The Coronavirus Hemagglutinin Esterase Glycoprotein

DAVID A. BRIAN, BRENDA G. HOGUE,
AND THOMAS E. KIENZLE

I. DISCOVERY OF THE HEMAGGLUTININ ESTERASE PROTEIN

Early reviews on coronavirus structure (Siddell *et al.*, 1983a,b; Sturman and Holmes, 1985) described coronaviruses as having three major structural proteins: a large-surface (or peplomer) glycoprotein of around 200 kDa, a phosphorylated nucleocapsid protein of around 50 kDa, and a glycosylated, multi-spanning membrane protein of around 30 kDa. This description was based primarily on studies of the prototypic avian infectious bronchitis virus (IBV) and the highly studied mouse hepatitis virus (MHV), strain A59. Although IBV was shown early on to have a weak hemagglutinating property, detection of the hemagglutinating activity required that the virus first be treated with phospholipase C or concentrated by centrifugation in sucrose gradients (Bingham *et al.*, 1975). Not all strains of IBV demonstrated hemagglutination, however, and hemagglutinating activity by IBV, as well as by the porcine transmissible gastroenteritis virus (TGEV) (Noda *et al.*, 1987, 1988) was probably a cryptic property

DAVID A. BRIAN • Department of Microbiology, University of Tennessee, Knoxville, Tennessee 37996-0845. BRENDA G. HOGUE • Department of Microbiology and Immunology, Division of Molecular Virology, Baylor College of Medicine, Houston, Texas 77030. THOMAS E. KIENZLE • John L. McClellan Memorial Veterans Administration Medical Research Service, Little Rock, Arkansas 72205; *present address*: Department of Ophthalmology, Baylor College of Medicine, Houston, Texas 77030.

The Coronaviridae, edited by Stuart G. Siddell, Plenum Press, New York, 1995.

of the spike protein (Cavanagh and Davis, 1986). The nature of this hemagglutinating activity is not well understood.

Several coronaviruses, unlike those described above, have been shown to have a strong hemagglutinating property. The activity requires no pretreatment of virus. These are the bovine coronavirus (BCV) (Sharpee *et al.*, 1976), the hemagglutinating encephalomyelitis virus of swine (HEV) (Callebaut and Pensaert, 1980), human coronavirus OC43 (HCV OC43) (and OC38 and other close relatives) (Kaye and Dowdle, 1969), the turkey coronavirus (TCV) (Dea and Tijssen, 1988), and the MHV-related diarrhea virus of infant mice (DVIM) (Sugiyama and Amano, 1980, 1981; Sugiyama *et al.*, 1986). Interestingly, in all cases where it has been examined, these viruses show a second, shorter fringe of peplomers by electronmicroscopy (Bridger *et al.*, 1978; Sugiyama and Amano, 1981).

The first report showing the possibility of a fourth major structural protein for the strongly hemagglutinating coronaviruses was that by Callebaut and Pensaert for HEV (1980). A glycoprotein of 140 kDa was described that reduced to subunits of 76 kDa after treatment with 2-mercaptoethanol. The gradient-purified 140-kDa glycoprotein appeared to have hemagglutinating properties. The character of the fourth major structural protein was more clearly shown by King *et al.* for the bovine coronavirus (King and Brian, 1982; King *et al.*, 1985). The 140-kDa glycoprotein was not removed from BCV by bromelain (whereas the surface protein was) and BCV retained its ability to hemagglutinate mouse erythrocytes. The property of hemagglutination was therefore associated with the 140-kDa glycoprotein. This association was further supported by four other observations. (1) Monospecific, polyclonal rabbit antiserum against the BCV hemagglutinin failed to identify a homologous protein on the nonhemagglutinating MHV A59 virion, an otherwise close antigenic relative of BCV (Hogue *et al.*, 1984). (2) Monoclonal antibodies to the BCV 140-kDa protein inhibited hemagglutination by BCV (in addition to showing neutralizing activity) (Deregt and Babiuk, 1987; Deregt *et al.*, 1989). (3) The presence of the 140-kDa protein on HCV OC43 correlated with hemagglutinating activity during protease digestion experiments (in a pattern similar to that for BCV) (Hogue and Brian, 1986). (4) The dimeric 140-kDa protein has been found on all of the strongly hemagglutinating coronaviruses studied to date.

II. DEDUCED AMINO ACID SEQUENCE AND PROPERTIES OF THE BOVINE CORONAVIRUS HEMAGGLUTININ ESTERASE PROTEIN

Extensive studies demonstrating the pattern of synthesis, assembly, and glycosylation of the hemagglutinin protein (Deregt *et al.*, 1987; Hogue *et al.*, 1989) were done prior to knowledge of the hemagglutinin gene sequence or knowledge of its acetyl esterase activity. These are discussed here, however, in the context of the deduced amino acid sequence for the protein.

The gene encoding the hemagglutinin esterase (HE) protein maps on the 5' side of the spike protein gene. This was predicted from the size of a mRNA (species 2b) that was present in BCV, but not in MHV A59 or some strains of

MHV-JHM, and from the 3' nested-set pattern of coronavirus mRNAs (Abraham *et al.*, 1990; Keck *et al.*, 1988; Siddell, 1983). Interestingly, the (defective) gene encoding the HE protein was first sequenced for the nonhemagglutinating MHV A59 (Luytjes *et al.*, 1988). Examination of the gene sequence revealed three important features. (1) The MHV-A59 genome contains a nonfunctional, apparently vestigial, hemagglutinin gene with no initiation codon for translation of the HE open reading frame (ORF). This protein, as demonstrated by Hogue *et al.* (1984), is not made in MHV-A59-infected cells. (2) There is no UCUAAC consensus intergenic sequence of the type thought to play a role in the initiation of transcription immediately upstream of the HE ORF. (3) Within the deduced HE amino acid sequence there is an F-G-D-S sequence identical to the acetyl esterase active site on the HE molecule of influenza C (Luytjes *et al.*, 1988).

Acetyl esterase activity, which functions to destroy a virus receptor (discussed in Section VI), was first demonstrated for the BCV hemagglutinin (Vlasak *et al.*, 1988a). The hemagglutinin was therefore renamed the hemagglutinin esterase protein.

