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FURTHER CHARACTERISATION OF THE CORONAVIRUS IBV ORF 1a PRODUCTS ENCODED BY THE 3C-LIKE PROTEINASE DOMAIN AND THE FLANKING REGIONS

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1. ABSTRACT

Coronavirus IBV encodes a piconarvirus 3C-like proteinase. In a previous report, this proteinase was shown to undergo rapid degradation *in vitro* in reticulocyte lysate due to a posttranslational event involving ubiquitination of the protein. Several lines of evidence presented here indicate that the proteinase itself is stable. Translation of the IBV sequence from nucleotide 8864 to 9787 resulted in the synthesis of a 33 kDa protein, representing the full-length 3C-like proteinase. Pulse-chase and time-course experiments showed that this protein was stable in reticulocyte lysate for up to 2 hours. However, a 45 kDa protein encoded by the IBV sequence from nucleotide 8693 to 9911 underwent rapid degradation in reticulocyte lysate, but was stable in wheat germ extract, suggesting that an ATP-dependent protein degradation pathway may be involved in the turnover of the 45 kDa protein. To identify the IBV sequence responsible for the instability of this 45 kDa protein species, the region from nucleotide 8693 to 9787 was translated both *in vitro* and *in vivo* , leading to the synthesis of a stable 43 kDa protein. These results suggest that a destabilising signal may be located in the IBV sequences between the nucleotides 9787 and 9911. Meanwhile, protein aggregation was observed when the product encoded by the IBV sequence from nucleotide 9911 to 10510 was boiled for 5 minutes before being analysed in SDS-PAGE; when the same product was treated at 37°C for 15 minutes, however, protein aggregation was not detected. Deletion studies indicate that the presence of a hydrophobic domain downstream of the 3C-like proteinase-encoding region may be the cause for the aggregation of the product encoded by this region of ORF 1a.

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2. INTRODUCTION

The 5'-unique region of IBV mRNA1 comprises two large ORFs (1a and 1b), overlapping by 42 nucleotides. ORF 1a potentially encodes a polypeptide of 441 kDa and ORF 1b potentially encodes a polypeptide of 300 kDa (Boursnell *et al.*, 1987). The downstream ORF 1b is produced as a fusion protein of 741 kDa with 1a by a mechanism involving ribosomal frame shifting (Brierley *et al.*, 1987). The 1a/1b fusion polyprotein is expected to be cleaved by viral or cellular proteinases to produce functional products associated with viral RNA replication. Three proteinase domains have been predicted to be encoded by ORF 1a, including two overlapping papain-like proteinase domains, and a trypsin-like proteinase domain of the picornavirus 3C proteinase group (Gorbalenya *et al.*, 1989). The papain-like proteinase domains are involved in proteolytic processing of the 1a polyprotein to an 87 kDa viral product (Liu *et al.*, 1995). More important role in the processing and maturation of the ORF 1 proteins is likely to be played by the 3C-like proteinase domain. In fact, processing of the 1a / 1b polyprotein to a 10 kDa and a 100 kDa protein species has been reported (Liu *et al.*, 1994, 1997). Meanwhile, internal deletion and substitution mutation studies have shown that the predicted nucleophilic cysteine residue (Cys^{2922}) and the histidine 2820 residue are essential for the activity of this proteinase, confirming that this proteinase belongs to the picornavirus 3C proteinase supergroup (Liu and Brown, 1995).

Recently, Tibbles *et al.* have reported that the 3C-like proteinase is subjected to rapid turnover when expressed in rabbit reticulocyte lysate due to a posttranslational event involving ubiquitination of the protein and degradation by an ATP-dependent system (Tibbles *et al.*, 1995). In this paper, we present experiments designed to verify the stability of the 3C-like proteinase. Data presented demonstrated that the 3C-like proteinase itself is stable both in reticulocyte lysate and in intact cells. A destabilising signal may be located downstream of the region encoding the 3C-like proteinase. In addition, deletion studies have confirmed that the extremely hydrophobic nature of the products encoded by the IBV sequence from nucleotide 10375 to 10450 causes protein aggregation.

