Cell-free Translation of Cricket Paralysis Virus RNA: Analysis of the Synthesis and Processing of Virus-specified Proteins

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SUMMARY

Translation of cricket paralysis virus (CrPV) RNA in a rabbit reticulocyte lysate yielded proteins which co-migrate on polyacrylamide gels with the structural proteins VP1 and VP3. These proteins were identified by partial proteolysis and a number of high mol. wt. proteins were shown to be precursors of the structural proteins. The third structural protein VP2 was not identified in vitro but was shown to be formed from the minor 'structural' protein VP0. The profile of high mol. wt. proteins in vitro differed from those found in infected cells, but processing was similar with precursors being cleaved sequentially to give a group of proteins with mol. wt. of 50000 to 63000. Processing proceeded in vitro to give VP1 and VP3 but synthesis of VP2 and its immediate precursor VP0 was not apparent. This is consistent with an inhibition of synthesis of VP0 found in infected cells treated with iodoacetamide and suggests that CrPV utilizes a different mechanism for the synthesis of VP0 and VP2 than it does for VP1 and VP3. A tentative model for the processing of CrPV-specified proteins is proposed.

INTRODUCTION

Cricket paralysis virus (CrPV) is an insect virus which has been included as a member of the Picornaviridae on the basis of its physical characteristics (Matthews, 1979). In common with mammalian picornaviruses it has a capsid diam. of 27 to 28 nm, sedimentation coefficient of 165S, a buoyant density in neutral CsCl of 1.34 g/ml and contains single-stranded RNA with a mol. wt. of 2.8 × 10⁶ (Reinganum, 1973; Reinganum et al., 1970). Despite these physical similarities, there are certain aspects of this virus which suggest that it is not a typical member of the Picornaviridae. It has three major capsid proteins with mol. wt. of 30 × 10³ to 35 × 10³ (Moore et al., 1980; Reinganum et al., 1970) which correspond to VP1, VP2 and VP3 in mammalian picornaviruses (Rueckert, 1976) but no capsid protein with a mol. wt. of 6000 to 10000 corresponding to VP4 could be found even in partially purified preparations of the virus (Moore et al., 1980). Study of the virus-induced polypeptides in Drosophila cells demonstrated the appearance of high mol. wt. proteins whose kinetic qualities suggested that they act as precursors to the virus structural proteins. The processing of these precursors differed from that found with mammalian picornaviruses in two respects. Firstly, cleavage of the precursors was extremely rapid and large amounts of the structural proteins were observed after short pulses of radioisotope in the cells. Secondly, the processing was asymmetrical with a rapid production of VP1 and VP3 but a much slower cleavage of VP0 to VP2 (Moore et al., 1980).

We have recently demonstrated that RNA extracted from purified CrPV could direct the synthesis of proteins in an in vitro system and that proteins were formed which co-migrated
with the structural proteins of the virus. However, the high mol. wt. proteins which were produced in vitro differed significantly from those found in infected cells (Reavy & Moore, 1981). We have now extended this initial analysis to study the relationships between the in vitro and in vivo proteins using partial proteolysis to characterize the proteins. Furthermore, we have examined the processing of the proteins in vitro in an effort to define the nature of the cleavages which are taking place.

METHODS

Materials [35S]methionine (1095 Ci/mmol) was purchased from The Radiochemical Centre, Amersham. Staphylococcus aureus V8 protease was obtained from Miles Laboratories, Slough, U.K.

Cells and virus. Drosophila cells (D. melanogaster) (Schneider, 1972) were grown as confluent monolayers at 28 °C in Schneider's Drosophila medium (Gibco-Biocult) supplemented with 10% foetal calf serum (FCS).

75 cm² plastic flasks (Nunc) were used for growth of virus and 25 cm² flasks for labelling of intracellular proteins. Virus was from a stock adapted to growth in tissue culture (Moore et al., 1980) and which had been plaque-purified (N. F. Moore & J. S. K. Pullin, unpublished results).

