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Identification of a new membrane-associated polypeptide specified by the coronavirus infectious bronchitis virus

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Nucleotide sequences from the third open reading frame of mRNA D (D3) of infectious bronchitis virus (IBV) were expressed in bacteria as part of a fusion protein with β-galactosidase. Antiserum raised in rabbits against this fusion protein immunoprecipitated from IBV-infected chick kidney or Vero cells a polypeptide of 12·4K, the size expected for a D3-encoded product. The D3 polypeptide is apparently non-glycosylated, and appears to be associated with the membrane fraction of infected cells, as judged by cell fractionation and immunofluorescence.

Introduction

Infectious bronchitis virus (IBV), a pathogen of chickens, is the prototype virus of the *Coronaviridae*, a group of enveloped viruses with a large, continuous, positive-stranded RNA genome. Expression of the genome of this class of viruses is mediated through the production in infected cells of a series of subgenomic mRNAs, and in the case of IBV, six major species of virus-specific mRNA are produced (Stern & Kennedy, 1980*a*). These have been designated mRNA A to mRNA F in order of increasing size [mRNA F corresponds to the size of the genomic RNA, i.e. approximately 27 kb (Boursnell *et al.*, 1987)]. These mRNAs form a nested set (Stern & Kennedy, 1980*b*), in which the sequences present in each of the subgenomic mRNAs are also present in each of the larger mRNAs; each mRNA species has a short 'leader' sequence corresponding to the 5′ end of the genomic RNA (Brown *et al.*, 1984) followed by progressively larger amounts of information derived from the 3′ end. It has been assumed that for each mRNA, the region of sequence not contained in the next smallest mRNA (the 'unique' region of each mRNA) is used for the translation of viral proteins (Stern & Kennedy, 1980*b*); *in vitro* translation studies on purified mRNAs have supported this view. Using this approach, mRNAs A, C and E have been identified as encoding the virus structural polypeptides, the nucleoprotein (N), membrane protein (M) and spike precursor (S), respectively (Stern & Sefton, 1984). However, the coding functions of mRNAs B, D and F have not yet been assigned, although it is generally assumed that mRNA F encodes the viral RNA-dependent RNA polymerase, as the naked genomic RNA is infectious.

Sequence analysis carried out previously (Boursnell *et al.*, 1985) has shown that the 'unique' region of mRNA D has three distinct open reading frames (ORFs) with the potential to encode polypeptides of M_r values 6·7K (D1), 7·4K (D2) and 12·4K (D3). Here we report the identification of a product of one of these ORFs (D3) in infected cells, and its preliminary characterization.

Methods

Viruses and cells. The Beaudette strain of IBV was a gift from D. Cavanagh (Houghton Poultry Research Station) and was grown in the allantoic cavity of 11-day-old embryonated chicken eggs, obtained from specific pathogen-free flocks; the virus was assayed by plaquing on chick kidney (CK) cell cultures prepared from 2- to 3-week-old birds. Vero cells were grown and maintained in Glasgow MEM supplemented with 10% tryptose phosphate broth and 2% newborn calf serum.

Analysis and purification of bacterial fusion proteins. Overnight cultures of bacteria carrying pEX-based expression plasmids were diluted 40-fold in fresh medium, grown to an optical density (O.D.) at 600 nm of approximately 0·2 at 30 °C, and incubated for a further 2 h at 42 °C. Cells were recovered from a 100 ml culture by centrifugation, incubated in 4 ml of lysozyme solution (0·1 mg/ml in 15% sucrose, 50 mM-Tris–HCl pH 8·0, 50 mM-EDTA) on ice for 40 min, and lysed by the addition of 5·6 ml Triton X-100 (0·2% in 10 mM-Tris–HCl pH 8·0, 1 mM-EDTA) for 5 min on ice. Insoluble material was recovered by centrifugation at 10000 *g* for 15 min, and the pellet was resuspended in 2·5 ml of 8 M-urea containing 2% 2-mercaptoethanol. An equal volume of gel sample buffer (Laemmli, 1970) was added and the samples were boiled for 5 min before electrophoresis on a 10% gel. Samples of 20 µl were analysed. For preparative electrophoresis, 2 ml samples were

fractionated on a single gel-width slot. Fusion proteins were purified by electroelution from bands cut from gels, which had been stained in 1 M-potassium acetate for 15 min at 4 °C to visualize the major proteins.

Preparation of anti-fusion protein antibodies. Rabbits were injected intramuscularly with approximately 100 μg of purified protein emulsified in Freund's complete adjuvant, and boosted at monthly intervals by the same route with similar doses of protein emulsified in Freund's incomplete adjuvant.

