PRODUCTS OF THE POLYMERASE-ENCODING REGION OF THE CORONAVIRUS IBV

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INTRODUCTION

The avian coronavirus infectious bronchitis virus (IBV) has a single-stranded, polyadenylated, positive-sense RNA genome some 27.6 Kb in length (Boursnell *et al.*, 1987). IBV replication almost certainly proceeds by the same mechanism that occurs in the more intensively studied coronavirus, mouse hepatitis virus (MHV). Genomic RNA is first translated to produce the viral RNA-dependent RNA polymerase, which transcribes the genomic RNA to generate a negative-stranded full-length copy of the virion RNA. This copy RNA then serves as a template for the production of a nested set of 3′-co-terminal transcripts from which virus polypeptides are translated (reviewed in Spaan *et al.*, 1988). These transcripts include a genomic-sized RNA (mRNA 1) and five subgenomic RNAs (mRNAs 2-6) of which mRNA 6 is the smallest (Stern and Kennedy, 1980). Almost all detailed analysis of IBV gene function to date has concentrated on the products of mRNAs 2, 4 and 6 which encode the viral structural proteins spike, membrane and nucleocapsid respectively (see Figure 1). Together, these mRNAs account for less than 30% of the genomic coding capacity; most of the remaining information is in two large, overlapping open-reading frames (ORFs) 1a and 1b, encoded by mRNA 1. These ORFs could potentially produce some 740 kD of protein and must encode the RNA polymerase (Boursnell *et al.*, 1987). A number of associated activities predicted to be involved in viral replication are also suspected to be encoded by mRNA 1 (Gorbalenya *et al.*, 1989). To date, however, the only product of this RNA that has been detected in coronavirus-infected cells is a 28 kD MHV polypeptide (p28) of unknown function (Denison and Perlman, 1987) which is derived from the 5′-end of the MHV genome (Soe *et al.*, 1987).

In this study, we have investigated expression of the 1a and 1b ORFs of IBV by using a panel of monospecific antisera raised against portions of the two ORFs. A number of polypeptides encoded by each ORF have been identified in infected cells and are likely to have arisen as a result of processing of larger precursor translation products. This is the first identification of products encoded by the polymerase-encoding region of IBV.

METHODS AND RESULTS

Expression of RNA 1 ORFs in bacteria

Our approach to the identification of polypeptides encoded by RNA 1 has been to prepare antisera against regions of ORFs 1a and 1b through expression of portions of these ORFs in bacteria, and to use these sera as probes for the analysis of infected cells.

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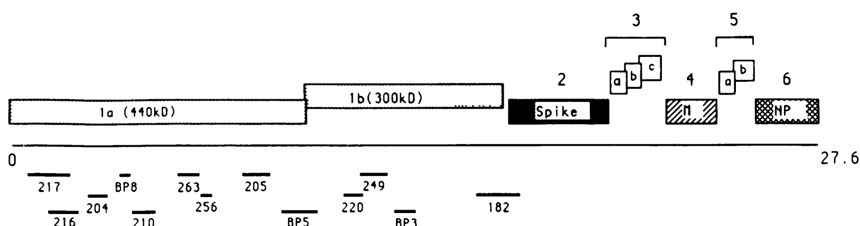
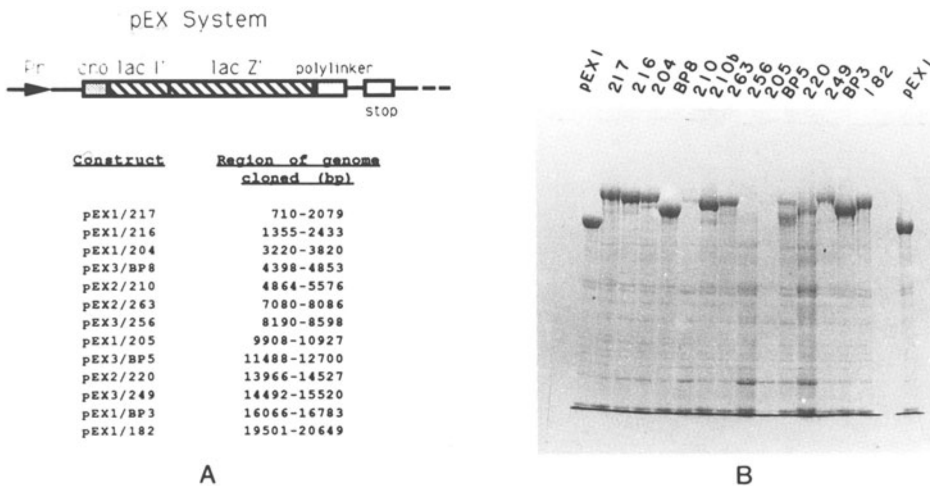