Radiolabelling of intracellular proteins. 25 cm² confluent monolayers of Drosophila cells were infected with 100 p.f.u./cell of CrPV. Following a 1 h attachment period, inocula were removed and cells overlaid with 2 ml Schneider's Drosophila medium supplemented with 2% FCS (maintenance medium; Moore et al., 1980) for 3 h. Cells were then washed twice with 2 ml methionine-deficient Schneider's Drosophila medium and incubated in 1 ml of this medium for 1 h prior to addition of [35S]methionine. Cells were pulsed with 500 μCi [35S]methionine for 30 min, harvested into 5 ml ice-cold maintenance medium and pelleted at 2000 g for 5 min at 4 °C. Pelleted cells were solubilized in 250 μl solubilization buffer containing 2% SDS, 2% 2-mercaptoethanol, 15% glycerol and 0.001% bromophenol blue in 1:10 concentration electrophoresis buffer, by boiling at 100 °C for 2 min. Alternatively, cells were pulsed for 30 min as above and the pulse was chased by washing the cells with 2 ml maintenance medium plus 15 mg/ml methionine and overlaying the cells with 5 ml of this medium for 3 h. All media contained actinomycin D at a concentration of 5 μg/ml.

Virus purification. Five x 75 cm² monolayers of Drosophila cells were infected with 10 p.f.u./cell of CrPV and following a 1 h attachment period, inocula were removed and the cells were overlaid with 5 ml maintenance medium. Complete cytopathic effect occurred by 48 h and virus was purified from the infected cells by differential centrifugation and CsCl density centrifugation as previously described (Moore et al., 1980).

Purification of virion RNA. RNA was extracted from purified virus by proteinase digestion, phenol extraction and ethanol precipitation as previously described (Reavy & Moore, 1981).

In vitro protein synthesis. Cell-free protein synthesis was performed using a commercial rabbit reticulocyte lysate (The Radiochemical Centre, Amersham) under conditions recommended by the manufacturers and as described in the figure legends.

Polyacrylamide gel electrophoresis. Proteins were analysed by polyacrylamide gel electrophoresis on 15 cm-long slab gels using the discontinuous tris–glycine buffer system of Laemmli (1970), as previously described (Moore et al., 1980). Gels were normally fixed for 1 h in 45% methanol, 10% acetic acid and impregnated with 22% 2,5-diphenyloxazole in dimethyl sulphoxide prior to drying for autoradiography (Laskey & Mills, 1975). Alternatively, if proteins were required for partial proteolysis, the gel was washed in water for 15 min following fixation and dried. Dried gels were exposed to Fuji X-ray film at −70 °C for
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Partial proteolysis. Partial proteolysis of proteins separated on polyacrylamide gels was performed by digestion with *S. aureus* V8 protease essentially by the method of Cleveland et al. (1977) as modified by Gorbalenya et al. (1979). Strips containing the desired proteins were excised from the dried gel and laid longitudinally on a 5% stacking gel overlaid on top of a 17.5% polyacrylamide separating gel. The entire assembly was placed in a tank containing electrophoresis buffer and the strips were allowed to swell for 90 min. Gel strips were then overlaid with 1 ml of a 200 µg/ml solution of *S. aureus* V8 protease in 125 mM-tris-HCl pH 7.2 containing 0.1% SDS, 1 mM-EDTA, 20% glycerol and 0.001% bromophenol blue. Electrophoresis was performed at 120 V until the marker dye reached the stacker/gel interface, whereupon it was stopped for 20 min to allow digestion of the proteins, and the resulting peptides were separated by electrophoresis at 80 V for 16 h.

