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Virus Research, 5 (1986) 253–263
Elsevier

VRR 00270

The peplomer protein sequence of the M41 strain of coronavirus IBV and its comparison with Beaudette strains

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(Accepted for publication 21 April 1986)

Summary

The amino acid sequence of the gene for the peplomer protein of the vaccine strain M41 and the Beaudette laboratory strain M42-Salk of avian infectious bronchitis virus (IBV) have been derived from cDNA sequences. As found with other coronaviruses, the peplomer protein carries the epitopes eliciting neutralizing antibodies. The gene encodes a primary translation product of 1162 amino acids with a molecular weight of 128079. The use of a recent algorithm to predict membrane–protein interactions led to the unambiguous localization of the signal peptide and a transmembrane anchor α-helix at the C-terminus. At 50 positions amino acid differences were found between M41 and two Beaudette strains (M42-Salk and M42-Houghton). They are partly clustered in two regions of the protein. These two regions are candidates for neutralization epitopes of the protein.

coronavirus IBV, peplomer protein, strain M41, M42

Introduction

Infectious bronchitis virus (IBV) of chickens is the prototype virus of the family Coronaviridae, a group of enveloped viruses with a single-stranded colinear RNA genome of positive sense, approximately 20 kb in length (Stern and Kennedy,

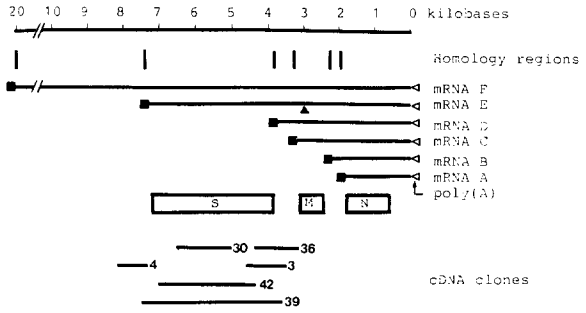


Fig. 1. Genomic organization of infectious bronchitis virus. The sequence relationships between the mRNAs are indicated, as well as the positions of the clones used for the sequencing of the gene encoding protein S of the M41 strain. The genes for the structural proteins are indicated by boxes. Homologies present in the genome at the intergenic boundaries are indicated with vertical bars. The leader sequence present at the 5′ end is indicated (■). The position of the oligonucleotide used to prime cDNA synthesis is marked by a triangle.

1980a,b). Virions contain three major structural proteins: the peplomer or surface protein (S), the nucleocapsid (N) and the membrane protein (M) (Cavanagh, 1981; Stern and Sefton, 1982; Stern et al., 1982).

The characteristic surface projections of the virions are made up from two copies of each of the two subunits of S: S1 (gp90) and S2 (gp84). Both S1 and S2 contain N-linked oligosaccharides of the high mannose type. They are non-covalently associated; the S2 protein is anchored to the membrane, while S1 is attached to S2 (Cavanagh, 1983a,b).

The most striking feature of coronavirus replication is the presence of a common leader in the genome and subgenomic mRNAs. The body and leader sequences of the mRNAs are spliced together by a hitherto unknown process of discontinuous transcription. The organization of the various genes of IBV in the genome and the subgenomic mRNAs have already been studied in detail and are summarized in Fig. 1 (Brown and Boursnell, 1984; Brown et al., 1984; Stern and Sefton, 1984; Binns et al., 1985). The peplomer protein is translated from mRNA E (Stern and Sefton, 1984). Neutralizing and hemagglutination inhibiting antibodies produced against the virion bind to the S1 subunit (Mockett et al., 1984; Niesters et al., unpublished data).

Recently, the sequence of the peplomer protein of the Beaudette strain (M42-Houghton) of IBV has been determined (Binns et al., 1985). Antibodies elicited by this laboratory strain fail to neutralize M41, another Massachusetts serotype virus, indicating that the two viruses have different neutralization epitopes (Chomiak et al., 1963). In this paper we present the peplomer sequence of this M41 strain, which is widely used as a vaccine to protect chickens against IBV infections, as well as from another Beaudette strain (M42-Salk). The comparison of the amino acid sequences of these viruses will be useful to localize epitopes eliciting neutralizing antibodies.

