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ABSTRACT

Title of Dissertation: Coronavirus Attachment and Replication
Susan Rowley Compton, Doctor of Philosophy, 1988
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Coronaviruses are enveloped RNA viruses which show marked tissue and species tropisms. Mouse hepatitis virus (MHV) is one example of the coronaviruses. In this dissertation I will discuss two aspects of coronaviruses: 1) the RNA polymerase of the A59 strain of MHV; and 2) the role of coronavirus receptors in coronavirus species specificity.

An in vitro replication system was developed to study the RNA dependent RNA polymerase of MHV-A59. Extracts of MHV-infected cells produced MHV-specific RNAs of genomic and subgenomic sizes. In vitro synthesized viral RNA became associated with the viral nucleocapsid protein to form ribonucleoprotein complexes. When cell lines of non-murine origin were inoculated with MHV, they produced no MHV RNAs or proteins. Therefore, species-specific host restriction for MHV may occur at the level of viral attachment or penetration.

MHV receptors in mouse strains susceptible, semi-resistant or resistant to MHV infection were compared on hepatocyte and intestinal brush border membranes. All strains tested except the fully resistant SJL/J strain expressed a 100-120 kilodalton MHV receptor, but C57BL/6 mice expressed a larger receptor on the intestine. MHV3 bound to the same receptor as MHV-A59 indicating that different MHV strains share a common receptor. The

species specificity of the MHV receptor was also investigated. Intestinal brush border membranes from nine other species did not express any MHV binding activity. Therefore, the marked species specificity of MHV appears to be determined by absence of the MHV-specific receptor in other species.

Solid phase assays to detect virus receptors on intestinal brush border membranes from normal host species were developed for canine (CCV), feline (FIPV), porcine (TGEV), human (HCV-229E), and bovine (BCV) coronaviruses. The antigenically related coronaviruses, CCV, FIPV, TGEV, and HCV-229E bound to intestinal brush border membranes of dog, cat, pig, and human. The presence of receptors for these viruses on multiple species, rather than on only one species, appears to reflect their host range which is broader than that of MHV.

CORONAVIRUS ATTACHMENT AND REPLICATION

by

SUSAN ROWLEY COMPTON

Dissertation submitted to the Faculty of the Department of Microbiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirement for the degree of Doctor of Philosophy, 1988

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LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
BBM	brush border membrane
BCV	bovine coronavirus
BME	beta-mercaptoethanol
BSA	bovine serum albumin
CCV	canine coronavirus
cDNA	complementary deoxyribonucleic acid
cm	centimeter
CsCl	cesium chloride
CTP,	cytidine triphosphate
DNA	deoxyribonucleic acid
DOC	deoxycholate
EBV	Epstein Barr virus
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EMC	encephalomyocarditis virus
Endo F	endoglycosidase F
FBS	fetal bovine serum
FeCV	feline enteric coronavirus
FIP	feline infectious peritonitis
FIPV	feline infectious peritonitis virus
g	gram
GTP	guanosine triphosphate
HCV	human coronavirus
HeCV	human enteric coronavirus

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEV	hemagglutinating encephalomyelitis virus
HIV	human immunodeficiency virus
HM	hepatocyte membrane
HRV	human rhinovirus
IBV	infectious bronchitis virus
IgG	immunoglobulin G
LIVIM	lethal intestinal virus of infant mice
µCi	microCurie
MEM	minimal essential medium
µg	microgram
mg	milligram
Mg	magnesium
MHV	mouse hepatitis virus
µl	microliter
ml	milliliter
µm	micromole or micromolar
mm	millimole or millimolar
Mn	manganese
MOI	multiplicity of inoculation
MOPS	3-(N-morpholine) propanesulfonic acid
mRNA	messenger ribonucleic acid
nm	nanometer
NP40	nonidet P-40
NTP	nucleotide triphosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PCA	procoagulant activity
PFU	plaque forming units
pi	post-inoculation
PMSF	phenylmethylsulfonyl fluoride
RBC	red blood cell
RbCV	rabbit coronavirus
RbECV	rabbit enteric coronavirus
RCV	rat coronavirus
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
rpm	revolutions per minute
RSB	reticulocyte standard buffer
SDAV	sialodacryadenitis virus
SDS	sodium dodecyl sulfate
SFV	Semliki Forest virus
SPA	staphylococcal protein A
STM	sample treatment mix
TCA	trichloroacetic acid
TCV	turkey coronavirus
TGEV	transmissible gastroenteritis virus
tRNA	transfer ribonucleic acid
UMP	uridine monophosphate
USDA	United States Department of Agriculture
USUHS	Uniformed Services University of the Health Sciences
UTP	uridine triphosphate
VOPBA	virus overlay protein blot assay
VSV	vesicular stomatitis virus

INTRODUCTION

History

Classification: The name coronavirus was introduced in 1968 to describe those enveloped viruses which by negative staining showed a characteristic "halo" or "corona", formed by the club shaped projections surrounding the virion (Tyrrell et al. 1968). Originally the coronaviruses group contained only the avian coronavirus infectious bronchitis virus (IBV), the murine coronavirus mouse hepatitis virus (MHV) and several human respiratory coronaviruses (Tyrrell et al., 1968). By 1975, when the family Coronaviridae was created by the International Committee on the Taxonomy of Viruses, several additional species had been discovered including canine coronavirus (CCV), feline infectious peritonitis virus (FIPV), bovine coronavirus (BCV), turkey coronavirus (TCV), rat coronavirus (RCV), sialodacryadenitis virus of rats (SDAV), and the porcine coronaviruses transmissible gastroenteritis virus (TGEV) and hemagglutinating encephalomyelitis virus (HEV) (Tyrrell et al., 1975).

Today, coronaviruses are also classified by several other criteria. They are enveloped viruses, 80-120 nm in diameter, surrounded by club shaped surface projections 14-26 nm long, and mature by budding through the membranes of the endoplasmic reticulum or Golgi apparatus. The genome of coronaviruses is a 6×10^6 dalton single-stranded message-sense RNA which is capped and polyadenylated. Coronavirus replication occurs in the cytoplasm and transcription generates a nested set of mRNAs which share a common 3' end. A small (70 base pair) leader RNA is found on the 5' end of all message and genomic RNAs. The virions contain three structural proteins, a 50-60 kilodalton nucleocapsid protein, a 20-30 kilodalton matrix-like glycoprotein

E1 and a 80-200 kilodalton peplomer glycoprotein E2 (Sturman and Holmes, 1983).

Several other viruses share many of these characteristics and are believed to be coronaviruses. They include the human coronaviruses SD and SK, human enteric coronavirus (HeCV), rabbit coronavirus (RbCV), rabbit enteric coronavirus (RbECV) and the porcine coronavirus CV777. SD and SK were isolated from brains of multiple sclerosis patients and passaged through mouse brain or mouse tissue culture cells, respectively. They are coronaviruses based on strong serologic and genetic relationships to MHV and HCV-OC43, but it is uncertain whether they are of human or murine origin (Gerdes et al., 1981; Weiss, 1983). HeCV is postulated to be a coronavirus based on two pieces of evidence: 1) Coronavirus-like particles are observed in stools of patients with gastroenteritis who seroconvert to HCV-OC43; and 2) Antiserum to these coronavirus-like particles purified from stools reacts with HCV-OC43 (Gerna et al., 1984 and 1985). However, it is uncertain whether HeCV is a new human coronavirus or a different strain of HCV-OC43. RbCV is a rabbit virus which causes pleuritis and myocarditis, and RbECV is a rabbit enteric virus. They are considered to be coronaviruses based on their serological cross-reactivity with the human coronaviruses (Small et al., 1979, Descoteaux, et al., 1985). CV777 is a porcine enteric coronavirus which causes a disease very similar to TGEV but does not show antigenic cross-reactivity with any other coronavirus (Pensaert and deBouck, 1978).

Diseases: Though coronaviruses have only been recognized as a family since 1975, the diseases they cause have been recognized since 1931 when "infectious bronchitis of baby chicks" was described (Schalk and Hawn, 1931). Coronaviruses cause many diseases but have tropisms primarily for

respiratory and intestinal epithelia (Table 1). Coronaviruses possess narrow host ranges, infecting only one species, and generally only causing severe disease in neonates of that species. Inapparent or persistent infections may often occur in adult animals infected with coronaviruses. This narrow host range is also reflected in the cell lines in which coronaviruses can be grown. The narrow host and tissue tropism of coronaviruses may be caused by one or more factors including presence of cell surface receptors, the requirement of cell factors for replication or assembly of the virus, and/or the types of immunological response a host generates against a virus. I investigated the roles of two of these factors in determining species specificity: cell surface receptors and RNA replication. Because a large portion of my dissertation research deals with a comparison of receptors for different coronaviruses, I will summarize the various coronaviruses and the diseases they cause. Later in the introduction, I will discuss the molecular biology of coronaviruses.

The prototype respiratory coronavirus is IBV. Although the disease infectious bronchitis was recognized in 1931, the causative agent, a coronavirus, was not isolated until 1937 (Beaudette and Hudson, 1937). IBV infects chickens of all ages via the aerosol route, but disease is most severe in young chicks. In young chicks, IBV causes gasping, coughing, tracheal rales, nasal discharge and occasionally death (Schalk and Hawn, 1931). In 1962, two investigators reported severe outbreaks of avian nephrosis or "uraemia" in the United States and Australia due to aberrant stains of IBV. It is now known that the primary cause of mortality in IBV-infected chickens is due to nephritis (Cosgrove, 1962; Cumming, 1962). In growing chickens, only mild respiratory symptoms are seen, but in laying flocks, IBV can infect the oviduct causing a severe drop in egg production and quality (Broadfoot and Smith, 1954; Sevoian and Levine, 1957) .

Table 1

Target Organs of Coronavirus Infections

	MHV	RCV	SDAV	BCV	HEV	HCV	TGEV	CCV	FIPV	IBV	TCV
Central nervous system	++*				++						
Blood vessels	+*								+*		
Ependyma									+		
Gonad										+	
Intestine	++			++	++	+	++	++	+		++
Kidney	+								+*	+*	
Liver	++*								++		
Lymphoid organs	+*								++*	+*	
Pancreas	+										
Parotid gland		+	++								
Peritoneum									++		
Respiratory tract	+	++	+	+	+	++	+		+	++	+

++ Major target organ

\+ Less frequently infected organ

* Organ involved in persistent/chronic disease

adapted from Wege et. al., 1982

RCV also has a tropism primarily for respiratory tissues, infecting the lungs and nasal mucosa of rats. RCV was isolated in 1970 by inoculation of infected rat lung homogenates onto primary rat kidney cells. Intranasal inoculation of newborn rats with RCV results in respiratory disease and death in 6-12 days due to interstitial pnuemonitis. Inoculation of 7-14 day old rats results in respiratory disease but no mortality and inoculation of rats 21 days or older results in an inapparent infection (Parker et al., 1970).

Two groups of human respiratory coronaviruses (HCV) have been identified which are serologically unrelated. The prototype virus for the first group is HCV-OC43. The first member (B814) of this group of viruses was isolated in 1965 from boy with a cold and passaged on human embryonic tracheal organ cultures (Tyrrell and Bynoe, 1965). Later, HCV-OC43 and several other viruses in this group were isolated on human embryonic tracheal organ cultures (McIntosh et al., 1967). The prototype virus for the second group is HCV-229E. It was isolated from a nasal swab from a student with an upper respiratory infection on human secondary kidney cell cultures (Hamre and Procknow, 1966). Both HCV-229E and HCV-OC43 infect the upper respiratory tract of humans and are responsible for causing about 15% of common colds (McIntosh et al., 1970; and Larson et al., 1980).

Many coronaviruses, including BCV, CCV, HEV, TCV ,TGEV and feline enteric coronavirus (FeCV), cause enteric diseases which are particularly severe in young animals. BCV was first identified in the spring of 1971 during a vaccination trial of newborn calves with a reo-virus like agent known to cause neonatal calf diarrhea. A significant reduction in the incidence of diarrhea and death was seen in most vaccinated herds, but in 6 of 35 herds, no reduction was seen. Electron microscopic examination of feces from infected calves in all of the herds which did not show a decreased

incidence of diarrheal disease showed coronavirus-like particles (Mebus et al., 1972). BCV subsequently was adapted to grow in fetal bovine kidney cell culture, and further characterization of the virus confirmed that it was a member of the coronavirus family (Mebus et al., 1973a; Sharpee et al., 1976). BCV infects cows of all ages but causes diarrhea in and can be lethal to young calves only. The most severe villous atrophy is seen in the small intestine but the large intestine may also be involved (Mebus et al., 1973b). Recently it has been discovered that BCV can also infect the upper respiratory tract causing mild respiratory symptoms (Reynolds, 1983). Intestinal and respiratory isolates of BCV have been shown to belong to the same serotype, and oral or intranasal inoculation of gnotobiotic calves produced both respiratory and intestinal infection (Reynolds et al., 1985; Saif et al., 1986). BCV is endemic in many herds of cattle and is known to be transmitted to calves via the fecal-oral route, but now it is postulated that BCV may also be transmitted via aerosols.

Transmissible gastroenteritis of swine was shown to have a viral etiology in 1946, although reports of the disease were made in 1935 and 1937 (Doyle and Hutchings, 1946; Smith,1956). TGEV causes vomiting and diarrhea in pigs of all ages but causes mortality only in piglets. (Doyle and Hutchings, 1946). The primary target of TGEV is the small intestine , but virus can be recovered from many organs of the pig. Within the intestine, villous atrophy is greatest in the jejunum and ileum, although the duodenum is also affected. Death in the piglet is probably due to dehydration and metabolic acidosis coupled with hyperkalemia due to increased sodium secretion by the cells repopulating the virus-damaged villi (Cornelius et al., 1968; Butler et al., 1974). TGEV is highly infectious and can be transmitted via the fecal-oral route. Inapparent respiratory infection of adult pigs may

also play a role in the transmission of the virus to piglets via aerosols (Underdahl et al., 1974).

A coronavirus of dogs serologically related with TGEV was suggested in 1970 when anti-TGEV antibodies were found in a large number of puppies and adult dogs which had had no contact with pigs (Norman et al., 1970). An outbreak of vomiting and diarrhea in both adult dogs and puppies was reported in 1972 in a breeding kennel. No bacteria or viruses were isolated, but all infected adult dogs showed rising titers to TGEV (Cartwright and Lucas, 1972). CCV was isolated in 1974 from United States military dogs during an epizootic of diarrheal disease in Germany using primary dog kidney cells and a canine thymus cell line (Binn et al., 1975). When neonatal dogs were challenged with CCV, they developed a self-limiting gastroenteritis of 1 to 2 weeks duration (Keenen et al., 1976). Pigs are resistant to CCV infection; therefore CCV is not just a different serotype of TGEV with a slightly different species tropism (Binn et al., 1975). Also, CCV has been shown by serologic and virological methods to infect coyotes. Dual infection with canine coronavirus and canine parvovirus causes fatal enteritis of coyotes (Appell et al., 1979).

TCV causes a disease known as bluecomb disease of turkeys, turkey infectious enteritis, or transmissible enteritis. The disease was identified in 1953 and is characterized by anorexia, weight loss, dehydration and watery diarrhea with low mortality (Pomeroy and Sieburth, 1953). In 1973, two groups identified the infectious agent responsible for bluecomb to be a coronavirus (Panigrahy et al., 1973; Ritchie et al., 1973). Infection of 1 day old poults produced disease but no gross lesions could be seen in the intestine. Infection of 3 week old poults produced the classic villous atrophy seen with other coronaviral infections (Gonder et al., 1976).

Two feline coronaviruses (FIPV and FeCV) have been identified which are antigenically closely related but have different tropisms. The disease feline infectious peritonitis was recognized in 1963 and was determined to be caused by an infectious agent in 1966 (Holzworth, 1963; Wolfe and Griesemer, 1966). Classification of the agent as a coronavirus was reported in 1970 based on morphology (Ward, 1970). FIPV causes two types of disease syndromes, though some infected cats show manifestations of both syndromes. Both types of syndromes are characterized by fever, weight loss and general depression. The first type is effusive or "wet" FIP and is also characterized by abdominal distention and/or dyspnea due to a peritonitis, and/or pleuritis. Involvement of the eyes and central nervous system is not usually seen (Holmberg and Gribble, 1973). The second type is non-effusive or "dry" FIP and is characterized by granulomatous lesions localized primarily in the parenchymatous organs such as the mesenteric lymph nodes, kidneys, eyes, meninges and ependyma of the spinal cord and brain. Little or no fluid exudation is seen (Montali and Strandberg, 1972). FIP occurs primarily in cats between 6 months and two years and in cats greater than 14 years old. Mortality is highest in cats less than 1 year old (Potkay et al., 1974). FIPV also infects wild cats, such as lions, leopards, jaguars and cheetahs, often with high mortality (Pfeifer et al., 1983). The immune system is believed to play an important role in the pathogenesis of FIP (Pedersen and Black, 1983). Infected cats which mount a strong humoral immune response without a cell-mediated immune response to FIPV are postulated to develop the effusive form of FIP while infected cats which develop a strong humoral immune response with a partial cell-mediated immune response to FIPV are postulated to develop the non-effusive form of the disease. Infected cats which develop both strong humoral and cell-mediated immune responses to

FIPV do not show any clinical symptoms and either recover completely from the infection, or develop an inapparent persistent infection. A cat with persistent FIPV may develop either form of FIP if it undergoes immunosuppression (Pedersen and Black, 1983). Antibodies against FIPV antigens are involved in the development of clinical disease, in that in experimentally infected seropositive and seronegative kittens, the seropositive kittens developed symptoms within 2 days while the seronegative kittens did not show symptoms till six days post infection, by which time anti-FIPV antibodies had been produced. Also, treatment of seronegative cats with purified anti-FIPV IgG results in aggravation of the disease (Pederson and Boyle, 1980; Weiss and Scott, 1981). In serological studies, many normal cats had serum antibodies to FIPV, though the frequency of clinical FIP was low (Pedersen, 1976a). It was postulated that clinical FIP was an uncommon secondary manifestation of FIPV infection.

With the isolation of FIPV in cell culture (Pedersen 1976b), it became clear that there was a second type of feline coronavirus antigenically closely related to FIP which caused enteric disease only and was ubiquitous in the cat population. The first isolate of FeCV was described in 1980 (Pedersen et al., 1981b). FeCV causes inapparent to mild intestinal infections in kittens 4 to 12 weeks of age. (Pederson et al., 1981b). FeCV infection by itself is of no clinical importance but some antibodies to FeCV cross-react with FIPV and may account for the high level of seropositivity to "FIPV " seen in normal cats. Also, preexisting anti-FeCV antibodies in a cat infected with FIPV can sensitize the cat, leading to a faster onset, and a more severe form of the disease (Pedersen and Boyle, 1980).

In 1957, an epidemic of "vomiting and wasting disease" was described in Canadian herds of nursing pigs. Generally, only vomiting and severe

depression was seen before the piglets died of starvation. Those piglets which survived were permanently stunted (Roe and Alexander, 1958). Almost concurrently, an epidemic viral encephalomyelitis occurred. Initially, the symptoms were similar to that of "vomiting and wasting disease", consisting of anorexia, vomiting and constipation, but within 1 to 3 days encephalomyelitis ensued. Hyperesthesia, muscle tremor, ataxia, and blindness were seen. Mortality in very young litters approached 100%, while mortality in litters over three weeks of age was low (Alexander et al., 1959). In 1961, a virus was isolated from the brain of a pig with encephalomyelitis. It was called hemagglutinating encephalomyelitis virus (HEV) due to its ability to hemagglutinate chicken RBCs (Grieg et al., 1971). In 1969, the virus was shown to be a coronavirus (Cartwright et al., 1965; and Phillip et al., 1971). Experimental inoculation of pigs with this virus produced both the vomiting and wasting syndrome and encephalomyelitis . It was, therefore, concluded that the two diseases were manifestations of the same virus. (Mengeling and Cutlip, 1976). Currently, clinical manifestations of HEV are infrequently seen, though high levels of seropositivity (up to 93%) exist in large swine herds due to inapparent infections of adult pigs. (Girard et al., 1964). Most suckling pigs are protected from severe disease by passive immunity from their seropositive mothers, and become subclinically infected. Only suckling pigs from seronegative mothers become ill (Andries and Pensaert, 1981). The primary sites of replication for HEV are the nasal mucosa, tonsils, lungs and small intestine. Virus spread via nerves towards the associated peripheral ganglia and to the central nervous system. Vomiting may be caused directly by viral replication in the vomiting center of the brainstem or indirectly by signals sent from infected peripheral neurons (Andries and Pensaert, 1981). Wasting may be due to neural signals to the stomach which greatly reduce the

gastric emptying mechanism resulting in food stagnation and anorexia (Andries and Pensaert, 1981).

Sialodacryadenitis of rats was recognized as a disease in 1961 and found to be caused by a coronavirus (SDAV) in 1972 (Innes and Stanton,1961; and Bhatt et al., 1972). SDAV was isolated by inoculation of infected organ homogenates into newborn mice (Bhatt et al., 1972). Intracerebral inoculation of newborn mice with SDAV causes neuronal degradation (Jonas et al., 1969). SDAV infects the nasopharynx, tracheobronchial tree, and parotid and submaxillary salivary glands, extraorbital gland, and Harderian gland of rats. Rhinitis occurs within the first 2 days post-inoculation, followed by necrosis in the salivary, extraorbital and Harderian glands. The disease is self-limiting and does not spread to other organs (Jacoby et al., 1975). Keratoconjunctivitis and ophthalmic lesions can also be associated with this disease, but these lesions may be secondary to bacterial invasion of the Harderian glands. (Lai et al., 1976).

Since most of my research involved the murine coronavirus MHV, I will describe MHV diseases in greater detail. MHV, of which there are greater than 20 strains, causes diseases of the liver, enteric tract and brain (Table 2). The type and severity of disease depends on virus strain, dose and route of inoculation, and the age, strain and immune status of the mouse. The first murine coronavirus described, MHV-JHM was isolated from a mouse with spontaneous flaccid paralysis, by passage through mouse brain (Cheever et al., 1949). Acute disease due to MHV-JHM is characterized by ruffled fur, hunching, general lassitude, and hindlimb paralysis leading to frank paralysis and death. Upon necropsy, necrotic lesions are seen in the hippocampus, olfactory lobes, and periependymal tissues; demyelination is seen primarily in the brainstem and spinal cord; and focal necrosis is seen in the liver (Weiner,

Table 2. Mouse strain susceptibility to different strains of MHV. S (susceptible) = high virus titers produced, severe disease and high mortality. SR^1 (semi-resistant) = moderate virus titers produced, moderate disease and moderate mortality. SR^2 (semi-resistant) = moderate virus titers produced, no or minimal disease and no mortality. R (resistant) = no virus produced, no disease or mortality. MHV2 and 3 were inoculated intraperitoneally; MHV-JHM was inoculated intracerebrally; and MHV-A59 was inoculated onto macrophages. References: a = Bang and Warwick, 1960; b = Le Prevost et al., 1975b; c = Stohlman and Frelinger, 1978 and Knobler et al., 1981; d = Smith et al., 1984.

Table 2

Mouse Strain Susceptibility

	2[a]	3[b]	JHM(4)[c]	A59[d]
PRI	S			
Balb/c		S	S	S
C57bl/6		S	S	S
DBA		S	S	S
CBA		SR[1]	S	S
C3H	SR[2]	SR[1]	S	S
A/J		SR[2]	S	S
A	SR[2]		S	
SJL/J			R	R

1973). During natural infection by the intranasal route, the virus replicates initially in the nasal mucosa and invades the central nervous system directly via the olfactory nerves (Goto et al., 1977). Mice which do not show clinical symptoms or which undergo remyelination and recover from the acute stage of the disease may develop a chronic infection. Small foci of active demyelination can be seen for up to 16 months due to persistent MHV-JHM infection (Herndon et al., 1975). MHV-JHM can also cause encephalomyelitis in weanling rats when inoculated intracerebrally (Cheever et al., 1949). MHV-JHM infects all strains of mice except SJL/J (Table 2). Resistance of SJL/J mice to MHV-JHM infection following intracerebral injection has been mapped to one recessive gene on mouse chromosome 7 (Stohlman and Frelinger, 1978; Knobler et al., 1981, Knobler et al., 1984a and 1984b).

In 1951, the first hepatotropic strain of MHV (MHV1) was isolated from in a colony of mice with acute fatal hepatitis (Gledhill and Andrewes, 1951). Hepatitis was shown to be the result of two agents, the parasite Eperythrozoon coccoides and MHV1. Susceptible mice were found to carry E. coccoides while resistant mice did not (Gledhill et al., 1955). MHV1 infection alone, causes only mild hepatic lesions in weanling or adult mice but causes an acute fatal hepatitis in suckling mice. Mild neurological symptoms are occasionally seen (Gledhill et al., 1955). Nelson discovered a second hepatotropic strain of MHV (MHV2) in an outbreak of murine hepatitis. Hepatitis was associated with dual infection by MHV2 and murine leukemia virus (Nelson, 1952). MHV2 was used for the first studies on mouse strain susceptibility (Bang and Warwick, 1960). Princeton (PRI) mice are fully susceptible and die from MHV2 infection. C3H mice are normally considered resistant since they do not die, but should be more appropriately termed semi-resistant since moderate viral titers are produced and mild disease is observed

(Table 2; Bang and Warwick, 1960). Semi-resistance of C3H mice to death by MHV-2 is determined by a single recessive gene (Bang and Warwick,1960).

MHV3, discovered in 1956, causes several types of diseases depending on the strain of mouse (Dick et al., 1956). In fully susceptible strains of mice (BALB/c, C57BL/6 and Swiss), small doses of MHV3 given parenterally, produce systemic infection leading to fulminant hepatitis and death due to massive liver necrosis in all ages of mice . Spleen, thymus, lymph nodes and Peyer's patches also show high levels of necrotic lesions (Virelizier, 1981). In semi-resistant strains of mice (C3H/He and A2G), no acute disease is seen when adult mice are infected, but virus can persist leading to a chronic illness with progressive neurologic symptoms. Three to six weeks post-inoculation, mice show loss of activity, failure to thrive, ruffled fur , incoordination, and paresis of one or more limbs. Animals die within 1 to 12 months post-inoculation (Virelizier et al., 1975). Adult A/Orl and A/J mice are generally considered resistant to MHV3 infection since they no not develop disease or die from the infection, but since they produce moderate titers of virus after infection, they will be referred to as semi-resistant (Table 2; Le Prevost et al., 1975b). Resistance to death by MHV3 infection in A/J mice has been mapped to a recessive gene which is not associated with the major histocompatability locus (H-2). Resistance to chronic disease in C3H mice has been mapped to one or two different recessive genes which are linked to the H-2 locus. Mice heterozygous or homozygous for the $H\text{-}2^f$ gene are resistant to chronic infections (Levy-Leblond et al., 1979). The induction of monocyte procoagulant activity (PCA) is believed to play a role in susceptibility to MHV3 disease. In response to MHV infection, increased levels of PCA correlate with the degree of susceptibility to disease. BALB/c mice, a susceptible strain of mouse, are induced to make large amounts of PCA, C3H

mice, a semi-resistant strain of mouse which develops disease, are induced to make moderate levels of PCA, and A/J mice, a semi-resistant strain of mouse which does not develop any disease, are not induced to make PCA (Levy et al., 1981). It is postulated that increased PCA levels in response to MHV infection are responsible for the severe and progressive hepatic microcirculatory abnormalities in BALB/c and C3H mice after MHV-3 infection (Levy et al., 1981, Dindzans et al., 1986).

MHV-A59 was isolated in 1961, from mice in a Moloney murine leukemia virus study which developed hepatic disease (Manaker et al., 1961). MHV-A59 is primarily hepatotropic but is also neuropathogenic. Via most routes of inoculation, acute fatal hepatitis due to destruction of liver parenchymal and Kupffer cells occurs (Hirano et al., 1981). Intracerebral inoculation of weanling mice results in acute meningoencephalitis or subacute spastic paralysis due to demyelination in the brain and in the spinal cord (Lavi et al.,1984; Woyciechowska et al., 1984). All strains of mice are susceptible to MHV-A59 except for SJL/J (Table 2). Resistance of SJL/J macrophages to infection by MHV-A59, like that of MHV-JHM, has been mapped to a recessive gene on mouse chromosome 7 (Smith et al., 1984).

The first enterotropic strain of MHV, termed lethal intestinal virus of infant mice (LIVIM), was isolated from a colony of infant C57BL/6 mice (Kraft, 1962). Infant mice infected with LIVIM and other enterotropic strains of MHV (MHV-D, S, DVIM) stop nursing, lose weight rapidly, become lethargic and die after a short period of cyanosis. Pathology is limited to the digestive tract, with the main features being decrease in villus size and number in the small intestine. Infection of adult mice results in inapparent infection (Kraft, 1962). Some enterotropic strains, MHV-D and -S, can also cause hepatitis in infant mice (Ishida et al., 1978).

Two very important factors in susceptibility to MHV infection are age and immune status of the mouse. All strains of neonatal mice are much more susceptible to MHV; some strains of mice, such as SJL/J and C3H, show a very sharp reduction in susceptibility to MHV at 3 weeks of age (Le Prevost et al., 1975b; Pickel et al., 1981). An intact immune system, particularly the cell-mediated arm, is important for resistance to MHV infection. Resistant strains of mice which have undergone immunosuppression by x-irradiation, thymectomy, graft-vs-host reaction, cortisone, cyclophosphamide, anti-lymphocyte serum treatment or infection with eperythrozoon become susceptible to MHV (Gallily et al., 1964; Lavelle and Bang, 1973; Willenborg et al., 1973; Dupuy et al., 1975). Some strains of susceptible neonatal mice can be rendered resistant by transfer of T cells and macrophages from resistant adult animals, and concanavalin A treatment can render susceptible mice or macrophages from susceptible mice resistant to MHV2 infection (Levy-Leblond and Dupuy, 1977; Weiser and Bang, 1977; Stohlman et al., 1980). It is unlikely that humoral immunity plays a major role in resistance to MHV, as transfer of immune serum is not effective in protecting susceptible animals, though it does prevent the chronic disease state in semi-resistant C3H animals infected with MHV-3 (Le Prevost et al., 1975a; Levy et al., 1981). Different MHV strains have differential susceptibilities to and differential abilities to induce interferon. This may play a role in the severity of disease produced by each strain of MHV (Virelizier et al., 1976 and 1977; Stohlman et al., 1978; Garlinghouse et al., 1984; Taguchi and Siddell, 1985)

Antigenic relationships: Coronaviruses have been separated into four antigenic groups based on their antigenic cross-reactivities (Pedersen et al., 1968; Table 3). The first antigenic group contains BCV, HCV-OC43,

Table 3

Antigenic Crossreactivity between Coronaviruses

	∝MHV	∝BCV	∝OC43	∝229E	∝CCV	∝FIPV	∝TGEV
MHV	+++	+/-	+++	-	-	-	-
BCV	++	+++	++	-	-	-	-
OC43	+++	++	+++	-	-	-	-
229E	-	-	-	+++	-	+/-	+
CCV	-	-	-	-	+++	+++	+++
FIPV	-	-	-	+	-	+++	+++
TGEV	-	-	-	++	-	+++	+++

from Pedersen et. al. 1978, established by immunofluorescence

- = negative
+/- = barely detectable
+ = weak
++ = moderate
+++ = strong

HEV, MHV, RCV, and SDAV. The second antigenic group contains CCV, FeCV, FIPV, HCV-229E, and TGEV. The third antigenic group contains IBV and the fourth antigenic group contains TCV. In 1969, McIntosh using neutralization, fluorescent antibody and complement fixation tests, showed that MHV and HCV-OC43 were antigenically related to one another but not to HCV-229E or IBV. IBV and HCV-229E were not antigenically related to each other, MHV or HCV-OC43 (McIntosh et al., 1969). Using complement fixation, gel diffusion tests and ELISAs, some weak cross-reactivity has been shown between MHV or HCV-OC43 and HCV-229E, but this is probably due to the stickiness of the nucleocapsid proteins of these viruses (Bradburne, 1970; Hasonry and Macnaughton, 1982). The antigenic cross-reactivities for eight coronaviruses were determined using immunofluorescence (Pedersen et al., 1978). The antigenic cross-reactivity of CCV, FIPV and TGEV was confirmed using neutralization tests, immunoblotting and immunoprecipitation. (Reynolds et al., 1980; Horzinek et al., 1982). The antigenic cross-reactivity of HCV-OC43 and BCV was confirmed using monospecific sera in ELISAs, hemagglutination-inhibition, neutralization tests, immunoblotting and immunoprecipitation (Gerna et al., 1981; Hogue et al., 1984). RCV and SDAV were shown to be antigenically related to each other and to MHV by neutralization and complement-fixation tests (Parker et al., 1970; and Bhatt et al., 1972). Immunoelectron microscopy studies showed that TCV is not antigenically related to IBV, HEV, TGEV, or BCV (Ritchie et al., 1973).