Some strains of MHV-JHM express a homologous 65-kDa protein (Makino *et al.*, 1983; Siddell, 1981; Taguchi *et al.*, 1986). However, MHV-JHM and also, for example, MHV-S isolates, hemagglutinate, if at all, only very poorly (Shieh *et al.*, 1989; Sugiyama *et al.*, 1986; Talbot, 1989; Yokomori *et al.*, 1989). The reasons for this are unclear, as the cloned MHV-JHM HE gene, expressed using a vaccinia virus/T7 RNA polymerase system, encodes a protein that dimerizes, has acetyl-esterase activity, and is able to hemadsorb rat erythrocytes (Pfleiderer *et al.*, 1991).

Studies on the synthesis and glycosylation of the BCV HE protein established that it is synthesized as a monomeric apoprotein of approximately 42 kDa that becomes cotranslationally glycosylated and rapidly disulfide-linked (Deregt *et al.*, 1987; Hogue *et al.*, 1989). Only asparagine-linked glycosylation is found, with six to seven sugar chains of either the high mannose or hybrid type and three to four chains of the complex type attached per subunit, as deduced from a final subunit size of 65 kDa. Since coronaviruses assemble by budding into transitional elements occurring between the endoplasmic reticulum and pre-Golgi (Tooze *et al.*, 1988), the maturation of HE carbohydrates to complex forms must take place on the virion during migration through the exocytic pathway to the cell surface.

The deduced amino acid sequence of the HE protein, as determined from cDNA clones of BCV genomic RNA (Fig. 1), shows it to be a 47,700-Da apoprotein of 424 amino acids (Kienzle *et al.*, 1990; Parker *et al.*, 1989; Zhang *et al.*, 1991). There are two very hydrophobic regions. The first encompasses the first 18 amino acids and has the properties of a signal peptide. Amino-terminal amino acid sequencing of the processed virion HE protein identifies a phenylalanine, indicating that the first 18 amino acids indeed function as a signal peptide for membrane translocation (Hogue *et al.*, 1989). The deduced molecular weight of the nonglycosylated protein without its signal peptide is 45 kDa. The second hydrophobic region extends from amino acid 390 through 415 and is known to serve as the membrane anchor for HE (Kienzle *et al.*, 1990; Parker *et al.*, 1990). Therefore, the resulting HE is a type I glycoprotein with an amino-

60
CTAAACTCAGTGAAATGTTTTGCTTCTTAGATTGTTCTAGTTAGCTGCATAATTGGT
 M F L L L R F V L V S C I I G
 HE →
 AGCCTAGGTTTTGATAACCCCTCTACCAATGTTGTTTCGATTAAATGGAGATTGGTTT
S L G F D N P P T N V V S H L N G D W F
 ↑
 M E I G F
 IORF1 →
 TTATTGGTGACAGTCGTTGAGATTGTAATCATGTTGTTAATACCAACCCCGTAATTAT
 L F G D S R S D C N H V V N T N P R N Y
 Y L V T V V Q I V I M L L I P T P V I I
 240
 TCTTATATGGACCTTAATCTGCCCTGTGTGATTCTGGTAAAATATCATCTAAAGCTGGC
 S Y M D L N P A L C D S G K I S S K A G
 L I W T L I L P C V I L V K Y H L K L A
 300
 AACTCCATTTTTAGGAGTTTCACTTTACCGATTTTTATATTACACAGGCGAAGGTCAA
 N S I F R S F H F T D F Y N Y T G E G Q
 T P F L G V F T L P I F I I T Q A K V N
 360
 CAAATTATTTTTATGAGGGTGTTAATTTTACGCCTTATCATGCCTTTAAATGCACCACT
 Q I I F Y E G V N F T P Y H A F K C T T
 K L F F M R V L I L R L I M P L N A P L
 420
 TCTGGTAGTAATGATATTTGGATGCAGAATAAAGGCTTGTTTTACACTCAGGTTTATAAG
 S G S N D I W M Q N K G L F Y T Q V Y K
 L V V M I F G C R I K A C F T L R I R
 480
 AATATGGCTGTGTATCGCAGCCTTACTTTTGTTAATGTACCATATGTTTATAATGGCTCT
 N M A V Y R S L T F V N V P Y V Y N G S
 I W L C I A A L L L M Y H M F I M A L
 540
 GCACAATCTACAGCTCTTTGTAAATCTGGTAGTTAGTTCTTAATAACCTGCATATATA
 A Q S T A L C K S G S L V L N N P A Y I
 H N L Q L F V N L V V *
 600
 GCTCGTGAAGCTAATTTTGGGGATTATTATTATAAGGTTGAAGCTGACTTTTATTGTCA
 A R E A N F G D Y Y Y K V E A D F Y L S
 660
 GGTGTGACGAGTATATCGTACCACCTTTGTATTTTAAACGGCAAGTTTTGTGCAATACA
 G C D E Y I V P L C I F N G K F L S N T
 720
 AAGTATTATGATGATAGTCAATATTATTTAATAAAGACACTGGTGTTATTTATGGTCTC
 K Y Y D D S Q Y Y F N K D T G V I Y G L
 780
 AATTCTACTGAAACCATTACCACTGGTTTTGATTTAATTGTCTATTATTAGTTTACCC
N S T E T I T T G F D F N C H Y L V L P
 840
 TCTGGTAATTATTAGCCATTTCAAATGAGCTATTGTTAACTGTTCTACGAAAGCAATC
 S G N Y L A I S N E L L L T V P T K A I
 900
 TGTCTTAACAAGCGTAAGGATTTTACGCCTGTACAGGTGTTGATTACGGTGAACAAT
 C L N K R K D F T P V Q V V D S R W N N
 960
 GCCAGGCAGTCTGATAACATGACGGCGGTGCTGTCAACCCCGTACTGTTATTTTCGT
 A R Q S D N M T A V A C Q P P Y C Y F R
 1020
 AATTCTACTACCAACTATGTTGGTGTGTTATGATATCAATCATGGGGATGCTGGTTTTACT
N S T T N Y V G V Y D I N H G D A G F T
 M L V F M I S I M G M L V L L
 IORF2 →

FIGURE 1.

The BCV HE gene contains within it two nonoverlapping ORFs, one (IORF 1) that could encode a 136 amino acid protein of 15,741 Da, and another (IORF 2) that could encode an 83 amino acid protein of 9,514 Da (Fig. 1). It is not known whether these are expressed during infection. The existence of these proteins is questionable since both are very hydrophobic throughout their length and there are no large internal ORFs within the HE gene of MHV-JHM or the HE pseudogene of MHV A59 [from our analysis of data in Luytjes *et al.* (1988) and Shieh *et al.* (1989)].