Figure 1. Organisation of the IBV genome. The boxes represent open reading frames and are numbered (1-6) with respect to the particular subgenomic mRNA from which they are expressed. Regions of the 1a and 1b ORFs which have been cloned into the pEX expression system are indicated by black bars and are numbered with respect to the original cDNA clone from which they were derived.

Our strategy was based on the pEX series of plasmids developed by Stanley and Luzio (1984), which allows the expression of "foreign" sequence information as a C-terminal fusion with β-galactosidase. Expression of the fusion protein is under the control of the bacteriophage lambda repressor; in bacterial cells containing a temperature sensitive repressor, expression is induced by raising the temperature (from 30°C to 42°C). Following induction, harvested bacteria are lysed and a Triton-insoluble pellet obtained which contains the crude fusion protein. The genomic position of each of the regions expressed in this study is shown in Figure 1 and the precise sequence information in Figure 2a. Each construct was numbered with reference to the original cDNA clone from which the IBV information was derived (detailed in Boursnell *et al.*, 1987). In total, some 65% of the 1a and 45% of the 1b coding regions were cloned and expressed in *E. coli.* Figure 2b shows the proteins synthesized in bacteria harbouring either wild-type (pEX1) or recombinant pEX plasmids following a one hour induction. All of the constructs expressed high levels of fusion protein (10-40 mg/litre) with the exception of constructs pEX256 and pEX205. The fusion proteins were purified by preparative SDS-polyacrylamide gel electrophoresis and electroelution from gel slices, and used to immunize rabbits. Animals were inoculated initially with approximately 0.1 mg of purified protein emulsified in Freund's Complete Adjuvant by intramuscular injection and at monthly intervals were boosted by the same route, using similar amounts of material in Freund's Incomplete Adjuvant.

Products of mRNA 1 ORFs 1a and 1b

We have investigated expression of mRNA 1 *in vivo* by testing the ability of the antisera raised against regions of ORFs 1a and 1b to recognize IBV-specified polypeptides in infected cell lysates in Western blotting experiments. Confluent chick kidney (CK) or VERO cell monolayers were inoculated with the Be-42 strain of IBV (at a multiplicity of infection of 1, 90 minutes at 37°C) and the cells harvested at various times post infection. Viral replication was essentially completed by about 16 hours post-infection in CK cells and some 24 hours post-infection in VERO cells. Samples were prepared for Western blot analysis as follows. Mock-infected or infected cell pellets were resuspended in phosphate-buffered saline (PBS) (at 5 x 10^6 cells/ml), mixed with an equal volume of twice-strength Laemmli's sample buffer (Laemmli, 1970), sonicated for 30 seconds in the presence of 1mM phenylmethylsulphonylfluoride and boiled for 4 minutes. Aliquots were loaded onto 4-20% gradient SDS-polyacrylamide gels and following electrophoresis, proteins were transferred to nitrocellulose by electroblotting (Bio-Rad Transblot). Following transfer, filters were probed with 1% solution of the relevant antiserum in 5% low-fat milk, 1% foetal calf serum in PBS, and bound antibodies detected using ^{125}I-labelled Staphylococcus protein A.



