IDENTIFICATION OF A NEW GENE PRODUCT ENCODED BY mRNA D OF INFECTIOUS BRONCHITIS VIRUS

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INTRODUCTION

The coronavirus Infectious Bronchitis Virus (IBV), a pathogen of chickens, has a genome structure typical of a coronavirus. The virus particle contains a single-stranded RNA molecule of approximately 27kb with positive polarity, and in infected cells six species of virus-specific mRNA are produced (1), designated RNA A to RNA F in order of increasing size (RNA F corresponding to the size of the genomic RNA). These RNAs form a nested set (2), in which the sequences in each of the subgenomic RNAs are also represented in all the larger RNAs; these RNA species each have a short 'leader' sequence corresponding to the 5' end of the genomic RNA (3) followed by progressively larger amounts of information derived from its 3'-end. For each mRNA, the region of sequence which is not contained in the next smallest RNA is assumed to be used for translation of viral proteins (2). Polypeptide products have been assigned for RNAs A, C and E (nucleoprotein, membrane protein and the spike protein precursor respectively) (4), but the coding function of the other RNAs has not so far been established, although it has generally been assumed that RNA F codes for the viral RNA-dependent-RNA-polymerase.

We report here studies designed to investigate the polypeptide coding function of RNA D. Sequence analysis carried out previously (5) has shown that RNA D has three distinct open reading frames (ORFs) (Fig. 1), with the potential to encode polypeptides of molecular weights 6.7K (D1), 7.4K (D2) and 12.4K (D3). Our approach has been to prepare antisera against these putative virus polypeptides through expression of their coding sequences in bacteria, and to use these sera as probes for the analysis of infected cells.

EXPRESSION OF RNA D ORFs IN BACTERIA

Our strategy for bacterial expression of IBV sequences was based on the pEX series of plasmids developed by Stanley and Luzio (6). Foreign sequences are inserted in a polylinker sequence at the C-terminal end of a β-galactosidase gene which is itself fused to the promoter, operator and

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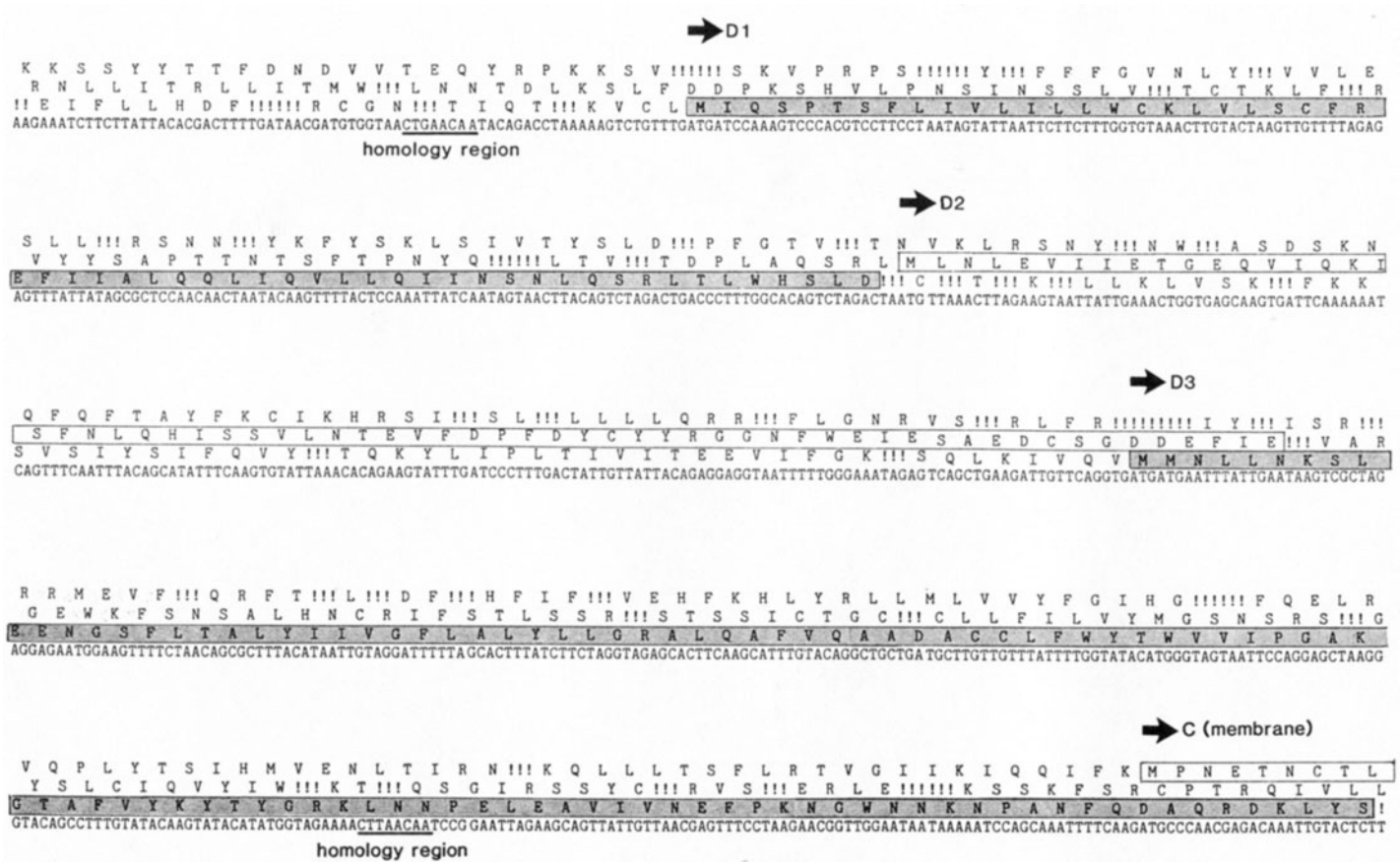

