Journal of General Virology (1991), 72, 1835–1843. *Printed in Great Britain*

Sequence analysis and expression of the host-protective immunogen VP2 of a variant strain of infectious bursal disease virus which can circumvent vaccination with standard type I strains

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The host-protective antigen VP2 of a variant strain of infectious bursal disease virus (IBDV) which emerged from a vaccinated flock and is able to circumvent vaccination with classic type I strains of IBDV, was cloned and its nucleotide sequence determined. Virus-neutralizing monoclonal antibodies (MAbs) raised against the Australian 002-73 strain of IBDV did not react or reacted only very weakly with the expression product of the variant virus. The deduced amino acid sequence of VP2 from the variant strain differed in 17 residues from that of the Australian strain and in eight positions from a consensus sequence compiled from six type I strains of IBDV. All the amino acid changes mapped within the central, variable region of VP2, which forms the conformational epitope recognized by virus-neutralizing MAbs. Changes in the two hydrophilic regions at either end of this fragment were unique to the variant virus and were crucial for its ability to escape the virus-neutralizing antibodies induced by vaccination with a standard type I vaccine.

Introduction

Infectious bursal disease virus (IBDV) is responsible for a severe immunodepressive disease in young chickens which causes significant losses to the poultry industry world-wide (reviewed in Kibenge *et al.*, 1988). Two distinct serotypes (I and II) of IBDV have been identified (McFerran *et al.*, 1980; Jackwood *et al.*, 1982); serotype II viruses are avirulent for chickens (Cummings *et al.*, 1986; Ismail *et al.*, 1988), and considerable variation in virulence occurs within serotype I (McFerran *et al.*, 1980; Saif *et al.*, 1987.

IBDV is a member of the *Birnaviridae* family (Brown, 1986) and its genome consists of two segments (A and B) of dsRNA (Dobos *et al.*, 1979). Genome segment B (2·9 kb) encodes a 90K protein, VP1 (Azad *et al.*, 1985), which is believed to be a dsRNA replicase (Spies *et al.*, 1987; Morgan *et al.*, 1988), and the larger segment, A (3·2 kb), encodes viral proteins VP2, VP3 and VP4 (Azad *et al.*, 1985), which are produced by autoproteolysis of a 106K precursor polyprotein (Hudson *et al.*, 1986; Azad *et al.*, 1987; Jagadish *et al.*, 1988). Structural protein VP2 (approximately 494 amino acid residues long) has been identified as the major host-protective antigen of IBDV (Azad *et al.*, 1987; Becht *et al.*, 1988; Fahey *et al.*, 1989); the sequence is highly conserved, the only variation between five different standard type I strains being in the central *Acc*I–*Spe*I fragment (residues 206 to 350) (Bayliss *et al.*, 1990; Kibenge *et al.*, 1990). Virus-neutralizing monoclonal antibodies (MAbs) (MAb 17-82, 39A and 9-6) raised against IBDV (strain 002-73) recognize a conformational epitope within this variable central region of VP2 (Azad *et al.*, 1987; Fahey *et al.*, 1991).

Vaccination of breeder hens with an inactivated serotype I virus vaccine protects their offspring against IBDV infection (Wyeth & Cullen, 1976), but recently, very virulent IBDV strains (Delaware variants) have been isolated from vaccinated flocks on the Delmarva peninsula (U.S.A.) (Rosenberger *et al.*, 1985). These variant strains can infect broiler chickens with relatively high levels of maternal antibodies and are antigenically different from the classic strains isolated before 1985 (Rosenberger *et al.*, 1985). It has been shown that vaccination with the variant strains of IBDV can protect against the variant strains as well as virulent type I strains (Rosenberger *et al.*, 1987); the Delaware variants had lost a neutralizing epitope, defined by MAb B69 (Snyder *et al.*, 1988). Subsequently another antigenic drift in IBDV was detected in the Delmarva area (Snyder *et al.*, 1989) and several outbreaks of very virulent IBDV have been reported from European countries (Van-Schaik, 1987; Stuart, 1989). To analyse the molecular

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basis of the emergence of disease in vaccinated flocks, the host-protective antigen gene, VP2, of the Delaware variant strain E was cloned and its nucleotide sequence determined. Site-directed mutagenesis of the VP2 from variant strain E, and VP2 hybrids consisting of sequences from variant strain E and Australian type 1 strain 002-73, were used to determine the influence of certain amino acid substitutions in the variant on its ability to bind virus-neutralizing MAbs which had been raised against VP2 of Australian strain 002-73.

