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A 100-Kilodalton Polypeptide Encoded by Open Reading Frame (ORF) 1b of the Coronavirus Infectious Bronchitis Virus Is Processed by ORF 1a Products

D. X. LIU,* I. BRIERLEY, K. W. TIBBLES, AND T. D. K. BROWN

Division of Virology, Department of Pathology, University of Cambridge, Cambridge CB2 1QP, United Kingdom

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The genome-length mRNA (mRNA 1) of the coronavirus infectious bronchitis virus (IBV) contains two large open reading frames (ORFs), 1a and 1b, with the potential to encode polypeptides of 441 and 300 kDa, respectively. The downstream ORF, ORF 1b, is expressed by a ribosomal frameshifting mechanism. In an effort to detect viral polypeptides encoded by ORF 1b in virus-infected cells, immunoprecipitations were carried out with a panel of region-specific antisera. A polypeptide of approximately 100 kDa was precipitated from IBV-infected, but not mock-infected, Vero cells by one of these antisera (V58). Antiserum V58 was raised against a bacterially expressed fusion protein containing polypeptide sequences encoded by ORF 1b nucleotides 14492 to 15520; it recognizes specifically the corresponding in vitro-synthesized target protein. A polypeptide comigrating with the 100,000-molecular-weight protein (100K protein) identified in infected cells was also detected when the IBV sequence from nucleotides 8693 to 16980 was expressed in Vero cells by using a vaccinia virus-T7 expression system. Deletion analysis revealed that the sequence encoding the C terminus of the 100K polypeptide lies close to nucleotide 15120; it may therefore be generated by proteolysis at a potential QS cleavage site encoded by nucleotides 15129 to 15135. In contrast, expression of IBV sequences from nucleotides 10752 to 16980 generated two polypeptides of approximately 62 and 235 kDa, which represent the ORF 1a stop product and the 1a-1b fused product generated by a frameshifting mechanism, respectively, but no processed products were observed. Since the putative picornavirus 3C-like proteinase domain is located in ORF 1a between nucleotides 8937 and 9357, this observation suggests that deletion of the picornavirus 3C-like proteinase domain and surrounding regions abolishes processing of the 1b polyprotein. In addition, the in vitro translation and in vivo transfection studies also indicate that the ORF 1a region between nucleotides 8763 and 10720 contains elements that down-regulate the expression of ORF 1b.

Avian infectious bronchitis virus (IBV) is the prototype species of the family *Coronaviridae*, a family of enveloped viruses with large positive-stranded RNA genomes. The genomic RNA of IBV is 27.6 kb in length and contains at least 10 distinct open reading frames (ORFs) (3). However, it appears that most of these ORFs are not translated from genomic RNA but are expressed from a set of subgenomic mRNAs. Available evidence suggests that five subgenomic mRNA species are produced in virus-infected cells. These mRNAs (designated 2 to 6) together with the genome-length mRNA (designated mRNA 1) (8) range in length from about 2 to 27.6 kb and have been shown to share a common 3′ terminus and to form a "nested" set structure (28, 29). Three of these, mRNAs 2, 4, and 6, encode the major virion structural proteins spike (S), membrane (M), and nucleocapsid (N), respectively, and in each case the expressed ORF is the only one which is not present on the next smallest mRNA in the nested set (30). Two of the other mRNAs, mRNA 3 and mRNA 5, which contain more than one ORF in their 5′-terminal unique regions, have recently been shown to encode three and two viral polypeptides, respectively (19, 21, 27).

Nucleotide sequence analysis of IBV genomic RNA has shown that the 5′-terminal unique region of mRNA 1 contains two large ORFs (1a and 1b), with ORF 1a having the potential to encode a polypeptide of 441 kDa and ORF 1b having the potential to encode a polypeptide of 300 kDa (3). The downstream ORF, ORF 1b, is likely produced as a fusion protein of 741 kDa with ORF 1a by a ribosomal frameshift (5, 7), a mechanism which has been proved to be universal in expression of ORF 1b in all coronaviruses studied so far (4, 16, 18). The 1a-1b fusion polyprotein is expected to be cleaved by viral or cellular proteinases to produce functional products associated with viral replication. Several putative functional domains were indeed predicted to be present in either ORF 1a or ORF 1b (15). For example, a picornavirus 3C-like proteinase domain was located in ORF 1a between nucleotides 8937 and 9357 and an RNA-dependent RNA polymerase domain was located in ORF 1b between nucleotides 14100 and 14798 (15). However, identification of gene products encoded by this mRNA has proved difficult. The only identified and characterized mRNA 1-specific product so far is a 28-kDa polypeptide from the coronavirus mouse hepatitis virus (MHV), which is encoded by the 5′ region of ORF 1a and proteolytically cleaved from the 1a polyprotein (1, 2, 10). In addition, polypeptides with sizes ranging from 50 to 290 kDa have been recently identified from MHV A59-infected cells by using antisera directed against fusion protein encoded in the first 6.5 kb of ORF 1a (11). In vitro translation of genomic RNA from MHV strain A59 (12) revealed a number of polypeptides with distinct electrophoretic mobilities, antiserum reactivities, and partial protease digestion patterns. However, these polypeptides are yet to be identified and characterized in virus-infected cells.