Materials and Methods

Virus

The M41 strain of IBV (obtained from Dr. B. Kouwenhoven, Poultry Health Institute, Doorn, The Netherlands) was cloned twice by end point dilution in the allantoic cavity of chicken embryos. Seed stocks were stored as allantoic fluid at −70°C. The origin of the M42-Salk strain has been described (Stern et al., 1984).

Virus growth and isolation of viral RNA

Approximately 100 egg infectious doses (EID_{50}) of the M41 virus stock were inoculated into the allantoic cavity of one hundred 10-day-old chicken embryos and incubated for 40 h at 37°C. The eggs were chilled for at least 4 h at 4°C before the allantoic fluid was harvested. After low speed centrifugation, sodium chloride and polyethylene glycol 6000 (BDH, Poole, England) were added to final concentrations of 2.33% (w/v) and 10% (w/v), respectively. The mixture was stirred overnight at 4°C and then centrifuged for 30 min at $10000 \times g$. The pellet as well as the fat at the meniscus were resuspended in 1/50 of the original volume of TESV-buffer (0.02 M Tris-HCl, 1 mM EDTA, 0.1 M NaCl, pH 7.4). The concentrated virus suspension was clarified (10 min at $10000 \times g$) and layered onto a 10% (w/v) sucrose cushion (10 ml) on top of a 20–50% (w/v) sucrose gradient (16 ml, both in TESV-buffer), and centrifuged for 6 h at 24000 rpm in an SW 27.0 rotor.

The light-scattering virus band was collected and diluted threefold in TESV-buffer. The virus was pelleted at $250000 \times g$ at 4°C for 90 min and incubated subsequently for 30 min at 37°C with 200 µl of TESV containing 0.5% SDS and 0.5 mg/ml proteinase K. Finally the RNA was phenol/chloroform extracted and the RNA was ethanol precipitated from the ether-extracted aqueous phase. About 35 µg RNA was obtained.

Cloning of IBV genomic RNA

cDNA synthesis was primed with the synthetic oligonucleotide 5'TGCATAGC-CATACTG3' on methylmercury hydroxide denaturated M41 RNA. First strand synthesis was carried out with 2.5 µg RNA and 6 µg primer in 60 µl of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 14 mM 2-mercaptoethanol and 600 U RNasin/ml (Amersham) for 60 min at 42°C. After addition of sodium acetate to 0.3 M, the cDNA was precipitated by the addition of 0.5 vol. isopropanol using Dextran 250 (Pharmacia) as carrier. The second cDNA strand was synthesized using the method of Gubler and Hoffman (1983). After dextran-isopropanol precipitation double stranded cDNA was tailed, using 4 U terminal transferase (Amersham) for 1 min with dC residues (Maniatis et al., 1982), annealed with PstI-cleaved, dG-tailed, pUC9 (Pharmacia) and subsequently used for transformation of *E. coli* strain JM109 (Yanish-Perron et al., 1985). White colonies hybridized with a few exceptions to a probe consisting of T4-polynucleotide-kinase labeled fragments of the genome prepared by alkali treatment. Restriction sites mapping of a number of clones resulted in a continuous map of 4.5 kb.

cDNA clones of the peplomer gene of strain M42-Salk were obtained using polyadenylated mRNAs from infected cells as template. Nucleotide sequencing was done following essentially the procedures described below. Full details will be presented elsewhere.

DNA sequencing

M41 restriction fragments of viral inserts in pUC9 were purified by agarose gel electrophoresis and isolated by binding to NA-45 paper (Schleicher and Schuell) before they were subcloned into M13mp9. Sequencing was carried out using the dideoxy method (Sanger et al., 1977) with α-^{32}P-dATP (Amersham) as label. Both an M13 sequencing primer as well as internal oligonucleotide primers complementary to IBV sequences were used. Nearly the whole sequence was determined on independently isolated cDNA clones. The data were analyzed on a DEC 20/60 computer, using the computer programs of Staden (1982).

Oligonucleotide synthesis

Oligonucleotides were prepared by solid phase synthesis as described previously (Seliger et al., 1982). The synthesis was carried out manually in a syringe (2 ml volume). The protected oligonucleotides were separated from truncated sequences by high performance liquid chromatography on a reversed phase column (Cp – Sm = Spher C18; Chrompack, The Netherlands). After deprotection and lyophilization, the oligonucleotides were ready to use as primers.