D F E Q S V Q L F K E Y N L F I T
T L N S Q F S F L K S I I Y L !!!
!! L !!! T V S S A F !!! R V !!! F I Y N
GACTTTGAACAGTCAGTTCAGCTTTTTAAAGAGTATAATTTATTTATAAC

Fig. 1. Complementary DNA sequence and coding potential of the D region of the IBV genome. The main open reading frames (ORFs) are boxed. The 'homology regions', sequences present in the genome at positions corresponding to the 5' termini of the bodies of the IBV mRNAs (in these cases RNA D and RNA C), are underlined.

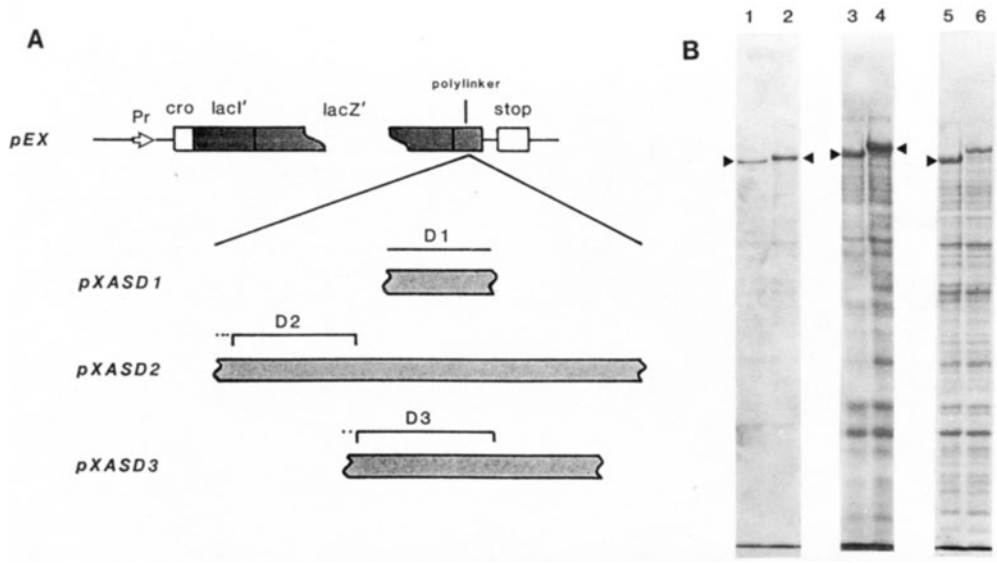

Fig. 2. (a) Plasmid constructions used for expression of RNA D ORFs in bacteria. Restriction fragments were inserted into the polylinker of the pEX plasmid to give expression of most of D1 (pXASD1), all of D2 (pXASD2) or all of D3 (pXASD3) as part of an insoluble fusion protein with β-galactosidase. (b) Polyacrylamide gel electrophoresis of proteins synthesised in bacteria carrying the wild type plasmid pEX (tracks 1,3,5) and pXAS D1 (track 2), pXAS D2 (track 4) and pXAS D3 (track 6) after heat induction. Separation was on a 10% polyacrylamide gel, and polypeptides were visualised by staining with Kenacid blue.

N-terminal region of the cro gene of phage λ. Expression of the fusion protein is therefore under the control of the λ repressor, and in cells carrying a temperature-sensitive repressor, may be induced simply by raising the temperature. In addition plasmid vectors are available with the polylinker sequence in all three reading frames. Restriction fragments, derived from a cDNA clone containing sequence from a unique region of RNA D (5), were cloned into the polylinker sequence of the appropriate plasmid to give pXAS D1, which contains the 