3. MATERIALS AND METHODS

3.1. Transient Expression of the IBV Sequences in Vero Cells by Using a Vaccinia Virus-T7 Expression System

ORFs placed under control of the T7 promoter were expressed transiently in eukaryotic cells as described in Fuerst *et al.*, 1986. Semiconfluent monolayers of Vero cells were infected with 10 pfu per cell of a recombinant vaccinia virus (vTF7–3) which expresses the T7 phage RNA polymerase and then transfected with appropriate plasmid DNA with Dosper (Boehringer Mannheim) according to manufacturer's instructions. After incubation of the cells at 37°C overnight (12 hours), the cells were incubated in methionine-free medium for 30 min prior to being labelled. After 4 hours of labelling with [^{35}S]methionine, the cells were lysed with radioimmunoprecipitation assay buffer (50 mM Tris-HCl [pH7.5], 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulphate [SDS]) and removed from the dishes. The cells were then precleared by centrifugation at 15,000 rpm for 5 min at 4°C and stored at -70°C.

3.2. Radioimmunoprecipitation

Radioimmunoprecipitation with polyclonal rabbit antisera was carried out as described previously (Liu *et al.*, 1994).

3.3. Polymerase Chain Reaction (PCR)

Appropriate primers and template DNAs were used in amplification reactions with *Pfu* DNA polymerase (Stratagene) under standard buffer conditions with 2mM $MgCl_2$.

3.4. Cell-Free Transcription and Translation

Plasmid DNA (5μg) was linearised at an appropriate restriction site downstream of the IBV sequence (unless stated otherwise) before they were transcribed and translated using the TNT transcription coupled translation system (Promega). Alternatively, linearised plasmid DNA was transcribed *in vitro* with T7 phage RNA polymerase as described before (Liu *et* al., 1994), with the dinucleotide ^{7m}GpppG incorporated to provide a 5' cap structure (Contreras, 1982). Product mRNA was recovered from the reaction mixtures by extraction with phenol-chloroform (1:1) and precipitation with ethanol before the RNA was translated in either the rabbit reticulocyte lysate system (Promega) or the wheat germ extract system (Promega) in the presence of [^{35}S]methionine as described previously (Liu *et al.*, 1994). Translation reactions were carried out at 30°C, typically for 90 min (unless stated otherwise), and were stopped by incubation with equal volume of translation stop buffer (1 mg/ml Rnase A, and 0.5M EDTA) for 20 min at room temperature. During pulse-chase experiments, excess cold methionine (5mM) was added at the times indicated for the individual experiments. Reaction products were separated by SDS-PAGE and detected by autoradioagraphy.

3.5. SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of virus polypeptides was carried out using 10% and 12.5% polyacylamide gels (Laemmli, 1970). Labelled polypeptides were detected by autoradiography or fluorography of dried gels.

3.6. Construction of Plasmids

Plasmids pIBV4, pIBV6, pIBV9 (formerly called pKT205), pIBV10, pIBV11 and pIBV3C, which cover the IBV sequences from nucleotides 10752 to 13896, 10510 to 12700, 8693 to 10925, 11952 to 13896, 13896 to 16980, and 8864 to 9787 respectively, were described before (Liu *et al.*, 1994, 1997).

Plasmid pBV9Æ2, which contains the IBV sequence from nucleotide 8693 to 9787 was made by cloning a *Bgl* II/*Spe* I restriction fragment covering the IBV sequence from nucleotide 8693 to 9608, into *Bgl* II/*Spe* I digested pIBV3C. *Spe* I cuts the IBV sequence at nucleotide 9608 and the *Bgl* II site is located between the T7 promoter and the IBV sequence.