In an effort to identify viral proteins encoded by IBV mRNA 1, a set of region-specific antisera against bacterial fusion proteins containing portions of IBV sequence were raised in

* Corresponding author. Mailing address: Division of Virology, Department of Pathology, University of Cambridge, Tennis Court Rd., Cambridge CB2 1QP, United Kingdom. Phone: 223 336918. Fax: 223 336926. Electronic mail address: DXL10@PHX.CAM.AC.UK.

rabbits (6). We show here the identification of a 100-kDa polypeptide from IBV-infected Vero cells by using one of these antisera (V58), which recognizes IBV sequence from nucleotides 14492 to 15520, suggesting it to be encoded by this region of mRNA 1. Expression of IBV sequence from nucleotides 8693 to 16980 in Vero cells by using a vaccinia virus-T7 expression system led to synthesis of a protein comigrating with the 100,000-molecular weight polypeptide (100K polypeptide). Deletion analysis revealed that this polypeptide is processed by the picornavirus 3C-like proteinase domain and surrounding regions; its C terminus may be generated by proteolysis at a potential QS cleavage site encoded by nucleotides 15129 to 15135. The nucleotide sequence encoding the 100K polypeptide therefore covers the putative polymerase domain, suggesting that it may represent the first identified polymerase protein of a coronavirus.

MATERIALS AND METHODS

Virus and cells. The Beaudette strain of IBV, adapted for growth in Vero cells, was the kind gift of D. Cavanagh. Virus working stocks were prepared by infection of Vero cells with IBV at a multiplicity of approximately 0.1 PFU per cell and incubation at 37°C, 5% CO_2 for 48 h. The virus was then assayed by plaque formation on Vero cells.

Vero cells were grown and maintained in Glasgow's modified minimal essential medium (GMEM) supplemented with 10% fetal calf serum.

Labelling of IBV-infected cells with [^{35}S]methionine. Confluent monolayers of Vero cells were infected with IBV at a multiplicity of approximately 1 PFU per cell. Before being labelled, the cells were incubated in medium free of methionine for 30 min. After 120 min of labelling with [^{35}S]methionine (60 μCi/ml), the cells were scraped off the dishes into saline, recovered by centrifugation, and stored at −70°C until required.

Cell-free transcription and translation. In vitro transcription from plasmid DNA with T7 phage RNA polymerase was carried out as described before (22), with the dinucleotide ^{7m}GpppG incorporated to provide a 5′ cap structure (9). Product mRNA was recovered from the reaction mixtures by extraction with phenol-chloroform (1:1) and precipitation with ethanol. Unincorporated nucleoside triphosphates were removed by gel filtration on Sephadex G-50, and the RNA was translated in the rabbit reticulocyte lysate cell-free system in the presence of 0.75 μCi of [^{35}S]methionine per μl as described previously (20). Translation of plasmid DNA in TnT T7-coupled reticulocyte lysate systems (TnT system) was carried out according to the manufacturer's instructions (Promega). Reaction products were separated by polyacrylamide gel electrophoresis (PAGE) and detected by autoradiography.

Transient expression of 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 by Fuerst et al. (13). Briefly, semiconfluent monolayers of Vero cells were infected (10 PFU per cell) with a recombinant vaccinia virus (vTF7-3) which expresses the T7 phage RNA polymerase and then transfected with appropriate plasmid DNA by using lipofectin (Gibco-BRL) according to the manufacturer's instructions. After incubation of the cells at 37°C for 4 h, 25 μCi of [^{35}S]methionine per μl was added directly to the medium. The radiolabelled cells were harvested at 18 h postinfection.

Radioimmunoprecipitation. IBV-infected or plasmid DNA-transfected Vero cells were lysed with radioimmunoprecipitation buffer (50 mM Tris HCl [pH 7.5], 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]) and precleared by centrifugation at 10,000 × *g* for 5 min at 4°C. Immunoprecipitation was carried out as described previously (19).

SDS-PAGE. SDS-PAGE of virus polypeptides was carried out by using between 12.5 and 17% polyacrylamide gels (17). Labelled polypeptides were detected by autoradiography or fluorography of dried gels.

PCR. The cDNA templates for PCR were prepared by using purified IBV virion RNA, a specific primer, and a first-strand 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 with 2 mM $MgCl_2$. PCR conditions were 92°C for 5 min, 56°C for 30 s, and 72°C for 6 min, followed by 30 cycles at 92°C for 30 s, 56°C for 30 s, and 72°C for 6 min.

Construction of plasmids. The original IBV clones 205 and BP5 (3) and several PCR fragments, obtained by amplification of the first-strand cDNA reverse transcribed from IBV genomic RNA, were used to construct three series of T7-based expression plasmids covering IBV sequences from nucleotides 8693 to 16980. The starting plasmid is a modified form (pKT0) of plasmid pING14 (19), in which a 23-bp fragment representing the T7 RNA polymerase promoter sequence was inserted between the SP6 promoter and the 5′ globin noncoding region by site-directed mutagenesis. To provide an efficient translation initiator ATG within pKT0 to enable expression of truncated IBV cDNA fragments, a 37-bp blunt-ended fragment containing the 5′-noncoding region and the authentic ATG triplet from the influenza A virus NS1 gene (a gift from S. C. Inglis) was inserted into *Pvu*II- and *Nco*I-digested pKT0 after end repairing of the vector with DNA polymerase I (PolI), giving plasmid pKT0/NS1 (Fig. 1).

Plasmid pKT0/NS1 was used to construct plasmids pKTBP5 and pKT205. For construction of plasmid pKT205, a 2,232-bp DNA fragment containing IBV sequence from nucleotides 8693 to 10925 was obtained by digestion of pBR322 clone 205 with *Stu*I and *Pst*I. This fragment was then cloned into *Eco*RV- and *Pst*I-digested pKT0/NS1 to give plasmid pKT205. Similarly, a 1,848-bp fragment covering IBV sequence from nucleotides 10752 to 12600 was excised from IBV clone BP5 with *Nsi*I and *Pst*I, end repaired with DNA PolI, and then cloned into *Pst*I-digested, PolI-end-repaired pKT0/NS1, giving plasmid pKTBP5.