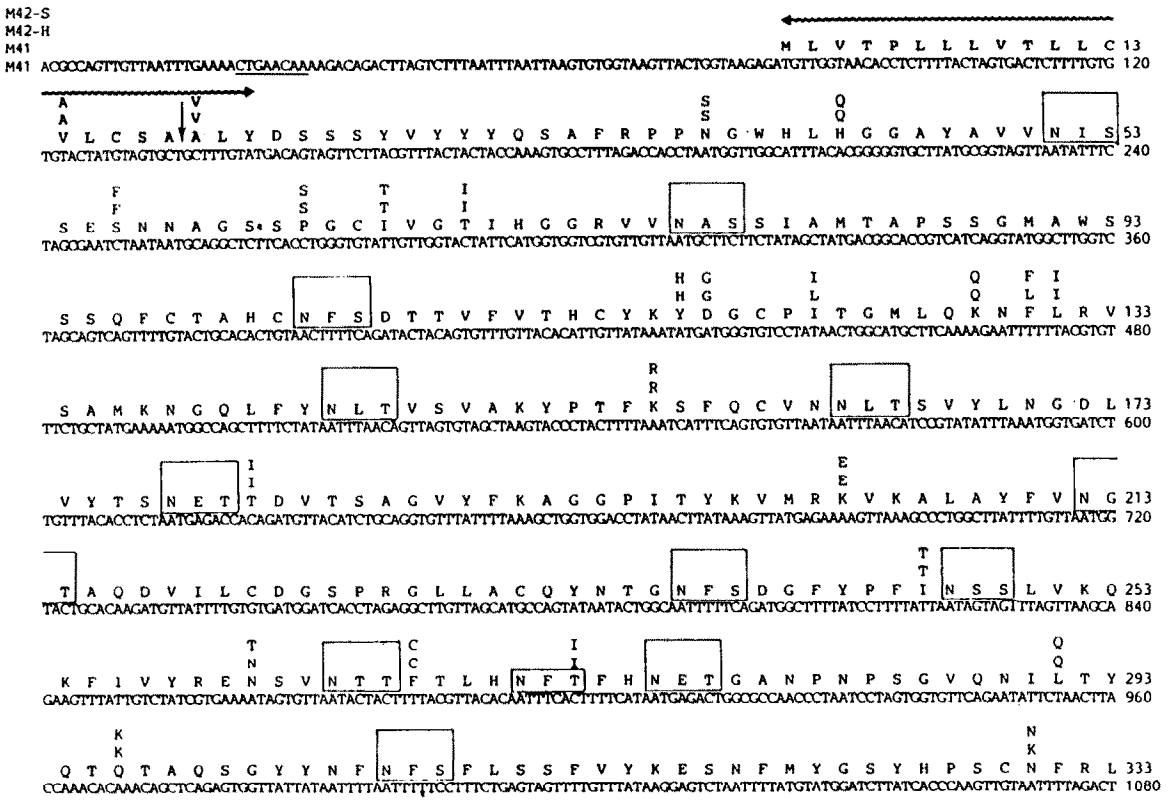
Results

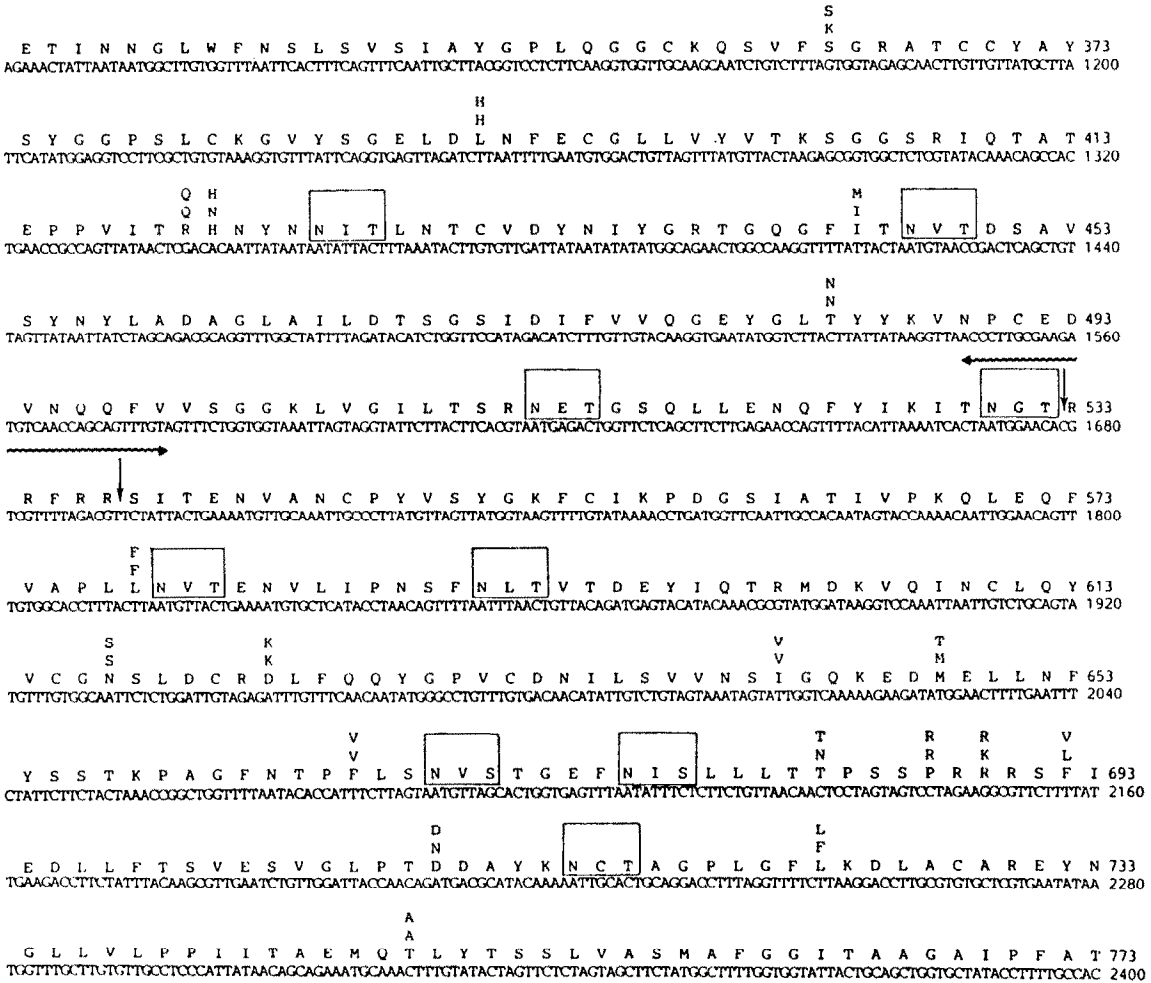
Preparation of M41 cDNA clones and their nucleotide sequencing

Unpublished sequence data from the membrane protein (M) of IBV Beaudette (M42-Salk) were available to us from Dr. John Rose, San Diego. An oligonucleotide primer complementary to a sequence stretch about 110 nucleotides downstream the initiating AUG codon (see Boursnell et al., 1984) was prepared to prime cDNA synthesis. Direct sequencing with this primer on genomic RNA of M41 and Beaudette virus revealed that it binds at the same position in the two strains (data not shown).

Forty-eight of the obtained recombinant DNA clones were further characterized by restriction site mapping. They contained inserts up to 4 kb with a mean length of 1 kb. Comparison with the restriction sites of the published sequence of the Beaudette strain (Binns et al., 1985) showed that one of them, p39, contains the genetic information of the complete peplomeric protein. To find more independent clones of the peplomer gene, overlapping clones were picked up by Southern blotting and restriction site analysis. In total 6 clones were used to determine the sequence of the gene. Their length and position within the gene are indicated in Fig. 1. The complete nucleotide sequence was determined after subcloning PstI fragments into the M13mp9 vector. Oligonucleotide primers were used to prime internally in long subcloned fragments.

Fig. 2. The nucleotide sequence and predicted amino acid sequence of the peplomeric protein of the IBV M41 strain. Amino acids substitutions in the two Beaudette strains (M42-Houghton and M42-Salk) are indicated. Potential posttranslational cleavage sites for the signal peptidase and the cleavage between the S1 protein (maximal 514 amino acids) and the S2 protein (maximal 625 amino acids) are indicated by arrows. Potential glycosylation sites (Asn-X-Ser or Asn-X-Thr, except when X = Pro) are boxed. Hydrophobic regions as well as the surface alpha helix predicted according to Eisenberg et al. (1984, Table 1) are indicated by a wavy line. The homology regions at the intergenic boundaries are underlined.

