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Identification, Expression, and Processing of an 87-kDa Polypeptide Encoded by ORF 1a of the Coronavirus Infectious Bronchitis Virus

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Nucleotide sequence analysis has shown previously that the genomic-length mRNA (mRNA1) of the coronavirus infectious bronchitis virus (IBV) contains two large open reading frames (ORFs), 1a and 1b, with the potential to encode polyproteins of approximately 441 and 300 kDa, respectively. We have characterized the specificity of a set of region-specific antisera raised against the 5′-portion of ORF 1a by immunoprecipitation of *in vitro*-synthesized, C-terminally truncated 1a polypeptides and used these antisera to detect virus-specific proteins in IBV-infected Vero cells. Two antisera, which had specificity for IBV sequences from nucleotides 710 to 2079 and 1355 to 2433, respectively, immunoprecipitated a polypeptide of approximately 87 kDa from IBV-infected Vero cells. *In vitro* translation of ORF 1a sequence terminating at nucleotide 5763 did not produce this protein unless the *in vitro* translation products were incubated with Vero cell S10 extracts prepared from either IBV-infected or mock-infected Vero cells. However, processing of the 87-kDa protein was also observed when the same region was expressed in Vero cells using the vaccinia virus/T7 expression system. This observation indicates that the 87-kDa polypeptide is encoded within the 5′-most 3000 nucleotides of mRNA 1 and that it might be cleaved from the 1a polyprotein by viral and cellular proteinases. © 1995 Academic Press, Inc.

INTRODUCTION

Avian infectious bronchitis virus (IBV) is the type species of the Coronaviridae, a family of viruses with a large, positive-stranded, monopartite RNA genome. The genomic RNA of these viruses is capped and polyadenylated and is infectious (Schochetmann *et al.*, 1977; Lomniczi, 1977), indicating that it can function directly in the infected cell as an RNA-dependent RNA polymerase-encoding messenger RNA. Determination of the complete nucleotide sequence of IBV genomic RNA has shown it to be 27.6 kb in length, with at least 10 distinct open reading frames (ORFs) (Boursnell *et al.*, 1987). However, it appears that most of these are not translated from genomic RNA, but are expressed from a set of subgenomic mRNAs. The available evidence indicates that, in virus-infected cells, five subgenomic mRNA species are produced. These mRNAs (designated 2 to 6), together with the genome-length mRNA (designated mRNA 1) (Cavanagh *et al.*, 1990), range in length from about 2 to 27 kb and have been shown to share a common 3′-terminus and to form a nested set structure (Stern and Kennedy, 1980a,b). Three of these, mRNAs 2, 4, and 6, have been shown to encode the major virion structural proteins, and in each case the expressed ORF is the only one which is not present in the next smallest mRNA in the nested set (Stern and Sefton, 1984). Two of the other mRNAs, mRNA 3 and mRNA 5, which contain more than one ORF in their 5′-unique regions, have recently been shown to encode three and two viral proteins, respectively (Smith *et al.*, 1990; Liu *et al.*, 1991; Liu and Inglis, 1992a).

Nucleotide sequence analysis of the genomic RNA has shown that the 5′-terminal sequence of mRNA 1 contains two large ORFs, 1a and 1b, which have the potential to encode two polypeptides of molecular weights 441 and 300 kDa, respectively (Boursnell *et al.*, 1987). *In vitro* transcription and translation studies have demonstrated that 1b may be produced as a fusion protein with 1a as a result of a ribosomal frameshift, leading to synthesis of a polyprotein of 740 kDa (Brierley *et al.*, 1987, 1989). This polyprotein is expected to be cleaved by viral or cellular proteinases to produce functional products associated with virus replication. Several putative functional domains containing well-characterized motifs and more complex homologies have been identified in the 1a or 1b regions by computer-aided techniques (Gorbalenya *et al.*, 1989; Lee *et al.*, 1991; Herold *et al.*, 1993). They include proteinase domains and viral RNA replication-related motifs commonly found in positive-strand RNA virus genomes (i.e., RNA-dependent RNA polymerase and RNA helicase motifs). For example, a papain-like domain and a picornavirus 3C-like proteinase domain were predicted to be located in IBV 1a (Gorbalenya *et al.*, 1989). However, in mouse hepatitis virus (MHV) and

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human coronavirus 229E, two papain-like proteinase domains were found to be located in the ORF 1a regions of the genomes (Lee *et al.*, 1991; Herold *et al.*, 1993). The first of these domains in MHV, which is absent in IBV, has been identified to be responsible for proteolytic cleavage of a p28 polypeptide from the 1a polyprotein (Baker *et al.*, 1989, 1993).

In an effort to identify viral polypeptides encoded by mRNA 1, Brierley and colleagues (1990) prepared a panel of region-specific antisera by immunizing rabbits with bacterially expressed 1a and 1b sequences (Brierley *et al.*, 1990). A number of polypeptides corresponding to putative 1a- and 1b-encoded proteins were detected in IBV-infected chick kidney cells and Vero cells by immunoblotting using these sera (Brierley *et al.*, 1990). We describe here studies in which a number of these sera have been used to detect viral polypeptides encoded by the 5′-portion of ORF 1a and expressed both in virus-infected Vero cells and in an *in vitro* translation system. Several antisera which were raised against this region of 1a were available. Four of these, designated V46, V52, V53, and V59 (Brierley *et al.*, 1990), were tested in an immunoprecipitation assay against an *in vitro*-synthesized polypeptide of 220 kDa prepared from the 5′-portion of ORF 1a. Three of the sera (V52, V53, and V59) were able to immunoprecipitate the 220-kDa protein and, in IBV-infected Vero cells, a protein of 87 kDa was detected upon immunoprecipitation with sera V52 and V59. *In vitro* and *in vivo* expression and processing studies demonstrate that this 87-kDa protein is encoded by the 5′-portion of ORF 1a within the first 3000 nucleotides of the virus genome and that it appears to be cleaved from the putative polyprotein by viral and cellular proteinases.

MATERIALS AND METHODS

Virus and cells

The Beaudette strain of IBV was used in this analysis. Virus stocks were prepared by infecting Vero cells at a multiplicity of approximately 0.1 PFU/cell and incubating for 48 hr. The virus was assayed by plaqueing on Vero cells.