Coronavirus Structure

General characteristics: Several reviews on the molecular biology of coronaviruses were published in the early 1980's (Siddell et al., 1982; Wege et

al., 1982; Sturman and Holmes, 1983). In this section of the Introduction, I will summarize the data in those reviews, and discuss new data which have more recently emerged. Negative stains of purified coronaviruses reveal pleomorphic spherical, enveloped particles of 80-160 nm in diameter (Berry et al., 1964; McIntosh et al., 1967; Okaniwa et al.,1968; Parker et al.,1970; Phillip et al., 1971; Adams et al., 1972; Stair et al., 1972; Osterhaus et al., 1976). On sucrose or potassium tartrate gradients, coronaviruses exhibit buoyant densities of 1.16-1.18 g/cm^3 (Cunningham, 1963; Witte et al., 1968; Kaye et al., 1970; Adams et al., 1972; Stair et al., 1972; Hierholzer, 1976; Osterhaus et al., 1976; Horzinek et al., 1977; Sturman and Holmes, 1977; Lai and Stohlman, 1978). Virions in general, are stable at pH 3.0, inactivated by pH 8.0 treatment and have the greatest stability between pH 6.0-6.5 (Quiroz and Hanson, 1958; Cartwright et al., 1964; Kapikian et al., 1969; Bhatt et al., 1972; Sharpee et al., 1976; Evermann et al., 1981). Their infectivity is destroyed by treatment with chloroform, ether, deoxycholate and other detergents (Cheever et al., 1949; Quiroz and Hanson, 1958; Greig and Girard, 1963; Hamre and Procknow, 1966; McIntosh et al., 1967; Harada et al., 1968; Kapikian et al., 1969; Parker et al., 1970; Bhatt et al., 1972; Pedersen, 1976c; Sharpee et al., 1976). Coronaviruses are inactivated by treatment at 56°C for 10-15 minutes but are stable at 37°C for up to several days and at 4°C for up to several months (Bay et al.,1949; Cheever et al., 1949; Young et al., 1955; Hofstad, 1956; Greig and Girard, 1963; Hamre and Procknow,1966; Parker et al.,1970; Bhatt et al.,1972; Pedersen,1976c; Sharpee et al.,1976). Different coronaviruses show different responses to trypsin, with the infectivity of some coronaviruses enhanced by trypsin treatment and the infectivity of others decreased by trypsin treatment (Buthala, 1956; Cartwright et al., 1965; Kaye et al., 1970; Sturman and Holmes, 1977; Evermann et al., 1981).

Nucleocapsids: Nucleocapsids can be visualized by electron microscopy of virions disrupted spontaneously or by non-ionic detergents. They appear as flexible helices 14-16 nm in diameter and up to 320 nm long (Kennedy and Johnson-Lussenburg, 1975-1976; Macnaughton et al., 1978). They have buoyant densities of 1.24-1.28 g/cm^3 (Wege et al., 1979; Sturman et al., 1980). Nucleocapsids are composed of the RNA genome and nucleocapsid proteins.

The coronavirus genome is a large ($5.4\text{-}6.9 \times 10^6$ daltons or 16,000-21,000 nucleotides) single stranded piece of RNA which is capped, polyadenylated , non-segmented and infectious (Lomniczi, 1977; Schochetman et al., 1977; Tannock and Hierholzer, 1977; Yogo et al., 1977; Macnaughton and Madge, 1977 and 1978; Wege et al., 1978; Lai and Stohlman,1978 and 1981; Guy and Brian, 1979; Brian et al., 1980).

The RNA genome is wrapped up in the nucleocapsid protein. The nucleocapsid protein is 50-60 kilodaltons, nonglycosylated, and phosphorylated (Hierholzer et al., 1972; Garwes et al., 1976; Hierholzer, 1976; Macnaughton et al., 1977; Pocock and Garwes, 1977; Sturman, 1977; Stohlman and Lai, 1979; Garwes and Reynolds, 1981; LaPorte and Bobulesco, 1981; Lomniczi and Morser, 1981; Storz et al., 1981; King and Brian, 1982; Boyle et al., 1984).

Envelope and Envelope Glycoproteins: By electron microscopy, large widely spaced club-shaped spikes or peplomers, 12-24 nm in length, can be seen in the viral envelope forming a "corona" (Berry et al., 1964; McIntosh et al., 1967; Okaniwa et al., 1968; Parker et al., 1970; Phillip et al., 1971; Adams et al., 1972; Stair et al., 1972; Osterhaus et al., 1976). In hemagglutinating coronaviruses (BCV, HCV-OC43 and HEV), a second shorter peplomer of 10 nm forms a second fringe (Grieg et al., 1971; Bridger et al., 1978). The viral envelope of coronaviruses contains lipid components in proportions

approximately the same as those of the host cell in which the virus is grown (Pike and Garwes, 1977). All carbohydrate in the virion is found in the form of membrane glycoproteins (Sturman and Holmes, 1985).

The large spikes found in all coronaviruses are formed by the E2 protein. It is a glycoprotein of 150-200 kilodaltons which can be cleaved to yield two fragments of approximately 90 kilodaltons (Hierholzer et al., 1972; Garwes et al., 1976; Hierholzer, 1976; Macnaughton et al., 1977; Pocock and Garwes, 1977; Sturman, 1977; Sturman and Holmes, 1977; LaPorte and Bobulesco, 1981; Storz et al., 1981; Garwes and Reynolds,1981; Stern and Sefton, 1982; Boyle et al., 1984). It is acylated and contains N-linked glycosylation on its external domain (Niemann and Klenk, 1981; Sturman, 1981; Niemann et al., 1982). The E2 glycoprotein has many functions including induction of cell fusion, binding to cell surface receptors and induction of neutralizing antibodies (Garwes et al., 1976; Sturman and Holmes, 1977; Holmes et al., 1981a and b; Macnaughton et al., 1981; Schmidt and Kenny, 1981).

The second structural glycoprotein of all coronaviruses is the E1 or matrix -like (M) protein. It is a glycoprotein of 20-30 kilodaltons (Hierholzer et al., 1972; Garwes et al., 1976; Hierholzer, 1976; Macnaughton et al., 1977; Pocock and Garwes, 1977; Sturman, 1977; Garwes and Reynolds, 1981; LaPorte and Bobulesco, 1981; Storz et al., 1981; Boyle et al., 1984). The external domain of the E1 protein of MHV contains O-linked glycosylation, while that of IBV contains only N-linked glycosylation (Sturman and Holmes, 1977; Holmes et al., 1981b; Niemann et al., 1982; Stern et al., 1982; Stern and Sefton, 1982). The E1 protein spans the viral envelope and functions as a matrix-like protein, linking the nucleocapsid to the viral envelope (Sturman et al., 1980; Sturman, 1981; Niemann et al., 1982).

It has recently become apparent that hemagglutinating (BCV, HCV-OC43, HEV and MHV-DVIM) coronaviruses possess a third membrane glycoprotein, the hemagglutinin. It is a 130-140 kilodalton membrane glycoprotein dimer which dissociates into a 65-70 kilodalton species upon reduction (Callebaut and Pensaert, 1980; King et al., 1985; Hogue and Brian, 1986). It is responsible for hemagglutination and hemadsorption of RBCs by BCV, HCV-OC43, HEV and one strain of MHV. BCV hemagglutinates and hemadsorbs rat, mouse and hamster RBCs at 4°C, 25°C, and 37°C (Sharpee et al., 1976). HCV-OC43 hemagglutinates human O and vervet monkey RBCs at 4°C only but chicken, rat and mouse RBCs at 4°C, 25°C, and 37°C (Kaye and Dowdle, 1969). HCV-OC43 hemadsorbs only mouse and rat cells efficiently (Kapikian et al., 1972). HEV hemagglutinates and hemadsorbs rat, chicken, turkey, mouse, and hamster RBCs at 22°C (Grieg and Girard, 1963; and Girard et al., 1964). The Japanese strain of MHV-DVIM hemagglutinates rat and mouse RBCs at 4°C (Sugiyama and Amano, 1980).

Coronavirus Replication

Adsorption, Penetration and Uncoating: At the time that this research was begun, very little was known about coronavirus adsorption, penetration and uncoating. Adsorption of virus to cell surface receptors is believed to occur via the E2 glycoprotein since treatment of virus with antiserum to the E2 glycoprotein inhibited attachment to susceptible cells (Garwes, 1978-79; Holmes et al., 1981b). Binding of MHV-A59 to L2 cells in spinner cultures was abolished by trypsin treatment of the host cells and occurred more rapidly at 37°C than at 4°C. Saturation of cell surface receptors on L2 cells was accomplished with approximately 700 virus particles per cell (Richter, 1976). HCV-229E bound randomly to MRC cell surfaces at 4°C and with warming to

33°C, virions were lost from the cell periphery by an energy-dependent mechanism (Patterson and Macnaughton, 1981).

One attractive hypothesis to explain the narrow host range and tissue tropism of coronaviruses is limited expression of coronavirus-specific cell surface receptors on membranes from cells of different tissues and species. Several studies have been done with MHV utilizing cells from susceptible and semi-resistant mice to determine the role of cell surface receptors in mouse strain specificity of MHV infection. MHV2 bound equally well to macrophages from genetically susceptible (PRI) and semi-resistant (C3H) mice (Shif and Bang, 1970). MHV3 bound equally well to macrophages, spleen cells, T lymphocytes, thymocytes, and hepatocytes from susceptible (C57BL/6) and semi-resistant (A/J) mice (Krystyniak and Dupuy, 1981; Arnheiter et al., 1982). These studies suggest that cell surface receptors do not play a role in mouse strain susceptibility, but all these studies used only susceptible and semi-resistant strains of mice. A new solid phase assay was recently developed in the laboratory of Dr. Kathryn Holmes to detect receptors for MHV (Boyle et al., 1987). The assay involves binding of intestinal brush border membranes (BBMs) or purified hepatocyte plasma membranes (HMs) from different strains of mice to nitrocellulose, incubation of nitrocellulose membranes with MHV followed by anti-MHV antisera, and then iodinated staphylococcal protein A. Results using this assay showed that membranes from susceptible BALB/c and semi-resistant C3H mice express a receptor for MHV while membranes from resistant SJL/J mouse did not express a receptor for MHV. Because SDS treatment of membranes did not reduce virus binding activity, membranes could be run on a SDS-PAGE gel, blotted to nitrocellulose and probed for virus binding to determine the size of the receptor. A broad band of virus binding activity was seen at 100 to 110

kilodaltons with BALB/c and C3H BBMs and HMs but not with SJL/J BBMs or HMs (Boyle et al., 1987). Thus, it appears that resistance of SJL/J mice to MHV-A59 infection is due to a lack of receptor for the virus. These studies suggested that the presence or absence of coronavirus receptors might play a role in determining the host range of coronaviruses.

Penetration of enveloped viruses occurs either by fusion of the viral envelope with plasma membranes or by endocytosis. Some evidence exists for the use of both mechanisms for coronavirus penetration, though the evidence is not conclusive. The evidence in support of envelope-plasma membrane fusion as a mechanism for coronavirus penetration consists of electron microscopy of BCV uptake by bovine microvilli which shows fusion of viral envelopes with plasma membranes (Doughri et al., 1976). However, electron microscopy has also shown that MHV, IBV, and CCV penetrate cells by endocytosis (Tanaka et al., 1962; David-Ferreira and Manaker, 1965; Sabesin, 1971; Chasey and Alexander, 1976; Patterson and Bingham, 1976; Takeuchi et al., 1976; Arnheiter et al., 1982).

The mechanism of viral uncoating is unknown. Treatment of cells with chloroquine, a lysosomotropic agent which elevates lysosomal pH, reduced MHV3 yields, possibly by blocking uncoating (Mallucci, 1966). However, MHV3 has been shown to infect cytochalasin B-treated murine macrophages and lymphocytes for which RBC phagocytosis and probably endocytosis is blocked (Krystyniak and Dupuy, 1981). More definitive studies will be required to resolve the mechanism of coronavirus penetration and uncoating.

Transcription and Replication: Coronaviruses replicate entirely in the cytoplasm as shown by electron microscopy and fluorescent antibody staining of infected cells (McIntosh, 1974) and replication in enucleated cells (Brayton

et al., 1981; Wilhelmsen et al., 1981). Several coronaviruses can replicate in cells treated with actinomycin D, an inhibitor of host cell RNA synthesis (Parker, 1970; Mishra and Ryan, 1973; Robb and Bond, 1979a; Stern and Kennedy, 1980a). Although some reports claim that alpha-amanatin or actinomycin D inhibits coronavirus virus yield, those experiments were flawed by the use of extremely long incubation periods (greater than 40 hours), or by the use of cell lines which produced only minimal amounts of virus (Kennedy and Johnson-Lussenburg, 1979; Evans and Simpson, 1980).

The coronavirus virion does not contain an RNA polymerase, like many other positive strand viruses, so the first step in replication after uncoating of the genomic RNA is the synthesis of an RNA-dependent RNA polymerase (Schochetman et al., 1977; Tannock and Hierholzer, 1977; Brayton et al., 1982; Dennis and Brian, 1982). Addition of protein synthesis inhibitors directly after adsorption blocks replication, presumably by blocking synthesis of the polymerase (Brayton et al., 1982; Mahy et al., 1983; Sawicki and Sawicki, 1986). The de novo polymerase synthesizes full length negative strand templates from which positive strand subgenomic mRNAs and new genomic RNAs are transcribed (Lai et al., 1982b). Six subgenomic mRNAs have been seen in MHV, TGEV, and HCV-229E infected cells, while only five are seen in IBV infected cells (Stern and Kennedy, 1980a; Cheley et al., 1981; Dennis and Brian, 1981; Lai et al., 1981; Leibowitz et al., 1981; Spaan et al., 1981; Wege et al., 1981; Weiss and Leibowitz, 1981). Subgenomic mRNAs are expressed in different quantities, but their ratios do not change during the course of virus infection (Stern and Kennedy, 1980a; Leibowitz et al., 1981). Most genome-sized RNA is present in EDTA-resistant structures, presumably nucleocapsids, while a small proportion of genome-sized RNA and the subgenomic RNAs are associated with the polysomes (Robb and Bond, 1979a; Spaan et al., 1981;

Wege et al. 1981; Lai et al., 1982). Since the combined size of the mRNAs is greater than the genomic length, the mRNAs must share some common sequences (Stern and Kennedy, 1980a; Spaan, et al., 1981). Using T1 mapping and Northern blotting, it was shown that the subgenomic RNAs form a nested set containing common 3' sequences, with each larger mRNA containing the sequences of the next smaller RNA plus additional unique sequences as shown in Figure 1 (Stern and Kennedy, 1980b; Cheley et al., 1981; Lai et al., 1981; Leibowitz et al., 1981). The subgenomic mRNAs also contain a stretch of 3' polyadenylation (Stern and Kennedy, 1980a; Cheley et al.,1981; Lai et al., 1981; Leibowitz et al., 1981; Spaan et al., 1981; Wege et al., 1981; Dennis and Brian, 1982). A cap and a small 50-70 nucleotide region of conserved sequence, known as the leader, is found at the 5' end of all the viral mRNAs and the genomic RNA (Lai et al.,1982a; Spaan et al., 1982; Brown et al., 1984).

For two reasons, the presence of the leader sequence at the 5' end of the mRNAs is believed to be due to a mechanism other than conventional splicing. First, conventional eukaryotic splicing occurs in the nucleus while coronaviruses replicate in the cytoplasm. Second, UV transcriptional mapping reveals that the UV target size of each subgenomic mRNA is approximately the same as its physical size, suggesting that each subgenomic mRNA is synthesized independently (Jacobs et al., 1981). Because of the leader's small size, the requirement for its synthesis would not effect the UV target size of the mRNAs. These data suggest that the subgenomic mRNAs are not derived by cleavage of a large precursor RNA. Several models for the transcription of the subgenomic messages have been proposed (Baric et al., 1983; Figure 2). The first is the loop out model in which portions of the negative stranded template are looped out during transcription. The second model is the leader-primed transcription model in which the leader RNA is

Figure 1. Intracellular RNAs of MHV. Transcription of genomic RNA (RNA 1) produces a nested set of MHV mRNAs (1 to 7) each with the same 5' leader sequence and common 3' ends with polyadenylation. The sizes of each mRNA ands its unique portion are shown in daltons on the left. The functions and sizes in kilodaltons of the proteins (A to G) encoded by each mRNA are shown on the right. NS, nonstructural; ▫, leader sequence; ∿∿, poly A; Adapted from Siddell et al., 1982.

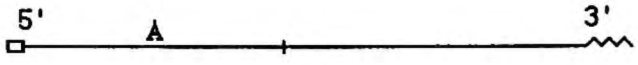
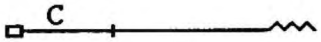
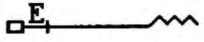
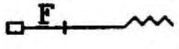
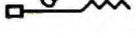
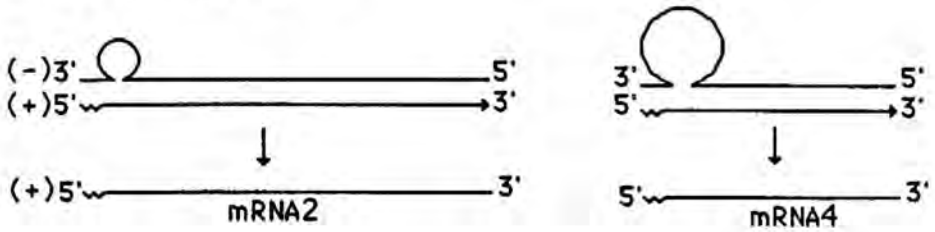
No.	RNA Size (total) ($\times 10^{-6}$)	RNA Size (unique) ($\times 10^{-6}$)	Gene	Protein Size (K)	Protein Function
1	6.3	2.9		200	NS
2	3.4	0.7		30	NS
3	2.7	1.4		120	E2 glycoprotein
4	1.3	0.2		15	NS
5	1.1	0.2		10,13	NS
6	0.9	0.3		23-24	E1 glycoprotein
7	0.6	0.6		54-60	Nucleocapsid

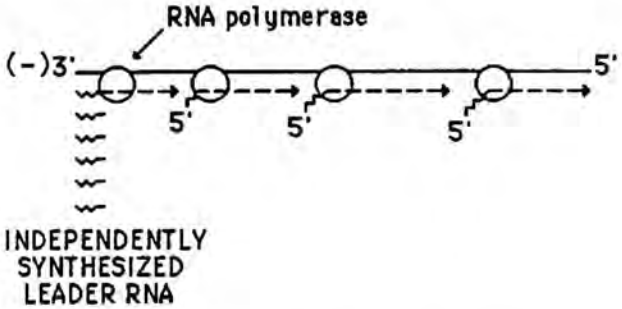
Figure 2. Models of MHV transcription strategy. Model 1 is the loop out model in which differing lengths of the negative strand template are looped out during positive strand synthesis to produce different length mRNAs. Model 2 is the leader-primed transcription model in which leader RNA is synthesized and then used to prime mRNA synthesis. Model 3 is the post-transcriptional processing model in which leader and mRNAs are synthesized independently and then spliced together. Adapted from Baric et al., 1983.

POSSIBLE MODELS OF MHV TRANSCRIPTION

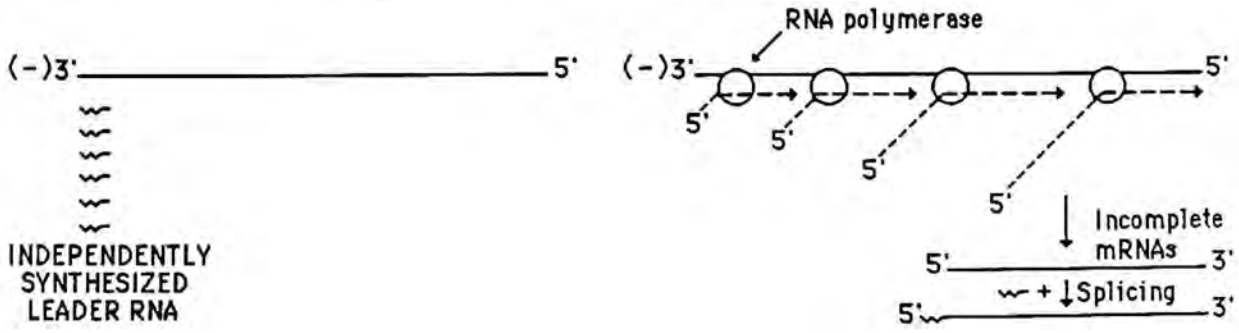
MODEL 1: "LOOP OUT"



MODEL 2: LEADER - PRIMED TRANSCRIPTION



MODEL 3: POST - TRANSCRIPTIONAL PROCESSING



transcribed, dissociates from the negative strand template, then rebinds to the template at the initiation sites of the different mRNAs and serves as a primer for mRNA transcription. The third model is the post-transcriptional processing model in which the leader RNA and the mRNA sequences are transcribed separately and then spliced together by an unknown mechanism. The first model seems unlikely since, no single stranded RNA loops have been detected in replicative intermediates (Spaan et al., 1982). The third model also seems unlikely, since leader sequences are present in replicative intermediates, suggesting that the leader is already present on the incomplete nascent strands. (Baric et al., 1983). At present, the leader primed transcription model is believed to be the correct model.

The RNA-dependent RNA polymerase of coronaviruses has been previously studied in vitro using crude fractionated cytoplasmic extracts (Dennis and Brian, 1981, 1982; Brayton et al., 1982, 1984; Mahy et al., 1983). Using these extracts, the general biochemical requirements of the polymerase and the time course of its appearance in infected cells were determined. When I started this research, many questions concerning the RNA polymerases of coronaviruses were still unanswered. It was not known which viral proteins composed the RNA polymerase, if any host factors were needed for RNA replication, the role of the leader RNA in replication or how the RNA polymerase could produce both negative strand genomic RNA as well as positive strand genomic and subgenomic RNAs. To give you a better feel of the systems available when I started my work, and the reasons that a new system was developed I will summarize the systems previously used to study coronavirus RNA polymerases.

The RNA polymerase of TGEV was the first coronavirus RNA polymerase to be investigated (Dennis and Brian, 1981, 1982). In vivo kinetics

of viral-specific RNA synthesis in actinomycin D-treated swine testicle cells showed one peak of RNA synthesis at 4-6 hours post-inoculation (pi). Using crude fractionated cytoplasmic extracts, the highest polymerase activity was found in the mitochondrial fraction of the infected cells. The polymerase required Mg^{2+} or Mn^{2+} and all four NTPs for activity and the product was destroyed by heating and treatment with RNase but not DNase. Polymerase activity increased steadily for the first 10 minutes and then deceased and remained steady for at least 50 minutes. The polymerase activity is believed to be located in a membrane bound complex, since deoxycholate treatment reduced the sedimentation rate of viral-specific RNA and the polymerase activity. Two peaks of polymerase activity were seen on sucrose gradients from infected cells 5 hours pi. Comparison of in vitro reaction products with those made in vivo revealed that they were approximately the same size (Dennis and Brian, 1981 and 1982).

The RNA-dependent RNA polymerase activity in MHV has been previously studied in vitro by two groups. Brayton et al. (1982 and 1984) using actinomycin D-treated DBT cells and MHV-A59, showed that in vivo viral RNA synthesis peaked at 2 and 5 hours pi and was inhibited by cycloheximide treatment. Using crude fractionated cytoplasmic extracts, polymerase activities were investigated at 1 and 6 hours pi and were found to be highest in the membrane fraction. Again, the polymerase(s) required Mg^{2+} and all four NTPs for activity and the product was destroyed by treatment with RNase but not DNase. The polymerase activities in the fractionated cell extracts from 1 and 6 hours pi had slightly different biochemical requirements. The early polymerase activity was stimulated by K^{+} while the late activity was not, and the early polymerase had a pH optimum of 7.5 whereas the late activity had a pH optimum of 8.0. Fractionation of cell

extracts from the early and late stages of MHV infection on sucrose gradients, revealed only one peak of activity for the early polymerase, but two peaks of activity for the late polymerase. The early polymerase synthesized negative sense, genome-sized RNA while the light peak of the late polymerase synthesized positive sense, genomic RNA and the heavy peak of the late polymerase synthesized positive sense, genomic and subgenomic mRNAs. It was postulated that the light peak of the late polymerase is the replication complex and the heavy peak is the transcription complex (Brayton et al., 1982 and 1984). Mahy et al. (1983) using Sac$^-$ cells and MHV-A59 showed that RNA synthesis was inhibited by anisomycin, a protein synthesis inhibitor but not by alpha-amanitin, a DNA-dependent RNA polymerase inhibitor. Only one temporal peak of activity was seen in vivo at 5 hours pi. In crude fractionated cytoplasmic extracts, RNA polymerase activity was found in the cytoplasmic fraction of the cell, and required Mg^{2+} and all four NTPs for activity. The pH optimum was between 8.0 to 8.4 and the activity was linear for at least 90 minutes.

While these systems were useful for determining the biochemical requirements of the MHV and TGEV RNA polymerase, the time frame of polymerase synthesis and the type of products made at various times during coronavirus infection, we believed that they would not be useful in determining which proteins were involved in RNA replication or how the RNA polymerase was regulated to produce different RNA products.

Translation: In general, each coronavirus mRNA is translated to yield only one protein from the open reading frame at the 5' end of the mRNA (Figure 1). The single exception to this rule is MHV mRNA 5 which is capable of producing more than one protein (Skinner et al., 1985, Budzilowicz and Weiss, 1987). The mRNAs of IBV are labeled A through F from smallest

to largest while the mRNAs of the other coronaviruses are labeled 1 through 7 from largest to smallest. In vitro translations showed that the nucleocapsid protein N is translated from the smallest mRNA (mRNA 7 or A) (Siddell et al., 1980). The matrix-like protein E1 is translated from mRNA 6 or C, and the E2 glycoprotein is translated from mRNA 3 or E (Rottier et al., 1981; Siddell et al., 1981; Stern and Sefton, 1984; Jacobs et al., 1986). Several nonstructural proteins of MHV have been mapped to the mRNAs which encode them. The genomic RNA encodes three nonstructural proteins greater than 200 kilodaltons, which are believed to be the polymerase proteins. A 15 kilodalton nonstructural protein is encoded by mRNA 4 , mRNA 5 encodes 10 and 13 kilodalton nonstructural proteins and mRNA 2 encodes a 30-35 kilodalton nonstructural protein (Leibowitz et al., 1982; Siddell, 1983; Skinner and Siddell, 1985; Skinner et al., 1985; Denison and Perlman, 1986; and Jacobs et al., 1986). The N protein and nonstructural proteins are synthesized on polysomes, whereas the E1 and E2 glycoproteins are synthesized on ribosomes bound to the rough endoplasmic reticulum (RER). E1 is post-translationally glycosylated in the Golgi apparatus at serine and threonine residues (Holmes et al., 1981b; Niemann et al., 1982). E2 is cotranslationally glycosylated on the RER at asparagine residues with core oligosaccharides and then transported to the Golgi apparatus for further glycosylation and acylation (Holmes et al., 1981b; Niemann et al., 1982).

Assembly and Release: Nucleocapsids are formed by binding of numerous copies of the N protein to the genomic RNA . Virions acquire their lipid envelope by budding into the RER, Golgi apparatus, and smooth-walled membranes in areas where E1 and E2 have accumulated (David-Ferriera and Manaker, 1965; Dubois-Dalcq et al., 1982; Massalaski et al., 1981; Tooze et al., 1984). The only glycoprotein necessary for virus budding is E1,

since in tunicamycin-treated cells, which make E1 but not E2, budding still occurs (Holmes et al., 1981b). It is postulated that the intracellular domain of E1 binds to the nucleocapsid (Holmes et al., 1981b). Following budding into intracellular membranes, the virions are apparently released from infected cells by exocytosis (Doughri et al., 1976; Holmes et al., 1984; Sturman and Holmes, 1985).

Introduction

Coronaviruses have a unique RNA transcription and replication strategy which has been described above. Although the nature of the genomic and mRNA species, the kinetics of their synthesis, and the mechanism of leader sequence addition to the mRNAs have been extensively studied, very little is known about the viral proteins involved in transcription and replication. Three viral structural proteins are made in large quantities in MHV-infected cells and have been well characterized. In addition, nonstructural proteins of greater than 200, 30, 15, 13 and 10 kilodaltons have been described. These proteins are made in much smaller quantities in MHV-infected cells and have not been well characterized. It is very likely that one or more of the nonstructural proteins is involved in MHV transcription and replication. The structural proteins also may be involved in MHV transcription and/or replication in a regulatory capacity as seen with VSV and Sendai viruses (Clinton et al., 1978; Hsu et al., 1984; Kingford and Emerson, 1980; Lamb and Choppin, 1977).

Systems previously used to study the RNA-dependent RNA polymerase of coronaviruses involved fractionation of infected cells and characterization of the cellular fractions enriched for polymerase activity (Dennis and Brian, 1981 and 1982; Brayton et al., 1982 and 1984; Mahy et al., 1983). Because these systems may not be ideal for determining the role(s) of different viral proteins in MHV transcription and replication, we chose to develop a new in vitro system for MHV replication based on an in vitro assay for replication of VSV RNA (Condra and Lazzarini, 1980; and Peluso and

Moyer, 1983). This VSV polymerase assay involves permeabilization of infected cells with lysolecithin to produce an extract containing the cytoplasmic contents of the infected cell (Miller et al., 1979). This system has proven particularly useful for the identification of the viral proteins involved in VSV and Sendai virus replication and transcription (Carlsen et al., 1985; and Peluso and Moyer, 1983). Using this new in vitro assay to study coronavirus RNA synthesis, we hoped to be able to identify the role(s) of viral proteins in coronavirus replication.

Materials and Methods

Virus and cell propagation: Sac$^-$, L2, and 17-Cl-1 cells were grown in Dulbecco modified Eagles minimal medium (MEM) with high glucose, 10% fetal bovine serum (FBS) and 2% antibiotic-antimycotic mixture. MHV-A59 obtained from Dr. L. Sturman, New York State Department of Health, Albany, N.Y. was grown in monolayers of 17-Cl-1 cells (Sturman and Takemoto, 1972). Supernatants were clarified at 10,000 g for 20 minutes at 4^{o}C and stored at -70^{o}C.

Antisera: Antisera against the MHV glycoproteins E1 and E2 respectively were made by immunization of rabbits with E1 or E2 separated from Nonidet P-40-disrupted virions by sucrose density centrifugation (Sturman et al., 1980). Anti-whole MHV antiserum was obtained by immunization of rabbits with detergent disrupted MHV virions using the same immunization strategy. This serum gave a positive reaction on Western blots with MHV E1, E2 and N proteins. Antiserum against the MHV nucleocapsid protein was made by immunization of rabbits with SDS-PAGE purified N protein. This serum is specific for the MHV N protein and smaller peptides derived from it. Immunoglobulins were precipitated with

ammonium sulfate, and protein concentrations were determined by the Bradford method (Bradford, 1976).

Agarose gel electrophoresis: RNA samples were prepared in 20 mM 3-(N-morpholine) propanesulfonic acid (MOPS), 1 mM EDTA, 8 mM sodium acetate, 50% formamide, 17% formaldehyde, 40 mM urea, heated to 50°C for 5 minutes and electrophoresed through denaturing formaldehyde 1% agarose (20 mM MOPS, 1 mM EDTA, 8 mM sodium acetate,17% formaldehyde, 1% agarose) gels at 50 volts for 6 hours (Lehrach et. al., 1977).

In vitro cell extracts for coronavirus RNA synthesis: Cell extracts were prepared from three 60-mm-diameter dishes of subconfluent monolayers of 17-Cl-1, L2 or Sac$^-$ cells infected with MHV-A59. At various times post inoculation (pi), cells were washed twice with wash buffer (100 mM sucrose, 33 mM ammonium chloride, 7 mM potassium chloride, 5 mM magnesium acetate, 30 mM Tris hydrochloride [pH 8.4]) and then treated with 75µg of lysolecithin per ml in wash buffer for one minute. Permeabilized cells were scraped into 300µl of reaction mixture containing 30 mM Tris hydrochloride (pH 8.4), 40 mM ammonium chloride, 140 mM potassium chloride, 5 mM magnesium acetate, 0.25 mM dithiothreitol, 1 mM adenosine triphosphate (ATP), 80 µM cytidine triphosphate (CTP), 80 µM guanosine triphosphate (GTP), 1 µM uridine triphosphate (UTP), 3 µM S-adenosylmethionine, 2.4 mM phoshoenolpyruvate, 80 µM spermine, 16 µg/ml pyruvate kinase, 5 mg/ml tRNA and 10 µg/ml actinomycin D. The cells were gently disrupted with a pipette and centrifuged for 5 seconds in a Microfuge. 20 to 50 µCi of [^{32}P]UTP was added to the resulting cell-free supernatant to a final concentration of 0.25 µM. Reaction mixtures were incubated at 37°C for 1 hour and then terminated by incubation at 37°C for 30 minutes with 1% SDS and 30 µg/ml proteinase K. RNA was extracted with

an equal volume of phenol saturated with Tris hydrochloride (pH 7.5) - 0.1 M sodium chloride - 1 mM EDTA and precipitated with 2.5 volumes of 95% ethanol (Miller et al., 1979; Peluso and Moyer, 1983).

Hybridization of MHV RNA to cDNA clones: ^{32}P-labeled RNA synthesized in vitro from MHV- or mock-infected cell extracts was hybridized to Gemini I, pBR322, the MHV cDNA clones c8 or g^{2}344. c8 contains the 3' portion of MHV gene 7 in pBR322 and g^{2}344 is the cDNA clone g344 containing the 3' portion of MHV genes 4, 5, and 6 and the 5' portion of gene 7 in pBR322, which I inserted into the Gemini I vector (Budzilowicz et al., 1985). Plasmid DNAs were denatured, dotted onto nitrocellulose, baked, and then prehybridized for 6 hours at 50°C in hybridization buffer (65% formamide, 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH, 6.4], 0.2% SDS, 0.4 M sodium chloride, 100µg/ml tRNA, 10% dextran sulfate). In vitro-synthesized RNA probes were generated from 100µl extracts of mock- or MHV-infected cultures which had been filtered through Sephadex G25 columns to remove free [^{32}]UTP. Approximately 2-3 x 10^5 cpm of labeled RNA product was used to probe the nitrocellulose sheets for 12 hours at 50°C in fresh hybridization buffer. The nitrocellulose sheets were washed 5 times with 2X SSC (1X SSC is 0.15 M sodium chloride, 0.015 sodium citrate, pH 7.0) containing 0.05% sodium pyrophosphate and 5 times with 0.1X SSC containing 0.05% sodium pyrophosphate, and then air dried. Hybridization was detected by autoradiography (Maniatis et al., 1982).

