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Host-cell Mediation Of Murine Hepatitis Virus (mhv) Persistence

Lee Alan Mizzen

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HOST-CELL MEDIATION OF MURINE HEPATITIS VIRUS (MHV) PERSISTENCE

by

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ABSTRACT

An *in vitro* study was undertaken to characterize the mechanisms by which the coronavirus, mouse hepatitis virus (MHV), mediates persistent infection. MHV persists in cultures of mouse fibroblast LM cells (designated LMTK$^-$), but produces a lytic infection in L-2 cells. Persistence in LMTK$^-$ cells was not accompanied by the production of virus mutants nor soluble antiviral factors. Rather, LMTK$^-$ cells possess two characteristics which allow cultures to support MHV persistence: a reduced level of initial infectability, and a resistance to virus-induced cell fusion. Similar host-cell determinants were found to be operative in persistent MHV infection of another mouse LM cell line, LM-ATCC. A semi-permissive category of host-cells was defined on the basis of reduced infectability by MHV. However, within this category, cell fusion-resistant (LMTK$^-$ and LM-ATCC) and permissive (mouse neuroblastoma; C-1300) hosts were identified. Evidence is presented that cell fusion by MHV is activated by a cellular chymotrypsin-like enzyme resulting in cleavage of the viral E_2 glycoprotein (180K MW) to a 90K MW form. Induction of cell fusion by proteolytically-activated E_2 was not observed in fusion-resistant host cells, due to some inherent property of the plasma membrane. Ammonium chloride, an agent reported to inhibit both virus penetration and cell fusion, was employed in an attempt to convert the acute MHV infection of L-2 cells to a state of persistence. Evidence for an endosomal uncoating mechanism by MHV was found on the basis of uncoating inhibition by ammonium chloride.

However, all other parameters of MHV replication, while chronologically displaced due to inhibition of virus uncoating were not otherwise inhibited by ammonium chloride, and the infection followed a lytic, fusogenic course. The expression of cell fusion in permissive L-2 cells was found to be associated with increased cellular permeability to sodium ions. The results of cell-free translation studies indicated that preferential synthesis of MHV proteins occurs in the presence of elevated sodium ion concentration.

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AMV	avian myeloblastosis virus
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
Ci	Curie(s)
CNS	central nervous system
cps	centipoises (viscosity)
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
dATP	deoxyadenosine 5'-triphosphate
DB	dissociation buffer
dCTP	deoxycytosine 5'-triphosphate
dGTP	deoxyguanosine 5'-triphosphate
ds	double-stranded
dTTP	deoxythymidine 5'-triphosphate
DI	defective interfering (particle or virus)
DTT	dithiothreitol
DMSO	dimethylsulfoxide
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis(2-amino ethylether)-N,N'-tetraacetic acid
ELISA	enzyme-linked immunosorbant assay
FCS	fetal calf serum
F.I.	fusion index
h	hour

HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSV	herpes simplex virus
IBV	infectious bronchitis virus
IP	immunoprecipitation
Kb	Kilobases
LCMV	lymphocytic choriomeningitis virus
MEM	minimal essential medium
MFM	methionine-free medium
MHV	mouse hepatitis virus
min	minutes
moi(s)	multiplicity (multiplicities) of infection
mRNA	messenger ribonucleic acid
MW	molecular weight
NDV	Newcastle disease virus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming unit(s)
pg	picogram
P.I.	post-infection
PML	progressive multifocal leucoencephalopathy
PMSF	phenylmethyl-sulfonylfluoride
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RSB	reticulocyte standard buffer
SDS	sodium dodecyl sulfate
SM	standard medium

Sp.act	specific activity
ss	single-stranded
SSC	standard saline citrate
SSPE	subacute sclerosing panencephalitis
TBS	Tris-buffered sorbitol
TEMED	N,N,N',N'-tetramethylenediamine
TLCK	N-∝-tosyl-L-lysyl-chloromethyl ketone
TPCK	N-∝-tosyl-phenylalanyl-chloromethyl ketone
tRNA	transfer ribonucleic acid
ts	temperature-sensitive
ug	micrograms
VSV	vesicular stomatitis virus
ZPCK	N-CBZ-L-phenylalanyl-chloromethyl ketone

CHAPTER 1

INTRODUCTION

Virus persistence can be considered to operate at three levels: persistence in the population, in the individual, and at the cellular level. The first aspect is more amenable to epidemiological study and will not be discussed here; the latter two aspects are applicable to the present work and will be discussed in turn.

1.1 Persistence in the Individual

1.1.1 Genome Integration and/or Maintenance of Viral Nucleic Acid

One mechanism by which virus can persist within the host is through integration into cellular DNA of somatic or germ line cells. Genome integration as a means of persistence has been well characterized for the retroviridae virus family (reviewed by Bishop, 1983). The retroviruses are RNA viruses that possess a virion-associated reverse transcriptase which copies the diploid ssRNA genome into a haploid dsDNA form (termed provirus) within the infected cell. Persistence in the provirus form does not require virus gene expression and so may circumvent host immune detection. By integration into germ line cells or by vertical transmission to offspring in sperm, egg or milk, these viruses can persist through generations.

Visna virus, a member of the lentivirinae (slow virus) subfamily of the retroviridae causes a slow, progressive disease of the central nervous system (CNS) of sheep. Virus can persist in the host,

sometimes for years, despite an immune response. Some of the best evidence for genome integration has come from studies with visna virus, where viral DNA can be detected by nucleic acid hybridization in the tissues of infected sheep, despite little or no viral antigen production (Haase et al., 1978). While acute infection is restricted by the host immune response, cells with integrated genomes go undetected. Periodically, latently infected cells release infectious virus, which, because of antigenic variation, is able to survive and trigger another episode of acute disease (Scott et al., 1979). Such antigenic variants, as well as variants arising in different animals, have point mutational changes clustered around the 3'-terminus of the RNA genome, apparently within a gene coding for a major glycoprotein antigen (Clements et al., 1980; 1982).

A further example of persistent virus infection involving maintenance of viral DNA is encountered with certain members of the herpesvirus group. Following primary infection with herpes simplex virus (HSV), latency may be established that enables the virus to persist throughout the lifetime of the host. HSV commonly establishes latent infection in the sensory ganglia, from which it may be reactivated at a later time, resulting in recrudescence of a lesion in the dermatome innervated by the sensory ganglion (Baringer et al., 1975).

Definitive evidence regarding the state of HSV or its DNA genome within latently infected sensory ganglion cells is lacking (Minson, 1983). Through explant culture of neural tissue in vitro, infectious virus can be reactivated from latently infected ganglia (Baringer et

al., 1975). In cultures which fail to release virus, viral DNA can be detected or virus release activated by superinfection with defined temperature-sensitive (ts) mutants (Brown et al., 1979; Lewis et al., 1984). It appears that HSV DNA expression is repressed in latently infected cultures, since, although DNA can be detected, little or no viral RNA is present (Puga et al., 1978). There is evidence that HSV DNA exists in a different form in latently infected ganglia than in acutely infected nervous tissue, where normal unit-length linear DNA was found (Puga et al., 1984; Rock and Fraser, 1983). These observations suggest that HSV latency may involve provirus integration as described for the retroviridae.

1.1.2. Immunomodulated Virus Persistence

The papovaviruses BK and JC are widespread in the human population (Padgett, 1980), and although BK virus has not been associated with disease, the presence of JC virus has been correlated with a rare demyelinating disease of the human CNS known as progressive multifocal leucoencephalopathy (PML; Walker and Padgett, 1983). While JC virus may remain latent in the general human population, it can be reactivated under conditions of immunosuppression to manifest PML disease. Studies on the nature of JC or BK virus DNA in normal and diseased human cadaver tissue have demonstrated that in fatal PML cases, up to 1000 copies per cell of JC virus DNA can be detected in affected areas of the brain, and much smaller numbers (0.2-10 copies/cell) in the kidney (Grinnell et al., 1983). When non-PML cases were examined, JC DNA could only be

detected in the kidney (Chesters et al., 1983). It has been suggested (Chesters et al., 1983) that JC infection of the kidney, during severe immunosuppression, can spread to the brain to cause PML. None of the studies reported thus far have detected integrated JC or BK DNA in human brain or kidney, and in this regard they resemble other papovaviruses associated with more acute infections, such as the papilloma viruses (Gissman, 1984).

A number of well-characterized animal virus infections are not associated with acute disease symptoms, but infectious virus can be recovered from serum or tissue throughout the life of the host. Such disease states are often associated with virus infection of lymphoreticular tissues (such as the spleen, liver and lymph nodes). However, some antiviral antibodies are induced, and in many cases, circulating immune complexes may eventually result in disease.

Probably the best characterized example of such infections is the life-long carrier state induced by lymphocytic choriomeningitis virus (LCMV) in mice. Persistent infection of the mouse with LCMV is a natural phenomenon, and many wild Mus musculus carry the virus (Lehmann-Grube et al., 1983). The virus is transmitted in utero from mother to offspring with essentially 100% efficiency; it appears that the viral genome is not integrated into either germ cells or differentiated tissue cells, but replicates in the cytoplasm, regulated by a mechanism not yet well understood. Studies involving in vitro LCMV replication have suggested that persistent infection may be a totally intracytoplasmic process involving transfer of infectious material within large, enveloped vesicles via cell-cell

contact (van der Zeijst et al., 1983). However, in vivo, the relevance of these findings remains to be determined; rather, two other mechanisms have been proposed to explain the lifelong persistence of LCMV in carrier mice: 1. T-cell unresponsiveness, and 2. DI particle generation. Of these two mechanisms, the first is generally recognized to be of primary importance.

After infection in utero, circulating antibodies or specific cell-mediated immunity to LCMV are not detectable; the mice appear normal for 9-12 months in spite of widespread viral multiplication that produces persistent viremia, with viral antigens present in most organs (Lehmann-Grube et al., 1983). However, infection of a non-carrier adult mouse results in a rapidly lethal infection of the CNS, associated with cell-mediated immune attack of affected tissues. The persistence of LCMV in mice receiving virus in utero is thought to be due to the absence of virus-specific cytotoxic T-cells (Cihak and Lehmann-Grube, 1974, 1978). This state of T-cell "tolerance" in carrier mice has been suggested to reflect the elimination of LCMV-reactive clones in neonatally or congenitally infected mice (Cihak and Lehmann-Grube, 1978). Later in life, many carrier mice develop a late-onset disease due to deposition, particularly in the kidneys, of immune complexes consisting of antibody and LCMV (Oldstone, 1975). Why these carrier mice eventually mount on antibody response to LCMV is not known, but presumably reflects a breakdown in the state of tolerance in adulthood.

During replication of LCMV both in vitro and in vivo, a class of virus particles are produced that are non-infectious but interfere

with infectious virus replication (Popescu et [redacted] 1976). Since these LCMV interfering particles (IP) are prod[redacted]n carrier mice (Popescu and Lehmann-Grube, 1977) they may play a role in the persistent infection. Unlike classical DI particles (Huang and Baltimore, 1970) these LCMV-IPs do not exhibit the amplification phenomenon (ie. ability to enrich itself at the expense of standard virus). Furthermore, unlike the random genome deletions of DI particles, these IPs specifically lack the S (small) RNA species of LCMV (Martinez-Peralta et al., 1981). It appears then, that these LCMV IPs are biologically active by-products of virus synthesis, being produced whenever infectious virus replicates (Lehmann-Grube et al., 1983). While these IPs are constantly produced in carrier mice, their role in LCMV persistence remains to be characterized (see section 1.2.1).

1.1.3. Persistence Associated With Defective Virus Replication

An interesting example of virus persistence which involves defective assembly/release of virus is seen in certain rare cases of measles virus infections. Normally, measles virus causes an acute febrile illness of children accompanied by a characteristic rash. However, two types of interaction between measles virus and the CNS are rare complications of the disease: acute encephalitis, and subacute sclerosing panencephalitis (SSPE). Encephalitis results from an acute cytopathic infection of certain brain areas, from which measles virus can be isolated. In SSPE, however, a period of 6-8 years elapses after measles infection before clinical manifestations

appear, after which a slow, progressive neurological impairment develops which is invariably fatal (Fraser and Martin, 1978). Associated with the disease are high levels of measles-specific oligoclonal IgG in the cerebrospinal fluid plus high antibody titres against measles virus in the serum. On post mortem, accumulations of measles-virus nucleocapsids within neurons, oligodendrocytes and astrocytes are observed as well as lymphocytic infiltration (ter Meulen et al., 1983). Viruses isolated from SSPE-neural tissue by cocultivation have been compared with measles virus from acute infection, but no significant differences were found that could explain the persistent infection (Wechsler and Meissner, 1982). Since antibodies made in response to acute measles infection neutralize measles or SSPE-derived measles virus to an equal extent, it has been proposed that either measles virus becomes established in the CNS at the time of original infection and remains latent for several years, or the virus persists in lymphoreticular cells from which it enters the CNS at a later time (ter Meulen et al., 1983). The mechanism by which SSPE virus persists in infected brain cells involves a defect in the expression of the viral matrix protein, which prevents formation of fully infectious virus (Hall and Choppin, 1979). Cell lines derived from SSPE brain tissue cultivated in vitro are also deficient in matrix protein, although other viral proteins are present (Machamer et al., 1981). In one such SSPE cell line, the matrix protein mRNA was present but inactive in an in vitro translation system (Carter et al., 1983). These results suggest that the matrix protein mRNA is functionally altered, perhaps by

frameshift or termination mutation so that matrix protein synthesis does not occur and virus assembly is prevented (Carter et al., 1983).

