprising since the other known small viral RNAs that also code for only a coat protein (components 4 of bromoviruses, cucumoviruses, and alfalfa mosaic virus) are all capped. Yet STNV is a highly efficient mRNA, and initiates translation well. That neither of these RNAs carries 5'-terminal poly(A) also puts them into a singular class. Altogether, TNV RNA appears to resemble the phage RNAs more than the eukaryotic mRNAs, both in terminal structural aspects and in that it may possibly become translated from three initiation sites, the relative availability of which would probably be determined by the conformation of the RNA.

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