Methods

Cloning of virus genomic RNA. DNA fragments containing the virus-neutralizing epitope of VP2 from IBDV variant strain E (Rosenberger *et al.*, 1985) were obtained by the polymerase chain reaction (PCR) using cDNA of the virus genomic RNA as a template and synthetic oligonucleotides homologous to conserved regions at the 5′ and 3′ ends of the VP2 gene as primers (Fig. 1). Restriction sites were incorporated at the 5′ ends of the primers to facilitate the subcloning of the amplified fragments into expression vectors. Briefly, the genomic RNA of variant strain E (obtained from Central Veterinary Laboratory, Weybridge, U.K.) was isolated from IBDV-infected bursae as described previously (Azad *et al.*, 1985). First strand cDNA was synthesized using avian myeloblastosis virus reverse transcriptase (Pharmacia LKB Biotechnology) from virus RNA which had been denatured by boiling and then snap frozen, priming with either oligonucleotide N527 (Fig. 1 *b*), to give the coding strand (reaction A), or oligonucleotide N526 (Fig. 1 *b*), to give the non-coding strand (reaction B). The RNA of the RNA–cDNA hybrid molecules was hydrolysed and VP2-specific sequences of the cDNA were amplified from the first strand by PCR using Taq polymerase (Biotech International). Oligonucleotides N527 and M526 were used as primers to obtain a full-length VP2 clone. The PCR conditions used were 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 2 min. The reaction products were phenol-extracted, ethanol-precipitated and analysed by agarose gel electrophoresis (Sambrook *et al.*, 1989). The amplified fragments were trimmed with the appropriate restriction enzymes (sites present at the 5′ ends of the primers), purified from the agarose gel by extraction with Geneclean (Bio101; BRESA) and inserted into the corresponding sites of vector pIP41 to replace the VP2 gene fragment of Australian strain 002-73.

Plasmids. Plasmid pIP41 (Fig. 2) contains the VP2 gene of Australian strain 002-73 under the control of the *tac* promoter. It was subcloned into the *Sma*I and *Xba*I sites of expression vector pTTQ18 (Amersham) as a 1·5 kb *Sma*I–*Xba*I fragment from plasmid pEX.POΔXhoI-PstI (Azad *et al.*, 1987). The fragment distal to VP2, containing the *LacZ* α fragment, was deleted and in its place the f1 intergenic region excised from pUC-f1 (Pharmacia) was inserted. Plasmid pIP77 was derived from pIP41 by oligonucleotide-directed mutagenesis and contains a 23 amino acid deletion spanning the second hydrophilic region of the *Acc*I–*Spe*I fragment.

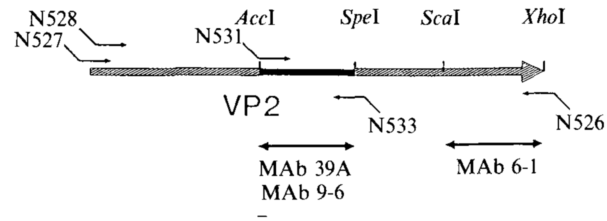
VP2 of variant strain E was cloned (Fig. 2) by replacing the VP2 gene of the Australian strain in pIP41 with the 1·5 kb *Eco*RI–*Xho*I fragment obtained from PCR of cDNA using primers N527 and N526 to give pIP201 (Fig. 1 *a*, *b*).

Hybrids between IBDV variant strain E VP2 and Australian strain 002-73 VP2 were obtained by exchanging homologous fragments between plasmids pIP201 and pIP41, as illustrated for plasmid pIP207 in Fig. 2; pIP207 contains a hybrid VP2 gene consisting of the N-terminal half of variant strain E VP2 fused at the *Sac*I site within VP2 to the C-terminal half of strain 002-73 VP2. The configuration of all the VP2 hybrids (pIP207 to pIP210) is illustrated in Fig. 3.

Amino acid substitutions in the VP2 of variant strain E (pIP203 and pIP204) were generated by oligonucleotide-directed mutagenesis of the single-stranded phagemid pIP201, using the Amersham mutagenesis kit.

DNA sequencing. Double-stranded DNA and ssDNA sequencing of recombinant pIP201, or M13mp18 and -mp19 clones containing *Eco*RI–*Spe*I and *Spe*I–*Xho*I inserts from pIP201, was carried out according to the manufacturer's instructions using either T7 polymerase (Pharmacia), the Taq polymerase system (Promega) or Gene-ATAQ (Pharmacia). The universal sequencing primer supplied in the

(*a*)



(*b*)

N527 *Eco*RI *Pvu*I *Cla*I
```
5'ATATATGAATTCGATCGCATCGATGACAAACCTGTCAGATCAAACCCAGCAG 3'
             >>>>>>>>*>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
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N528 *Sma*I *Bam*HI
```
5'TTAACCCGGGGATCCAACCCAGCAGATTGTTCCGTTTATACGGAGCC 3'
            >>>>@>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
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N531 *Bam*HI *Acc*I
```
5'TTAAGGATCCGTCTACACCATAACTGCCGCAGATGATTACCAATTCTC 3'
            >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
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N533 *Hin*dIII *Spe*I
```
5'TTAAAAGCTTGGCTACTAGTGTGACGGGGCGGAGGGCACC 3'
            >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
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N526 *Pst*I *Xho*I
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5'TTAACTGCAGGCTCGAGCAGTTCCTGAAGCGGCCTGGGCCTCATCGCCC 3'
           >>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>>
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Fig. 1. Primers for cDNA synthesis and PCR amplification of VP2. The hatched arrow represents full-length VP2 of Australian strain 002-73. The variable region (*Acc*I–*Spe*I fragment) is shown as a black bar and the epitopes for various MAbs are indicated below VP2 (*a*). The homology to VP2 of oligonucleotides N526 to N533 is indicated by their position (*a*) and is shown by > > > in (*b*). Restriction sites were incorporated at the non-homologous 5′ ends of the primers (*b*). The homologous nucleotide sequences of primers N527, N528 and N531 are complementary to the non-coding strand, and those of primers N526 and N533 are complementary to the coding strand of IBDV. In N527, the asterisk marks the G to T nucleotide substitution introduced to create the *Cla*I site and the ATG initiation codon of native VP2, which forms part of the *Cla*I site, is underlined. In N528, the * marks the A to C nucleotide substitution introduced to create the *Bam*HI site, which changes the corresponding amino acid in position 7 from glutamine to proline.