Cesium chloride gradient analysis of in vitro-synthesized RNA: ^{32}P-labeled RNA synthesized in vitro was immunoprecipitated with antiserum against MHV virions or treated with RNase and layered onto 20 to 40% cesium chloride gradients in TMS buffer (0.05 M Tris hydrochloride [pH 6.0], 0.25 M maleic acid, 0.1 M sodium chloride). Gradients were centrifuged 20

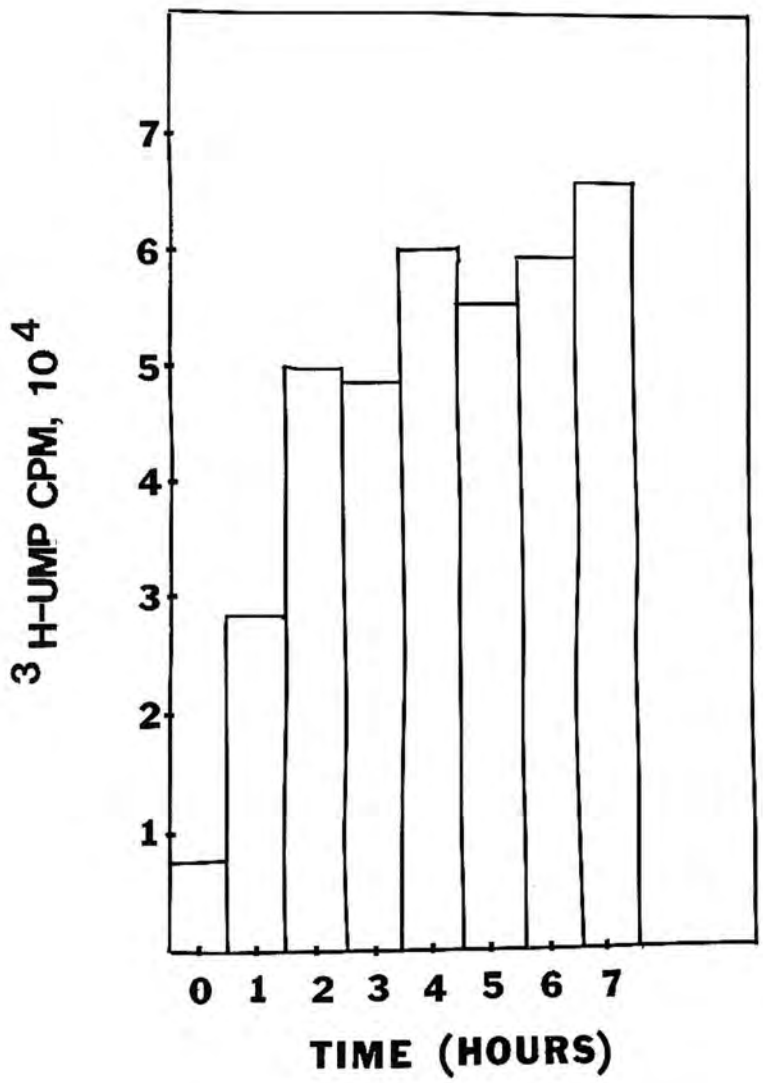
hours at 36,000 rpm in a Beckman SW41 rotor at 15°C. Fractions (0.2 ml) were collected from the bottom of the gradient, and the amount of trichloroacetic acid (TCA)-precipitable radioactivity in each was determined by scintillation spectrophotometry.

Results

In vivo kinetics of MHV-A59 RNA synthesis: Prior to attempting to develop an in vitro assay for MHV RNA synthesis, the kinetics of the MHV RNA polymerase were investigated in infected Sac⁻ cells, to determine the best time to make infected cell extracts. To synchronize expression of MHV RNA polymerase, virus was adsorbed to monolayers of Sac⁻ cells which had been preincubated 2 hours with actinomycin D, to inhibit cellular RNA polymerases, at 4°C with a multiplicity of infection (MOI) of 10 plaque forming units (PFU) (Brayton et al., 1982). Synthesis of virus-specific RNA was detected beginning at 1 hour pi and the rate of viral RNA synthesis steadily increased from 1 to 4 hours pi (Figure 3). The rate of viral RNA synthesis remained constant from 4 to 7 hours pi Therefore, for subsequent studies we chose to make cell extracts at 5 to 6 hours pi when maximal viral RNA synthesis was occurring, and syncytium formation had begun.

Kinetics of RNA synthesis in lysolecithin treated cell extracts: The lysolecithin technique of permeabilizing cells used to study VSV replication was modified to optimize the incorporation of [^{32}P] UMP into genome-sized coronavirus RNA (Condra and Lazzarini, 1980; Miller et al., 1979; Peluso and Moyer, 1983). Briefly, extracts from actinomycin D-treated MHV-infected Sac⁻ cells were prepared by treatment of monolayers with lysolecithin at 6 hours pi, followed by centrifugation to remove cell debris from the soluble fraction. Depending on the cell line used, lysolecithin concentrations of 75 to 200 μM

Figure 3. Kinetics of viral RNA synthesis in MHV-A59 infected Sac$^-$ cells. One hour before inoculation, 5 μg/ml actinomycin D was added to subconfluent 60-mm-diameter plates of Sac$^-$ cells. Cells were infected with 400 μl of MHV-A59 virus at a MOI of 10 at 4°C for one hour, after which 3 ml of minimal essential medium-fetal calf serum with 2 μg/ml actinomycin D was added to each plate. For each time point, 100 μCi of [^{3}H]uridine /ml was added to two plates, and the plates were incubated for one hour at 37°C. The cells were then washed, scraped into 5 ml of reticulocyte lysis buffer, solubilized with 1% SDS, and precipitated with TCA. TCA pellets were resuspended in 100 μl of distilled water and quantitated by scintillation spectrophotometry.


^{3}H-UMP CPM, 10^4
7
6
5
4
3
2
1
0 1 2 3 4 5 6 7
TIME (HOURS)

were needed to permeabilize the cells. Extracts from Sac⁻ cells consistently gave more reliable results than extracts from 17 Cl-1 or L2 cells and were therefore used in all subsequent work. The kinetics of RNA synthesis in MHV-infected extracts were measured by incorporation of [^{32}P] UMP into acid-precipitable material. In the presence of 5 μg/ml actinomycin D, MHV-A59 infected cell extracts made 6 hours pi incorporated [^{32}P] UMP into newly synthesized viral RNA at a linear rate for at least 60 minutes (Figure 4). In the presence of actinomycin D, mock-infected extracts showed less than 10% of the [^{32}P] UMP incorporation seen in MHV-infected extracts. Subsequently, extracts were routinely incubated for 60 minutes.

Characteristics of the in vitro RNA products: The products of the in vitro reaction were compared with those synthesized in vivo (Figure 5). In MHV-infected cells (lane A) both genomic RNA and varying amounts of subgenomic RNAs were made. In contrast, the predominant species of RNA made in vitro was genomic size. Synthesis of genome sized RNA was detected in extracts made from 2-6 hours pi (lanes C-G) but not in extracts made 1 hour pi (lane B) or in extracts from mock-infected cells (lane H). The temporal increase in RNA synthesis seen in vitro reflected that seen in vivo (Figure 3). Small amounts of subgenomic RNAs were also made as shown by the arrows, which may represent mRNA species. Clearly, the in vitro MHV polymerase reaction favors replication over transcription.

To determine if the products of the in vitro reaction were MHV specific, ^{32}P labeled RNA synthesized in vitro in MHV- or in mock-infected cell extracts was hybridized to plasmid DNAs which did or did not contain MHV specific sequences (Figure 6). In vitro synthesized RNA from mock-infected cell cultures did not hybridize to any of the plasmids. RNA products from MHV-infected cell extracts hybridized only to those plasmids containing

Figure 4. Kinetics of the in vitro incorporation of radionucleotides into acid precipitable material. Extracts from MHV-A59- or mock-infected Sac$^-$ cells were made 6 hours post inoculation as described in Material and Methods. [^{32}P]UTP was added, and the reaction mixture was incubated at 31^{o}C. At 15 minute intervals, 20 µl portions were removed from the reaction mixture and the amount of radiolabel incorporated into TCA precipitable material was determined.

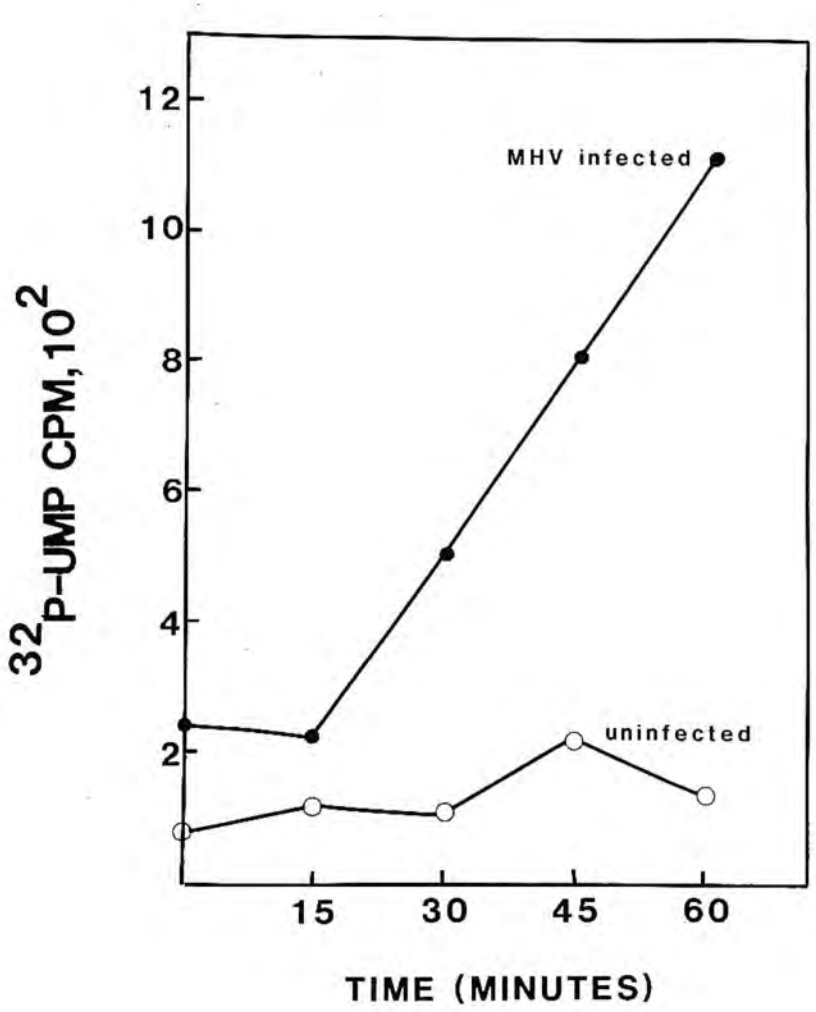

12
10
8
6
4
2
MHV infected
uninfected
15
30
45
60
TIME (MINUTES)
32P-UMP CPM, 10^2

Figure 5. Analysis of the coronavirus-specific RNAs synthesized in vitro by agarose gel electrophoresis. At one hour intervals after MHV-A59 infection of Sac$^-$ cells, extracts were made as indicated in Materials and Methods and were incubated for one hour at 37°C with [^{32}P]UTP. The resulting RNA products were resolved on denaturing formaldehyde-1% agarose gels. Lanes: A, RNA labelled in vivo with $^{32}P_i$ in MHV-A59 infected Sac$^-$ cells, showing the relative migration and quantity of the seven MHV mRNAs (1-7); B to G, RNA made from in vitro extracts of MHV-A59 infected Sac$^-$ cells, and harvested at one hour intervals (B-1 hour; C-2 hours; D-3 hours; E-4 hours; F-5 hours; G-6 hours); H, RNA from in vitro extracts of uninfected Sac$^-$ cells. Arrows indicate subgenomic RNAs.

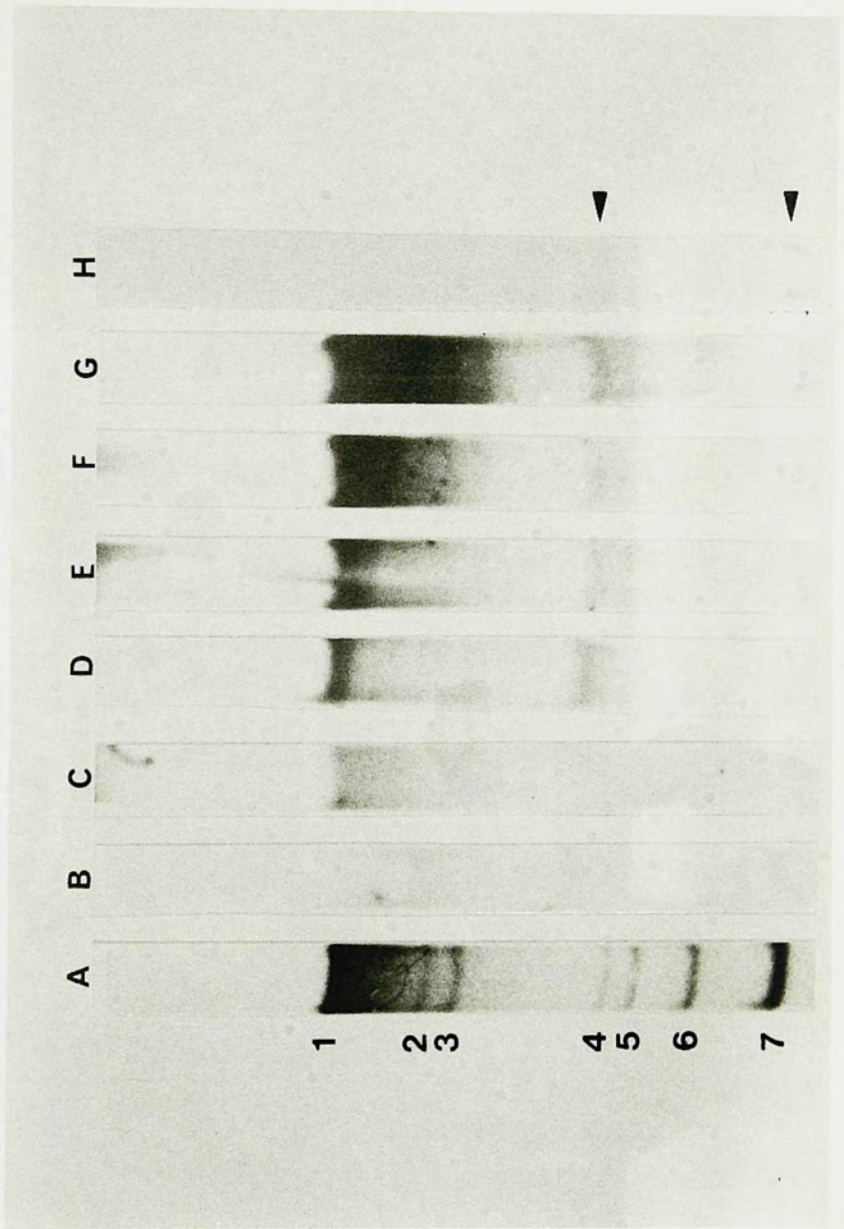
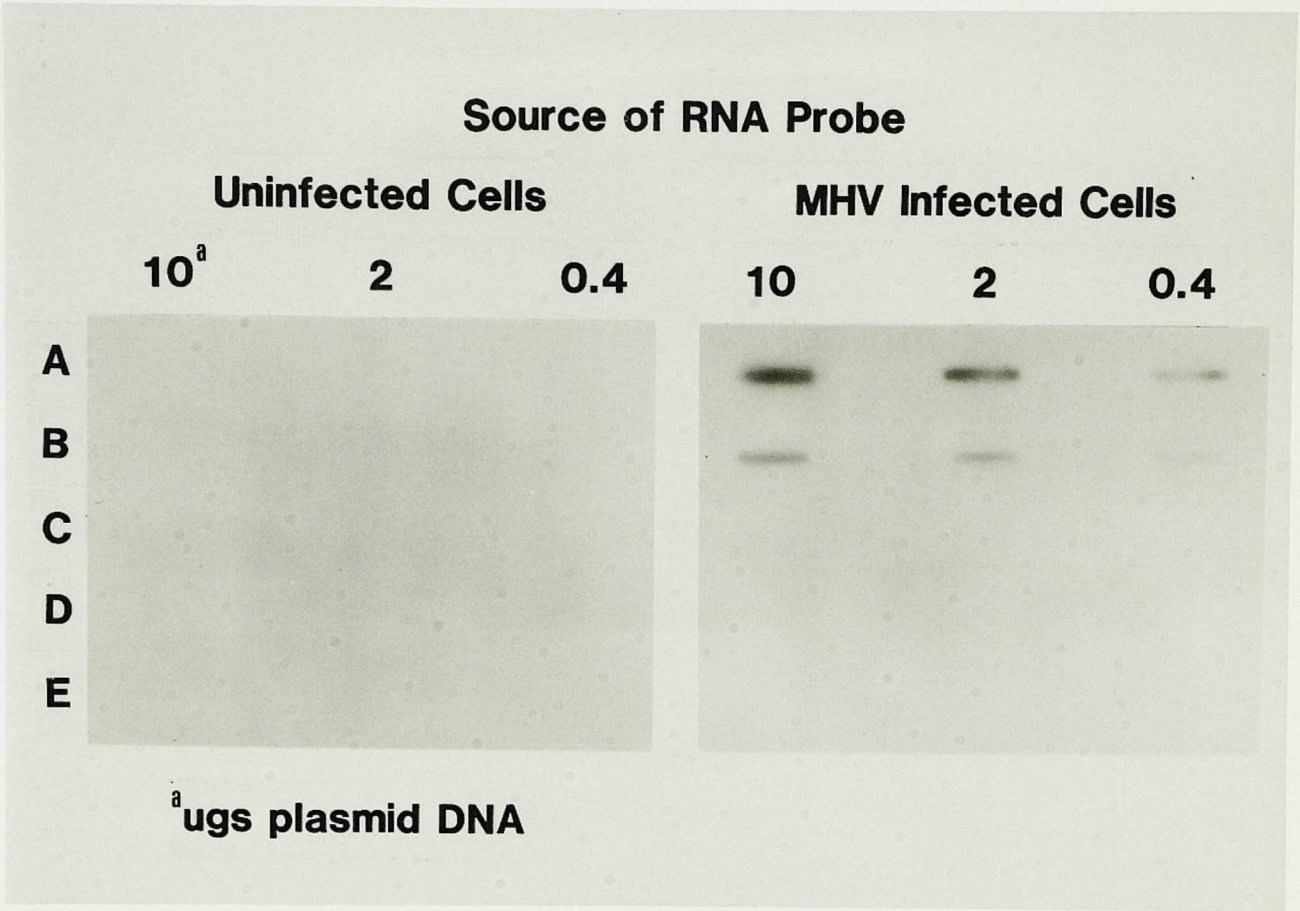

A
B
C
D
E
F
G
H
1
2
3
4
5
6
7

Figure 6. MHV specificity of in vitro synthesized RNA products. DNA from plasmids containing MHV-specific cDNA or control plasmids was bound to nitrocellulose in the amounts shown and probed with ^{32}P-labelled RNA synthesized in 100µl extracts of mock- or MHV-infected cells. Rows: A and B, g^{2}344 in Gemini I and c8 in pBR322, respectively, containing MHV sequences; C and D, Gemini I and pBR322, respectively, lacking MHV sequences; E, no DNA. [a] micrograms of plasmid DNA.

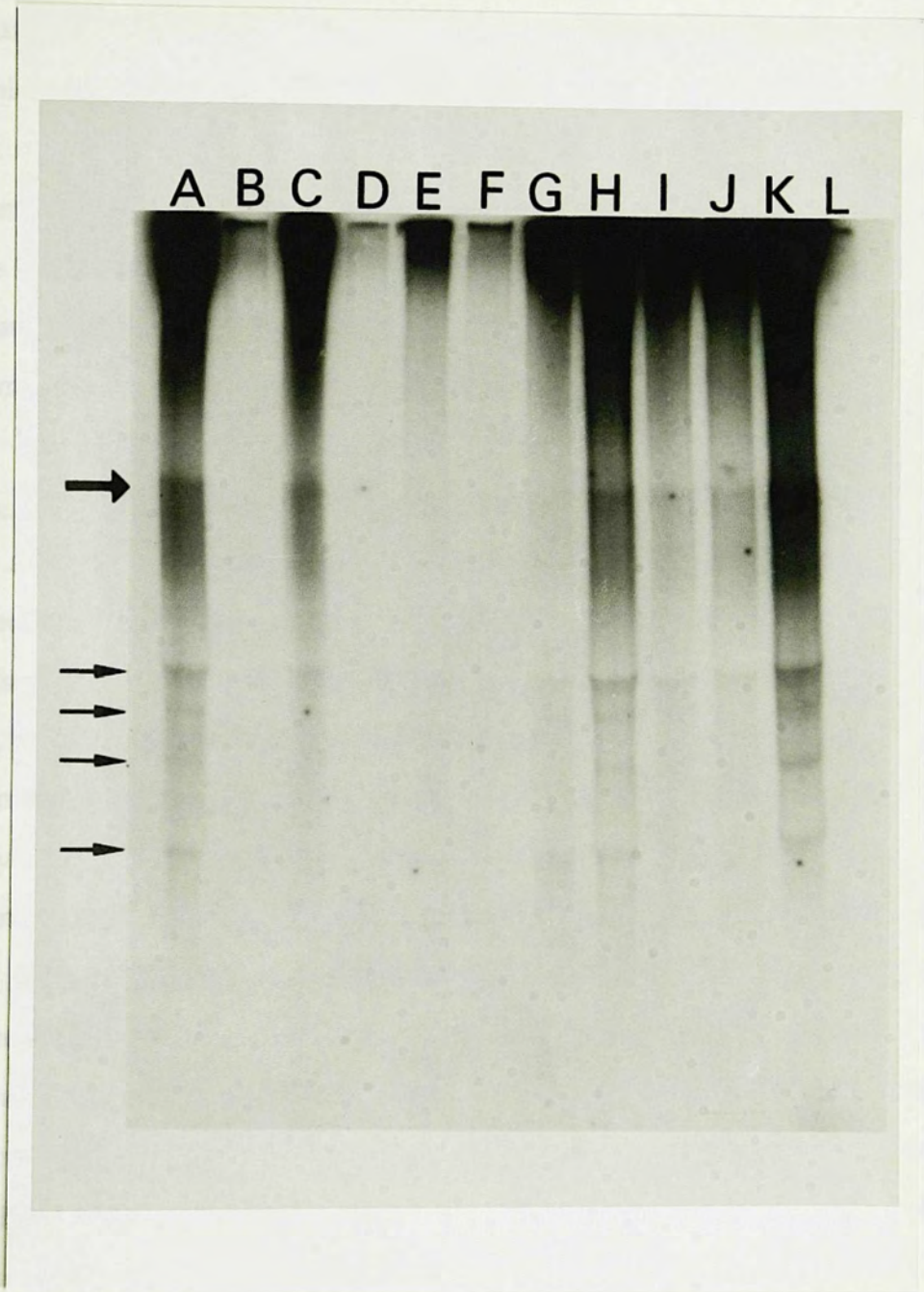

Source of RNA Probe
Uninfected Cells
MHV Infected Cells
10[a]
2
0.4
10
2
0.4
A
B
C
D
E
[a]ugs plasmid DNA

MHV-specific sequences and not to the control plasmids. Therefore, the RNA synthesized in MHV-infected cell extracts is MHV-specific, and primarily genome sized.

Characteristics of the in vitro polymerase reaction: In vitro extracts were prepared under a variety of conditions to determine the optimal conditions for the MHV polymerase reaction. The MHV RNA polymerase activity is greatest at pH 8.2 to 8.5. For Sac$^-$ cells, the RNA polymerase was most active in vitro at ion concentrations of 0 to 50 mM sodium, 140-160 mM potassium, 10 to 100 mM ammonium, and 2 to 8 mM magnesium, with optimal activity occurring in a mixture containing 140 mM potassium, 40 mM ammonium and 5 mM magnesium (data not shown).

The nucleotide triphosphate (NTP) requirements of the polymerase reaction were determined. Since the MHV-infected cell extracts are crude cytoplasmic extracts, to analyze the NTP requirements of the MHV polymerase, the endogenous NTPs were first removed by filtering extracts through Sephadex G25 columns preequilibrated with reaction mix lacking all four NTPs. When all four NTPs were added to filtered or unfiltered extracts, both genome sized and some smaller subgenomic sized RNAs were detected (Figure 7, lane A and K). Extracts lacking all four NTPs were inactive (lanes B and L). No incorporation of [^{32}P]UTP was seen into viral RNAs when UTP, GTP or CTP were added back singly to the filtered extracts (Lanes D, E, and F, respectively). However, a small amount of incorporation of [^{32}P]UTP into genome sized RNA was detected when unlabeled ATP was added back singly to the filtered extract (Lane C). This result suggests that a small quantity of residual NTPs remain after filtration and that the addition to the extract of ATP, which served as an energy source and a NTP source, permitted a small amount of replication to occur. To determine if the RNA polymerase

Figure 7. Nucleotide triphosphate requirements of the MHV RNA polymerase reaction. Extracts from MHV-infected Sac$^-$ cells were filtered through Sephadex G25 columns preequilibrated with a reaction mixture lacking nucleotide triphosphates. Portions (25µl) of the extract were incubated for one hour at 37°C with 10 µCi of [^{32}P]UTP, and unlabeled nucleotide triphosphates (1 mM ATP, 0.08 mM CTP, 0.08 mM GTP and 0.001 mM UTP) were added singly or in combinations of three or four. Extracts were resolved on denaturing formaldehyde-1% agarose gels. Lanes: A through J, RNA made in filtered extracts with the addition of nucleotide triphosphates (A, all four; B, none; C, ATP; D, UTP; E, GTP; F, CTP; G, CTP, GTP, UTP; H, ATP, CTP, GTP; I, ATP, CTP, UTP; J, ATP, GTP, UTP); K and L, RNA made in unfiltered extracts with the addition of all four or no nucleotide triphosphates respectively. Heavy arrow, genome-sized RNA; Light arrows, smaller RNA species which may represent MHV-specific mRNAs.

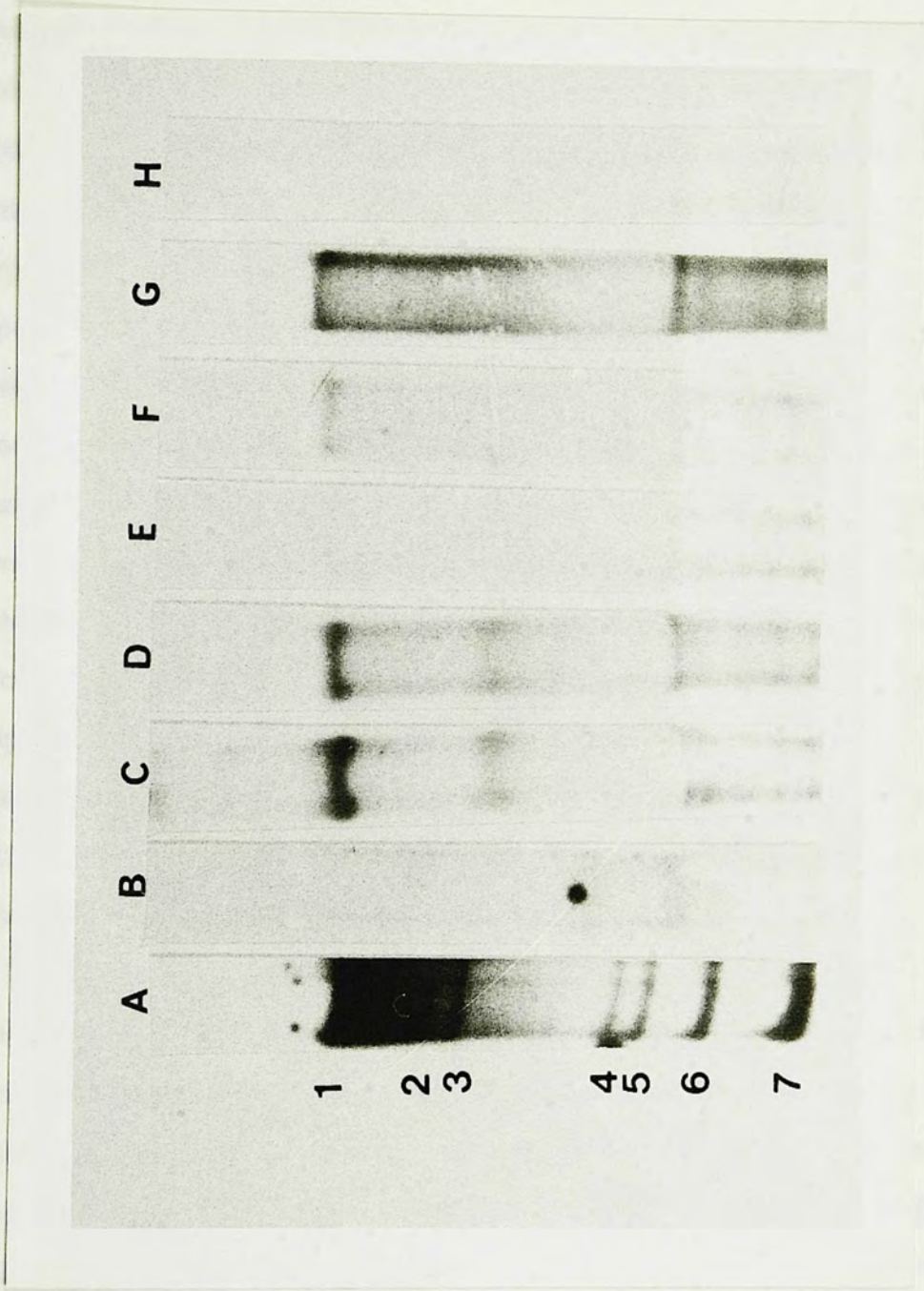

A B C D E F G H I J K L

required all four nucleotides, as would be expected of a heteropolymerase, three of the four NTPs were added back to filtered extracts. Omission of either ATP, CTP or GTP from filtered extracts resulted in only a very small amount of [^{32}P]UTP incorporation (Lanes G, I, and J, respectively). Omission of unlabeled UTP from the extract resulted in only a very small decrease in RNA synthesis when compared to the complete reaction (Lane H) due to the presence of [^{32}P]UTP as the source of radiolabel in the extract. Taken together, these results show that the MHV RNA polymerase requires all four NTPs and supports the hypothesis that the product of the in vitro MHV RNA polymerase reaction is a heteropolymer.

Effect of protein synthesis inhibitors and antiviral antisera on in vitro RNA polymerase activity: The in vitro replication of negative strand viruses such as VSV and Sendai virus, which like coronaviruses have helical nucleocapsids, is greatly reduced if ongoing protein synthesis is inhibited (Carlsen et al., 1985, Davis and Wertz, 1982, and Peluso and Moyer, 1983). Also, in vivo coronavirus RNA synthesis has been shown to be inhibited by treatment of cells with protein synthesis inhibitors (Brayton et al., 1982; and Mahy et al., 1983). Therefore it was of interest to determine whether puromycin, an inhibitor of polypeptide chain elongation, could inhibit in vitro RNA synthesis in extracts from MHV-infected cells. When puromycin was added to MHV-infected cells 30 minutes prior to extract preparation, the synthesis of genome sized MHV RNA was reduced to less than 10% of that observed in untreated MHV-infected extracts (Figure 8, Lane H). Therefore, ongoing protein synthesis is needed for in vitro as well as for in vivo MHV RNA synthesis.

The effects of antisera to several viral proteins on viral RNA synthesis in extracts from MHV-infected cells were analyzed to elucidate which

Figure 8. Effects of antisera to MHV-A59 proteins or puromycin on the in vitro synthesis of MHV RNA. Six hours pi, extracts were made as indicated in Materials and Methods from MHV-infected Sac$^-$ cells and assayed in the presence of 1.3 to 1.5 μg of antisera or 50 μg of puromycin. The resulting RNA products were resolved on denaturing formaldehyde-1% agarose gels. Lanes: A, RNA labelled with $^{32}P_i$ from MHV-A59 infected Sac$^-$ cells; B, RNA synthesized in mock-infected Sac$^-$ cells; C, untreated extracts from MHV-infected Sac$^-$ cells; D, MHV-infected extract treated with preimmune IgG; E, MHV-infected extract treated with IgG against the MHV-A59 N protein; F, MHV-infected extract treated with IgG against the MHV-A59 E1 protein; G, MHV-infected extract treated with IgG against the MHV-A59 E2 protein; H, MHV-infected extract treated with puromycin. The relative positions of the seven MHV mRNAs are noted at the left.


A
B
C
D
E
F
G
H
1
2
3
4
5
6
7

proteins might be required for MHV RNA synthesis in vitro. Extracts from MHV-infected cells were assayed in the presence of 1.3 to 1.5 μg immunoglobulin G (IgG) from sera of preimmune rabbits or rabbits immunized with purified preparations of each of the major MHV structural proteins N, E1, or E2. The addition of preimmune serum, caused a slight inhibition of coronavirus RNA synthesis (Figure 8, Lane D). However, the addition of antibody to the N protein of MHV (Lane E) reduced the incorporation of [^{32}P]UTP into MHV genome sized RNA by more than 90% compared with that of the control preimmune serum (Lane D). In contrast, addition of antibodies to the envelope glycoproteins E1 or E2 (Lanes F and G) did not significantly inhibit MHV RNA synthesis (5-20% and <2% respectively in repeated experiments) compared with that of the control preimmune serum. As a control for possible nonspecific inhibitory effects of anti-N IgG, anti-N IgG was added to in vitro assays for the transcription and replication of VSV. The level of VSV RNA synthesized, as measured by acid-precipitable counts, remained unchanged (data not shown). These data suggest that the inhibition of RNA synthesis by anti-N antibody is virus specific, and that the MHV N protein may play an important role in the synthesis of MHV genomic RNA.

Encapsidation into nucleocapsids of in vitro synthesized RNA: RNA synthesized in vitro in extracts from MHV-infected cells was examined for association with coronavirus proteins. If the newly made RNA formed ribonucleoprotein complexes with the proper ratio of RNA to N protein, then these complexes might band in cesium chloride (CsCl) gradients at 1.24 g/cm^3, the density of MHV nucleocapsids isolated from MHV virions (Sturman et al., 1980). Antibody specific for the structural proteins of MHV was utilized to immunoprecipitate RNA-protein complexes produced in vitro in MHV-

infected cell extracts. The immunoprecipitated complexes were run on a CsCl gradient, and only one peak of radioactivity was obtained at a buoyant density of approximately 1.2 g/cm^3 (Figure 9). No significant amount of radioactivity was obtained with immunoprecipitates from mock-infected cells or from MHV-infected extracts immunopreciptitated with preimmune serum (data not shown).

