Comparison of the strategies of expression of five tymovirus RNAs by \textit{in vitro} translation studies

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Total nucleotide sequencing of the RNA genome of various tymoviruses has demonstrated that the overall genome organization of these viruses is identical. Furthermore, the strategies of expression of the turnip yellow mosaic virus (TYMV) genome have been established by \textit{in vitro} translation studies; these include the synthesis of a subgenomic RNA, the utilization of overlapping open reading frames (ORFs) and maturation of a polyprotein. In the experiments described here, the strategies of expression of other tymovirus (eggplant mosaic virus, ononis yellow mosaic virus, belladonna mottle virus and physalis mottle virus) genomes have been compared to those used by the TYMV genome, in particular to determine whether these tymoviruses also resort to the expression of overlapping ORFs and maturation of a polyprotein.

Tymoviruses are a genus of positive-stranded RNA viruses containing a monopartite genome, the type member of which is turnip yellow mosaic virus (TYMV). Translation studies performed using various \textit{in vitro} systems have already helped in defining the strategies of expression used by TYMV RNA. The genomic RNA (Morch \textit{et al.}, 1988) directs the synthesis of two non-structural proteins (Bénicourt \textit{et al.}, 1978; Weiland & Dreher, 1989). The larger protein, the 206K protein, is produced by translation of an open reading frame (ORF) that includes over 90\% of the genome. Here, this protein is designated the replicase protein (RP) because it is the postulated replicase polyprotein. Its ORF nearly completely overlaps the ORF of the smaller protein, the 69K protein, the function of which is unknown; this protein is designated the out-of-phase protein (OP). A subgenomic RNA species derived from the 3' region of the genomic RNA and encapsidated into virus particles directs the synthesis of the viral coat protein (CP).

In addition to expressing two overlapping ORFs and generating a subgenomic RNA species, TYMV also resorts to the strategy of proteolytic maturation of the primary translation product, RP, for the generation of its final translation products (Morch \textit{et al.}, 1989). Proteolytic maturation of RP is temperature-sensitive; it occurs at 30°C, but is inhibited at 37°C. At the lower temperature it yields two polypeptides, one corresponding to the N-terminal region of RP (N-RP), of about 150K, and the other to the C-terminal region of RP (C-RP), of about 78K. Cleavage is inhibited by several cysteine and serine proteinase inhibitors.

A comparison of the genomic sequence of TYMV (Morch \textit{et al.}, 1988) with that of the TYMV-Club Lake isolate (Keese \textit{et al.}, 1989), eggplant mosaic virus (EMV; Osorio-Keese \textit{et al.}, 1989), ononis yellow mosaic virus (OYMV; Ding \textit{et al.}, 1989) and kennedya yellow mosaic virus (Ding \textit{et al.}, 1990) reveals common features of genome organization as well as sequence similarities that may be shared by all tymoviruses. In all cases, the RNA genome contains three ORFs: the two longer ORFs overlap and are 5'-proximal, whereas the short ORF corresponding to the CP gene is 3'-proximal. A subgenomic RNA is most likely responsible for the synthesis of CP in all tymoviruses (Hirth & Givord, 1988).

To determine whether tymoviruses other than TYMV use the same strategies of expression as the type member, in particular whether they resort to proteolytic maturation and whether OP can be detected among the translation products, a series of comparative translation studies were undertaken using the rabbit reticulocyte lysate system. The RNAs of TYMV, OYMV, EMV, belladonna mottle virus (BdMV) and physalis mottle virus (PhMV) were compared. It should be stressed that the total nucleotide sequence of the genomic RNAs of the latter two viruses has not been published.

The profiles of the virus RNAs were compared first by agarose gel electrophoresis. Two types of translation experiments were then done using the viral RNAs as templates: temperature shift experiments performed in

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Fig. 1. Analysis of tymovirus RNAs by agarose gel electrophoresis. RNA (1 μg/well) was loaded except for EMV RNA for which 2 μg was loaded. After electrophoresis, the RNAs were stained with ethidium bromide and photographed under u.v. light. Lanes 1 to 6, TYMV, EMV, RNA markers (BRL), OYMV, PhMV and BdMV RNAs. Sizes and positions of RNA markers are indicated to the right. The size and position of the TYMV subgenomic RNA is indicated to the left.

the presence or absence of proteinase inhibitors, and C-terminal labelling experiments of the translation products.

TYMV was isolated (Leberman, 1966) from infected Chinese cabbage leaves kindly supplied by S. Astier-Manifacier (Versailles, France). EMV (bottom component) and OYMV were gifts from A. Gibbs (Canberra, Australia) and BdMV was a gift from R. Peter (Strasbourg, France). The RNA from these viruses was extracted as described (Porter et al., 1974). PhMV (bottom component) was isolated from infected Nicotiana glutinosa plants (Savithri et al., 1987) and the viral RNA was isolated (Jacob et al., 1991). The RNA concentrations were estimated from the absorbance at 260 nm, and RNA was analysed by 1·5% agarose gel electrophoresis (Sambrook et al., 1989).

