The Stability of Cowpea Mosaic Virus VPg in Reticulocyte Lysates

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SUMMARY

The ability of the genome-linked protein (VPg) of cowpea mosaic virus (CPMV) to survive incubation in rabbit reticulocyte lysates was investigated. In contrast to the results obtained with picornavirus RNAs, there was no evidence for the specific removal ('unlinking') of the VPg from CPMV RNA during incubation. While linked to RNA, CPMV VPg was protected from proteolytic degradation; if the RNA was first digested with nuclease P1, rapid degradation of the VPg occurred. However if as few as 17 nucleotides were left attached to the VPg, stability was retained.

INTRODUCTION

In common with those of the Picornaviridae and several other groups of viruses, the 5' termini of both the genomic RNAs of cowpea mosaic virus (CPMV) are covalently linked to a small, basic protein (VPg). In picornaviruses the RNA–protein linkage consists of an O4-(5'-uridylyl) tyrosine bond (Ambros & Baltimore, 1978; Rothberg et al., 1978; Vartapetian et al., 1980; King et al., 1980) whereas there is strong, but circumstantial, evidence that the VPg of CPMV is attached to the RNA via a serine residue (Zabel et al., 1984). It has been demonstrated that rabbit reticulocyte lysates contain an 'unlinking' activity which can specifically cleave the VPg–RNA bond of both poliovirus (Ambros et al., 1978; Dorner et al., 1981) and foot-and-mouth disease virus (FMDV) (Sangar et al., 1981). Once released, the 'free' VPg is rapidly degraded by a proteinase present in the lysates (Dorner et al., 1981; Sangar et al., 1981). These findings imply that the RNA which is translated in vitro, like that found on polyribosomes in vivo, lacks the VPg.

In view of the apparent differences between picornaviruses and CPMV in both the protein–RNA linkage and the amino acid sequences of the VPgs, we have investigated the stability of both the VPg–RNA bond and of 'free' VPg during incubation in rabbit reticulocyte lysates. The results show that the 'unlinking' activity is not active on CPMV RNA, but that if the VPg is released prior to incubation, it is rapidly degraded unless a small portion of RNA is left attached.

METHODS

Preparation of labelled RNA. CPMV virion RNA was specifically labelled at its VPg by reaction with Na125I (Amersham, 100 mCi/ml) in the presence of Iodogen (Pierce Chemical, Rockford, Ill., U.S.A.). The reaction conditions and subsequent recovery of the RNA were as previously described (Lomonossoff et al., 1985). Uniformly 32P-labelled virion RNA was prepared from CPMV-infected cowpeas (Vigna unguiculata var. Blackeye Early Ramshorn) grown hydroponically in the presence of 32PO4. The conditions for plant growth and the method of virus purification were as described by Stanley et al. (1978). The nucleoprotein components were separated by a single cycle of caesium chloride density gradient centrifugation and the RNAs were extracted as described by Zimmern (1975).

Assay of unlinking activity. A natural mixture of 125I-labelled CPMV RNAs or separated 32P-labelled M or B RNAs were incubated in message-dependent rabbit reticulocyte lysates at a final concentration of 0.1 to 0.2 mg/ml. The salt concentrations in the lysate (0.5 mM-MgCl2, 80 mM-KCl) were identical to those described by Harbison et al. (1984), conditions which were found to support efficient translation of both RNAs (data not shown). After incubation for various times at 30 °C, samples were removed and analysed either directly or after

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isolation of the RNA by phenol extraction and ethanol precipitation. For direct analysis of \(^{125}\!)I\)-labelled RNA, samples were mixed with 8 vol. 6 M-urea, 1% (w/v) SDS, 1% 2-mercaptoethanol, 0.01% bromophenol blue in 10 mM-sodium phosphate, pH 7.2 and heated to 100°C for 2 to 3 min. Electrophoresis was in 15% polyacrylamide gels containing 6 M-urea and 0.1% SDS and buffered with 100 mM-sodium phosphate, pH 7.2. After electrophoresis the gels were fixed in 30% (v/v) ethanol, 12% acetic acid, dried and autoradiographed.