1.2 Persistence in the Cell

Virus persistence at the cellular level has been extensively investigated through establishment of persistent virus infections in tissue culture cells (reviewed by Friedman and Ramseur, 1979; Youngner and Preble, 1980). Mechanisms by which persistence is induced and maintained can be studied more readily in vitro; such mechanisms usually involve one or a combination of the following three mechanisms: generation of defective interfering (DI) virus particles, generation of temperature-sensitive (ts) virus mutants or induction of endogenous interferon in the host cell.

1.2.1 Defective Interfering Particles

Defective interfering (DI) particles represent a major controlling element of virus replication. They are constantly generated at low levels by infectious virus and only amplify to interfering levels when the parent helper virus is abundant. This autointerference phenomenon was first recognized by von Magnus (1954) in preparations of influenza virus propagated by serial undiluted passage of virus in eggs. The widespread occurrence of DI particles, particularly among RNA viruses has since been recognized (Huang and Baltimore, 1970; Holland et al., 1980). It is generally agreed that RNA viruses (with the exception of retroviruses) lack the ability to integrate dsDNA copies or fragments of their genome into eucaryotic host cell chromosomes. RNA viruses may therefore rely on alternative

mechanisms of attenuation, such as DI particles, to achieve long term virus-host cell association. As defined by Huang and Baltimore (1970), the criteria for the definition of DI particles are as follows: 1. antigenic similarity to standard virus, 2. requirement for homologous parent virus as helper for replication, 3. preferential replication of DI over standard virus (amplification), and 4. interference with the replication of the standard virus. Another important characteristic of DI particles is that their generation is very dependent upon host-cell type (Huang and Baltimore, 1970; Holland et al., 1976; Perrault and Holland, 1972). In general, the requirement for helper virus as well as interfering activity is limited to the homologous parent or serologically related strains. The nature of the helper function supplied by the standard virus varies in different virus systems. For example, wild-type vesicular stomatitis virus (VSV) provides all five virus-coded proteins for replication and encapsidation of a DI RNA template which does not code for any translation products (Huang and Baltimore, 1970). In poliovirus, however, the standard virus helper, in some cases, provides the missing capsid proteins to a DI RNA which encodes all other viral proteins (Phillips et al., 1980).

Because of the antigenic similarity of DI and parent virus, the biological properties of DI particles are attributable to the genome deletions they contain. Their interfering and preferential replication properties are due to the nature of the DI template itself, which, intracellularly, competes for the viral polymerase (Perrault, 1981).

Thus far, all DI particles examined critically contain deletions in a part of the genome essential for infectivity. Trivial explanations for the generation of DI RNAs, such as RNA chain scission or premature termination during synthesis are insufficient to explain the conservation of genomic ends or the presence of inverted terminal complementarity, characteristic of DI genomes (Holland et al., 1980). These unique features of DI particles have led to the proposal of a "template-switching" and "copy-back" model for DI generation in negative-strand viruses (Huang, 1977 ; Leppert et al., 1977). The essential feature of this model is a viral replicase detaching from its template (while remaining attached to the nascent chain), and then binding back near the 5'-end of the nascent chain to initiate copying in the reverse direction. This model accounts for many features of DI RNAs, including the retention by most of a large fragment from the 5'-end of the standard genome. However, to account for other features of DI RNAs, such as internal genome deletions, a more general mechanism has been proposed (Perrault, 1981), designated the "leaping replication complex" model, whereby the "copy-back" mechanism operates in conjunction with a transcribing replicase complex that can leap to downstream sites either on the template strand or resume synthesis on the nascent chain carried by the leaping polymerase complex. Many of the current concepts regarding the origin and replication of DI particles are derived from the VSV system which has been studied in most detail, and serves as a model for negative-strand RNA viruses in general.

In the positive-strand RNA virus group, both togavirus and

picornavirus DI particles have been studied in some detail. Briefly, this group, including the double-stranded members such as reovirus, appear to all generate DI particles with internally deleted genomes with retention of 5'- and 3'-terminal sequences (Ahmed and Fields, 1981; Cole, 1975; Kennedy et al., 1976). Furthermore, in the case of viruses with segmented genomes, such as the negative-strand influenza and positive-strand reovirus, partial or complete segment deletions have been mapped to polymerase-encoding genes (Davis et al., 1980; Nonoyama and Graham, 1970). Therefore the mechanisms proposed above for DI genome generation may be applicable, in one form or another, to all RNA viruses.

Since DNA viruses commonly mediate persistent infections through genome integration and less well defined latenoy states, the role of DI particles in these infections is considered to be of lesser importance in nature than with RNA viruses. Nonetheless, DI particles have been demonstrated for all DNA viruses, with the possible exception of adenoviruses and poxviruses, although their role in persistence is at present unclear (Perrault, 1981). Unlike the DNA viruses, DI particles of RNA viruses have been recognized for some time, and are firmly linked to persistent infections, as studied in vitro. The pioneering studies of Holland and Villareal (1974) provided the first clear evidence for a DI requirement in establishing stable VSV carrier cultures in vitro. They established a persistently infected BHK cell culture by coinfection with a temperature-sensitive (ts) mutant of VSV and its homologous DI particle. These cultures have continuously shed low levels of

infectious virus and DI particles. The DI particles are similar, if not identical, in structure and activity to DIs generated by high multiplicity passage in permissive cell lines (Holland et al., 1980, 1982). Another important feature of these cultures is that the DI particles act as a selection pressure for the rapid and extensive mutational drift of standard virus during long-term persistence (Holland et al., 1979). This is exemplified by the rapid emergence of standard virus mutants no longer subject to interference by the original DI particle used to initiate the culture. The new standard virus, however, can generate new DI particles which interfere with its own growth (Holland et al., 1982; Horodyski and Holland, 1984). This important regulatory phenomenon has been observed in other virus systems, including rabies virus (Honda et al., 1985), Sindbis virus (Weiss and Schlesinger, 1981) and West Nile virus (Brinton and Fernandez, 1983).

Modulation of the infectious process by DI particles has been demonstrated in vivo for a variety of viruses. Pre-or coinfection of mice with large amounts of purified DI particles provided prophylaxis against challenge with standard virus for VSV (Jones and Holland, 1980), Semliki Forest virus (Barrett and Dimmock, 1984), and reovirus (Spandidos and Graham, 1976). Interestingly, UV-inactivated VSV DI particles possessing double-stranded RNA were somewhat protective (Jones and Holland, 1980), presumably due to their capacity to induce interferon (Marcus and Sekellick, 1977). However, in all these protection studies, the large dosages of DI particles required for protection were also immunogenic, and so the relative contribution of

DI particles *in vivo* to early protection via humoral antibody vs. true interference with standard virus replication remains unclear. As mentioned in section 1.1.2, the persistent LCMV infection of mice may be, in part, due to DI particle-like virus. These interfering particles can be demonstrated in the organs (Popescu and Lehmann-Grube, 1977) as well as peritoneal macrophages of carrier mice (Welsh and Oldstone, 1977). Coupled with the finding that LCMV DI particles protect against acute cerebellar disease (Welsh *et al.*, 1977), these results suggest a possible involvement of DI particles in persistent LCMV infections, *in vivo*.

1.2.2 Temperature-sensitive mutants

The appearance of temperature-sensitive (ts) virus mutants is another common finding in persistently infected tissue culture cells. The ts lesion is usually a missense mutation in the viral genome which results in production of an altered, but functional protein at permissive temperatures, but at non-permissive temperatures, either no protein or a non-functional protein is formed. If the lesion is within a structural gene, the mutant virus may be heat-labile compared to wild-type virus (reviewed by Youngner and Preble, 1980).

A large body of evidence implicates ts viral mutants as the persisting species in a variety of chronic infections initially established with wild-type virus. In early studies on persistently infected cell cultures, an L cell line chronically infected with Newcastle disease virus (NDV) was maintained, and virus isolated from such cultures displayed temperature-sensitive replication, increased

thermal lability, and reduced virulence when administered to susceptible mice (Preble and Youngner, 1973). Biochemical characterization of these NDV ts mutants showed that they did not synthesize viral RNA (RNA(-)) at non-permissive temperatures due to defective RNA polymerase activity. Using a mixture of standard and DI particles of VSV, Youngner et al. (1976) established a persistent infection in L cells which, with time, shed an ever increasing proportion of ts phenotypes, until after two months all released virus was ts. The ts virus was all of one complementation group (group 1, defective in RNA synthesis) and was able to induce new persistent infections in L cells. Interestingly, DI particles were not detected, suggesting that these were not necessary for initiation and maintenance of persistence in this system. Futher evidence for the singular role of ts mutants in some persistent infections comes from the demonstration of Youngner et al. (1981) that in persistently infected BHK cell cultures, which are interferon-incompetent, ts mutants accumulated under conditions where DI particle production was restricted. However, some ts mutants can interfere with standard virus replication, much like DI particles (Youngner and Quagliana, 1976), and so the role of interference, per se, cannot be immediately ruled out in persistent infection with ts virus.

Extensive genetic characterization of VSV and other rhabdovirus ts mutants has been carried out by Pringle (1977). About 90 % of spontaneous or induced ts mutants map to the gene encoding the viral polymerase (L protein). At non-permissive temperatures, this mutant polymerase is either non-functional (hence, an RNA(-) phenotype) or

transcribes RNA with increased mutational frequency. Translation of such RNA could give rise to proteins with altered structure and biological activity (Holland et al.,1982). While the vast majority of isolated VSV ts mutants are polymerase defective (complementation group 1), other ts mutants have been mapped to the genes coding for the matrix protein (M; group 3) and the spike protein (G; group 5; Youngner et al., 1978).

Although best characterized in the rhabdovirus system, examples of in vitro persistence involving production of ts mutants exist for a wide variety of RNA viruses.

Like the rhabdovirus ts mutants, isolates from cultures persistently infected with alphaviruses often display the RNA(-) phenotype at non-permissive temperatures (Keranen et al., 1977; Maeda et al., 1979). Furthermore, alterations in the expression or biological activity of structural proteins of ts mutants, like those known for VSV, have been clearly demonstrated in chronic infections involving corona- (Baybutt et al.,1984) and paramyxoviruses (Roux and Waldvogel, 1982).

In cultures persistently infected with the coronavirus, mouse hepatitis virus (MHV), a ts mutant was isolated which had structural alterations in the spike glycoprotein, E2. This mutant displayed decreased cytopathic effect in vitro and reduced neurovirulence in vivo, two properties ascribed to the E2 protein (Baybutt et al., 1984). A Sendai virus ts mutant, isolated from persistently infected BHK cells was blocked at a late stage of maturation at non-permissive temperatures due to a ts lesion in the viral matrix (M) protein,

which must co-operatively interact with other structural proteins for proper virion assembly at the plasma membrane (Yoshida et al., 1979). Associated with the ts lesion in the M protein was a reduced or improper expression of the viral hemagglutinin-neuraminidase (HN) protein at the surface of infected cells. Reduced HN expression has been reported in cells chronically infected with measles virus (Fisher and Rapp, 1979), and was interpreted as a means whereby the persisting virus might escape the antibody-mediated defense system in vivo. In connection with these studies, a chemically-induced ts mutant of the orthomyxovirus family, influenza virus, was also determined to possess a ts lesion in the viral M protein (Ghendon et al., 1983). At non-permissive temperatures, hemagglutinin (HA) cleavage was reduced, functionally active HA and neuraminidase (N) were absent, and virions were not formed. Therefore, such ts lesions can dramatically alter the maturation process of virions, and thus contribute to the maintenance of persistence in vitro.

In the VSV system, the dominance of the polymerase-defective (RNA(-)) phenotype over the maturation-defective phenotype (structural protein lesions) was demonstrated in studies where persistent infections initiated with maturation-defective virus (complementation group 3 or 5) were monitored over time for the phenotype of released virus. In each case, with prolonged persistence, virus with the RNA(-) phenotype appeared, and with time, their proportion of the total increased , until by two to three months post-infection , over 80 % of the virus was of group 1 phenotype (Youngner et al., 1978). These results were interpreted by

the authors to indicate that the apparent selective advantage for ts mutants with "early" defects (RNA(-) phanotype) over those with "late" defects (structural protein lesions) may be explained by the limited ability of RNA(-) ts mutants to produce virus-specific RNA and cytotoxic proteins at the semi-restrictive temperature.

1.2.3 Interferon

In addition to DI particles and ts mutants, endogenous interferon production has also been implicated in maintenance of virus persistence *in vitro*. It should be noted from the outset, however, that interferon induction does not appear to represent a primary mechanism through which persistence can be maintained; rather, it has been found to operate in those systems where DI particles or ts mutants are generated. That interferon production is not necessary for the establishment or maintenance of persistent infections *in vitro* comes from the observation that two cell lines which are defective interferon producers (Vero or BHK) have been persistently infected by a variety of viruses.