The characteristics of ribonucleoprotein complexes produced in MHV-infected extracts were investigated. Products from MHV-infected cell extracts were treated with RNase T1 and analyzed on CsCl gradients (Figure 10A). RNase T1 treatment of in vitro reaction products did not affect detection of a peak of radioactivity at 1.2 g/cm^3, indicating that the RNA was protected from degradation. When MHV-infected cell extract products were treated with SDS and proteinase K and phenol extracted prior to RNase T1 treatment, the amount of radiolabel seen at 1.2 g/cm^3 was greatly reduced (Figure 10B). No peak of radioactivity was seen at 1.2 g/cm^3 when extracts were treated with RNase T1 prior to incubation with [^{32}P]UTP (data not shown). Therefore, at least some of the newly synthesized RNA is being incorporated in RNase resistant ribonucleoprotein complexes which may be analogous to nucleocapsids.

To determine what host cell proteins might be involved in MHV replication, several cell lines were tested for their ability to replicate MHV-A59. BHK (hamster), BSC (monkey), XTC (frog), and HeLa and KB (human) cells were tested to see if it could be determined at what level MHV replication was blocked in these cell lines. No cytopathic effects could be seen in BHK, KB or XTC cells inoculated with MHV-A59. No MHV RNA synthesis could be detected by Northern blots of "MHV-infected" extracts with MHV containing cDNA clones or by in vivo labelling of RNA in "MHV

Figure 9. Encapsidation of in vitro-synthesized MHV RNA. MHV-A59 infected Sac$^-$ cell extract products (200µl) were mixed with 33% washed staphylococccal protein A beads (400µl) and 1% Nonidet P-40 (900 µl) and then precleared by centrifugation at 1,000 g for 10 minutes. Anti-MHV-A59 serum (30µl) was added to the supernatant, and the mixture was incubated on ice for 60 minutes. After, one hour, 200µl of staphylococcal protein A beads were added and the mixture was pelleted. The pellet was resuspended in PBS and resolved on a 20-40% CsCl gradient. Circles, CsCl densities in grams per cubic centimeter; triangles, radiolabel in counts per minute.

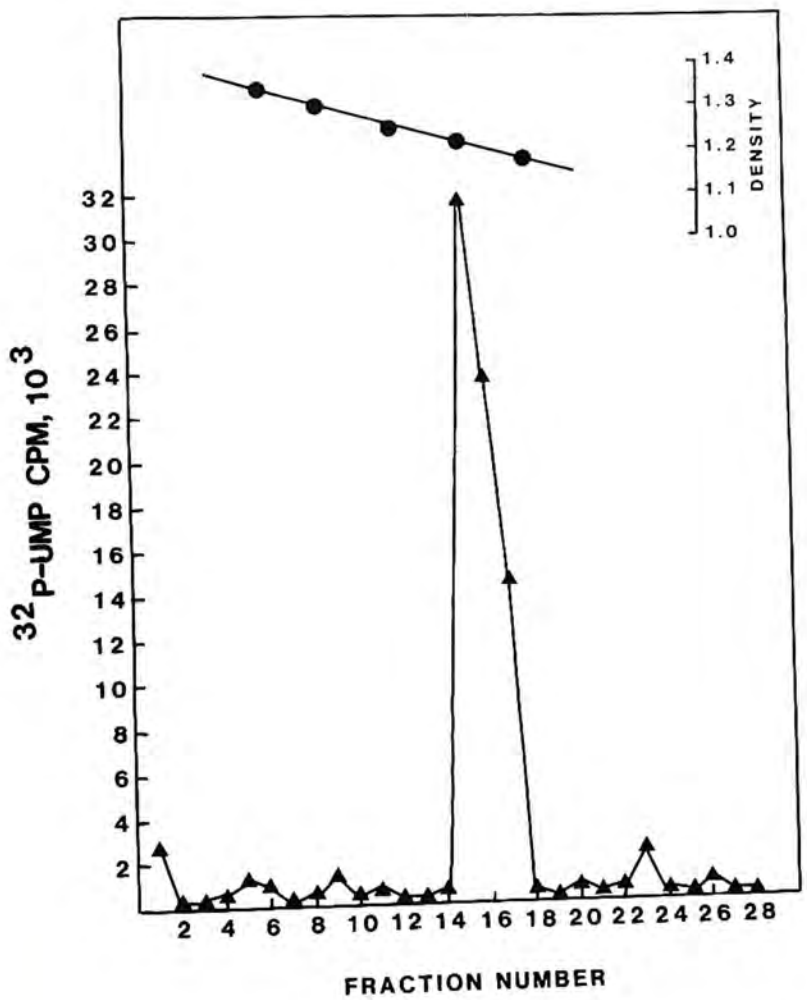
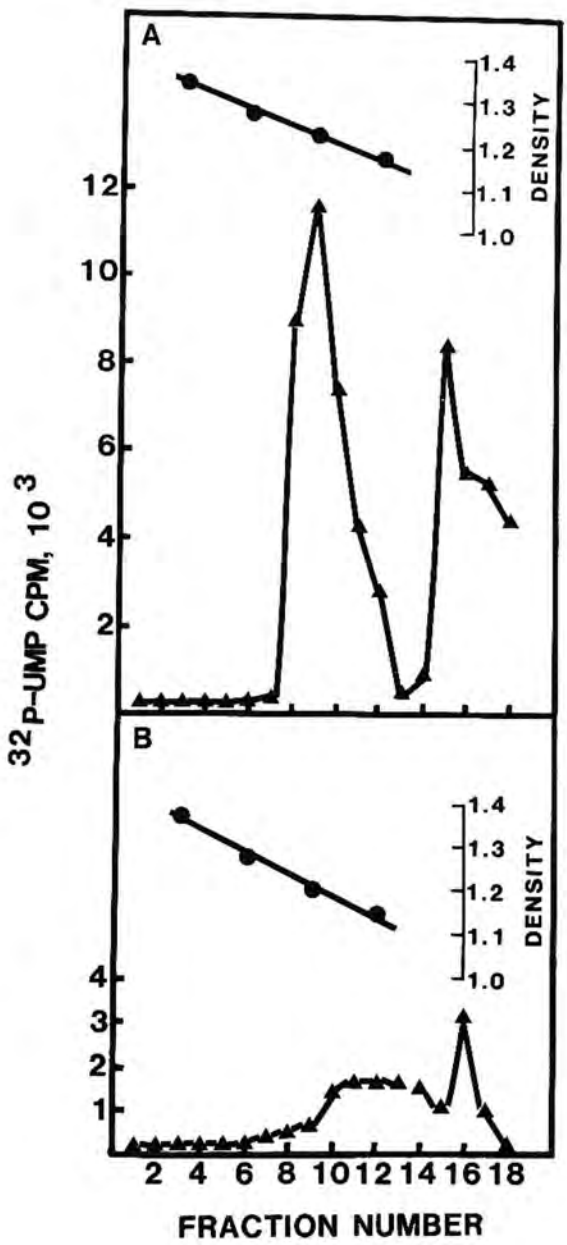

1.4
1.3
1.2
1.1
1.0
DENSITY
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
^{32}P-UMP CPM, 10^{3}
2 4 6 8 10 12 14 16 18 20 22 24 26 28
FRACTION NUMBER

Figure 10. Effect of RNase on the ribonucleoprotein complexes synthesized in vitro. Panel A: MHV-infected Sac⁻ cellular extract (100µl) was incubated with [^{32}P]UTP and treated with 5 U of RNase T_1 for 30 minutes at 37°C. A portion of the extract was resolved on a 20%-40% CsCl gradient. Panel B: MHV-infected Sac⁻ cellular extract (200µl) was incubated with [^{32}P]UTP and treated with 40 µl of 10 mg/ml proteinase K and 30 µl 20% SDS. The resulting RNA was then treated with 5 U of RNase T_1 for 30 minutes at 37°C, and a portion of the mixture was resolved on a 20%-40% CsCl gradient. Circles, CsCl densities in grams per cubic centimeter; triangles, radiolabel in counts per minute.


A
B
1.4
1.3
1.2
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1.0
DENSITY
12
10
8
6
4
2
4
3
2
1
^{32}P-UMP CPM, 10^3
2 4 6 8 10 12 14 16 18
FRACTION NUMBER

infected" BHK, BSC, HeLa, KB or XTC cells. No MHV proteins could be detected in "MHV-infected" BHK, BSC, HeLa, KB or XTC cells using immunofluorescence techniques (data not shown). In contrast to cell lines of non-murine origin, mouse cell lines L and Sac$^-$ infected with MHV-A59 showed evidence of MHV infection by all of the above methods. It therefore appears that the restriction for MHV infection of these non-murine cell lines occurs early in the replication cycle, possibly at the level of virus binding or penetration.

Discussion

Coronavirus-specific RNA dependent RNA polymerases have been studied in TGEV- and MHV-infected cell extracts (Dennis and Brian, 1981 and 1982; Brayton et al., 1982 and 1984; Mahy et al., 1983; Sawicki and Sawicki, 1986). The present experiments demonstrated a rise in activity from 1 to 6 hours pi with one temporal peak of MHV RNA polymerase activity at 4 to 6 hours pi This agrees with the kinetics of RNA synthesis seen by Dennis and Brian in TGEV-infected cells and Mahy in MHV-infected cells but not with Brayton's results of two peaks of activity at 2 and 6 hours pi We were unable to determine the strandedness of the RNA products made in our in vitro system with the available probes. However, it is likely that the in vitro products of our extracts are primarily positive stranded, since other studies have shown that at 6 hours pi, the ratio of positive strand to negative strand RNA synthesis is large, both in vitro and in vivo (Lai et al., 1982; Brayton et al., 1984; Sawicki and Sawicki, 1986). Whether any of the RNA products of this in vitro system represent newly initiated products is not known. Most of the [^{32}P] UTP incorporation in this system is probably due to elongation of previously initiated products, since the addition of exogenous MHV genomic

or leader RNA to the system did not stimulate in vitro RNA synthesis to any greater extent than the addition of equivalent amounts of tRNA (data not shown). In general, the salt and pH requirements of the RNA polymerase activity described here were similar to those determined for the TGEV RNA polymerase and the late RNA polymerase of MHV, except that potassium and ammonium ions were used in this in vitro assay instead of sodium (Dennis and Brian, 1981; Brayton et al., 1982; Mahy et al., 1983). This in vitro system allows for efficient replication but not efficient transcription of MHV RNA. The relative levels of replicase versus transcriptase activities exhibited by the MHV RNA polymerase may be affected by salt or pH conditions, or by the availability or ratio of viral proteins required for each activity.

Six complementation groups with RNA negative phenotypes have been identified, using temperature sensitive mutants of MHV-JHM (Leibowitz et al., 1982). Therefore, the MHV transcription process probably involves many viral proteins. Given the large number of RNA negative phenotypes, structural as well as nonstructural proteins may be involved in MHV RNA synthesis, probably in a regulatory role, and the proteins involved in transcription may not all be the same as those involved in replication. The sensitivity of the MHV RNA polymerase in vivo and in vitro to protein synthesis inhibitors suggests that a newly made or labile protein may be needed for MHV specific RNA synthesis. Synthesis of negative strand MHV RNA has been reported to be 3 to 4 fold more sensitive to cycloheximide than positive strand RNA synthesis, and the switch from early to late RNA polymerase activity seen by Brayton is inhibited by cycloheximide (Brayton et al., 1982; Sawicki and Sawicki, 1986). In some other virus groups, cycloheximide inhibits replication preferentially. Sendai virus does not require ongoing protein synthesis for viral RNA synthesis in vivo

or in vitro, but pre-treatment with cycloheximide 1 to 3 hours prior to preparation of lysolecithin extracts of Sendai-infected cells inhibits Sendai virus replication and encapsidation, but not transcription. This is believed to be due to a depletion of the preexisting pool of viral proteins found in the infected cell necessary for replication but not transcription (Robinson, 1971; Carlsen et al., 1985). VSV replication also can be supported in vitro by the pool of viral proteins present at the time of extract preparation, but VSV replication is more efficient if an in vitro translation system is coupled to the in vitro replication system. Cycloheximide treatment of in vitro VSV replication systems inhibits VSV replication and encapsidation, while slightly stimulating VSV transcription (Davis and Wertz, 1982; Peluso and Moyer, 1983). In our system, pre-treatment of extracts with puromycin or cycloheximide (data not shown) inhibited in vitro synthesis of genome sized RNA, probably by depletion of the pool of proteins necessary for replication.

Inhibition of MHV replication in vitro by antiserum to the N protein suggests an important role for the N protein in MHV replication. The N protein may play a role in MHV RNA synthesis in a manner similar to that of the VSV N protein in VSV replication, where genomic RNA synthesis is regulated by the amount of available N protein (Patton et al., 1984). Alternatively, if MHV replication occurs, as postulated, on templates coated with N protein as with VSV, binding of anti-N sera to the N protein could inhibit the interaction of the MHV RNA polymerase with the template (Emerson and Wagner, 1972). The observation that antibody to the E1 protein did not inhibit MHV RNA replication in vitro, and therefore does not play an important role in replication, is interesting given that the matrix (M) protein of VSV which has a function homologous to E1 down regulates VSV

transcription both in vivo and in vitro (Clinton et al., 1978; Carroll and Wagner, 1979; Holmes et al., 1981b).

In extracts made from MHV-infected cells, newly made genome size RNA is rapidly incorporated into RNase resistant ribonucleoprotein structures. These ribonucleoprotein structures could be either encapsidated genome or genome present in replication complexes. It is more likely that these ribonucleoprotein complexes are encapsidated genome, since they possess the same buoyant density as nucleocapsids. In this in vitro assay, the high proportion of genomic RNA seen in comparison to subgenomic mRNAs may be due to selective protection from RNase degradation of encapsidated genomic RNA but not of mRNAs. Thus, when anti-N sera is added to in vitro MHV replication extracts, the decrease in the pool of N protein available to encapsidate the genomic RNA may result in a decrease in genomic RNA seen due to degradation of newly made genomic RNA by endogenous RNases.

This lysolecithin in vitro MHV replication system should also be useful for identifying the other viral proteins involved in MHV replication. Using monoclonal antibodies to the N protein, additional insights into the role of the N protein in replication may be elucidated. As antibodies to the nonstructural proteins become available, the role(s) of these proteins in MHV replication can be determined also.

CORONAVIRUS RECEPTORS

Introduction

Virus receptors and their role as determinants of species specificity have been studied since the late 1950's (Holland, 1961). Early receptor research focused on the species of animals and cell types which could be infected by different viruses, the chemical nature of viral receptors, and whether viruses which bound to the same cells bound to the same receptor. More recently, with the identification of cell surface markers, the development of monoclonal antibodies, anti-idiotype antibodies, and x-ray crystallography a new interest in viral receptors has emerged. Since the next section of my dissertation focuses on coronavirus receptors and their role in species specificity of coronaviral infection, I will briefly summarize what is known about other viral receptors and their role in species specificity (Table 4).

Viruses vary widely in their species specificity: e.g. polio and coronaviruses exhibit narrow host ranges and rabies virus exhibits a wide host range. These species specificities are often reflected in binding to tissue culture cells. In vivo, poliovirus infects only primates (Bodian, 1959). For the development of poliovirus vaccines, attempts were made to find a non-primate cell line in which to grow poliovirus. Cells from many primate and non-primate origins were tested for their ability to bind poliovirus, but only cells of primate origin and one rabbit cell line bound poliovirus (McLaren et al., 1959). Coronaviruses normally infect only one species. Although experimental inoculation of a few coronaviruses by an artificial route (intracerebrally), or at a highly susceptible age (neonates), can result in infection, the infection is often asymptomatic (Table 5). Coronaviruses also

Table 4

Cellular Molecules Proposed as Viral Receptors

Enveloped viruses	
Human immunodeficiency	CD4 (T4) antigen
Epstein Barr	Complement receptor C3d
Rabies	Acetylcholine receptor
Semliki Forest	H-2K and H-2D antigens
Lactate dehydrogenase	Ia antigen
Vaccinia	Epidermal growth factor
Influenza A	Glycophorin A
Sendai	Gangliosides
Mouse hepatitis	110 K glycoprotein
Moloney murine leukemia	110 K glycoprotein
Vesicular stomatitis	Phospho- or glycolipid
Nonenveloped viruses	
Reovirus type 3	Beta-adrenergic hormone receptor
Encephalomyelitis	Glycophorin A
Coxsackie B	49.5 K glycoprotein
Rhinovirus	90 K glycoprotein
Adenovirus	42 K glycoprotein
Polyomavirus	25, 50, 95 K glycoproteins

adapted from Crowell, 1987

Table 5. Infection of animals with coronaviruses. Bold + indicates coronavirus infection in the normal host, + indicates infection of an abnormal host, and +/- indicates a very mild infection in an abnormal host. Methods of determining productive infection are as follows: A = infected animals produced antiviral antibody, D = disease developed, E = virus was visualized in tissues by EM, H = histological changes developed, I = virus was reisolated from inoculated animal, T = virus was transmitted from feces of inoculated animal to normal host. s = infection developed in suckling animals only and * = one case of accidental infection of a laboratory worker. References: a, Cheever et al., 1949; b, Kaye et al., 1975; c, Storz and Rott et al., 1981; d, Akashi et al., 1981; e, McIntosh et al., 1967; f ,Barlough et al., 1985; g, Barlough et al., 1984; h, Woods et al., 1981; i, McClurkin et al., 1970; j, Larson et al., 1979; k, Reynolds et al., 1979

Table 5

Infection of Animals with Coronaviruses

	Mouse	Cow	Human	Dog	Cat	Pig	Rat
MHV	+						+[a,s] D
BCV	+[b,s] D	+	+[c,*] D				+[d,s] D
OC43	+[e,s] D		+				
229E			+		+[f] A		
CCV				+	+[g] A	+/-[h,s] A,H	
FIPV					+	+[h,s] A,D,H	
TGEV				+[i,j] A,E,T	+[k] A,I	+	

exhibit a high level of species specificity in cell culture, infecting only cells derived from their normal host and occasionally from species that are susceptible to antigenically related coronaviruses (Table 6). Rabies in contrast, infects all mammals and cells in culture derived from many species (Tierkell, 1959; Murphy, 1977).

Virologists have been interested in determining the chemical nature of viral receptors. Enzymatic treatments of host cells or red blood cells (RBCs) which inhibited virus binding have been identified for several viruses. Many viruses, such as influenza, Sendai, and encephalomyocarditis (EMC) viruses hemagglutinate RBCs (Hirst, 1941). Incubation of these viruses with RBC membranes or cell debris from susceptible cells inhibits hemagglutination. Neuraminadase or periodate but not protease treatment of RBC membranes or cell debris from susceptible cells prior to incubation with these viruses abolishes this inhibition of hemagglutination (Springer and Ansell, 1958; Mori et al., 1962). Sendai, EMC, and influenza viruses were postulated to bind to sialic acid containing receptors. In contrast, enterovirus receptors were postulated to be protein molecules because protease but not neuraminadase or lipase treatment of host cells inhibited poliovirus and other enterovirus binding (Holland and McLaren, 1959). Coronavirus binding to L cells is also inhibited by protease treatment (Richter, 1976). Rhabdovirus binding to host cells was inhibited by phosholipase and neuraminadase, but not protease treatment of host cells. Therefore the rhabdovirus receptor was postulated to be a glyco- or phospholipid (Wunner et al., 1984).

More recently, virus binding studies have been performed on purified gangliosides or proteins. Glycophorin A, the main sialoglycoprotein of RBCs, has been identified as the influenza and EMC receptor on RBCs (Marchesi and

Table 6. Infection of cell lines with coronaviruses. + indicates productive infection of a cell line, * indicates animal source of the cell line. References: a, Manaker et al., 1961; b, Sturman and Takemoto, 1972; c, Hirano et al., 1974; d, Wege et al., 1978; e, McIntosh et al., 1969; f, Dea et al., 1980; g, Inaba et al., 1976; h, King and Brian, 1982; i, Gerna et al., 1981; j, Kapikian et al., 1972; k, Schmidt et al., 1979; l, Hogue et al., 1984; m, Hamre and Procknow, 1969; n, Bradburne, 1969; o, Laporte et al., 1980; p, Binn et al., 1980; q, Woods, 1982; r, Evermann et al., 1981; s, O'Reilly et al., 1979; t, Black, 1980; u, Pederson et al., 1981; v, Barlough et al., 1983; w, Welter, 1965; x, McClurkin and Norman, 1966; y, Horzinek et al., 1982; z, Lee, 1956.

Table 6

Infection of Cell Lines with Coronaviruses

	Mouse *	Cow	Human	Monkey	Dog	Cat	Pig
MHV	+ a,b,c,d		+ e				
BCV		+ f,g	+ h,i	+ f			+ f
OC43			+ j,k,l	+ j			
229E			+ k,m,n				
CCV			+ o		+ p	+ q,r	
FIPV						+ q,s,t,u	
TGEV					+ v,w	+ q,y	+ x,z

Andrews, 1971; Enegren and Burness, 1977). In chymotrypsin digests of glycophorin A, only one peptide inhibited EMC and influenza virus hemagglutination, and it had no unique pattern of glycosylation, indicating that the specific binding domain for both EMC and influenza contained both protein and sialic acid residues (Burness and Pardoe, 1983). Sendai virus was shown to bind preferentially to the ganglioside GD1a and its higher homologs GT1b and GQ1b when they are bound to plastic or inserted in liposomes (Holmgren et al., 1980, Markwell and Paulson, 1980; Markwell et al., 1981). Liposomes containing the HLA-A and HLA-B antigens bind Semliki Forest virus (SFV) and compete with cells for SFV binding. Purified SFV coat proteins were isolated as a complex with HLA or H2 antigens from host cells by immunoprecipitation or affinity chromatography (Helenius et al., 1978).

Many viruses replicate in the same cell lines, and the question was asked whether these viruses bound to the same or different viral receptors on these cells. Competition studies in which an unlabeled virus is allowed to bind to a cell and then binding of a second radiolabeled virus is measured, were useful in determining if two viruses which bound to the same cell types bound to the same receptor. In competition studies, the three types of polioviruses bound to the same receptor on HeLa cells, but Coxsackie B virus, the echoviruses and rhinoviruses bound to different receptors (Crowell and Siak, 1978). Herpes simplex viruses 1 and 2 did not compete for receptor sites on several cell lines suggesting that they expressed strain-specific receptors (Vahlne et al., 1979). Rabies virus and VSV competed for binding on cultured neural and non-neural cells, suggesting a common receptor for rhabdoviruses (Wunner et al., 1984). Monoclonal antibodies confirmed the presence of different receptors for Coxsackie B virus, the polioviruses and

rhinoviruses (Campbell and Cords, 1983; Minor et al., 1984; Norbis, et al., 1985; Crowell et al., 1986).

As viral receptors are not present on cells for the sole purpose of allowing virus infection, identification of the normal function of these receptor molecules was of great interest. With the identification of many cell surface markers, studies were undertaken to determine if viruses bound to any of these known markers. EBV receptors on B lymphocytes, were shown to be closely associated with the complement components C3b and C3d by co-capping and immunofluorescence studies (Yefenof et al., 1976). Purified C3d receptor (CR2) binds both C3d and EBV. Many, but not all, anti-CR2 monoclonal antibodies block EBV binding, indicating that while both EBV and C3d bind to CR2, they bind to different epitopes (Fingeroth et al., 1984; Nemerow et al., 1985 and 1986). Rabies virus is believed to use acetylcholine receptors to bind to mouse and chicken muscle cells because rabies virus and acetylcholine receptors are co-localized by immunofluorescence, and treatment of cells with acetylcholine receptor blockers decreased rabies viral replication in susceptible cells (Lentz et al., 1982). Treatment of cells with monoclonal antibody to the alpha subunit of the acetylcholine receptor blocked attachment of radiolabeled rabies virus to cells (Burrage et al., 1985). The receptor for HIV has been identified as the T4 antigen CD4 (Dalgleish et al., 1984; and Klatzmann et al., 1984). Receptors for HIV were seen only on cells which expressed CD4 and anti-CD4 antibodies blocked binding to and infection of cells by HIV (Dalgleish et al., 1984; and Klatzmann et al., 1984).

With the identification of viral receptors for EBV, SFV, HIV and rabies, it was possible to determine the distribution of these molecules and whether their distribution correlated with susceptibility to infection. For EBV and HIV, the limited distribution of the C3d receptor on B lymphocytes only, and

of CD4 on T4 lymphocytes and a small proportion of macrophages, correlates with susceptibility of these cells only to infection (Eden et al., 1973; Reinherz et al., 1979). For rabies, which is neurotropic, the presence of acetylcholine receptors at neuromuscular junctions correlates with the known pathology of rabies (Anderson and Cohen, 1974; Murphy, 1977). A 100-120 kilodalton receptor for MHV has been identified in Dr. K. Holmes' laboratory using new receptor assays (Boyle et al., 1987). Its presence or absence in mice correlated with susceptibility or resistance to MHV infection. I used these new assays to investigate the role of MHV receptors in mouse and virus strain specificity of MHV infections. I have also modified these assays for other coronaviruses so that the role of other coronavirus receptors in species specificity could be investigated.

Materials and Methods

Cell culture and virus propagation: Human rectal tumor (HRT18) cells were obtained from Dr. D. Brian, University of Tennessee, Knoxville TN and were propagated in D-MEM with 8% FBS. Felis catus whole fetus (fcwf-4) cells were obtained from Dr. N. Pedersen, University of California, Davis CA and were propagated in D-MEM with 10% FBS. Canine tumor (A72) cells were obtained from Dr. L. Binn, Walter Reed Army Institute of Research, Washington, D.C. and were propagated in D-MEM with 20% FBS. Human lung fibroblast (IMR-90) cells were obtained from American Type Culture Collection, Rockville, MD and propagated in DMEM with 10% FBS. Rhabdomyosarcoma (RD) cells were obtained from Dr. A. Collins, State University of New York, Buffalo and were propagated in DMEM with 10% FBS. Bovine coronavirus was obtained from Dr. D. Brian and grown in HRT18 cells (King and Brian, 1982). Canine coronavirus was obtained from

Dr. L. Binn and grown in A72 cells (Binn et al.,1980). Feline infectious peritonitis virus and feline enteric coronavirus were obtained from Dr. N. Pedersen and were grown in fcwf-4 cells (Pedersen et al., 1981a). Human coronavirus OC43 was obtained from Dr. A. Collins and was grown in RD cells (Schmidt et al., 1979). Human coronavirus 229E was obtained from Dr. M. Johnson-Lussenburg, University of Ottawa, Canada and grown on IMR-90 cells. MHV-A59 was obtained from Dr. L. Sturman, State Department of Health, Albany, NY and MHV-3 was obtained from Dr. A. Smith, Yale University, New Haven, CT and were grown in 17-Cl-1 cells (Sturman and Takemoto, 1972). Transmissible gastroenteritis virus stocks were obtained from Dr. D. Brian.

Animal sources: BALB/c , SJL/J, A/J, C57BL/6 and (C57BL/6 x BALB/c) F1 mice were obtained from the National Cancer Institute or Jackson Laboratories (Bar Harbor, ME). C3H mice were obtained from Dr. F. Bang, Johns Hopkins University, Baltimore MD, and maintained by brother-sister matings in filter-topped cages in laminar flow incubators at USUHS. Wistar Furth rats were obtained from Charles River breeding laboratories (Wilmington, MA). Tissues from the following species were obtained from investigators sacrificing animals after performing surgery for other purposes. Sprague-Dawley rats were obtained courtesy of Dr. J. Anders, Department of Anatomy, USUHS from Charles River Breeding Laboratories. Rabbit intestine was obtained from Laboratory Animal Medicine, USUHS. Cotton rat intestine was obtained from Dr. G. Prince at NIH. Chicken intestine and embryos were obtained from Dr. D. Snyder, University of Maryland. Cat intestine was obtained from Dr. C. Bahn , Department of Surgery, USUHS. Dog intestine was obtained from Dr. F. Haddy, Department of Physiology, USUHS. Pig intestine was obtained from Dr. J. Kishel, Department of

Pathology, USUHS. Cow intestine was obtained from Trueth Meats, Catonsville MD and Dr. M. Solomon, USDA, Beltsville, MD. Small samples of normal human small intestine were obtained from surgery patients at Malcolm Grow Air Force Hospital via Dr. T. Scott, Department of Surgery, USUHS.

Antisera: Goat anti-MHV E2 glycoprotein was raised by immunization of a goat with MHV E2 isolated from detergent disrupted virions by sucrose density gradient ultracentrifugation (Sturman et al., 1980). Rabbit anti-CCV and rabbit anti-HCV-229E were raised by immunization of a rabbit with detergent disrupted virions purified by sucrose density gradient ultracentrifugation using the procedure of Sturman et al. (1980). Anti-FIPV immune ascites was obtained courtesy of Dr. N. Pedersen. Rabbit anti-TGEV, rabbit anti-BCV, and rabbit antibody directed against the BCV hemagglutinin (anti-BCV HA) were obtained courtesy of Dr. D. Brian. Polyclonal anti-MHV receptor antibody was obtained by immunization of SJL/J mice with BALB/c brush border membranes (Holmes et al., in preparation). Those mice which produced anti-receptor antibodies received intraperitoneal inoculations with Sarcoma 180 cells, obtained from Dr. W. Brandt, Walter Reed Army Institute of Research, Washington, DC, to induce ascites fluid. Monoclonal anti-MHV receptor antibodies were generated by immunization of SJL/J mice with deoxycholate extracted BALB/c brush border membranes. Spleens from mice producing anti-receptor antibodies were fused with Sp2/0 cells to produce anti-receptor hybridomas (Shulman et al., 1978). When anti-receptor monoclonal antibody CC1 was used in Western blots, rabbit anti-mouse IgG was used to amplify the CC1 signal because CC1, being of the immunoglobulin subclass IgG1, did not bind staphylococcal protein A well (Holmes et al., in preparation).

Brush border membrane preparation: Intestinal brush border membranes (BBMs) were prepared from frozen intestine or intestinal mucosa by the method of Kessler et al. (1978). Briefly, mouse, rat, cotton rat and rabbit intestines were flushed with cold PBS, held on ice, snap frozen in liquid nitrogen, and then stored at - 70°C. Cat, dog, pig, cow, chicken and human intestinal mucosa were scraped from the intestine prior to being snap frozen in liquid nitrogen. Intestinal tissue was homogenized in 15 volumes/gram whole intestine or 30 volumes/gram intestinal mucosa of homogenization buffer (300 mM mannitol, 2 mM Tris hydrochloride, pH 7.2). Extraneous material was removed by centrifugation at 3000 g following addition of calcium chloride to a final concentration of 10 mM. BBMs were pelleted by centrifugation at 15,000 rpm in the Beckman SW28 rotor for 45 minutes and were resuspended in TE and stored frozen at - 70°C.

Hepatocyte plasma membrane preparation: Hepatocyte plasma membranes (HMs) were prepared by the established procedure of Neville (1976). Livers were harvested, snap frozen in liquid nitrogen and stored at -70°C. Livers were homogenized in 1 mM sodium carbonate, pH 7.5 and filtered through cheese cloth to remove remaining large debris. The homogenate was centrifuged 15 minutes at 1500 g. Pellets were resuspended in 44% sucrose, overlaid with 42.3% sucrose and centrifuged at 22340 rpm in the Beckman SW28 rotor for 2 hours. The material floating on top of the gradient was harvested, resuspended in 1 mM sodium carbonate, loaded onto a step gradient of 3, 27, and 50% sucrose and centrifuged 1 hour at 1746 rpm in the Beckman SW28. Material at the 27-50% interface was harvested, centrifuged 4 minutes at 12,000 rpm in the Beckman microfuge, and the packed pellets were stored at -70°C.

Chorioallantoic membrane preparation: Chorioallantoic membranes were removed from 14 day old chicken embryos and washed 3 times in cold phosphate buffered saline (PBS). Membranes were swollen in reticulocyte standard buffer (RSB: 1.5 mM magnesium chloride, 10 mM potassium chloride, 10 mM Tris hydrochloride pH 8.0) with 1µg/ml PMSF (phenylmethylsulfonylfluoride) for 15 minutes and then homogenized. The homogenate was centrifuged 5 minutes at 1000 g to remove large debris and the resulting supernatant was centrifuged 2 hours at 24,000 rpm in the Beckman SW28 rotor. Pellets were resuspended in PBS and stored at -70°C.

Solid-phase virus-binding assay: 5-25 µg of intestinal BBMs or HMs in TE buffer were bound to nitrocellulose sheets in a 96-well minifold apparatus (Schleicher and Schuell). For MHV, the sheets were blocked with 2% bovine serum albumin (BSA) in dilution buffer (50 mM Tris hydrochloride, pH 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.05% Tween 20, and 0.1% BSA) and then incubated for 1 hour with virus in MEM with 10% FBS and 10 mM HEPES . Nitrocellulose sheets were washed 4 times for 5 minutes each with dilution buffer and incubated for 1 hour with appropriate dilutions of preimmune or immune sera in dilution buffer. Sheets were washed 4 times with dilution buffer again and bound antibody was detected by incubation for 1 hour with radioiodinated staphylococcal protein A (^{125}I-SPA) in dilution buffer. Sheets were washed 4 times with dilution buffer, air dried and autoradiographed (Boyle et al., 1987). For solid phase virus binding assays with BCV, CCV, FIPV, TGEV or 229E, the same procedure was followed except nitrocellulose sheets were blocked with 10% non-fat dry milk in dilution buffer, no HEPES was added to the virus and washes were conducted with 1% non-fat dry milk in dilution buffer. In some experiments BBMs were boiled in 5% B-mercaptoethanol prior to being applied to the

nitrocellulose sheets to denature endogenous antibodies present on the brush border membranes. In other experiments BBMs were treated with detergents prior to being applied to the nitrocellulose sheets; samples were diluted such that detergent concentrations were below the levels which interfered with binding of the BBMs to the nitrocellulose.