RNA which was phenol-extracted after incubation in lysate was digested with either ribonuclease T1 (for \(^{32}\!)P\)-labelled RNA) or nuclease P1 (for \(^{125}\!)I\)-labelled material). For nuclease P1 digestion, the solution containing radiolabelled RNA was made 50 mM in ammonium acetate pH 5.3 and nuclease P1 was added to a final concentration of 10 \(\mu\)g/ml. Incubation was for 30 min at 37°C. For ribonuclease T1 digestion, samples were dissolved in 10 mM-Tris–HCl pH 7.4, 0.1 mM-EDTA, heated to 100°C briefly and digested with ribonuclease T1 (final concentration 1 to 2 units per \(\mu\)g RNA). Nuclease P1-digested \(^{125}\!)I\)-labelled samples were run in 15% polyacrylamide gels containing 6 M-urea and 0.1% SDS as described above. Ribonuclease T1 digests of \(^{32}\!)P\)-labelled RNA were electrophoresed through 20% polyacrylamide thin gels containing 7 M-urea as previously described (Lomonossoff et al., 1985).

Assay of VPg degradation. \(^{125}\!)I\)-labelled CPMV RNA was digested with either nuclease P1 or ribonuclease T1 using the conditions described above. One-half \(\mu\)g of each digest was incubated in 20 \(\mu\)l of reticulocyte lysate at 30°C supplemented with either MgCl\(_2\) (5 mM) or EDTA (20 mM). Samples were withdrawn after various times and were analysed in 15% polyacrylamide gels containing 6 M-urea and 0.1% SDS as described above.

Preparation of pCp-labelled ribonuclease T1 fragments. Fifty \(\mu\)g of unlabelled CPMV RNA was digested with ribonuclease T1 (20 units) for 30 min at 37°C. The digest was then made 10 mM with respect to MgCl\(_2\) and 0.5 units of calf intestinal phosphatase was added. Incubation was continued for a further 30 min after which the digest was electrophoresed in a 10% polyacrylamide thin gel as previously described (Lomonossoff et al., 1985). As a marker, a sample of \(^{125}\!)I\)-labelled RNA was also digested and electrophoresed in adjacent lanes of the gel. After electrophoresis, the position of the \(^{125}\!)I\)-labelled marker was determined by autoradiography and the area corresponding to the unlabelled VPg-linked T1 oligonucleotide (VPg–T1) was excised. The VPg–T1 fragments were eluted overnight and purified by binding to, and elution from, a small DEAE-cellulose column (Brownlee, 1972). The purified fragments were then labelled using [5-\(^{32}\!)P\]pCp (Amersham, 3000 Ci/mmol) and RNA ligase exactly as described previously (Lomonossoff et al., 1985) and were re-purified from a 10% polyacrylamide gel. The pCp-labelled T1 products were incubated in reticulocyte lysate and the material was analysed exactly as described for the \(^{125}\!)I\)-labelled fragments.

RESULTS

Assay of VPg removal with \(^{125}\!)I\)-labelled RNA

Treatment of CPMV RNA with \(^{125}\!)I\) in the presence of Iodogen results in the specific labelling of VPg, enabling the removal of VPg to be monitored by following loss of label from \(^{125}\!)I\)-labelled RNA. When \(^{125}\!)I\)-labelled RNA was incubated in rabbit reticulocyte lysate for various times under translation conditions and the samples analysed directly in a 15% polyacrylamide gel, the label only just entered the resolving gel even when the sample had been incubated in the lysate for 60 min (Fig. 1). No radioactivity migrated to the position expected of 'free' VPg which would run near the position of the VPg–pU marker produced by nuclease P1 digestion of \(^{125}\!)I\)-labelled CPMV RNA (Daubert et al., 1978). The accumulation of radioactivity at the top of the gel strongly suggests that most, if not all, of the labelled VPg was still linked to high molecular weight RNA. Electrophoresis of the samples shown in Fig. 1 in 1% agarose gels confirmed that the radioactivity was indeed associated with high molecular weight RNA though the average length of the RNA decreased during the incubation period (data not shown). These results strongly suggest that CPMV RNA is not a substrate for the lysate 'unlinking' activity.