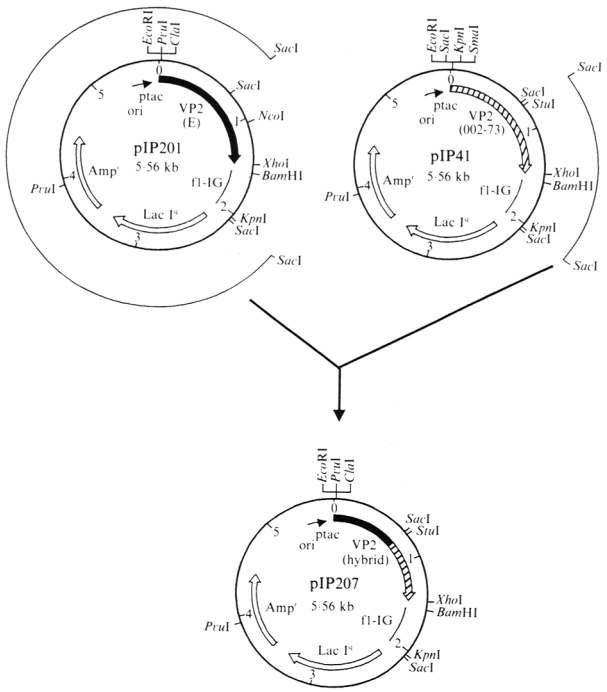

Fig. 2. Construction of a VP2 hybrid. Plasmid pIP207 contains a hybrid of variant strain E and the Australian strain 002-73 VP2s and was constructed by replacing the small *Sac*I fragment of pIP201 with that of pIP41.

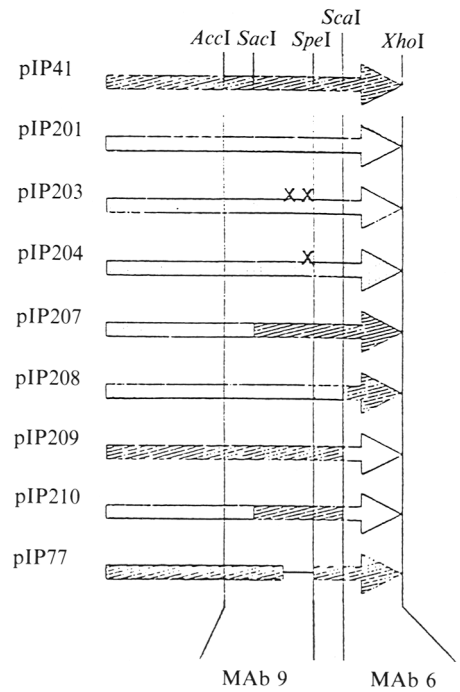

Fig. 3. Configuration of VP2 in plasmids. Genetic material from the Australian strain is indicated as a hatched bar and DNA from variant strain E as an open bar. The positions of the back-mutations in pIP203 and pIP204 are indicated by X.

kits was used for ssDNA sequencing of M13 clones and synthetic oligonucleotides homologous to sequences of IBDV strain 002-73 were used for dsDNA sequencing.

Expression and characterization of recombinant VP2 protein. Plasmids were maintained in *Escherichia coli* DH5α (BRL) or NM522 (Pharmacia) in Luria-Bertani medium containing 0·4% glucose and 100 μg/ml ampicillin. Expression of VP2 was induced by growth for between 1 and 2 h in the presence of 0·5 mM-IPTG, omitting glucose. Bacteria were lysed by lysozyme treatment and sonication, and proteins were analysed by Western blotting (instruction manual, Mini Trans-Blot cell; Bio-Rad) of SDS–polyacrylamide gels and by dot blot immunoassay (DBIA) (instruction manual, Bio-Dot microfiltration apparatus; Bio-Rad) of nitrocellulose filters (Schleicher & Schuell).

Results

Cloning of the host protective antigen of variant strain E

Synthetic oligonucleotides homologous to conserved regions at the N and C termini of the VP2 gene were used as primers for the synthesis of cDNA from virus genomic RNA, and for amplification of the cDNA by PCR. Five different primers were synthesized (Fig. 1 *b*) to enable the PCR amplification of either the full-length VP2 gene (using primers N527 and N526), or smaller fragments corresponding to the *Sma*I–*Xho*I (N528 and N526), *Sma*I–*Spe*I (N528 and N533), *Acc*I–*Xho*I (N531 and N526), or *Acc*I–*Spe*I (N531 and N533) regions of the VP2 gene of Australian stain 002-73. A 1·5 kb fragment containing the full-length VP2 gene of the Delaware variant strain E was amplified using primers N527 and N526 (Fig. 1 *a*). Owing to the method of construction, the N termini of the recombinant VP2 proteins expressed from plasmids pIP41 and pIP201 are slightly different. VP2 of the Australian strain expressed from pIP41 is fused at the aspartic acid residue in position 6 to eight residues of the vector, which replace the first five amino acids of the native VP2 protein. In pIP201, containing VP2 of the variant strain, the methionine initiation codon of the native VP2 protein is preceded by six residues from the primer/vector. The clones could be distinguished by restriction analysis because unique *Cla*I and *Pvu*I sites were introduced into the VP2 gene of pIP201 by primer N527.