The finding that a heterologous antiviral state exists in many persistently infected cultures suggests, however, that interferon may be operative. Furthermore, detection of interferon in such culture media, and the abolishment of persistance with anti-interferon serum clearly demonstrates that interferon can play a role in persistence (Nishiyama, 1977; Ramseur and Freidman, 1977). It has been proposed (primarily by one laboratory) that initiation and maintenance of persistent infection in cell cultures competent for the interferon system involves evolution of either special DI particles or ts

mutants with an increased capacity to induce interferon in infected cells (Marcus and Sekellick, 1977; Sekellick and Marcus, 1978, 1979). Interferon induction by DI particles was demonstrated using a VSV DI particle which contained covalently-linked message (+) and antimessage (-) RNA genome (Perrault and Leavitt, 1977). A conventional (-) RNA particle did not induce interferon under the same conditions. This double-stranded DI particle was also shown to protect interferon-competent cultures from cytopathic effect when challenged with standard VSV; no protective effect was observed in Vero or BHK cells (Sekellick and Marcus, 1978). Since DI particles appear frequently in wild-type stocks of VSV (Perrault and Leavitt, 1977), but are not selected for during persistent infection (Youngner et al., 1978; Holland et al., 1980), these particles may play a role early in establishment of the persistent state by reducing infectious (cell-killing) virus through homotypic interference and interferon induction (Marcus and Sekellick, 1980). In their study of interferon induction by Newcastle disease virus (NDV), Marcus et al. (1983) identified a non-infectious interferon-inducing particle (IFP) present in NDV released from "aged" chick embryo cells. This IFP, present in large excess over wild-type NDV, was postulated to possess a double-stranded character at the immediate 3'-end of the genome. Thus, this NDV IFP may have a structure like that described for the interferon-inducing VSV ((+/-)-strand) DI particle.

Temperature-sensitive mutants of VSV have also been implicated in interferon induction. Such ts mutants are the most consistently implicated in the maintenance of persistent infection in interferon-

competent cells (reviewed by Youngner and Preble, 1980). The RNA(-) ts mutants, commonly isolated from chronic infections in vitro, were found to be excellent inducers of interferon in mouse L cells (Marcus and Sekellick, 1979). Such ts mutants also protected cell cultures from cytopathic effect when challenged with standard VSV. The authors suggested that only a small fraction of the VSV genome need be transcribed to produce an interferon inducer moiety (as hypothesized for NDV above).

Clearly, interferon appears to play a subsidiary role in persistent infections in vitro; these studies indicate that only under exceptional circumstances can interferon function as a primary determinant in establishing the persistent state.

1.3 Coronavirus Persistence

Coronaviruses are important agents of both acute and persistent infection (reviewed by Wege et al., 1982; Sturman and Holmes, 1983). Murine coronaviruses, exemplified by mouse hepatitis virus (MHV), have been the most widely studied member of this virus family, and within the past decade, considerable progress has been made towards characterizing, in molecular terms, the events involved in the coronavirus replication cycle (see section 1.6). With these newly gained insights into coronavirus replication, it is now possible to investigate individual parameters in the infectious cycle to determine which factors play a key role in determining the outcome of infection. A number of strains of MHV have received much interest as a result of their demonstrated ability to become persistent under

certain conditions, both in vivo and in vitro (Lucas et al., 1977, 1978; Robb and Bond, 1979; Sorensen et al., 1980). A variety of mechanisms implicated in in vitro MHV persistence have been cited in the literature, and have been shown to operate at the level of the persisting virus (ie. temperature-sensitive mutants and DI particles (Baybutt et al., 1984; Holmes and Behnke, 1981; Makino et al., 1984, 1985)) or the host cell (Lucas et al., 1977, 1978; Mizzen et al., 1983).

The present work was undertaken to further characterize the nature of the supporting host cell in MHV persistence in vitro to provide insight into mechanisms by which the virus can mediate persistent infection in vivo.

1.4 Coronaviruses: Overview

Coronaviruses are large, enveloped RNA viruses that have been found to cause a wide variety of disease states in natural vertebrate hosts such as fowl (Schalk and Hawn, 1931), rodents (Cheever et al., 1949; Gledhill and Andrews, 1951), cattle (Kaye et al., 1975), cats (Holzworthy, 1963) and man (Mc Intosh et al., 1967). In man, an estimated 15 % of common colds are caused by human respiratory tract coronaviruses (Mc Intosh et al., 1970). Thus, coronaviruses are clearly of both economic and clinical importance.

The first coronavirus isolates were thought to be related to the ortho- and paramyxoviruses, which were similar in morphology and were also commonly isolated from respiratory infections (Berry et al. 1964). However, it became increasingly apparent that coronaviruses

represented a separate taxonomic family with many unique characteristics (Tyrell et al., 1975). The previously unrecognized virus family Coronaviridae began to emerge in the late 1960's when viruses isolated from human patients were noted to have similar morphological appearance to viruses associated with disease in fowl (infectious bronchitis virus; IBV) and mice (mouse hepatitis virus; MHV; Almeida and Tyrell, 1967; McIntosh et al., 1967). Furthermore, antigenic relatedness was demonstrated between MHV and the human coronavirus HCV-OC43 (McIntosh et al., 1969; Tyrell et al., 1968). For each virus, a characteristic fringe of large, widely-spaced, petal-shaped glycoprotein spikes or peplomers were observed to project from the envelope of the virion in negatively-stained preparations. The name coronavirus derives from the resemblance of these spikes to the corona spinarum, or crown of thorns, found in religious art (Tyrell et al., 1968).

1.5 Virion Structure and Composition

Negative-stained preparations of coronavirus virions appear as pleiomorphic, enveloped, generally spherical particles ranging in diameter of 60-200 nm and bearing unique surface peplomers of about 20 nm in length (McIntosh, 1974). The genomic RNA is a non-segmented, single-stranded molecule that is about 18 kilobases in length (or 6 x 10^6 MW), as estimated by a number of methods (reviewed by Siddell et al., 1982). The genome contains a poly-A tract at the 3'-end, that in the case of mouse hepatitis virus, MHV, is estimated to be 90 adenylate residues in length (Yogo et al., 1977). The coronavirus

genome is infectious and functions as messenger RNA (mRNA; Wege et al., 1982).

Many molecules of a basic phosphoprotein, N (nucleocapsid; 50-60K MW), encapsidate the genomic RNA to form a long, flexible nucleocapsid with helical symmetry (MacNaughton et al., 1978; Stohlman and Lai, 1979). The nucleocapsid lies within a lipoprotein envelope derived from the smooth or rough endoplasmic reticulum of the host cell (Alenso-Caplen et al., 1984; Massalski et al., 1982). The envelope consists of a lipid bilayer with two viral glycoproteins, E_1 and E_2; the ratio of structural proteins in the MHV virion was shown to be 8N: $16E_1$: $1E_2$ (Sturman et al., 1980).

The E_1 glycoprotein (20-30K MW) is a transmembrane molecule that is deeply embedded in the viral envelope, so that only a small, glycosylated N-terminal region is exposed on the outer surface of the lipid bilayer (Rottier et al., 1984). Nucleotide sequence of the cloned E_1 gene suggests that the protein penetrates the lipid bilayer via two hydrophobic domains, and a large domain lies beneath the bilayer (Armstrong et al., 1984). In the case of MHV, E_1 is glycosylated through serine or threonine residues at the N-terminal domain of the molecule (Niemann and Klenk, 1981).

The second coronavirus glycoprotein, E_2 (180-200K MW), the structural protein of the peplomer, is embedded in the lipid bilayer by a small anchor region and is glycosylated at asparagine residues (Cheley and Anderson, 1981; Niemann and Klenk, 1981). The E_2 has also been shown to contain covalently linked fatty acid (Schmidt, 1982).

Like other plus-stranded RNA viruses, coronaviruses do not

applied $mRNA_N$. Since the [^{32}P]-cDNA probe used in these studies was made against $mRNA_N$, and all MHV RNAs contain the $mRNA_N$ sequence (Cheley et al., (1981a)), there is a direct correlation between autoradiographic image intensity and amount of total MHV RNA in the applied dot-blot samples. Therefore, the relative levels of MHV RNA per infected cell calculated above were expressed as pg MHV RNA per infected cell using this calibration procedure.

2.20 [5-^{3}H]-uridine Labelling of MHV-infected L-2 cell RNA

Cultures of L-2 cells (10^7 cells per 100 mm dish) inoculated with MHV at an moi of 20 were adsorbed 30 min. at 4° then incubated in SM at 37°. AT 0 (immediately after virus adsorption was complete), 1, 2 or 3 h P.I. culture medium was changed to fresh SM containing 20 mM ammonium chloride. Cultures were supplemented with actinomycin D (2 µg/ml) at 4 h P.I. and subsequently labelled at 5 h P.I. with [5-^{3}H]-uridine in SM (10 µCi/ml). Cultures were harvested at 6 h P.I. by the guanidine-HCl procedure (section 2.17), and the RNA pellets taken up in 1 ml of 50% dimethyl sulfoxide (DMSO), 7 M urea (ultra pure, Schwarz-Mann), 10 mM Tris-acetate (pH 5.0). Samples were heated at 50° for 15 min. and then placed on ice immediately prior to loading on the agarose gel (section 2.21).

2.21 Agarose Gel Electrophoresis

A solution consisting of 0.7% agarose (Seakem) in 50% DMSO, 7 M urea, 10 mM Tris-acetate (pH 5.0) was heated at 68° for 45 min. to dissolve the agarose. Gels were poured into the same vertical system used for protein gels (glass plates with 1.0 mm spacers; section

1.6.3 Transcription of Minus-Strand RNA

Viral RNA polymerase activity can be detected in membrane fractions within 2 to 3 hours post-infection (Brayton et al., 1982). The genomic RNA is transcribed by this enzyme to form a complementary, full-length, minus-strand RNA with a poly-U sequence at the 5'-end (Baric et al., 1983; Lai et al., 1982).

1.6.4 Transcription of Plus-Strand RNA

The minus strand serves as a template for the synthesis of both new genomic RNA and six capped, polyadenylated subgenomic mRNAs ranging in size from 0.6 to 4.0 x 10^6 MW (Cheley et al., 1981a; Lai et al., 1982a). Oligonucleotide mapping of the genomic and subgenomic mRNAs showed that they form a nested set of overlapping molecules with common 3'-ends (Spaan et al., 1982; Stern and Kennedy, 1980). Each mRNA contains all of the nucleotide sequences of the next smaller mRNA plus one additional gene at the 5'-end. Only the 5'-proximal sequence of each mRNA is translated (Siddell et al., 1982). At the 5'-end of the genomic RNA, the cap is attached to a leader sequence of about 70 bases. Although it is not present elsewhere in the genome, this leader is also found at the 5'-end of each of the subgenomic mRNAs (Baric et al., 1983; Lai et al., 1982a; Spaan et al., 1983). This leader is transcribed from the 3'-end of the minus-strand template and acts as a primer for synthesis of plus -strand RNAs, through recognition of specific (AU)-rich intergenic sequences on the minus strand template (Spaan et al., 1983). The properties of the RNA-dependent RNA polymerase associated with the

synthesis of plus-strand RNAs have been noted to differ from those of the enzyme which produces the minus-strand RNA, on the basis of ionic and pH requirements (Brayton et al., 1982).

1.6.5 Viral Protein Synthesis

In vitro translation of each virus-specific mRNA yields only a single polypeptide that is encoded at the 5'-end of the mRNA (Leibowitz et al., 1982; Rottier et al., 1981; Siddell, 1983). The nucleocapsid protein (N) and several non-structural proteins (30, 14 and 200K MW) are apparently made on cytoplasmic polysomes (Leibowitz et al., 1982). The functions of the non-structural proteins are unknown. The synthesis of the E_1 and E_2 glycoproteins occurs on polysomes associated with the rough endoplasmic reticulum (RER), but there are several important differences in their processing and transcription (Niemann and Klenk, 1981; Niemann et al., 1982). The E_2 is cotranslationally glycosylated at the RER through asparagine residues on the growing polypeptide chain. Treatment of cells with tunicamycin prevents E_2 glycosylation (Cheley and Anderson, 1981; Niemann and Klenk, 1981). As E_2 is transported through the Golgi apparatus to the plasma membrane, cellular enzymes trim the oligosaccharides and acylate the glycoprotein by addition of fatty acid residues (Schmidt, 1982). There appear to be two or three E_2 molecules per peplomer (Cavanaugh, 1983), but the site of assembly of E_2 monomers to form a peplomer is unknown. Recent evidence suggests that the 180K MW E_2 of MHV is cleaved into two 90K MW species either in the Golgi apparatus or at the plasma membrane (Frana et al.,

1985). The E_2 molecule has many biological activities, including cell receptor binding (Collins et al., 1982), induction of neutralizing antibody (Collins et al., 1982; Garwes et al., 1978-1979) and activation of cell-fusion (Collins et al., 1982; Sturman et al., 1985).

The E_1 protein, as mentioned, is also synthesized on RER-bound polysomes, but is post-translationally glycosylated through serine or threonine residues in the Golgi apparatus (Niemann et al., 1982). This O-linked glycosylation, demonstrated for MHV is not a universal feature of coronaviruses; the E_1 of infectious bronchitis virus has N-linked oligosaccharides like those found on E_2 (Stern and Sefton, 1982). The intracellular transport of E_1 appears to be restricted beyond the Golgi apparatus, where it accumulates (Holmes et al., 1981).

1.6.6 Virion Assembly

Unlike most other enveloped RNA viruses which bud from the plasma membranes of infected cells, progeny coronavirus virions are formed by budding from smooth or rough endoplasmic reticulum membranes (Alonso-Caplen et al., 1984; Massalski et al., 1982). At the budding site, strands of nucleocapsid align in an orderly array on the cytoplasmic surface of these membranes, presumably in areas that contain the E_1 and E_2 (Dubois-Dalcq et al., 1982; Massalski et al., 1982). The interaction of the nucleocapsid with the endoplasmic reticulum is presumed to occur via the cytoplasmic domain of the E_1 glycoprotein; this is supported by the demonstrated interaction of

nucleocapsids with E_1 *in vitro* (Sturman *et al.*, 1980), and the localization of E_1 to the endoplasmic reticulum and Golgi apparatus within infected cells (Holmes *et al.*, 1981).