Vero cells were grown at 37° in 5% CO_2 and maintained in Glasgow's modified minimal essential medium supplemented with 10% fetal calf serum.

Purification of genomic RNA

Isolation and purification of RNA from IBV virions was carried out essentially as described (Liu *et al.*, 1991).

Labeling of IBV-infected cells with [^{35}S]methionine

Confluent monolayers of Vero cells were infected with IBV at a multiplicity of 1 PFU/cell. Prior to labeling, the cells were incubated in methionine-free medium for 30 min. After 120 min of labeling with [^{35}S]methionine (60 μCi/ml), the cells were scraped off the dishes in phosphate-buffered saline (PBS), pH 7.4, recovered by centrifugation, and stored at −70°.

Radioimmunoprecipitation

IBV-infected Vero cells were lysed with RIPA buffer (50 m*M* Tris–HCl, pH 7.5, 150 m*M* NaCl, 1% sodium deoxycholate, 0.1% SDS) and precleared by centrifugation at 12,000 *g* for 5 min at 4°. Radioimmunoprecipitation with polyclonal rabbit antisera was carried out as described previously (Liu *et al.*, 1991).

SDS–polyacrylamide gel electrophoresis

SDS–polyacrylamide gel electrophoresis (PAGE) of virus polypeptides was carried out using a range of polyacrylamide concentrations (12.5 to 17%) (Laemmli, 1970). Labeled polypeptides were detected by autoradiography or fluorography of dried gels.

Cell-free transcription and translation

In vitro transcription of plasmid DNA using T7 bacteriophage RNA polymerase was carried out as described (Liu and Inglis, 1992b), incorporating the dinucleotide ^{7m}GpppG to provide a 5′ cap structure (Contreras *et al.*, 1982). Product mRNA was recovered from the reactions by extraction with phenol–chloroform (1:1) and precipitation with ethanol. Remaining unincorporated nucleotides were removed by gel filtration on Sephadex G-50 spin columns. The purified mRNAs were translated in the rabbit reticulocyte lysate cell-free translation system in the presence of 0.75 μCi/μl [^{35}S]methionine as described (Liu *et al.*, 1993). Reaction products were separated on SDS–polyacrylamide gels and detected by autoradiography.

Polymerase chain reaction (PCR)

Complementary DNA templates for PCR were prepared from purified IBV virion RNA using a specific primer and a first-strand cDNA synthesis kit (Pharmacia) according to the manufacturer's instructions. Appropriate primers and template DNAs were then used in amplification reactions with Pfu DNA polymerase (Stratagene) under standard buffer conditions using 2 m*M* $MgCl_2$. PCR reaction conditions were 92° for 5 min, 56° for 30 sec, 72° for 6 min, then 30 cycles of 92° for 30 sec, 56° for 30 sec, and 72° for 6 min.

Preparation of Vero cell S10 extracts

IBV-infected or mock-infected Vero cell S10 extracts were prepared essentially as described for the preparation of the HeLa cell S10 extracts (Dorner *et al.*, 1984). Briefly, 5 × 10^8 Vero cells were infected with IBV at a

multiplicity of 1 PFU/cell or were mock infected. After 6 hr, the cells were harvested and washed with cold PBS. An equal volume of cold lysis buffer (10 m*M* KCl, 1.3 m*M* $Mg(OAc)_2$, 2.5 m*M* DTT, 10 m*M* HEPES, pH 7.4) was added, and the cells were left on ice for 20 min. The cells were then broken by Dounce homogenization. Cell debris and nuclei were removed by centrifugation at 2500 *g* for 5 min. Mitochondria were removed from the supernatant by centrifugation at 10,000 rpm for 15 min in a Sorvall SS34 rotor. The supernatant was adjusted to 20% glycerol and stored in small portions at −70° until required.

Construction of plasmids

The IBV cDNA clones 217, 216, 204, and BP8, which cover IBV genomic RNA sequences from nucleotide 212 to 2079, 1183 to 2433, 2080 to 3820, and 3150 to 4858, respectively (Boursnell *et al.*, 1987), were used to construct the expression plasmid pKT1a1 (containing IBV genomic sequence from nucleotide 364 to nucleotide 4858) using three cloning steps (see Fig. 1). First, a 1715-bp cDNA fragment containing the IBV genomic sequence from nucleotide 364 to nucleotide 2079 was obtained by digestion of pBR322 clone 217 with *Bst*E11, end-repair with DNA polymerase I, and redigestion with *Pst*I. This fragment was cloned into *Pvu*II- and *Pst*I-digested pKT0 to give plasmid pKT217. Plasmid pKT0, which is a modified form of plasmid pING14 (Liu *et al.*, 1991), was prepared by inserting, using site-directed mutagenesis, a sequence corresponding to the T7 RNA polymerase promoter just downstream of the SP6 promoter. Second, plasmid pKT217 was extended to include the *Esp*I site at position 2127 by replacing a *Pvu*II (position 1358)−*Pst*I (position 2079) fragment with a 1079-bp cDNA fragment (position 1358 to 2433) excised with *Pvu*II and *Pst*I from clone 216, to yield plasmid pKT 217-216. The final step in the construction of plasmid pKT1a1 involved a coligation of fragments excised from clones 204 (*Esp*I−*Dra*III) and BP8 (*Dra*II−*Pst*I) into the *Esp*I and *Pst*I sites of pKT217-216, the two fragments joining internally at the common *Dra*III site (position 3509).

Plasmid pKT1a2 was constructed by extension of pKT1a1 to include IBV sequence information up to nucleotide 5753. This was achieved by insertion of a *Stu*I−*Nhe*I (position 4620 to 5753)-digested PCR fragment into *Stu*I- and *Xba*I (which cuts within the polylinker immediately downstream of the IBV sequence)-digested pKT1a1.