Expression of the cloned BCV HE gene, both *in vitro* and *in vivo*, has established that the HE protein alone contains all the necessary information for disulfide-linked homodimerization, membrane translocation, anchorage, transport to the cell surface as an integral membrane protein, erythrocyte-binding activity, and esterase activity (Kienzle *et al.*, 1990; Parker *et al.*, 1990; Yoo *et al.*, 1992). Cell surface expression of HE may explain hemadsorption by infected cells, although other explanations such as binding by nonreleased surface-

adherent viruses have been given (Bucknall *et al.*, 1972; Kapikian *et al.*, 1972). Cells infected with an adenovirus recombinant expressing the BCV HE exhibited both hemadsorption and esterase activities, both of which could be blocked by monoclonal antibodies having neutralizing activity (Parker *et al.*, 1990, Yoo *et al.*, 1992).

III. COMPARISON OF THE HES OF BCV, HCV-OC43, MHV, AND INFLUENZA C VIRUS

A remarkable conservation of amino acid sequence between the deduced HE proteins of MHV A59 (Luytjes *et al.*, 1988) and influenza C virus (Nakada *et al.*, 1984) suggested a common evolutionary origin for these proteins (Luytjes *et al.*, 1988). A similarly high degree of homology among the HEs of influenza C virus, BCV, and HCV-OC43 (Parker *et al.*, 1989; Kienzle *et al.*, 1990; Zhang *et al.*, 1992) and a striking conservation in the position of the putative acetylsterase active site and several of the cysteine residues (Fig. 2) further supported the idea of a common origin for the HE proteins. Although the HEs of BCV and HCV OC43 are quite similar to each other, they as a group appear to be no closer to influenza C virus HE in sequence similarity than does MHV-JHM HE (Fig. 2), suggesting they are equidistant in evolutionary time. Because of the ability of coronaviruses to undergo recombination at a high frequency (Lai *et al.*, 1987), it is conceivable that during coinfection with influenza C virus, a coronavirus (e.g., HCV OC43) could have undergone a similar recombination to give rise to the HE gene in the coronavirus genome (Luytjes *et al.*, 1988). One major interesting difference between the HEs of BCV and influenza C is that the influenza C HE is formed by the proteolytic cleavage of a large precursor (Herrler *et al.*, 1981), whereas the BCV HE is not.

Interestingly, the HE of influenza C virus (Nakada *et al.*, 1984) with which BCV HE shows 29% amino acid sequence homology does form disulfide-linked dimeric structures (Herrler *et al.*, 1981). Comparative structural analyses might also suggest the site of the hemagglutinating (cell binding) domain, assuming it is common among the various HEs.

IV. MODEL OF THE BCV HE ON THE VIRION

From evidence of the membrane orientation and dimeric structure of BCV HE, we have drawn a tentative model for the dimeric protein (Fig. 3). Placement

FIGURE 2. Alignment of the deduced amino acid sequences for the HE genes of MHV (JHM), BCV (Mebus), HCV-OC43, MHV-JHM, and influenza C virus (C/Cal/78). The MHV (Shieh *et al.*, 1989) and BCV (Kienzle *et al.*, 1990), HCV-OC43 (Zhang *et al.*, 1992), and influenza C (Nakada *et al.*, 1984) sequences were each aligned using the PileUp program in the Wisconsin GCG Sequence Analysis Package. The sequences begin with the signal peptide of each protein. The influenza C sequence contains all of the HA1 sunbunit and the first 26 amino acids of the HA2 sunbunit. Gaps (identified by dots) were introduced to maximize alignment. Aligned cysteine residues in all four sequences are boxed. The esterase site (FGDS) is boldfaced.

	1				50
BCV		..MFL.....	...LRFVLVS	CIIGSLGFDN	PPTNVVSHLN
OC43		..MFL.....	...PRFILVS	CIIGSLGFYN	PPTNVVSHVN
JHM	MGSTCIAMAP	RTLLLL....	...IGCQLV.	FGF.N	EPLNIVSHLN
FluC	MFF	SLLMLGLTE	AEKIKICLQK	QVNSSFSLHN	GFGGNLYATE
	51				100
BCV	GDWFL <u>FGDSR</u>	SDCNHVNTN	PRNYSYMDLN	PALCDSG...	.KISSKAGNS
OC43	GDWFL <u>FGDSR</u>	SDCNHIVNIN	PHNYSYMDLN	PVLCDSG...	.KISSKAGNS
JHM	DDWFL <u>FGDSR</u>	SDCTYVENNG	HPKLDWLDLD	PKLCNSG...	.KISAKSGNS
FluC	EKR.MFELVK	PKAGASVLNQ	STWIG <u>FGDSR</u>	TDQSNSAFPR	SLMSAKTADK
	101				150
BCV	IFRSFHFTDF		..YNYTGEGQ	QIIFYEGVNF	TPYHAFKTT
OC43	IFRSFHFTDF		..YNYTGEGQ	QIIFYEGVNF	TPYHAFKVR
JHM	LFRSFHFTDF		..YNYTGEGD	QIVFYEGVNF	SPNHGFMCLA
FluC	.FRSLSGGSL	MLSMFGPPGK	VDYLYQCGCK	HKVIFYEGVNW	SPHAAIDCYR
	151				200
BCV	SGSNDIWMQN	KGLFYTQVYK	NMAVYRSLTF	VNVVPYVYNGS	AQSTALCK.S
OC43	SGSNDIWMQN	KGLFYTQVYK	NMAVYRSLTF	VNVVPYVYNGS	AQSTALCK.S
JHM	YGDNKRWMGN	KARFYARVYE	KMAQYRSLSF	VNVPIAYGGK	AKPTSICK.H
FluC	KNWTDIKLN.	...FQKSIYE	LASQSHCMSL	VNALDKTIPL	QVTKGVAKNC
	201				250
BCV	GSLVLNNPAY	IAREANFGD.	YYY	KVEADFYLSG	QDEYIVPIC.
OC43	GSLVLNNPAY	IAPQANS GD.	YYY	KVEADFYLSG	QDEYIVPIC.
JHM	KTLTLNNPTF	ISKESNYVD.	YYY	ESEANFTLAG	QDEFIVPIC.
FluC	NNSFLKNPAL	YTQEVKPLEQ	ICGEENLAFF	TLPTQFGTYE	CKLHLVASCY
	251				300
BCV	.IFNGKFLSN	T.....KYYD	DSQYFKNKDT	GVIYGLNSTE	TIT.....TG
OC43	.IFNGKFLSN	T.....KYYD	DSQYFKNKDT	GVIYGLNSTE	TIT.....TG
JHM	.VFNGHSGKS	SSDPANKYYM	DSQSYNNMDT	GVLYGFNSTL	DVGNTAKDPG
FluC	FIYDSKEVYN	KRGCGNYF..	..QVIYDSSG	KVVGGLDNRV	SPYTGNSGDT
	301				350
BCV	FDFNCHYLVL	PSGNYLAISN	ELLTVPTKA	ICLNKRKDFT	PVQVDSRWVN
OC43	FDLNCHYLVL	PSGNYLAISN	ELLTVPTKA	ICLNKRKDFT	PVQVDSRWVN
JHM	LDLTCRYLAL	TPGNYKAVSL	EYLLSLPSKA	ICLRKPKRFM	PVQVDSRWVN
FluC	PTMQDMLQL	KPGRYSVRSS	PRFLMPERS	YCFDM.KEKG	PVTAVQSIWG
	351				400
BCV	NARQSDNMTA	VACQPPYCYF	RNSTTNY.VG	VYDINHGDAG	FTSILSGLLY
OC43	NARQSDNMTA	VACQPPYCYF	RNSTTNY.VG	VYDINHGDAG	FTSILSGLLY
JHM	STRQSDNMTA	VACQLPYCFF	RNTSADYSGG	THDVHGHGDFH	FRQLLSGLLL
FluC	KGRKSDYAVD	QACQLSTPGCM	LIQKQKPYIG	EADDHHGDQE	MRELLSGLDY
	401				450
BCV	DSPCFSQQGV	FRYDNVSSVW	PLY....SYG	RCPTAA.DIN	TPDVPICVYD
OC43	NSPCFSQQGV	FRYDNVSSVW	PLY....PYG	RCPTAA.DIN	NPDLPICVYD
JHM	NVSCIAQQGA	FLYNNVSSSW	PAY....GYG	CCPTAA.NIG	YMA.PVCIYD
FluC	EARCISQSG.	.WVNETSPFT	EEYLLPPKFG	RCPLAAKEES	IPKIPDGLLI
	451				500
BCV	PLPLILLGI.	LLGVA	VIIIVVLLLY	FMVDN.....	.GTRLHDA
OC43	PLPVILLGI.	LLGVA	VIIIVVLLLY	FMVDN.....	.GTRLHDA
JHM	PLPVVLLGV.	LLGIA	VLIIVFLILY	FMTDS.....	.GVRLHEA
FluC	PTSGTDTTVT	KPKSRIFGID	DLIIGLLFVA	IVEAGIGGYL	LGSRKESGGG