Spherical budding virions that have incorporated a complete nucleocapsid are pinched off into the lumen of the RER and Golgi apparatus. Although some virions may be released by cell lysis (Chasey and Alexander, 1976), coronaviruses are also released from intact cells apparently via the cellular exocytosis pathway (Doughri *et al.*, 1976). After virions migrate through the Golgi apparatus, they are transported into smooth-walled vesicles which migrate to the cell periphery and fuse with the plasma membrane to effect virion release into the external environment.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cells and Medium

The L-2 subline of mouse L-929 fibroblasts (Rothfels et al., 1959) was used for propagating virus and as the model permissive host for comparative studies of virus replication among different cell lines. The other cell lines used in these studies were as follows: LM-ATCC mouse fibroblast (Merchant and Hellman, 1962) (from the American Type Culture Collection, Rockville, Maryland), a thymidine-kinase deficient LM cell line designated LMTK$^-$ (Kit et al., 1963), a mouse neuroblastoma cell line, C-1300 (Augusti-Tocco and Sato, 1969), a mouse astrocytoma cell line, C-6 (Benda et al., 1968) and an African green monkey kidney cell line (Vero) (Yasumura and Kawakita, 1963). All cells were propagated at 37° as monolayers in standard medium (SM) composed of Eagles (1959) minimal essential medium (MEM) supplemented with 5% (v/v) fetal calf serum (FCS), 100 units/ml penicillin and 100 ug/ml streptomycin. The L-2 cells were also grown in suspension at 37° with rotary shaking (140 rpm; New Brunswick Psychrotherm) in SM plus 0.1% methyl cellulose (15 cps). For virus replication studies involving ammonium chloride, a 200 mM stock solution in SM was added to the culture media to a 20 mM final concentration.

2.2 Viruses

The murine hepatitis virus (MHV) strain A59 (Manaker et al., 1961), obtained from the American Type Culture Collection, was used

in all studies of virus replication.

2.3 Propagation of Virus

The propagation of stock MHV-A59 was routinely performed in L-2 cell monolayers (175 cm^2 tissue cultures flasks (2 x 10^7 cells) (Nunc)) or in suspension culture (0.5 - 1.0 x 10^6 cells/ml) at 37°. For infection of 175 cm^2 monolayers, 5 ml of pre-titred virus was adsorbed for 30 min. at room temperature, excess inocula removed and 30 ml SM added to the flask prior to incubation at 37°. To infect suspension cultures, cells were pelleted by centrifugation at 650 g for 10 min., then resuspended in a minimal volume of pre-titred virus (10 ml virus in SM per 2 x 10^8 cells). After 30 min. adsorption at room temperature, the cells were resuspended to a density of 2 x 10^6 cells per ml in SM plus 0.1% methylcellulose (15 cps) and incubated at 37°. Supernatant virus was harvested when approximately 50% of the fused monolayer had detached from the substrate. For monitoring infected suspension cultures, 10^6 cells were removed from suspension after virus adsorption was complete and set up as a monolayer in a 35 mm dish incubated at 37° (referred to as an "Indicator plate"). To remove cellular debris, virus-containing media was centrifuged at 2000 g for 20 min. at 4°, then aliquoted out and stored frozen at -70°.

For concentration of virus, this clarified supernatant virus stock was centrifuged at 70,000 g for 1 h at 4° and the virus pellet gently resuspended on ice in a small volume (typically 1-2 ml) of SM and stored frozen at -70°.

All virus stock were quantitated for infectious virus by plaque assay as described in section 2.4.1

2.4 Virus Assays

To quantitate both released and cell-associated virus, a variety of assays were performed on monolayer cell cultures. While the plaque assay (section 2.4.1) and infectious centre assay (2.4.5) were routinely performed in all studies of virus replication, all five types of virus assay (sections 2.4.1 to 2.4.5) were used in the studies involving ammonium chloride.

2.4.1 Virus Titration (Plaque Assay)

To quantitate released (extracellular) virus from either the virus stocks or from virus replication studies, the L-2 cell line was used for seeding the plaque indicator plate (Lucas et al., 1978). Confluent 35 mm multiwell (Nunc) monolayer cultures of L-2 cells (10^6 cells per well) were adsorbed for 1 h at room temperature with 0.25 ml virus inoculum (or serial 10 fold dilutions therefrom). Unadsorbed inoculum was removed; monolayers were washed three times in SM and then incubated at 37° for 24 h in fresh SM containing 0.5% methylcellulose (4000 cps). Monolayers were subsequently fixed for 10 min. in phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH_2PO_4, 137 mM NaCl, 8 mM Na_2HPO_4) containing 10% formaldehyde, then stained by addition of 0.1% crystal violet solution for visualization of plaques. The monolayers were then washed extensively by immersion in water, air dried and individual plaques scored. In this manner, the sample inoculum was quantitated for infectious virus by

determining plaque forming units per ml (pfu/ml).

2.4.2 Intracellular Virus Assay

Cultures of L-2 cells in 35 mm plates were adsorbed for 30 min. at 4° with MHV at a multiplicity of infection (moi) of 20, excess inocula removed and the cultures incubated at 37° in SM with or without 20 mM ammonium chloride. To assay intracellular MHV, the monolayers were washed several times in SM and harvested on ice in 2 ml SM by scraping with a Teflon policeman. Harvests were forced twice through a 21 gauge needle fitted to a 3 ml plastic syringe and subjected to a single cycle of freezing (-70°) and rapid thawing. The thawed samples were finally forced through a 30 gauge needle and the clarified supernatant assayed for infectious virus by plaque assay (section 2.4.1).

2.4.3 Virus Internalization Assay

To assay the process of virus internalization, cultures of L-2 cells were adsorbed for 30 min. at 4° with MHV at an moi of 5, washed with cold SM to remove unadsorbed inoculum, then warmed to 37° for varying intervals in the presence or absence of 20 mM ammonium chloride. Cultures were subsequently treated with proteinase K (0.5 mg/ml) in PBS for 45 min. at 4° to remove external, cell-associated virus (Helenius et al., 1980). After terminating the protease treatment with 1 mM phenylmethyl-sulfonylfluoride (PMSF), 3% bovine serum albumin (BSA) in PBS, cells were transferred to a centrifuge tube, spun into pellets (1 min. at 650 g), and washed twice with 0.2% BSA in PBS. The final cell pellets were assayed for internalized

virus by infectious centre assay on L-2 cell monolayers (section 2.4.5).

2.4.4 Infectivity of Internalized Virus

Cultures of L-2 cells in 35 mm plates were infected with MHV exactly as above (section 2.4.3), washed and incubated for 20 min. at 37° in SM with or without 20 mM ammonium chloride. Cells were then treated with proteinase K (as above), washed, and then returned to incubation at 37° in SM with or without 20 mM ammonium chloride. At various intervals, cells were harvested by centrifugation, washed twice, and assayed for intracellular MHV as described in section 2.4.2.

2.4.5 Infectious Centre Assay

To determine the number of infected cells in MHV-inoculated cultures, whether maintained in SM (sections 3.1 and 3.8) or SM with 20 mM ammonium chloride (section 3.16), an infectious centre assay (Lucas et al., 1978) was performed. Cells were harvested at 2 h P.I. by trypsinization (0.25% trypsin in citrate saline (100 mM sodium chloride, 50 mM sodium citrate)) after washing three times with citrate saline. Harvested cells were freed from trypsin by centrifugation (1 min. at 650 g) and resuspended in SM or SM plus 20 mM ammonium chloride where indicated. Aliquots of the resuspended cells and dilutions therefrom were plated onto confluent L-2 cell monolayers (10^6 cells per 35 mm dish), allowed to attach for 2 h at 37°, then overlaid with SM plus 0.5% methylcellulose (4000 cps), and, where indicated, including 20 mM ammonium chloride. Plaques were

read after 24 h at 37° as described in section 2.4.1.

2.5 Contact Fusion Assay

In the comparative studies of MHV replication in various cell lines (L-2, LMTK⁻, LM-ATCC and C-1300), a contact fusion assay was performed to detect the presence of fusion-active MHV protein(s) at the surface of infected cells. The presence of such activity is indicated when a given infected cell line can rapidly (within 3 h) induce cell fusion with a neighboring uninfected cell. To ensure that the infected cells were not in contact, they were sparsely seeded in 35 mm plates at 10^5 cells/plate and then inoculated with MHV at appropriate moi, so that equal numbers of infected cells could be produced among the various cell lines (see Tables 1 and 2). Following adsorption and washing, these cultures were incubated 5 h at 37°. At this time 10^6 uninfected cells ("indicator cells") were added to complete the monolayer, and incubation continued for a further 3 h at 37°. Cultures were scored either positive or negative for cell fusion at this time as indicated by syncytium formation.

2.6 Fusion Index (F.I.) Calculation

To semi-quantitate the level of virus-induced cell fusion, a fusion index (F.I.) was determined for each cell culture at various times P.I.

Monolayer cultures of the various cell lines in 35 mm dishes were inoculated with MHV at the indicated moi and maintained in SM (sections 3.3 and 3.7) or where indicated, SM with 20 mM ammonium chloride (section 3.15). At various time intervals P.I., the

cultures were fixed in 100% methanol, air dried, then Giemsa stained for 20 min. The cultures were washed in 95% ethanol, and then air dried. Stained cultures were viewed through a Leitz light microscope using a 12.5X eyepiece and 50X objective lens. To calculate the F.I., four representative fields of view were taken and the average F.I. determined using the formula:

$$F.I. = 1 - \frac{\text{number of mononuclear cells per field (infected culture)}}{\text{number of mononuclear cells per field (uninfected culture)}}$$

2.7 $[^{22}Na]^+$ Influx Assay

Confluent monolayer cultures of L-2 cells in 35 mm dishes (10^6 cells per dish) were MHV infected at an moi of 20. After 30 min. adsorption at room temperature, the cells were incubated in SM at 37°. At various times P.I. one series of plates were processed for calculation of the F.I. (section 2.6) while another identical series were prepared for the $[^{22}Na]^+$ influx assay. Cells were washed three times in Tris-buffered sorbitol (TBS; 20 mM Tris-HCl (pH 7.4), 0.25 M sorbitol; prewarmed to 37°) and then incubated in the presence of $[^{22}Na]^+$ (20μCi/ml in TBS) for 30 min. at 37°. Cultures were then rapidly washed five times with PBS followed by harvesting in 0.5 ml 0.1M NaOH. Intracellular $[^{22}Na]^+$ was determined by scintillation counting in Atomlite (NEN).

2.8 Isolation of Cellular Membranes

The procedure of Atkinson (1973) was used, with modification, for the isolation of L-2 cell membranes. Typically, each preparation

was obtained from 10^9 MHV-infected L-2 cells maintained in suspension (section 2.1). At 50% cell liftoff in the "indicator plate" (section 2.3) the cells were pelleted by centrifugation at 1000 g for 30 min., then resuspended in a small volume of SM and the pellets pooled and repelleted. The final cell pellet (wet volume 2 ml) was resuspended in 20 ml reticulocyte standard buffer (RSB) (10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1.5 mM $MgCl_2$) and the cells allowed to swell for 5 min. at 4°. The cells were disrupted by dounce homogenization on ice (10 strokes in a tight-fitting glass dounce) and completion of cell breakage confirmed by checking an aliquot by phase microscopy. The nuclei were pelleted by centrifugation at 650 g for 1 min. and the post-nuclear supernatant (approximately 16 ml) was layered onto discontinuous sucrose gradients (buffered with 10 mM Tris-HCl (pH 7.4)) prepared in 30 ml capacity glass Corex (Dupont) tubes. The gradients consisted of a 6 ml 45% sucrose cushion overlaid with 20 ml of 30% sucrose, on top of which 3 ml of sample (diluted in RSB) was layered. Typically, each 30 ml gradient received the post-nuclear supernatant from the equivalent of 10^8 infected cells. The gradients were centrifuged at 6000 g for 20 min. at 4° in an HB-4 rotor (Dupont) and the cloudy band visible at the 30%/45% sucrose interface removed with a 3 ml syringe fitted with an 18 g needle. These plasma-membrane enriched fractions (approximately 3 ml per gradient) were further enriched by diluting them 5 fold in PBS and pelleting only the largest membrane fragments (particularly plasma membrane ghosts) by centrifugation at 6000 g for 5 min. The pellets were pooled and this process repeated once more. The sample composition

was then examined by phase microscopy to confirm the presence of plasma membrane ghosts. The final pellet was resuspended in 2 ml MEM and stored frozen at -70°.

2.9 Membrane Fusion Assay

The ability of the MHV-infected L-2 cell membranes prepared above to induce fusion in monolayer cultures of the various cell lines was tested as follows. From the 2 ml membrane sample, 600 ul was used for each membrane fusion assay. To half of this sample (300 µl) was added 300 µl of 20 µg/ml trypsin (or chymotrypsin) in MEM (10 µg/ml final concentration), while the other half received 300 µl MEM. Both control and protease containing samples were incubated 1 h at 37° and protease digestion terminated by addition of N-∝-tosyl-L-lysyl-chloromethyl ketone (TLCK) (or N-∝-tosyl-phenylalanyl-chloromethyl ketone (TPCK)) at a final concentration of 10^{-4}M. These membrane preparations were then added to confluent monolayer cultures (200 µl per well) of L-2, LM-ATCC and Vero cells grown in multiwell trays (4×10^5 cells per well). After incubation at 37° for 1 h, the cells were extensively washed with MEM, Giemsa stained (section 2.6) and photographed using a Leitz Dialux 20 microscope.