5'-terminal 143 nucleotides (ntds) of the D1 ORF (out of a total of 169 ntds), pXAS D2 which carries a complete copy of the D2 ORF, and pXAS D3 which contains the entire D3 coding sequence (Figure 2(a)). Figure 2(b) shows the polypeptides synthesised in E. coli host cells carrying these plasmids after heat induction, and it is evident that in each case a protein larger than that directed by the wild type plasmid is synthesised. These fusion proteins were purified by preparative SDS-polyacrylamide gel electrophoresis and electro-elution from gel slices, and used to immunise rabbits. Animals were inoculated initially with approximately 100μg 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.

Sera produced from these animals were then tested for their ability to recognise IBV-specified polypeptides by immunoprecipitation experiments using infected cell lysates. Lysates were prepared as follows: chick kidney (CK) or VERO cells were inoculated with the Be-42 strain of IBV (at a multiplicity of infection >10), and harvested at 8h post infection (p.i.) or

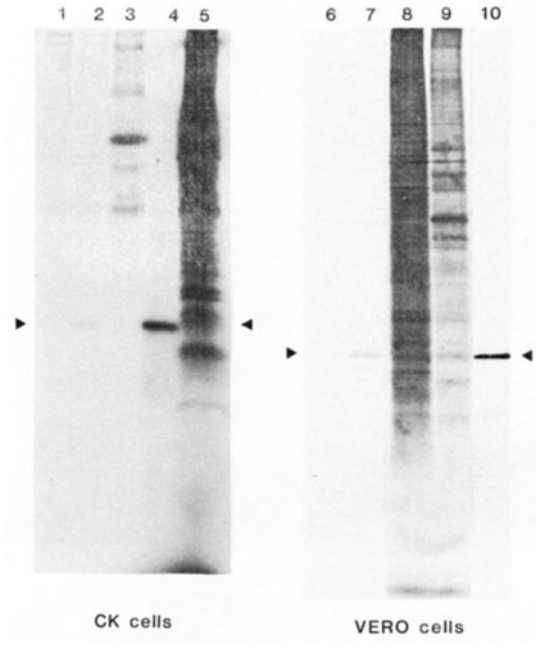

Fig. 3.

Polyacrylamide gel electrophoresis of ^{35}S-labelled polypeptides immunoprecipitated from IBV-infected chick-kidney (CK) cells (tracks 1,2,3) or VERO cells (tracks 6,7) with preimmune (tracks 1,6), anti-D3 (tracks 2,7) or anti-IBV virion (track 3) antisera. Tracks 5,9 - unprecipitated lysates from IBV-infected CK and VERO cells respectively. Track 8 - unprecipitated lysate from mock-infected VERO cells. Tracks 4,10 - D3 polypeptide prepared in vitro (translation of pIBS1-directed RNA (see Figure 4(b), track 4). Labelled polypeptides were detected by autoradiography.