Affinity purification of antibodies. Anti-D3 antibodies were purified from crude sera by affinity chromatography using a two-stage procedure. Initially the serum was passed over a column consisting of purified β-galactosidase coupled to CNBr-activated Sepharose (Pharmacia), which retained activity directed against the bacterial component of the fusion protein. Subsequently the flowthrough was applied to a second column consisting of Sepharose coupled to the D3–β-galactosidase fusion protein. The fusion protein was prepared for coupling by the following procedure. A 100 ml culture of *Escherichia coli* POP2136 cells containing the pEXD3 recombinant plasmid was incubated for 1 h at 42 °C to induce synthesis of the fusion protein. Bacteria were recovered by centrifugation and lysed by incubation for 40 min on ice with lysozyme (0·5 mg/ml) in 4 ml of buffer containing 15% sucrose, 50 mM-Tris–HCl pH 8·0, followed by addition of 5·6 ml of Triton X-100 solution (see above) for 5 min. After vigorous mixing, the solution was centrifuged at 10000 *g* for 15 min, and the pellet was resuspended in 10 ml of 8M-urea containing 2% β-mercaptoethanol. This material was dialysed for approximately 2 h against three changes of buffer (0·1 M-$NaHCO_3$, pH 8·3, 0·5 M-NaCl, 5 mM-β-mercaptoethanol), and 2·5 ml (containing approximately 500 μg of the fusion protein) was coupled to 1 ml (bed volume) of CNBr-activated Sepharose according to the manufacturer's instructions. Purified β-galactosidase (Sigma) was coupled by the same procedure. Columns consisting of 1 ml of protein bound to Sepharose were washed extensively with 0·1 M-glycine (pH 2·1) and with phosphate-buffered saline (PBS) containing NP40 (0·1%) before use. Sera (0·5 ml samples) were applied in 5 ml of PBS–NP40 and washed through with PBS. Bound antibodies were eluted by reverse flushing with 10 ml of 0·1 M-glycine pH 2·1, and 0·5 ml fractions were collected in tubes containing 40 μl of 2M-Tris–HCl pH7·6. The procedure was monitored by an ELISA of anti-β-galactosidase activity in the flowthrough and eluted fractions, which indicated greater than 90% recovery.

Production of [^{35}S]cysteine-labelled infected cell lysates. Confluent monolayers of CK or Vero cells were infected with IBV at an m.o.i. of >10. The cells were incubated in cysteine-free medium for 30 min before labelling with [^{35}S]cysteine (300 μCi/ml) for 90 min. Cells were harvested, lysed with RIPA buffer (0·05 M-Tris–HCl pH 7·3, 0·15 M-NaCl, 1% sodium deoxycholate, 0·1% SDS, 1% Triton X-100) and the lysates were pre-cleared by centrifugation at 50000 *g* for 60 min at 4 °C.

Radioimmunoprecipitation. Immunoprecipitation was carried out by standard procedures using Sepharose–Protein A, as described before (Brierley *et al.*, 1987), and with non-fractionated antiserum.

Cell fractionation. The procedure of Hay (1974) was used to fractionate [^{35}S]cysteine-labelled IBV-infected CK cells, which had been harvested at 6·5 h post-infection (p.i.). Briefly, labelled cells were harvested, washed with hypotonic solution and lysed using a Dounce homogenizer. Nuclei were pelleted by centrifugation at 1500 *g* for 5 min, and then centrifuged through a 60% sucrose cushion to remove contaminating membranes. The superatant from the first centrifugation was centrifuged again at 45000 *g*, to remove large membrane fragments, and then at 200000 *g* to pellet small membrane particles and microsomes. The pelleted material was solubilized using RIPA buffer, and the final supernatant was taken as the cytoplasmic fraction.

SDS–PAGE. SDS–PAGE of virus polypeptides was carried out using 20% acrylamide gels (Laemmli, 1970) with an acrylamide : bis-acrylamide ratio of 300 : 1 according to previously described procedures (Inglis *et al.*, 1976). Labelled polypeptides were detected by autoradiography of dried gels.

Indirect immunofluorescence. To detect polypeptides on the surface, cells were used without fixation and all procedures were carried out at 4 °C to prevent capping of immune complexes. To detect polypeptides within cells, fixation was carried out using 2% paraformaldehyde (in isotonic PBS) for 15 min, and the cells were permeabilized by washing in a solution of 1% Triton X-100, 10% sucrose in IFW (1% newborn calf serum in PBS) for 20 min. Indirect immunofluorescence was carried out as described (Dreyfuss *et al.*, 1984). Cells grown on glass coverslips (fixed or unfixed) were washed three times with IFW, and the first stage antiserum was applied (diluted 1 : 50 in IFW). The cells were incubated for 60 min (at 4 °C for unfixed cells or 20 °C for fixed cells) and then washed three times in IFW. The second stage antiserum, sheep anti-rabbit IgG–FITC conjugate (Wellcome Foundation), was added, diluted 1 : 50 in IFW, and incubated for 45 min. The cells were washed four times in IFW, and the coverslips were mounted on glass slides. The mountant, Cityfluor, contained a quenching agent to increase the stability of the FITC under u.v. illumination. Cells were viewed under u.v. light and photographed using Kodak Ektachrome 400 colour reversal film.

Cell-free transcription and translation. Transcription from plasmid DNA using the SP6 phage RNA polymerase was carried out as described previously (Melton *et al.*, 1984), incorporating the dinucleotide ^{7}mGpppG to provide a 5′ cap structure (Contreras *et al.*, 1982). Product RNA was extracted from the reactions with phenol–chloroform (1 : 1), precipitated with ethanol, and further purified by gel filtration on Sephadex G50. The integrity of the RNA was checked by electrophoresis on 2% agarose gels containing formaldehyde and staining with ethidium bromide, before addition to the cell-free system. RNA was translated in the wheatgerm cell-free system in the presence of [^{35}S]methionine as described previously (Inglis *et al.*, 1977), and analysed by gel electrophoresis.