To confirm that the radioactivity associated with high molecular weight material in Fig. 1 still resided in intact VPg, RNA was isolated from the incubated samples, digested with nuclease P1 and the digest was analysed in a 15% polyacrylamide gel. It is clear from Fig. 2 that 'intact' VPg–pU can be recovered from RNA which has been incubated in reticulocyte lysates for as long as 60 min. This result confirms that VPg is not 'unlink'ed from CPMV RNA in reticulocyte lysates and demonstrates that while linked to high molecular weight RNA, the CPMV VPg is not degraded by any proteases present in the lysate.

Assay of VPg removal using \(^{32}\!)P\)-labelled RNA

There are two possible criticisms of using \(^{125}\!)I\)-labelled RNA to assay VPg removal. The first is that the removal of a modified (iodinated) VPg is being monitored and the second is that there
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Fig. 1. Autoradiogram of a 15% polyacrylamide gel of 125I-labelled CPMV RNA after incubation in rabbit reticulocyte lysate. Lanes 1 to 4 contain samples taken after 0, 10, 30 and 60 min incubation respectively. Lane 5 contains a nuclease P1 digest of 125I-labelled CPMV RNA which provides the VPg-pU marker.

Fig. 2. Autoradiogram of a 15% polyacrylamide gel of nuclease P1 digests of 125I-labelled CPMV RNA. Lanes 1, 2 and 3 contain digests of RNA which had been incubated in rabbit reticulocyte lysate for 0, 10 and 60 min respectively prior to nuclease P1 digestion. The position of VPg-pU is marked.

are no internal controls by which sample recovery can be monitored. To circumvent these problems a series of experiments using uniformly 32P-labelled fragments was carried out. When either CPMV M or B RNA is digested with ribonuclease T1, the slowest migrating discrete product in acrylamide gels is a 17 base RNA fragment linked to VPg. Removal of most of the VPg by proteinase K digestion leads to an increase in mobility of this fragment (Stanley & van Kammen, 1979). Thus the potential removal of VPg could be followed by analysing the ribonuclease T1 products derived from 32P-labelled M or B RNA which had been incubated in reticulocyte lysates. Fig. 3 shows the result of such an analysis for M RNA. The heterogeneous material near the top of the gel represents the 3' poly(A) tail and the two slowest migrating discrete bands represent the VPg-linked T1 fragment (upper) and a 41 nucleotide-long T1 fragment comprising bases 49 to 89 of the M RNA sequence (Najarian & Bruening, 1980). That
Fig. 3. Analysis of RNase T1 digests of uniformly $^{32}$P-labelled middle component RNA. Lanes 1 and 2 contain digests of native and proteinase K-treated M RNA respectively. Lanes 3 to 7 contain digests of RNA which had been incubated in reticulocyte lysate for 0, 5, 10, 30 and 60 min prior to digestion. Electrophoresis was in a 20% polyacrylamide thin gel. The position of the VPg-linked RNase T1 product is indicated.
the slowest migrating band was indeed the VPg-linked terminal T1 oligonucleotide was confirmed by its sensitivity to digestion with proteinase K (compare lanes 1 and 2 in Fig. 3) and also by eluting the material and subjecting it to complete digestion with alkali or RNase A. Total alkali digestion gave uridine 3',5'-bisphosphate (pUp) as well as the expected nucleoside 3' monophosphates; pUp is characteristic of the VPg-RNA linkage (Stanley et al., 1978) and its presence is diagnostic for the VPg-linked RNase T1 oligonucleotide. Ribonuclease A digestion also yielded the expected catalogue of products (Stanley & van Kammen, 1979).