A 2,190-bp DNA fragment covering IBV sequences from nucleotides 10510 to 12700 was obtained from a PCR-amplified fragment by digestion with *Sau*I and *Sma*I. This fragment was cloned into plasmid p205, which was first digested with *Pst*I, end repaired with DNA PolI, and redigested with *Sau*I, giving plasmid pIBV6. Plasmid pIBV6 was digested with *Ksp*I and *Xba*I, which cut the IBV sequence at nucleotide position 11952 and the polylinker sequence located immediately downstream of the cloned viral sequence, and religated with a *Ksp*I- and *Xba*I-digested PCR fragment covering IBV sequence from nucleotides 11952 to 13896, giving plasmid pIBV10. Plasmid pIBV11 was made by insertion of an *Xba*I- and *Sma*I-digested PCR fragment containing IBV sequences from nucleotides 13896 to 16980 into *Xba*I- and *Sma*I (located in the polylinker)-digested pIBV10 (Fig. 1).

For construction of plasmids pIBV4 and pIBV5, which cover IBV sequences from nucleotides 10752 to 13896 and 10752 to 16960, respectively, plasmid pBP5 was digested with *Ksp*I and *Xba*I and ligated with a *Ksp*I- and *Xba*I-digested PCR fragment (covering IBV sequence from nucleotides 11952 to 13896), giving plasmid pIBV4. Plasmid pIBV5 was made by insertion

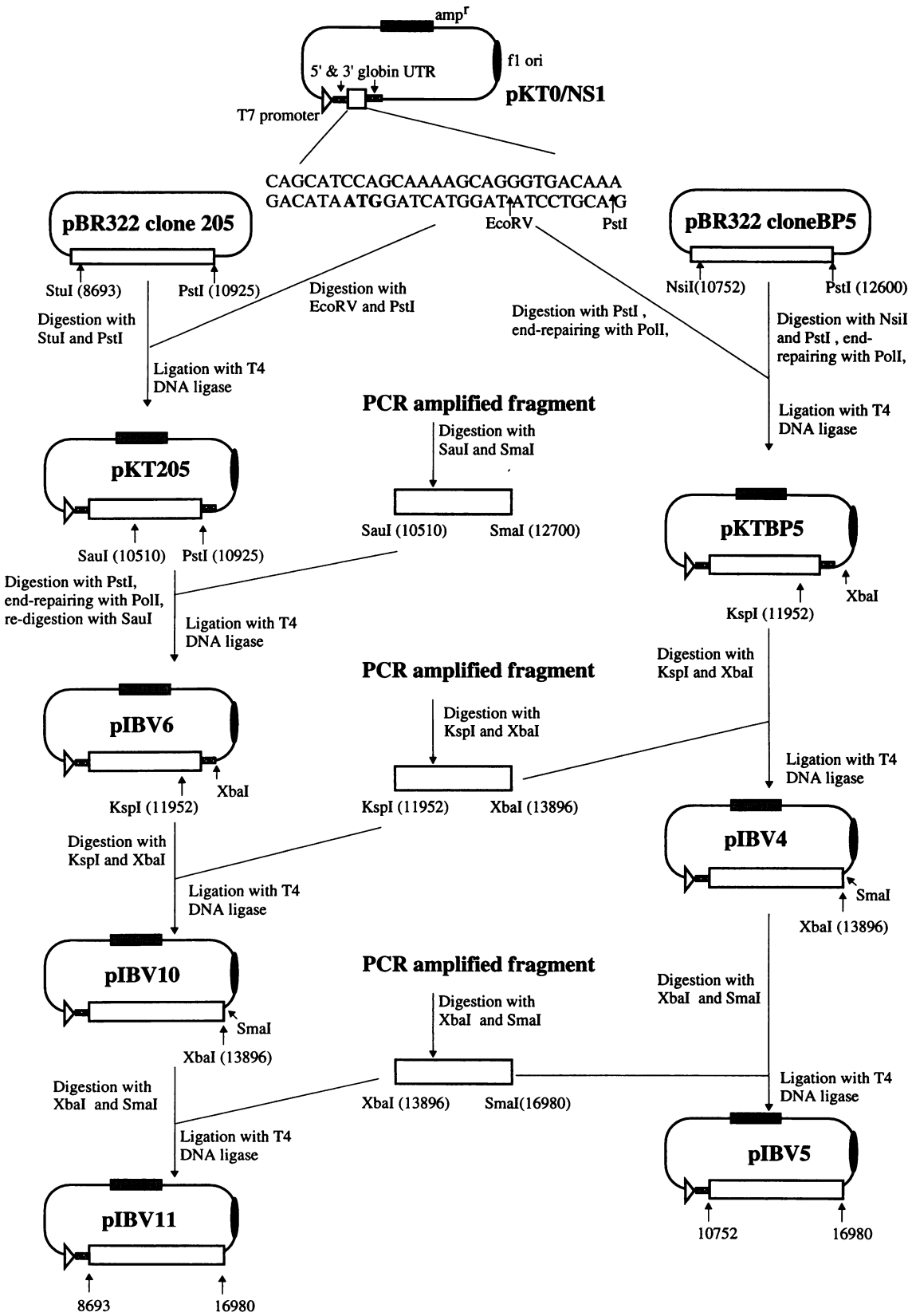

FIG. 1. Construction of plasmids pKT205, pKTBP5, pIBV4, pIBV5, pIBV6, pIBV10, and pIBV11. See also Materials and Methods. UTR, untranslated region.

of an *Xba*I-*Sma*I PCR fragment (IBV sequence from nucleotides 13896 to 16980) into *Xba*I- and *Sma*I-digested pIBV4 (Fig. 1).

Plasmids pIBV1b1 and pIBV1b2, which cover IBV sequences from nucleotides 12625 to 13896 and 12625 to 16980, respectively, were made as follows. Plasmid pIBV4 was digested with *Eco*RV and *Pvu*II, which cut at a position 10 nucleotides downstream of the initiator ATG triplet and IBV sequence at nucleotide position 12264, respectively, and religated with T4 DNA ligase, giving plasmid pIBV1b1. Plasmid pIBV1b2 was made by insertion of an *Xba*I-*Sma*I PCR fragment (covering IBV sequence from nucleotides 13896 to 16980) into *Xba*I- and *Sma*I-digested pIBV1b1.