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H
H
S Y G G P S L C K G V Y S G E L D L N F E C G L L V Y V T K S G G S R I Q T A T 413
TTCATATGGAGGTCCTTCGCTGTGTAAAGGTGTTTATTCAGGTGAGTTAGATCTTAATTTTGAATGTGGACTGTTAGTTTATGTTACTAAGAGCGGTGGCTCTCGTATACAAACAGCCAC 1320
Q H
Q N
M
I
E P P V I T R H N Y N N I T L N T C V D Y N I Y G R T G Q G F I T N V T D S A V 453
TGAACCGCCAGTTATAACTCGACACAATTATAATAATATTACTTTAAATACTTGTGTTGATTATAATATATATGGCAGAACTGGCCAAGGTTTTATTACTAATGTAACCGACTCAGCTGT 1440
N
N
S Y N Y L A D A G L A I L D T S G S I D I F V V Q G E Y G L T Y Y K V N P C E D 493
TAGTTATAATTATCTAGCAGACGCAGGTTTGGCTATTTTAGATACATCTGGTTCCATAGACATCTTTGTTGTACAAGGTGAATATGGTCTTACTTATTATAAGGTTAACCCTTGCGAAGA 1560
V N Q Q F V V S G G K L V G I L T S R N E T G S Q L L E N Q F Y I K I T N G T R 533
TGTCAACCAGCAGTTTGTAGTTTCTGGTGGTAAATTAGTAGGTATTCTTACTTCACGTAATGAGACTGGTTCTCAGCTTCTTGAGAACCAGTTTTACATTAAAATCACTAATGGAACACG 1680
R F R R S I T E N V A N C P Y V S Y G K F C I K P D G S I A T I V P K Q L E Q F 573
TCGTTTTAGAAGTTCTATTACTGAAAATGTTGCAAATTGCCCTTATGTTAGTTATGGTAAGTTTTGTATAAAACCTGATGGTTCAATTGCCACAATAGTACCAAAACAATTGGAACAGTT 1800
F
F
V A P L L N V T E N V L I P N S F N L T V T D E Y I Q T R M D K V Q I N C L Q Y 613
TGTGGCACCTTTACTTAATGTTACTGAAAATGTGCTCATACCTAACAGTTTTAATTTAACTGTTACAGATGAGTACATACAAACGCGTATGGATAAGGTCCAAATTAATTGTCTGCAGTA 1920
S
S
K
K
V
V
T
M
V C G N S L D C R D L F Q Q Y G P V C D N I L S V V N S I G Q K E D M E L L N F 653
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V
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Y S S T K P A G F N T P F L S N V S T G E F N I S L L L T T P S S P R R R S F I 693
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D
N
L
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E D L L F T S V E S V G L P T D D A Y K N C T A G P L G F L K D L A C A R E Y N 733
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A
A
G L L V L P P I I T A E M Q T L Y T S S L V A S M A F G G I T A A G A I P F A T 773