2.10 Protease Digestion of [^{35}S]-methionine Labelled MHV-infected L-2 Cell Membranes

A confluent monolayer culture of L-2 cells in a 100 mm plate (10^7 cells) was inoculated with MHV (moi = 10) and incubated at 37° in SM. When approximately 80% of the monolayer had undergone cell fusion (6 h P.I.) the cells were labelled with [^{35}S]-methionine (100

μCi/ml in 2 ml methionine-free MEM (MFM)) for 1 h at 37°, by which time virtually 100% of the monolayer had fused. The cells were washed with PBS, harvested in PBS by scraping with a teflon policeman and pelleted by centrifugation at 650 g for 2 min. at 4°. The pellet was resuspended in 2 ml RSB, swollen for 5 min. at 4° and dounce homogenized. Nuclei were pelleted at 650 g for 30 sec and the supernatant (2 ml) layered onto a 30%/45% discontinuous sucrose gradient (as described in section 2.8) prepared in a 30 ml capacity polyallomer centrifuge tube (Beckman). The gradient was spun in an SW-27 rotor (Beckman) at 6000 g for 10 min. at 4°. The centrifuge tube was punctured at the bottom and 2 ml fractions drop-collected and aliquots therefrom checked for [^{35}S]-methionine activity by scintillation counting in Atomlite (NEN). The peak of [^{35}S]-methionine incorporation corresponding to material from the 30%/45% sucrose interface was identified and then pelleted as small aliquots in 1.5 ml Eppendorf microfuge tubes after being diluted 5 fold in PBS. The samples were spun 30 min. at 13,000 g at 4° and then resuspended in various concentrations of trypsin or chymotrypsin in 200 μl of MEM. After 1 h incubation at 37°, the protease inhibitors TLCK and TPCK, respectively, were added to a final concentration of 10^{-4} M. The samples were then frozen at -70°, lyophilized and resuspended in dissociation buffer (section 2.12) prior to analysis by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; section 2.13).

2.11 Isotopic Labelling of Proteins in MHV-Infected Cell Cultures

For standard isotopic labelling, confluent monolayer cell

cultures were MHV infected at the indicated moi, washed and incubated at 37° in SM. At the indicated time P.I., the SM was removed and replaced with methionine-free SM (MFM) plus [^{35}S]-methionine (sp.act. 524-1048 Ci/mmole; Amersham) at the given concentration (typically 100 μCi/ml), and incubated for 30 min. to 1 h at 37°.

For pulse-chase studies (section 3.13), a 15 min. pulse-labelling period was used, followed by a chase period where the isotopic medium was removed and replaced by MFM and incubation at 37° continued for varying intervals of time.

2.12 Preparation of Cell Extracts for SDS-PAGE

Cell monolayers were washed with PBS or MEM and harvested by scraping with a teflon policeman in dissociation buffer (DB) (1 mM PMSF, 7 mM Tris-HCl (pH 6.8), 6% SDS, 3.46 M 2-mercaptoethanol, 30% glycerol and 0.006% bromophenol blue) (typically 200 μl per 10^6 cells). Harvests were transferred to small plastic tissue culture tubes (Falcon #2054; 5 ml capacity) or 1.5 ml Eppendorf microfuge tubes. If samples were viscous, gentle, repeated aspiration through a syringe fitted with a 21 g needle was performed to shear cellular DNA. All samples were stored frozen at -70°.

2.13 Linear Gradient SDS-PAGE

The vertical discontinuous system of Laemmli (1970) was used except that the separating gel consisted of a continuous 5-18% polyacrylamide gradient. Such gradients were generated using a two-chambered gradient former, each chamber of which contained 17 ml of the appropriate concentration of stock acrylamide/bisacrylamide in a

ratio of 30 to 0.8 in 1.5 M Tris-HCl (pH 8.8) containing 0.1% SDS. Polymerization was initiated by the addition of 50 µl ammonium persulfate and 5 µl of N, N, N', N'-tetramethylenediamine (TEMED).

The stacking gel consisted of 4.5% acrylamide/bisacrylamide in a ratio of 30 to 0.8 in 0.625 M Tris-HCl (pH 6.8) containing 0.1% SDS. A constant current of 30 mA was applied for the duration of a run (approximately 3.5 h) until the bromophenol blue dye marker reached the bottom of the gel. Gels (1.0 mm thick) were then either fixed and stained (section 2.14) or prepared for fluorography (section 2.23).

2.14 Silver Staining Procedure

Non-isotopically labelled proteins resolved by SDS-PAGE were visualized using the silver stain procedure of Wray et al., (1981). The gels were soaked in 50% methanol overnight, with several changes of methanol, on a rocking platform. The methanol was discarded, and the gel soaked for 15 min., with rocking, in a solution containing 0.8% $AgNO_3$, 0.76% NaOH in 0.2 M NH_4OH prepared with distilled H_2O. The gel was then washed in distilled H_2O for 10 min. and then developed in 0.02% formaldehyde, 26 µM citric acid for 5-10 min. The development was stopped by soaking the gel in H_2O followed by soaking in a 45% methanol, 10% acetic acid solution.

The wet gel was then analyzed by densitometric scanning (section 2.24).

2.15 Immunoprecipitation of [^{35}S]-methionine Labelled Proteins

The procedure of Lee et al., (1981), was used with slight

modification for radioimmunoprecipitation. MHV infected cell monolayers (4 x 10^5 or 10^6 cells) which had been labelled with [^{35}S]-methionine (section 2.11) were harvested by scraping in immunoprecipitation buffer (IP buffer) (Nusse et al., 1978; composed of 10 mM Na_2HPO_4, 0.15 M NaCl, 1.0% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS (pH 7.2)).

Anti-MHV polyclonal antibody was prepared by three successive intra-peritoneal inoculations (1 week apart) of lysed MHV-infected L-2 cells in complete Freund's adjuvant (2-3 mg total protein per injection) into adult Swiss white mice. One week following the third injection, mice were bled by cardiac puncture and the serum fraction exhaustively absorbed against lysed L-2 cells. Following centrifugation at 13,000 g for 30 min., antibody was aliquoted out and stored frozen at -70°.

In a typical immunoprecipitation, 5-10 µg of [^{35}S]-labelled protein in 20 µl IP buffer was pre-adsorbed for 1 h against 2 µl of a 10% suspension of protein-A bearing Staphylococcus aureus (Cowan 1 strain) prepared according to Kessler (1981). This mixture was then washed once in buffer A (0.15 M NaCl, 0.5% Triton-X-100 (Kodak), 5 mM EDTA, 50 mM Tris-HCl (pH 7.4) and 0.02% NaN_3) followed by three washes in buffer B (same as buffer A except 0.05% Triton-X-100). The mixture was pelleted in a minifuge at 13,000 g for 5 min. and the supernatant antigen transferred to a fresh microfuge tube containing 20 µl of a 1/500 dilution of mouse anti-MHV polyclonal antibody.

The antibody-antigen mixture was incubated overnight at 4° with gentle rocking. Then, to this was added 20 µl of a washed 10%

suspension of Staphylococcus protein-A and incubation continued for 30 min. at room temperature. The pellet was washed several times with 0.5 ml buffer C (0.5 M NaCl, 0.1% SDS, 50 mM Tris-HCl (pH 7.4) and 1.0% Triton-X-100) and pelleted at 13,000 g, each time discarding the supernatant. The final pellet was taken up in 30 µl DB (section 2.12), immersed in boiling water for 3 min., microfuged at 13,000 g for 5 min. and then the supernatant labelled protein analyzed by SDS-PAGE (section 2.13) and fluorography (section 2.22).

2.16 Enzyme-Linked Immunosorbant Assay (ELISA)

The ELISA procedure of Buchanan et al., (1981) was used with modification. Monolayer cultures of L-2 cells, grown in flat-bottom 96 well trays (approximately 8 X 10^4 cells per well), were infected with MHV at the indicated moi (40 µl per well), and incubated at 37°. At various times P.I., cultures were fixed with 4% formaldehyde in PBS for 5 min., washed three times in PBS and permeabilized with 1.0% Triton-X-100 in PBS for 5 min. at room temperature. One further wash in 0.05% Tween-20 (polyoxyethylene sorbitan monolaurate) in PBS was followed by incubation in a 30% normal goat serum (Gibco) solution, prepared in 1.0% Triton-X-100 in PBS, for 30 min. at 37°. This was followed by incubation in a 1/2000 dilution (in 5% normal goat serum) of mouse anti-MHV antibody (section 2.15) overnight at 4°. The monolayers were then washed five times in 1.0% Triton-X-100 in PBS and incubated for 1 h at 37° with a 1/1000 dilution (in 5% normal goat serum) of goat anti-mouse IgG (heavy and light chain) conjugated to alkaline phosphatase (Bio Can Scientific Inc.). Following

extensive washing, the monolayers were incubated for 30 min. to 1 h at 37° with p-nitrophenyl-phosphate substrate (1 mg/ml in carbonate buffer (10 mM Na_2CO_3, 35 mM $NaHCO_3$, 1 mM $MgCl_2$)) for color development. The colorimetric reaction was stopped by addition of 1/10th volume of 5N NaOH and the absorbance of each well read at 405 nm in a Dynatech ELISA reader (Alexandria, Virginia).

For quantitation of MHV protein synthesis per infected cell, 35 mm cultures (10^6 cells) of L-2 cells were MHV-infected at a moi of 0.01 and incubated at 37° in SM. At 6, 9 and 12 h P.I., one series of infected cultures were harvested for assay of total protein content by the method of Lowry et al. (1951), while a duplicate series was harvested in DB (section 2.12) and subjected to SDS-PAGE analysis (section 2.13) using immunoprecipitated MHV proteins (section 2.15) as MW standards. The specific proportion of MHV protein in each sample total was assessed by a densitometric scan (section 2.24) of the silver-stained gel (section 2.14). The ELISA absorbance values obtained for MHV-infected L-2 cells (moi = 0.01) were then correlated with amounts of synthesized MHV protein, and in combination with the infectious centre data (Table 2), converted to pg of MHV protein synthesized per infected cell.

2.17 Guanidine-hydrochloride Extraction of RNA

For agarose gel electrophoresis (section 2.21) and in vitro translation (section 2.22), MHV-infected L-2 cell RNA was extracted using a modified procedure of Strohman et al, (1977). Cell monolayers were washed with PBS, harvested by scraping in a solution of 7.6 M guanidine-HCl, 0.1 M potassium acetate (pH 5.0) and

homogenized in a tight-fitting glass dounce on ice. After addition of 0.6 volume 95% ethanol, mixing and cooling at -20° overnight, the mixture was centrifuged at 3000 g for 10 min. The pellet was taken up in buffer containing 7.6 M guanidine-HCl, 0.02 M EDTA and vortexed at room temperature. Following addition of 2 M potassium acetate (pH 5.0) to a final concentration of 0.1 M, 0.5 volume 95% ethanol was added and the mixture was placed at -20° overnight. The precipitate was then centrifuged at 3000 g for 10 min. The pellet was dissolved in 20 mM EDTA (pH 7.0) and extracted at room temperature with 3 volumes chloroform/butanol (4:1, v/v). The aqueous phase was combined with two additional aqueous washings of the organic phase and made 3 M in sodium acetate using 4.5 M sodium acetate (pH 6.0). After cooling at -20° overnight, the precipitate was centrifuged at 8700 g for 60 min. to yield an RNA pellet which was subsequently precipitated twice from ethanol.

For use in dot-blotting (section 2.19) this extraction procedure was simplified (Cheley and Anderson, 1984). Cell monolayers were harvested directly with 1 ml of 7.6 M guanidine-HCL in 0.1M potassium acetate (pH 5.0). The viscous mixture was homogenized by aspiration 5 times through a 1.0 ml plastic syringe fitted with a 21 g needle. Syringe contents were transferred to small plastic tissue culture tubes (Falcon #2054), mixed with 0.6 ml 95% ethanol, cooled at -20° overnight and centrifuged at 5000 g for 20 min. to produce the final RNA pellet.

The concentration of extracted RNA was determined by measuring absorbance at 260 nm in a spectrophotometer (Bausch and Lomb).

$$\text{Concentration of RNA} = \frac{1}{\text{dilution factor}} \times 40\ \mu g\ RNA/A_{260}$$

2.18 Preparation of Complementary DNA

Complementary DNA (cDNA) was synthesized in the presence of actinomycin D, calf-thymus primers and purified AMV polymerase using the procedure of Shank et al. (1978). Purified MHV (strain A59) nucleocapsid protein messenger RNA ($mRNA_N$) isolated from preparative formamide polyacrylamide gels (Cheley et al., 1981a), was kindly provided by Stephen B. Cheley and served as the template for the cDNA reactions.