Plasmids pIBV14 and pIBV15, which cover IBV sequences from nucleotides 8693 to 15537 and 8693 to 15117, respectively, were made as follows. Plasmid pIBV11 was digested with *Pst*I, which cut the IBV sequences at nucleotide positions 15537 and 16788, and religated with T4 DNA ligase, giving plasmid pIBV14. Plasmid pIBV15 was made by digestion of pIBV11 with *Sac*I, which cut IBV sequence at position 15117 and polylinker sequence immediately downstream of the viral sequence, and religation with T4 DNA ligase.

RESULTS

Identification of gene products encoded by mRNA 1 in IBV-infected cells. A panel of region-specific antisera was used in immunoprecipitation studies of IBV-infected, [^{35}S]methionine-labelled Vero cell lysates for detection of gene products encoded by the C-terminal region of ORF 1a, the junction of 1a-1b, and the N-terminal portion of 1b. These antisera were raised in rabbits against bacterial fusion proteins prepared by expression of viral sequences in bacteria as fusions with β-galactosidase (6). The cell lysates prepared from IBV-infected Vero cells harvested at 8 h postinfection were analyzed by immunoprecipitation with four antisera, V47, V57, V58, and V60, which should recognize IBV sequences from nucleotides 11488 to 12600, 13966 to 14527, 14492 to 15520, and 16066 to 16783, respectively. The results of this study showed that a polypeptide with an apparent molecular mass of approximately 100 kDa on SDS-PAGE was precipitated specifically from IBV-infected, but not from mock-infected, Vero cell lysates by antiserum V58 (Fig. 2). Antiserum V57 could also precipitate the same polypeptide, but the specific protein band was very faint, even after a prolonged exposure (data not shown). No specific protein bands, however, were detected from the same lysates with antisera V47 and V60 (Fig. 2).

Reactivities of the antisera to their in vitro-synthesized target products. The studies described above suggest that the reactivities of the antisera used to their target products may be different. To address this possibility directly, we tested the specificity and avidity of these four antisera for the appropriate in vitro-synthesized target proteins. For this purpose, the corresponding IBV sequences were first cloned into a T7 promoter-based vector and synthetic RNAs were obtained by transcription of plasmid DNA by using T7 phage RNA polymerase. The artificial RNA transcripts were then tested with the rabbit reticulocyte lysate cell-free translation system for their ability to direct synthesis of protein products. Accordingly, two plasmids, pIBV4 and pIBV1b2 (Fig. 3A), were digested with restriction endonucleases *Dra*II and *Hin*dIII, respectively, and transcribed in vitro by using T7 RNA polymerase. RNA transcripts from *Dra*II-digested pIBV4 plasmid DNA, which should contain IBV sequence from nucleotides 10752 to 12874, directed the synthesis of two major polypeptides of approximately 60 and 75 kDa, which represent the

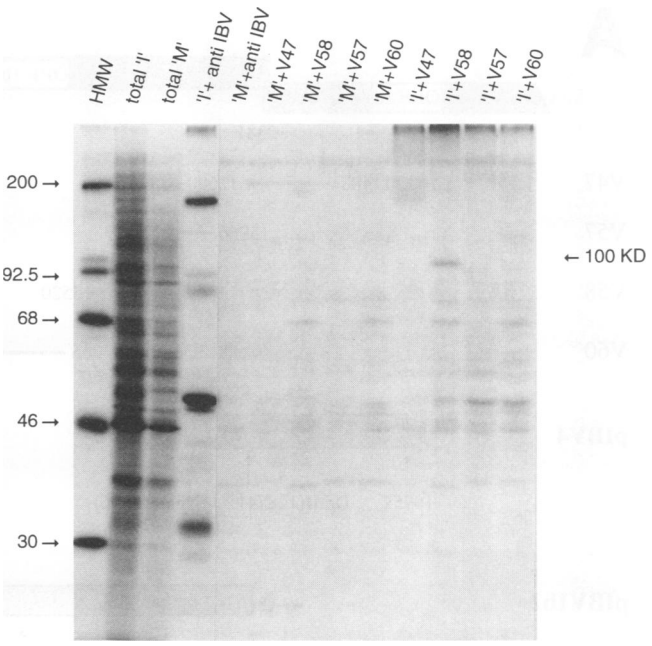

FIG. 2. Detection of polypeptides encoded by ORF 1b in IBV-infected and mock-infected Vero cells by immunoprecipitation with region-specific antisera raised in rabbits against bacterial fusion proteins. Cells were labelled with [^{35}S]methionine, lysates were prepared, and polypeptides were either analyzed directly or immunoprecipitated with the antisera indicated above each lane as described in Materials and Methods. Polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. The lane labelled HMW contains high-molecular-mass markers (Amersham). 'M', mock-infected Vero cell lysate; 'I', IBV-infected Vero cell lysate.

ORF 1a stop product and the 1a-1b fusion product generated by a ribosome frameshifting mechanism, respectively (Fig. 3B). Similarly, RNA transcripts from *Hin*dIII-digested pIBV1b2 plasmid DNA, containing IBV sequence from nucleotides 12625 to 16091, directed synthesis of a major polypeptide of 125 kDa (Fig. 3B). No processed products were observed from the in vitro translation of these two plasmids.