15 h.p.i. (CK or VERO cells respectively) after labelling for 90 min with ^{35}S-cysteine (100 µCi/ml); the cells were incubated in cysteine-free medium for 30 min prior to labelling. Cysteine was used as the radioactive label because the D1, D2 and D3 polypeptides should each contain two cysteine residues. Cells were disrupted in RIPA buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100), and the lysates were cleared by centrifugation at 50,000 x g for 1 hour. Immunoprecipitation was carried out according to standard methods (7) using protein A-sepharose, and the products were analysed by gel electrophoresis. Antisera raised against the D1 and D2 ORFs did not appear to precipitate specifically any polypeptides from the IBV-infected cell, although a very faint band of approximately the expected size for D2 was observed in some experiments (data not shown). However the anti-D3 sera clearly recognised specifically a polypeptide of the expected size (12.4K) in both infected CK and VERO cells (Figure 3), and this polypeptide comigrated exactly with the cell-free translation product of artificially synthesised D3 mRNA (see next section).

The results therefore indicate that the ORF which is furthest downstream from the start of RNA D, is expressed in infected cells. Our failure to detect products of the upstream D1 and D2 ORFs in infected cells could be interpreted in a number of ways. The polypeptides may be produced at very low levels if at all. Alternatively however, the antisera raised against the bacterial fusion proteins may simply not react with the native polypeptides, perhaps because the fusion protein is not properly antigenic or possibly because the native polypeptide is modified in some way in the infected cell. To investigate further these possibilities we tested the ability of the antisera to recognise polypeptides synthesised in vitro by cell-free translation of artificially synthesised mRNAs.

EXPRESSION OF D2 AND D3 ORFs USING RNA TRANSCRIBED IN VITRO

Restriction fragments containing the D2 and D3 ORFs were cloned into the plasmid pSP65, which contains a promoter for the RNA polymerase of the bacteriophage SP6 (8), such that the sequences of these ORFs could be transcribed in vitro as mRNA (Figure 4(a)). Two new plasmids were