DNA sequence analysis

The DNA sequence of the variant VP2 gene (Fig. 4) was determined from the 1·5 kb *Eco*RI–*Xho*I fragment of pIP201 and by ssDNA sequencing of M13 subclones in both orientations; the sequence of the central *Acc*I–*Spe*I fragment was confirmed by dsDNA sequencing of pIP201. The N and C termini of the VP2 gene sequence from pIP201 correspond to sequences of Australian

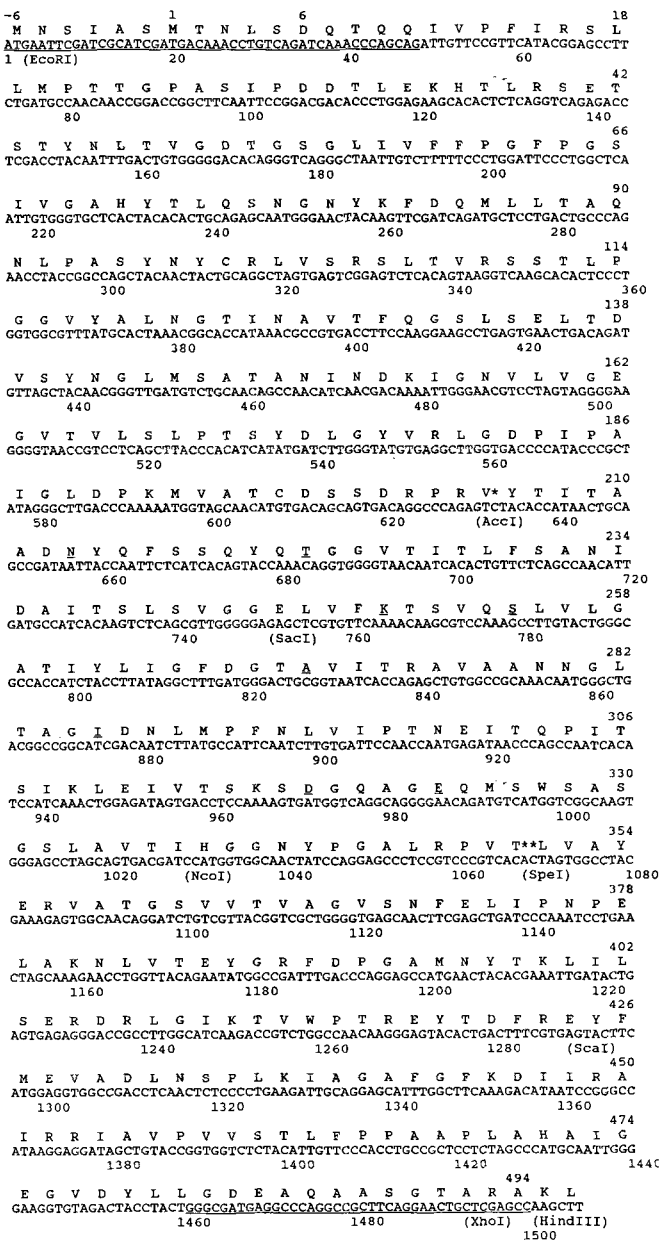

Fig. 4. Nucleotide sequence and deduced amino acid sequence of VP2 from IBDV variant strain E. The N and C termini of VP2 (underlined) correspond to primers N527 and N526 respectively. The beginning and end of the variable *Acc*I–*Spe*I fragment are marked in the protein sequence by a single asterisk at the *Acc*I site and a double asterisk at the *Spe*I site. The numbering of the nucleotide sequence starts with the first initiation codon preceding the native VP2 sequence, whereas the numbering of the amino acid sequence starts with the first methionine of the native VP2 and not that of primer N527. The amino acid residues which differ from the IBDV consensus sequence are underlined.

strain 002-73 (underlined in Fig. 4) and were derived from PCR primers N527 and N526 (Fig. 1). The nucleotide sequence of the variant contained an *Nco*I restriction site (at nucleotide position 1029) and three *Pst*I sites (at positions 238, 312 and 644) which are not present in the Australian strain; the *Stu*I site (at position 777) present in strain 002-73 had been lost. The nucleotide sequence contained one uninterrupted open reading frame from which the amino acid sequence was deduced.