FIGURE 3. Model of the BCV HE protein. Identical chains representing the 65-kDa subunits (without N-terminal signal peptides) are shown as disulfide-linked monomers. Open circles represent hydrophilic amino acid residues (Asp, Glu, Lys, Arg, His, Asn, Gln), dotted circles represent intermediate amino acid residues (Gly, Ser, Thr, Tyr, Try, Pro), and solid circles represent hydrophobic amino acid residues (Ala, Val, Leu, Ile, Phe, Cys, Met). Amino acids were classified by the scale of Kyte and Doolittle (1982). Amino acid position numbers are shown (beginning with the N terminus of the signal peptide, not shown). Disulfide bridges between cysteine residues are represented by connected solid circles. The potentially glycosylated Asn residues in the ectodomain are identified by open diamonds. Triangles identify the putative active site of the esterase (F-G-D-S).

of disulfide bonds and protein loops is purely arbitrary but done in a way to maximize exposure of the hydrophilic, sugar-containing regions and the F-G-D-S esterase active site. It remains to be determined whether higher-order noncovalent oligomeric forms of the dimeric HE exist.

V. HEMAGGLUTININATING ACTIVITY AND ITS POSSIBLE ROLE IN VIVO

It has been proposed that HE may function as a second attachment protein that, in addition to the spike protein, serves to initiate infection (Parker *et al.*, 1989; Vlasak *et al.*, 1988a). This proposal was based primarily on the following observations: (1) The process of hemagglutination itself is a cell-binding process that may mimic cell binding during infection. (2) The HE homologue on influenza C virus was known to be an attachment protein that recognizes 9-O-acetylated sialic acid-containing cell receptors (Herrler *et al.*, 1985; Herrler and Klenk, 1987; Rogers *et al.*, 1986; Vlasak *et al.*, 1987, 1989). (3) A panel of monoclonal antibodies to the BCV HE protein exhibited neutralization of infection in cell culture and in the calf (Deregt *et al.*, 1989). With these antibodies, four neutralizing epitopes could be identified on the HE molecule (Deregt and Babiuk, 1987).

The idea that HE may function as a second attachment protein seemed to be further strengthened by the striking correlation between its presence and the neuronotropism expressed by the virus (Table I). Targeting of the HE-containing coronaviruses to the brain of experimentally infected mice was demonstrated for BCV (Akashi *et al.*, 1981; Kubo *et al.*, 1982; Barthold *et al.*, 1990), HCV OC43 (Pearson and Mims, 1983; Barthold *et al.*, 1990), and HEV (Yagami *et al.*, 1986, 1993). Except for the restricted growth of HE-less MHV A59 in neurons of the subthalamic nucleus and substantia nigra of mice (Fishman *et al.*, 1985) and the neuronotropism demonstrated by the HE-less MHV3 (Talbot, 1989; Tardieu *et al.*, 1986), it is particularly striking that coronaviruses possessing HE are neu-

TABLE I. Neuronotropism of HE-Containing Coronaviruses Cell Tropism in Central Nervous System

Coronavirus	Hemagglutinin form	Astrocytes	Oligodendrocytes	Neurons	References
MHV A59	No HE	+	+	— ^a	b
MHV-JHM	Dimer	+	+	+	b–e
DVIM	Dimer	?	?	?	f–h
BCV	Dimer	?	?	+	i
HCV OC43	Dimer	—	+	+	j–l
HEV	Dimer	?	?	+	m–o
TCV	Dimer	?	?	?	p

^aThere is restricted growth in neurons of the subthalamic nucleus and substantia nigra in mice (Fishman *et al.*, 1985).