Immunoprecipitation of the in vitro-synthesized products showed that antisera V57 and V58 reacted with the relevant target sequences and precipitated specifically the corresponding 125K protein products, though antiserum V57 showed only weak reactivity with the target protein (Fig. 3B). This may explain the low level of reactivity of V57 to the 100K polypeptide detected in the virus-infected cells. Surprisingly, however, antiserum V47 reacted strongly with both the 60K ORF 1a stop polypeptide and the 75K 1a-1b fusion protein generated from translation of the pIBV4-*Dra*II-derived transcripts (Fig. 3B). This was unexpected, since immunoprecipitation of virus-infected Vero cell lysates with this antiserum showed no detection of any specific viral products (Fig. 2). Antiserum V60 was also shown to react specifically with the related in vitro-synthesized target (data not shown); once again, no protein products from virus-infected cells were detected with this antiserum. Why certain antisera react strongly against in vitro-synthesized material but do not recognize virus-infected cell products is uncertain, but it raises the possibility that processing may be complex and that control mechanisms may exist to regulate expression and accumulation of gene products encoded by the corresponding regions of mRNA 1.

Expression and processing of the 100K polypeptide in a

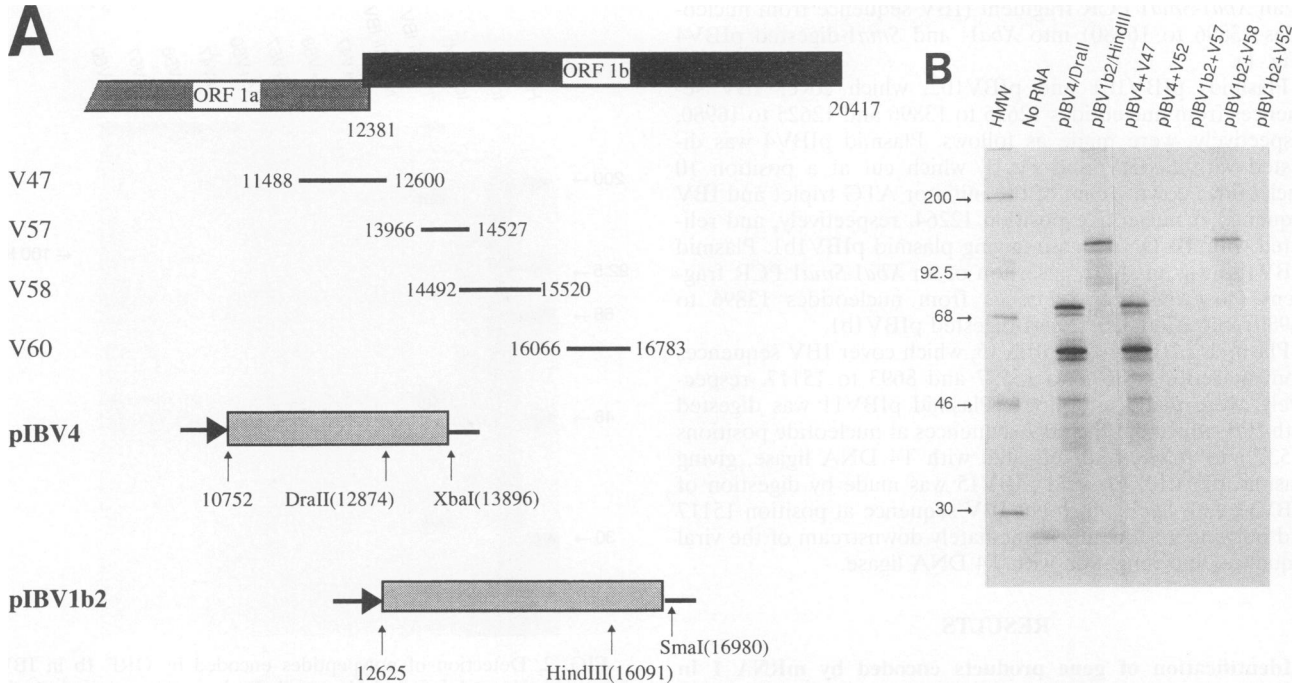

FIG. 3. (A) Four regions of ORFs 1a and 1b which have been expressed in *Escherichia coli* as bacterial fusion proteins and used to raise antisera in rabbits. The expressed region for each antiserum is indicated by a black bar, and numbers indicate the nucleotide positions. Also shown are the structures of plasmids pIBV4 and pIBV1b2, with the T7 promoter and the restriction enzyme sites used to linearize the plasmids for in vitro transcription. (B) Testing the specificities of four antisera by immunoprecipitation against the in vitro-synthesized target polypeptides. In vitro-transcribed RNAs derived from *Dra*II-digested pIBV4 and *Hin*dIII-digested pIBV1b2 were translated in a reticulocyte lysate cell-free translation system, and the in vitro-translated polypeptides were analyzed directly or immunoprecipitated with the antisera indicated above each lane. V52, negative control antiserum. Polypeptides were separated on an SDS–16% polyacrylamide gel and detected by fluorography. The lane labelled HMW contains high-molecular-mass markers (numbers indicate kilodaltons).

transient eukaryotic expression system. Detection of the 100K polypeptide in IBV-infected Vero cells with region-specific antiserum V58 indicated that this protein is encoded by the corresponding region of IBV ORF 1b and proteolytically cleaved from the 1a-1b polyprotein. In vitro translation of IBV sequence from nucleotides 12625 to 16091, however, showed that the final product of 125 kDa could not be cleaved (Fig. 3b), indicating that additional viral sequence(s) is required for processing of the 100K polypeptide. Comparisons of the amino acid sequences of IBV ORFs 1a and 1b by using a computer-aided program predicted that a picornavirus 3C-like proteinase domain was located at nucleotide positions 8963 to 9360 of the ORF 1a region (15). It was possible, therefore, that this domain may be responsible for processing of the 100K protein. To test this, two plasmids, pIBV10 and pIBV11, which cover IBV sequences from nucleotides 8693 to 13896 and 8693 to 16980, respectively, were constructed (Fig. 4A). In vitro translation of pIBV10- and pIBV11-derived RNAs by using the conventional reticulocyte lysate cell-free translation system failed to produce distinct full-length protein products, although full-length RNA transcripts were used to program the reticulocyte lysate (data not shown). Therefore, we decided to express the two plasmids in intact eukaryotic cells, using the system described by Fuerst et al. (13), by which Vero cells were infected with a recombinant vaccinia virus expressing the T7 phage RNA polymerase and subsequently transfected with plasmid DNAs from pIBV10 and pIBV11. The results of this experiment are shown in Fig. 4B. No specific protein products were detected by immunoprecipitation of pIBV10-transfected Vero cell lysates with antisera V47 and V58, but a polypeptide comigrating with the 100K protein identified from virus-infected cells was clearly detectable on immunoprecipitation of pIBV11-transfected cells with antiserum V58, although the expression efficiency was consistently low. No specific products were detected from the same lysates with antiserum V47 (Fig. 4B). These results confirm that the 100K polypeptide is encoded and processed by IBV sequence information within nucleotides 8693 to 16980.