The amino acid sequence of VP2 from variant strain E (Fig. 4) was compared with the published sequences of five different pathogenic or non-pathogenic standard type I strains and a consensus sequence was compiled (Fig. 5). Australian strain 002-73 (Hudson *et al.*, 1986) and European strain CU1 (Bayliss *et al.*, 1990) are of low pathogenicity; strain STC (Kibenge *et al.*, 1990) is the pathogenic challenge strain of the U.S. Department of Agriculture and strain 52/70 (Bayliss *et al.*, 1990) is a pathogenic European strain; strain PBG 98 (Bayliss *et al.*, 1990) is a non-pathogenic European strain. Differences in the deduced amino acid sequence clustered in the region encoded by the *Acc*I–*Spe*I fragment (Fig. 5), which contains the virus-neutralizing epitope (Azad *et al.*, 1987) and has been identified as a variable region in VP2 (Bayliss *et al.*, 1990). Outside the *Acc*I–*Spe*I-encoded region, there was only a single residue difference between the sequence of the variant strain and that of the Australian strain 002-73 (besides the changes at the N terminus of VP2 introduced by primer N527), and none compared with the other strains; Australian strain 002-73 contained a methionine at position 74 instead of a leucine residue as in all the other strains. Within the *Acc*I–*Spe*I-encoded region, the sequence of variant strain E differed in nine residues from strain 57/70, in 11 residues from strain STC, in 12 residues from strain CU1, in 13 residues from strain PBG98 and in 17 residues from Australian strain 002-73 (Fig. 5); the variant strain differed in only eight amino acid residues from the consensus sequence. Two substitutions occurred in the first hydrophilic region of the variable fragment [residue 213, from aspartic acid to asparagine (D213N), and residue 222, from proline to threonine (P222T)], two substitutions in the second hydrophilic region [residue 318, from glycine to aspartic acid (G318D), and residue 323, from aspartic acid to glutamic acid (D323E)] and four amino acid substitutions in the region between the two hydrophilic regions [residue 249, from glutamine to lysine (Q249K), residue 254, from glycine to serine (G254S), residue 270, from threonine to alanine (T270A), and residue 286, from threonine to isoleucine (T286I)] (Fig. 5). Changes in the two hydrophilic domains (Azad *et al.*, 1987) (boxed in Fig. 5) of the *Acc*I–*Spe*I-encoded region were unique to the variant strain.

By comparing the amino acid sequences of VP2 of the virulent strains 52/70, STC and variant E with those of strains 002-73 and CU1 of low pathogenicity, and the

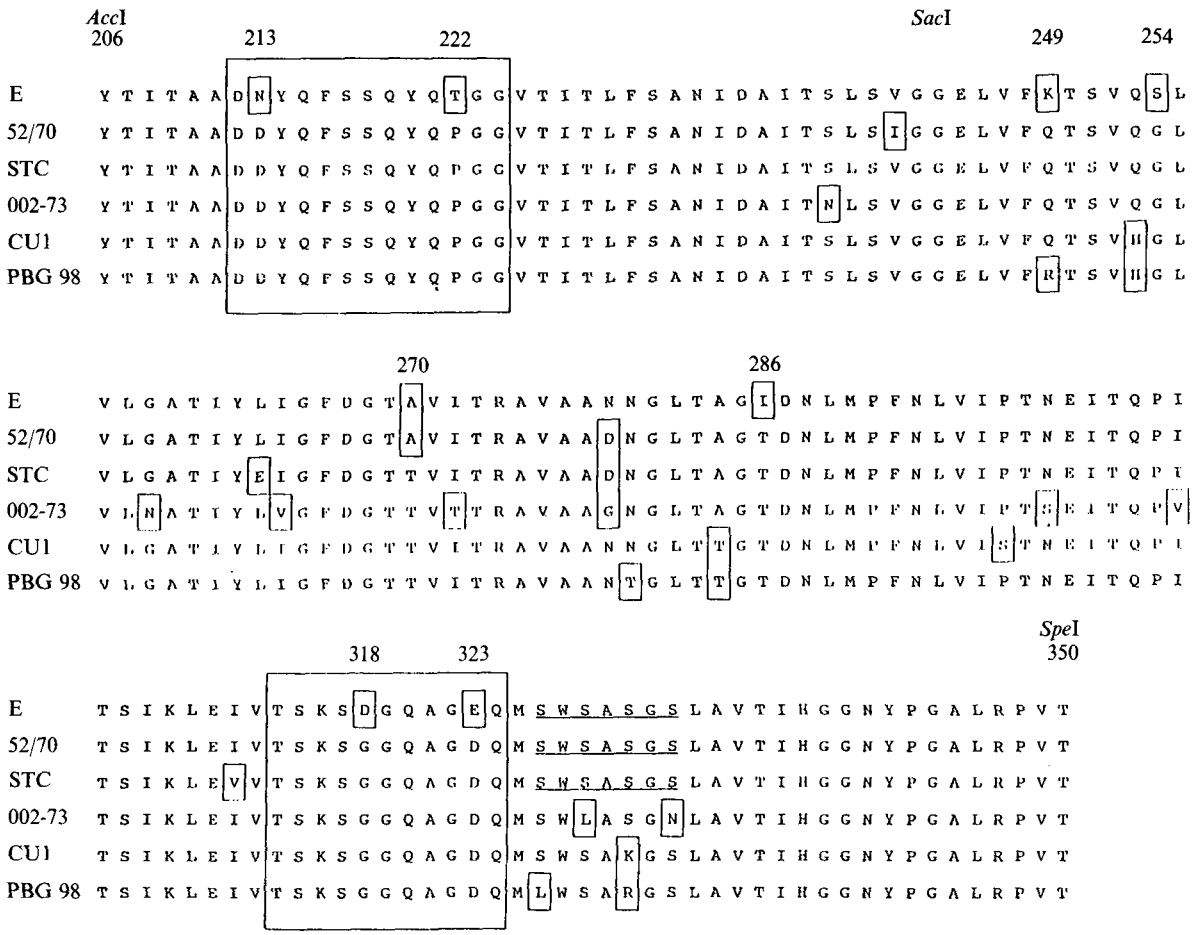

Fig. 5. Comparison of the amino acid sequences of the central *Acc*I–*Spe*I region of VP2 of variant strain E and type I strains 002-73 (Hudson *et al.*, 1986), STC (Kibenge *et al.*, 1990), CU1, PBG 98 and 52/70 (Bayliss *et al.*, 1990). Both hydrophilic regions are surrounded by a box and the residues which differ from the consensus sequence are boxed individually. A heptapeptide conserved in virulent strains only is underlined. The *Acc*I, *Sac*I and *Spe*I sites indicated are present in strain 002-73 and in variant strain E.