The standard translation conditions (total volume 10 μl) using a rabbit reticulocyte lysate and 370 kBq of L-[^35]S)cysteine (>22·2 TBq/mmol; Amersham) were as described (Morch et al., 1989). Incubation was at 30 °C or 37 °C for the times indicated. The translation products were analysed on 12·5% acrylamide-0·1% bis-acrylamide-SDS gels (modified from Laemmli, 1970). The gels were then fixed (Sambrook et al., 1989), fluorographed (Bonner & Laskey, 1974), dried and submitted to autoradiography.

Temperature shift experiments were performed as described by Morch et al. (1989). The concentrations of the proteinase inhibitors correspond to those which completely inhibit cleavage of TYMV RP (Morch et al., 1989); total incubation time was 90 min.

For the C-terminal labelling experiments, the volume of the incubation reaction was 20 μl. The translation reactions were initiated in the absence of radiolabelled cysteine at either 30 °C or 37 °C. After 10 min, edeine (20 μM, final concentration) was added to block further initiation of polypeptide chains. Twenty or 30 min (for incubations at 37 °C or 30 °C respectively) after the onset of incubation, L-[^35]S)cysteine was added so as to label the C-terminal regions of the proteins, and incubation was continued for a total of 90 min without temperature shift.

The migration pattern of each of the five tymovirus RNAs is shown in Fig. 1. An intense band migrating between 6 and 7·5 kb and corresponding to genomic RNA is visible in the lanes containing TYMV, PhMV and BdMV RNA. In the RNA samples from EMV and OYMV, the intensity of the corresponding band is very low, whereas a particularly intense smear appears that is probably due in part to uncontrolled degradation of these RNAs. The band corresponding to the CP subgenomic RNA is particularly intense in the case of OYMV and almost invisible in the other samples; indeed, the EMV and PhMV preparations used in the experiments described here contained only the 'bottom component', which contains little subgenomic RNA (Szybiak et al., 1978; Jacob & Savithri, 1991).

To determine the optimal conditions for protein synthesis, in particular that directed by EMV and OYMV RNA, a series of preliminary experiments were carried out using different concentrations of virus RNA, rabbit reticulocyte lysate, and MgCl2 and KCl. Kinetic experiments were also performed to determine the time necessary to synthesize RP. These experiments (not shown) demonstrated that the optimum conditions required by EMV and OYMV RNA do not differ significantly from those required by TYMV RNA. However, regardless of the conditions and the temperature (30 °C or 37 °C) of incubation, and probably because of the low amount of full-length RNA in the preparations, the level of synthesis of RP remained low with EMV and OYMV RNA, as compared to the level reached with TYMV, BdMV and PhMV RNA. For the sake of simplicity, the translation conditions adopted for
abundant in the C-terminal third than in the N-terminal two-thirds of the RPs of the tymoviruses for which such information is available.

The inhibitors efficiently hindered the appearance of the N-RP band with all the virus RNAs tested except with PhMV RNA, for which only a decrease in the intensity of this band was observed. These results suggest that N-RP could be one of the cleavage products of RP in all the tymoviruses analysed. However, except for the situation encountered with TYMV RNA, the presence of C-RP could not be unambiguously detected among the translation products obtained using the other tymovirus RNAs. As expected, the intensity of the OP band observed when TYMV RNA served as template remained virtually unaltered whether proteins were synthesized in the presence or in the absence of proteinase inhibitors. By comparison, the intense band that is insensitive to the proteinase inhibitors and occupies a relative position comparable to that of TYMV OP could correspond to the OPs produced by the other tymovirus RNAs.

With the aim of better detecting the C-terminal cleavage product of RP when the various tymovirus RNAs served as mRNA in the translation experiments, C-terminal labelling experiments were carried out in which the radioactive amino acid was added after the onset of protein synthesis. Under these conditions OP should not be labelled because its synthesis is complete prior to the addition of L-[35S]cysteine. On the other hand, RP should be labelled because its C-terminal region will have incorporated the radiolabelled amino acid. Thus, if cleavage of RP produced from the other tymovirus RNAs occurs as it does with TYMV RNA, the appearance of a labelled band corresponding to C-RP would be expected.

Fig. 3 presents the results of total or C-terminal labelling experiments performed at 30 °C or 37 °C using the various virus RNAs. As expected, in all cases OP was detected easily by total labelling and could not be detected in the samples obtained by C-terminal labelling; the intensity of this band produced by total labelling was not modified dramatically when incubation was at 30 °C or 37 °C. In total labelling experiments the N-RP band was more intense at 30 °C than at 37 °C in all cases, and in C-terminal labelling experiments it was faint (or virtually absent with OYMV and EMV) at 30 °C and absent at 37 °C. With TYMV RNA, C-RP was clearly detected at 30 °C and was very faint at 37 °C under total and C-terminal labelling conditions. However with no other tymovirus RNA could C-RP be detected, whatever the incubation conditions. Such a result is unexpected with BdMV in particular, the RP of which is produced in considerable amounts under total labelling conditions.
The results reported here indicate that the five tymovirus RNAs tested give rise to a similar pattern of proteins in a rabbit reticulocyte lysate. These are essentially the proteins designated RP (approx. 200K), N-RP (approx. 150K), OP (approx. 70K) and CP (approx. 20K). However, the size of the proteins produced differs somewhat depending on the virus RNA serving as the template. Basically the same protein patterns were obtained using a wheatgerm extract (not shown). It has been demonstrated previously that the RNA contained in the bottom component of EMV directs the synthesis of major proteins, the sizes of which are approximately 180K, 150K and 70K (Szybiak et al., 1978). Translation of the PhMV (bottom component) RNA also yields products of similar sizes (Jacob & Savithri, 1991).