RESULTS

Specificity and affinity of monospecific antibodies against the *in vitro*-synthesized products of ORF 1a

A set of monospecific antibodies against the predicted products encoded by the 5′-portion of mRNA 1 ORF 1a (Fig. 2a) was available in this laboratory. These antisera had been raised in rabbits using bacterially expressed fusion proteins; the fusion proteins contained viral sequences fused to the carboxy-terminus of β-galactosidase (Brierley *et al.*, 1990). The specificity and affinity of these sera for their putative protein targets, however, had not previously been evaluated thoroughly by immunoprecipitation assay. We therefore tested these antisera for their ability to immunoprecipitate radiolabeled 1a-encoded polypeptides synthesized by *in vitro* translation of a synthetic mRNA derived from plasmid pKT1a1. This plasmid contains ORF 1a sequence from the presumed initiator AUG at nucleotide position 537 up to nucleotide 4858 and is expected to encode a polypeptide of 170 kDa (see below). As can be seen in Fig. 2b, translation of pKT1a1 gave rise to a major protein species of approximately 220 kDa. Although this is somewhat larger than the predicted 170 kDa, it does indeed represent the full-length expected product (see Discussion). The results of this experiment indicated that three sera (V52, V53, and V59) were indeed capable of recognizing specifically the appropriate target sequences and immunoprecipitating the 220-kDa polypeptide (Fig. 2b). These antisera were used to test for viral products in immunoprecipitation experiments with [^{35}S]methionine-labeled lysates prepared from IBV-infected Vero cells.

Identification of gene products encoded by the ORF 1a in IBV-infected cells

Confluent monolayers of Vero cells were infected with IBV, labeled with [^{35}S]methionine at either 6 or 15 hr p.i. and, after a further 2 hr, the cells were harvested. Cell lysates were prepared and immunoprecipitated with V52, V53, or V59. Figure 3 shows the results of this experiment using lysates prepared from Vero cells harvested at 8 hr p.i. As can be seen, antisera V52 and V59 precipitated specifically a protein with an apparent molecular weight of approximately 87 kDa from IBV-infected, but not from mock-infected, Vero cell lysates; antiserum V53, however, did not precipitate any specific protein band from the same lysates. No obvious difference in abundance of the 87-kDa protein and in the appearance of any intermediate cleavage products of the 1a polyprotein was observed from lysates prepared from cells harvested at 8 and 17 hr p.i. (data not shown).

Expression of pKT1a1 and pKT1a2 in reticulocyte lysates

The detection of the 87-kDa polypeptide in IBV-infected Vero cells with region-specific antisera V52 and V59 indicated that this polypeptide is encoded by the corresponding region of ORF 1a and that it is likely to be cleaved from the 1a polyprotein. However, RNA transcripts from *Bam*HI-digested pKT1a1 plasmid DNA, which contain IBV

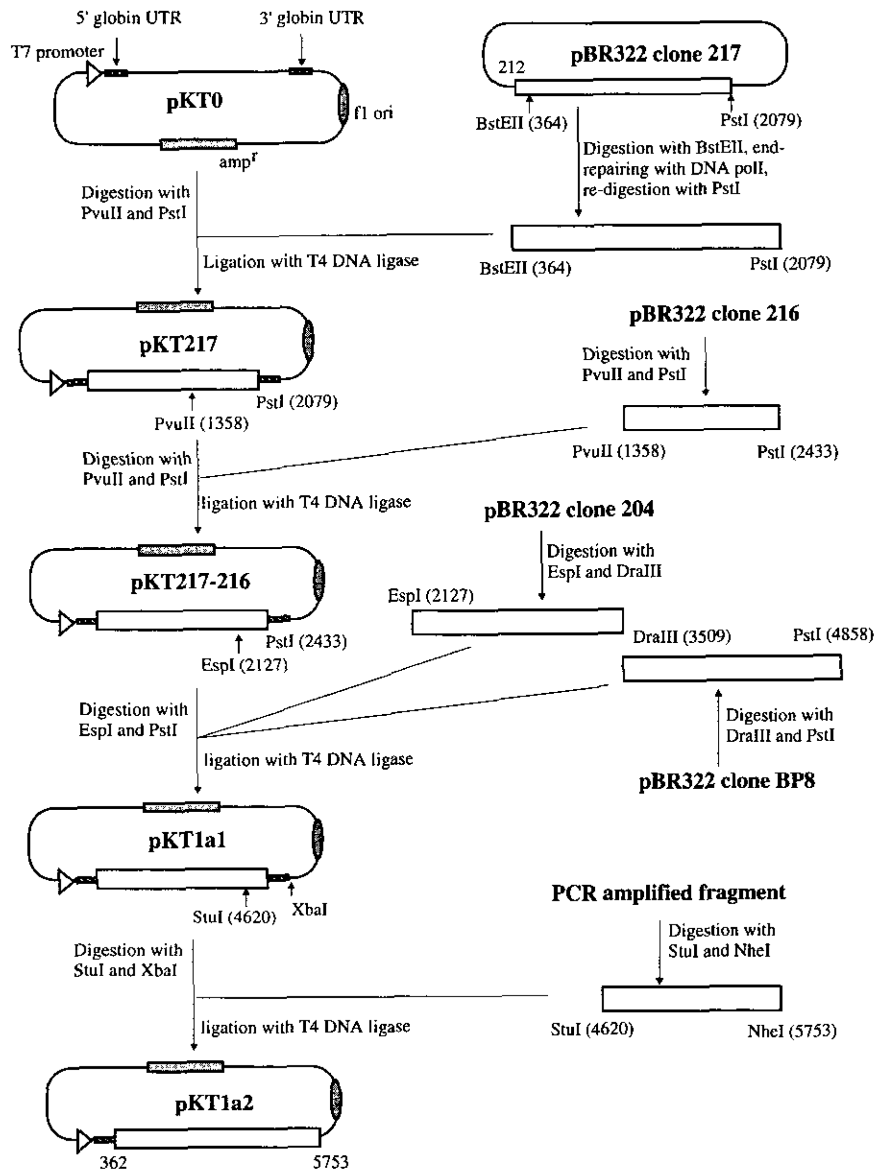

FIG. 1. Construction of plasmids pKT1a1 and pKT1a2.