Virus overlay protein blot assay: Proteins from BBMs or HMs were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Gels were electroblotted onto nitrocellulose sheets with a Trans-blot cell using transfer buffer containing 25 mM Tris, 0.192 M glycine, pH 8.6, with 20% methanol (Towbin et al., 1979). Subsequent steps of the virus overlay protein blot assay (VOPBA) were performed as described for the solid-phase virus-binding assay above, starting with the step of blocking the nitrocellulose with 2% BSA in dilution buffer (Boyle et al., 1987).

Endo F digestion of gel-eluted MHV receptor: 6 mg of BALB/c or C57BL/6 BBMs were each run on a preparative 8% SDS-PAGE gel. Each gel was incubated for 20 minutes in 500 ml of TE pH 7.4 with 0.05% lubrol and 0.05% Tween 20, 20 minutes in TE pH 7.4 with 0.05% lubrol and 20 minutes in TE pH 7.4 alone. Each gel was cut into approximately 50 0.375 cm wide slices and each slice was placed in 2.5 ml TE pH 7.4. Slices were frozen at -70°C, then thawed in a 37°C water bath and homogenized 30 seconds at 70% capacity using a Techmar homogenizer. Acrylamide was removed from the fractions by centrifugation at 1500 rpm in a table top centrifuge, and MHV binding activity of each fraction was assayed by a solid phase receptor assay as described above. The 4 gel fractions with the highest MHV binding activities were pooled and stored at -70°C. Forty ul of gel-eluted receptor in 50 mM sodium acetate pH 8.0, 25 mM EDTA, 0.05 % NP40 was boiled for 5 minutes to denature the proteins and then was digested with 0 to 64 mU of

Endoglycosidase F (Boehringer Mannheim Biochemica) with 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF) for 18 hours. The reaction was stopped by the addition of 1/4 volume 4X sample treatment mix (STM: 62.5 mM Tris hydrochloride, pH 6.7, 10% glycerol, 2% SDS, 5% BME, 0.1% bromphenol blue) and freezing at -70°C. Samples were boiled 5 minutes and were analyzed on 8% SDS-PAGE gels. Gels were electroblotted onto nitrocellulose sheets with a Trans-blot cell using transfer buffer containing 25 mM Tris, 0.192 M glycine, pH 8.6, with 20% methanol (Towbin et al., 1979). Receptor activity was detected in a Western blot with monoclonal anti-MHV receptor antibody.

Chloroform-methanol extraction: 1.5 mg of BALB/c mouse or dog BBMs were resuspended in 2 ml of methanol and incubated 10 minutes at room temperature. 2 ml of chloroform were added and the BBMs were allow to incubate at room temperature for another 10 minutes, followed by the addition of 2 more ml of chloroform and another 5 minute incubation at room temperature. The mixture was centrifuged 5 minutes at 2000 K , and the pellet was resuspended in 3 ml of TE. The supernatant was brought up to 6 ml with 2:1 chloroform-methanol. 1.2 ml of 0.1M KCl was added to the mixture, the mixture was vortexed extensively, and allowed to incubate at room temperature for 15 minutes. The aqueous and chloroform phases were separated by centrifugation at 1000g for 5 minutes (Folch, et al., 1957).

Sucrase assay: Sucrase assays were performed on brush border, hepatocyte and chicken chorioallantoic membranes (Messer and Dahlvist, 1966). Two hundred µl PGO reagent (0.49M sodium phosphate buffer pH 6.0, 100µg/ml o-dianisidine, 10µg/ml horseradish peroxidase, 5U/ml glucose oxidase [Sigma, type V-S]) was mixed with 200 µl of 7.75 mM glucose-free sucrose and 20 µl of sample to be tested. Reaction mixtures were incubated at

37°C for 75 minutes. At 15 minute intervals, 75µl aliquots were added to 35 µl of 50% sulfuric acid in a microtiter plate to stop the reaction. Adsorbance was read at 540 nm. Protein concentrations were determined by the Bradford method (Bradford, 1976).

SDS-PAGE electrophoresis: Protein samples were prepared in STM and boiled 5 minutes. Samples were electrophoresed through 8-10% SDS-polyacrylamide gels with 5% stacking gels (separating gel: 375 mM Tris hydrochloride, pH 8.8, 10-12% glycerol, 8-10% acrylamide, 0.213-0.266% bis-acrylamide, 0.1% SDS, 0.05% ammonium persulfate, and 0.6% TEMED, stacking gel: 125 mM Tris hydrochloride,pH 6.8, 5% acrylamide, 0.133% bis-acrylamide, 0.1% SDS, 0.03% ammonium persulfate, and 0.1% TEMED) in PAGE buffer (25 mM Tris, 192 mM glycine, and 0.1% SDS) (Laemmli, 1970).

Hemadsorption and hemagglutination: Mouse red blood cells (RBCs) were harvested from BALB/c mice, washed in cold phosphate buffered saline (PBS) and stored in Alsever's solution (60 mM dextrose, 30 mM citric acid, 70 mM sodium chloride). Hemadsorption was performed by replacing media on tissue culture cells infected with HCV-OC43 or BCV with 0.2% v/v mouse RBCs in PBS. Cells were incubated 1 to 2 hours at 4°C. Monolayers were washed twice with cold PBS and hemadsorption was visually observed. Hemagglutination assays were performed by mixing 100µl of diluted virus supernatant with 100 µl 0.4% v/v mouse RBCs in microtiter plates. Plates were incubated for 2-4 hours at 4°C, and hemagglutination was quantitated visually (Hierholzer, et al., 1969).

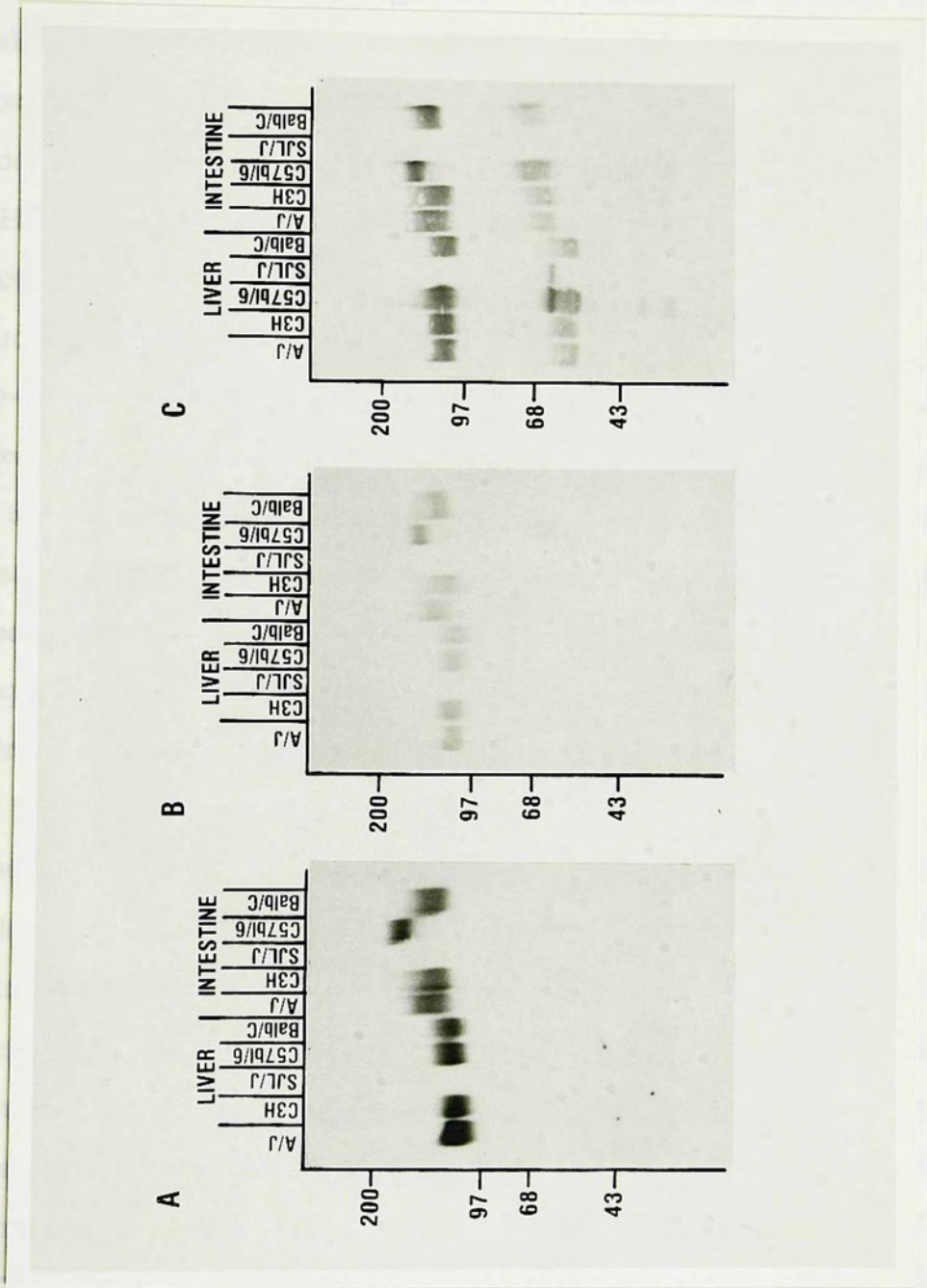
Results

Mouse strain specificity of the MHV receptor: The fully susceptible BALB/c mouse and the semi-resistant C3H mouse were shown to express

receptors for MHV-A59 on their intestinal BBMs which range in size from 100 to 120 kilodaltons (Boyle et al., 1987). A slightly smaller MHV receptor which ranges in size from 90 to 110 kilodaltons was present on their hepatocyte plasma membranes (HMs) (Boyle et al., 1987). The fully resistant SJL/J mouse lacked MHV-A59 receptors on these tissues. Therefore, the absence of a receptor for MHV on tissues from SJL/J mice could account for their complete resistance to MHV infection.

I wanted to determine if there were quantitative differences in the MHV receptor between the fully susceptible and semi-resistant strains of mice, and whether these differences could explain the different susceptibilities to MHV infection. VOPBAs were performed on BBMs and HMs from fully susceptible BALB/c and C57BL/6 , semi-resistant A/J and C3H and fully resistant SJL/J mice (Figure 11, Panel A). In liver plasma membranes, I confirmed the published results of Boyle et al. (1987) with BALB/c, C3H and SJL/J mice and showed that A/J and C57BL/6 mice also expressed MHV receptors of equal size and quantity to those seen on BALB/c liver plasma membranes. In intestinal BBMs, I again confirmed the published results of Boyle et al. (1987) with BALB/c, C3H and SJL/J mice and showed that A/J and C57BL/6 mice expressed MHV receptors on their BBMs in amounts equal to that on BALB/c BBMs. However, while the A/J mice expressed on their BBMs a MHV receptor the same size as on BALB/c BBMs, the C57BL/6 MHV receptor on BBMs was larger than that seen on BALB/c BBMs, ranging in size from 130 to 150 kilodaltons. Results similar to those seen with MHV were obtained in Western blots of BBMs and HMs from these five mouse strains with polyclonal anti-MHV-receptor antibody or monoclonal anti-MHV-receptor antibody CC1 (Figure 11, Panels B and C). These antibodies detected MHV receptors on BBMs and HMs from BALB/c,

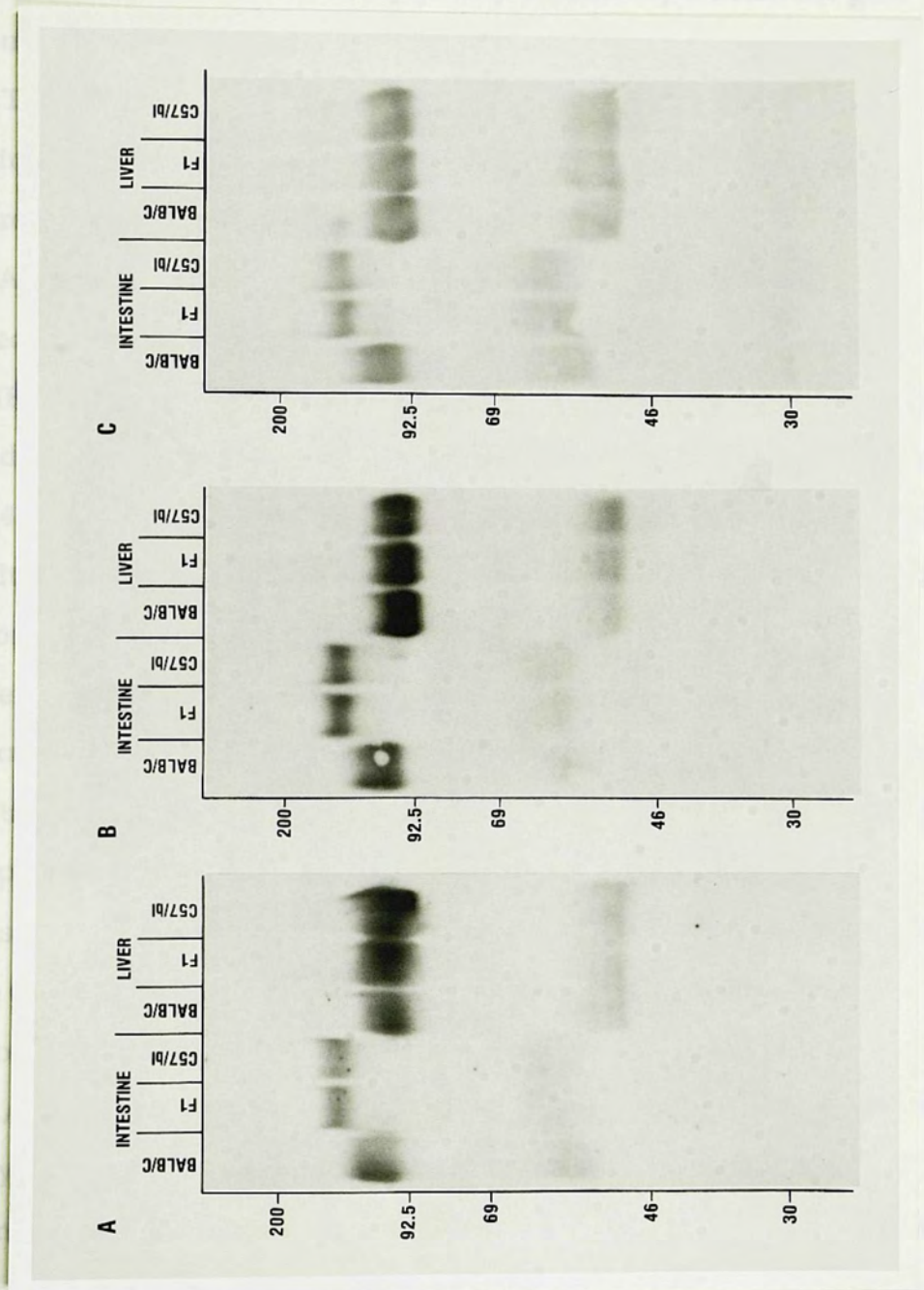
Figure 11. VOPBA of MHV-A59 binding to intestinal brush border and hepatocyte membranes from different strains of mice. 100 μg of HMs or BBMs from A/J, C3H, SJL/J, C57BL/6 and BALB/c mice were analysed on SDS-PAGE gels, transferred to nitrocellulose and probed with: Panel A, MHV-A59 and anti-MHV E2 antibody; Panel B, polyclonal anti-MHV receptor antibody; Panel C, monoclonal anti-MHV receptor antibody CC1 and rabbit anti-mouse immunoglobulin. ^{125}I-SPA was used to detect bound antibody. Molecular weights in kilodaltons are shown by the numbers on the left of the panels.


A
B
C
LIVER
INTESTINE
A/J
C3H
SJL/J
C57bl/6
Balb/C
200
97
68
43

A/J, C3H and C57BL/6 mice but not from SJL/J mice. Again, the MHV receptor on the C57BL/6 intestine was larger than that of other mouse strains. Controls with rabbit anti-mouse IgG and ^{125}I-SPA, CC1 and ^{125}I-SPA or ^{125}I-SPA alone did not reveal any bands (data not shown). A second band at approximately 58 kilodaltons is seen by the monoclonal anti-MHV-receptor antibody and occasionally by the polyclonal anti-MHV-receptor antibody and by MHV. The second band from C57BL/6 BBMs was larger than that seen with other mouse strains. This extra band probably represents a cleavage product of the MHV receptor, as it becomes more prevalent after multiple freeze-thaws of the membrane preparations (data not shown). A light band at approximately 58 kilodaltons was seen in SJL HMs in this experiment, but in more than 20 other experiments this band was not observed. The level of expression of MHV receptor does not explain the difference in susceptibility between susceptible and semi-resistant strains. Instead, a block in a subsequent step in viral replication or a difference in the host's response to the virus must be responsible for this difference in susceptibility.

To determine whether the strain-specific tissue-dependent size difference in the MHV receptor on C57BL/6 intestine was manifested at the gene level or at the level of post-translational modification, BBM and HM proteins from (C57BL/6 x BALB/c) F1 mice were analyzed by VOPBA and by Western blots with polyclonal and monoclonal anti-MHV-receptor serum (Figure 12). As expected, MHV receptors for F1 mice on HMs were the same size as the liver MHV receptors from both of their parents. However, MHV receptors on intestinal BBMs from F1 mice were identical in size to those of the C57BL/6 parent, being markedly larger than the BALB/c intestinal MHV receptor. Since the larger intestinal MHV receptor phenotype was expressed by 100% (6/6) of the F1 offspring, it is likely that the larger receptor size seen

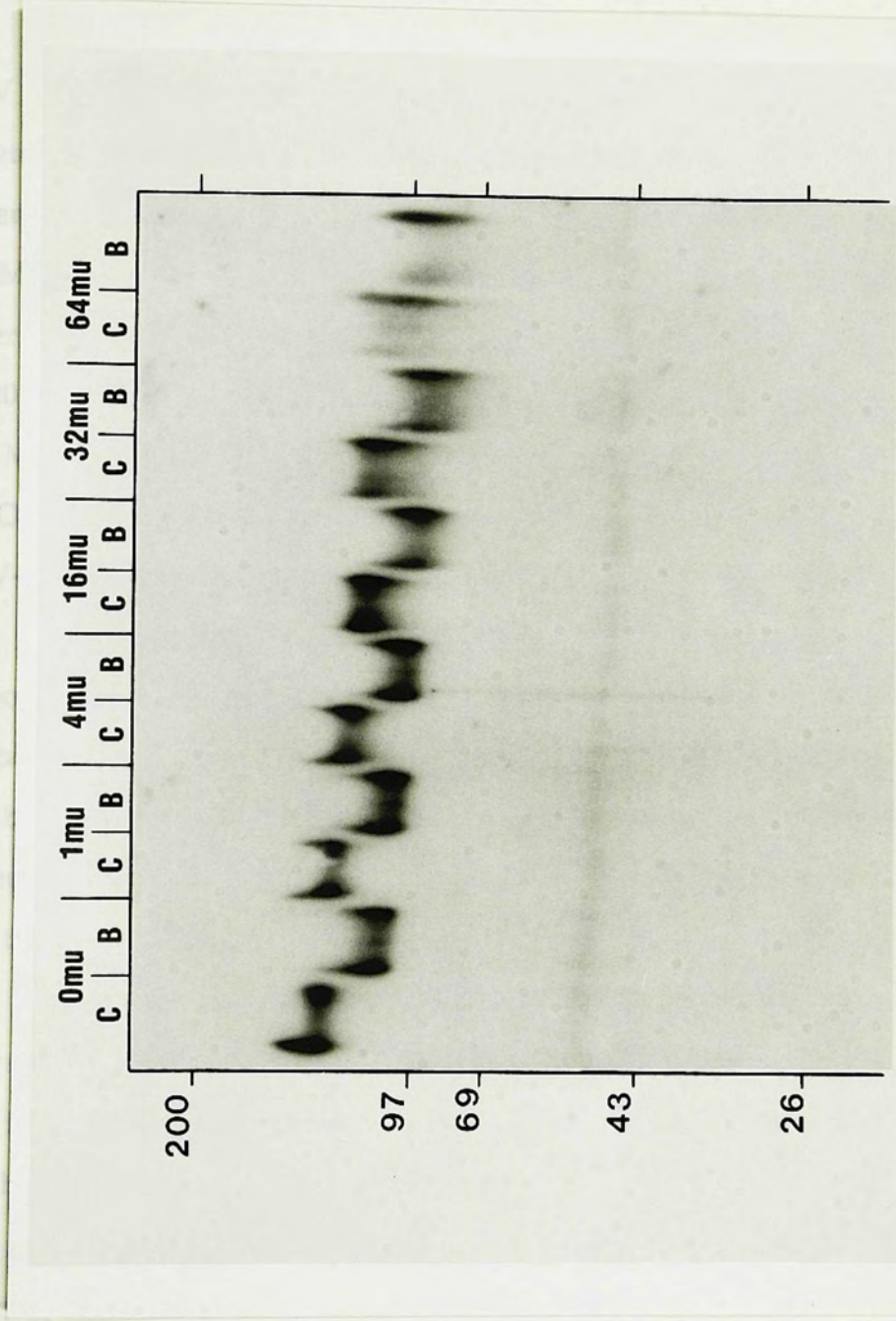
Figure 12. Assays of MHV-A59 binding to intestinal brush border and hepatocyte membranes from BALB/c, C57BL/6 and (BALB/c x C57BL/6) F1 mice. 100 µg of BBMs or HMs prepared from six 4 to 6 week old BALB/c, C57BL/6 or (BALB/c x C57BL/6) F1 mice were run on SDS-PAGE gels, transferred to nitrocellulose and probed with: Panel A, MHV-A59 and anti-MHV antibody; Panel B, polyclonal anti-MHV receptor antibody; Panel C, monoclonal anti-MHV receptor antibody CC1 and rabbit anti-mouse immunoglobulin. ^{125}I-SPA was used to detect bound antibody. Molecular weights in kilodaltons are shown by the numbers on the left side of the panels.


A
B
C
INTESTINE
LIVER
BALB/C
F1
C57/bl
200
92.5
69
46
30

in the C57BL/6 intestine is due to a tissue-specific post-translational modification of the receptor, such as enhanced glycosylation. The enzyme responsible for the modification is probably encoded by a dominant gene active only in the intestine of C57BL/6 mice.

To determine if the tissue-dependent differential modification occurring in C57BL/6 mice intestines was due to enhanced glycosylation, MHV receptors from BALB/c and C57BL/6 intestinal BBMs were eluted from SDS-PAGE gel bands and digested with Endoglycosidase-F (Endo-F). The digested proteins were analyzed on SDS-PAGE gels, blotted to nitrocellulose, and MHV receptor activity was detected with monoclonal anti-MHV receptor antibody CC1. A representative blot is shown in Figure 13. After treatment with 64 mU of Endo-F, a decrease in receptor size of approximately 20 to 25 kilodaltons was seen for the C57BL/6 receptor and of 10 to 15 kilodaltons was seen for the BALB/c receptor. Use of higher levels of Endo-F, to ensure complete deglycosylation, resulted in greater smearing and a loss of MHV receptor activity. This decrease in MHV receptor activity could possibly be due to contaminating proteases in the enzyme mixture, even though PMSF and ortho-phenanthroline (ONP) were included in the reaction to inhibit proteases. The decrease in receptor size seen with Endo-F was slightly greater for the C57BL/6 receptor than for the BALB/c receptor but the C57BL/6 receptor was still larger than the BALB/c receptor, suggesting that the C57BL/6 intestinal receptor's larger size may not be due solely to increased glycosylation. The explanation for the larger size of the MHV receptor in intestines of C57BL/6 mice may become evident after cloning of the MHV receptor gene and the analysis of the mRNAs for the MHV receptor from C57BL/6 and and BALB/c liver and intestines. Comparison of the C57BL/6 and BALB/c intestinal MHV receptors on two dimensional gels could also be

Figure 13. Endoglycosidase F digestion of MHV intestinal receptor. 40 ul of MHV receptor eluted from SDS-PAGE gels of BALB/c (B) or C57BL/6 (C) intestinal BBMs was digested with 0, 1, 4, 16, 32, or 64 mU of Endoglycosidase F for 18 hours at 37°C. Sample treatment mix was added to each digest and proteins were electrophoresed on a 8% SDS-PAGE gel, and blotted to nitrocellulose. MHV receptor activity was detected with monoclonal anti-receptor antibody followed by rabbit anti-mouse immunoglobulin and ^{125}I-SPA. Molecular weights in kilodaltons are shown on the left and right side of the figure.


0mu
C
B
1mu
C
B
4mu
C
B
16mu
C
B
32mu
C
B
64mu
C
B
200
97
69
43
26

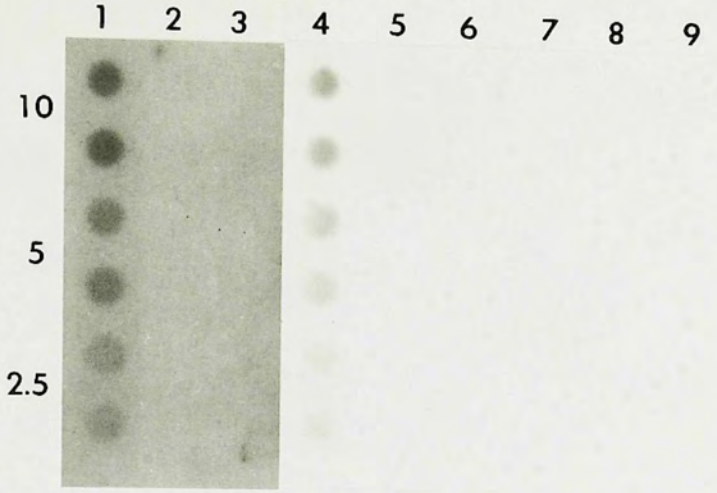
useful in determining the reason for the larger size of the C57BL/6 receptor, as modifications which include charged groups such as phosphate, sulfate or sialic acid residues would greatly alter the pI of the receptor.

Virus strain specificity of the MHV receptor: Since different strains of MHV exhibit marked differences in tissue tropisms in vivo, I was interested in determining whether differences in tissue tropisms were due to the presence of different receptors for the different MHV strains. Figure 14 shows that MHV3 binds to BBMs from BALB/c mice by solid phase assay (Figure 14, upper panel) and that the MHV3 receptor on BALB/c intestinal BBMs is a 100 to 120,000 dalton protein, like the MHV-A59 receptor (Figure 14, lower panel). Like MHV-A59, MHV3 did not bind to SJL/J BBMs in the solid phase assay or VOPBA (data not shown). Therefore, these data suggest that MHV3 and MHV-A59 bind to the same receptor on BALB/c intestinal BBMs.

Experiments in the laboratory of Dr. Abigail Smith at Yale University support this conclusion. She has shown that our polyclonal and CC1 monoclonal anti-receptor antibodies completely blocked infection of 17-Cl-1 cells by five strains of MHV, though the dilution of antibody needed to protect cells varied with virus strain used. Thus, it appears that on 17-Cl-1 cells there exists only one receptor for all 5 strains of MHV tested.

Since the immunoglobulin class of CC1 is IgG1, and therefore does not bind staphylococcal protein A efficiently, this monoclonal antibody could be used in a blocking assay to determine whether CC1 could prevent infection of cells by blocking the binding of MHV to the MHV receptor. BALB/c BBMs were applied to nitrocellulose, blocked with BSA, then incubated with differing dilutions of CC1, washed and incubated with MHV-A59 or MHV3 and the remainder of the solid phase assay steps were performed. Figure 15, columns 1 and 2 show that at a dilution of 1:50 or 1:100, CC1 completely

Figure 14. MHV3 binding to intestinal brush border membranes from BALB/c mice. Upper panel: 10, 5 or 2.5 μg of BALB/c BBMs were bound to nitrocellulose. Nitrocellulose sheets were incubated with MHV-A59 (columns 1-3), MHV3 (4-6), or with DMEM (7-9). Virus binding was detected with goat anti-MHV E2 (1,4,7). Controls included samples incubated with normal goat serum (2,5,8), or with dilution buffer (3,6,9) instead of goat anti-MHV E2. Bound antibody was detected with ^{125}I-SPA. Lower panel: 100 μg of BALB/c BBMs were run on a SDS-PAGE gel, transferred to nitrocellulose, probed with MHV3, goat anti-MHV E2 serum and ^{125}I-SPA. Controls included samples incubated with MHV3 and normal goat serum, MHV3 and no serum, DMEM and goat anti-MHV E2 serum, DMEM and normal goat serum, and DMEM and no serum. Molecular weights in kilodaltons are shown by the numbers on the left side of the panel.


1
2
3
4
5
6
7
8
9
10
5
2.5

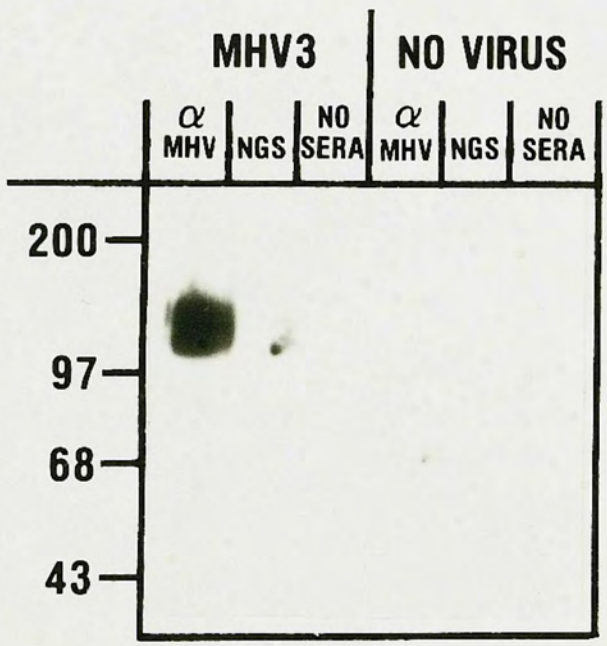
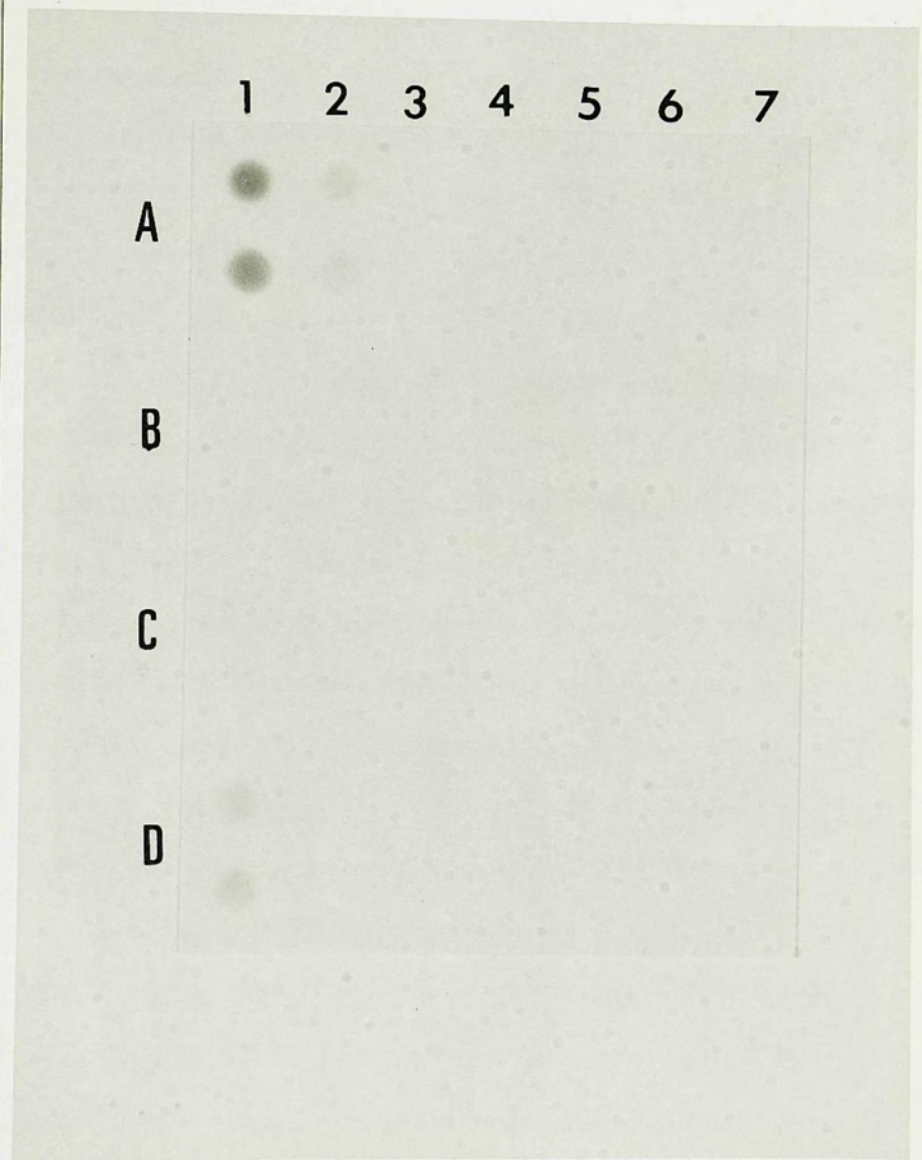

MHV3
NO VIRUS
α MHV
NGS
NO SERA
α MHV
NGS
NO SERA
200
97
68
43

Figure 15. Blocking of MHV-A59 binding to BALB/c intestinal brush border membranes by monoclonal anti-MHV receptor antibody CC1. 10 μg of BALB/c BBMs were loaded to each dot in column 1, and 5 μg of BALB/c BBMs were loaded to each dot in columns 2 to 7. Row A was untreated and rows B - D were incubated for one hour with the following dilutions (B - 1:50 , C - 1:100, D - 1:200) of monoclonal antibody CC1. Columns 1 and 2 were incubated with MHV-A59 followed by anti-MHV E2 antiserum; column 3 was incubated with MHV-A59 followed by normal goat serum; column 4 was incubated with MHV-A59 only; column 5 was incubated with anti-MHV E2 antiserum only; column 6 received normal goat serum only; and column 7 was not incubated with either virus or antiserum. All columns were then incubated with ^{125}I-SPA to detect bound antibodies.