In view of the similarity in the protein patterns observed with the different virus RNAs, it seemed of interest to examine the origin of these protein bands. The position of OP produced using TYMV RNA as template has been established unequivocally by Weiland & Dreher (1989) by mutagenesis of the corresponding initiator AUG. The aberrant position of this protein on polyacrylamide–SDS gels with respect to its calculated size is most likely due to its very basic nature (Morch et al., 1988). The main characteristics of the TYMV OP as determined by in vitro translation experiments are (i) the protein is insensitive to the presence of proteinase inhibitors, (ii) the level of its synthesis is virtually the same at 30 °C and 37 °C and (iii) it is barely visible (if at all) when C-terminal labelling experiments are performed. Consequently, the protein produced when the other virus RNAs serve as template, and which is designated OP in Fig. 2 and 3, fully complies with these criteria. Therefore, it seems reasonable to consider this protein to be OP.

It should be pointed out that with EMV RNA the protein considered as OP always appears as a doublet band. We presently have no explanation for this phenomenon. The initiator AUG of OP occupies positions 102 to 104 on the EMV RNA. The slightly faster migrating band cannot have resulted from initiation at the next AUG in the OP reading frame (positions 1260 to 1262), because the calculated size of the resulting protein would be less than half that of OP. Another possibility would be suppression of the UGA

incubation time being 90 min (C-terminal labelling). Incubation mixture (2 μl, 100000 c.p.m.; 18 μl, 400000 c.p.m.) was layered onto the wells in the total labelling and C-terminal labelling experiments respectively. The positions of RP, N-RP, OP and CP synthesized from the various tymovirus RNAs are indicated by brackets to the left of each panel, as is the position of TYMV C-RP (arrow). To the right of (b) the size and position of the 14C-labelled protein markers (Amersham) analysed on an adjacent lane of the same gel are given.
codon located at the end of the OP reading frame. If the context surrounding this termination codon were to favour suppression, an OP with an extra 11 amino acids would be produced, the position of which could correspond to the slightly slower migrating band of the doublet. Although rather unlikely, the possibility that the doublet band results from a contaminant, for instance a mutant in the EMV preparation, cannot be excluded. Finally, the OP of EMV could undergo a partial post-translational modification of an unknown nature, resulting in the appearance of a doublet band.

Using TYMV RNA as template for translation experiments \textit{in vitro}, it has been demonstrated previously that the primary translation product, RP, is processed to yield N-RP and C-RP, presumably by a temperature-sensitive proteinase (Morch et al., 1989). Similar studies carried out using EMV, OYMV, BdMV and PhMV RNA suggest that, as with TYMV RNA, RP is the primary translation product which produces N-RP at 30 °C but not at 37 °C (Fig. 3), and as a function of time at 30 °C (not shown). Furthermore, the appearance of N-RP is inhibited by proteinase inhibitors, and appears to be accompanied by a concomitant decrease in RP (Fig. 2). However, to our surprise the C-terminal cleavage product designated C-RP could only be detected using TYMV RNA. Neither comparative kinetic studies performed at 30 °C and 37 °C (not shown), temperature shift experiments in the presence of proteinase inhibitors, nor C-terminal labelling experiments, which all clearly detected C-RP with TYMV RNA as template, enabled the detection of a corresponding protein among the translation products using the other four tymovirus RNAs as mRNAs.

Consequently, although circumstantial evidence is in favour of maturation of RP in the case of all five tymovirus RNAs tested, the absence of C-RP from the translation products obtained using OYMV, EMV, BdMV or PhMV RNA as template does not allow us to conclude unequivocally that maturation of RP is a common strategy of expression used by all five tymoviruses. One can postulate that C-RP is rapidly degraded during \textit{in vitro} translation. A similar situation is encountered regarding nsP4 from Sindbis virus (Shirako & Strauss, 1990). This polypeptide is produced by cleavage of a precursor polyprotein, but appears to be degraded rapidly during \textit{in vitro} translation experiments using Sindbis virus RNA as template. Likewise, it is present in smaller amounts in Sindbis virus-infected cells than in cells infected with Semliki Forest virus, another alphavirus (Li & Rice, 1989).

As the tymovirus C-RP (as does Sindbis virus nsP4) contains the conserved consensus sequence Gly-Asp-Asp (GDD) motif related to all virus RNA-dependent RNA polymerases analysed to date (Argos, 1988; Candresse et al., 1990), one would have to assume that this polypeptide is at least partly preserved \textit{in vivo}, even by those tymoviruses for which it is not detected \textit{in vitro}.

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