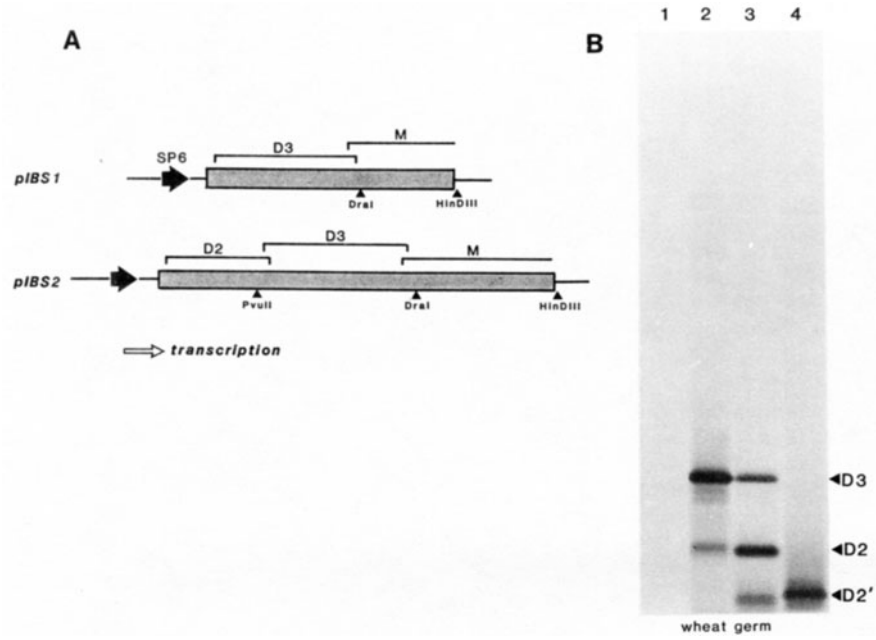

Fig. 4. (a) Plasmid constructions used for in vitro transcription of ORFs D2 and D3 of mRNA D. Restriction fragments containing the D3 coding sequence alone (pIBS1), or both the D2 and D3 ORFs (pIBS2) were inserted downstream of the SP6 promoter in plasmid pSPS5.
(b) Wheat germ cell-free translation products (10) of RNA transcribed using the SP6 RNA polymerase from DraI-digested pIBS1 (track 2), DraI-digested pIBS2 (track 3), or PvuII-digested pIBS2 (track 4). Track 1 - no added RNA. Translation products were labelled with ^{35}S-methionine, analysed on a 17.5% polyacrylamide gel, and detected by autoradiography.

constructed - pIBS1 which contains the entire D3 coding sequence adjacent to the SP6 promoter, and pIBS2 which contains both the D2 and D3 ORFs, with the D2 sequence proximal to the promoter. As yet we have been unable to construct a plasmid carrying the D1 ORF adjacent to the SP6 promoter because there are no convenient restriction sites just upstream of its iniation codon. Plasmids pIBS1 and pIBS2 were linearised with DraI, which cleaves immediately downstream of the D3 ORF and were transcribed in vitro using the SP6 RNA polymerase (8); the dinucleotide ^{7}mGpppG was included in the reaction in order to generate an authentic 5' cap structure on the transcripts (9). The product RNAs were checked for their integrity by gel electrophoresis, and translated in the wheat germ cell-free translation system (Figure 4(b)). Translation of RNA transcribed from DraI-digested pIBS1 produced a major polypeptide of approximately 12K, as expected for the product of the D3 ORF, and some minor bands of lower M.W., which are almost certainly premature termination products. The 12K polypeptide was also synthesised in response to RNA transcribed from DraI-digested pIBS2, but in this case the major translation product was a polypeptide of M.W. about 7.5K, which presumably represents the product of the D2 ORF. Consistent with this conclusion, RNA transcribed from PvuII-digested pIBS2 produced

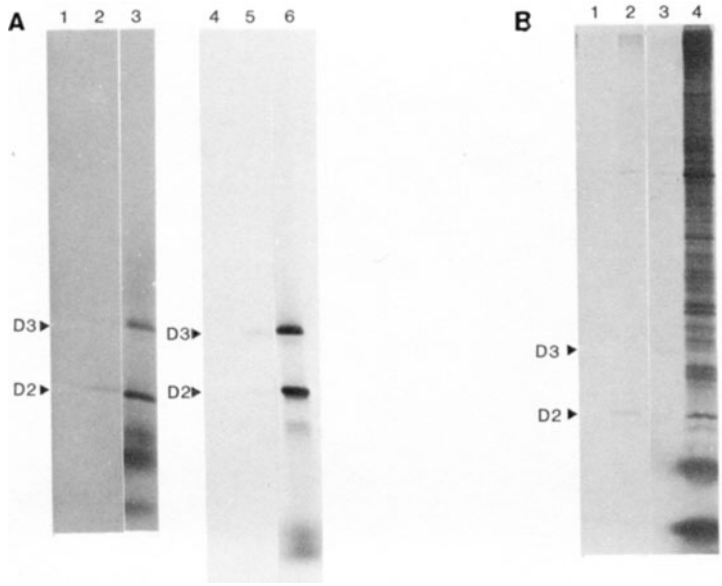

Fig. 5. (a) Polypeptides immunoprecipitated from the cell-free translation product of RNA transcribed from DraI-digested pIBS2 (see Figure 4(b)) using preimmune (tracks 1,4), anti-D2 (track 2) or anti-D3 (track 5) sera. Tracks 3,6 - equivalent amounts of the unprecipitated products. Polypeptides were separated on a 20% polyacrylamide gel, and detected by autoradiography.
(b) Polypeptides immunoprecipitated from a mixture of IBV-infected CK cell lysate (see Figure 3) and in vitro translation products of DraI-digested pIBS2 (see Figure 4(b)), using pre-immune (track 1), anti-D2 (track 2) and anti-D3 (track 3). Track 4 - unprecipitated material. Polypeptides were separated on a 20% polyacrylamide gel and detected by autoradiography.

a smaller product, presumably corresponding to the truncated D2 ORF.