sequence from nucleotide 365 to nucleotide 4858 including the putative initiator AUG at position 537, directed the synthesis of a protein of approximately 220 kDa in an *in vitro* translation system derived from rabbit reticulocyte lysate, and no processing to the 87-kDa polypeptide occurred (Figs. 2b and 4a). This result is perhaps not surprising since no proteinase domain is predicted to be located in this region of the IBV ORF 1a (Gorbalenya *et al.,* 1989). To investigate the possibility that a papain-like proteinase domain thought to be located between nucleotides 4680 and 5550 of ORF 1a (Gorbalenya *et al.,* 1989) is involved in cleavage of the 1a polyprotein, plasmid pKT1a2 was constructed. This plasmid covers IBV sequence from nucleotide 365 to 5753 and hence contains the papain-like proteinase domain. Expression of pKT1a2 *in vitro* gave rise to the synthesis of a polypeptide which migrated more slowly than the 220-kDa polypeptide encoded by pKT1a1 and was estimated to have a molecular weight of 250 kDa (Fig. 4a). Once again, no processing to the 87-kDa polypeptide was observed (Fig. 4a). As with the pKT1a1 translated product, the pKT1a2 polyprotein migrates somewhat more slowly (250 kDa) than predicted from amino acid sequence (205 kDa). This is considered under Discussion.

In addition to the full-length products, two major species of approximately 43 and 92 kDa were observed following *in vitro* translation of pKT1a1 and pKT1a2. To investigate the possibility that these proteins were derived from proteolytic processing of the full-length products, the kinetics of polypeptide synthesis from pKT1a2 was

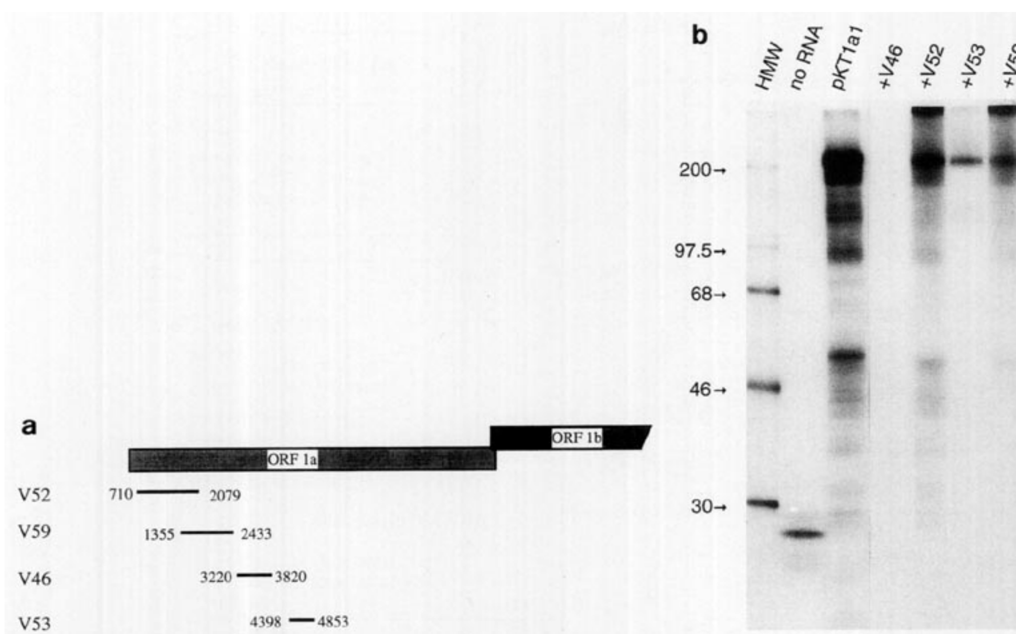

FIG. 2. (a) Regions of ORF 1a expressed in *Escherichia coli* as β-galactosidase fusion proteins and used to raise antisera from rabbits (Brierley *et al.*, 1990). The expressed region for each antiserum is indicated by a black bar and the numbered nucleotide positions. (b) Specificity of four antisera in immunoprecipitation against *in vitro*-synthesized target polypeptides. *In vitro*-transcribed RNA derived from *Bam*HI-digested pKT1a1 was translated in reticulocyte lysate and the *in vitro*-translated polypeptides analyzed directly or after immunoprecipitation with the antiserum indicated above each lane. Polypeptides were separated on a 16% SDS–polyacrylamide gel and detected by fluorography. The lane labeled HMW contains high-molecular-weight markers (Amersham).

studied by a pulse–chase experiment. In this experiment, a 10-fold excess of unlabeled methionine was added to the *in vitro* translation reaction after a 10-min incubation at 30°; aliquots were taken from the translation reaction mixture after incubation for 0, 10, 20, 30, 40, 50, 60, 80, 100, and 120 min, and the polypeptides were analyzed on 15% SDS–PAGE. As can be seen in Fig. 4b, synthesis of the 43- and 92-kDa polypeptides appeared at 20 min, which was prior to the appearance of the full-length product (Fig. 4b). Furthermore, no increasing accumulation of these polypeptides was observed during the time course of the experiment (Fig. 4b). This suggests strongly that these products arise following premature termination of translation and are not produced by proteolysis of the full-length products.

Transient expression of pKT1a1 and pKT1a2 in a eukaryotic system

To explore the possibility that proteolytic cleavage of the 1a polyprotein may require an intact cellular environment, plasmids pKT1a1 and pKT1a2 were expressed in Vero cells using the system described by Fuerst *et al.* (1986). Vero cells were infected with a recombinant vaccinia virus expressing the T7 phage RNA polymerase and subsequently transfected with pKT1a1 DNA. As shown in Fig. 5, transfection of pKT1a1 led to the synthesis of a polypeptide migrating above the 200-kDa marker, which was specifically precipitated by antisera V52 and V59. No processing to the 87-kDa polypeptide was observed (Fig. 5). Expression of pKT1a2 in Vero cells gave rise to synthesis of a polypeptide which migrated more slowly on 15% SDS–PAGE than the 220-kDa polypeptide expressed from pKT1a1 and was estimated to have a molecular weight of 250 kDa (Fig. 5). This polypeptide could be specifically precipitated by antisera V52 and V59. In addition, two polypeptides of approximately 87 and 160 kDa were observed. Both proteins were immunoprecipitated with antisera V52 and V59, indicating that they are derived from the N-terminus of the 250-kDa polyprotein. The 87-kDa species comigrated with the 87-kDa protein identified in IBV-infected Vero cells (data not shown), indicating it was one of the final cleavage products of the 250-kDa polyprotein. The 160-kDa species probably represents an intermediate cleavage product. As no processing of the pKT1a1-derived 220-kDa polyprotein was seen in these experiments, it seems likely that cleavage of the pKT1a2-derived polyprotein occurred as a result of including the papain-like proteinase domain in this construct. Nevertheless, the inclusion of this domain did *not allow complete processing of the 250-kDa precursor* in the vaccinia/T7 virus-infected cells. This is considered under Discussion.