A typical reaction contained 0.1 µg $mRNA_N$, 0.45 µg each of dATP, dGTP and dTTP (Boehringer), 30 mM KCl, 4.6 mM $MgCl_2$, 1.0 mM dithiothreitol (DTT), 30 mM Tris·HCl (pH 8.1), 100 µg/ml actinomycin D (Calbiochem), 100 µCi [^{32}P]-dCTP (> 400 Ci/mmole; Amersham), 10 µg calf-thymus DNA primers and 50-100 units AMV reverse transcriptase (Dr. J. Beard, Life Sciences, St. Petersburg, Fla.). The above mixture was incubated for 45 min. at 40°, base treated for 2 h by making the reaction 0.6 M in NaOH using 3 M NaOH and then neutralized by making the reaction 50 mM in Tris-HCl (pH 7.4) and an equal volume of 3 M HCl was added as in the base-treatment. The pH was checked by micro-dotting 1/2 µl aliquots onto pH indicator strips. The above reaction mixture was applied to a 2.5 x 30 cm Sephadex G-75 (Pharmacia) column and eluted with buffer (containing 0.3 M NaCl, 0.02 M Tris-HCl, (pH 7.4), 0.3 mM EDTA and 0.1% SDS) in order to separate cDNA from radiolabel according to the procedure of Cohen et. al., (1979). Peak fractions were pooled and the cDNA precipitated

with 100 µg yeast tRNA (Sigma) and 2 volumes of 95% ethanol.

In order to remove contaminating cellular sequences, the cDNA product was annealed with 500 µg of uninfected L-2 cell RNA in 0.6 M NaCl at 68° for 45 min. The annealing mixture was then made 0.12 M in phosphate buffer (pH 6.8) and the single-stranded sequences were eluted from an hydroxyapatite column at 60° with 0.12 M sodium phosphate buffer (pH 6.8). The fractions containing single-stranded [^{32}P]-cDNA (usually 80-85% of the total radioactivity applied) were collected and pooled.

2.19 Dot-blot Hybridization

RNA pellets (each from 10^6 cells) prepared as outlined in section 2.17 were taken up in 250 µl 15% formaldehyde to which was added 250 µl 20X standard saline citrate (SSC; 1 X SSC is 0.15 M sodium chloride, 0.015 M trisodium citrate). Samples were subsequently heated for 15 min. at 50° then placed on ice. For dot-blot hybridization, 50 µl aliquots or serial 10 fold dilutions therefrom were applied in a Schleicher and Schuell "Minifold" filtration apparatus to nitrocellulose sheets (0.45 µm, Millipore) supported on three layers of slab dryer filter paper (Bio-Rad Laboratories). Both nitrocellulose sheets and filter paper had been previously equilibrated with 10X SSC. After sample application the sheets were baked 1 h at 80° and kept at 4° until needed.

Prior to application of the [^{32}P]-cDNA, the sheets were pre-annealed for 24 h at 39.5° with annealing buffer consisting of 50% formamide, 1 mg/ml yeast tRNA, 200 µg/ml salmon sperm DNA (see

below), 3X SSC and 1X Denhardt's buffer (0.02% w/v bovine serum albumin, 0.02% polyvinyl pyrrolidine and 0.02% Ficoll; Denhardt, 1966). Salmon sperm DNA was made 0.6% w/v in 20 mM Tris-HCl, (pH 7.4). The DNA was sheared by passage through an 18 then 20 g needle. To 10 ml of sheared DNA were added 5 ml 1N HCl, and the solution boiled for 25 min. After neutralization with NaOH, 2 volumes of 95% ethanol were added and precipitated DNA recovered by centrifugation for 30 min. at 10,000 g. The DNA pellet was lyophilized and resuspended in 30 mls of 20 mM Tris-HCl (pH 8.0).

After pre-annealing, annealing buffer containing 0.5-2 x 10^6 dpm/ml [^{32}P]-cDNA was applied and the sheets were incubated for 48 h at 39.5° and then washed according to the procedure of Thomas (1980). The air-dried sheets were autoradiographed for 2-5 days with a Cronex intensifying screen (Dupont) to yield the dot-blots.

For quantitation of MHV-specific RNA synthesis, densitometric scanning of dot-blot autoradiographic images was performed to assess relative levels of viral RNA synthesis. In combination with the infectious center data (Table 2), the relative levels of MHV RNA per infected cell was calculated at the indicated times P.I. To convert this data to pg MHV RNA per infected cell, the following standardization procedure was performed. Using purified MHV nucleocapsid protein mRNA ($mRNA_N$) from 10^9 cells (kindly provided by Stephen B. Cheley), the yield of $mRNA_N$ per cell was calculated by spectrophotometry (section 2.17). Aliquots of this $mRNA_N$ were included as an internal standard on all dot-blot hybridizations to calibrate the intensity of the autoradiographic image with amount of

applied $mRNA_N$. Since the [^{32}P]-cDNA probe used in these studies was made against $mRNA_N$, and all MHV RNAs contain the $mRNA_N$ sequence (Cheley et al., (1981a)), there is a direct correlation between autoradiographic image intensity and amount of total MHV RNA in the applied dot-blot samples. Therefore, the relative levels of MHV RNA per infected cell calculated above were expressed as pg MHV RNA per infected cell using this calibration procedure.

2.20 [5-^{3}H]-uridine Labelling of MHV-infected L-2 cell RNA

Cultures of L-2 cells (10^7 cells per 100 mm dish) inoculated with MHV at an moi of 20 were adsorbed 30 min. at 4° then incubated in SM at 37°. AT 0 (immediately after virus adsorption was complete), 1, 2 or 3 h P.I. culture medium was changed to fresh SM containing 20 mM ammonium chloride. Cultures were supplemented with actinomycin D (2 μg/ml) at 4 h P.I. and subsequently labelled at 5 h P.I. with [5-^{3}H]-uridine in SM (10 μCi/ml). Cultures were harvested at 6 h P.I. by the guanidine-HCl procedure (section 2.17), and the RNA pellets taken up in 1 ml of 50% dimethyl sulfoxide (DMSO), 7 M urea (ultra pure, Schwarz-Mann), 10 mM Tris-acetate (pH 5.0). Samples were heated at 50° for 15 min. and then placed on ice immediately prior to loading on the agarose gel (section 2.21).

2.21 Agarose Gel Electrophoresis

A solution consisting of 0.7% agarose (Seakem) in 50% DMSO, 7 M urea, 10 mM Tris-acetate (pH 5.0) was heated at 68° for 45 min. to dissolve the agarose. Gels were poured into the same vertical system used for protein gels (glass plates with 1.0 mm spacers; section

2.13) and allowed to gel slowly at room temperature for 2-3 h. Small aliquots of the tritiated RNA (10 µl from 1 ml sample) were mixed with 10 µl of 30% glycerol plus 0.006% bromophenol blue and loaded onto the gel. Samples were electrophoresed at 20 mA constant current for 5 h in running buffer consisting of 50% DMSO, 7 M urea in 10 mM Tris-acetate (pH 5.0). Gels were washed for 1 h in a 10% methanol-10% acetic acid solution to remove DMSO and urea and then processed for fluorography using EN^{3}HANCE (NEN) (section 2.23).

2.22 In Vitro Translation

MHV-infected L-2 cell RNA extracted by the guanidine-HCl procedure (section 2.17) was translated in a cell-free rabbit reticulocyte lysate system (BRL) using a modification of the manufacturer's protocol. Components of the translation cocktail consisted of the reticulocyte lysate (3 X; containing 3.5 mM $MgCl_2$, 0.05 mM EDTA, 25 mM KCl, 70 mM NaCl, 0.5 mM DTT, 25 µM hemin, 50 µg/ml creatine kinase, 1 mM $CaCl_2$, 2 mM EGTA), a protein biosynthesis reaction mixture (10X; containing 250 mM HEPES (pH 7.2), 400 mM KCl, 100 mM creatine phosphate, 19 L-amino acids (0.5 mM each minus methionine)), potassium acetate (2 M (pH 7.2)), magnesium acetate (2 M (pH 7.2)), sterile H_2O, [^{35}S]-methionine (15 µCi/µl) and RNA (suspended in sterile H_2O).

To determine optimal K^+ and Mg^{++} concentrations for translation of the RNA trial reactions were performed varying either K^+ or Mg^{++} concentrations while other parameters were kept constant. The optimal concentrations of K^+ and Mg^{++} were found to be 87 mM and 1.0 mM respectively.

Therefore, each standard translation reaction (30 µl volume) consisted of 1.3 µl potassium acetate, 1.6 µl magnesium acetate, 3.0 µl biosynthesis mix, 10 µl reticulocyte lysate, 2 µl [^{35}S]-methionine (30 µCi), and 12 µl of water containing the desired amount of RNA (0.5 µg/µl).

RNA was routinely lyophilized prior to use and then taken up in deionized water, heated at 50° for 20 min. and then chilled on ice prior to use in the reactions. The mixtures were vortexed and incubated at 30° for 1 h with intermittent vortexing. Translation was terminated by addition of 30 µl DB (section 2.12) and the [^{35}S]-methionine labelled products analyzed by SDS-PAGE (section 2.13) and fluorography (2.23).

For the translations involving addition of NaCl, a concentrated stock solution was used so that a 30 µl final reaction volume was maintained. All other parameters were identical to above.

2.23 Autoradiography and Fluorography

Isotopically labelled samples resolved by SDS-PAGE (section 2.13) and agarose gel electrophoresis (section 2.21) were processed for fluorography (Bonner and Laskey, 1974) in order to enhance autoradiographic imaging. Gels were prepared for fluorography by impregnation with EN^{3}HANCE (NEN) for 1 h followed by soaking in H_2O for 1 h. Gels were dried on Whatman filter paper 3 in a slab gel drier (Bio-rad model 224) under heat and vacuum for 1 h. The dried gels were exposed to Kodak X-omat XAR film at -70° and developed according to standard procedure. Dot blots which had been annealed

with [^{32}P]-cDNA probe were processed for autoradiography by exposure to Kodak film at -70° and standard development.

2.24 Densitometric Scanning

To quantitate relative levels of protein and RNA densitometric scans of developed X-ray films and silver-stained gels was performed on an LKB laser densitometer (model 2202) interfaced with a Hewlett-Packard integrator (model 3392A).

CHAPTER 3

RESULTS

3.1 Comparative Infectability of L-2 and LMTK⁻ Cells with MHV

To perform valid comparisons between L-2 and LMTK⁻ cells with respect to their ability to support replication of MHV, it was initially necessary to standardize results on an infected cell basis. Toward this end, cultures of L-2 and LMTK⁻ cells were inoculated with the A59 strain of MHV at moi's ranging from 0.01 to 10 and, following a two hour incubation period (to permit virus internalization), numbers of infected cells were determined by infectious center assay (section 2.4.5). At a given moi, MHV infection of L-2 cells gave rise to a greater number of infected cells than LMTK⁻ cells (Table 1). At high moi's, (moi = 5 and 10) the L-2 cells were approximately one hundred-fold more infectable than LMTK⁻ cells. Under non-saturating conditions, that is, when the moi was less than 1.0, the LMTK⁻ cells were found to be approximately five-hundred fold less infectable than correspondingly inoculated L-2 cells. Also evident from Table 1 is that comparable numbers of infected cells are produced, for example, when L-2 cells are inoculated at an moi of 0.01 and LMTK⁻ cells are inoculated at an moi of 10.

Therefore, for the purposes of studying the various parameters of MHV replication in these two cell lines, one can produce equal numbers of infected L-2 and LMTK⁻ cells by appropriate choice of moi.

TABLE 1

Comparative Infectability of L-2 and LMTK$^-$ Cells with MHV

moi[a]	Number of cells infected: L-2 cells	LMTK$^-$ cells	Ratio
10	1.1×10^6	1.4×10^4	78
5	9.3×10^5	6.4×10^3	145
1	6.0×10^5	1.8×10^3	333
0.5	3.4×10^5	8.1×10^2	420
0.1	9.0×10^4	1.9×10^2	474
0.01	1.1×10^4	2.1×10^1	524

[a] As determined by plaque assay (section 2.4.1) on L-2 cells.

[b] Cultures of 10^6 cells in 35 mm dishes were inoculated with MHV at the moi indicated, washed, and numbers of infected cells determined by infectious center assay (section 2.4.5).

3.2 Yields of Virus and Viral RNA from MHV-Infected L-2 and LMTK$^-$ Cells are Comparable

In collaboration with S. Cheley, (Mizzen, et al., 1983) the output of progeny virus and synthesis of viral RNA in L-2 and LMTK$^-$ cells was determined.

Since infectious center assay indicated that a much higher moi was required to produce comparable numbers of infected LMTK$^-$ as L-2 cells (Table 1), virus yields (section 2.4.1) were determined on cultures inoculated with a range of moi's. If L-2 and LMTK$^-$ cells were equally efficient virus producers, virus outputs from the two cell types would be comparable, once cultures were standardized on the basis of equal numbers of infectious centers. On this basis, virus production from LMTK$^-$ cells (moi = 10) would be expected to most closely parallel that seen in L-2 cells inoculated at an moi of 0.01. In fact, in terms of both virus yields and chronological similarity, the LMTK$^-$ virus titration curve was most comparable to that of L-2 cells inoculated at an moi of 0.1. Hence, on an infected cell basis, LMTK$^-$ cells are more efficient than L-2 cells in producing infectious progeny MHV.

In long-term cultures of MHV-infected L-2 (moi = 0.01) and LMTK$^-$ (moi = 10) cells, peak virus output in both cell lines (10^7 pfu/ml) occurred by 24 h P.I.

In contrast to MHV-infected L-2 cells, which were terminated after 48 h P.I. due to cell death, MHV-infected LMTK$^-$ cells continued to produce progeny virus in an undulating fashion over the seven day period of study. Longer-term studies (data not shown) indicate that

infected LMTK$^-$ cell cultures continue to produce progeny virus over a period of several weeks, in a cyclical fashion similar to that previously described for a number of persistently infected cell lines (Lucas et al., 1978). Periodic infectious center assays of such long term cultures revealed that between 1 and 10% of the cells were infected at any given time point, suggesting that MHV persistence in LMTK$^-$ cells is mediated by a carrier-culture mechanism, involving virus propagation and transmission among a relatively small fraction of the total cell population.