non-virulent strain PBG98, the heptapeptide S-W-S-A-S-G-S was identified, which was conserved only in strains of high pathogenicity. In the non-pathogenic strains and strains of low pathogenicity, one or two serines were substituted for residues occupying more space in the protein structure. In the primary sequence this heptapeptide (underlined in Fig. 5) includes amino acid residues 326 to 332 and lies immediately after the second hydrophilic region encoded by the *Acc*I–*Spe*I fragment. According to secondary structure predictions (Garnier *et al.*, 1978), it is highly probable that the second hydrophilic region has a random coil structure, whereas the adjacent heptapeptide motif probably has a β-sheet structure.

Antigenic properties of recombinant VP2 products

Three virus-neutralizing MAbs and one non-neutralizing MAb which were raised against the Australian IBDV strain 002-73 have been used to analyse the antigenic properties of recombinant VP2 proteins expressed in *E. coli*. Virus-neutralizing MAbs 17-82, 39A and 9-6 (Azad *et al.*, 1987; Fahey *et al.*, 1991) bind to the central variable region (*Acc*I–*Spe*I fragment) in VP2 (Fig. 1*a*); non-neutralizing MAb 6-1 recognizes a conserved region (*Sca*I–*Xho*I fragment) at the C terminus of VP2 (Azad *et al.*, 1987) and was used as an internal control to assess the level of expression of VP2. This would allow specific effects of mutations on MAb binding to be discriminated from non-specific effects of mutations on levels of protein expression and/or stability.

VP2 of the variant strain, expressed from pIP201, was not recognized in DBIA by the virus-neutralizing MAbs 17-82 and 39A, and only weakly by MAb 9-6 (Table 1). The influence of particular amino acid residues on the formation of the conformational virus-neutralizing epitope was analysed in hybrids between Australian strain 002-73 and variant strain E VP2s, and in point mutants

Table 1. *Reactivity of MAbs in DBIA**

	MAb			
	17-82	39A	9-6	6-1
IBDV	+++	+++	+++	++
pIP41	+++	+++	+++	+++
pIP201	−	−	++	++
pIP203	+++	+++	+++	++
pIP204	−	+++	+++	++
pIP207	+++	+++	+++	+++
pIP208	−	−	+	+++
pIP209	+++	+++	+++	++
pIP210	+++	+++	+++	++

* −, No reaction; +, ++ and +++, increasing levels of reactivity.

of the variant strain E VP2, which restored the consensus sequence at particular positions.

The hybrid VP2 molecules from pIP207 and pIP210, which contained only the first hydrophilic region from the *Acc*I–*Spe*I fragment of the variant strain and most of the virus-neutralizing epitope of the Australian strain, reacted with the virus-neutralizing MAbs in DBIA as strongly as the VP2 of the Australian strain expressed from pIP41 (Table 1). The influence of the two changes in the second hydrophilic region from this fragment in the variant strain on the formation of the virus-neutralizing epitope was analysed by site-directed mutagenesis. In mutant pIP203, both amino acid substitutions observed in the variant VP2 sequence (D318G and E323D), and in mutant pIP204 only a single substitution (E323D), were mutated back to the sequence of the Australian strain, which is the same as the consensus sequence of all other strains in this region (Fig. 5). The double mutation in pIP204 completely restored the binding activity of the three virus-neutralizing MAbs in DBIAs. The single E323D mutation in pIP204 restored the binding of MAbs 39A and 9-6 in DBIAs, but not that of MAb 17-82 (Table 1). Of the three virus-neutralizing MAbs, only MAb 9-6 is reactive in Western blot experiments (Azad *et al.*, 1987; Fahey *et al.*, 1991), in which it reacted strongly with VP2 of the Australian strain but not with VP2 of the variant strain (Fig. 6). Surprisingly even the 'back-mutants' of the variant strain VP2 (pIP203 and pIP204), which reacted strongly in DBIA, did not react in Western blots. The VP2 hybrids (pIP207 and pIP210) which contained only the two changes of the variant strain in the first hydrophilic region of the variable fragment and otherwise had the same amino acid sequence as the Australian strain, reacted only very weakly in Western blots with MAb 9-6, although they were fully reactive in DBIA.