Involvement of cellular factors in processing the polyprotein encoded by ORF 1a

It is clear that the *in vitro* translation products of pKT1a1 and pKT1a2 are not processed in reticulocyte

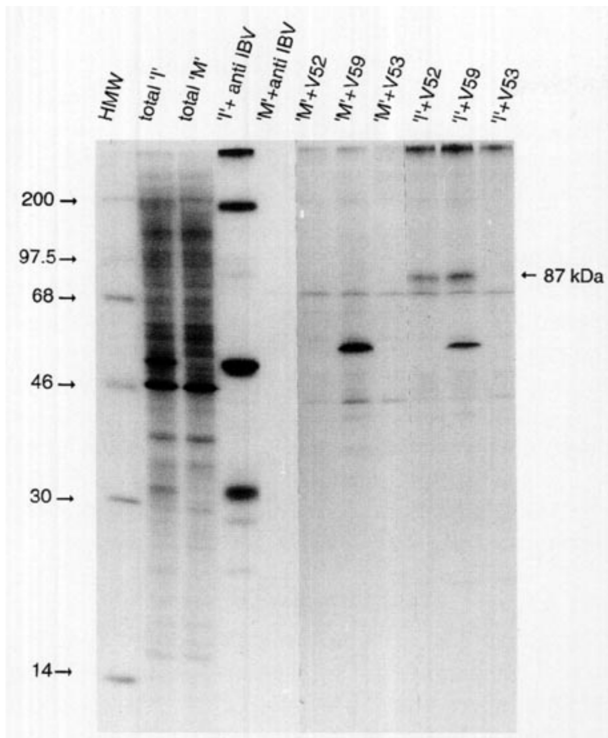

FIG. 3. Detection of polypeptides encoded by ORF 1a in IBV-infected and mock-infected Vero cells by immunoprecipitation with region-specific antisera. Cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were analyzed directly or immunoprecipitated with the antiserum indicated above each lane. Polypeptides were separated on a 17.5% SDS–polyacrylamide gel and detected by fluorography. I and M denote infected or mock-infected cell lysates.

lysates. We wished to explore the possibility that proteinase activities could be provided *in trans* by incubating the *in vitro* translation products with an IBV-infected Vero cell lysate in an attempt to achieve cleavage of the *in vitro*-synthesized polyprotein. For this purpose, Vero cell S10 extracts were prepared from mock-infected and IBV-infected Vero cells and incubated with the *in vitro* translation products for 1 hr at 37°. As shown in Fig. 6a, incubation of the translation products derived from *Bam*HI-digested pKT1a1 transcripts with lysis buffer alone did not induce cleavage of either the full-length 220-kDa or the minor polypeptides arising from premature termination. However, cleavage was observed following incubation with IBV-infected Vero cell extracts and furthermore following incubation with extracts prepared from mock-infected cells. In both cases, two major products migrating at about 90 and 87 kDa were observed; both products were specifically recognized by antiserum V52 (Fig. 6a), indicating that they contain sequences present in the region of 1a encoding the polypeptide used to raise the antiserum. Several minor protein species migrating more slowly than the 90- and 87-kDa polypeptides were also observed occasionally; these might represent intermediate cleavage products. In addition, the 43-kDa premature termination product was also processed to form two products with sizes differing by about 3 kDa (Fig. 6a). The 92-kDa protein is also cleaved, possibly to the 90- and 87-kDa forms.

Similarly, incubation of the translation products from pKT1a2 with Vero cell extracts prepared from mock-in-

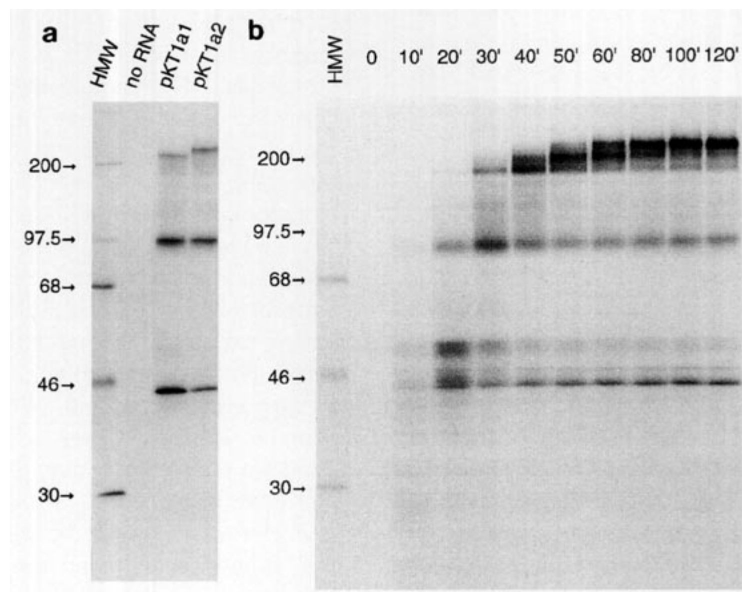

FIG. 4. (a) Analysis of cell-free translation products of mRNAs obtained by *in vitro* transcription of *Bam*HI-digested pKT1a1 and pKT1a2. RNA was added to the reticulocyte lysate cell-free system, as indicated above each lane. [^{35}S]Methionine-labeled translation products were separated on a 15% SDS–polyacrylamide gel and detected by fluorography. (b) Kinetic analysis of the polypeptide synthesis in reticulocyte lysates from pKT1a2-derived mRNA by pulse–chase experiment. A 10-fold excess of unlabeled methionine was added to the *in vitro* translation reaction after a 10-min incubation at 30°, and aliquots were taken from the translation reaction mixture after incubation for 0, 10, 20, 30, 40, 50, 60, 80, 100, and 120 min. [^{35}S]Methionine-labeled translation products were separated on a 15% SDS–polyacrylamide gel and detected by fluorography.