Virus isolated from infected LMTK$^-$ cells displayed no appreciable temperature sensitivity when assayed for growth in L-2 cells at 32.5, 37 or 39.5°C. Plaque morphology on L-2 cells was also unaltered from that seen with stock MHV. As with previously described persistent MHV infections (Lucas et al., 1978), it therefore seems unlikely that MHV persistence necessitates the production of ts virus mutants.

Supernatant MHV-infected LMTK$^-$ culture fluids, which had been freed from virus by centrifugation at 100,000 g for 1 h had no inhibitory effect on virus replication when incubated with VSV inoculated L-2 cells. Therefore, no evidence was found for the production of soluble antiviral factors which might suppress virus production and contribute to persistence.

Using an MHV-specific [^{32}P]-labeled cDNA probe (section 2.18) (Cheley et al., 1981a) the relative amounts of positive-sense viral RNA were determined in MHV-infected L-2 and LMTK$^-$ cells by dot-blotting (section 2.19) of guanidine-HCl extracted RNA (section 2.17)

(Mizzen et al., 1983). LMTK⁻ cultures inoculated with an moi of 10 showed a pattern of viral RNA synthesis most closely resembling that seen in L-2 cells inoculated at an moi of 0.01, that is, a one thousand-fold less moi than that employed for the LMTK⁻ cells. This value is in reasonably good agreement with the difference in infectability of the two cell lines as determined by infectious center assay (Table 1). There would, therefore, appear to be no significant difference in the levels of MHV RNA synthesis, on an infected cell basis between L-2 and LMTK⁻ cells.

3.3 Differential Expression of MHV-Induced Fusion in L-2 and LMTK⁻ Cultures

The expression of virus-induced cell fusion, a characteristic cytopathic effect of MHV in cell culture (Figure 1), was found to differ markedly between L-2 and LMTK⁻ cells with respect to both kinetics of syncytium formation and syncytial morphology.

The progression of MHV-induced cell fusion, expressed as a Fusion Index (F.I.) (section 2.6) in long-term cultures of L-2 and LMTK⁻ cells is shown in Figure 2. The Fusion Index, calculated at the indicated time P.I. in L-2 (moi's of 10, 1.0, 0.1, 0.01) and LMTK⁻ (moi = 10) cells, semi-quantitates the proportion of the monolayer which has undergone fusion to produce multi-nucleate syncytia. An F.I. value of 1.0 indicates the absence of mononuclear cells; that is, complete fusion of the monolayer into a collection of syncytia. Conversely, an F.I. value of 0 indicates that all the cells in the monolayer exist in their normal, mononuclear state.

Figure 1: Light micrograph of MHV-induced cell fusion in L-2 cells. Uninfected (panel A) and MHV-infected (panel B) monolayer cultures of L-2 cells were fixed, Giemsa stained (section 2.6) and photographed using a Leitz Dialux 20 microscope. Note the numerous syncytia (panel B) characteristic of MHV infection in L-2 cells, each containing a large collection of nuclei from the fused cells. Interspersed among the syncytia are single mononuclear cells that have not yet fused with the surrounding syncytia.

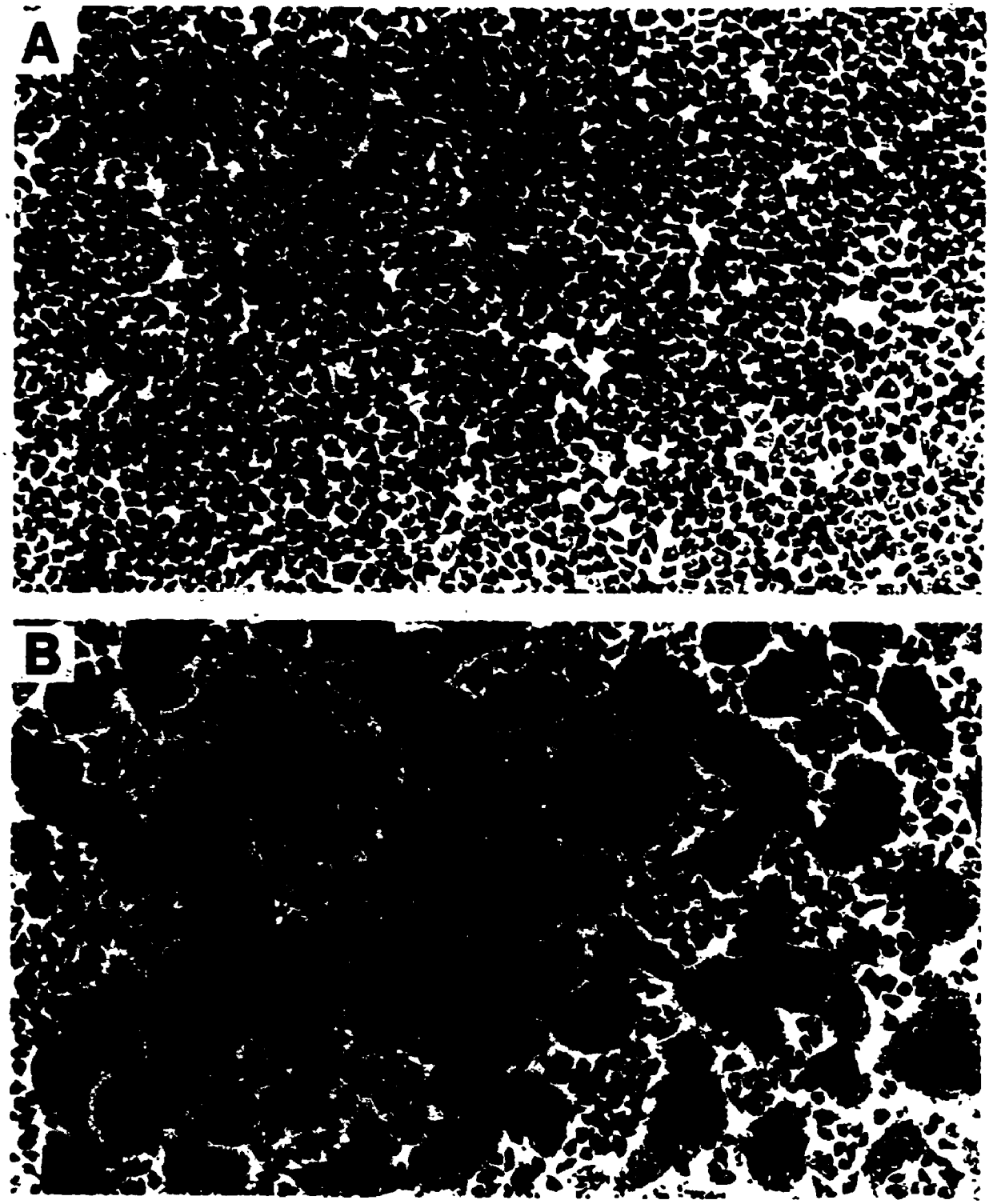
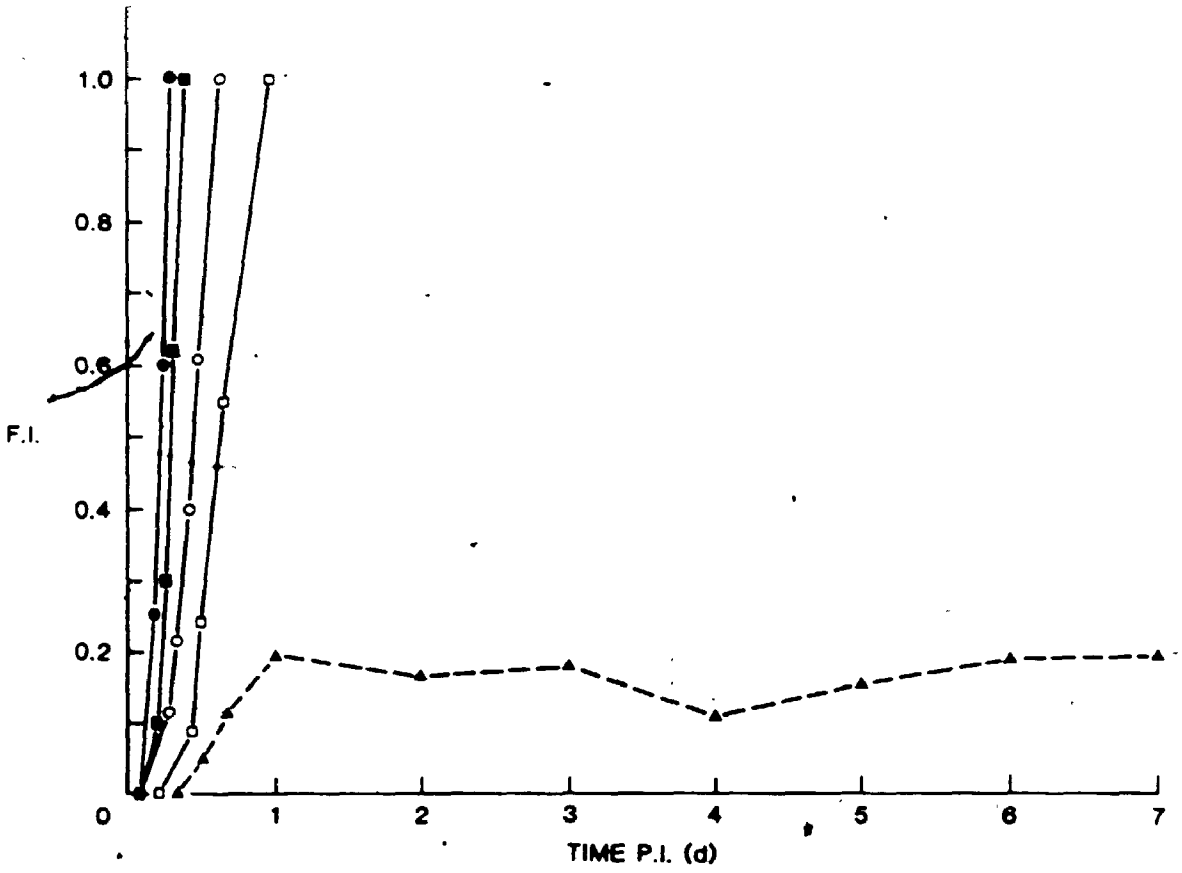

A
B

Figure 2: Progression of MHV-induced cell fusion in long term cultures of L-2 and LMTK$^-$ cells. Monolayer cultures of L-2 (solid lines) and LMTK$^-$ (dashed lines) cells were inoculated with MHV at various moi's and monitored for cell fusion over a seven day period. Cultures were fixed and Giemsa stained, followed by calculation of the Fusion Index (F.I.) of each sample (section 2.6). L-2 cells were inoculated at an moi of 10 (●), 1.0 (■), 0.1 (○) and 0.01 (□). LMTK$^-$ cells were inoculated at an moi of 10 (▲).

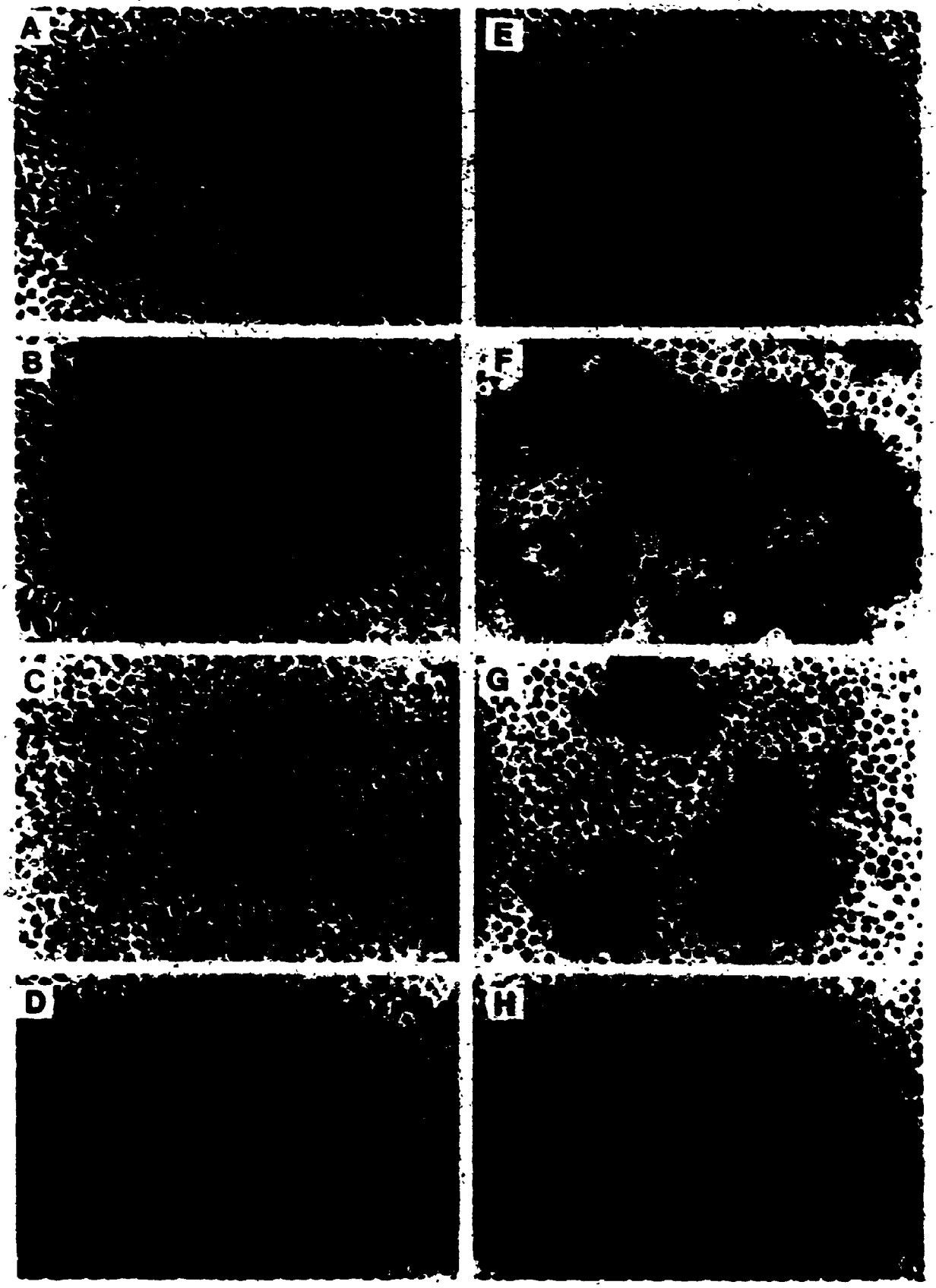

1.0
0.8
0.6
F.I.
0.4
0.2
0
1
2
3
4
5
6
7
TIME P.I. (d)

The induction and spread of fusion in infected L-2 cells is very rapid and complete. In response to increasing moi's this process is accelerated, and in every case, completely consumes the monolayer (F.I. = 1.0). In contrast, the F.I. in LMTK$^-$ cells is seen to increase slowly and reach a maximal value of 0.2 by 24 h P.I. Moreover, there was no increase in the F.I. with time; rather, it fluctuated around this ceiling value over the seven day period of study.