Plasmid pIBV9Q^{2779}-E covering the IBV sequence from nucleotide 8693 to 10637 and containing a mutation of the Gln^{2779} residue to a Glu was created by PCR with pIBV9 as the template. The sequence of the mutation primer is 5'-GTTAGTAGATTA-GAGTCTGGTTTTAAG-3', and the sequence of the complementary primer is 5'-GTCACCACCAATTCCCTCTATAAGTAT-3'.

Plasmid pCAT-205, which covers the IBV sequence from nucleotides 9911 to 10925 tagged downstream to a gene coding for the bacterial chloramphenicol acetyltransferase (CAT), was made by cloning a 516 bp *Bam* HI/*Sca* I digested CAT fragment into *Bgl* II/Pvu II digested pIBV9.

4. RESULTS

4.1. *In Vitro* Expression of IBV Sequence Containing the 3C-Like Proteinase Domain and the Flanking Regions

We have previously reported that translation of the IBV sequence containing the 3C-like proteinase domain and the surrounding regions in an *in vitro* expression system led to the poor expression of the viral proteins (Liu *et al.*, 1994). To extend this study, four plasmids, pIBV9, pIBV6, pIBV10 and pIBV11, containing the IBV sequences from nucleotide 8693 to 10925, 8693 to 12700, 8693 to 13896 and 8693 to 16980 respectively, together with pIBV4 (10752 to 13896) were translated *in vitro* by using the TNT transcription cou-

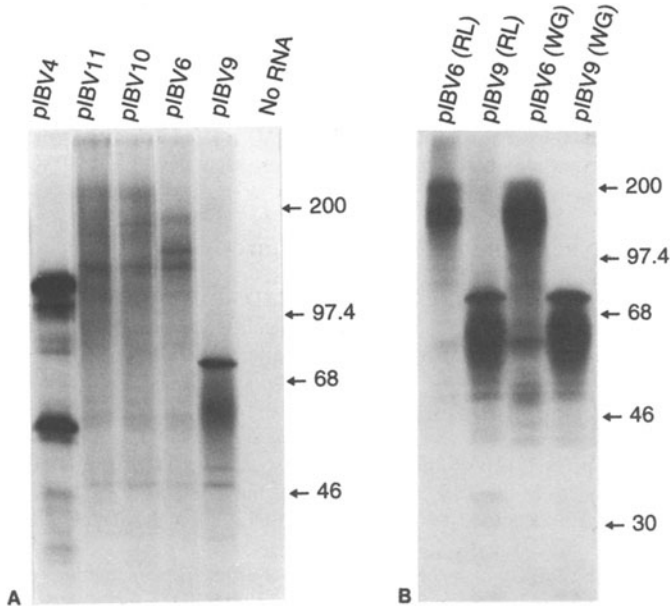

Figure 1. (A). Analysis of *in vitro* translation products produced from plasmids pIBV9, pIBV6, pIBV10, pIBV11, and pIBV4 in reticulocyte lysate by using the TNT coupled translation system (Promega). Plasmid DNA was added to reticulocyte lysate at 200 μg/ml, and the ^{35}S-labelled products were separated on an SDS-12.5%-polyacrylamide gel and detected by fluorography. (B). Comparison of *in vitro* translation products produced from plasmids pIBV9 and pIBV6 in both reticulocyte lysate and wheat germ extract systems (Promega). Capped transcripts were generated from the mentioned plasmids and then translated in either reticulocyte lysate or wheat germ extract. Translation reactions were stopped after 60 min incubation by the addition of translation stop buffer. The samples were analysed on an SDS-12.5%-polyacrylamide gel.

pled translation system (Promega). As shown in Figure 1a, a full-length product of 82 kDa from translation of pIBV9 was observed; transcription and translation of pIBV6, pIBV10, and pIBV11 led to the synthesis of a 138 kDa protein, representing the ORF1a stop product. Two proteins of approximately 150 and 195 kDa, representing 1a-1b fusion products, were also detected from the expression of pIBV6 and pIBV10, respectively (Figure 1a). No full-length product, however, was seen from the translation of pIBV11(Figure 1a). A heterogenous smear of polypeptides covering a large size range was also seen from expression of plasmids pIBV6, pIBV10 and pIBV11(Figure 1a), indicating that this region may contain elements that affect the expression of downstream sequence or accumulation of the gene products.