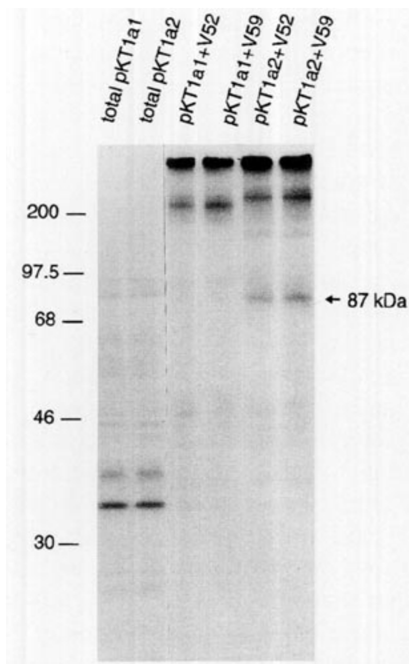

FIG. 5. Analysis of transiently expressed ORF 1a products from plasmids pKT1a1 and pKT1a2, using the system described by Fuerst *et al.* (1986), in which Vero cells were infected with a vaccinia recombinant virus expressing the T7 RNA polymerase and subsequently transfected with plasmid DNAs. Cells were labeled with [^{35}S]methionine, lysates were prepared, and polypeptides were analyzed directly or immunoprecipitated with the antisera V52 and V59. Polypeptides were separated on a 15% SDS–polyacrylamide gel and detected by fluorography.

fected cells led to processing of the full-length product to the 90- and 87-kDa polypeptides (Fig. 6b). The 87-kDa polypeptide was shown to migrate at the same position as the 87-kDa polypeptide detected from pKT1a2-transfected cells (Fig. 6b), suggesting that they might represent the same cleavage products from the N-terminal portion of the 1a polyprotein. In addition to the 90- and 87-kDa polypeptides, a protein migrating at about 60 kDa was also seen on a longer exposure of the same gel (data not shown, see below).

Determination of the C-terminal boundary of the 87-kDa protein

To define approximately the C-terminal boundary of the 90- and 87-kDa polypeptides within the ORF 1a polyprotein, pKT1a1 was linearized separately with five restriction enzymes (Fig. 7a) and a set of target polypeptides with common amino-termini was prepared by *in vitro* transcription and translation. These products were then tested for processing with the mock-infected Vero S10 extract. As shown in Fig. 7b, translation of the RNAs derived from templates linearized by *Nci*I, *Mlu*I, and *Bam*HI (which cut the IBV sequence at nucleotide positions 3002, 3997, and 4858, respectively) resulted in synthesis of full-length products with sizes greater than 90 kDa, which were then processed down to the 90- and 87-kDa forms upon incubation with the Vero cell S10 extract. This supports the idea that both the 90- and the 87-kDa polypeptides are derived from within the first 3000 nucleotides of ORF 1a. Incubation of the products translated from pKT1a2, as indicated in Fig. 6b, gave rise to the 90-, 87-, and 60-kDa polypeptides (Fig. 7b). The 60-kDa polypeptide was clearly detectable (Fig. 7b). Immunoprecipitation studies indicated that it could be precipitated specifically by antiserum V53 (data not shown), suggesting that it is encoded in ORF 1a between nucleotides 3000 and 5000. The failure to detect this polypeptide in IBV-infected Vero cells (Fig. 3) may be due to poor incorporation of [^{35}S]methionine during the labeling procedure; the deduced amino acid sequence of the ORF 1a in the region between nucleotides 3300 and 4920 contains only three methionine residues.

DISCUSSION

Nucleotide sequence determination of the Beaudette strain of IBV, carried out by Boursnell *et al.* (1987), indicated that the region of the RNA genome corresponding to the 5′-unique portion of IBV mRNA 1 contained two large ORFs with the potential to encode functional virus proteins associated with viral replication. In this study, we have identified an 87-kDa polypeptide expressed in IBV-infected Vero cells using region-specific antisera. The evidence presented suggests that this novel polypeptide is encoded by the 5′-most 3000 nucleotides of ORF 1a and is cleaved from the 1a polyprotein by viral and cellular proteinases. First, the 87-kDa polypeptide is recognized by N-terminal antisera. V52 and V59, which recognize IBV sequences from nucleotides 710 to 2079 and 1355 to 2433, respectively, and overlap by 724 nucleotides, immunoprecipitate the 87-kDa polypeptide from both virus-infected cells and the *in vitro* translation and processing reactions. Second, expression of ORF 1a up to nucleotide 5763 (pKT1a2), which includes the presumed papain-like proteinase domain (Gorbalenya *et al.*, 1989), leads to synthesis of a final polypeptide product of approximately 250 kDa. This protein appears to represent the expected full-length translation product encoded by this plasmid. In addition, an inefficient processing of this polyprotein to the 87-kDa polypeptide was also observed. However, the 87-kDa polypeptide is not produced simply by translating ORF 1a *in vitro*. Translation of pKT1a1 and pKT1a2 *in vitro* in reticulocyte lysates results in synthesis of the 220- and 250-kDa polypeptides, respectively, which are clearly not processed to mature products. Incubation of these *in vitro* translation products with either IBV-infected or mock-infected Vero cell lysates leads to processing of the *in vitro*-synthesized polyproteins and the appearance of the 87-kDa polypeptide. This suggests strongly that cellular factors participate in processing of the 1a polyprotein.