1 2 3 4 5 6 7
A
B
C
D

blocked the binding of MHV-A59 to 10 and 5 μg of BALB/c BBM (rows B and C). At a dilution of 1:200, CC1 blocked completely blocked binding of MHV to 5 μg of BALB/c BBMs, but only partially blocked binding of MHV to 10 μg of BALB/c BBMs (row D). Columns 3 through 7 contain controls (see legend, Figure 15). Similar results were obtained with MHV3 (data not shown). Since monoclonal antibody CC1 can block binding of both MHV-A59 and MHV3 to intestinal BBMs, it is probable that both virus strains utilize the same MHV receptor. This experiment shows that CC1 probably protects cells from infection by MHV by blocking the receptor on the cell membrane so that virions can not attach.

Species specificity of MHV receptors: In nature, MHV infects only mice and cells of mouse origin. Experimentally, it is possible to infect suckling rats if relatively large doses of MHV are injected intracerebrally (Cheever et al., 1949). Since MHV has such a narrow host range, I was interested in determining whether absence of receptors for MHV on other species accounted for their resistance to MHV. I therefore, attempted to detect MHV receptors on intestinal BBMs from other species. Rat, rabbit, cat, dog, pig, cow, man and chicken were chosen because each of these species is the natural host for one or more coronaviruses. Cotton rat (Sigmodon hispidus) was also included in my studies because of its susceptibility to infection by human respiratory syncytial virus and other human respiratory viruses (Prince et al., 1976). Intestinal BBMs were chosen as the tissue on which to look for MHV receptors for two reasons. First, most of the coronaviruses which infect the above species are enterotropic (Table 1). Second, the procedure for preparing BBMs had been successfully used in many of these species, and assays were available with which to standardize the different

BBM preparations. Chicken chorioallantoic membranes were also prepared since IBV has a respiratory tropism .

Different mouse BBM preparations were normally standardized by concentrations of membrane proteins using the Bradford method. However, since it was unclear whether it was reasonable to use membrane protein concentrations as a means of comparing membrane preparations from different species, sucrase assays were performed. Sucrase activity is generally only found on the brush border membrane of the intestine, and sucrase assays are a standard means of comparing the purity of BBM preparations. Table 7 shows that sucrase levels were fairly comparable (3-20 µmoles glucose released / ml / minute / mg protein) between BBM preparations from all species tested except for cows and infant rats. In rat brush border membranes, sucrase activity does not reach its peak until weaning, and cows do not exhibit any sucrase activity in their intestinal brush borders so it was not unexpected that sucrase levels in infant rat and cow BBMs were low or non-detectable (Rubino et al., 1964; Siddons, 1968; Toofanian et al., 1974). As expected, no sucrase activity was detectable in mouse liver plasma membrane or chicken chorioallantoic membrane preparations. Since sucrase levels were fairly comparable in BBM preparations of comparable protein concentration from most species, in subsequent experiments BBM preparations were standardized by membrane protein concentrations.

A solid phase assay for MHV binding was performed on BBMs from 10 species (Figure 16). MHV-A59 bound only to BALB/c BBMs and all controls for the specificity of the solid phase MHV-receptor assay were negative. A VOPBA and Western blots with polyclonal and monoclonal anti-receptor antisera confirmed the lack of MHV binding to BBM membrane proteins from 8 other mammalian species and showed that the MHV receptor was

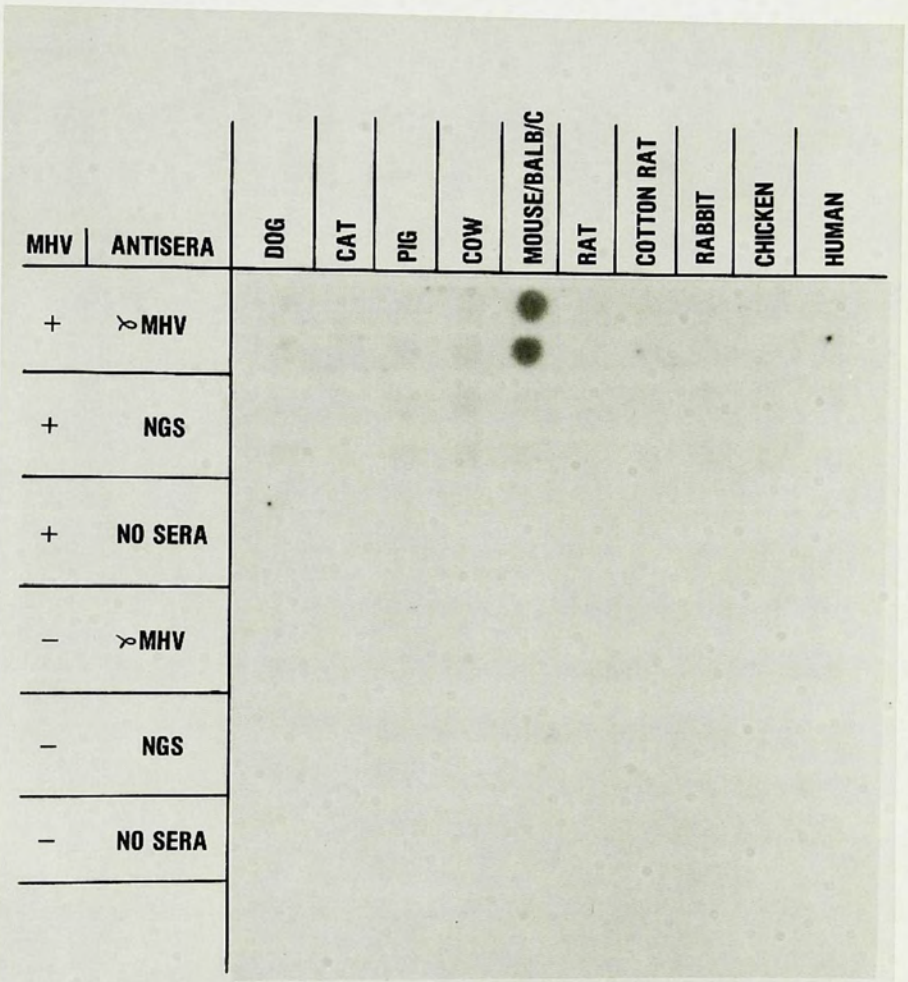
Table 7

Comparison of Sucrase Activity In Membranes from Tissues of Different Species

Tissue	Species, Strain, Age	Sucrase activity
Intestine:	Mouse - BALB/c	7.4, 6.9, 5.8
	- SJL/J	7.4
	- A/J	7.4
	- C3H	5.6
	- C57BL/6	8.8
	Rat - Sprague Dawley 1 day old	0.2
	11 day old	0.8
	adults	7.4, 9.3, 12.2
	Cotton rat	7.9
	Rabbit	19.3
	Chicken	4.6
	Human	3.7
	Cow	≤0.005
	Cat - Duodenum	4.7
	- Jejunum	4.5
	- Ileum	5.0
	- Colon	4.2
	- Mixed	1.9, 4.6
	Pig - Duodenum	9.0
	- Jejunum	6.6
	- Ileum	10.9
	- Mixed	4.9, 15.4
	Dog - Duodenum	10.9
	- Jejunum	12.9
	- Ileum	7.7
	- Colon	4.5
	- Mixed	2.9, 8.7
Liver:	Mouse - BALB/c	≤ 0.01
	- SJL/J	≤ 0.01
Other:	Chicken chorioallantoic membranes	≤ 0.01

* umoles glucose released/ml/minute/mg protein

Figure 16. Solid phase assay of MHV-A59 binding to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, chicken or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with MHV-A59 (+) and virus binding was detected with goat anti-MHV E2 antiserum, and ^{125}I-SPA. Controls included samples incubated with medium (-) instead of MHV-A59, or with normal goat serum (NGS), or dilution buffer (no sera) in place of anti-MHV E2 antiserum.


MHV
ANTISERA
DOG
CAT
PIG
COW
MOUSE/BALB/C
RAT
COTTON RAT
RABBIT
CHICKEN
HUMAN
+
≻MHV
+
NGS
+
NO SERA
−
≻MHV
−
NGS
−
NO SERA

only present on intestinal membranes from susceptible mouse strains (Figure 17). Chicken BBMs also did not bind MHV or the monoclonal anti-MHV receptor antibodies in VOPBAs and Western blots (Figure 18). The lack of MHV receptors on these 9 other probably explains why MHV exhibits such a limited host range, infecting only mice and murine cell lines (Tables 5 and 6).

Although intestinal infection of rats with MHV has never been demonstrated, MHV is antigenically closely related to RCV and SDAV, and certain strains of neonatal rats can be infected by MHV if injected intracerebrally with relatively large doses of MHV (Cheever et al., 1949; Parker et al., 1970; Bhatt et al., 1972). I studied several strains and ages of rats to determine if the data in Figure 17 was specific only for adult Sprague Dawley rats, or if it was indicative of a lack of MHV receptors in intestines of all ages and strains of rats. BBMs were prepared from adult and neonatal Sprague Dawley and Wistar Furth rats. Sprague Dawley rats were used to determine whether age could play a role in the absence of MHV receptors from rat BBMs. Wistar Furth rats were used because they appear to be the rat strain most susceptible to MHV infection, sustaining greater than 90% mortality when inoculated intracerebrally with MHV-JHM before 10 days of age (Sorensen et al., 1980). A VOPBA and Western blot with monoclonal anti-MHV receptor antibody did not reveal any MHV receptors on adult or neonatal rat BBMs of either strain (Figure 18). Thus, the lack of detectable MHV receptors in these two strains of rats both in adult or neonatal intestinal BBMs may reflect the tissue tropism of MHV which does not infect rat intestine, but rather infects rat brains where MHV receptors should be found. The nature of the MHV receptor in the brains of mice has not yet been determined but is, at present, being investigated in our laboratory. Studies on MHV receptors in the brains of susceptible BALB/c and resistant SJL/J mice

Figure 17. Binding of MHV-A59 intestinal brush border membranes from different species. 100 μg of BBMs from BALB/c and SJL/J mice, cow, human, dog, cat, pig, rat, cotton rat, rabbit intestines or chicken chorioallantoic membranes were run on SDS-PAGE gels, transferred to nitrocellulose and probed with: Panel A, VOPBA using MHV-A59 and anti-MHV E2 antibody; Panel B, polyclonal anti-MHV receptor antibody; Panel C, monoclonal anti-MHV receptor antibody CC1 and rabbit anti-mouse immunoglobulin. ^{125}I-SPA was used to detect bound antibody. Molecular weights in kilodaltons of protein standards are shown on the left of the panels.

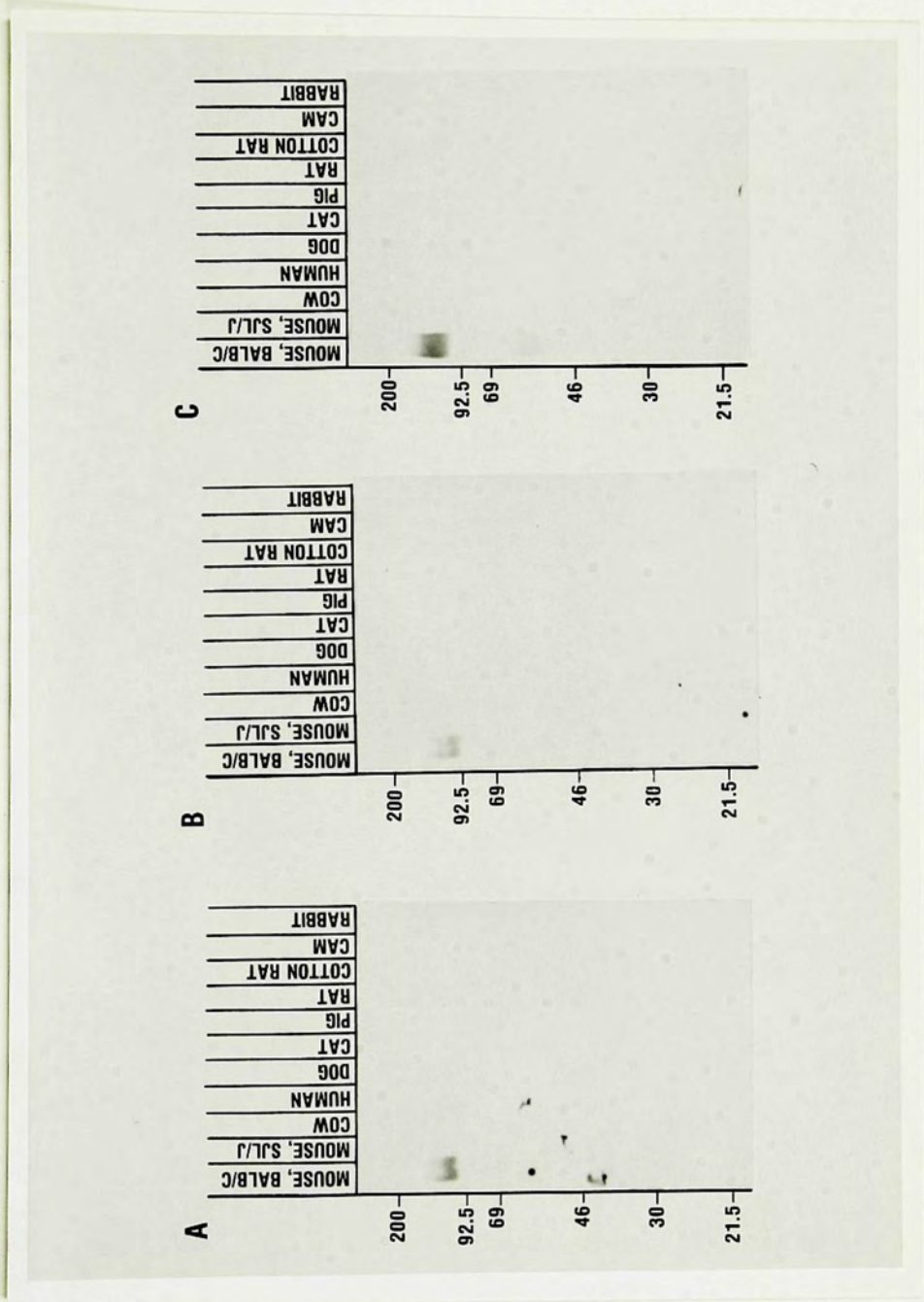
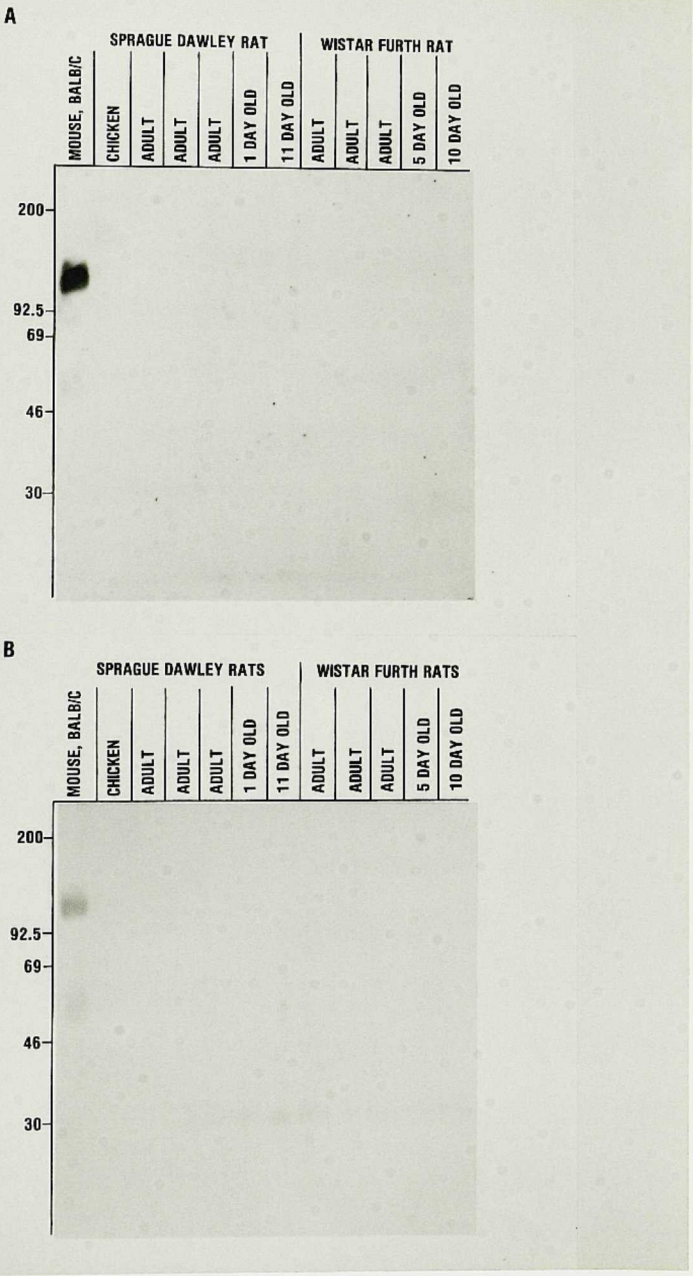

A
B
C
200
92.5
69
46
30
21.5
MOUSE, BALB/C
MOUSE, SJL/J
COW
HUMAN
DOG
CAT
PIG
RAT
COTTON RAT
CAM
RABBIT

Figure 18. Binding of MHV-A59 to intestinal brush border membranes from chicken and adult and suckling rats. 100 μg of BBMs from BALB/c mice, chicken, three female adults or pools of 1 or 11 day old Sprague Dawley rats, and three female adult or pools of 5 or 10 day old Wistar Furth rats were run on SDS-PAGE gels, transferred to nitrocellulose and probed with: Panel A, VOPBA using MHV-A59 and anti-MHV E2 antibody; Panel B, monoclonal anti-MHV receptor antibody CC1 and rabbit anti mouse immunoglobulin. ^{125}I-SPA was used to detect bound antibody. Molecular weights in kilodaltons are shown by the numbers on the left side of the panels.


A
SPRAGUE DAWLEY RAT
WISTAR FURTH RAT
MOUSE, BALB/C
CHICKEN
ADULT
ADULT
ADULT
1 DAY OLD
11 DAY OLD
ADULT
ADULT
ADULT
5 DAY OLD
10 DAY OLD
200
92.5
69
46
30
B
SPRAGUE DAWLEY RATS
WISTAR FURTH RATS
MOUSE, BALB/C
CHICKEN
ADULT
ADULT
ADULT
1 DAY OLD
11 DAY OLD
ADULT
ADULT
ADULT
5 DAY OLD
10 DAY OLD
200
92.5
69
46
30

should precede the analysis of MHV receptors in rat brains, as it is likely that the number of receptors in the rat brain may be much lower than in the murine brain, since it takes 1000-fold more virus to infect neonatal rats than it does to infect neonatal mice (Sorensen et al., 1980; Pickel et al., 1981; Barthold and Smith, 1984).

Development of solid phase receptor assays for other coronaviruses: To investigate the role of coronavirus receptors in species specificity I developed solid phase virus binding assays for coronaviruses of several other species (CCV, FIPV, TGEV, HCV-229E and BCV). Several changes had to be made in the solid phase assay to be able to detect specific binding of these viruses. First, BBMs from cat, dog, pig and man apparently contain bound immunoglobulins which bind ^{125}I-SPA (data not shown). BBMs were boiled 5 minutes in 5% BME prior to loading onto nitrocellulose, to reduce the disulfide bonds in the bound immunoglobulins, thus eliminating the binding of radiolabeled SPA. Second, several of these coronaviruses bound to BSA which was used to block non-specific binding to nitrocellulose in the solid phase receptor assay for MHV. Therefore, the protein used to block non-specific binding of virus to nitrocellulose, had to be changed. Several alternative blockers were tested, and 10% nonfat dry milk gave the lowest background binding of virus to blocked nitrocellulose (data not shown). Third, the anti-viral antibodies used in these new solid phase receptor assays were made in rabbits or for anti-FIPV was derived from ascites fluid from an acutely infected cat, unlike the goat anti-MHV E2 sera used in the MHV solid phase receptor assay. Although these rabbit and cat sera initially bound non-specifically to the BBMs, BSA and non-fat dry milk, the non-specific antibody binding activity was subsequently adsorbed out of the antiserum by serial

adsorptions with BBMs, and with nitrocellulose strips pre-adsorbed with 10% non-fat milk.

With these new solid phase receptor assays I was able to detect binding of each of these five coronaviruses to intestinal BBMs isolated from its normal host species as described below. Future experiments may permit development of virus specific VOPBAs for each of these coronaviruses.

CCV receptor on dog BBMs: CCV binding was detected on BME pre-treated dog intestinal BBMs using a new solid phase receptor assay (Figure 19). No CCV binding was seen to BBMs from BALB/c mice, or when CCV or rabbit anti-CCV were left out of the reaction. CCV binding to dog BBM was not reduced by extraction of BBMs with 0.25 % deoxycholate (Figure 19, Upper panel), or by pre-treatment with 0.4% NP40, 0.4% lubrol, or 0.4% octyl B-d glucopyranoside (data not shown). Initial attempts to develop a VOPBA assay with CCV have so far been unsuccessful. One explanation for CCV binding to BBMs in the solid phase receptor assay but not in a VOPBA could be the inactivation of the CCV receptor on dog BBMs by SDS or another component of the standard treatment mix in which the BBMs were resuspended for SDS-PAGE electrophoresis. Pre-treatment of the dog BBMs with 0.1 % SDS or 1X STM did not destroy CCV binding to the dog BBMs (Figure 19, Lower panel). Therefore, the lack of binding observed in attempted VOPBAs with CCV was not due to an inactivation of the CCV receptor by SDS or other components of STM.

Like other receptor-ligand interactions, CCV binding to dog BBMs decreased in proportion to the amounts of BBMs on each dot (Figure 20). A second possible explanation for the lack of CCV binding observed on attempted VOPBAs with CCV was that the CCV binding component on dog BBMs might not be a protein but rather a lipid, like the receptor(s) for VSV

Figure 19. Solid phase binding assay for canine coronavirus binding to dog and mouse brush border membranes. Upper panel: 5 μg BALB/c mouse BBMs, 25 μg mixed dog BBMs from dog duodenum, jejunum, ileum and colon pre-treated with 5% BME, or 25 μg mixed dog BBMs solubilized with 0.25% deoxycholate and pre-treated with 5% BME, Lower panel: 25 μg mixed dog BBMs pre-treated with 5% BME, 25μg mixed dog BBMs pre-treated with 0.1 % SDS and 5% BME, or 25μg mixed dog BBMs pre-treated with standard treatment mix. Nitrocellulose sheets were incubated with CCV or medium (no virus), and virus binding was detected with rabbit anti-CCV antiserum, normal rabbit serum (NRS), or dilution buffer (no sera) and ^{125}I-SPA.

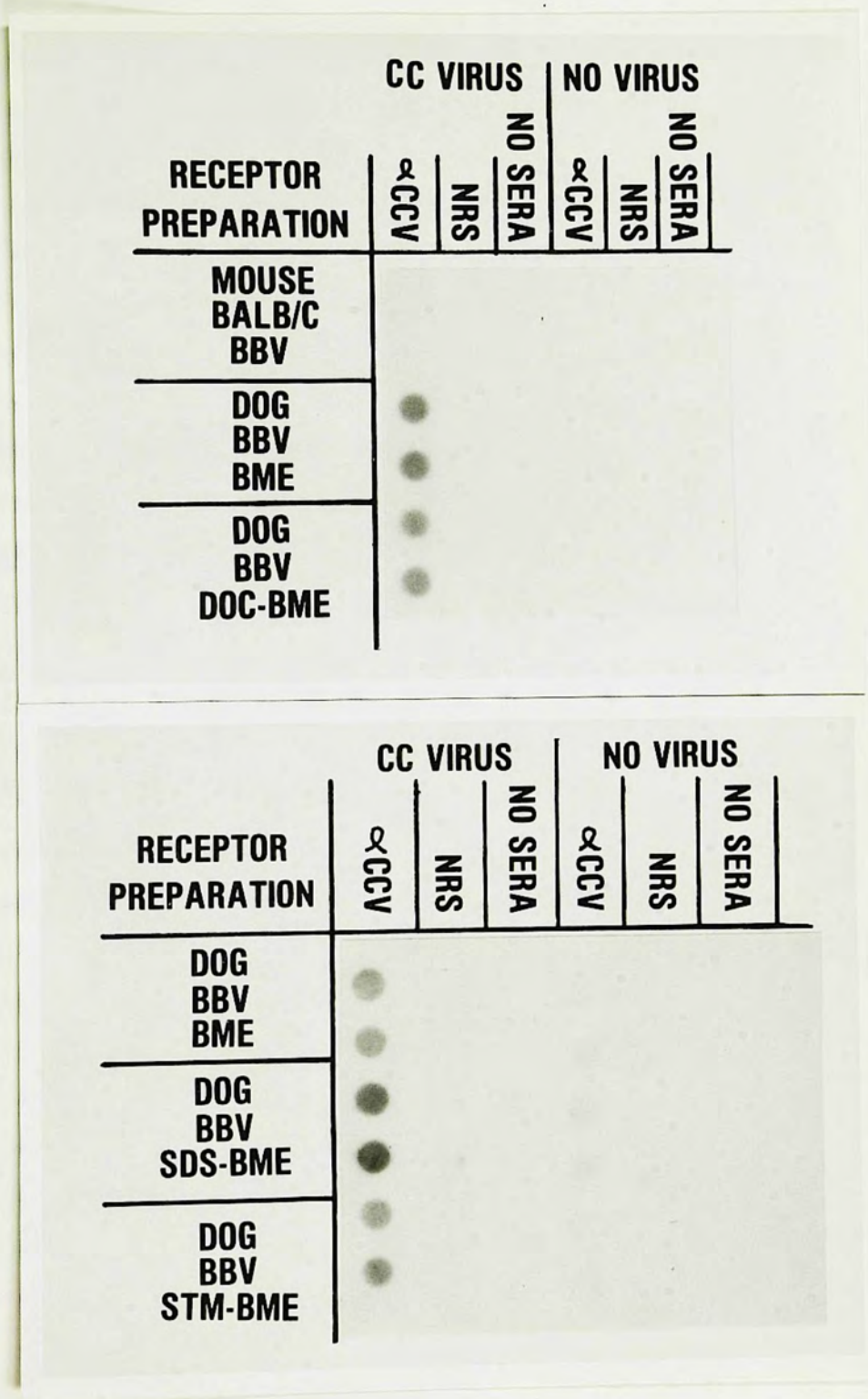
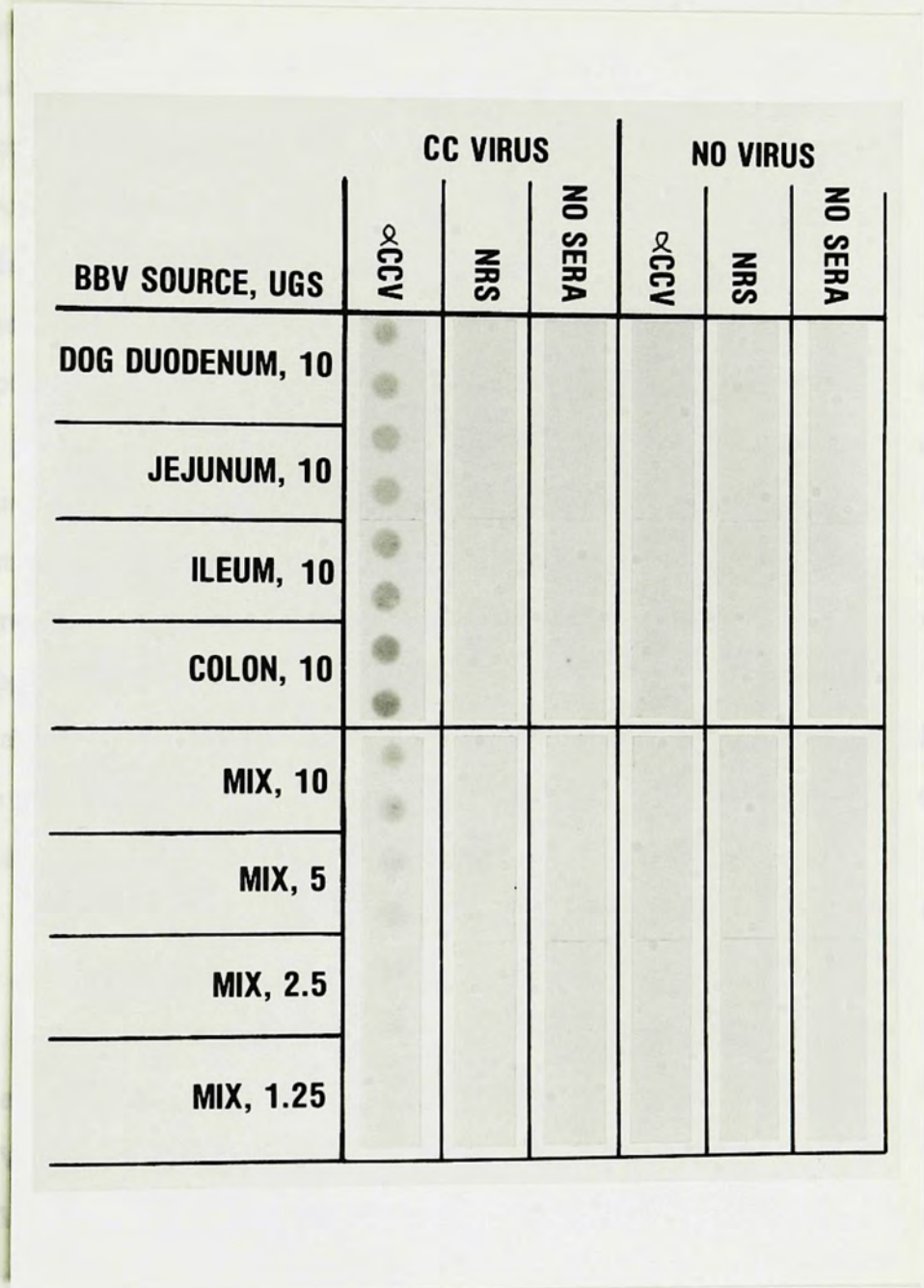

CC VIRUS
NO VIRUS
RECEPTOR PREPARATION
αCCV
NRS
NO SERA
αCCV
NRS
NO SERA
MOUSE BALB/C BBV
DOG BBV BME
DOG BBV DOC-BME
CC VIRUS
NO VIRUS
RECEPTOR PREPARATION
αCCV
NRS
NO SERA
αCCV
NRS
NO SERA
DOG BBV BME
DOG BBV SDS-BME
DOG BBV STM-BME

Figure 20. Solid phase binding assay for canine coronavirus binding to BBMS from different intestinal segments of the dog and titration of dog BBMs. 10 µgs of BBMs from the duodenum, jejunum, ileum or colon of a dog or 1.25 to 10 µg of mixed (duodenum, jejunum, ileum and colon) dog BBMs were bound to nitrocellulose. Nitrocellulose sheets were incubated with CCV or medium (no virus), and virus binding was detected with diluted rabbit anti-CCV antiserum, normal rabbit serum (NRS), or dilution buffer (no sera) and ^{125}I-SPA.

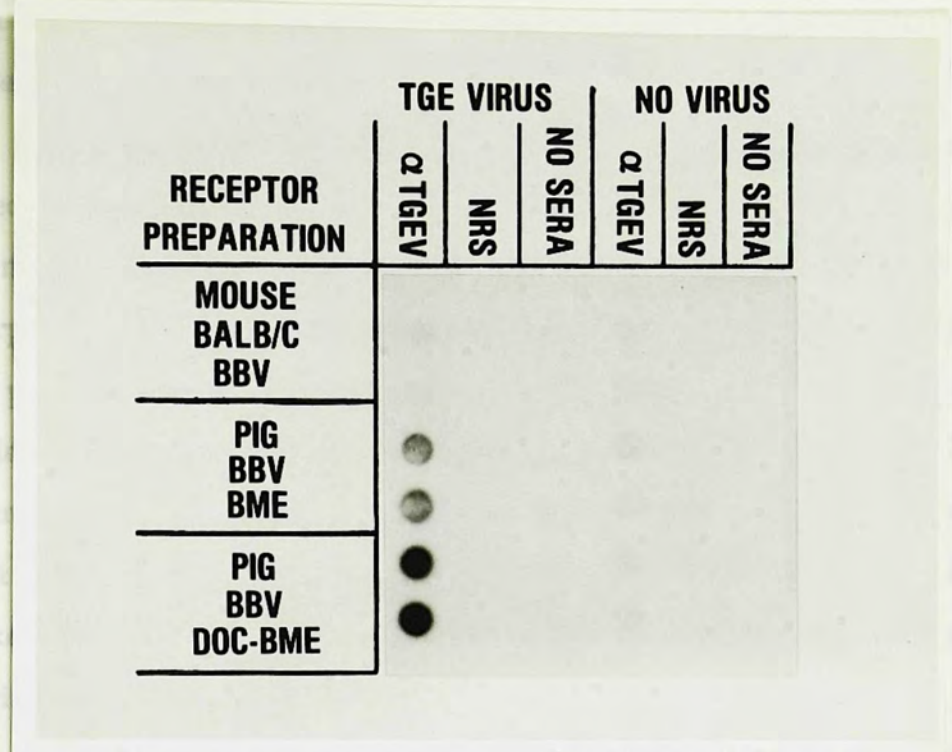

CC VIRUS
NO VIRUS
BBV SOURCE, UGS
αCCV
NRS
NO SERA
αCCV
NRS
NO SERA
DOG DUODENUM, 10
JEJUNUM, 10
ILEUM, 10
COLON, 10
MIX, 10
MIX, 5
MIX, 2.5
MIX, 1.25

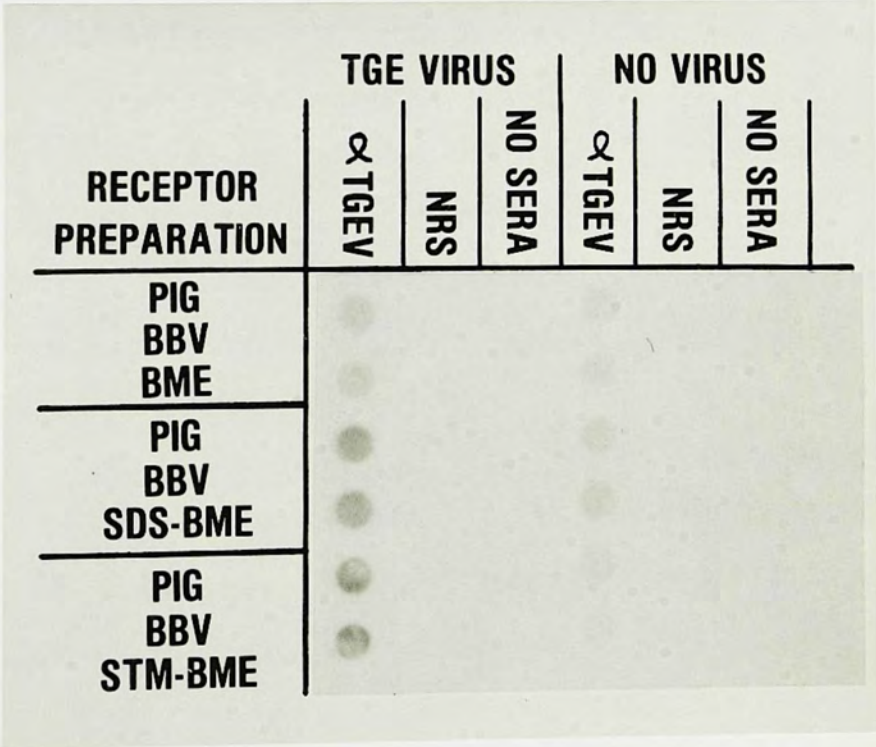
and other rhabdoviruses (Wunner et al., 1984). Lipid receptors would not be resolved on a SDS-PAGE gel. Therefore, chloroform-methanol extractions of dog BBMs were performed to determine the chemical nature of the CCV receptor. CCV bound only to the pellet from the chloroform-methanol extracted dog BBMs, which contained released membrane proteins and sugars. No CCV binding was observed to the polar and nonpolar lipids in the aqueous or organic layers of the extraction, respectively (data not shown). Similarily, MHV bound only to the pellet of the chloroform-methanol extracted mouse BBMs (data not shown). These data suggest that the CCV receptor, like the MHV receptor is a protein, not a glyco- or phospholipid.