Results

Synthesis of the D3 ORF product in vitro *using artificially synthesized mRNA*

We first tested whether RNA containing the D3 ORF (Fig. 1) could be translated *in vitro* into a polypeptide of the expected size for D3. The observation of such a polypeptide product would lend credence to the idea that the D3 ORF represented a genuine gene, but in addition would provide a convenient marker for recognition of authentic D3 in infected cells. For this purpose, a DNA fragment containing the entire D3 ORF (together with a portion from the 5′ end of the distal membrane ORF) was cloned into the plasmid pSP65 (Fig. 2*a*) which contains a promoter for the bacteriophage SP6 RNA polymerase (Melton *et al.*, 1984). The resulting plasmid (pIBS1) was transcribed *in vitro* after digestion with *Dra*I (to produce an RNA containing only D3 coding sequences) and the RNA product was translated in a cell-free system derived from wheatgerm (Fig. 2*b*). A single major translation

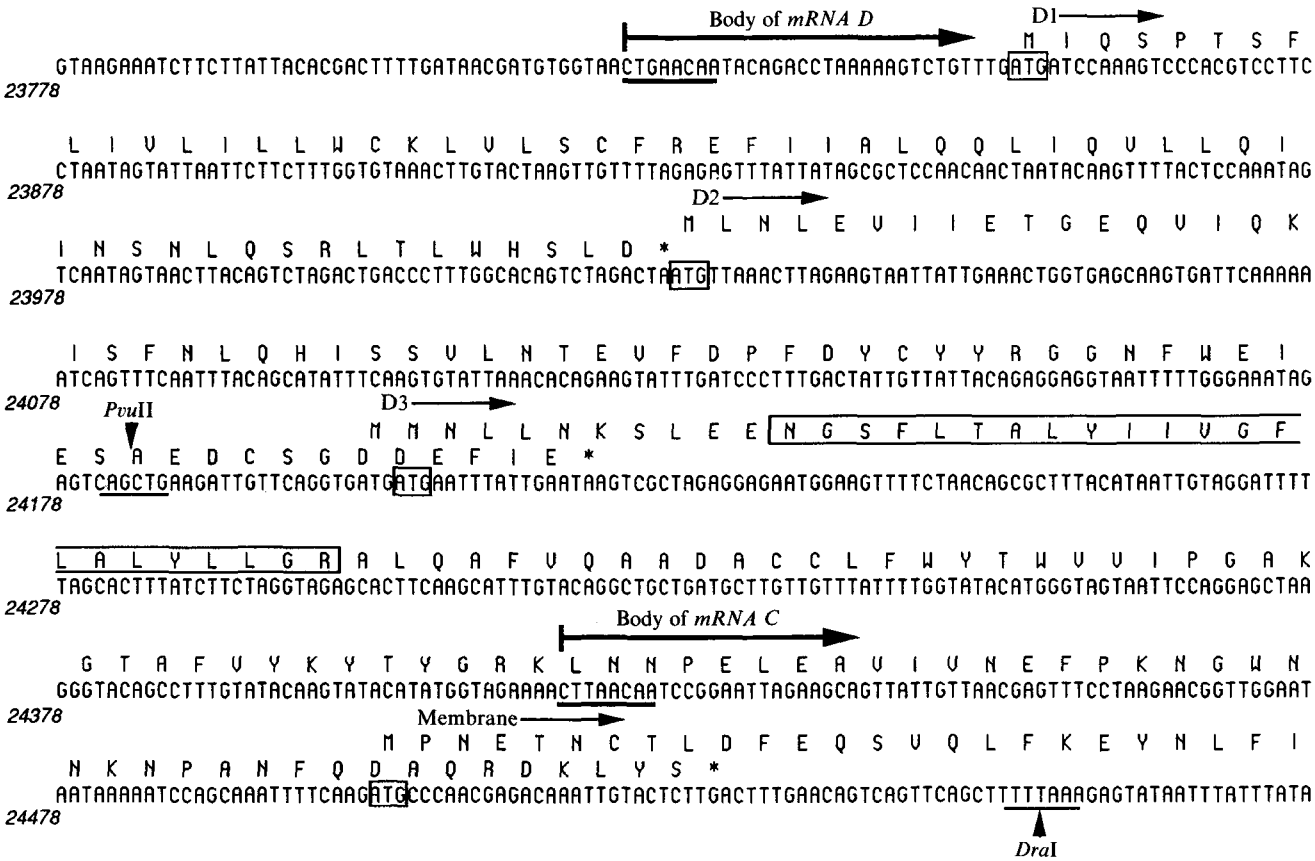

Fig. 1. cDNA sequence of the D region of the IBV genome (Boursnell *et al.*, 1985). Predicted amino acid sequences are shown for the three ORFs contained within the 'unique' region of mRNA D and the start of the coding sequence for the membrane protein gene. Also shown are the 'homology regions' associated with the fusion of the 5′ leader sequence in the synthesis of the subgenomic mRNAs (heavy underlining). A stretch of 21 uncharged amino acids in D3, thought to be a potential membrane-spanning region, is boxed. Nucleotides are numbered from the 5′-end of genomic RNA, based on the sequence information given in Boursnell *et al.* (1987).

product was observed which had a mobility in SDS–PAGE consistent with its predicted M_r of 12·4K. Synthesis of this polypeptide by the cell-free system was quite efficient; saturating amounts of RNA produced a stimulation of radioactive methionine incorporation approximately 20-fold over background incorporation (i.e. no added RNA), providing support to the idea that the D3 ORF may represent a genuine gene. To investigate this possibility further, we set out to produce antisera against the predicted D3 polypeptide by expressing its coding sequences in bacteria.

Production of specific antibodies against the predicted product of the D3 ORF

Our approach was to construct a recombinant plasmid which would allow the expression of D3 coding sequences as a bacterial fusion protein, which could be used to immunize animals, and therefore produce specific antisera. The expression system chosen was that developed by Stanley & Luzio (1984) which involves the insertion of foreign sequences at the C terminus of a β-galactosidase gene, which is itself fused to the promoter, operator and N terminal region of the *cro* gene of bacteriophage λ. Expression of the fusion protein is under the control of the λ repressor and, in cells carrying a temperature-sensitive repressor, may be induced by temperature shift from 30 °C to 42 °C. The plasmid vectors (pEX series) are available for expression from all three reading frames, and a restriction fragment derived from a cDNA clone containing the sequence from the unique region of mRNA D (Boursnell *et al.*, 1985) was cloned into the appropriate pEX vector to give the plasmid pXASD3 which contains the entire D3 ORF fused, in frame, to the bacterial β-galactosidase gene (Fig. 3*a*).

