Translation of Tobacco Ringspot Virus RNA in Reticulocyte Lysate: Proteolytic Processing of the Primary Translation Products

By STEPHEN A. JOBLING† AND K. ROGER WOOD*

Department of Microbiology, University of Birmingham, Birmingham B15 2TT, U.K.

(Accepted 14 August 1985)

Summary

Tobacco ringspot virus (TobRV) RNA was translated efficiently in rabbit reticulocyte lysate and directed the synthesis of two principal polypeptides, Mr 207 × 10^3 (207K) and 116K, corresponding to the translation products of RNA-1 and RNA-2 respectively. In addition, a 112K RNA-2-encoded polypeptide was sometimes detected. The 116K polypeptide was immunoprecipitated with anti-TobRV serum, suggesting that it was a precursor to coat protein. When translations were performed in the presence of dithiothreitol, the 207K polyprotein was apparently cleaved to yield 37K and 180K polypeptides, with additional processing into 65K and 128K polypeptides. Cleavage of the RNA-2-encoded polypeptide also occurred, although to a much lesser extent than that of the 207K polyprotein; polypeptides of 88K and 54K, both immunoprecipitated with antiviral serum, were identified as RNA-2-encoded cleavage products.

Introduction

The genome of tobacco ringspot virus (TobRV), the type member of the nepovirus group, consists of two species of plus-sense single-stranded RNA (Stace-Smith, 1970; Harrison & Murant, 1977). Both RNA-1 (M, 2.8 × 10^6) and RNA-2 (M, 1.3 × 10^6) (Murant et al., 1981) possess a genome-linked protein (VPg) attached at the 5' terminus (Mayo et al., 1979b) and a polyadenylate tail at the 3' terminus (Mayo et al., 1979a). Each RNA species of two other nepoviruses, tomato black ring virus (TBRV) and grapevine fanleaf virus (GFLV) has been translated in vitro to produce a single large polypeptide (Fritsch et al., 1978, 1980; Morris-Krsinich et al., 1983). In addition, the RNA-2-encoded polypeptide of GFLV is proteolytically cleaved in vitro to yield coat protein (Morris-Krsinich et al., 1983). Based on these in vitro results it has been proposed that the genome strategy of nepoviruses involves proteolytic processing, and, in general agreement with this view, there have been suggestions of in vitro processing of the translation products of the RNAs of the nepoviruses arabis mosaic, tobacco ringspot, tomato ringspot and strawberry latent ringspot (Forster & Morris-Krsinich, 1984). The results presented here demonstrate that TobRV RNA is translated into high molecular mass polypeptides which appear to be extensively processed in vitro, indicating the involvement of proteolytic processing in the genome strategy of this virus.

Methods

Virus purification. An isolate of TobRV from cherry (C; Stace-Smith & Hansen, 1974) was propagated in Nicotiana tabacum cv. White Burley. Leaves were harvested 7 days after inoculation and virus was purified as described by Mayo et al. (1982). Virus which was to be used for the production of antisera was further purified by centrifugation (3.5 h at 45000 g, 4 °C) in a density gradient of 5 to 20% sucrose in 0.07 M-sodium phosphate pH 7. Gradients were fractionated by upward displacement through an absorptiometer set at 254 nm. Fractions containing virus were pooled, diluted with 4 vol. 0.07 M-sodium phosphate pH 7, and concentrated by centrifugation (2 h at 150000 g).

† Present address: Division of Health Sciences and Technology, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, U.S.A.
Preparation of TobRV RNA. RNA was extracted by shaking purified virus (5 mg/ml in 10 mM-Tris–HCl pH 7.5, 50 mM-NaCl, 1 mM-EDTA, 1% SDS) at room temperature with an equal volume of buffer-saturated phenol containing 0.1% 8-hydroxyquinoline. After 5 min, the same volume of chloroform/isoamyl alcohol (24:1, v/v) was added and the mixture was shaken for a further 5 min. The phases were separated by centrifugation (2500 g, 10 min, 4 °C) and the aqueous phase was re-extracted with an equal volume of phenol/chloroform (1:1, v/v) and then twice with chloroform alone. The aqueous phase was made to 0.2 M-sodium acetate, mixed with 2.5 vol. ethanol and kept overnight at −20 °C. The precipitated RNA was pelleted by centrifugation at 12500 g for 20 min, washed with cold ethanol and dissolved in distilled water at a concentration of 1 to 4 mg/ml.