References: b, Dubois-Dalcq *et al.* (1982); c, Knobler *et al.* (1981); d, Parham *et al.* (1986); e, Taguchi *et al.* (1986); f, Sugiyama and Amano (1980); g, Sugiyama and Amano (1981); h, Sugiyama *et al.* (1986); i, Kubo *et al.* (1982); j, Pearson and Mims (1983); k, Pearson and Mims (1985); l, Barthold *et al.* (1990); m, Andries and Pensaert (1981); n, Yagami *et al.* (1986); o, Yagami *et al.* (1993); p, Dea and Tijssen (1988).

ronotropic in either the natural infection (e.g., HEV) or during experimental infection in mice (Table I). One possible explanation for this neuronotropism might be the high concentration of 9-*O*-acetylated sialic acids on brain gangliosides (Haverkamp *et al.*, 1977; Herrler and Klenk, 1987). It will be important to determine whether the neuronotropism exhibited by some, but not all, strains of MHV-JMH (Dubois-Dalcq *et al.*, 1982; Fleming *et al.*, 1987; Goto *et al.*, 1986; Knobler *et al.*, 1981; Massa *et al.*, 1988; Morris *et al.*, 1989; Parham *et al.*, 1986; Taguchi *et al.*, 1986; Wege *et al.*, 1982) is a function of the variability in expression of the HE protein (Shieh *et al.*, 1989). One recent study showed that JHM-infected mice were protected when passively immunized with HE-specific nonneutralizing monoclonal antibodies, suggesting that this protein does play a role in infection by MHV-JHM (Yokomori *et al.*, 1992).

Recently the potential role of HE as an attachment protein for initiating infection has required reevaluation since the peplomeric spike proteins on BCV have also been shown to bind 9-*O*-acetylated sialic acid, the receptor determinant for BCV infection, and to cause hemagglutination (Kinkel and Herrler, 1993; Schultze *et al.*, 1991a; Schultze and Herrler, 1992; Storz *et al.*, 1991). Whether the S protein of BCV also recognizes proteins similar to the carcino-embryonic antigen as does MHV or the aminopeptidase N protein as does HCV 229E or TGEV (see Chapter 4, this volume) remain to be determined. But the fact that S binds to the same receptor determinant as HE would suggest that they both use the same receptor protein to initiate infection, if in fact they both do initiate infection. In a study where separately purified S and HE proteins from BCV were used to measure hemagglutination, a difference in the agglutinability of erythrocytes from different animal species was found (Schultze *et al.*, 1991a,b). Both S and HE were able to agglutinate mouse and rat erythrocytes; however, only S could agglutinate chicken erythrocytes. This was thought to reflect a more powerful binding by S since the chicken erythrocytes have fewer surface sialic acid molecules. These observations have led Herrler and co-workers to suggest that S and HE recognize the same receptor determinant, but that S is the important molecule for initiating infection (Schultze, 1991a,b). HE, it was proposed, may function primarily as a receptor-destroying enzyme to remove glycoconjugates that act as false receptors.

VI. ESTERASE ACTIVITY AND ITS POSSIBLE ROLE *IN VIVO*

The enzymatic activity associated with the HE is neuraminate-*O*-acetyl-esterase, which destroys the receptor determinants on erythrocytes bound by BCV, HCV OC43, and HEV (Vlasak *et al.*, 1988a,b). The receptors therefore contain 9-*O*-acetylated sialic acid and the activity is a serine esterase indistinguishable from the esterase of influenza C virus (Herrler *et al.*, 1985, 1988; Vlasak *et al.*, 1987, 1988a,b). The only other viruses possessing receptor-destroying activity are the orthomyxovirus types A and B and the paramyxoviruses, for which the receptor-destroying enzyme is neuraminidase.

Enzymatic association with the hemagglutinin molecule was shown in studies using diisopropyl fluorophosphate (Vlasak *et al.*, 1988a), which binds to

the serine in the active site and acts as a serine esterase inhibitor (Muchmore and Varki, 1987). Diisopropyl fluorophosphate completely inhibited the receptor-destroying activity of BCV, and 3H-diisopropyl fluorophosphate treatment of BCV with subsequent sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the protein revealed that the 140-kDa hemagglutinin molecule was specifically labeled. Presence of the F-G-D-S esterase-active site in the amino acid sequence of the hemagglutinin protein (Kienzle *et al.*, 1990; Parker *et al.*, 1989) and the existence of esterase activity on HE expressed from the cloned gene (Parker *et al.*, 1990) confirmed this association. The studies by Vlasak *et al.* (1988a) suggested that the active esterase may be required for either endocytosis of the virus and/or uncoating with subsequent release of the virus into the cytoplasm during the initial stages of infection. This notion comes from the fact that diisopropyl fluorophosphate treatment of BCV destroyed the esterase activity and at the same time reduced the infectious titer of the virus 100- to 400-fold. This treatment, however, did not prevent binding of the virus to sialic acid-containing receptors. The acetyl esterase, however, may act at the end of the replication cycle by releasing progeny virus from infected cells. In this way it would behave analogously to the neuraminidase of influenza A virus (Palese *et al.*, 1974).

VII. CONCLUDING REMARKS

Many interesting features of HE expression, structure, and function remain to be characterized. It remains to be established, for example, what genetic mechanisms cause variable expression of HE in some strains of MHV. One hypothesis still needing direct proof is that it is the number of UCUA repeat units at the 5' end of the HE gene that regulates transcription (Shieh *et al.*, 1989). It also remains to be shown whether the two ORFs within the BCV HE gene (IORF 1 and IORF 2) are expressed during virus replication, and, if so, what the functions of their products might be. Is their function related to those of HE?

Regarding HE structure, it remains to be determined where the intramolecular and intermolecular disulfide bridges occur in the dimeric molecule, which asparagine residues become glycosylated, and the exact nature of the carbohydrate modifications. Detailed mapping will be required to identify the sites on the HE molecule involved in hemagglutination, cell binding (if different from hemagglutination), recognition by neutralizing monoclonal antibodies (Deregt and Babiuk, 1987), and interaction (if any) with other structural and nonstructural proteins. Do higher-level noncovalent multimeric forms of HE exist? Ultimately it will be important to determine the three-dimensional structure of HE and establish the antigenic epitopes on this structure.

The full range of functions of HE in virus replication remains to be determined. Further studies are needed to test whether HE is, in fact, required for replication in those viruses that express it constitutively. Is it responsible for expanded tissue tropism or host range in those viruses that possess it? If so, by what mechanism? Is it responsible for the strong neuronotropism exhibited by the HE-containing coronaviruses? If so, then special importance should be

placed on understanding its role since the HE-containing HCV OC43 (and its close relatives) is a common human respiratory pathogen and has the potential for involving the CNS in human disease. The precise role(s) of the esterase in the infection process remains to be determined.