Construct	Region of genome cloned (bp)
pEX1/217	710-2079
pEX1/216	1355-2433
pEX1/204	3220-3820
pEX3/BP8	4398-4853
pEX2/210	4864-5576
pEX2/263	7080-8086
pEX3/256	8190-8598
pEX1/205	9908-10927
pEX3/BP5	11488-12700
pEX2/220	13966-14527
pEX3/249	14492-15520
pEX1/BP3	16066-16783
pEX1/182	19501-20649

Figure 2. pEX expression system. A. Regions of the IBV genome which have been cloned into the pEX polylinker and expressed in *E. coli* are shown. B. Expression of β-galactosidase-IBV fusion proteins in *E. coli.* Bacteria harbouring wild-type (pEX1) or recombinant pEX plasmids were grown for 2 hours and 30oC then induced for 1 hour at 42oC. Following lysis, a Triton-insoluble pellet was obtained and a small aliquot ran on a 7.5% SDS-polyacrylamide gel. Proteins were stained with Coomassie brilliant blue R. The major product in each track is the fusion protein.

In Figure 3, a western blot experiment is shown in which both CK and VERO mock-infected or infected cell lysates from varying times post-infection have been probed with antiserum 216 (α-216), a serum raised against a 5′-proximal region of 1a. In CK cells, a large number of non-specific bands common to both mock-infected and infected cells were seen. However, two proteins were detected which were unique to infected cells and accumulated during viral replication, one of 75 kD, the other 220 kD. In IBV-infected VERO cells, the 75 kD and 220 kD products were also detected, although the 220 kD product was less abundant. Two additional low-abundance proteins of 90 kD and 180 kD were detected in VERO cells. No specific bands were seen when similar filters were probed with pEX 216 pre-immune serum or an irrelevant hyperimmune serum raised against the influenza PA protein (not shown). Because of the background of non-specific bands associated with CK cells, the investigation was continued with VERO cells only.

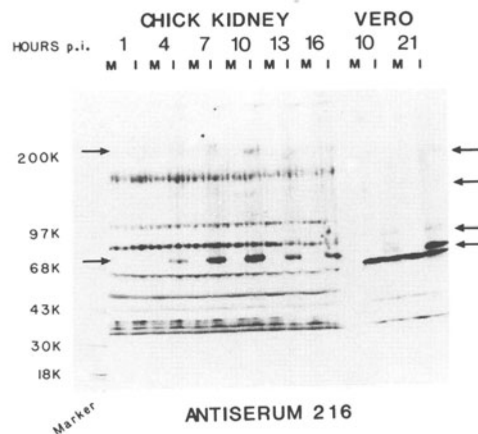


Figure 3. Detection of 1a expression products with antiserum 216. IBV-infected (I) or mock-infected (M) chick kidney or Vero cells from various times post-infection were probed in a Western blot with antiserum 216. Products unique to infected cells are indicated by arrows.

The western blot in Figure 4 shows the products seen in infected VERO cells when filters were probed with antisera α204 and α210, sera raised against central portions of 1a. The patterns of reactivity seen were highly similar; four specific proteins of approximate molecular weights 62kD, 80kD, 140kD and 180kD were detected by both antisera. Antiserum α210 recognised, in addition, a 40kD protein. The regions of cDNA from which α204 and α210 were raised are closed spaced on the IBV genome; it is likely, therefore that the sera are recognising the same group of polypeptides. When an identical filter was probed with pEX210 pre-immune serum (at four times the immune serum concentration), no specific products were recognised. This was also the case for pre-immune serum from pEX204 (not shown). Our preliminary analysis of 1a expression, therefore, suggests strongly that the primary translation product of the ORF is processed to yield a number of cleavage products which accumulate during infection. Whether the proteins detected in these experiments represent the final products of processing is not yet clear since this type of immunological analysis enables only the detection of accumulated products of ongoing viral replication.

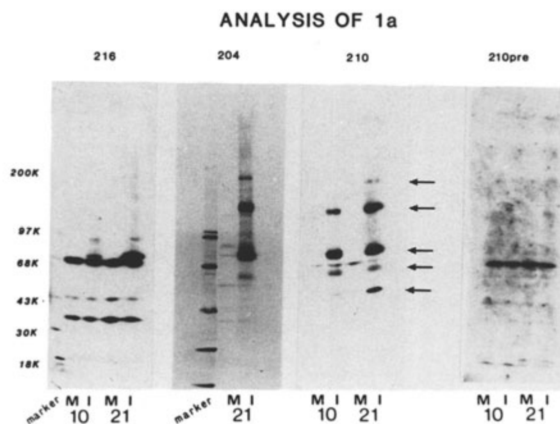


Figure 4. Further analysis of 1a expression. IBV-infected (I) or mock-infected (M) Vero cells were probed with antiserum 216, 204, 210 or with 210 pre-immune sera in a Western blot experiment.