To define roughly the C-terminal boundary of the 100K protein within the polyprotein encoded by this region of mRNA 1, two C-terminal deletion constructs (pIBV14 and pIBV15) were made. Plasmids pIBV14 and pIBV15 were constructed by deletion of pIBV11 viral sequences from nucleotides 15537 to 16788 and 15117 to 16980, respectively (Fig. 4A). As shown in Fig. 4B, expression of pIBV14 in Vero cells directed the synthesis of a polypeptide comigrating with the 100K polypeptide identified from IBV-infected cells. However, transfection of pIBV15 yielded a polypeptide that migrated slightly more slowly than the 100K polypeptide did on SDS-PAGE (Fig. 4b), suggesting that this deletion may block the C-terminal cleavage and therefore lead to addition of some extra amino acids to the 100K polypeptide. Examination of the C-terminal sequence of pIBV15 revealed that 15 additional nucleotides derived from the vector sequence were fused with ORF 1b before reaching a stop codon, resulting in synthesis of a fusion polypeptide with five extra amino acids. This result suggested that the C-terminal cleavage site lies close to nucleotide position 15120.

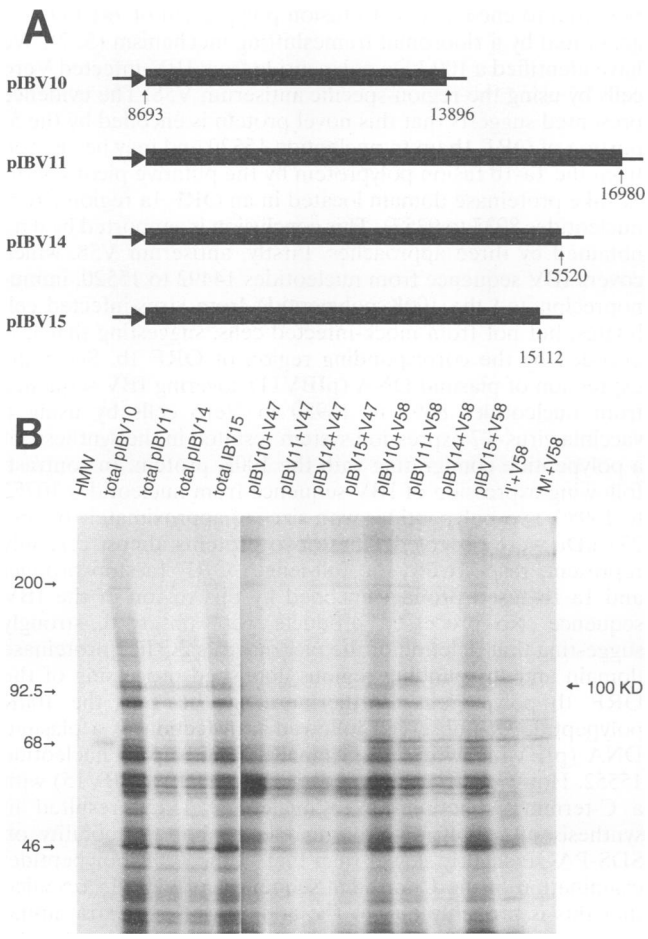

FIG. 4. (A) Structures of plasmids pIBV10, pIBV11, pIBV14, and pIBV15. (B) Analysis of transiently expressed ORF 1a and 1b products from plasmids pIBV10, pIBV11, pIBV14, and pIBV15 by the system described by Fuerst et al. (13), by which Vero cells were infected with a recombinant vaccinia virus expressing the T7 RNA polymerase and subsequently transfected with plasmid DNAs. Cells were labelled with [^{35}S]methionine, lysates were prepared, and polypeptides were analyzed directly or immunoprecipitated with the antisera indicated above each lane. Polypeptides were separated on an SDS–12.5% polyacrylamide gel and detected by fluorography. The lane labelled HMW contains high-molecular-mass markers. 'M', mock-infected Vero cell lysate; 'I', IBV-infected Vero cell lysate.