When the RNase T1 products of M RNA which had been incubated in reticulocyte lysate for various times were examined, there was no sign of any selective loss of radioactivity from the VPg-linked RNase T1 fragment (Fig. 3). The persistence of the VPg-T1 band indicates that the VPg is not cleaved from CPMV RNA even after 60 min incubation in the lysate. In addition to the analysis in one-dimensional gels, ribonuclease T1 digests of untreated M RNA, proteinase K-treated M RNA and M RNA which had been incubated in reticulocyte lysates for 60 min were also analysed by two-dimensional gel electrophoresis. Proteinase K treatment was found to result in the expected change in mobility of the VPg-linked RNase T1 product (Stanley & van Kammen, 1979), whereas no change in mobility was found with the lysate-treated sample (data not shown). This confirms that CPMV VPg is not unlinked from its RNA by incubation in reticulocyte lysates.

In a series of experiments similar to those described above, uniformly labelled B RNA was used. Apart from the fingerprints being more complex, the results were essentially identical, showing that the VPg is not unlinked from either RNA.

Degradation of VPg by reticulocyte lysate

Because intact 125I-labelled VPg can be recovered from CPMV RNA incubated in reticulocyte lysate for up to 60 min, we conclude that while attached to virion RNA, CPMV VPg is resistant to proteolysis by any enzymes present in the lysate. To investigate whether the presence of RNA is necessary for this resistance, 125I-labelled VPg linked to either one or 17 nucleotides was produced by digesting 125I-labelled CPMV RNAs with nuclease P1 or ribonuclease T1 respectively. These digests were then incubated in lysates either under conditions which allow translation or in the presence of 20 mM-EDTA (final concentration). Samples were removed at various times and directly analysed in 15% polyacrylamide gels. From Fig. 4 it is clear that 125I-labelled VPg-pU (produced by nuclease P1 digestion) was very rapidly degraded in both the presence and absence of EDTA. Indeed the degradation was so rapid that some radioactivity had disappeared while the zero time sample was being taken from the lysate as soon as possible after mixing. No degraded VPg fragments could be detected, presumably because they had diffused rapidly from the gel during the fixation process. In comparison with the rate of degradation of VPg-pU, loss of radioactivity from VPg linked to 17 nucleotides was considerably slower even in the absence of EDTA. By including EDTA the slow loss of label from VPg-T1 was almost entirely abolished. The difference between the behaviour of the two digests suggests that the presence of as few as 17 bases linked to the VPg is sufficient to provide considerable protection for the protein. The ability of EDTA to reduce the rate of degradation of VPg-T1 but not that of VPg-pU suggests that it may act by inhibiting the action of divalent cation-dependent nucleases in the lysate. Thus, in the absence of EDTA, the RNA portion of VPg-T1 was slowly reduced in size to a point where it could no longer protect the protein. This hypothesis is supported by presence of faster migrating bands in the time zero lane in the −EDTA samples (Fig. 4, lane 1); such bands are present to a much lesser extent when EDTA is present and were not present before VPg-T1 was added to the lysate (data not shown). The disappearance of these bands at longer times in both the presence and absence of EDTA confirms their inability to offer the VPg significant protection. The mode of action by which EDTA protects VPg-T1 was verified by experiments using pCp-labelled VPg-T1 fragments.

Assay of VPg degradation using 3'-labelled VPg-T1 fragments

To investigate further the degradation of the VPg-linked ribonuclease T1 fragments, a preparation of such fragments was specifically 3'-labelled with [5'-32P]pCp. Such material (designated VPg–T1–pCp) was incubated in reticulocyte lysates for various times and the
products were analysed in 15% polyacrylamide gels containing 0.1% SDS and 6 M-urea (Fig. 5). In the absence of EDTA, most of the label was converted to a faster migrating product after 10 min incubation and essentially all the radioactivity had disappeared after 60 min. This conversion and the eventual disappearance of the radioactivity was slowed down markedly in the presence of EDTA, the fast-migrating material only appearing in significant amounts after 60 min incubation. Though we were unable to carry out a detailed analysis of the fast-migrating material, we can rule out the possibility that it was produced by cleavage of VPg from the pCp-labelled RNase T1 fragment or by degradation of the VPg itself. Comparison of its mobility with that of the proteinase K-treated VPg-T1-pCp marker (Fig. 5, lane 8) suggests that it was considerably shorter than 17 nucleotides. It must therefore have arisen by nucleolytic degradation of the RNA portion of VPg-T1-pCp. The appearance of such a fast-migrating product suggests that this degradation starts by cleavage of the RNA near its 3' terminus. Since the rate of this cleavage is sensitive to the presence of EDTA, the nuclease responsible for it is probably divalent cation-dependent.