Separation of the viral RNA species. The viral RNAs were separated in a submerged agarose gel (0.7%, w/v) in 40 mM-Tris-HCl pH 7.4, 20 mM-sodium acetate, 1 mM-EDTA. RNA (up to 1 mg in 1 ml) was loaded in the same buffer containing 10% glycerol and 0.001% bromophenol blue and electrophoresed for approximately 5 h at 100 V. RNA bands were visualized by staining with ethidium bromide (5 μg/ml in distilled water) for 15 min and after brief washing in distilled water, RNA was extracted from the excised gel strips using the freeze-squeeze method of Tautz & Rentz (1983).

Cell-free translation. Nuclease-treated rabbit reticulocyte lysate was prepared and translations were performed as described by Pelham & Jackson (1976). [35S]Methionine (>1000 Ci/mmol, New England Nuclear) was used at approximately 250 μCi/ml, and incubation was at 30 °C.

SDS–PAGE. Polypeptides were analysed by SDS–PAGE using the discontinuous buffer system of Laemmli (1970). After electrophoresis, the gels were fluorographed using the APEX method (Jen & Thach, 1982), dried and exposed at −70 °C to preflashed Kodak XRP5 film (Laskey & Mills, 1975). The molecular masses of the translation products were estimated by comparison with those of 14C-labelled protein standards (Bethesda Research Laboratories: myosin, 200K; phosphorylase b, 97.4K; bovine serum albumin, 68K; ovalbumin, 43K; α-chymotrypsinogen, 25.7K; β-lactoglobulin, 18.4K; cytochrome c, 12.3K).

Immunoprecipitation. Translation products were immunoprecipitated as described by Rosen et al. (1983) using a 10% (w/v) suspension of formalin-fixed Staphylococcus aureus (Immunoprecipitin, Bethesda Research Laboratories) as the immunoadsorbent.

Antiserum. Antiserum was raised against TobRV in New Zealand white rabbits by injecting 100 μg (0.2 ml) of purified virus mixed with an equal volume of Freund’s complete adjuvant into one footpad. Four weeks later a further 1.5 mg of virus with incomplete adjuvant was injected into the other footpad. The rabbit was bled 4 weeks after the last injection. The titre of the antiserum was at least 1/2048 as determined by double diffusion.

RESULTS

Translation of unfractionated viral RNA

The optimum ionic conditions for the translation of TobRV RNA in reticulocyte lysate were 125 to 140 mM-K+ and 1.6 to 1.8 mM-Mg2+. Under these conditions, when TobRV RNA was added at a concentration of 100 to 200 μg/ml, the incorporation of [35S]methionine was stimulated up to 200-fold (8 × 104 d.p.m./μl lysate) above that of the control (no RNA added).

A time course of the appearance of the translation products in the lysate is shown in Fig. 1. After 60 min incubation, unfractionated TobRV RNA had directed the synthesis of two principal polypeptides of Mr 207K and 116K (Fig. 1). In addition, a prominent polypeptide of Mr 195K was detected in some experiments (compare Fig. 1 and Fig. 2). The sequential appearance of polypeptides with increasing molecular mass does not suggest a precursor-product relationship between the larger and smaller polypeptides. Coat protein-sized polypeptides were not detected among the translation products under these conditions.

Translation of the separated viral RNAs

When individual RNA species were translated (Fig. 2), the 116K polypeptide was the major translation product of RNA-2 (Fig. 2, lane 2) and although RNA-1 was contaminated with RNA-2, it is evident that polypeptides larger than 116K were RNA-1-encoded (Fig. 2, lane 1). However, when the translation products were analysed on gels with an acrylamide concentration lower than 10%, the 116K polypeptide was sometimes resolved into two polypeptides of Mr 116K and 112K, both of which were RNA-2-encoded (Fig. 2, lane 5). When observed, these two polypeptides were produced in equal amounts and this ratio did not change significantly over a wide range of RNA concentrations during translation. Thus, ratios of the concentrations of the 116K and 112K polypeptides synthesized at RNA concentrations of 6-25, 25, 100 and 400 μg/ml were 0.90, 0.94, 0.97 and 0.97 respectively.
Fig. 1. Polypeptides translated from unfractionated TRSV RNA in rabbit reticulocyte lysate. At the indicated times (min) after the addition of TobRV RNA, samples were removed from the translation mixture, and analysed in a 10% polyacrylamide gel. The numbers at the right refer to the molecular masses (×10–3) of the major translation products.