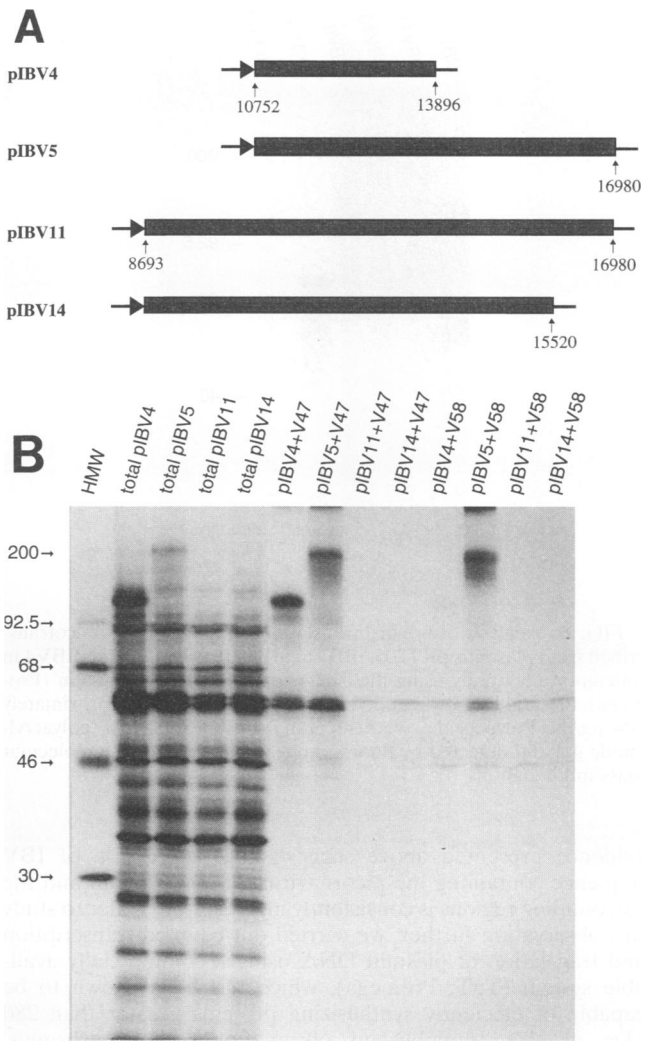

FIG. 5. (A) Structures of plasmids pIBV4, pIBV5, pIBV11, and pIBV14. (B) Analysis of transiently expressed ORF 1a and 1b products from plasmids pIBV4, pIBV5, pIBV11, and pIBV14 with the vaccinia virus-T7 recombinant virus expression system. Cells were labelled with [^{35}S]methionine, lysates were prepared, and polypeptides were analyzed directly or immunoprecipitated with the antisera indicated above each lane. Polypeptides were separated on an SDS–15% polyacrylamide gel and detected by fluorography. The lane labelled HMW contains high-molecular-mass markers (numbers indicate kilodaltons).

Involvement of the putative picornavirus 3C-like proteinase domain in processing of the 100K polypeptide. The results described above confirm that the 100K polypeptide is generated from the N-terminal portion of ORF 1b up to nucleotide 15120 and suggest that it is cleaved from the 1a-1b polyprotein by proteolytic processing. Since the 100K polypeptide is generated when IBV sequences covering the putative picornavirus 3C-like proteinase domain are present, it is tempting to speculate that this domain and its surrounding regions may be involved in processing of this polypeptide. To address this possibility directly, two additional plasmids, pIBV4 and pIBV5, which cover IBV sequences from nucleotides 10752 to 13896 and 10752 to 16980, respectively (Fig. 5A), and do not contain the 3C region were expressed in Vero cells. As shown in Fig. 5B, transfection of plasmids pIBV4 and pIBV5 directed efficient synthesis of polypeptides with sizes of approximately 60 and 118 kDa (pIBV4) and 60 and 235 kDa (pIBV5). These polypeptides represent the ORF 1a stop product (60K) and the 1a-1b fusions (118K and 235K). As expected, all three polypeptides were immunoprecipitated efficiently by antiserum V47, whereas the 235K protein was precipitated only by V58. Significantly, no processed products were observed, indicating the involvement of the picornavirus 3C-like domain and the surrounding regions in processing of the 100K polypeptide. In comparison with the levels of protein synthesis seen with the transfection of pIBV4 and pIBV5, expression of the 100K polypeptide from pIBV11 and pIBV14 occurs with very low efficiency (Fig. 5B); the 100K protein band was seen only after a considerably longer exposure (data not shown).

In vitro translation of plasmid DNA covering the picornavirus 3C-like proteinase domain by using the TnT system. The

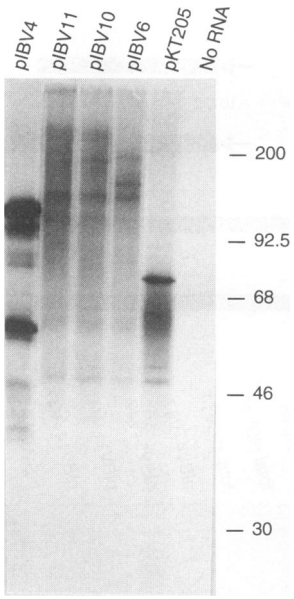

FIG. 6. Analysis of in vitro translation products of RNAs cotranscribed from plasmids pKT205, pIBV6, pIBV10, pIBV11, and pIBV4 in reticulocyte lysate by using the TnT coupled translation system (Promega). Plasmid DNA was added to reticulocyte lysate at approximately 200 μg/ml. Polypeptides were separated on an SDS–15% polyacrylamide gel and detected by fluorography. Numbers indicate molecular mass in kilodaltons.

evidence presented above suggests that expression of IBV sequence containing the picornavirus 3C-like domain and the surrounding regions is consistently inefficient. In order to study this observation further, we carried out coupled transcription and translation of plasmid DNA using a commercially available system (TnT; Promega), which has been shown to be capable of efficiently synthesizing proteins greater than 280 kDa in size (unpublished observations). Four plasmids, pKT205, pIBV6, pIBV10, and pIBV11, which contain IBV sequences from nucleotides 8693 to 10925, 8693 to 12700, 8693 to 13896, and 8693 to 16980, respectively, together with pIBV5, were translated in vitro by this system. As shown in Fig. 6, pKT205 directed synthesis of the full-length product of 82 kDa. Transcription and translation of pIBV6, pIBV10, and pIBV11 led to synthesis of a 138-kDa polypeptide, representing the ORF 1a stop product. In addition, two polypeptides of approximately 150 and 195 kDa, which may represent 1a-1b fused products, were clearly observed from expression of pIBV6 and pIBV10, respectively (Fig. 6). No full-length products from translation of pIBV11 were observed. As can be seen from the gel (Fig. 6), expression from plasmids pIBV6, pIBV10, and pIBV11 shows greatly reduced synthesis of proteins of the expected size in comparison with expression from pIBV4, and in addition, there is a heterogenous smear of polypeptides covering a large size range, confirming the in vivo observation and indicating that this region may contain elements that down-regulate or otherwise influence the expression of downstream sequence or the accumulation of the gene products.