RESULTS

Translation of CrPV RNA in vitro

RNA extracted from purified CrPV was translated in a rabbit reticulocyte lysate and the proteins specified were separated by polyacrylamide gel electrophoresis (Fig. 1, lane 3). These were compared with the proteins labelled in vivo (Fig. 1, lanes 1 and 2). Proteins were synthesized in vitro that co-migrated with the virus structural proteins VP1 and VP3. A protein which we previously thought could be VP2 (Reavy & Moore, 1981), clearly migrated differently from the VP2 found in cells which had been pulsed and chased. The highest mol. wt. protein synthesized in vitro (protein 1) had a lower mol. wt. than protein A in infected cells, which disappeared upon chasing the initial pulse with excess methionine. The lower mol. wt. of protein 1 was either due to incomplete translation of the genome or possibly extremely rapid processing of any higher mol. wt. proteins. The other high mol. wt. proteins produced in vitro again differed significantly from those found in vivo, the only similarity being a group of proteins (4) which co-migrated with those in region D which, like protein A, disappeared upon chasing. These differences in high mol. wt. proteins were probably due to the combined effects of premature termination and the processing activity which exists in rabbit reticulocyte lysates. No proteins were synthesized in vitro that co-migrated with the minor structural protein VP0. Since VP0 is the immediate precursor to VP2 in mammalian picornaviruses (Rueckert, 1976), and VP2 was absent in vitro, it was possible that the processing pathway giving rise to these proteins did not occur in vitro. There was, however, a group of proteins (5) synthesized in vitro which migrated immediately above VP0 and which could be uncleaved forms of that protein. From the autoradiograph (Fig. 1) it is apparent that at least one of the low mol. wt. proteins (protein 8) synthesized in vitro co-migrated with a protein labelled in infected cells. Densitometer scans of the autoradiograph (Fig. 2a, b) showed that three low mol. wt. proteins (proteins 7, 8 and 9) synthesized in vitro co-migrated with those synthesized in vivo. Of these, protein 6 disappeared in infected cells upon chasing with excess methionine and protein 9 was produced as a result of the chase, showing that they are involved in processing in vivo. The relative amounts of proteins also differed in vitro and in vivo. The major proteins in vivo were the structural proteins VP0, VP1 and VP3 whereas in vitro the major proteins were the high mol. wt. species and VP1 and VP3 were not as prominent (Fig. 2).

Analysis of proteins by partial proteolysis

Comparison of the proteins synthesized in vitro with those in vivo was performed by partial proteolysis (Fig. 3, 4). The proteins synthesized in vitro which co-migrated with VP1
and VP3 had the same peptide profiles as those proteins. The protein which co-migrated with VP1 contained the same three major peptides as VP1 in both Fig. 3 and 4 and similarly the three major peptides produced by VP3 in vitro were found in the in vitro product with which it co-migrated. The minor peptides were not observed in digests of the in vitro proteins probably because the smaller amounts of the proteins made resolution of those peptides very difficult. This procedure clearly showed that VP1 and VP3 were being faithfully translated in vitro. The protein which we previously tentatively identified as VP2 could not be shown to be identical to VP2 in infected cells (Fig. 4). The in vitro protein appeared to contain two peptides in common with two of the major peptides of VP3. This similarity also existed between VP2 and VP3 in vivo but a number of peptides unique to either protein clearly showed that they were different. Proteolysis of VP2 resulted in a larger number of peptides than VP1 or VP3 but none was present in as large quantities as the major peptides of VP1 or VP3. Subsequently, identification of VP2 in vitro was not possible because of the previously mentioned difficulty of resolving minor peptides with the small amounts of in vitro proteins synthesized. It therefore remains to be determined whether the protein we tentatively identified as VP2 previously, is in fact related to this protein or whether it is an uncleaved precursor of VP3.
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Fig. 2. Densitometer scans of the autoradiogram shown in Fig. 1. (a) Comparison of the proteins produced *in vitro* and those labelled in infected cells at 4 to 4.5 h post-infection. (b) Proteins produced *in vitro* and those from infected cells after pulsing for 30 min at 4 h post-infection and chasing the pulse with excess unlabelled methionine. The structural proteins (VP1 and VP3) are indicated by arrows and labelled. The protein previously believed to be VP2 is indicated by an arrow between VP1 and VP3. The high mol. wt. precursors A, B and C are indicated as is the group of intermediate precursors (D).