Because infection by CCV and other enteric coronaviruses is seen most frequently in the duodenum, followed later in the course of the disease by infection of the jejunum and ileum and rarely with infection of the colon (Keenen et al., 1976) I investigated whether the prevalence of CCV receptors in dog BBMs from each of these intestinal segments correlated with prevalence of CCV infection in vivo in each region. CCV binding levels were approximately equivalent on BBMs from all three sections of the small intestine and from the large intestine (Figure 20). Thus, the prevalence of CCV receptors on the different intestinal segments does not correlate with their different infection rates.

TGEV receptor on pig BBMs: TGEV binding was detected on BME pre-treated pig intestinal BBMs using a new solid phase assay (Figure 21). No TGEV binding was observed on BBMs from BALB/c mice. Controls lacking TGEV or anti-TGEV were negative. TGEV binding to pig BBMs was not reduced by solubilization of BBMs with 0.25% deoxycholate (Figure 21, Upper panel), or pre-treatment with 0.1% NP40, 0.1% lubrol (data not shown), 0.1% SDS or 1X STM (Figure 21, Lower panel) much like CCV binding to dog

Figure 21. Solid phase binding assay for transmissible gastroenteritis virus binding to pig and mouse brush border membranes. Upper panel: 5 μg BALB/c mouse BBMs, 25μg mixed pig BBMs from the duodenum, jejunum and ileum pre-treated with 5% BME, or 25 μg mixed pig BBMs solubilized with 0.25% deoxycholate and pre-treated with 5% BME, Lower panel: 25 μg mixed pig BBMs pre-treated with 5% BME, 25 μg mixed pig BBMs pre-treated with 0.1% SDS and 5% BME, 25 μg mixed pig BBMs pre-treated with standard treatment mix. Nitrocellulose sheets were incubated with TGEV or medium (no virus), and virus binding was detected with rabbit anti-TGEV antiserum, normal rabbit serum (NRS), or dilution buffer (no sera) and ^{125}I-SPA.


TGE VIRUS
NO VIRUS
RECEPTOR PREPARATION
αTGEV
NRS
NO SERA
αTGEV
NRS
NO SERA
MOUSE BALB/C BBV
PIG BBV BME
PIG BBV DOC-BME


TGE VIRUS
NO VIRUS
RECEPTOR PREPARATION
αTGEV
NRS
NO SERA
αTGEV
NRS
NO SERA
PIG BBV BME
PIG BBV SDS-BME
PIG BBV STM-BME

BBMs. BBMs prepared from the jejunum of the pig bound about twice as much TGEV/μg BBM protein as BBMs prepared from the ileum or duodenum of the pig (data not shown).

FIPV receptor on cat BBMs: FIPV binding was detected on BME pre-treated cat intestinal BBMs using a new solid phase assay (Figure 22). Some binding was seen of anti-FIPV ascites to cat BBMs, even after extensive adsorptions with cat BBMs but at a level less than that seen with the complete solid phase reaction. Solubilization of cat BBMs with 0.25% deoxycholate did not destroy FIPV binding activity, but did remove the non-specific binding of the anti-FIPV antibodies to the BBMs. In repeated experiments, pre-treatment of cat BBMs with 1% SDS substantially inhibited FIPV binding to the BBMs, unlike MHV, CCV and TGEV receptors which retain their virus binding activity after SDS treatment.

HCV-229E receptor on human BBMs: HCV-229E binding was detected on BME pre-treated human intestinal BBMs using a new solid phase assay (Figure 23). Controls lacking HCV-229E or rabbit anti-HCV-229E were negative. HCV-229E binding to human BBMs was not reduced by pre-treatment of BBMs with 0.4 % SDS, 0.4 % lubrol, 0.4 % NP40, 0.4 % deoxycholate or 0.4 % octyl B-d glucopyranoside (data not shown). Therefore, like CCV and TGEV receptors, HCV-229E intestinal receptor was not affected by pre-treatment with detergents or SDS.

BCV receptor on adult and fetal bovine BBMs: Initial attempts to detect BCV binding in a solid phase receptor assay to bovine BBMs from intestine from a beef steer obtained from Trueth meats were unsuccessful (data not shown). The lack of BCV binding to these particular BBMs could have been caused by several factors, strain or age of the cow, region of intestine processed, or time from death to processing of intestine. Intestines

Figure 22. Solid phase binding assay for feline infectious peritonitis virus binding to cat brush border membranes. 10 µg mixed cat BBMs from cat duodenum, jejunum, ileum pre-treated with 5% BME, 10 µg mixed cat BBMs solubilized with 0.25% deoxycholate and pre-treated with 5% BME or 10µg mixed cat BBMs pre-treated with 0.1 % SDS and 5% BME were bound to nitrocellulose. Nitrocellulose sheets were incubated with FIPV or medium (no virus), and virus binding was detected with anti-FIPV ascites from an acutely infected cat, or dilution buffer (no sera) and ^{125}I-SPA.

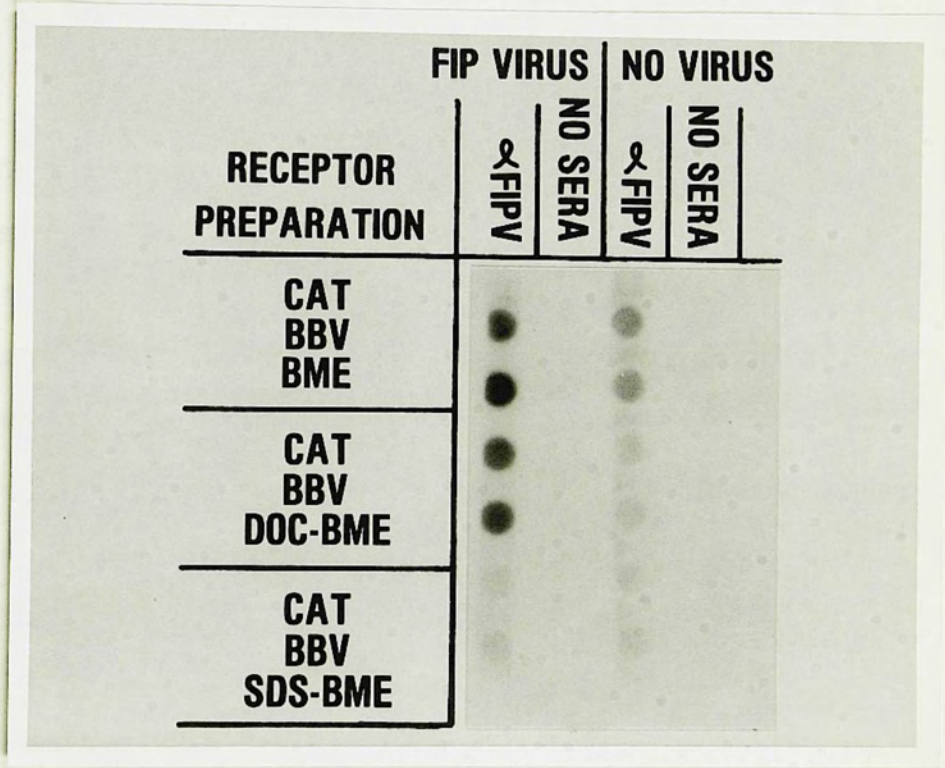
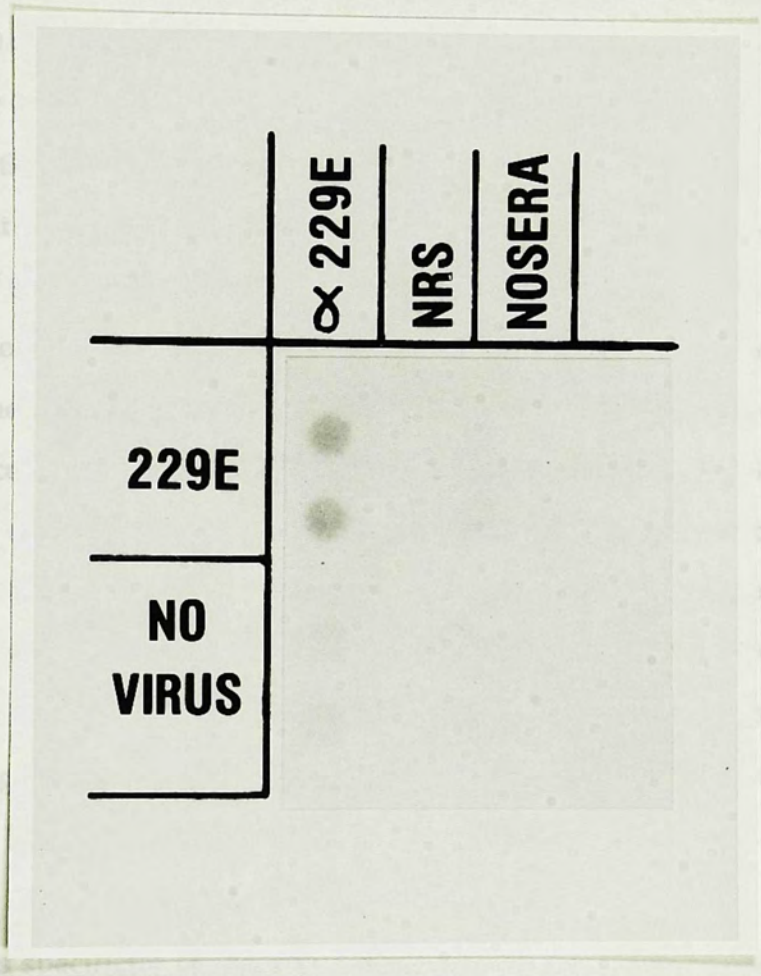

FIP VIRUS
NO VIRUS
RECEPTOR PREPARATION
αFIPV
NO SERA
αFIPV
NO SERA
CAT BBV BME
CAT BBV DOC-BME
CAT BBV SDS-BME

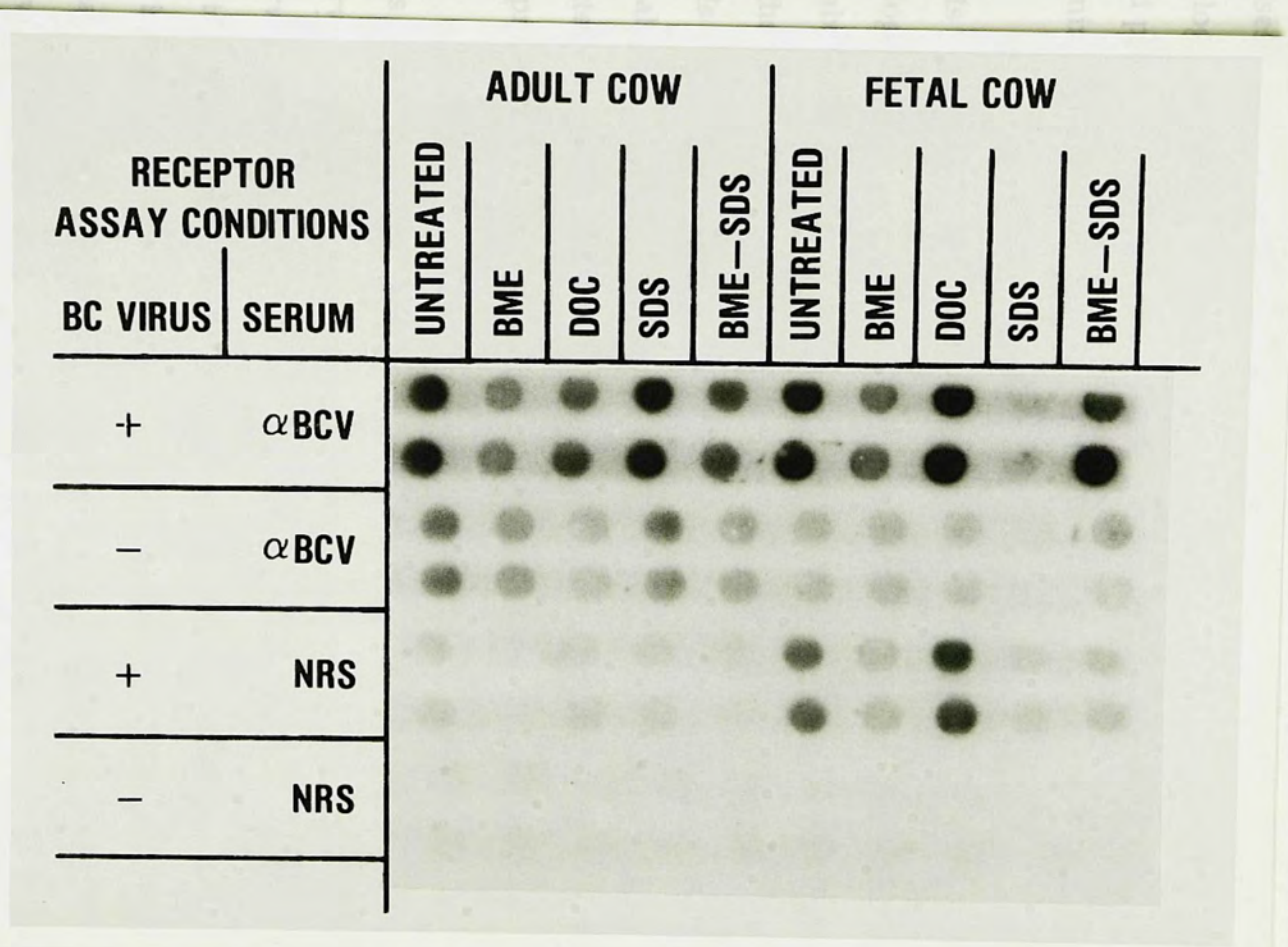
Figure 23. Solid phase binding assay for human coronavirus HCV-229E binding to human brush border membranes. 10 μg human BBMs pre-treated with 5% BME were bound to nitrocellulose. Nitrocellulose sheets were incubated with HCV-229E or medium (no virus), and virus binding was detected with rabbit anti-HCV-229E, normal rabbit serum (NRS) or dilution buffer (no sera) and ^{125}I-SPA.


∝ 229E
NRS
NOSERA
229E
NO VIRUS

were subsequently obtained from a Holstein cow, and her 37 week-old fetus (normal gestation: 40 weeks). BCV binding was observed to intestinal BBMs from both of these animals (Figure 24). Even after extensive adsorptions with bovine BBMs, anti-BCV serum still bound to bovine BBMs, but at a level less than that seen in the complete solid phase BCV receptor assay. BCV binding to BBMs from the adult cow were slightly diminished by pre-treatment with 5% BME or 0.0625% deoxycholate, but not by treatment with 0.25% SDS, while BCV binding to BBMs from the fetal calf was greatly diminished by pre-treatment with 0.25% SDS, slightly diminished by 5% BME and not affected by pre-treatment with 0.0625% deoxycholate (Figure 24). The intermediate sensitivity of fetal bovine BBMs to BME and of adult bovine BBMs to BME and deoxycholate suggests that more than one BCV receptor may exist on these intestines, and that the receptors differ in their sensitivity to detergents. The difference in sensitivity of fetal and adult bovine BBMs to SDS suggests the existence of different BCV receptor(s) on fetal and adult brush border membranes. Since, BCV contains two potential receptor binding proteins, the E2 peplomer glycoprotein and the hemagglutinin glycoprotein, it was not surprising to find multiple receptors on the BBMs of cow intestines. It also is not surprising to observe differences in the BCV receptors found on fetal and adult bovine BBMs as susceptibility to BCV decreases during the development of the calf (Langpap et al., 1979).

Species specificity of receptor recognition of the antigenically-related coronaviruses CCV, FIPV, TGEV and HCV-229E. As shown above, MHV receptors are only present in intestinal BBMs from susceptible mouse strains and have not been detected in the resistant mouse strain SJL/J or in 9 other species. Therefore, availability of MHV receptors plays an important role in determining the narrow host range of MHV. In nature, CCV, FIPV, TGEV

Figure 24. Solid phase binding assay for bovine coronavirus binding to adult and fetal cow brush border membranes. 10μg of adult or fetal cow BBMs were untreated, pre-treated with 5 % BME, pretreated with 0.25 % SDS, extracted with 0.0625 % DOC, or pretreated with 5 % BME and 0.25 % SDS and loaded onto nitrocellulose. Nitrocellulose sheets were incubated with BCV (+) or medium (-), and virus binding was detected with rabbit anti-BCV serum, or normal rabbit serum (NRS), and ^{125}I-SPA.


ADULT COW
FETAL COW
RECEPTOR ASSAY CONDITIONS
BC VIRUS
SERUM
UNTREATED
BME
DOC
SDS
BME—SDS
UNTREATED
BME
DOC
SDS
BME—SDS
+
αBCV
–
αBCV
+
NRS
–
NRS

and HCV-229E are also quite species specific. They normally only infect dog, cat, pig or humans respectively or cells derived from these species. I was interested in investigating whether coronaviruses receptors for these four viruses could be found on BBMs from other species susceptible to serologically related or unrelated coronaviruses. Consequently, I used the solid phase receptor assays for each of these coronaviruses, described above, to examine the species specificity of coronavirus binding.

Solid phase CCV receptor assays were performed on BME pre-treated BBMs from 10 species (Figure 25). High levels of CCV binding were observed on dog, cat, pig, cow, and human BBMs . CCV binding to cotton rat BBMs was variable from strong, in some experiments, as shown here, to barely present in others. No CCV binding was observed to mouse, rat, rabbit or chicken BBMs (Table 8). Thus, CCV can bind to receptors on BBMs from dog intestine and also to receptors on BBMs from intestines of other species normally infected by antigenically related coronaviruses (FIPV, TGEV and HCV-229E). The presence of CCV receptors on bovine BBMs was unexpected.

A solid phase FIPV receptor assay was performed on BME pre-treated BBMs from the same 10 species (Figure 26). High levels of FIPV binding were observed on dog, cat, pig, cow, and human BBMs. No FIPV binding was observed on mouse, rat, rabbit or chicken BBMs (Table 8). Thus, FIPV can bind to receptors on BBMs from cat intestine and also to receptors on BBMs from intestines of other species normally infected by antigenically related coronaviruses (CCV, TGEV and HCV-229E). The presence of FIPV receptors on cow BBMs was again unexpected. A pattern appeared to be emerging in which coronaviruses in the second antigenic group bind to BBMs from all the species susceptible to coronaviruses in that group.

Figure 25. Solid phase assay of binding of canine coronavirus to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, chicken or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with CCV (+) or medium (-), and virus binding was detected with diluted rabbit anti-CCV antiserum, or normal rabbit serum (NRS), and ^{125}I-SPA.

RECEPTOR
ASSAY CONDITIONS
CC VIRUS
SERUM
DOG
CAT
PIG
COW
MOUSE, BALB/C
RAT
COTTON RAT
RABBIT
CHICKEN
HUMAN
+
∝CCV
–
∝CCV
+
NRS
–
NRS

Table 8

Coronavirus Receptor Binding

	Balb/c Mouse	Cow	Human	Dog	Cat	Pig	Rat	Chicken	Cotton Rat	Rabbit
MHV	+/+/+	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-	-/-/-
BCV	(±)	++	-	+	-	++	(±)		(±)	(±)
OC43			++							
229E	(±)	+	++	+	+	+	(-)	-	(±)	-
CCV	-	++	++	++	++	++	(-)	-	(±)	-
FIPV	-	++	++	++	++	++	(-)	-	(-)	-
TGEV	-	++	++	++	+	++	-	-	(±)	-

Dot blot/VOPBA/Anti-receptor western blot

++ Strong receptor binding on BBMs from normal host

++ Strong receptor binding equal to homologous reaction

+ Moderate receptor binding

+/- Weak receptor binding

- No receptor binding detected

( ) level of receptor binding varied between experiments

A solid phase TGEV receptor assay was performed on BME pre-treated BBMs from 10 species (Figure 27). In repeated experiments, high levels of TGEV binding were observed on dog, pig, cow and human BBMs. In this particular experiment, the signal for dog BBMs were abnormally low. A lower level of TGEV binding was seen on cat BBMs and a variable (low to none) level of binding was seen to cotton rat BBMs (Table 8). Thus, TGEV binds to receptors on BBMs from pig intestine and also to receptors on BBMs from intestines of other species normally infected by antigenically related coronaviruses (CCV, FIPV and HCV-229E).

A solid phase HCV-229E receptor assay was performed on BME pre-treated BBMs from 10 species (Figure 28). Human BBMs gave the strongest signal and dog, cat, cow and pig gave weaker signals for HCV-229E binding. Cotton rat, and mouse BBMs gave variable levels of binding from fairly strong to none in different experiments (Table 8). Levels of nonspecific binding of rabbit anti-HCV-229E sera to BBMs even after extensive adsorption with human BBMs were often high but always less than the specific binding seen in the complete solid phase assay. Thus, HCV-229E can bind to receptors on BBMs from human intestine and also to receptors on BBMs from intestines of other species normally infected by antigenically related coronaviruses (CCV, FIPV, and TGEV).

These data with CCV, FIPV, TGEV and HCV-229E support the theory of one (or more) cross-reactive receptors for coronaviruses in the second antigenic group on intestinal BBMs of species normally infected by these viruses. To determine if these coronaviruses bind to the same receptor or to different receptors on the same BBMs, the SDS sensitivities of CCV and FIPV receptors on dog, cat, pig, cow and human BBMs were compared. BBMs were pre-treated with BME alone or BME and SDS. Similar patterns of SDS

Figure 26. Solid phase assay of binding of feline infectious peritonitis virus to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, chicken or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with FIPV (+) or medium (-), and virus binding was detected with diluted anti-FIPV ascites from and acutely infected cat, or dilution buffer (No Sera) and ^{125}I-SPA.

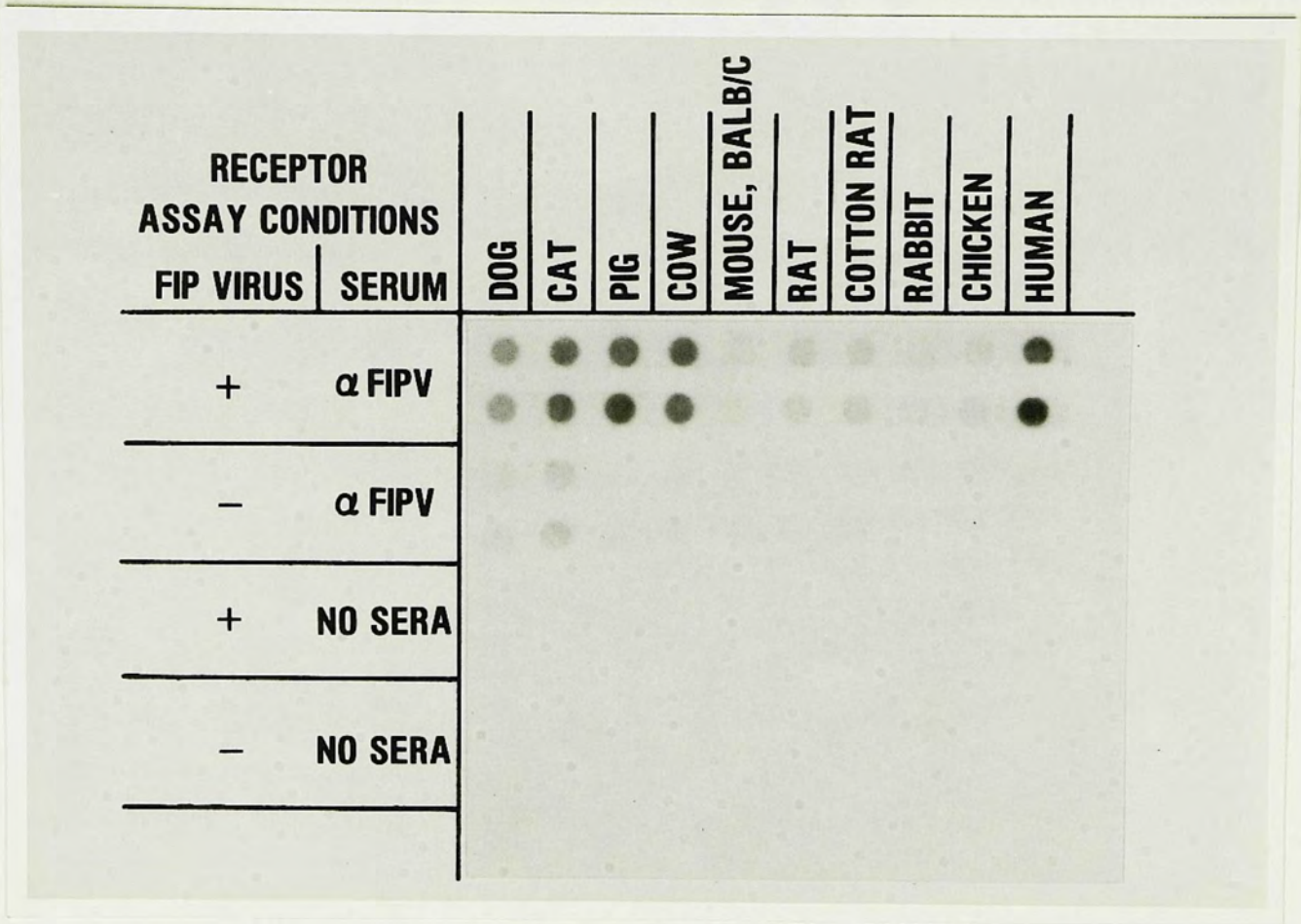

RECEPTOR
ASSAY CONDITIONS
FIP VIRUS
SERUM
DOG
CAT
PIG
COW
MOUSE, BALB/C
RAT
COTTON RAT
RABBIT
CHICKEN
HUMAN
+
α FIPV
−
α FIPV
+
NO SERA
−
NO SERA

Figure 27. Solid phase assay of binding of transmissible gastroenteritis virus to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with TGEV (+) or medium (-), and virus binding was detected with diluted rabbit anti-TGEV antiserum, normal rabbit serum (NRS), or dilution buffer (no sera) and ^{125}I-SPA.

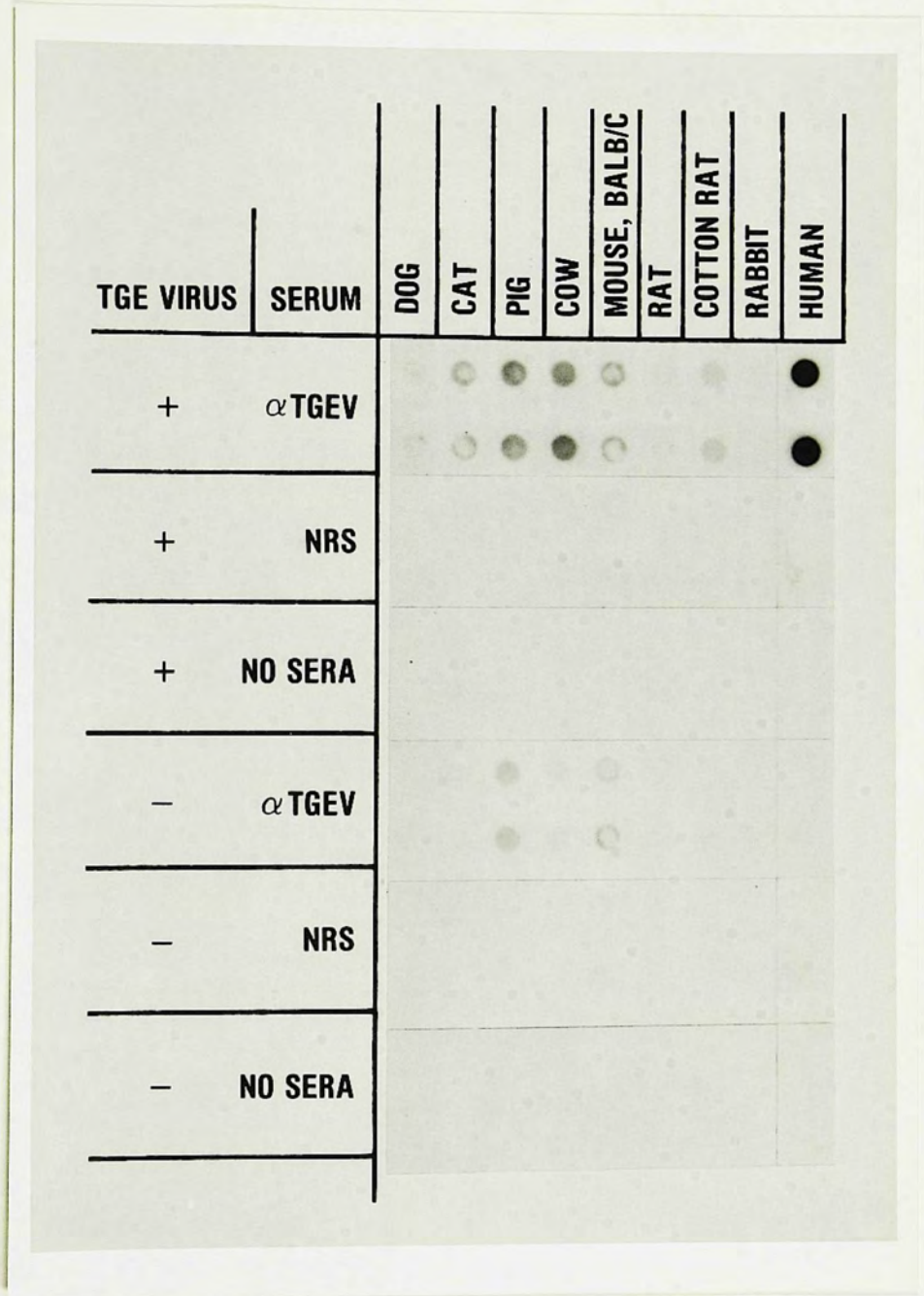
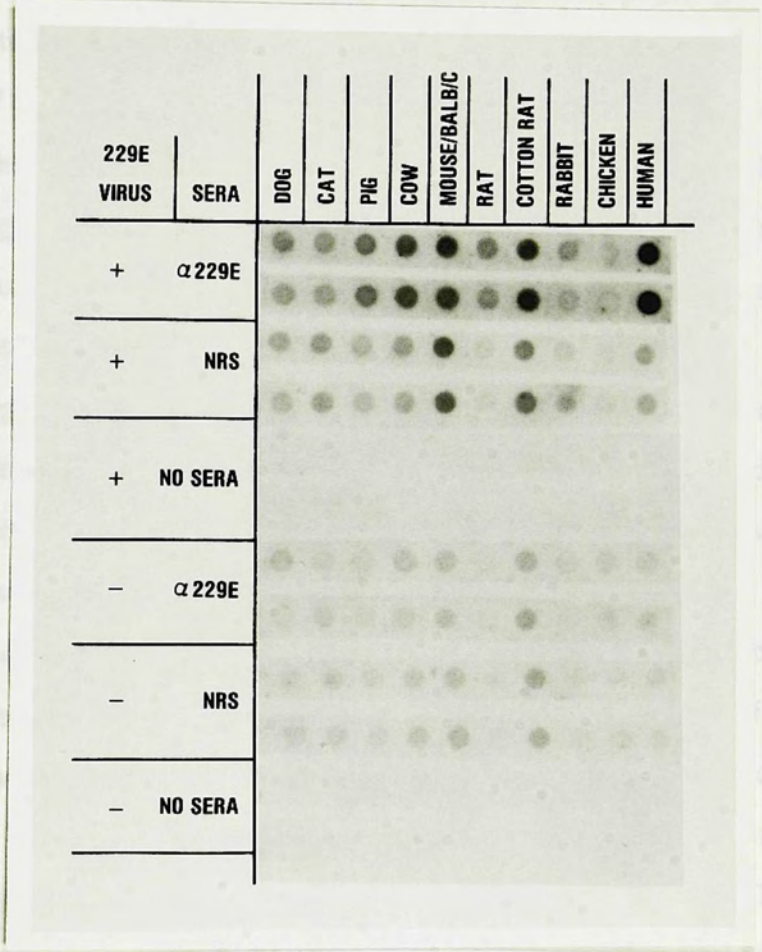

TGE VIRUS
SERUM
DOG
CAT
PIG
COW
MOUSE, BALB/C
RAT
COTTON RAT
RABBIT
HUMAN
+
αTGEV
+
NRS
+
NO SERA
–
αTGEV
–
NRS
–
NO SERA

Figure 28. Solid phase assay of binding of human coronavirus 229E to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, chicken or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with HCV-229E (+) or medium (-), and virus binding was detected with diluted rabbit anti-HCV-229E antiserum, normal rabbit serum (NRS), or dilution buffer (no sera) and ^{125}I-SPA.