Processing of the translation products

Under normal translation conditions (Fig. 3, lanes 1, 3, 5, 7 and 9), no new polypeptides appeared when the translation products were incubated for a further 15 h at 30 °C. However, when translations were performed in the presence of the reducing agent dithiothreitol (DTT, 2 mM) (Fig. 3, lanes 2, 4, 6, 8 and 10), extensive processing of the 207K polypeptide was observed; new polypeptides of M, 180K, 128K, 65K and 37K appeared during further incubation, with the concomitant disappearance of the 207K product. Processing appeared to occur only on completion of the 207K polypeptide, as no cleavage products were seen after 30 min translation, at which time polypeptides up to 195K were synthesized (Fig. 3, lane 2). However, by the end of the normal translation period (60 min), a large proportion of the 207K polypeptide had been processed (Fig. 3, lane 4). After 2 h incubation, almost all of this polypeptide had disappeared and the 180K and 37K polypeptides had reached their maximum levels (Fig. 3, lane 6). Upon further incubation, the level of the 180K polypeptide decreased considerably, concomitant with an increase in the levels of the 128K and 65K polypeptides (Fig. 3, lanes 8 and 10). It should be noted that the general reduction in the intensity of the majority of the bands upon prolonged incubation (Fig. 3, lanes 7 to 10) resulted from failure of material to enter the resolving gel, rather than from proteolytic processing. It may be that selective retention of the higher molecular mass polypeptides is responsible for the change in ratio of the quantities of 128K and 65K polypeptides.

The results of a similar experiment are shown in Fig. 4 and confirm and extend these findings. In this experiment, some processing occurred in the samples translated in the absence of DTT; a low level of the 128K polypeptide was detected from 1 h onwards (Fig. 4, lanes 10 to 14). In
addition to the previously detected cleavage products, polypeptides of \( M_r \) 88K and several of approximately 20K to 25K were apparent in the samples translated in the presence of DTT (Fig. 4, lanes 1 to 6), although the 88K polypeptide was also apparent in the samples translated without DTT (Fig. 4, lanes 10 to 14). The observation that the polypeptides were not present after 30 min incubation, by which time polypeptides up to 195K had been synthesized, would suggest that they are cleavage products. The 88K polypeptide, as well as the 116K and 112K polypeptides, was immunoprecipitated with anti-TobRV serum from the translation products which had been incubated for 5 h (Fig. 4, lanes 8 and 9). A small amount of a 54K polypeptide, which co-migrated with coat protein, was also immunoprecipitated from the sample which had been translated in the presence of DTT (Fig. 4, lane 8), although a polypeptide of this size could not be distinguished in the original sample (Fig. 4, lane 5). No polypeptides were precipitated by normal rabbit serum (Fig. 4, lane 7).
Translation of TobRV RNA in vitro

Fig. 3. Effect of DTT on the stability of the translation products of TobRV RNA during prolonged incubation at 30 °C. Unfractionated TobRV RNA was translated in reticulocyte lysates that contained either no added DTT (lanes 1, 3, 5, 7 and 9) or 2 mM-DTT (lanes 2, 4, 6, 8 and 10). Samples were removed after 0.5 h (lanes 1 and 2), 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), 5 h (lanes 7 and 8) or 16 h (lanes 9 and 10) and analysed in a 9.2% polyacrylamide gel. The numbers at the right refer to the molecular masses (×10⁻³) of the processed and unprocessed translation products.

DISCUSSION

The results presented here have demonstrated that in reticulocyte lysates TobRV RNA-1 and RNA-2 are each translated into a high molecular mass polypeptide (207K and 116K respectively) and that each appears to be a polyprotein which is proteolytically processed in vitro, yielding polypeptides of Mr 180K, 128K, 88K, 65K, 54K, 37K and several of approximately 20K to 25K.

The data suggest that the extent of cleavage of the RNA-1-encoded polyprotein (207K) was far greater than that of the RNA-2-encoded polyprotein and that, of the four major cleavage products which were detected (180K, 128K, 65K and 37K), three were encoded by RNA-1. The 180K and 128K polypeptides appear to be too large to have been translated from RNA-2; if the 65K polypeptide had been processed from the RNA-2 translation product, it might have been expected that either it or the corresponding cleavage product would have contained coat protein sequences, and therefore would have been immunoprecipitated. In addition, the kinetics of the appearance of the 37K polypeptide, together with the molecular masses and relative quantities of the processed polypeptides, suggest that it too was RNA-1-encoded. It is possible, therefore, that the 207K polyprotein was rapidly cleaved to yield the 180K and 37K polypeptides and that the 180K polypeptide was subsequently processed to give the 128K and 65K polypeptides.