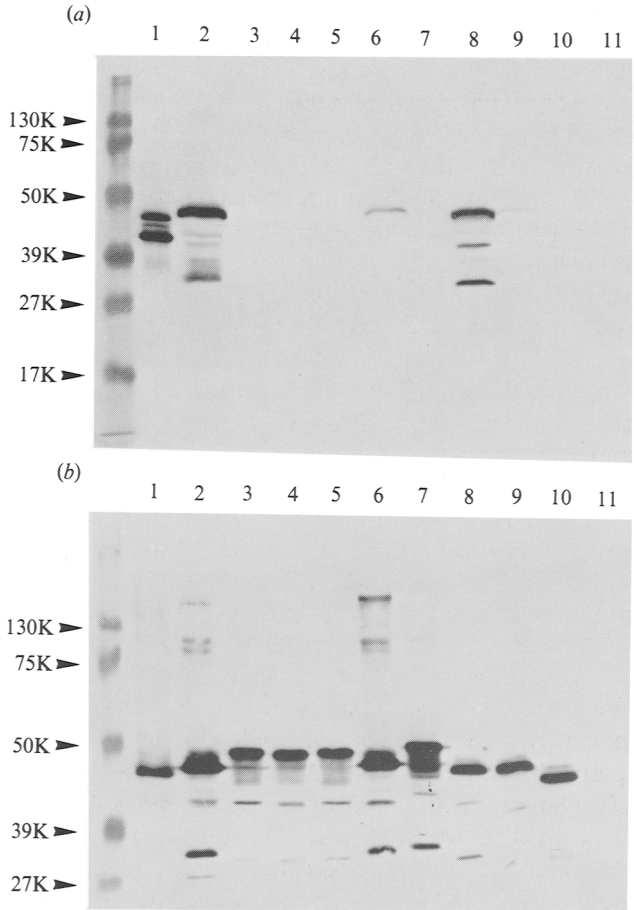

Fig. 6. Western blots of recombinant VP2 proteins. Lysates from IPTG-induced cultures of *E. coli* DH5α containing pIP41 (lane 2), pIP201 (lane 3), pIP203 (lane 4), pIP204 (lane 5), pIP207 (lane 6), pIP208 (lane 7), pIP209 (lane 8), pIP210 (lane 9), pIP77 (lane 10) and no plasmid (lane 11), were run on 11% SDS–polyacrylamide gels and Western-blotted with MAb 9-6 (*a*) and MAb 6-1 (*b*). Prestained markers (Bio-Rad low range standards) were run in lane M and 5 μg of IBDV protein from strain 002-73 was run in lane 1.

MAb 6-1 reacted in DBIA (Table 1) and Western blotting (Fig. 6*b*) experiments with the VP2 protein of Australian strain 002-73 expressed from pIP41, and that of variant E expressed from pIP201. In Western blots with MAb 6-1, the apparent M_r of the VP2 protein expressed from pIP201 (approximately 44 K) was slightly greater than that of VP2 expressed from pIP41 (approximately 42K) (Fig. 6*b*, lanes 2 and 3), which can partly be explained by the presence of three additional amino acids at the N terminus of the VP2 expressed from pIP201; the lower M_r products in the Western blot are degradation products of VP2. The reactivity in Western

blotting (Fig. 6*b*) and dot blot hybridization experiments (Table 1) of MAb 6-1 with the VP2 protein of the variant strain appeared to be diminished compared with that of the VP2 protein from strain 002-73, despite the identity of the amino acid sequences at the C termini of both proteins. The hybrid VP2 proteins expressed from pIP207 and pIP208 contain the same modified N terminus as the VP2 gene from pIP201, but did not show diminished reactivity with MAb 6-1. The expression levels of VP2 were therefore not influenced by differences in the N terminus of the VP2 gene. The full reactivity with MAb 6-1 of the hybrid VP2 protein expressed from pIP208 was restored (Table 1) by replacing the C-terminal *Sca*I–*Xho*I fragment of the VP2 gene from the variant strain with the corresponding sequence of the Australian strain. The cause of the diminished reaction of MAb 6-1 with VP2 fragments derived from variant strain E must therefore be encoded at the 3′ end of the VP2 gene. This was surprising as no amino acid changes had been detected in this region. We therefore analysed the nucleotide sequence within the *Sca*I–*Xho*I fragment at the C terminus of the VP2 gene in more detail and found a change in the codon usage of the tandem Arg at residues 452 and 453, which might explain the reduced reactivity with MAb 6-1. In the Australian strain the tandem Arg at positions 452 and 453 is encoded by AGAAGG, whereas in the variant VP2 gene there is a single nucleotide substitution, producing AGGAGG, which does not result in an amino acid change. In *E. coli* the use of the AGG codon for Arg is avoided because there is little tRNA for this codon, and the use of tandem AGG codons can produce a 50% translational frameshift (Spanjaard & van Duin, 1988; Spanjaard *et al.*, 1990). Although we have no direct proof for the effect of the tandem AGG codon in the VP2 gene, the reduced reactivity with MAb 6-1 could be explained by a frameshift which decreases the level of expression of the relevant eiptope without affecting the level of expression of the virus-neutralizing epitope.

Discussion

The host-protective antigen VP2 of an IBDV variant strain (Delaware variant E), which had emerged as a very virulent virus in vaccinated flocks, was cloned by PCR using primers homologous to the N- and C-terminal flanking regions of Australian strain 002-73. The DNA sequence of VP2 from the variant virus is highly conserved and, in comparison with five type I strains, changes affecting the amino acid sequence are found only in the central region (*Acc*I–*Spe*I fragment) of VP2, which contains the host-protective conformational epitope (Azad *et al.*, 1987). Antigenic variation between different strains must therefore be determined by residues in the variable central region of VP2, and the cloning procedure described here could be used to deal quickly with any variant strains which might arise in the future as it would allow the insertion of the host-protective epitope as a cassette into existing expression vectors suitable for the production of a recombinant vaccine (Macreadie *et al.*, 1990; Azad *et al.*, 1990).