E. coli host cells carrying this plasmid synthesized a polypeptide larger than that produced by cells carrying the wild-type pEX3 plasmid following heat induction (Fig. 3*b*). This fusion protein was purified by gel

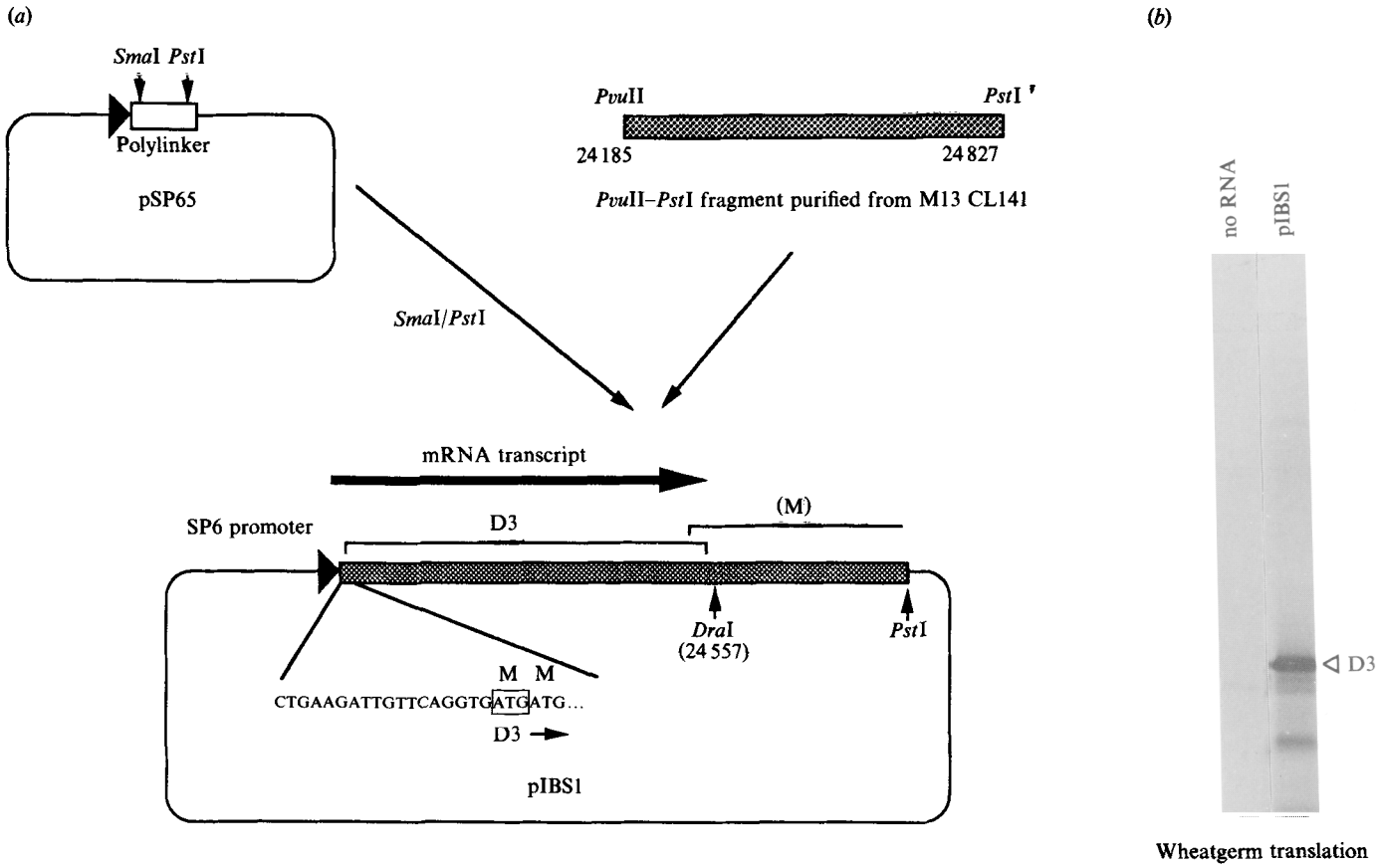

Fig. 2. (*a*) Construction of the plasmid (pIBS1) used to express the D3 ORF *in vitro*. A *Pvu*II/*Pst*I restriction fragment of the cDNA insert contained within the M13 clone CL141, containing the D3 ORF coding sequence was purified by preparative agarose gel electrophoresis and ligated into plasmid pSP65 DNA (Melton *et al.*, 1984) which had been digested with the restriction endonucleases *Sma*I and *Pst*I, and treated with calf intestinal phosphatase. Ligated material was transformed into *E. coli* (strain JM101) and the resulting colonies were screened for the presence of the correct plasmid by restriction endonuclease digestion of purified plasmid DNA. Nucleotides are numbered as for Fig. 1. (*b*) Analysis of cell-free translation products of mRNA obtained by *in vitro* transcription from *Dra*I-digested pIBS1, using phage SP6 RNA polymerase. RNA was added to the wheatgerm cell-free system, as indicated, at approximately 100 μg/ml. Translation products were labelled with [^{35}S]methionine, and separated on a 17·5% SDS–polyacrylamide gel. Labelled polypeptides were detected by autoradiography. Unlabelled M_r markers were included in an adjacent lane and were detected by Coomassie Brilliant Blue staining.

electrophoresis and inoculated into rabbits in order to produce specific antiserum.