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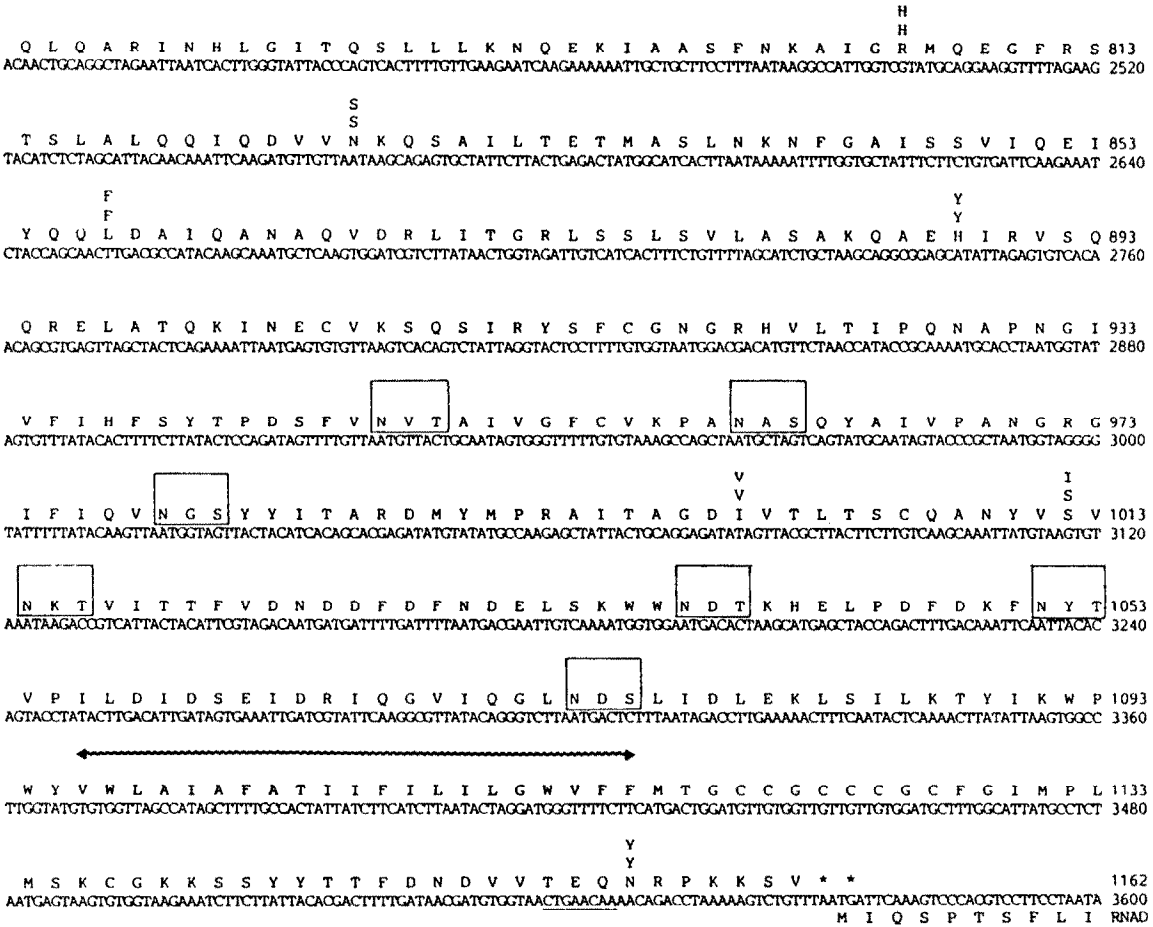
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S
S
T S L A L Q Q I Q D V V N K Q S A I L T E T M A S L N K N F G A I S S V I Q E I 853
TACATCTCTAGCATTACAACAAATTCAAGATGTTGTTAATAAGCAGAGTGCTATTCTTACTGAGACTATGGCATCACTTAATAAAAATTTTGGTGCTATTTCTTCTGTGATTCAAGAAAT 2640
F
F
Y
Y
Y Q Q L D A I Q A N A Q V D R L I T G R L S S L S V L A S A K Q A E H I R V S Q 893
CTACCAGCAACTTGACGCCATACAAGCAAATGCTCAAGTGGATCGTCTTATAACTGGTAGATTGTCATCACTTTCTGTTTTAGCATCTGCTAAGCAGGCGGAGCATATTAGAGTGTCACA 2760
Q R E L A T Q K I N E C V K S Q S I R Y S F C G N G R H V L T I P Q N A P N G I 933
ACAGCGTGAGTTAGCTACTCAGAAAATTAATGAGTGTGTTAAGTCACAGTCTATTAGGTACTCCTTTTGTGGTAATGGACGACATGTTCTAACCATACCGCAAAATGCACCTAATGGTAT 2880
V F I H F S Y T P D S F V N V T A I V G F C V K P A N A S Q Y A I V P A N G R G 973
AGTGTTTATACACTTTTCTTATACTCCAGATAGTTTTGTTAATGTTACTGCAATAGTGGGTTTTTGTGTAAAGCCAGCTAATGCTAGTCAGTATGCAATAGTACCCGCTAATGGTAGGGG 3000
V
V
I
S
I F I Q V N G S Y Y I T A R D M Y M P R A I T A G D I V T L T S C Q A N Y V S V 1013
TATTTTTATACAAGTTAATGGTAGTTACTACATCACAGCACGAGATATGTATATGCCAAGAGCTATTACTGCAGGAGATATAGTTACGCTTACTTCTTGTCAAGCAAATTATGTAAGTGT 3120
N K T V I T T F V D N D D F D F N D E L S K W W N D T K H E L P D F D K F N Y T 1053
AAATAAGACCGTCATTACTACATTCGTAGACAATGATGATTTTGATTTTAATGACGAATTGTCAAAATGGTGGAATGACACTAAGCATGAGCTACCAGACTTTGACAAATTCAATTACAC 3240
V P I L D I D S E I D R I Q G V I Q G L N D S L I D L E K L S I L K T Y I K W P 1093
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W Y V W L A I A F A T I I F I L I L G W V F F M T G C C G C C C G C F G I M P L 1133
TTGGTATGTGTGGTTAGCCATAGCTTTTGCCACTATTATCTTCATCTTAATACTAGGATGGGTTTTCTTCATGACTGGATGTTGTGGTTGTTGTTGTGGATGCTTTGGCATTATGCCTCT 3480
Y
Y
M S K C G K K S S Y Y T T F D N D V V T E Q N R P K K S V * * 1162
AATGAGTAAGTGTGGTAAGAAATCTTCTTATTACACGACTTTTGATAACGATGTGGTAACTGAACAAAACAGACCTAAAAAGTCTGTTTAATGATTCAAAGTCCCACGTCCTTCCTAATA 3600
M I Q S P T S F L I RNAD