Fig. 3. Partial proteolysis of the proteins labelled *in vivo* at 4 to 4.5 h post-infection and the proteins specified by *CrPV* *in vitro*. The regions excised from the gel in Fig. 1 are shown and the peptides produced by digestion with *S. aureus* V8 protease are immediately below. The structural proteins VP0, VP1 and VP3 are indicated as are the precursor proteins. T, Top; B, bottom.
Fig. 4. Partial proteolysis of proteins labelled in infected cells after pulsing for 30 min at 4 h post-infection and chasing the pulse with unlabelled methionine for 3 h and the proteins specified by CrPV RNA in vitro. The regions excised from the gel in Fig. 1 are shown and the peptides produced by digestion with S. aureus V8 protease are immediately below. The structural proteins VP0, VP1, VP2 and VP3 are indicated as are the precursors. T, Top; B, bottom.

The high mol. wt. proteins synthesized in vitro (proteins 1, 2, 3, 4) contained the same peptides as those found in both VP1 and VP3 (Fig. 3, 4). In addition, they contained a peptide similar to the major peptide of VP0 but positive identification of VP0 was again difficult due to the large number of minor peptides which comprised its peptide profile. The protein synthesized in vitro which migrated immediately above VP0 (protein 5 in Fig. 1) also contained the major peptide of VP0 and possibly may be an uncleaved precursor of that protein. Furthermore, protein 5 lacked the upper peptide of VP3 and the lower peptide of VP1 and therefore was unlikely to be a precursor to either of these proteins. Thus, it appeared that processing in vivo proceeded as protein 1 → 2 → 3 → 4 and thence diverged to give VP1 and VP3 separate from protein 5. Comparison of the peptide profiles of VP0 and VP2 in vivo (Fig. 4) showed that they had at least five peptides in common identifying VP0 as being the immediate precursor of VP2 as is the case with mammalian picornaviruses. Protein A was related to both proteins B and C and also to at least one of the intermediate proteins in region D (Fig. 3, 4) indicating a cleavage pattern of A → B/C → D in vivo.

Kinetics of proteins synthesized in vitro

The sequence of appearance of proteins in vitro was examined by initiating translation and removing aliquots from the incubation mixture at different times (Fig. 5). A number of low mol. wt. products including VP1 were the first products synthesized, probably as a result of premature termination. The appearance of VP1 can be explained as arising from processing of early termination products. Complete translation of the virus genome did not occur until 30 min after the start of the incubation and no new high mol. wt. proteins were synthesized after that time. Following complete translation of the genome at 30 min some processing of
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Fig. 5. Autoradiogram of a 17.5% polyacrylamide gel showing the proteins specified by CrPV RNA in a rabbit reticulocyte lysate at: 0 min (1), 5 min (2), 10 min (3), 15 min (4), 30 min (5), 60 min (6), 90 min (7) and 120 min (8). Translation was performed in an assay comprising 50 μl lysate, 65 μCi [35S]methionine (6-64 mCi/ml, 1095 Ci/mmol) and 3 μl RNA. Five μl samples were removed at the times indicated, added to 100 μl of solubilization buffer and boiled for 2 min. Thirty μl of each sample were added to the gel which was impregnated with 2,5-diphenyloxazole and exposed to X-ray film for 5 days.

...the proteins was apparent (Fig. 6). There was a decrease in the amounts of the initial precursors and an accumulation of the intermediate precursors including protein 5. This indicated that the immediate precursors were being cleaved to form the intermediate precursors but no substantial accumulation of VP1 and VP3 could be observed. There was, however, an accumulation of the tentative VP2 protein which did not appear until after 60 min and increased slightly in amount by 90 min.