To determine if the poor expression of pIBV6 and pIBV9 was due to the instability of the translated products in reticulocyte lysate system, plasmids pIBV6 and pIBV9 were expressed in wheat germ extract. As can be seen in Figure 1b, transcription and translation of pIBV6 and pIBV9 led to the synthesis of full-length proteins of approximately 138 kDa and 82 kDa, respectively. No significant differences in the translational pattern of the two constructs were observed from wheat germ extract, although more efficient translation of both plasmids was seen in this system (Figure 1b). Once again, translation of both plasmids showed the formation of the heterogenous high molecular mass species. These results indicate that products expressed from reticulocyte lysate may be unstable, and tend to aggregate.

4.2. Products Encoded by the IBV Sequences Flanking the 3C-Like Proteinase Domain Are Unstable

The data presented suggest that proteins expressed from plasmid pIBV6 and derivatives may be unstable in reticulocyte lysate. To investigate this further, pIBV6 was linearised separately with two restriction enzymes, *Hinc* II and *Sma* I, which digest the IBV sequences at nucleotide positions 10438 and 12677, respectively, and analysed by a pulse-chase experiment. Aliquots were taken from the translation reaction mixture after incubation for 0, 5, 10, 15, 20, 30, 40, 50, and 60 minutes, and the polypeptides were analysed by 12.5% SDS-PAGE. As shown in Figure 2a, translation of *Sma* I digested pIBV6 showed that no product of the predicted full-length of 160 kDa was detected. Instead, premature termination products of approximately 48 kDa and 65 kDa were observed. The products were seen to degrade rapidly and convert into high molecular mass forms, becoming trapped at the top of the gel. Translation of *Hinc* II digested pIBV6 led to the detection of the full-length product of 67 kDa; however, once again accumulation of high molecular mass protein species and decreasing of the 67 kDa protein species were seen over the 60 minute time course (Figure 2b). These results indicate that some regions flanking the 3C-like proteinase may contain destabilising sequences, which promote rapid protein degradation.

To define further the destabilising signals-containing region, plasmid pIBV9 was linearised with restriction enzyme *Pvu* II which cuts the IBV sequence at nucleotide position 9911, and analysed by pulse-chase experiment in both reticulocyte lysate and wheat germ extract systems. As presented in Figure 3, the full-length protein of 45 kDa was detected after 10 min chase; it then underwent rapid degradation in reticulocyte lysate. Interestingly, the protein was stable in wheat germ extract over the time course, suggesting that an ATP-dependent proteolytic system present in reticulocyte lysate may be responsible for the rapid degradation of this protein. These results indicate that a protein destabilising signal may be located in the IBV sequence between nucleotides 8693 and 9911.