Finally, the usefulness of HE as a vaccine immunogen for the control of BCV-induced gastrointestinal disease should be explored. The existence of HE as an integral membrane protein on the surface of infected cells (Kienzle *et al.*, 1989), as well as a viral structural protein that can induce neutralizing antibodies (Deregt *et al.*, 1989), suggests that both cellular and humoral immune responses are important in protection against BCV. BCV-neutralizing antibodies can be induced in mice inoculated with an adenovirus recombinant expressing HE (Yoo *et al.*, 1992), raising the possibility that protective mucosal immunity can be induced by HE. The usefulness of HE in vaccines given by the mucosal route should be assessed.

VIII. REFERENCES

- Abraham, S., Kienzle, T. E., Lapps, W., and Brian, D. A. 1990, Deduced sequence of the bovine coronavirus spike protein and identification of the internal proteolytic cleavage site, *Virology* **176**:296.
- Akashi, H., Inaba, Y., Minra, Y., Sato, K., Tokuhisa, S., Asagi, M., and Hayashi, Y., 1981, Propagation of the Kakagawa strain of bovine coronavirus in suckling mice, rats, and hamsters, *Arch. Virol.* **67**:367.
- Andries, K., and Pensaert, M., 1981, Vomiting and wasting disease, a coronavirus infection of pigs, *Adv. Exp. Med. Biol.* **142**:399.
- Barthold, S. W., de Souza, M. S., and Smith, A. L., 1990, Susceptibility of laboratory mice to intranasal and contact infection with coronaviruses of other species, *Lab. Anim. Sci.* **40**:481.
- Bingham, R. W., Madge, M. H., and Tyrrell, D. A. J., 1975, Hemagglutination by avian infectious bronchitis virus—A coronavirus, *J. Gen. Virol.* **28**:381.
- Bridger, J. C., Caul, E. O., and Egglestone, S. I., 1978, Replication of an enteric bovine coronavirus in intestinal organ cultures, *Arch. Virol.* **57**:43.
- Bucknall, R. A., Kalica, A. R., and Chanock, R. M., 1972, Intracellular development and mechanism of hemadsorption of human coronavirus, OC43, *Proc. Soc. Exp. Biol. Med.* **139**:811.
- Callebaut, P. E., and Pensaert, M. B., 1980, Characterization and isolation of structural polypeptides in hemagglutinating encephalomyelitis virus, *J. Gen. Virol.* **48**:193.
- Cavanagh, D., and Davis, P. J., 1986, Coronavirus IBV: Removal of spike glycopolypeptide S1 by urea abolishes infectivity and hemagglutination but not attachment to cells, *J. Gen. Virol.* **67**:1443.
- Dea, S., and Tijssen, P., 1988, Identification of the structural proteins of the turkey enteric coronavirus, *Arch. Virol.* **99**:173.
- Deregt, D., and Babiuk, L. A., 1987, Monoclonal antibodies to bovine coronavirus: Characteristics and topographical mapping of neutralizing epitopes on the E2 and E3 glycoproteins, *Virology* **161**:410.
- Deregt, D., Sabara, M., and Babiuk, L. A., 1987, Structural proteins of bovine coronavirus and their intracellular processing, *J. Gen. Virol.* **68**:2863.
- Deregt, D., Gifford, G. A., Ijaz, M. K., Watts, T. C., Gilchrist, J. E., Haines, D. M., and Babiuk, L. A., 1989, Monoclonal antibodies to bovine coronavirus glycoproteins E2 and E3: Demonstration of *in vivo* virus-neutralizing activity, *J. Gen. Virol.* **70**:993.
- Dubois-Dalcq, M. E., Doller, E. W., Haspel, M. V., and Holmes, K. V., 1982, Cell tropism and expression of mouse hepatitis viruses (MHV) in mouse spinal cord cultures, *Virology* **119**:317.
- Fishman, P. S., Gass, J. S., Swoveland, P. T., Lavi, E., Highkin, M. K., and Weiss, S. R., 1985, Infection of the basal ganglia by a murine coronavirus, *Science* **229**:877.