Prior to this study, the only product that had been detected from the polymerase-encoding region of a coronavirus was a 28kD polypeptide of MHV expressed from the extreme 5′-end of the 1a coding sequence (Denison and Perlman, 1987; Soe *et al.*, 1987). We have attempted to determine whether a similar protein is expressed in IBV-infected cells by using antiserum α217. This serum was raised against a 1.4kb region at the extreme 5′-end of the IBV 1a ORF. As yet, however, we have no evidence that p28 is produced in IBV-infected cells, since the α217 serum did not detect any specific proteins. The most likely explanation for the lack of reactivity seen, however, is that the pEX217 immunization has failed to produce a suitable immune response in the experimental animals. As the α217 serum was raised against a cDNA which overlapped considerably with that used to raise antiserum α216, we expected that the α217 serum would recognise the 75kD and 220kD proteins seen with α216. Thus expression of the extreme 5' end of the IBV 1a ORF remains to be characterized.

In addition to the 1a analysis, we have also carried out a preliminary analysis of 1b expression in a similar manner. Mock-infected or IBV-infected VERO cells harvested at twenty hours post-infection were blotted and probed with three anti-1b sera, α220, α249 or α182. As can be seen in Figure 5, no high molecular weight proteins were detected with any of the three 1b antisera tested. The most C-terminal serum, α182 detected three specific proteins of approximate molecular weights 30kD, 34kD and 36kD. Antisera α220 and α249 recognised only a single polypeptide of 49kD. As these antisera were raised against overlapping 1b cDNAs, the sera are probably recognising the same

polypeptide. It therefore appears that the primary translation of 1b is also processed post-translationally.

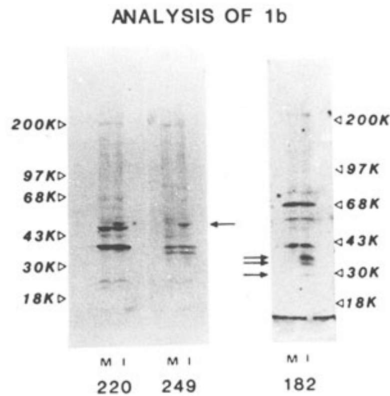


Figure 5. Detection of 1b expression products. IBV-infected (I) or mock-infected (M) Vero cells 21 hours post infection were probed with antiserum 220, 249 or 182 in a Western blot experiment. Products unique to infected cells are indicated by arrows.

DISCUSSION

The complete sequence of IBV (Boursnell *et al.*, 1987) has revealed that the 'unique' region of the genomic RNA contains two extremely large, briefly overlapping ORFs 1a and 1b, which have a 740kD protein coding capacity. In this study we have prepared a panel of 13 monospecific antisera against selected portions of the two ORFs and used a number of these in Western blots to identify products in infected cells. Our results indicate that the primary translation products of the two ORFs appear to be processed proteolytically, giving rise to a number of polypeptides in infected cells. The largest abundant species identified was a 220kD protein encoded within 1a. Recent *in vitro* work on the mechanism of 1a/1b expression has suggested that *in vivo*, 1b is probably expressed as a fusion with the overlapping 1a ORF following a ribosomal frameshift event during translation of the genomic RNA (Brierley *et al.*, 1987;1989; Inglis *et al.*, this volume). Thus the predicted primary translation products are a 440kD product corresponding to 1a, and a 740kD product corresponding to a 1a-1b fusion protein. In this study, we were not able to detect proteins of such size in any quantity. Longer exposures of the 1a blots, however, did reveal a number of minor species with molecular weights considerably in excess of 220kD. These may represent intermediates produced early in a cleavage pathway.

A more rigorous analysis of mRNA 1 expression has been hindered by our inability to detect reproducibly products by radioimmuneprecipitation. The reason for this is uncertain, but may reflect the method of production of the sera, since rabbit antibodies were raised against denatured protein in this study. Nevertheless, when the regions of cDNA used in the pEX constructs were cloned into SP6-based transcription vectors, and synthetic mRNAs derived from these vectors translated *in vitro*, a number of the sera were able to specifically immunoprecipitate the relevant target antigen (not shown). Thus the problem may simply be one of sensitivity.