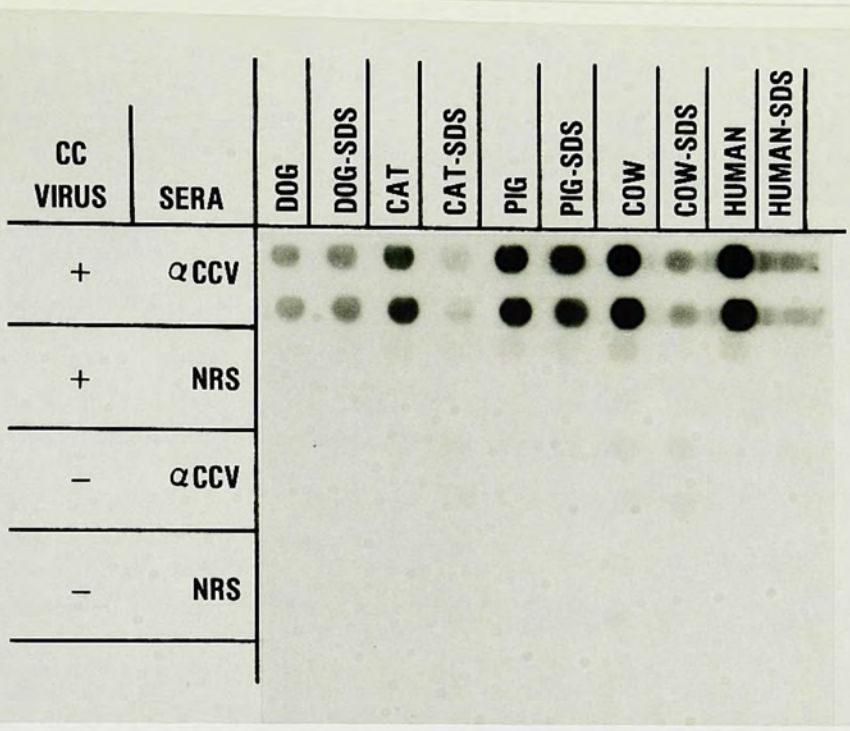

229E VIRUS
SERA
DOG
CAT
PIG
COW
MOUSE/BALB/C
RAT
COTTON RAT
RABBIT
CHICKEN
HUMAN
+ α229E
+ NRS
+ NO SERA
– α229E
– NRS
– NO SERA

sensitivity were seen for CCV and FIPV binding to BBMs from these 5 species (Figure 29). SDS treatment of BBMs from cat, cow and human diminished both CCV and FIPV binding to these BBMs while treatment of BBMs from dog and pig did not affect CCV or FIPV binding to these BBMs. The untreated dog BBMs gave an unusually weak signal with CCV in this particular experiment, but other experiments show equal binding in the untreated and SDS pre-treated dog BBMs with CCV (data not shown). Therefore, it appears that the CCV and FIPV receptor(s) on each of these five species may be the same, or at least share the common characteristic of sensitivity to SDS. These data also suggest that at least two classes (SDS-sensitive and SDS-insensitive) of coronavirus receptors for the coronaviruses in the second antigenic group exist in different species.

Species specificity of BCV receptors: A solid phase receptor assay was performed on BME pre-treated BBMs from 10 species for BCV binding (Figure 30). Levels of nonspecific binding of rabbit anti-BCV serum to BBMs, even after extensive adsorption with bovine BBMs were often high, but specific binding could be detected above the level of nonspecific binding for some BBMs. BBMs from adult and fetal cow and pig gave high levels of BCV binding. Lower levels of binding were observed with BBMs from dog and variable low levels of binding were seen with BBMs from mouse, rat, cotton rat and rabbit. No binding was seen to BBMs from human or cat (Table 8). Due to very high non-specific binding, it is unclear whether any BCV binding occurred with chicken BBMs (data not shown).

BCV is a member of the first antigenic group which also includes MHV, HCV-OC43 and HEV. MHV is very species specific and I have shown above that MHV receptors play a role in the narrow species tropism of MHV. BCV is also quite species specific, but it is unclear from this study what the

Figure 29. Solid phase assay of binding of canine coronavirus and feline infectious peritonitis virus to intestinal brush border membranes pre-treated with SDS. 10 μg of BBMs from dog, cat, pig, cow and human were treated with 5% BME only or 5% BME and 0.25% SDS. Upper panel: nitrocellulose sheets were incubated with CCV (+) or medium (-), and virus binding was detected with diluted rabbit anti-CCV antiserum, or normal rabbit serum (NRS), and ^{125}I-SPA. Lower panel: nitrocellulose sheets were incubated with FIPV (+) or medium (-), and virus binding was detected with diluted anti-FIPV ascites from and acutely infected cat, or dilution buffer (no sera) and ^{125}I-SPA.


CC VIRUS
SERA
DOG
DOG-SDS
CAT
CAT-SDS
PIG
PIG-SDS
COW
COW-SDS
HUMAN
HUMAN-SDS
+
αCCV
+
NRS
–
αCCV
–
NRS

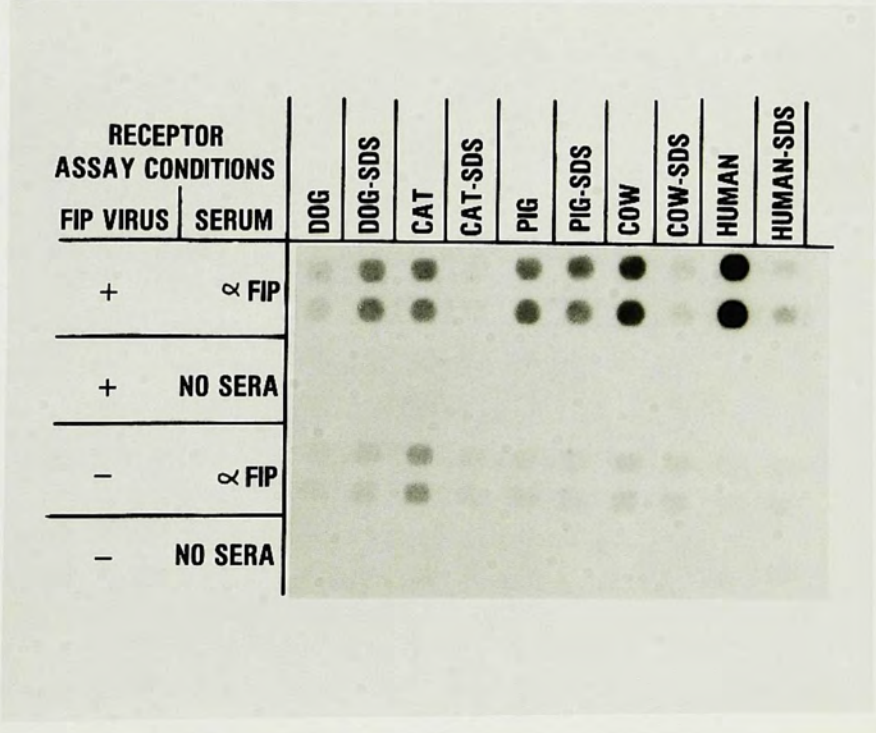
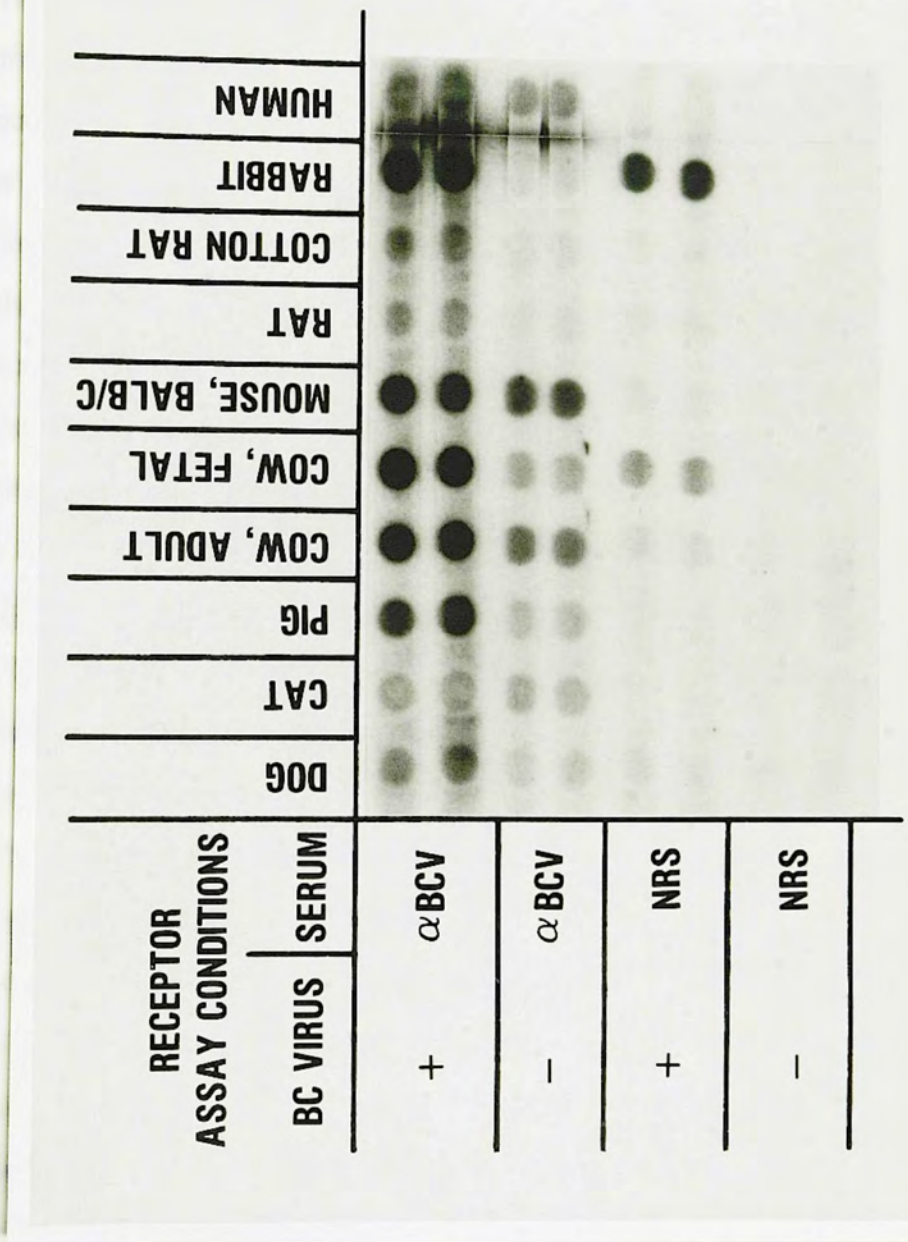

RECEPTOR ASSAY CONDITIONS
FIP VIRUS
SERUM
DOG
DOG-SDS
CAT
CAT-SDS
PIG
PIG-SDS
COW
COW-SDS
HUMAN
HUMAN-SDS
+
α FIP
+
NO SERA
–
α FIP
–
NO SERA

Figure 30. Solid phase assay of binding of bovine coronavirus to intestinal brush border membranes from different species. 10μg of BBMs pre-treated with 5% BME from dog, cat, pig, cow, BALB/c mice, rat, cotton rat, rabbit, chicken or human intestines were bound to nitrocellulose. Nitrocellulose sheets were incubated with BCV (+) or medium (-), and virus binding was detected with diluted rabbit anti-BCV antiserum, or normal rabbit serum (NRS), and ^{125}I-SPA.


RECEPTOR ASSAY CONDITIONS
BC VIRUS
SERUM
+
αBCV
−
αBCV
+
NRS
−
NRS
DOG
CAT
PIG
COW, ADULT
COW, FETAL
MOUSE, BALB/C
RAT
COTTON RAT
RABBIT
HUMAN

role of BCV receptors is in the species tropism of BCV, since BCV binding was detected on pig and dog BBMs as well as on cow BBMs. The presence of a receptor which binds BCV on pig BBMs is not unexpected since the serologically related HEV infects pig intestines, and BCV can be grown in vitro in pig cells. However, BCV binding to dog BBMs was unexpected. The lack of a BCV receptor on human and mouse BBMs was somewhat unexpected, as BCV has been frequently grown in the human cell line HRT18 and in suckling mouse brains. Receptors for BCV must be present on at least some human and mouse cells to explain these experimental infections, even though intestinal BBMs from these animals do not express detectable BCV receptors. VOPBAs or purified peplomer and hemagglutinin proteins will be necessary to separate the different binding specificities of the BCV peplomer and hemagglutinin proteins.

Discussion

The presence of MHV receptors only on BBMs and HMs from fully susceptible and semi-resistant mice and not on BBMs or HMs from fully resistant mice indicates that complete resistance to MHV is mediated by a lack of MHV receptor. However, partial resistance to MHV is mediated by a different mechanism such as inability of the host to replicate the virus or the type of host response generated in response to MHV infection in different mouse strains. For example in MHV3 infection the production of monocyte PCA activity in response to MHV infection in different strains of mice correlates with the different disease manifestations (Levy et al., 1981). To explore the hypothesis that the limitation in MHV tropism is at the level of MHV receptors, the MHV receptor is being cloned in our laboratory. Transfer of the cloned receptor to receptor negative cells and demonstration of

resulting infectability of these cells would definitively prove the role of the MHV receptor in virus attachment and penetration. This method has been used with EBV in which EBV receptors from Raji cell membranes were purified and transferred to murine lymphocytes or to cells from a human T cell line which are normally resistant to EBV infection. After transfer of EBV receptors to these cells, EBV penetration and replication could be seen (Volsky et al., 1980). While SJL/J mice do not express MHV receptors on its intestinal BBMs and HMs, SJL/J mice may express a gene for a homologous protein, or this gene may not be expressed in SJL/J mice, or may code for a protein that is changed in such a way as to abolish MHV binding, though other functions of the protein may be retained. Clones of the MHV receptor will allow us to determine if SJL/J mice lack MHV receptors because they lack the gene which encodes the MHV receptor or because a cryptic gene exists which can not be transcribed or because the gene product of a functional homologous gene is altered, degraded or not produced. Further research is needed to determine why the MHV receptor is larger in C57BL/6 intestine. Endo F digestions of BALB/c and C57BL/6 intestinal MHV receptors did not conclusively prove whether differential glycosylation occurred between the BALB/c and C57BL/6 intestinal MHV receptors. The smearing and reduction of MHV receptor activity seen after treatment with higher concentrations of Endo F is probably due to protease digestion rather than loss of MHV binding sugars. Loss of receptor binding activity with Endo F digestion would normally imply that the virus was binding to sugar moieties, but two pieces of evidence indicate that MHV-A59 does not bind to sugars. First, Endo F digestions done by Dr. Richard Williams on gel-eluted BALB/c MHV receptor yielded MHV receptor activity in a protein of approximately 65 kilodaltons, and no concanavalin A binding could be detected on this protein; therefore, it was probably

completely deglycosylated. Second, anti-receptor antibody binding has been detected to non-glycosylated MHV-receptor-B-galactosidase fusion proteins made in E. coli (personal communication, K. Holmes and C. Stephensen). Clones of the MHV receptor will be useful in determining whether the size difference is due to a difference between the C57BL/6 and BALB/c MHV receptor genes or is the result of action on the MHV receptor by a gene product from a gene other than the receptor gene.

While Dr. A. Smith has shown that five strains of MHV bind to the same receptor in tissue culture cells, we still do not know if they all bind to the same receptor in vivo. Rhinoviruses appear to recognize the same receptor in vivo and in vitro. Administration of anti-receptor antibodies to rhinoviruses in chimpanzees prior to rhinoviruses challenge has proven useful in blocking rhinovirus infection (Colonno et al., 1987). Currently, our anti-MHV antibodies are being tested in mice for their ability to block MHV infection. The fact that MHV-A59 and MHV3 bind to the same size receptor on BALB/c BBMs, indicates that the different strains of MHV probably bind to the same MHV receptor in intestinal brush borders.

The role of MHV receptors in tissue tropism still needs to be investigated. Immunoelectron microscopy of sections from BALB/c and SJL/J mice is in progress. While binding to tissue culture cells of all five MHV strains tested were inhibited by anti-MHV receptor antisera, the titer of antibodies needed for inhibition differed depending on the the virus strain used. This suggests that the different MHV strains may have different affinities for the MHV receptor. Differences in the type and number of different MHV receptors in different tissues may account for the different tropisms. For example, neurotropic strains may have a higher affinity for MHV receptors in the brain than hepatotropic strains, and vice versa.

I have shown that MHV receptors are absent from intestinal BBMs from all species tested except susceptible mouse strains. The lack of MHV receptors on other species appears to determine the narrow host range of MHV. The fact that MHV can infect neonatal rats when injected intracerebrally in fairly large doses, indicates that some form of MHV receptors are probably present in rat brain, though they may be limited in number. These receptors may or may not be similar to those present on intestinal brush borders or livers of mice. While anti-MHV receptor antisera did not detect any proteins antigenically related to the MHV receptor, the epitope on the receptor to which MHV binds may be present in other species on a completely different protein. MHV receptor clones will be used to determine if any gene(s) exist in other species which share significant homology with the MHV receptor gene.

The ability to develop the solid phase receptor binding assays for 6 other coronaviruses (CCV, FIPV, TGEV, HCV-229E, HCV-OC43 and BCV) implies that this type of assay may be adaptable for studies of other viral receptors. Development of better immunological reagents for these other coronaviruses comparable to goat anti-MHV E2, should permit the development of VOPBA assays for these other coronaviruses. I found that rabbit antisera contained very high levels of antibodies cross-reactive with BBMs of all species, while goat anti-MHV-E2 did not contain antibodies which cross-reacted with BBMs. Early attempts at VOPBAs using rabbit anti-MHV serum were unsuccessful (Boyle and Holmes, personal communication). At present, we are producing goat antisera against HCV-OC43 and HCV-229E in the hope that these antisera will allow us to develop VOPBA assays for these human coronaviruses. Development of anti-receptor antibodies for any of the other coronaviruses would also be very useful but at

present we do not have the convenience of a receptor negative strain of these animals, like we had for MHV, in which to raise anti-receptor antibodies. Therefore it may be necessary to employ other strategies such as development of monoclonal antibodies to BBMs which could potentially block coronavirus infection.

While MHV receptors appear to be very species-specific, being present only in mice, receptors for the antigenically related CCV, FIPV, TGEV and HCV-229E appear much less species specific. CCV, FIPV, TGEV and HCV-229E bound to BBMs from dog, cat, pig and human intestines. The presence of receptors for all four of these viruses on the intestines of these four species may account for the greater degree of cross infection seen in vivo and in vitro with these viruses compared to MHV (Tables 5 and 6). At present it is not clear whether all of the these viruses bind to a common receptor expressed in intestines of different species or if different receptors exist for each of these viruses on each intestine. It is clear that the receptor(s) for CCV and FIPV on dog and pig intestines are SDS insensitive like the MHV receptor, while the receptor(s) for CCV and FIPV on cat, human and cow are SDS sensitive. This suggests that there are at least two receptors or receptor classes for coronaviruses in antigenic group 2. Further characterization of these receptors is needed. Competition studies, in which one virus is allowed to bind and then binding of a second labeled virus is measured could possibly determine whether these viruses recognize the same or different receptors. Development of VOPBAs with these viruses or development of anti-receptor antibodies against these virus receptors, will also be useful in determining whether these virus bind to the same or different receptors. If the coronavirus receptors for these viruses turn out to be the same, then the species specificity seen in these coronaviruses is probably due to differences

in the host cell's ability to replicate the virus, or the host's response to the virus. While each of these viruses binds to BBMs from each of these species, it is not yet certain whether this binding could lead to infection. To determine if this binding leads to infection, anti-receptor antibodies must be developed and tested to see if they block infection in vitro, as was done with anti MHV-receptors on MHV-infected L cells in our laboratory, and with anti-rhinovirus receptor antibodies on rhinovirus infected-HeLa cells in the laboratory of Dr. R. Colonno (Colonno et al., 1986). The ability of an antibody to block infection in vitro does not imply that it will also block infection in vivo, as some viral receptors may be lost when cells are cultured. For example, HLA antigens have been identified as receptors for SFV on some cell lines and acetylcholine receptors have been identified as receptors for rabies on neural cells, but these viruses replicate in cells in vitro which lack these molecules (Oldstone et al., 1980; Reagan and Wunner, 1985). This suggests that alternative viral receptors other than the HLA antigens and the acetylcholine receptor exist for these viruses on some but not all cell lines.

I was particularly interested in studying the receptors for the human coronaviruses HCV-229E and HCV-OC43. In particular I would like to determine whether they bind to the same or different receptors. HCV-229E and HCV-OC43 are serologically unrelated with HCV-229E belonging to the second antigenic group for which cross-reactive receptors may exist, and HCV-OC43 belongs to the first serological group with MHV for which virus receptors appear to be species-specific. While both HCV-229E and HCV-OC43 have tropisms primarily for human respiratory tissues, both viruses can be grown in cell lines whose origins are intestinal, and the existence of human enteric coronaviruses which may be variants of HCV-OC43 encouraged me to look for coronavirus receptors on human BBMs. In the future, it would also

be desirable to look for HCV receptors on respiratory tissues. I developed a solid phase receptor assay for HCV-229E (Figure 23), and C. M. Hay developed one for HCV-OC43 (data not shown). HCV-OC43 and HCV-229E differ in one major way and that is that HCV-OC43, but not HCV-229E, possesses a hemagglutinin spike on its surface. This third viral glycoprotein may serve as a second or alternative receptor binding protein. The existence of a hemagglutinin on HCV-OC43 complicated the development of the solid phase receptor assay causing the virus to bind to most blocking agents and resulting in a very high background. Because of limited human intestinal tissue, only one human coronavirus receptor could be more fully investigated , so I chose to pursue investigations on the HCV-229E receptor.

While I was unable to investigate fully HCV-OC43 receptors, I did investigate receptors for another coronavirus in the first antigenic group, BCV. Like HCV-OC43, BCV virions contain a hemagglutinin glycoprotein which as well as the peplomer protein may recognize cell surface receptors. At present, it is not clear which of these viral glycoproteins is responsible for binding to bovine BBMs observed in the solid phase assay for BCV. To identify which viral glycoproteins the cell surface receptor is binding to, assays must be performed with purified peplomer and hemagglutinin in VOPBAs, or in VOPBAs with whole virus which has been incubated with anti-peplomer or anti-hemagglutinin sera. The ability of BCV to bind to porcine as well as bovine BBMs is not too surprising since HEV, an antigenically related virus, infects porcine intestines and because BCV grows quite well in porcine cell lines. Even though BCV is frequently grown in human cell lines and in suckling mouse brains, and has been reported to have caused a case of BCV- induced diarrhea in a human laboratory worker, no binding was seen to human or mouse BBMs. The lack of binding to mouse

BBMs may be a reflection of the fact that BCV replication in mice is in the brain not the intestine. Lack of detectable BCV binding to human BBMs may indicate that while receptors for BCV may exist on human intestine, they are at a level below detection by the solid phase receptor assay. Unexpectedly high levels of BCV binding were seen to dog BBMs. The HCV-OC43 hemagglutinin binds to many proteins on RBCs and probably on host cells most likely via sugar moieties on glycoproteins (C. M. Hay and K. V. Holmes, personal communication). The BCV hemagglutinin may also bind to sugar moieties present on many glycoproteins, and therefore, the BCV binding seen on dog BBMs may be due to binding by the hemagglutinin to sugar moieties found in high concentration on dog BBMs. Further research is needed to elucidate the difference in binding by the hemagglutinin and peplomer proteins of BCV and HCV-OC43 to target tissues.

The presence of receptors for CCV, FIPV, TGEV and HCV-229E on cow BBMs was quite surprising as I had previously only seen binding of coronaviruses to species which were susceptible to antigenically related coronaviruses. There could be several explanations for this finding. First, cows may express a molecule on their intestines to which these viruses bind but which does not serve as a classical receptor, in that it is not used to internalize the virus. Second, these viruses could bind to an epitope of the BCV receptor which is also present on CCV, FIPV, TGEV and HCV-229E receptors but that the portion of the E2 protein which bind this epitope does not induce antibodies.

In the last few years, very exciting discoveries have been made concerning reovirus and rhinovirus receptors which could influence future research on coronavirus receptors. Reovirus strains 1 and 3 have very different tropisms which are determined by the hemagglutinin protein of

these viruses. Reovirus type 1 binds to ependymal cells from mouse brain, while reovirus type 3 binds to murine neurons and human and murine lymphocytes (Weiner and Fields, 1977; Weiner et al., 1980). A large panel of anti-idiotype monoclonal antibodies was made against murine lymphocytes. These monoclonal antibodies blocked binding of ^{125}I-labeled reovirus type 3 to lymphocytes. (Nepom et al., 1982; and Tardieu et al., 1982). Using anti-idiotype antibodies, a reovirus type 3 cell surface receptor was isolated which is present on cells from diverse tissue types from rat, mouse, human and monkey (Co et al., 1985a). The reovirus type 3 receptor is believed to be the same as the beta-adrenergic hormone receptor based on identical molecular weights, pIs and tryptic peptide sizes, the ability of anti-reovirus receptor antibody to immunoprecipitate purified beta-adrenergic receptor, and the ability of purified reovirus receptor to bind the beta-adrenergic antagonist [^{125}I]-iodohydroxybenzlpindolol (Co et al., 1985b). While, anti-idiotype antibodies have been used in identifying receptors for drugs, this is the first instance of anti-idiotype antibodies being used to identify a virus receptor. Anti-idiotype antibodies might also be useful in identifying coronavirus receptors.

A 90 kilodalton receptor for 78 of 88 serotypes of human rhinoviruses (HRVs) was identified using blocking of HRV infection of HeLa cells by anti-HeLa cell monoclonal antibodies. These monoclonal antibodies also protected HeLa cells by infection with three Coxsackie A virus serotypes and bound only to human and chimpanzee cells (Colonno et al., 1986). The atomic structure of the rhinovirus attachment protein has been elucidated and the receptor binding site is believed to be a "canyon" with five-fold symmetry (Rossmann et al., 1985). This "canyon" is too small for an antibody to fit into, which explains how the 78 of 88 rhinovirus serotypes which share

no common antigenically recognized site can bind a common receptor. This information is being used to design anti-viral drugs which can fit in the "canyon" and block viral attachment. Another anti-viral "drug" which blocks viral attachment, soluble CD4 has been shown to block infection of lymphocytes by HIV in culture and is scheduled to be tested in humans soon (Deen et al., 1988; Fisher et al., 1988; Hussey et al., 1988; Traunecker et al., 1988). The potential clinical use of anti-receptor antibodies or anti-viral drugs targeted at viral receptors is very exciting. However, an important factor to keep in mind with the use of anti-receptor drugs or antibodies is that virus receptors may have important functions (such as the acetylcholine receptor to which rabies viruses binds) in the cell which when blocked may lead to massive side effects.

In summary, our new solid phase receptor assays have been very useful in the study of coronavirus species specificity. The lack of MHV receptors on all species except mice appear to account for MHV's narrow host range. All MHV strains appear to bind to the same receptor on tissue culture cells and probably also on BBMs. CCV, FIPV, TGEV, and HCV-229E express receptors on BBMs from all four species naturally susceptible to these viruses, and BCV binds to BBMs from cow and pig. Using these new solid phase assays and VOPBA assays, it should be possible to determine whether CCV, FIPV, TGEV, and HCV-229E bind to the same receptor, whether serologically unrelated viruses, like HCV-229E and HCV-OC43, which infect the same host, bind to the same receptor, and to determine the individual roles of the hemagglutinin and peplomer proteins of BCV and HCV-OC43 in binding to susceptible tissues. These assays may also be able to be modified for the identification of virus receptors for other viruses.

DISCUSSION

I developed a in vitro replication for MHV-A59 utilizing lysolecithin. With this assay I showed that antiserum to the N protein inhibited RNA replication, and that the RNA synthesized in vitro is present in a RNase ribonucleoprotein complex. Experiments performed in the laboratory of Dr. M. Lai, after most of my work on replication was completed, showed that the leader primed model of coronavirus replication is probably correct and that MHV RNA undergoes recombination at high frequency (Baric et al., 1985; Budzilowicz et al., 1985; Lai et al., 1985, Makino et al., 1986a and 1986b, Shieh et al., 1987). Evidence for the leader-primed model of RNA replication in coronaviruses is as follows: free leader, 50-90 nucleotides in length has been found in the cytoplasm of cells infected with a RNA minus mutant of MHV; second, temperature sensitive mutants exist which make only leader RNA at nonpermissive temperatures ; third, in mixed infections with MHV-A59 and JHM, progeny viruses have been recovered with leader from one strain and the body of the mRNA from the other strain ; and last, intergenic regions have been shown to possess regions of homology with the leader at which the leader could bind and act as a primer for transcription, with the degree of homology being postulated to be one mechanism for regulating the quantities of different mRNAs produced (Baric et al., 1985; Budzilowicz et al., 1985; Makino et al., 1986b, Shieh et al., 1987).

RNA recombination in coronaviruses was demonstrated by infection of cells at non-permissive temperatures with two strains of MHV of which one or both of the strains were temperature sensitive. Progeny virus was shown by T1 mapping to possess sequences from both strains of virus (Lai et al., 1985, Makino et al., 1986a). Recombination rates may be as high as 10%,

based on the yield of progeny virus (Makino et al., 1986a). The new model for MHV replication involves synthesis of leader RNA, dissociation of the polymerase-leader RNA complex from the template, reassociation at intergenic sequences based on sequence homology between the leader and intergenic regions, followed by transcription. During transcription, the RNA polymerase may pause at areas of high secondary structure and sometimes dissociate from the template. Later reassociation of the leader primed polymerase with the negative strand template occur, and recombination may result using the copy choice mechanism (Baric et al., 1987). While evidence for leader primed synthesis of coronavirus RNA, and the existence of recombination in coronaviruses is good, the mechanism of recombination is not yet clear.

While I have shown that virus receptors for MHV are present only on tissues from susceptible mice and probably play a role in the species specificity of MHV infection, the coronaviruses CCV, FIPV, TGEV, and HCV-229E bind to tissues from dog, cat, pig and human. Receptors for these viruses may be cross-reactive. These differences in species specificity of these coronaviruses is probably manifested in differences in the E2 protein. I have looked at the cellular aspect of virus binding, but studies on the differences between the E2 proteins of different coronaviruses have also recently been done. The E2 proteins of IBV, FIPV and MHV, which infect completely different species have been compared. They share approximately 30% homology at their carboxyl ends and no detectable homology was found in the rest of the molecule (deGroot et al., 1987). The carboxyl end of the E2 is the end of the molecule anchored in the membrane. It would be interesting to compare the E2 proteins of FIPV with CCV, TGEV or HCV-229E, since they bind to tissues from the same species. Studies by Dr. S. Snyder with fusion proteins

containing different portions of the E2 protein of MHV, obtained from Dr. W. Spaan, are in progress to determine the domain of E2 which binds to the MHV receptor.

RNA viruses are known to undergo rapid evolution, primarily via high rates of errors by RNA polymerases, but also via reassortment of viral segments as seen in reoviruses or by recombination as seen in picornaviruses and now in coronaviruses (Holland et al., 1982). Given that coronaviruses undergo recombination, it is interesting to speculate on how recombination has affected the evolution of coronaviruses, and in particular the viral attachment proteins. Recombination may permit rapid incorporation of new RNA sequences into the viral genome from other viruses, or from the cell. CCV, FIPV, TGEV and HCV-229E which bind to tissues from the same species, may have recently evolved from each other and still possess epitopes on their E2 molecules which react with receptors from other species, while MHV may have branched off from the other coronaviruses a longer time ago and may have evolved to bind only to molecules expressed on mouse cells. Also, it is interesting to speculate on the role of the hemagglutinin in virus binding. Hemagglutinins in general do not confer species specificity, and why some coronaviruses which possess E2 proteins which can confer species specificity also have hemagglutinins is unclear. The hemagglutinin of BCV, HCV-OC43, and HEV may be a recent addition to these viruses acquired via recombination from another virus. Recently it has been shown that BCV and OC43 bind, probably via the hemagglutinin, to sialic acid containing receptors similar to those bound by influenza C (W. Spaan, personal communication). Therefore, BCV and HCV-OC43 may have acquired their hemagglutinins from influenza C. MHV may have previously possessed a hemagglutinin which it lost, as it was not necessary for MHV's entry into susceptible cell

types. It is also interesting to speculate how more than one coronavirus evolved which infect the same species but which are antigenically different, such as HCV-229E and HCV-OC43. Did one evolve from the other, or did they evolve separately from coronaviruses which infected other species? With the development of VOPBAs for these two human coronaviruses, we hope to answer this question. While MHV receptors play a role in the narrow species tropism of MHV, it is still unclear whether the receptors play a role in tissue tropisms of MHV. Immunoelectron microscopy with anti-MHV receptor antibodies of several tissues from the mouse may help us to determine if MHV receptors are determinants of MHV tissue tropism. Unlike MHV receptors, the receptors for the antigenically related CCV, FIPV, TGEV and HCV-229E do not appear to be species specific, but they may play a role in the tissue tropisms of these viruses. Other tissues from dogs, cats, pigs and humans will be investigated for the presence of coronavirus receptors, in the future, using solid phase assays, and VOPBAs and anti-receptor antibodies when they become available.

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