Amino acid sequence

The sequence contains an open reading frame of 3486 nucleotides with a coding capacity for an apoprotein with an M_r of 128079. The open reading frame is followed by two stop codons (Fig. 2). The same open reading frame was found in the Beaudette M42-Salk strain (data not shown). This sequence differs at 20 positions from the Beaudette M42-Houghton strain and at 64 positions from the M41 strain. The differences in the predicted amino acid sequences of the M41, M42-Salk and M42-Houghton strains are indicated in Fig. 2.

To identify and characterize the segments of the polypeptide interacting with a lipid bilayer, we used the algorithm of Eisenberg et al. (1984). In this algorithm, hydrophobicities of a 21-residue moving segment are used to predict α-helices of proteins which penetrate with the lipid bilayer. Their positions for the M41 strain, together with their mean hydrophobicities, are given in Table 1. An α-helix was predicted at the N-terminus of the S proteins of all three strains from positions 1–21. Another monomeric transmembrane anchor was predicted at the carboxy terminus of the S2 protein (amino acid 1096–1116, bold face in Fig. 2), within a region of 44 non-polar amino acids (amino acid 1092–1135). This region is followed by a cluster of cysteine residues and a number of charged amino acids, which have to be situated within the interior of the virion.

The Eisenberg program also indicates possible surface α-helices; they are amphophilic (low hydrophobicity, high hydrophobic moment) and seek a surface between hydrophilic and hydrophobic phases. Such a surface helix was predicted at amino acids 528–538, the region where the proteolytic cleavage between S1 and S2 occurs (see below).

TABLE 1

TRANSMEMBRANE AND SURFACE ALPHA HELICES IN THE PEPLOMERIC PROTEIN S OF IBV STRAIN M41

	21-residue segment with highest ⟨H⟩		11-residue segment with max. ⟨μH⟩		
	Amino acids [a]	⟨H⟩ [b]	Amino acids [c]	⟨μH⟩ [d]	⟨μH⟩ [e]
1st helix	1– 21	0.68	8– 18	0.67	0.26
2nd helix	1096–1116	1.00	1102–1112	1.00	0.14
Surface helix			528– 538	−0.86	0.82

[a] Transmembrane helices localized on the basis of the maximal mean hydrophobicity (⟨H⟩) according to Eisenberg et al. (1984); numbers represent the residue numbers in the protein. Paired hydrophobic helices more than 100 amino acids apart from each other have been ignored.

[b] Corresponding mean hydrophobicity values. A single hydrophobic transmembrane segment should have a mean hydrophobicity more than 0.68.

[c] Regions with maximal mean hydrophobic moment.

[d] Corresponding mean hydrophobicity values.

[e] Corresponding mean hydrophobic-moment values.