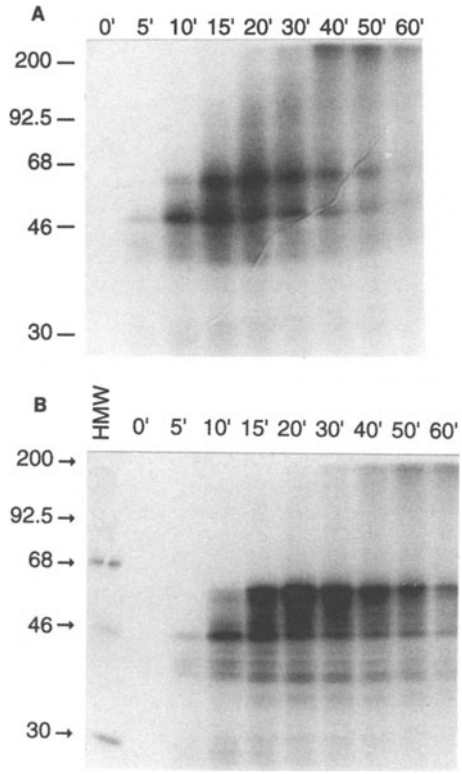

Figure 2. (A). Pulse-chase analysis of the accumulation of [^{35}S]methionine-labelled products from *Sma* I digested pIBV6. 50µl reaction was performed and 10-fold excess of unlabelled methionine was added to the reaction after 10 min incubation at 30°C. Aliquots were taken at intervals (min) as indicated. Polypeptide products were separated on an SDS-12.5%-polyacrylamide gel and detected by fluorography. (B). Pulse-chase experiment of *Hinc* II digested pIBV6. Reaction was performed as described above and polypeptide products were separated on an SDS-12.5%-polyacrylamide gel and detected by fluorography.

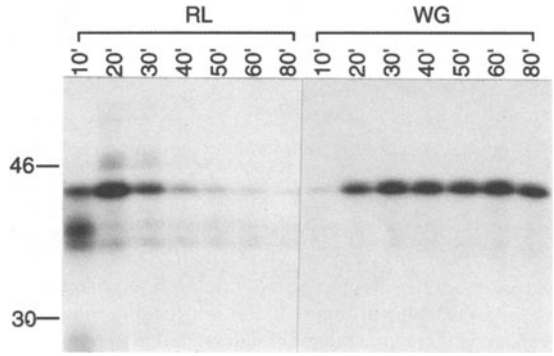

Figure 3. Pulse-chase analysis of cell-free translation products of mRNA obtained by *in vitro* transcription of *Pvu* II digested pIBV9. RNA was added to reticulocyte lysate and wheat germ extract as indicated. Samples were taken from the translation reaction mixtures after incubation for 10, 20, 30, 40, 50, 60, and 80 min, and separated on an SDS-12.5%-polyacrylamide gel and detected by fluorography.

4.3. Stable Expression of the 3C-Like Proteinase *in Vitro* and *in Vivo*

To define further the destabilising signal, pIBV9Δ2, which covers the IBV sequence from 8693 to 9787, was cloned and analysed by *in vitro* pulse-chase assay. As shown in Figure 4a, a full-length protein of 43 kDa was detected after 10 min incubation, and was seen to remain stable till the end of the pulse-chase experiment. The stability of the 43 kDa protein was further studied by *in vivo* expression of plasmid pIBV9Q^{2779}-E, which has