To study the processing of proteins in vitro in the absence of further protein synthesis, a pulse–chase experiment was performed using excess methionine, and ribonuclease T1. The same profile of proteins was synthesized as previously (Fig. 7) with the exception of a band co-migrating with VP0 which appeared 24 h after the initial pulse (Fig. 7, graph 5). The relative intensities of the bands indicated the processing of the proteins synthesized during the initial pulse. VP1 and VP3 started as minor bands (Fig. 7, graph 1) but had increased in amount by 15 min after the pulse (Fig. 7, graph 2) and by 2 h post-pulse had become major protein species (Fig. 7, graph 3). This coincided with a decrease in the amounts of precursor proteins 1 and 2, an increase in the protein 3 region and the appearance of a new protein (51) above protein 5. Therefore, it appeared that proteins 1 and 2 were cleaved to give VP1 and VP3, and also protein 5, via the intermediate precursors in region 3. Increasing the length of chase led to a further decrease in the protein 2 region with the higher mol. wt. species being processed first until by 6 h only a single lower mol. wt. protein remained in that region (Fig. 7, graph 4) and eventually that was also processed (Fig. 7, graph 5). The proteins in regions 4 and 5 appeared to be cleaved extremely slowly but by 24 h there was a significant decrease in the proteins of region 4. Furthermore, all the proteins in region 5 along with the upper band
Fig. 6. Densitometer scan of lanes 3 (1), 4 (2), 5 (3), 6 (4) and 7 (5) in Fig. 5 showing synthesis of proteins specified by CrPV RNA *in vitro*. Scans of lanes 1 and 2 are not shown. Structural proteins VP1 and VP3 are indicated by arrows, as is the position of VP2.

Fig. 7. Densitometer scan of the autoradiogram of a 12.5% polyacrylamide gel showing the processing of CrPV-specified proteins *in vitro*. Translation was initiated in an assay comprising 40 μl lysate, 40 μCi [35S]methionine (5.27 mCi/ml, 1000 Ci/mmol) and 2 μl RNA. After 30 min 5 μl of a chase solution containing 20 mM-methionine, 1.3 mM-magnesium acetate, 80 mM-potassium acetate and 1000 units/ml ribonuclease T1 from *Aspergillus oryzae* (Boehringer, Mannheim) was added to give final concentrations of 2 mM-methionine and 100 units/ml ribonuclease T1. Five μl samples were removed into 100 μl of solubilization buffer at 0 min (1), 15 min (2), 120 min (3), 360 min (4) and 24 h (5) after the pulse. Thirty μl of each sample were added to the gel and electrophoresed as described in the text. The gel was impregnated with 2,5-diphenyloxazole prior to drying and was exposed to X-ray film for 4 days.

(protein S1) which appeared after 2 h (Fig. 7, graph 3) had been processed. Concomitant with this was the appearance of a protein which co-migrated with VP0 and another protein (S2) which migrated slightly higher (Fig. 7, graph 5). Protein S2 appeared to be synthesized very slowly and appeared after 6 h (Fig. 7, graph 4). It appeared therefore that proteins in region 5, including proteins S1 and S2 and perhaps proteins in region 4, were cleaved very slowly to give a protein which co-migrated with VP0.

**DISCUSSION**

CrPV RNA directed the synthesis *in vitro* of the structural proteins VP1 and VP3 but with the exception of proteins co-migrating with those found in region D of intracellular proteins, the high mol. wt. proteins differed significantly from those found in infected cells. This is not entirely unexpected as *in vitro* translation of poliovirus and encephalomyocarditis (EMC)
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In vitro

\begin{align*}
\text{A} & \quad \text{B/C} \\
\text{DDD} & \\
\text{VP0} & \quad \text{VP2} \quad \text{VP1} \quad \text{VP3}
\end{align*}