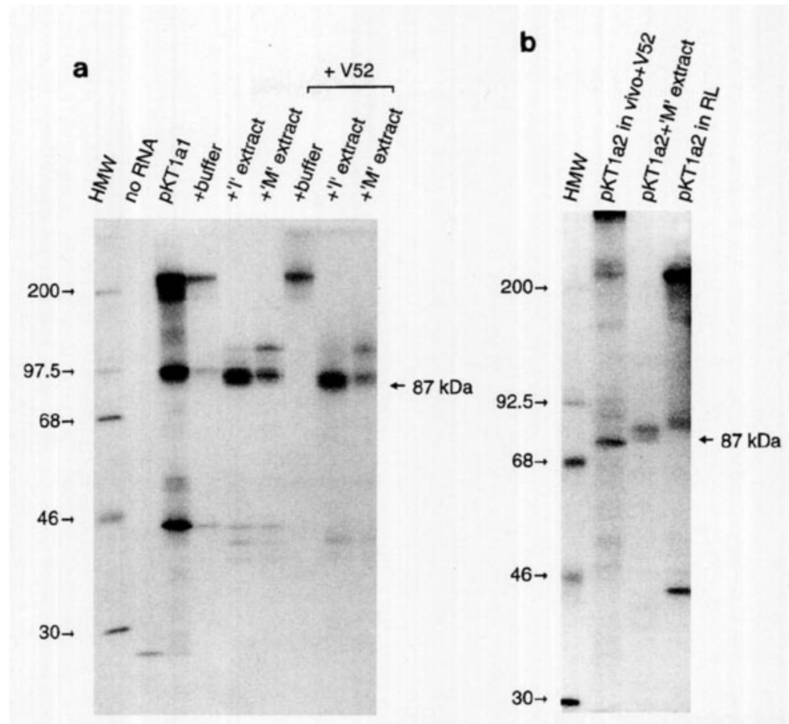

FIG. 6. (a) *In vitro* translation and processing of polypeptides encoded by pKT1a1-derived transcripts. *Bam*HI-digested pKT1a1 DNA was transcribed *in vitro* using T7 RNA polymerase, and the resulting transcript was translated in the reticulocyte lysate cell-free translation system. Translation products were incubated with buffer, IBV-infected Vero cell S10 extract, or mock-infected Vero cell extract. The translated and processed materials were analyzed directly or immunoprecipitated with antiserum V52 as indicated above each lane. Polypeptides were separated on a 17.5% SDS–polyacrylamide gel and detected by fluorography. (b) *In vitro* and *in vivo* expression and processing of polypeptides encoded by plasmid pKT1a2. *Bam*HI-digested pKT1a2 DNA was transcribed *in vitro* using T7 RNA polymerase, the resulting transcript was translated in the reticulocyte lysate cell-free translation system (lane pKT1a2 in RL), and the translation products were incubated with mock-infected Vero cell extract (lane pKT1a2+'M' extract). Plasmid pKT1a2 was also expressed in Vero cells using the vaccinia virus/T7 system as described in the legend for Fig. 5, and the products were immunoprecipitated with antiserum V52 (lane pKT1a2 in vivo+V52). Polypeptides were separated on a 10% SDS–polyacrylamide gel and detected by fluorography.

Throughout this report, we have referred to the full-length translation products from pKT1a1 and pKT1a2 as the 220- and 250-kDa species. However, their amino acid sequences suggest that they should migrate more rapidly on SDS–PAGE as 170- and 205-kDa species, respectively. The increased apparent molecular weight of the proteins is probably a reflection of their conformation. Brierley and colleagues (unpublished observations) have obtained evidence that the inclusion in 1a-derived proteins of amino acid residues encoded by ORF 1a between nucleotides 3220 and 3820 can dramatically reduce the mobility of such proteins in SDS–PAGE. The reasons for this are as yet unclear.

We have recently reported that a 100-kDa polypeptide encoded by IBV ORF 1b was processed by a region of ORF 1a related to the picornavirus 3C-like proteinase domain (Liu *et al.,* 1994). No cleavage of the *in vitro*-synthesized polyprotein was observed when IBV sequences containing the 3C-like proteinase domain were expressed *in vitro* in reticulocyte lysates. However, proteolytic processing to the 100-kDa polypeptide from the polyprotein did occur when this region was expressed in Vero cells using the vaccinia virus/T7 system (Liu *et al.,* 1994). The data presented here indicate that processing of the 1a polyprotein encoded by the N-terminal 5.7-kb segment of ORF 1a does not occur efficiently even when this region is expressed in intact cells using the vaccinia virus/T7 system. However, processing was observed following incubation of the *in vitro*-synthesized polyprotein with either IBV-infected or mock-infected Vero cell S10 extracts. Currently, we are uncertain why the proteinase(s) present in the Vero cell S10 extract does not appear to function when the truncated 1a polyprotein is expressed in intact Vero cells using the vaccinia virus/T7 system. One possible explanation is that infection of Vero cells with vaccinia virus may lead to inhibition of the proteinase activities required for cleavage of the polyprotein. It is well documented that infection of host cells by vaccinia virus leads to shut off of host cell protein synthesis, resulting in a general reduction of host protein concentration in infected cells. More specifically, three serine proteinase inhibitors (serpins) are produced during virus infection, leading to an inhibition of normal cellular proteinase activities (Smith, 1993). Alternative expression systems are currently being used to explore this possibility further.