Identification of a gene product encoded by the D3 ORF in IBV-infected cells

Antisera raised against the D3-specific bacterial fusion protein were tested for their ability to immunoprecipitate the *in vitro* translation product of the D3 ORF (data not shown) and a serum that was found to be positive by this criterion was further tested against [^{35}S]cysteine-labelled lysates prepared from IBV-infected CK and Vero cells (Fig. 4). This antiserum recognized a polypeptide, in both infected cell types, which had exactly the same electrophoretic mobility as the *in vitro* translation product from the D3 ORF and which presumably represents the predicted D3 polypeptide. A combination of poor radiolabelling of D3 and the relatively weak reactivity of the anti-D3 antiserum (suggested by its inefficiency against *in vitro* synthesized D3; data not shown) make it difficult to assess the production of D3 relative to other virus polypeptides. In some experiments using Vero cells, it was just possible to discern a band corresponding to D3 directly in unprecipitated lysates. Given the small size of the polypeptide, and the number of cysteine residues (two) which it contains, this would suggest that the molar quantity of this polypeptide is not dissimilar to that of the other virus structural proteins; more careful analysis will be required to resolve this point.

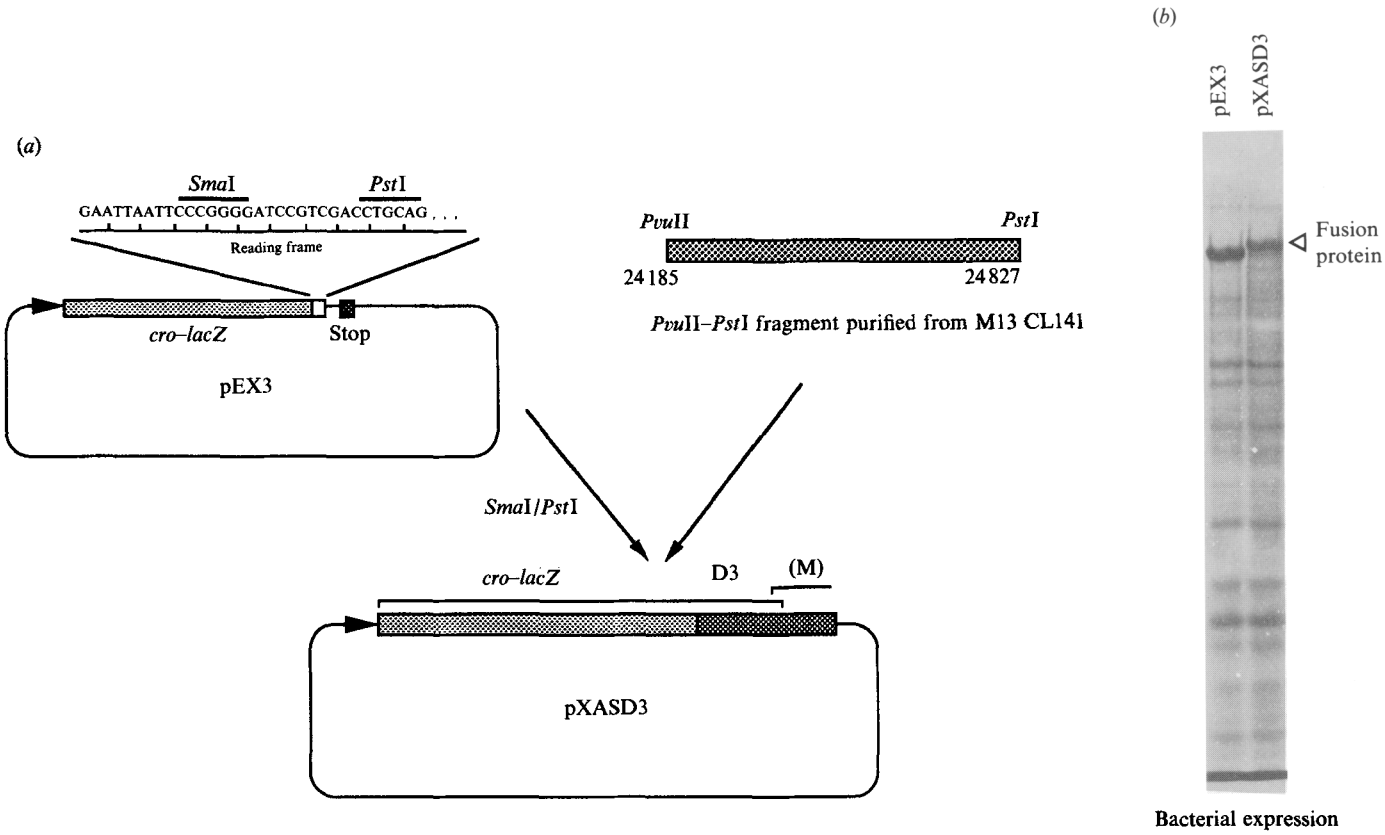

Fig. 3(*a*) Construction of the plasmid used to express the D3 ORF in *E. coli* as a fusion protein with β-galactosidase. A *PvuII*/*PstI* restriction fragment of the cDNA insert within the M13 clone CL141, containing the D3 ORF coding sequence, was purified by preparative agarose gel electrophoresis and ligated into plasmid pEX3 DNA which had been digested with the restriction endonucleases *SmaI* and *PstI*, and treated with calf intestinal phosphatase. Ligated material was transformed into *E. coli* (strain POP2136) and the resulting colonies were screened for the presence of the correct plasmid by restriction endonuclease digestion of purified plasmid DNA. Nucleotides are numbered as for Fig. 1. (*b*) Proteins induced in *E. coli* (POP2136) cells carrying either plasmid pEX3 or pXASD3, after incubation at 42 °C. Polypeptides were separated on a 10% SDS–polyacrylamide gel, and detected by staining with Coomassie Brilliant Blue.