Antiserum raised against the D2 ORF was tested for its ability to immunoprecipitate in vitro translation products of pIBS2-directed RNA (Figure 5(a)). The results indicate that antibodies to the D2 polypeptide are indeed present in the anti-fusion protein antiserum. In a further experiment the in vitro translation products were mixed with lysate from IBV-infected CK cells prior to immunoprecipitation (Figure 5(b)), and once again it was clear that the D2 polypeptide was specifically precipitated. We estimate that the reaction between the anti-D2 serum and in vitro translated D2 is about as efficient as that observed between the anti-D3 serum and the D3 polypeptide. Thus it seems likely that if the D2 polypeptide is present in infected cells, it exists in much lower quantities than D3, although we cannot rule out the possibility that it takes a form immunologically different from the in vitro translated material. Unless the D2 polypeptide is inherently unstable in infected cells, it therefore seems likely that during infection, ribosomes translate the downstream cistron (ORF D3) of RNA D more efficiently than the upstream D2 cistron. Examination of the

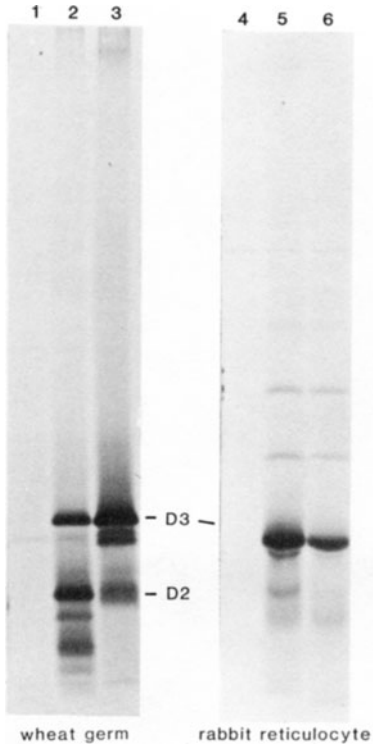

Fig. 6.
Cell-free translation products of RNA transcribed from HindIII-digested pIBS1 (tracks 2,5) and pIBS2 (tracks 3,6) synthesised in wheat-germ extracts (tracks 1-3) and rabbit reticulocyte lysate (tracks 4-6). Tracks 1,4 - no added RNA. Translation products were labelled with ^{35}S-methionine, separated on a 20% polyacrylamide gel and detected by autoradiography.

sequences surrounding the initiation codons for translation of D2 and D3 lends some support for this, with a pyrimidine residue at position -3 for the D2 ORF implying weak initiation, and a purine at position -3 for both of the AUG codons at the start of the D3 ORF, implying stronger initiation (11). We do not know whether or not the D1 ORF is translated in the cell, but it appears that this too has a 'weak' context for ribosome initiation according to current ideas (11). The pattern obtained from translation of artificially synthesised RNA in the wheat germ cell-free system however does not support the idea that the D2 ORF has a weaker initiation site than that of D3, since RNA transcribed in vitro from pIBS2 directs the synthesis of at least as much D2 as D3 (Figure 4(b)). However when the same RNA was translated in lysates of rabbit reticulocytes (Figure 6), which may be more representative of the situation in infected cells, the D2 polypeptide appeared to be synthesised poorly, if at all.

In summary we have identified, through the preparation of specific antisera against cloned IBV sequences expressed in bacteria, a new IBV gene product of M.W. 12.4K (D3), which is translated from the third open reading frame of mRNA D. Preliminary experiments, based on translation of artificially synthesised mRNAs, suggest that this ORF may be expressed more efficiently than the upstream D1 and D2 ORFs and further studies are in progress to investigate the mechanism by which this occurs.

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