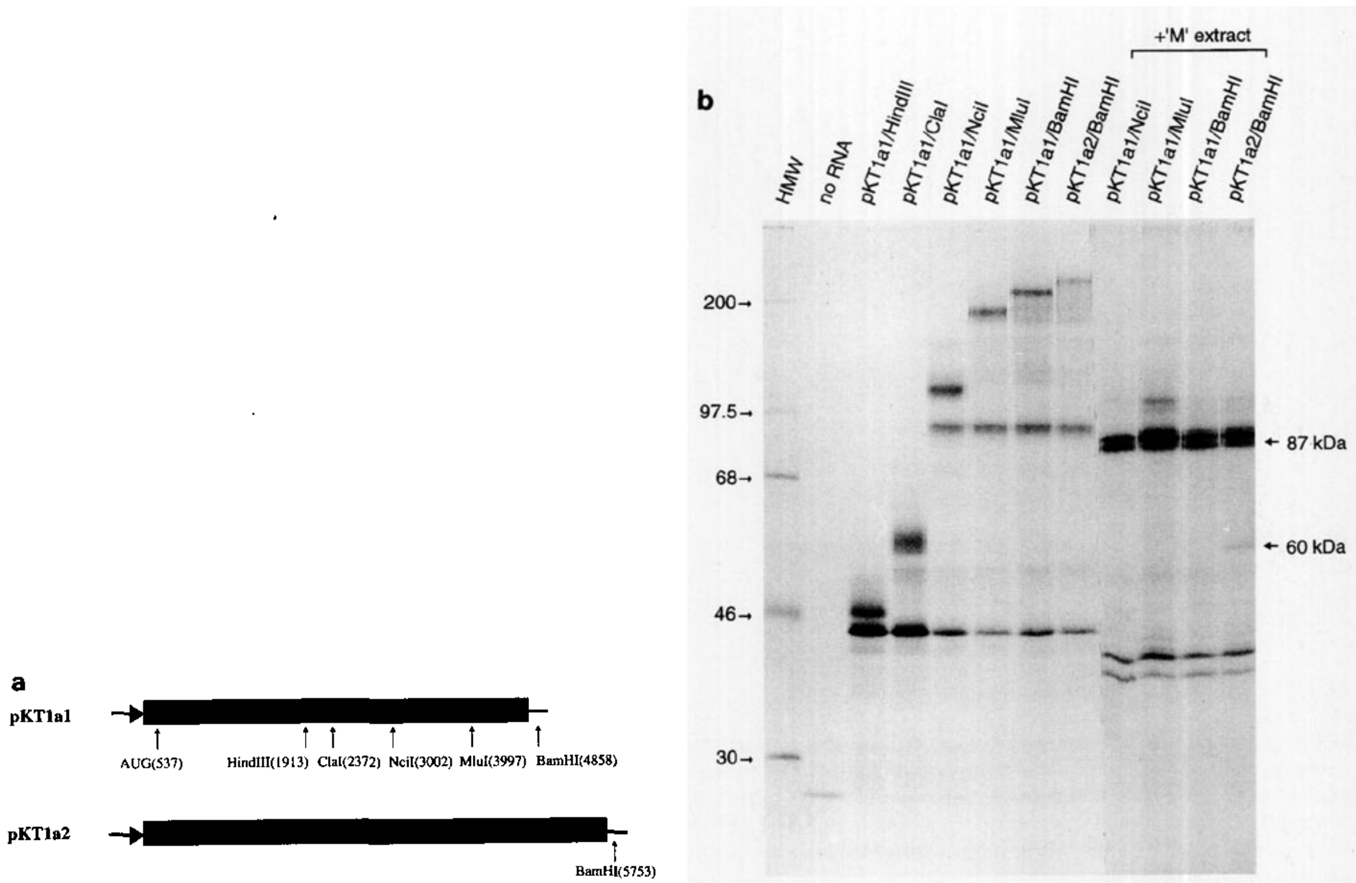

FIG. 7. (a) Diagram of plasmids pKT1a1 and pKT1a2, showing the T7 promoter site and the restriction enzyme sites used to linearize the plasmids for *in vitro* transcription. (b) Analysis of cell-free translation products of mRNAs obtained by *in vitro* transcription of *Hin*dIII-, *Cla*I-, *Nci*I-, *Mlu*I-, and *Bam*HI-digested pKT1a1 and from *Bam*HI-digested pKT1a2. RNA was added to the reticulocyte lysate cell-free system, as indicated above each lane. Translation products from *Nci*I-, *Mlu*I-, and *Bam*HI-digested pKT1a1 and from *Bam*HI-digested pKT1a2 were processed by incubation with mock-infected Vero cell S10 extract as described in Fig. 4. [^{35}S]Methionine-labeled translation products and the processed species were separated on a 12.5% SDS–polyacrylamide gel and detected by fluorography.

It was previously reported that Western blots of IBV-infected chicken kidney cells and Vero cells with antisera V59 led to the detection of two virus-specific polypeptides of approximately 75 and 220 kDa (Brierley *et al.*, 1990). Similar Western blot experiments repeated recently have indicated that the 75-kDa polypeptide migrates on linear SDS–PAGE at the same position as the 87-kDa polypeptide reported here (Brierley *et al.*, unpublished observation). The underestimation of the molecular weight of this polypeptide in the original report is possibly due to the gradient gel system used in the original experiment.

In vitro processing of truncated 1a polyproteins of either 220 or 250 kDa produced a major species of 90 kDa (Fig. 5b), which was not detectable in virus-infected cells. It is likely that this 90-kDa polypeptide has the same C-terminus as the 87-kDa polypeptide since processing of the 43-kDa premature termination product also gave rise to two products that differed in size by about 3 kDa. It is tempting to speculate that inefficient cleavage of the N-terminus of the polyprotein occurs in both cases, giving rise to a small, N-terminal cleavage product which could not be detected by the SDS–PAGE system used. An N-terminal cleavage of the 1a polyprotein occurs in MHV; a 28-kDa polypeptide, derived from the 5′-terminal region of ORF 1a, is cleaved from the 1a polyprotein by a papain-like proteinase encoded by ORF 1a (Baker *et al.*, 1989, 1993).

Involvement of cellular proteinases in the processing of viral polyproteins has been observed in other virus families, including togaviridae, flaviviridae, reoviridae, herpesviridae, poxviridae, and retroviridae (Dougherty and Semler, 1993). For example, cellular signal peptidases are responsible for cleavage of alpha-, flavi-, and pestivirus structural polypeptides from polyproteins; some cleavages of nonstructural polypeptides from structural polypeptides also involve signal peptidases (Chambers *et al.*, 1990). Cellular cofactors have recently been found to be required for efficient cleavage of the poliovirus 3CD polyproteins in virus-infected cells (Blair *et al.*, 1993). *In vitro* translation studies also indicate that

multiple proteolytic activities may be required for processing of the 1a polyprotein encoded by MHV (Denison *et al.*, 1992).

It is currently unclear what role the 87-kDa polypeptide may play in the viral RNA replication and life cycle. No functional domains have so far been identified in this region of the viral genome by computer-aided methods (Gorbalenya *et al.*, 1989; Lee *et al.*, 1991). However, moderate similarity in this segment of the 1a polyprotein between IBV and MHV has been demonstrated (Lee *et al.*, 1991). Determination of the functional role of the 87-kDa polypeptide will be difficult to achieve until systems for the molecular genetic analysis of the coronavirus replication machinery are developed.

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