- Fleming, J. O., Trousdale, M. D., Bradbury, J., Stohlman, S. A., and Weiner, L. P., 1987, Experimental demyelination induced by coronavirus JHM (MHV-4): Molecular identification of a virus determinant of paralytic disease, *Microbial Pathogen* **3**:9.
- Goto, N., Makino, S., and Fujiwara, K., 1986, Neuropathogenicity of mutant strains of mouse hepatitis virus, 1a and 2c, from DBT cells persistently infected with JHM strain, *Adv. Exp. Med. Biol.* **218**:439.
- Haverkamp, J., Veh, R. W., Sander, M., Shauer, R., Kamerling, J. P., and Vliegthart, J. F. G., 1977, Demonstration of 9-O-acetyl-N-acetylneuraminic acid in brain gangliosides from various vertebrates including man, *Hoppe-Seyler Physiol. Chem.* **358**:1609.
- Herrler, G., and Klenk, H.-D., 1987, The surface receptor is a major determinant of the cell tropism of influenza C virus, *Virology* **159**:102.
- Herrler, G., Nagele, A., Meier-Ewert, H., Bhowan, A. S., and Compans, R. W., 1981, Isolation and structural analysis of influenza C virion glycoproteins, *Virology* **113**:439.
- Herrler, G., Rott, R., Klenk, H. D., Muller, H. P., Shukla, A. K., and Schauer, R., 1985, The receptor-destroying enzyme of influenza C virus is neuraminidase, *EMBO J.* **4**:1503.
- Herrler, G., Durkop, I., Becht, H., and Klenk, H.-D., 1988, The glycoprotein of influenza C virus is the hemagglutinin, esterase, and fusion factor, *J. Gen. Virol.* **69**:839.
- Hogue, B. G., and Brian, D. A., 1986, Structural proteins of human respiratory coronavirus OC43, *Virus Res.* **5**:131.
- Hogue, B. G., King, B., and Brian, D. A., 1984, Antigenic relationships among proteins of bovine coronavirus, human respiratory coronavirus OC43, and mouse hepatitis coronavirus A59, *J. Virol.* **51**:384.
- Hogue, B. G., Kienzle, T. E., and Brian, D. A., 1989, Synthesis and processing of the bovine enteric coronavirus hemagglutinin protein, *J. Gen. Virol.* **70**:345.
- Kapikian, A. Z., James, H. D., Kelly, S. J., King, L. M., Vaughn, A. L., and Chanock, R. M., 1972, Hemadsorption by coronavirus strain OC43, *Proc. Soc. Exp. Biol. Med.* **139**:179.
- Kaye, H. S., and Dowdle, W. R., 1969, Some characteristics of hemagglutination of certain strains of "IBV-like" virus, *J. Infect. Dis.* **120**:576.
- Keck, J., Hogue, B. G., Brian, D. A., and Lai, M. M. C., 1988, Temporal regulation of bovine coronavirus RNA synthesis, *Virus Res.* **9**:343.
- Kienzle, T. E., Abraham, S., Hogue, B. G., and Brian, D. A., 1990, Structure and orientation of expressed bovine coronavirus hemagglutinin esterase protein, *J. Virol.* **64**:1834.
- King, B., and Brian, D. A., 1982, Bovine coronavirus structural proteins, *J. Virol.* **42**:700.
- King, B., Potts, B. J., and Brian, D. A., 1985, Bovine coronavirus hemagglutinin protein, *Virus Res.* **2**:53.
- Knobler, R. L., Dubois-Dalcq, M., Haspel, M. V., Claysmith, A. P., Lampert, P. W., and Oldstone, M. B. A., 1981, Selective localization of wild type mutant mouse hepatitis (JHM strain) antigens in CNS tissue by fluorescence, light and electron microscopy, *J. Neuroimmunol.* **1**:81.
- Kubo, M., Akashi, H., Inaba, Y., Osada, M., and Konno, S., 1982, Pathological studies on encephalitis in mice experimentally inoculated with bovine coronavirus, *Natl. Inst. Anim. Health Q.* **22**:45.
- Kunkel, F., and Herrler, G., 1993, Structural and functional analysis of the surface protein of human coronavirus OC43, *Virology* **195**:195.
- Kyte, J., and Doolittle, R. F., 1982, A simple method for displaying the hydrophobic character of a protein, *J. Mol. Biol.* **157**:105.
- Lai, M. M. C., Makino, S., Soe, L. H., Shieh, C.-K., Keck, J. G., and Fleming, J. O., 1987, Coronavirus: A jumping RNA transcription, *Cold Spring Harbor Symp. Quant. Biol.* **52**:359.
- Luytjes, W., Bredenbeek, P., Noten, A., Horzinek, M., and Spaan, W., 1988, Sequence of mouse hepatitis virus A59 mRNA2: Indications for RNA recombination between coronaviruses and influenza C virus, *Virology* **166**:415.
- Makino, S., Taguchi, F., Hayami, M., and Fujiwara, K., 1983, Characterization of small plaque mutants of mouse hepatitis virus, JHM strain, *Microbiol. Immunol.* **27**:445.
- Massa, P. T., Wege, H., and ter Meulen, V., 1988, Growth pattern of various JHM coronavirus isolates in primary rat glial cell cultures correlates with differing neurotropism *in vivo*, *Virus Res.* **9**:133.
- Morris, V. L., Tieszer, C., Mackinnon, J., and Percy, D., 1989, Characterization of coronavirus JHM variants isolated from Wistar Furth rats with a viral-induced demyelinating disease, *Virology* **169**:127.

- Muchmore, E. A., and Varki, A., 1987, Selective inhibition of influenza C esterase: A probe for detecting 9-O-acetylated sialic acids, *Science* **236**:1293.
- Nakada, S., Creager, R. S., Krystal, M., Aaronson, R. P., and Palese, P., 1984, Influenza C virus hemagglutinin: Comparison with influenza A and B virus hemagglutinins, *J. Virol.* **50**:118.
- Noda, M., Yamashita, H., Koide, F., Kadoi, K., Omori, T., Asagi, M., and Inaba, Y., 1987, Hemagglutination with transmissible gastroenteritis virus, *Arch. Virol.* **96**:109.
- Noda, M., Koide, F., Asagi, M., and Inaba, Y., 1988, Physicochemical properties of transmissible gastroenteritis virus hemagglutinin, *Arch. Virol.* **99**:163.
- Palese, P., Tobita, K., Ueda, M., and Compans, R. W., 1974, Characterization of temperature sensitive influenza A virus mutants defective in neuraminidase, *Virology* **61**:397.
- Parham, D., Tereba, A., Talbot, P., Jackson, D., and Morris, V., 1986, Analysis of JHM central nervous system infections in rats, *Arch. Neurol.* **43**:702.
- Parker, M. D., Cox, G. J., Deregt, D., Fitzpatrick, D. R., and Babiuk, L. A., 1989, Cloning and *in vitro* expression of the gene for the E3 hemagglutinin glycoprotein of bovine coronavirus, *J. Gen. Virol.* **70**:155.
- Parker, M. D., Yoo, D., and Babiuk, L. A., 1990, Expression and secretion of the bovine coronavirus hemagglutinin-esterase glycoprotein by insect cells infected with recombinant baculovirus, *J. Virol.* **64**:1625.
- Pearson, J., and Mims, C. A., 1983, Selective vulnerability of neural cells and age-related susceptibility to OC43 virus in mice, *Arch. Virol.* **77**:109.
- Pearson, J., and Mims, C. A., 1985, Differential susceptibility of cultured neural cells to the human coronavirus OC43, *J. Virol.* **5**:1016.
- Pfleiderer, M., Routledge, E., Herrler, G., and Siddell, S. G., 1991, High-level transient expression of the murine coronavirus haemagglutinin-esterase, *J. Gen. Virol.* **72**:1309.
- Rogers, G., Herrler, G., Paulson, J., and Klenk, H.-D., 1986, Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as high affinity receptor determinant for attachment to cells, *J. Biol. Chem.* **261**:5947.
- Schultze, B., and Herrler, G., 1992, Bovine coronavirus uses N-acetyl-9-O-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells, *J. Gen. Virol.* **73**:901.
- Schultze, B., Gross, H.-J., Brossmer, R., Klenk, H.-D. and Herrler, G., 1990, Hemagglutinating encephalomyelitis virus attaches to N-acetyl-9-O-acetylneuraminic acid-containing receptors on erythrocytes: Comparison with bovine coronavirus and influenza C virus, *Virus Res.* **16**:185.
- Schultze, B., Gross, H. J., Brossmer, R., and Herrler, G., 1991a, The S protein of bovine coronavirus is a hemagglutinin recognizing 9-O-acetylated sialic acid as a receptor determinant, *J. Virol.* **65**:6232.
- Schultze, B., Wahn, K., Klenk, K., and Herrler, G., 1991b, Isolated HE-protein from hemagglutinating encephalomyelitis virus and bovine coronavirus has receptor-destroying and receptor-binding activity, *Virology* **180**:221.
- Sharpee, R. L., Mebus, C. A., and Bass, E. P., 1976, Characterization of a calf diarrheal coronavirus, *Am. J. Vet. Res.* **37**:1031.
- Shieh, C.-K., Lee, H.-J., Yokomori, K., Monica, N. L., Makino, S., and Lai, M. M. C., 1989, Identification of new transcriptional initiation site and the corresponding functional gene 2b in the murine coronavirus RNA genome, *J. Virol.* **63**:3729.
- Siddell, S., 1983, Coronavirus JHM: Coding assignments of subgenomic mRNAs, *J. Gen. Virol.* **64**:113.
- Siddell, S. G., Wege, H., Barthel, A., and ter Meulen, V., 1981, Coronavirus JHM: Intracellular protein synthesis. *J. Gen. Virol.* **53**:145.
- Siddell, S. G., Anderson, R., Cavanagh, D., Fujiwara, K., Klenk, H. D., Macnaughton, M. R., Pensaert, M., Stohlman, S. A., Sturman, L., and van der Zeijst, B. A. M., 1983a, Coronaviridae, *Intervirology* **20**:181.
- Siddell, S., Wege, H., and ter Meulen, V., 1983b, The biology of coronaviruses, *J. Gen. Virol.* **64**:761.
- Storz, J., Herrler, G., Snodgrass, D. R., Hussain, K. A., Zhang, X. M., Clark, M. A., and Rott, R., 1991, Monoclonal antibodies differentiate between the haemagglutinating and the receptor-destroying activities of bovine coronavirus, *J. Gen. Virol.* **72**:2817.
- Sturman, L., and Holmes, K., 1985, The novel glycoproteins of coronaviruses, *Trends in Biological Science* **10**:17.