Amino acid changes in the two hydrophilic regions at either end of the variable fragment (*Acc*I–*Spe*I) of VP2 are unique to variant strain E. Deletion studies have indicated that these hydrophilic regions may be involved in the formation of the virus-neutralizing epitope (Azad *et al.*, 1987); although these hydrophilic regions lie in the variable region of VP2, they are completely conserved in standard type I strains (Bayliss *et al.*, 1990). Virus-neutralizing antibodies cross-react with different standard IBDV strains (Wood *et al.*, 1988), indicating that at least part of the virus-neutralizing epitope is conserved between those strains. In contrast, VP2 from the variant strain contains two amino acid substitutions in each of these hydrophilic regions (Fig. 5) and virus-neutralizing MAbs 17-82, 39A and 9-6, raised against the Australian strain of IBDV, do not react or react very weakly with VP2 from variant strain E (Fig. 6 and Table 1).

An amino acid sequence motif, S-W-S-A-S-G-S, has been identified which is conserved only in virulent strains and could therefore be involved in virulence. The S-W-S-A-S-G-S motif lies next to the second hydrophilic region in the variable region of VP2 and has a predicted β-sheet secondary structure in which all four serine residues are exposed on one side. It is hypothesized that such a serine-containing surface could form hydrogen bonds which might be involved in intra- or intermolecular interactions important for virulence (through involvement in the virus attachment or maturation processes). In the non-pathogenic strains and strains of low pathogenicity the substitution of one or two serines for residues occupying more space in the protein structure might prevent such an interaction and interfere with virulence.

To analyse the molecular basis for the emergence of variants escaping vaccination with standard type I strains, we created hybrid proteins between the variant and Australian strain VP2s, as well as the variant VP2s containing point mutations. The involvement of particular residues in the formation of the host-protective epitope was then assessed by determining their ability to bind to virus-neutralizing MAbs raised against Australian strain 002-73 and cross-reacting with other standard type 1 strains of IBDV.

The two substitutions (D213N and P222T) in the first hydrophilic region of the variable central fragment (present in the hybrid VP2 expressed from pIP207 and

pIP210) do not themselves cause the loss of binding activity in DBIAs using MAbs 17-82, 39A and 9-6, although their activity in Western blot experiments is very much reduced. We argue that the substituted residues in the first hydrophilic region are not involved directly in binding to MAbs, but play a role in stabilizing the conformation of the epitope; the VP2 hybrids expressed from pIP207 and pIP210 have reduced reactivity in Western blot experiments after SDS–PAGE compared to that of the VP2 protein of the Australian strain expressed from pIP41.

The amino acid substitutions in the second hydrophilic region of the variable fragment of VP2 appear to be crucial for the ability of variant strain E to escape virus-neutralizing antibodies; the mutation of both residues back to those present in the consensus sequence restores the binding activity. This region is also important for the specificity of the binding to MAbs because for two of the MAbs (39A and 9-6) just the single back-mutation E323D was sufficient to restore reactivity in DBIAs, whereas for MAb 17-82 both changes in the second hydrophilic region had to be back-mutated to restore the ability to bind virus-neutralizing MAbs. The failure of these back-mutations (VP2 expressed from pIP203 and pIP204) to restore the reactivity with MAb 9-6 in Western blot experiments is a clear indication of the importance of other regions for the stabilization of the conformational epitope. We therefore conclude that the two hydrophilic regions are part of the virus-neutralizing epitope, as suggested earlier (Azad *et al.*, 1987), and especially that changes in the second hydrophilic region account for the resistance of variant strain E to neutralization by antibodies induced by classic vaccines.

The binding of MAb 6-1, which recognizes the C terminus of VP2, with VP2 from the variant strain was slightly reduced, although no amino acid changes had occurred in this region. This decrease in reactivity was not due to altered levels of protein expression or reduced stability of the protein caused by differences in the N termini of VP2 from pIP201 and pIP41. The causes of the reduction in binding of MAb 6-1 remain unclear, but the change in the usage of codons encoding the tandem Arg at positions 452 and 453 from AGAAGG in the Australian strain to AGGAGG in the variant strain could explain the reduced reactivity. Although we have no direct proof for the effects of the tandem AGG codon on the expression of VP2 in *E. coli*, it has been reported that the introduction of AGG codons in tandem can lead to a 50% translational frameshift in *E. coli* (Spanjaard & van Duin, 1988; Spanjaard *et al.*, 1990).

The data presented in this paper imply that the two hydrophilic regions within the variable central fragment of VP2 are involved in the formation of the major virus-neutralizing epitope; both hydrophilic regions are conserved in type I strains and only the variant strain differed in these regions. The substituted amino acids in the second hydrophilic region in the variant VP2 appear to be directly involved in binding to virus-neutralizing MAbs, whereas the substitutions in the first hydrophilic region are more likely to be involved in stabilizing the conformation of the epitope. The influence of other substitutions in the hydrophobic region lying between these hydrophilic regions has not been examined, and their involvement in the formation and stabilization of the conformational epitope cannot be ruled out without the analysis of further mutants.

We wish to thank Anthony Chapman for providing monoclonal antibodies and Nick Bartone for synthesizing the oligonucleotides. We gratefully acknowledge the contribution of the Central Veterinary Laboratory, Weybridge, U.K. in providing the variant strain E and use of their facilities to isolate the viral RNA. This work was supported in part by grants from the Generic Technology Component of the Industrial Research and Development Act 1986, and by funds from Arthur Webster Pty. Ltd., Sydney, Australia.

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(Received 14 December 1990; Accepted 11 April 1991)