The product of the D3 ORF co-fractionates with viral membrane-associated proteins.

Analysis of the predicted amino acid sequence of the D3 polypeptide indicates that it contains a hydrophobic region of 21 amino acids near its N terminus (Boursnell *et al.*, 1985) suggesting that it may be associated with, or integrated into, membranes in the infected cell. This possibility was investigated through cell fractionation studies. IBV-infected CK cells which had been labelled with [^{35}S]cysteine were fractionated into four components by the method described by Hay *et al.* (1974): nuclei (fraction 1), cytoplasm (fraction 2), a fraction consisting mainly of microsomes and other small membranes (200000 *g* pellet, fraction 3) and a fraction containing predominantly large membranes (45000 *g* pellet, fraction 4). Equivalent samples from these fractions were analysed by gel electrophoresis directly, or after immunoprecipitation with anti-D3 serum, as well as with anti-IBV and control sera (Fig. 5). The major IBV virion core protein, N, was found predominantly in the microsomal fraction (fraction 3), and in trace amounts in the cytoplasm and large membranes (fractions 2 and 4); the presence of the nucleoprotein mainly in the microsomal membrane fraction probably reflects the packaging of the nucleoprotein into virions and the localization of these virions within the Golgi apparatus (Tooze *et al.*, 1984). The viron envelope proteins M and S appeared mainly, as expected, in the large membranes, with some detected in the microsomal fraction. As before, D3 was not strongly radiolabelled, but a clear D3-specific band was observed in the large membrane fraction and a faint band in the microsomal fraction. Thus D3 showed the same kind of subcellular distribution as the known IBV envelope glycoproteins, suggesting that it is likely to be membrane-associated.

Analysis of the predicted amino acid sequence of the D3 polypeptide suggested also that it might be glycosy-

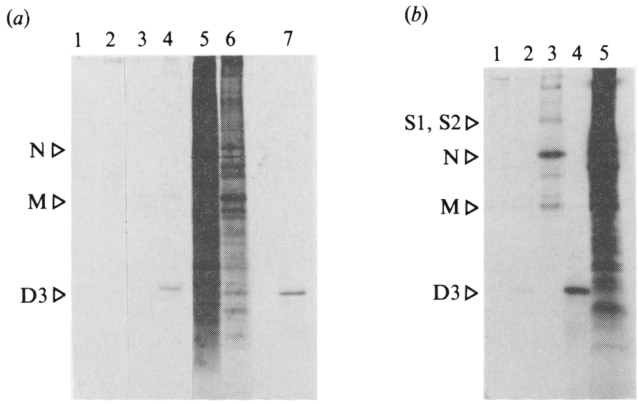

Fig. 4. Detection of a D3-encoded polypeptide in IBV-infected cells. Cells were labelled with [^{35}S]cysteine, separated on a 20% SDS–polyacrylamide gel, and labelled polypeptides were detected by autoradiography. Unlabelled M_r markers were included in an adjacent lane and detected by Coomassie Brilliant Blue staining. (*a*) Vero cells. Infected cells (lanes 3, 4 and 6) and mock-infected cells (lanes 1, 2 and 5) were analysed directly (lanes 5 and 6) or after immunoprecipitation with anti-D3 antiserum (lanes 2 and 4) or a control preimmune antiserum (lanes 1 and 3). Lane 7 shows D3 synthesized by *in vitro* translation of artificial pIBS1-derived mRNA (see Fig. 2). (*b*) CK cells. IBV-infected cells were analysed directly (lane 5) or after immunoprecipitation with anti-D3 (lane 2), anti-IBV (lane 3) or control serum as above (lane 1). *In vitro* translated D3 is shown in lane 4.

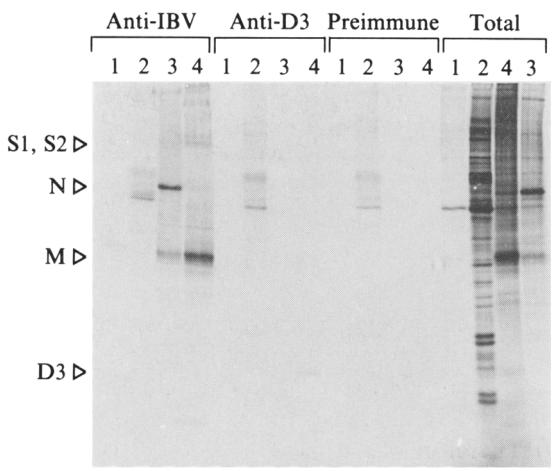

Fig. 5. Subcellular fractionation of the D3-encoded polypeptide. [^{35}S]Cysteine-labelled IBV-infected CK cells were fractionated into nuclei (lane 1), cytoplasm (lane 2), small membranes (lane 3) and large membranes (lane 4), by the procedure outlined in Methods. Samples of each fraction were analysed directly (total) or after immunoprecipitation with anti-D3 antiserum, anti-IBV antiserum or control preimmune serum as indicated. Polypeptides were separated on a 20% SDS–polyacrylamide gel, and were detected by autoradiography. [^{35}S]Methionine-labelled influenza virus polypeptides were used as M_r markers.

lated, because there are two potential *N*-linked glycosylation sites near the N terminus (Boursnell *et al.*, 1985). The observation that *in vitro* synthesized D3 comigrates exactly with the equivalent polypeptide in infected cells conflicts with this suggestion, because glycosylation would be expected to alter the gel mobility of the polypeptide, and it is very unlikely that such processing would occur in a cell-free system not supplemented with microsomal membranes. Furthermore, the electrophoretic mobility of the D3 polypeptide was not altered by treatment of infected cells with tunicamycin (data not shown) which inhibits the *N*-linked glycosylation of proteins by blocking the formation of dolichol-linked precursor oligosaccharides (Hubbard & Ivatt, 1981).