- Sugiyama, K., and Amano, Y., 1980, Hemagglutination and structural polypeptides of a new coronavirus associated with diarrhea in infant mice, *Arch. Virol.* **66**:95.
- Sugiyama, K., and Amano Y., 1981, Morphological and biological properties of a new coronavirus associated with diarrhea in infant mice, *Arch. Virol.* **67**:241.
- Sugiyama, K., Ishikawa, R., and Fukuhara, N., 1986, Structural polypeptides of the murine coronavirus DVIM, *Arch. Virol.* **89**:245.
- Taguchi, F., Massa, P. T., and ter Meulen, V., 1986, Characterization of a variant virus isolated from neural cell culture after infection of mouse coronavirus JHMV, *Virology* **155**:267.
- Talbot, P., 1989, Hemagglutination by murine hepatitis viruses: Absence of detectable activity in strains 3, A59, and S grown on DBT cells, *Intervirology* **30**:117.
- Tardieu, M., Boespflug, O., and Barbe, T., 1986, Selective tropism of a neurotropic coronavirus for ependymal cells, neurons, and meningeal cells, *J. Virol.* **60**:574.
- Tooze, S. A., Tooze, J., and Warren, G., 1988, Site of addition of N-acetyl-galactosamine to the E1 glycoprotein of mouse hepatitis virus-A59, *J. Cell Biol.* **106**:1475.
- Vlasak, R., Krystal, M., Nacht, M., and Palese, P., 1987, The influenza C virus glycoprotein (HE) exhibits receptor-binding (hemagglutinin) and receptor-destroying (esterase) activities, *Virology* **160**:419.
- Vlasak, R., Luytjes, W., Leider, J., Spaan, W., and Palese, P., 1988a, The E3 protein of bovine coronavirus is a receptor-destroying enzyme with acetyl esterase activity, *J. Virol.* **62**:4686.
- Vlasak, R., Luytjes, W., Spaan, W., and Palese, P., 1988b, Human and bovine coronaviruses recognize sialic acid-containing receptors similar to those of influenza C viruses, *Proc. Natl. Acad. Sci. USA* **85**:4526.
- Vlasak, R., Muster, T., Lauro, A. M., Powers, J. C., and Palese, P., 1989, Influenza C virus esterase: Analysis of catalytic site, inhibition, and possible function, *J. Virol.* **63**:2056.
- Wege, H., Siddell, S., and ter Meulen, V., 1982, The biology and pathogenesis of coronaviruses, *Curr. Top. Microbiol. Immunol.* **99**:165.
- Yagami, K., Hirai, K., and Hirano, N., 1986, Pathogenesis of hemagglutinating encephalomyelitis virus (HEV) in mice experimentally infected by different routes, *J. Comp. Pathol.* **96**:645.
- Yagami, K., Isumi Y., Kajiwarra, N., Sugiyama, F., and Sugiyama, Y., 1993, Neurotropism of mouse-adapted haemagglutinating encephalomyelitis virus, *J. Comp. Pathol.* **108**:21.
- Yokomori, K., Monica, N. L., Makino, S., Shieh, S.-K., and Lai, M. M. C., 1989, Biosynthesis, structure, and biological activities of envelope protein gp65 of murine coronavirus, *Virology* **173**:683.
- Yokomori, K., Baker, S. C., Stohlman, S. A., and Lai, M. M. C., 1992, Hemagglutinin-esterase-specific monoclonal antibodies alter the neuropathogenicity of mouse hepatitis virus, *J. Virol.* **66**:2865.
- Yoo, D., Graham, F. L., Prevec, L., Parker, M. D., Benko, M., and Babiuk, L. A., 1992, Synthesis and processing of the haemagglutinin-esterase glycoprotein of bovine coronavirus encoded in the E3 region of adenovirus, *J. Gen. Virol.* **73**:2591.
- Zhang, X., Kousoulas, K. G., and Storz, J., 1991, The hemagglutinin/esterase glycoprotein of bovine coronaviruses: Sequence and functional comparisons between virulent and avirulent strains, *Virology* **185**:847.
- Zhang, X., Kousoulas, K. G., and Storz, J., 1992, The hemagglutinin/esterase gene of human coronavirus strain OC43: Phylogenetic relationships to bovine and murine coronaviruses and influenza C virus, *Virology* **186**:318.