DISCUSSION

In this paper we have addressed ourselves to two questions. Can the reticulocyte ‘unlinking activity’ which operates on picornavirus RNA also operate on CPMV RNA, and how does attachment to RNA effect the stability of VPg in lysates? The answer to the first question appears to be negative, no ‘unlinking’ of VPg being detectable after incubation in lysates for 60 min. This contrasts with picornavirus RNA where removal is essentially complete after 30 min incubation (Ambros et al., 1978; Dorner et al., 1981; Sanger et al., 1981). The reason for this
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Fig. 5. Autoradiogram of a 15% polyacrylamide gel of pCp-labelled VPg-linked RNase T1 fragment after incubation in reticulocyte lysate. Incubation was carried out in the absence (lanes 1, 2 and 3) or presence (lanes 4, 5 and 6) of 20 mM-EDTA for 0 (lanes 1 and 4), 10 (lanes 2 and 5) and 60 (lanes 3 and 6) min. Lanes 7 and 8 contain samples of untreated and proteinase K-treated pCp-labelled VPg-linked RNase T1 fragment.

difference in behaviour is uncertain, though the likely differences in the nature of the VPg–RNA linkages seems the most plausible explanation. When the experiments involving $^{125}$I-labelled RNA were repeated in a wheat germ translation system, the results were identical, no unlinking activity being observed (data not shown).

The significance of the removal of the VPg from poliovirus and FMDV RNA during incubation in reticulocyte lysates is unknown. It is, however, noteworthy that poliovirus-specific RNA isolated from polyribosomes does not possess the VPg at its 5' end (Hewlett et al., 1976; Nomoto et al., 1976) and an 'unlinking activity' has been found in HeLa cells (Ambros et al., 1978). Why VPg is removed from the polysomal RNA is somewhat mysterious as the presence of VPg does not appear to interfere with ribosome binding (Golini et al., 1980). In the case of CPMV, the structure of the virus-specific polysome RNA is unknown but our results demonstrate that VPg removal is not a prerequisite for translation of the viral RNAs, at least in vitro using either an animal- or a plant-derived cell-free system.

Once 'unlinked' from the RNA, picornavirus VPgs are rapidly degraded by proteinase(s) present in the lysate (Ambros et al., 1978; Dorner et al., 1981; Sangar et al., 1981). In addition, Dorner et al. (1981) showed that poliovirus VPg linked to one or nine nucleotides was also susceptible to proteolysis whereas VPg linked to intact RNA was stable provided the 'unlinking activity' was first inhibited. In a similar set of experiments we have shown that while attached to high molecular weight RNA, CPMV VPg is stable but that if the RNA is first degraded with nuclease P1, the resulting VPg–pU is rapidly proteolysed. However if an RNA 'tail' of as little as 17 nucleotides is left attached to the VPg, the rate of proteolysis is dramatically reduced. Furthermore we have shown that the degradation of this VPg–T1 fragment which does occur
can be ascribed to the action of a divalent cation-dependent nuclease rather than direct proteolysis of the protein component.

The ability of a full-length CPMV RNA to protect the VPg from proteolysis in reticulocyte lysates could simply have been the consequence of a general steric phenomenon, the large RNA molecules simply surrounding the VPg and preventing access to it by proteases. However, the ability of a stretch of RNA as short as 17 nucleotides to offer a similar degree of protection argues that there is a much more specific interaction between the VPg and the 5' end of the RNA. Such an interaction is possibly to be expected in view of the basic (positively charged) nature of the VPg. Our results also suggest that 17 bases may be about the minimum length of RNA required to stabilize the VPg, since 125I-labelled fragments bearing a shorter length of RNA have only a transient existence (see Fig. 4). This conclusion is supported by the inability of a nine base 'tail' to protect the similarly sized and charged poliovirus VPg from proteolysis (Dorner et al., 1981).

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