Detection of the D3 ORF product on the surface of infected cells

If D3 is indeed membrane-associated, it might be expected to span the membrane with an extracellular and an intracellular domain, as the hydrophobic region identified by sequence analysis is flanked on both sides by hydrophilic regions (see Fig. 1). We tested the possibility that part of the D3 polypeptide was exposed on the surface of infected cells by indirect immunofluorescence on intact and permeabilized IBV-infected Vero cells. For this purpose, antibodies specific for the D3 polypeptide were purified from crude serum by immunoaffinity chromatography (see Methods). At 18 h p.i., permeabilized cells showed clear positive fluorescence with anti-D3 serum (Fig. 6). The fluorescence was rather granular, and was observed mainly around the nucleus, with some evidence of polarization into structures which could represent the Golgi apparatus, the site of coronavirus assembly (Tooze *et al.*, 1984). Positive, but weak, staining was observed also in unfixed infected cells, in a pattern suggesting the presence of at least part of the D3 polypeptide on the surface of infected cells.

Discussion

Sequence analysis of the IBV genome has revealed a number of ORFs not previously assigned a protein coding function (Boursnell *et al.*, 1985, 1987). We have confirmed the coding potential of one of these, D3, which has the potential to encode a 12·4K polypeptide, by *in vitro* transcription of the cloned D3 gene and translation of the resulting artificial mRNA in a wheatgerm cell-free system. Furthermore, we have identified a D3-encoded polypeptide in IBV-infected cells, using serum raised against a bacterial fusion protein containing D3 amino acid sequences.

Available evidence suggests that the D3 polypeptide is expressed from a 3·8 kb subgenomic mRNA, mRNA D, which consists of a 65 nucleotide 5′ leader sequence corresponding to the 5′ end of the genomic RNA, fused to a 'body' which initiates 23832 nucleotides from the

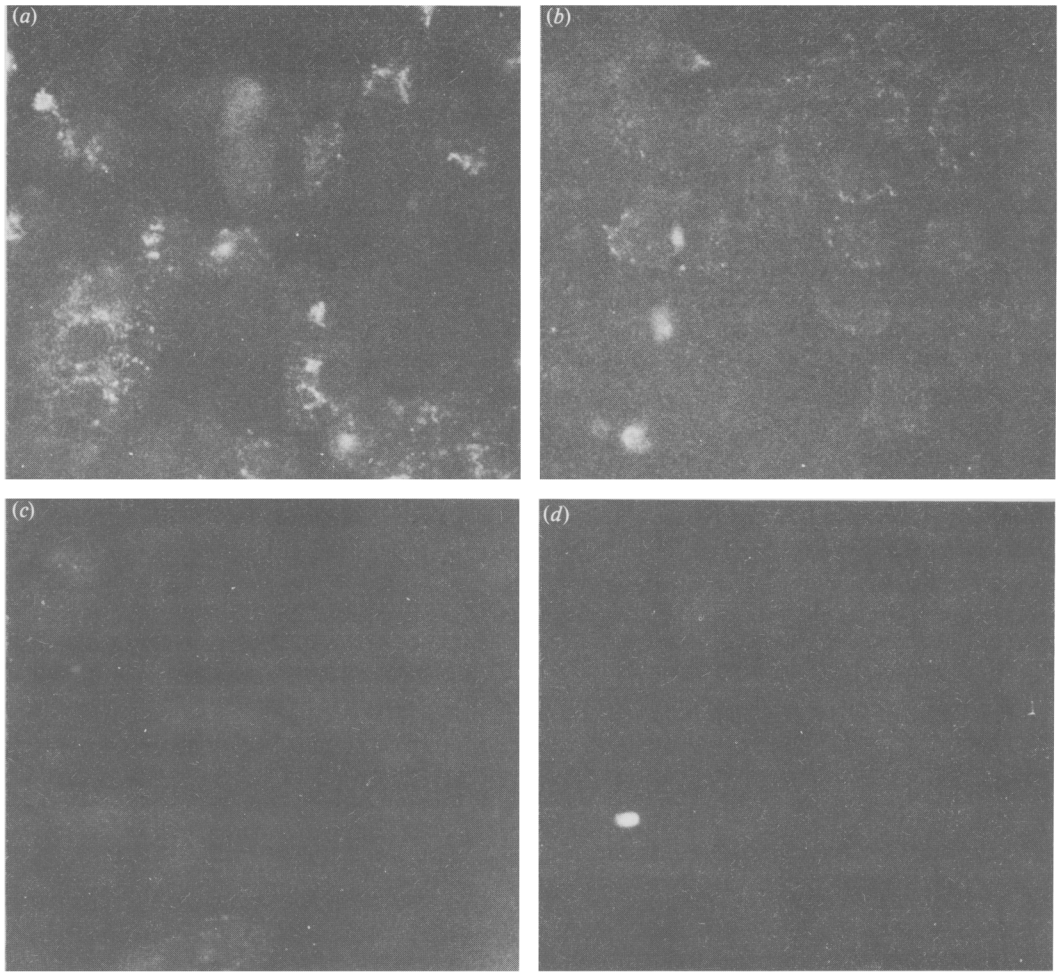

Fig. 6. Indirect immunofluorescence of IBV-infected (*a* and *b*) or mock-infected (*c* and *d*) Vero cells at 18 h p. i. using antiserum raised against affinity-purified D3. Cells were either permeabilized, i.e. fixed (*a* and *c*) or left intact, unfixed; (*b* and *d*) before analysis (see Methods).