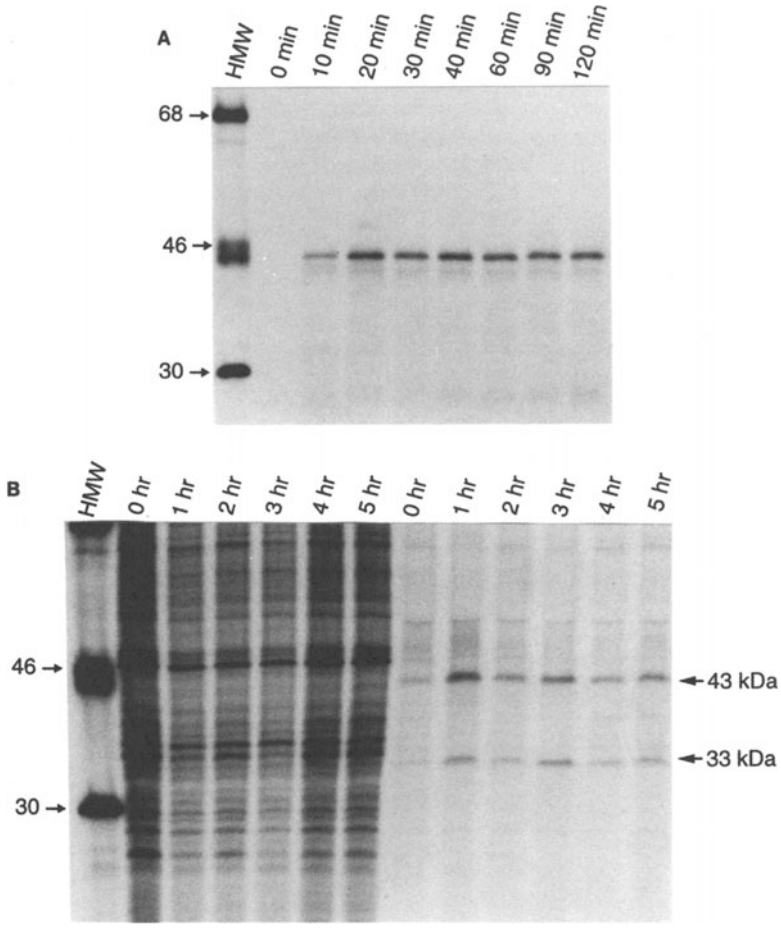

Figure 4. (A). *In vitro* pulse-chase analysis of pIBV9Æ2. Aliquots were removed at intervals as indicated, and polypeptide products were separated on an SDS-12.5%-polyacrylamide gel and detected by fluorography. (B). *In vivo* expression and stability analysis of pIBV9 ^{Q2779}-E in Vero cells. At 13 hours postinfection, infected cells were incubated in methionine-free medium for 30 min prior to being labelled with [^{35}S]methionine for 4 hours. 10-fold excess unlabelled methionine was added and cells were harvested at intervals (hour) as indicated. Total cell lysates were immunoprecipitated with anti-3C antiserum, and separated on an SDS-12.5%-polyacrylamide gel.

the $Q^{2779}S$ site mutated to ES. As expected, the 43 kDa protein was detected and seen to remained stable 4 hours after addition of 10-fold excess of cold methionine. These results demonstrate that product encoded by the IBV sequence from nucleotide 8693 to 9787 is stable. It is therefore suggested that the 3C-like proteinase, predicted to be encoded by the IBV sequence from nucleotide 8864 to 9787 should be stably expressed (Figure 4b).

To support this conclusion further, plasmid pIBV3C, which contains the sequence encoding the 3C-like proteinase, was analysed by pulse-chase experiment. As shown in Figure 5a, a 33 kDa protein representing the full-length product encoded by this construct, was observed after incubation for 10 min and remained stable in the reticulocyte lysate up to 120 min after adding 10-fold excess of cold-methionine in the reaction mixture.

The stability of the 3C-like proteinase was finally assessed by expression of pIBV9 *in vivo*. Expression of pIBV9 *in vitro* showed the synthesis of a product of 82 kDa (Figure 1b and 1c). *In vivo* expression of this plasmid, however, showed processing of full-length protein to smaller products. As shown in Figure 5b, instead of the 82 kDa protein, a product with an apparent molecular weight of approximately 33 kDa was immunoprecipitated by anti-3C antiserum. This 33 kDa protein co-migrated with the 33 kDa expressed from