DISCUSSION

The 27.6-kb genome-length mRNA 1 of the coronavirus IBV has previously been shown to contain in its 5′-terminal unique region two large ORFs (ORFs 1a and 1b) with the potential to encode a 1a-1b fusion polyprotein of 741 kDa (3) generated by a ribosomal frameshifting mechanism (5, 7). We have identified a 100-kDa polypeptide from IBV-infected Vero cells by using the region-specific antiserum V58. The evidence presented suggests that this novel protein is encoded by the 5′ portion of ORF 1b up to nucleotide 15520 and may be cleaved from the 1a-1b fusion polyprotein by the putative picornavirus 3C-like proteinase domain located in an ORF 1a region (from nucleotides 8937 to 9357). This conclusion is supported by data obtained by three approaches. Firstly, antiserum V58, which covers IBV sequence from nucleotides 14492 to 15520, immunoprecipitated the 100K polypeptide from virus-infected cell lysates, but not from mock-infected cells, suggesting that it is encoded by the corresponding region of ORF 1b. Secondly, expression of plasmid DNA (pIBV11) covering IBV sequence from nucleotides 8693 to 16980 in Vero cells by using a vaccinia virus-T7 expression system resulted in the synthesis of a polypeptide comigrating with the 100K protein. In contrast, following expression of IBV sequence from nucleotides 10752 to 16980, two polypeptides with sizes of approximately 60 and 235 kDa were observed. These two proteins almost certainly represent, respectively, the full-length ORF 1a stop product and 1a-1b fused product encoded by this region of the IBV sequence. No processed products were observed, strongly suggesting that deletion of the picornavirus 3C-like proteinase domain and surrounding regions abolished processing of the ORF 1b polyprotein. Furthermore, synthesis of the 100K polypeptide was observed followed transfection of a plasmid DNA (pIBV14) with a C-terminal deletion up to nucleotide 15552. However, transfection of plasmid DNA (pIBV15) with a C-terminal deletion up to nucleotide 15120 resulted in synthesis of a polypeptide with electrophoretic mobility on SDS-PAGE slightly slower than that of the 100K polypeptide; examination of the C-terminal sequences of pIBV15 revealed that this is probably due to the addition of five extra amino acids from the vector sequence to the 100K protein, thereby altering the mobility of the polypeptide. This result suggests that the sequence encoding the C terminus of the 100K polypeptide lies close to nucleotide 15120.

The observation that the C terminus of the 100K polypeptide is specified by ORF 1b sequences close to nucleotide 15120 raises the possibility that this polypeptide may be generated by proteolysis at a potential QS cleavage site encoded by nucleotides 15129 to 15135 (15). A potential QS cleavage site is also present from nucleotides 12310 to 12315 (15). Cleavage at both QS sites would result in the production of a polypeptide with a calculated molecular mass of 107.9 kDa, which is close to the size of the 100K polypeptide identified in this study. Further deletion and site-directed mutagenesis studies to investigate potential QS cleavages are under way.

It was previously reported that Western immunoblots of IBV-infected Vero cells with antisera V57 and V58 led to detection of a polypeptide of approximately 49 kDa (6). However, no similar product was identified in this study. One possible explanation is that the 49K polypeptide represents a product of further processing of the 100K protein and contains epitopes recognized by the antisera only after denaturation of the protein. However, no additional proteolytic cleavage sites are predicted within the 100K polypeptide-encoded region (15), arguing against the likelihood that further processing of the 100K polypeptide occurs. The reason for this discrepancy is not yet understood.

Evidence presented shows that production of the 100K polypeptide was consistently inefficient both in IBV-infected cells and in a transient eukaryotic expression system. In vitro

translation and in vivo transfection studies indicate that the ORF 1a region between nucleotides 8763 and 10720 contains elements that down-regulate the expression of ORF 1b. The mechanisms that may account for this down-regulation are currently unclear, but there are a number of possibilities. The ORF regions of some mRNAs have been shown to contain destabilizing sequences, which can cotranslationally promote rapid RNA decay, possibly initiated by translocation of ribosomes through these regions. Examples of this type of regulatory mechanism include expression of mRNAs encoding β-tubulin (14, 24), c-*fos* (26), and the yeast MATα1 (25). It is yet to be determined if a similar destabilizing signal is located in this region of IBV mRNA 1. Alternatively, rapid turnover of the protein products encoded by this region of the IBV sequences may limit the expression of ORF 1b or control the accumulation of the gene products. The encephalomyocarditis virus 3C proteinase, together with some polyproteins containing a mutated, inactive 3C proteinase catalytic site, has recently been shown to be rapidly and selectively degraded by an ATP-dependent proteolytic system present in reticulocyte lysate (23).

It is currently uncertain what role the 100K polypeptide may play in viral RNA transcription and replication. Preliminary observations from cellular fractionation studies of IBV-infected Vero cells indicate that the 100K polypeptide is located mainly in the membrane fraction (unpublished observation). The putative polymerase domain is predicted to be located in the ORF 1b region between nucleotides 14100 and 14798 (15), a sequence which is contained within the nucleotide region encoding the 100K polypeptide. This strongly suggests that the 100K polypeptide is the viral protein associated with viral RNA-dependent RNA polymerase activity. Although we have been able to detect only low levels of poly(A)-dependent oligo(U)-primed poly(U) polymerase activity in IBV-infected Vero cells (unpublished observation), the identification, expression, and processing of the 100K polypeptide in a eukaryotic expression system described in this study should facilitate further investigation of coronavirus RNA transcription and replication.

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