In vivo

\begin{align*}
\text{A} & \quad \text{B/C} \\
\text{DDD} & \\
\text{VP0} & \quad \text{VP1} \quad \text{VP3}
\end{align*}

Fig. 8. Preliminary proposed pathway for processing of CrPV-specified proteins.

virus RNAs both give rise to high mol. wt. proteins not observed in infected cells but with the properties of precursor proteins. They probably arise as a result of premature termination and the processing activity in vitro (Shih et al., 1978, 1979; Brown & Ehrenfeld, 1979). It must be borne in mind that processing conditions in rabbit reticulocyte lysates probably differ significantly from those in Drosophila cells. Evidence has accumulated that processing of EMC virus-specified proteins involves a virus-specified protease and that the protease activity resides in a protein derived from a virus-coded component of the virus RNA polymerase (Palmenberg et al., 1979; Gorbalenya et al., 1979; Svitkin et al., 1979; Shih et al., 1979). It will therefore be of interest to convert D. melanogaster cells into a cell-free translation system to determine if processing is different from that in rabbit reticulocyte lysates and also to determine whether CrPV codes for its own protease.

Despite differences between high mol. wt. proteins in vitro and in vivo, it was apparent that those formed in vitro still acted as precursors to the structural proteins as shown by partial proteolysis and pulse-chase data. Processing of the precursors in vitro led to the formation of VP1 and VP3 but there appeared to be a blockage in the processing of VP0 and VP2. It seems likely that processing of VP0 and VP2 was inhibited at the stage of proteins found in region 4, which could be chased over a long time to give a protein which co-migrated with VP0 and also protein 5. We propose that VP0 is cleaved from one or more proteins in region 4 via protein 5 which is not observed in infected cells due to its rapid processing and we outline a tentative processing pathway in Fig. 8. The proteins in region 4 in vitro correspond to region D in vivo and inhibition of VP0 formation, similar to that found in vitro, has been observed upon treatment of infected cells with iodoacetamide prior to pulsing. A large decrease in VP0 could be observed as a result of this treatment together with the formation of a new protein in region D suggesting that cleavage of a precursor of VP0 in region D was being inhibited (N. F. Moore et al., unpublished results). We suggest that it is that precursor in region D or a related protein which is being cleaved very inefficiently in reticulocyte lysates. It would therefore appear that CrPV utilizes a different mechanism of processing for VP0 and VP2 as it does for VP1 and VP3 and this is supported by the asymmetrical cleavage in infected cells (Moore et al., 1980). The inhibition of synthesis of VP0 and VP2 in vitro suggests that processing of these proteins is possibly performed by host cell proteases and that proteases in rabbit reticulocyte lysates perform the processing of VP0 and VP2 much less efficiently. Synthesis of VP1 and VP3 could be explained as processing by a virus-coded protease. More detailed peptide mapping is required, however, to identify VP2 and its precursors especially in region D which contains a number of proteins before a complete pathway for the processing of CrPV-specified polypeptides can be determined.
Picornaviruses exploit a number of different translational strategies. Poliovirus requires complete assembly of virions as a prerequisite for cleavage of VP0 to VP2 which does not take place in vitro (Shih et al., 1978). Poliovirus can also utilize two different initiation sites on the genome to give a low mol. wt. protein or a high mol. wt. protein which has been identified as the capsid protein precursor (Celma & Ehrenfeld, 1975; Brown & Ehrenfeld, 1979; Knaurert & Ehrenfeld, 1979; Humphries et al., 1979). Translation of EMC virus RNA gives all the structural proteins but there is a delay in elongation which allows the accumulation of capsid protein precursors before the synthesis of a protease which processes them (Shih et al., 1979). CrPV shares with picornaviruses the common feature of having its structural proteins cleaved from high mol. wt. precursors but it remains to be seen how unique the separate processing of VP0 and VP1: VP3 is and what function it serves.

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REFERENCES


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