Discussion

The nucleotide as well as the amino acid sequence of the unique region of mRNA E of IBV strain M41 reported here contain the characteristic features for the coronavirus peplomer protein S (Binns et al., 1985). Upstream from the AUG initiation codon at position 84, as well as near the end of the open reading frame, a sequence 5′CTGAACAAA3′ is located, which is almost identical to the homology regions that have been postulated to be present at the putative 5′ ends of the bodies from mRNA A, B, C, D and E of the Beaudette strain (Brown and Boursnell, 1984; Brown et al., 1984; Binns et al., 1985). These sequences are underlined in Fig. 2. The open reading frame extends 21 nucleotides beyond the homology region between the genes of mRNA E and D. The coding information for gene D starts at position 3571 in another reading frame within the double stop codon of gene E. The apoprotein contains a characteristic signal sequence, which is cleaved off between Ala-18 and Ala-19 (Von Heijne, 1983; Cavanagh et al., 1986). At the carboxyterminus a monomeric transmembrane anchor is present. The M41 precursor has 29 potential glycosylation sites, assuming that the Asn-Pro-Ser signal is not used (Neuberger et al., 1972), 1 on S1 and 12 on S2. The two M42 strains lack the glycosylation site in S1 of M41 at amino acid position 271.

The recent elucidation of the NH_2 terminal amino acid sequence of S2 as Ser-Ile-Thr-Glu by Cavanagh et al. (1986) proves that S2 starts at Ser-538. As is often found, this cleavage is next to two basic amino acids (Strauss and Strauss, 1983). It is possible that a Golgi carboxypeptidase trims away the 5 preceding amino acids Arg-Arg-Phe-Arg-Arg after the cleavage. A similar removal of a connecting peptide occurs in the hemagglutinin of fowl plague virus (Porter et al., 1979) and during the processing of the complement C4 protein (Belt et al., 1984). In the peplomer of IBV, the polypeptide chain may form a single α-helix in this region (Table 1, Fig. 2).

The main reason to undertake this study was to be able to compare the amino acid sequence of the S1 proteins of related IBV-strains. Although excellent vaccines are available for IBV, the disease is still widespread due to the occurrence of new variants. These variants probably arise from random mutations in the field giving rise to strains escaping herd immunity. So far as is known, only antibodies directed against the S1 protein are able to neutralize virus infectivity (Mockett et al., 1984; Niesters et al., unpublished data). This is in agreement with the model of Cavanagh (1983b), where S1 is on the outside of the virion and attached to the membrane by way of the S2 protein. Therefore, S1 is probably responsible for the attachment of the virion to the cell, an interaction which can be prevented by neutralizing antibodies. Thus amino acid substitutions in S1 are likely to be responsible for antigenic differences between the various strains of IBV. Comparison of the sequence of M41 virus with the published amino acid sequence of the Beaudette strain (M42-Houghton) as well as with the other Beaudette strain (M42-Salk) shows that the proteins have the same length and a 96.2% conservation of the amino acid residues. There are 29 positions where amino acid differences between S1 of M41 and the two Beaudette strains were observed; for the S2 proteins 20 differences were

found. In the S1 sequence two regions of clustered amino acid substitutions have been found (residues 56–69, and residues 117–131). The clustering is statistically significant using the χ-square test ($r = 0.98$). In the S2 protein only one cluster of changed amino acids is observed (residues 683–692) when one Beaudette strain (M42-Houghton) is considered.

It seems likely that at least one of the clusters in S1 reflects the difference in neutralization epitopes between M41 and M42 virus; they contain the only two amino acid substitutions requiring 2 mutations (Pro-63 and Lys-128). The occurrence of a neutralizing monoclonal antibody recognizing a conformation dependent epitope in the S1 protein of M41 (unpublished results) indicates that both clusters, brought together by disulfide linkages, might be part of the neutralization epitope.

The availability of the sequences reported here together with a panel of monoclonal antibodies allows extensive studies on the localization of the various epitopes of the S protein, both by screening expression libraries containing fragments of the S1 protein (Nunberg et al., 1984; Stanley and Luzio, 1984) or by monitoring the reaction of the antibodies with synthetic peptides representing the whole protein (Geysen et al., 1984). In addition we will use this sequence as a prototype for comparison with other IBV variant strains.

Acknowledgements

We thank Dr. Albert Maagdenberg for setting up the computer programs. The synthetic primer 5'TGCATAGCCATACTG3' was a gift from Dr. J.H. van Boom. We thank Drs. John Rose and David Cavanagh for sharing unpublished data, and Dorothee Brandenburg for assistance with the oligonucleotide synthesis. W.J.M.S. was supported by NACEE and The Netherlands Organization for the Advancement of Pure Research (Z.W.O.). The analysis of M42-Salk was performed at the Salk Institute, San Diego, California. This work was supported by a research grant from Duphar BV, Weesp, The Netherlands.

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(Manuscript received 6 January 1986)