There are similarities between the suggested pattern of proteolytic processing of the TobRV RNA-1-encoded polyprotein and the B-RNA-encoded polyprotein of cowpea mosaic virus (CPMV) (Franssen et al., 1984; Peng & Shih, 1984). These are reflected in the similarities in sizes of the putative cleavage products and the requirements for DTT. Processing of the CPMV product, however, appears to take place on nascent chains, while that of TobRV does not.
Fig. 4. Processing and immunoprecipitation of the translation products of TobRV RNA in rabbit reticulocyte lysate. Unfractionated TobRV RNA was translated in the presence (lanes 1 to 6) or absence (lanes 10 to 14) of DTT (2 mM). Samples were removed after incubation for 0.5 h (lanes 1 and 10), 1 h (lanes 2 and 11), 1.5 h (lane 3), 2 h (lanes 4 and 12), 5 h (lanes 5 and 13) and 21 h (lanes 6 and 14) and analysed in an 8.33% polyacrylamide gel. Lanes 8 and 9 represent polypeptides which were immunoprecipitated with anti-TobRSV serum from samples 5 and 13 respectively. Lane 7 represents the combined control immunoprecipitation (normal rabbit serum) of samples 5 and 13. The numbers at the left refer to the molecular masses \((\times 10^{-3})\) of the processed and unprocessed translation products. Lanes 7 to 9 and the lower portion of the remaining lanes were exposed for three times as long as the rest of the fluorograph in order to reveal the minor bands.

The primary translation product of RNA-2 was a polyprotein of \(M_r\) 116K, which was immunoprecipitated by anti-TobRV serum and therefore contained coat protein sequences. In addition, a slightly smaller polypeptide, \(M_r\) 112K, was apparent in some experiments. It seems unlikely that the 116K polypeptide was derived from the 112K polypeptide by readthrough of a leaky termination codon, since the ratio of the two polypeptides did not change when translations were performed over a wide range of RNA concentrations, and if readthrough was occurring this should have led to a reduction in the level of the 116K polypeptide. The 112K product could, however, be a premature termination product of the 116K polypeptide. Alternatively, it may be that, like comovirus M-RNA (e.g. van Wezenbeek et al., 1983) TobRV RNA-2 has two initiation sites close to the 5' end.

In contrast to the polyprotein encoded by RNA-1, processing of the 116K RNA-2-encoded product was limited, although an immunoprecipitable 88K polypeptide and a coat protein-sized 54K polypeptide, also immunoprecipitable, were detected as putative cleavage products. It is possible that the 20K to 25K polypeptides also represent cleavage products of the 116K/112K polypeptide, although further experiments will be required to substantiate this. These observations are not, however, at variance with a recent preliminary report indicating that the TobRV RNA-2 translation product can be processed \textit{in vitro} to shorter species which include the coat protein (Forster & Morris-Krsinich, 1984).

In contrast to the results presented here for TobRV, it was shown that the RNA-2-encoded polyprotein of GFLV (125K) was cleaved at a single site yielding a 68K polypeptide and a 58K
polypeptide characteristic of coat protein (Morris-Krsinich et al., 1983). Processing of the GFLV RNA-2-encoded polyprotein was also more evident than that of the RNA-1-encoded polyprotein (220K), where polypeptides of 195K and 190K were the only cleavage products detected. In addition, DTT had no effect on the translation products of GFLV RNA. There appear, therefore, to be several differences in the processing of the polyproteins of TobRV and GFLV, and it will be interesting to see how the processing of other nepovirus polyproteins compares with that of the two investigated to date.

Although these data suggest that the translation products of TobRV RNA-1 and RNA-2 undergo proteolytic processing in vitro in the manner described, conclusions are necessarily tentative and further experiments are required before the processing pattern can be established completely and with certainty.

After this paper had been submitted for publication, a communication expanding on results described previously in outline (Forster & Morris-Krsinich, 1984) was published (Forster & Morris-Krsinich, 1985). These authors also report the translation of the two genomic RNAs of TobRV into polyproteins which are subsequently processed to smaller polypeptides, although processing in the absence of DTT was rather more extensive than observed in the experiments described here. The size and number of the putative cleavage products are similar. They suggest, however, that there are two primary translation products from RNA-1, with apparent Mr 225K and 205K, and a major product from RNA-2 of apparent Mr, 116K. In common with the 116K polypeptide described here, that described by Forster & Morris-Krsinich also appeared to contain the coat protein sequence.

The authors wish to thank Dr Stephen Oldfield for preparation of the lysate and the Science and Engineering Research Council for providing a studentship to S.A.J.

REFERENCES


(Received 22 April 1985)