As the genomic RNA of IBV is infectious (Schochetman *et al.*, 1977) the RNA-dependent RNA polymerase(s) of the virus is almost certainly encoded by 1a/1b; indeed a region in the 1b coding sequence has been predicted to encode an RNA polymerase activity based on a sequence homology with the RNA polymerases of other positive-stranded RNA viruses (Hodgman, 1988; Gorbalenya *et al.*, 1989). To date, no RNA polymerase activity has been detected in IBV-infected cells. However, such an activity has been reported in MHV-infected (Brayton *et al.*, 1982; Mahy *et al.*, 1983) and in transmissible gastroenteritis virus (TGEV)-infected cells (Dennis and Brian, 1982).

Recent sequence analysis of MHV (Bredenbeek *et al.*, this volume) has revealed that MHV, like IBV, has two large overlapping ORFs in the unique region of the genomic RNA and, in addition, the RNA polymerase 'homology' sequence found in IBV, is conserved in MHV. In our preliminary analysis of IBV 1a/1b expression described here, two antisera (α220 and α249) which were raised against the region of the genome containing this polymerase homology region reacted with a 49kD protein in infected VERO cells. Whether this protein has any polymerase activity remains to be determined.

ACKNOWLEDGEMENTS

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REFERENCES

Boursnell, M.E.G., Brown, T.D.K., Foulds, I.J., Green, P.F., Tomley, F.M. and Binns, M.M., 1987, Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus, J. gen. Virol., 68:57-77.

Brayton, P.R., Lai, M.M.C., Patton, D.F. and Stohlman, S.A., 1982, Characterisation of two RNA polymerase activities induced by mouse hepatitis virus, J. Virol., 42:847-853.

Brierley, I., Boursnell, M.E.G., Binns, M.M., Bilimoria, B., Blok, V.C., Brown, T.D.K. and Inglis, S.C., 1987, An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV, EMBO J., 6:3779-3785.

Brierley, I., Digard, P. and Inglis, S.C., 1989, Characterisation of an efficient coronavirus ribosomal frameshifting signal: requirement for an RNA pseudoknot, Cell, 57:537-547.

Denison, M. and Perlman, S., 1987, Identification of a putative polymerase gene product in cells infected with murine coronavirus A59, Virology, 153:565-568.

Dennis, D.E. and Brian, D.A., 1982, RNA-dependent RNA polymerase activity in coronavirus-infected cells, J. Virol., 42:153-164.

Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. and Blinov, V.M., 1989, Coronavirus genome: prediction of putative functional domains in the non-structural polyprotein by comparative amino acid sequence analysis, Nucl. Acids Res., 17:4847-4860.

Hodgman, T.C., 1988, A new superfamily of replicative proteins, Nature, 333:22-23.

Laemmli, U.K., 1970, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature, 227:680-685.

Mahy, B.W.J., Siddell, S., Wege, H. and ter Meulen, V., 1983, RNA dependent RNA polymerase activity in murine coronavirus infected cells, J. gen. Virol., 64:103-111.

Schochetman, G., Stevens, R.H. and Simpson, R.W., 1977, Presence of infectious polyadenylated RNA in the coronavirus avian infectious bronchitis virus, Virology, 77:772-782.

Soe, L.H., Shieh, C.K., Baker, S.C., Chang, M.F. and Lai, M.M.C., 1987, Sequence and translation of the murine coronavirus 5'-end genomic RNA reveals the N-terminal structure of the putative RNA polymerase, J. Virol., 61: 3968-3976.

Spaan, W., Cavanagh, D. and Horzinek, M.C., 1988, Coronaviruses: structure and genome expression, J. gen. Virol., 69:2939-2952.

Stanley, K.K. and Luzio, J.P., 1984, Construction of a new family of high efficiency bacterial expression vectors: identification of cDNA clones coding for human liver proteins, EMBO J, 3:1429-1434.

Stern, D.F. and Kennedy, S.I.T., 1980, Coronavirus multiplication strategy. I. Identification and characterisation of virus-specified RNA, J. Virol., 34:665-674.