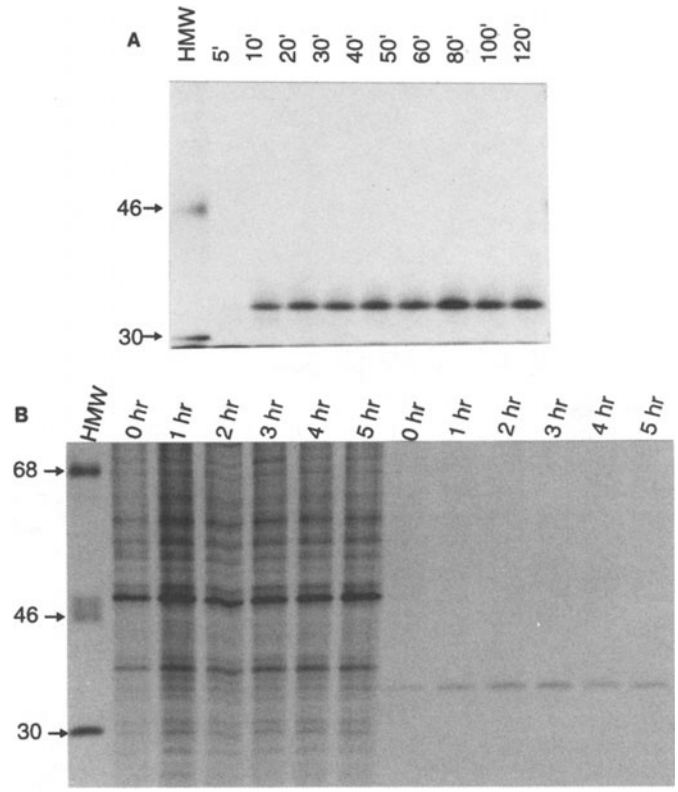

Figure 5. (A). *In vitro* pulse-chase analysis of pIBV3C. (B). *In vivo* expression, processing and stability analysis of pIBV9 in Vero cells.

pIBV3C and was shown to be released from the 1a polyprotein by autocleavage at two QS dipeptide bonds ($Q^{2779}S$ and $Q^{3086}S$) (Ng and Liu, unpublised observations), suggesting that it is the protein associated with the 3C-like proteinase activity. Time-course and pulse-chase experiments showed that this protein remained stable after chasing with 10-fold excess of cold-methionine up to 5 hours (Figure 5b).

4.4. Products Encoded by the IBV Sequence from 10438 to 10900 Tend to Aggregate

During the expression of plasmid pIBV6 and its derivatives, heterogenous high molecular mass protein species were consistently observed when the IBV sequence from 10438 to 10900 were included (Figure 2 and unpublished data). Examination of the deduced amino acid sequence showed that this region encoded a stretch of 39 hydrophobic amino acid residues. To investigate the possibility that the formation of the heterogenous smear of high molecular mass protein species is due to protein aggregation caused by the hydrophobic amino acid sequences, the IBV sequence from nucleotide 9911 to 10925 was tagged to the C-terminal end of the CAT gene, giving plasmid pCAT-205. Plasmid pCAT-205 was then linearised with *Hinc* II, *Sau* I, *Hin*d III, and *Bam* HI, which cut the IBV sequences at 10438, 10510, 10721 and 10925 respectively, and transcribed and translated. The polypeptides were treated separately with two different temperatures, at 100°C for 4 minutes, and at 37°C for 15 minutes before subjected to SDS-PAGE analysis. Data presented in Figure 6 showed that products translated from *Hin*d III and *Bam* HI digested

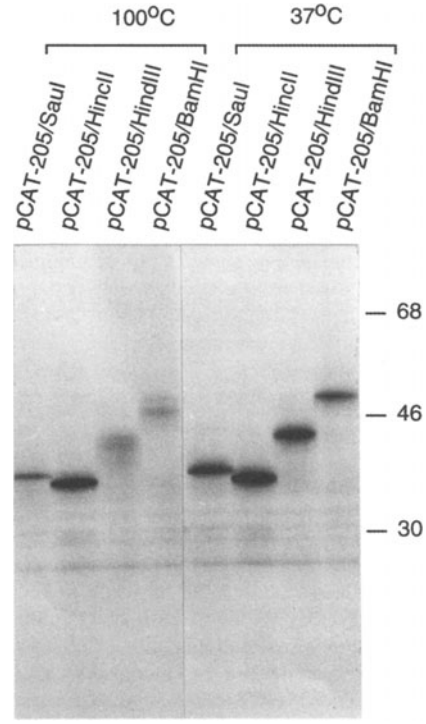

Figure 6. C-terminal deletion analysis of translation products of pCAT-205. Plasmid pCAT-205 was linearised at the restriction sites described in the text and transcribed *in vitro* with T7 RNA polymerase. Translation was performed in the presence of [^{35}S]methionine in reticulocyte lysate, and labelled products were analysed on an SDS-10%-polyacrylamide gel after treatment either at 37°C for 15 min or at 100°C for 4 min.