genomic 5′ end (see Fig. 1), and runs through to its extreme 3′ terminus (Boursnell *et al.*, 1985). This is based on the observation that the next smallest subgenomic mRNA in the nested set, mRNA C, does not contain the D3 ORF, and that the next largest, mRNA E, encodes the virus S glycoprotein. Thus the D3 ORF is initiated a considerable distance (370 nucleotides) downstream of the site at which the leader sequence joins the body of mRNA D (Boursnell *et al.*, 1985). Upstream of the D3 ORF there are two other potential initiation sites, both at the origin of ORFs of significant size (6·7K and 7·4K coding potential), which overlap each other by a few nucleotides. The D3 initiation codon is in a relatively good context for recognition by ribosomes, with a purine at position −3 for both the methionine codons at the start of the ORF (Kozak, 1984), whereas the upstream AUG codons are in less favourable contexts with pyrimidines at position −3 in both cases. The presence of AUG codons upstream of an ORF can suppress the translation of a given ORF, and it has been observed that translation of an upstream, non-overlapping ORF on a polycistronic mRNA can severely reduce the level of translation of a downstream ORF (reviewed in Kozak, 1989). Therefore, there may be selective pressure against the presence of 'non-functional' sequences upstream of a functional ORF if they contain AUG triplets. However there are several examples where the major translation product of a eukaryotic mRNA initiates downstream of the 5′-proximal AUG triplet (Kozak, 1989), and so this is not a strong argument in favour of the functional significance of D1 and D2. Nevertheless it remains possible that the D mRNA is polycistronic because, although their codon usage conforms poorly to that observed for the IBV structural proteins, both the D1 and D2 ORFs are conserved in a different IBV strain, M41 (Boursnell *et al.*, 1985). Further work is being carried out, using the approach described here, for the identification of the D3-encoded product, to determine whether these

ORFs are indeed functional.

Among the other coronaviruses, there is also some suggestion that subgenomic mRNAs are functionally polycistronic. Nucleotide sequencing of both the A59 and JHM strains of murine hepatitis virus (MHV) indicates that mRNA 5 contains two overlapping ORFs (Skinner *et al.*, 1985; Budzilowicz & Weiss, 1987), and a 9·6K product from the more distal of these has been detected in infected cells (Leibowitz *et al.*, 1988).

Our results suggest that D3 is a membrane-associated polypeptide, and this finding is consistent with its predicted amino acid sequence, which contains a stretch of 21 uncharged amino acids near the N terminus (Fig. 1). The hydrophobic domain is bounded on both sides by hydrophilic regions, suggesting a transmembrane orientation, with parts of the protein exposed on either side of the membrane. A portion of D3 is exposed on the external surface of the infected cell, indicated by the pattern of surface fluorescence observed using anti-D3 antiserum. However it is not clear whether this would represent the N- or the C-terminal region of the polypeptide. A possible implication of these findings is that D3 could represent a structural polypeptide present in the virus envelope, and experiments are in progress to test this possibility.

Small membrane-associated proteins have also been observed in other enveloped RNA viruses. For example influenza A virus encodes an integral membrane protein (M2) of M_r 15K, which is present on the surface of infected cells (Lamb *et al.*, 1985; Zebedee *et al.*, 1985) and, in small amounts, in virions (Zebedee & Lamb, 1988). It has an amino-terminal region of about 15 amino acids which is exposed on the cell surface, a hydrophobic stretch of 19 amino acids which spans the cell membrane and a hydrophilic domain within the cell. A protein of similar size is present in influenza B virus-infected cells; this 18K glycoprotein, termed NB, is an integral membrane protein with several similarities to M2 in gross structure, although without a high degree of homology in its amino acid sequence (Williams & Lamb, 1986). Other small membrane proteins encoded by enveloped viruses include the 15K transmembrane SH protein and the 7·5K 1A protein of the paramyxoviruses simian virus 5 and respiratory syncytial virus, respectively (Hiebert *et al.*, 1988; Olmsted & Collins, 1989). The role of these proteins is not yet clear [although the influenza virus M2 protein has been implicated indirectly in virus entry (Hay *et al.*, 1985)], but it seems possible that their overall similarity might reflect a common function.

There is as yet no direct evidence that other coronaviruses contain a small envelope protein with the characteristics of D3, but it is noteworthy that the polypeptide encoded by the downstream ORF of MHV mRNA 5 has a long stretch of uncharged amino acids near the N terminus (Skinner & Siddell, 1985; Leibowitz *et al.*, 1988), and that some similarity between this coding sequence and D3 has been suggested on the basis of sequence comparison (Boursnell *et al.*, 1985). The 15K protein encoded by MHV mRNA 4 (Ebner *et al.*, 1988) also contains a potential transmembrane region (Skinner & Siddell, 1985) although again, as yet, there is no direct evidence of membrane association.

Thus small transmembrane envelope proteins may be a general feature of coronaviruses, and could, by analogy with the influenza virus M2 protein (Hay *et al.*, 1985), be involved in virus entry or assembly. However it remains possible that the polypeptides could serve some completely different function, for example to provide a membrane anchor for virus RNA polymerase activity (which is thought to occur in association with membranes in the virus-infected cell) as suggested previously (Skinner *et al.*, 1985; Boursnell *et al.*, 1985).

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