pCAT-205 tended to aggregate after treatment at 100°C for 4 min; no aggregation was observed after the samples were treated at 37°C. However, protein aggregation was not observed when products translated from *Sau* I and *Hinc* II digested pCAT-205 were analysed either after treatment at 37°C for 15 min or at 100°C for 4 min. These results confirm that the hydrophobic amino acid stretch encoded by the IBV sequence from 10375 to 10450 caused the protein aggregation at 100°C.

5. DISCUSSION

Coronavirus gene expression involves proteolytic processing of the mRNA 1-encoding polyproteins by viral and cellular proteinases. Recent reports have demonstrated that an ORF 1a-encoding proteinase domain of the picornavirus 3C proteinase group is responsible for proteolytic processing of the IBV 1a/1b fusion polyprotein to a 100 kDa and a 10 kDa protein species (Liu *et al.*, 1994 ,1997). It is likely that this proteinase may play a major role in processing of the 1a/ 1b polyprotein during the viral replication cycle. In an attempt to characterise this proteinase, Tibbles *et al.* observed that products encoded by the 3C-like proteinase domain and the flanking regions were unstable. They suggested that the 3C-like proteinase domain may be a target for the ubiquitin-mediated ATP-dependent degradation present in the reticulocyte lysate (Tibbles *et al.*, 1995). In this report, systematic analysis of the stability of the 3C-like proteinase was carried out using constructs covering the 3C-like proteinase domain and the surrounding regions. It was observed that *in vitro* transcription and translation of plasmids covering both upstream and downstream regions of the 3C-like proteinase were poorly expressed in reticulocyte lysate system. Products encoded by this region of ORF 1a tend to be aggregated to form heterogenous smear of high molecualr mass protein species. Construction and expression of a series of deletion constructs confirmed that a 33 kDa protein, representing the 3C-like proteinase is stably expressed in reticulocyte lysate and in Vero cells. However, the IBV sequence between 9787 to 9911 may code for a destabilising signal promoting rapid protein degradation. The protein aggregation may be caused by the presence of a stretch of 39 hydrophobic amino acid residues encoded by the IBV sequence from 10375 to 10450.

Evidence presented in this communication shows that the 33 kDa protein expressed and processed from pIBV9 as well as expressed from pIBV3C is stable both in reticulocyte lysate and in intact cells. As the 33 kDa protein is actually the 3C-like proteinase, this study suggested that the level of the 3C-like proteinase may be stably maintained during, at least, the early stage of the viral RNA replication cycle. This is understandable as the 3C-like proteinase is a major proteinase responsible for cleaving the polyprotein encoded by ORF 1b. Considering that the physical distance between the 3C-like proteinase-encoding region and the last two scissile bonds encoded by ORF 1b is approximately 10 kb, it is reasonable to assume that stable maintenance of the proteinase is a prerequisite for efficient cleavage of the target product at these positions. Kinetic study of the synthesis and accumulation of the 3C-like proteinase in virus-infected cells is underway to address this issue further.

The observation that products encoded by the IBV sequence between nucleotides 10375 and 10450 tend to form aggregation highlights a classical difficulty in analysis of the extremely hydrophobic proteins. As attempts to raise antisera against proteins encoded by this region of ORF 1a failed, we are currently lacking tools to extend our study on this region to intact cells.

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