Cyclical variations of cell fusion over periods of several weeks have been a common feature of MHV-infected LMTK$^-$ cultures maintained in our laboratory.

A light micrographic comparison of MHV-induced cell fusion (Figure 3) in L-2 (panels E to H) and LMTK$^-$ (panels A to D) cells illustrates the differences in syncytial morphology that are also indicative of depressed fusion levels in infected LMTK$^-$ cells. Individual syncytial foci in infected L-2 cultures (e.g. panels G and H) were roughly circular and rapidly expanding, suggesting facile recruitment of neighboring uninfected cells. In contrast, LMTK$^-$ syncytia displayed little radial expansion. Rather, they tended to remain narrow and spindle-shaped, thus preserving relatively large areas of uninfected cells. The progression of fusion throughout the infected L-2 culture is suggested by the sequence of panels H to F in Figure 3, which represent parallel MHV-infected cultures (moi = 0.01, 0.1 and 1.0 respectively) photographed at 8 h pi. For comparison, panels D to B show fusion induced in LMTK$^-$ cultures (moi = 0.1, 1.0 and 10) in the same experiment.

Figure 3: Light micrographic comparison of MHV-induced cell fusion in L-2 and LMTK$^-$ cells. Cultures of LMTK$^-$ (panels A to D) and L-2 (panels E to H) cells were mock-infected (A and E) or MHV-infected at moi's of 10 (B), 1.0 (C and F) 0.1 (D and G) and 0.01 (H). Photographs of representative fields of the live cultures were taken at 9 h P.I. using a Leitz inverted microscope. Arrow indicates syncytium.


A
E
B
F
C
G
D
H

Taken together, these observations on virus-induced cell fusion are highly suggestive of a dynamic balance between infected and uninfected cells in MHV-infected LMTK$^-$ cultures, which would contribute to a persistent state.

3.4 Demonstration of Viral Structural Polypeptides in MHV-Infected L-2 and LMTK$^-$ Cells

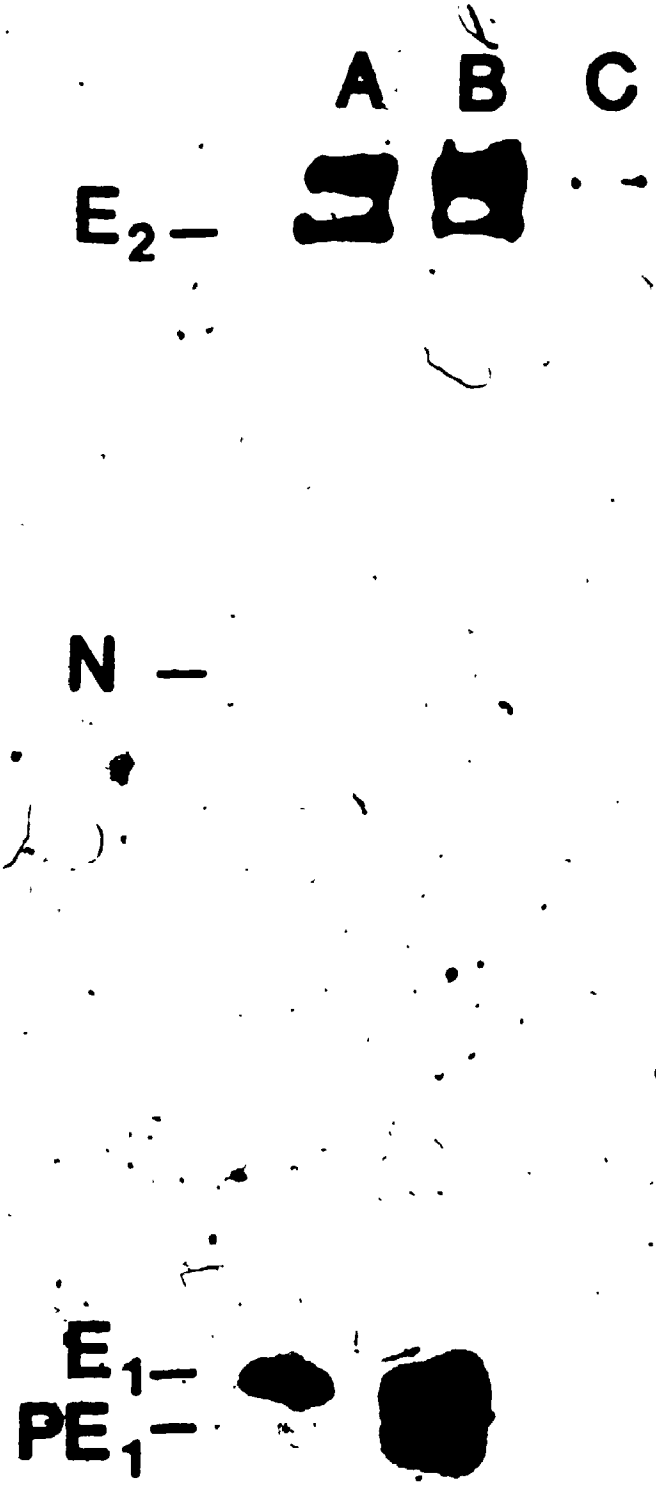
To test for the presence of viral structural proteins, including the protein responsible for cell fusion (Collins et al., 1982), in MHV-infected L-2 and LMTK$^-$ cells, immunoprecipitation (section 2.15) was performed on extracts prepared from cultures labelled with [^{35}S]-methionine (section 2.11) at 8 h P.I. The resultant radioimmunoprecipitates were analyzed by SDS-PAGE (section 2.13) and fluorography (section 2.23).

As shown in Figure 4, all three viral structural polypeptides, E_1 (and precursor PE_1), N and E_2 were present in both L-2 and LMTK$^-$ cells in similar proportions. Therefore, the observed lack of fusion in MHV-infected LMTK$^-$ cells is not likely due to defective synthesis of the viral fusion protein, which has been shown by Collins et al., (1982) to be the E_2 protein.

3.5 Depressed LMTK$^-$ Syncytiogenesis is Due to Membrane Resistance to MHV-Induced Fusion

To determine whether the reduced level of cell fusion observed in MHV-infected LMTK$^-$ vs. L-2 cultures was a consequence of inherent membrane fusion resistance or a lack of expression of viral fusion protein at the cell surface, a contact fusion assay was performed

Figure 4: Demonstration of MHV structural polypeptides in MHV-infected L-2 and LMTK$^-$ cells. Culture dishes (35 mm, 10^6 cells) of LMTK$^-$ (lane A) or L-2 (lanes B and C), inoculated at an moi of 10, were labelled with [^{35}S]-methionine (section 2.11) for 1 h at 0 (lane C) or 8 (lanes A and B) h P.I. Cell extracts were immunoprecipitated with anti-MHV serum (section 2.15) and equal aliquots subjected to fluorographic SDS-PAGE (sections 2.13 and 2.23). MHV polypeptide designations (E_2, N, E_1 and PE_1) are by the convention of Sturman et al., (1980).


A
B
C
E_2
N
E_1
PE_1

(section 2.5). The ability of infected L-2 or LMTK$^-$ cells to induce fusion with neighboring, uninfected "indicator" L-2 or LMTK$^-$ cells was examined (Figure 5). Fusion was assayed within 3 h after addition of the indicator cells to monitor contact fusion with the infected cells and to avoid fusion expression as a result of possible infection of the indicator cells.

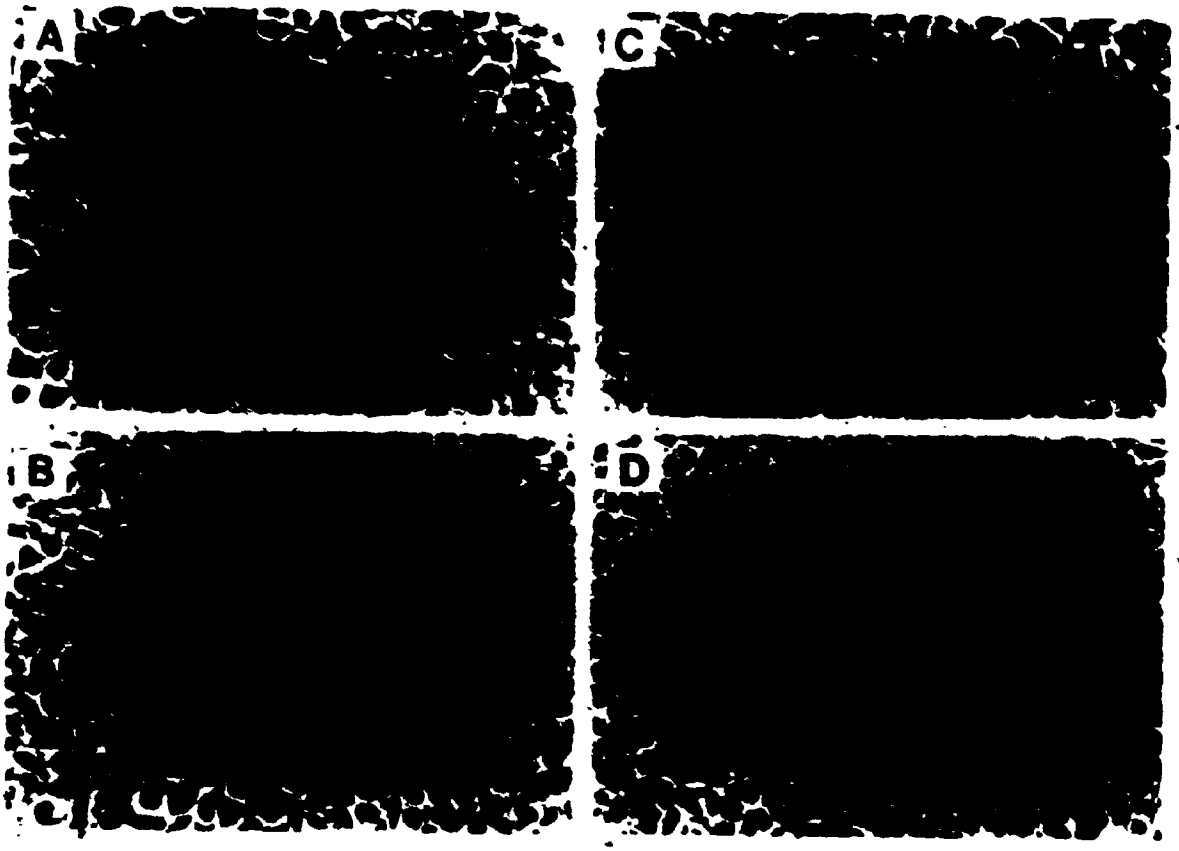
The addition of indicator L-2 cells to sparsely-seeded cultures of either infected L-2 (panel C) or LMTK$^-$ (panel A) cells resulted in the rapid spread of fusion throughout the indicator cells. However, when LMTK$^-$ cells were used as indicators, no fusion was observed (panels B and D).

Since infected LMTK$^-$ cells induce fusion with neighboring uninfected L-2 cells, there is no lack of viral fusion protein at the infected LMTK$^-$ cell surface. However, uninfected LMTK$^-$ cells do not undergo rapid fusion with either infected L-2 or LMTK$^-$ cells, indicating that the difference in cell fusion between infected L-2 and LMTK$^-$ cultures is due to resistance of uninfected LMTK$^-$ cells to undergo fusion with infected neighbors. This fusion resistance must be due to an inherent property of the LMTK$^-$ cell membrane. Such a conclusion is also inferred by observations made earlier (Figure 3), namely that LMTK$^-$ syncytia remain narrow and display little radial expansion when compared to L-2 syncytia.

3.6 Characteristics of MHV Replication in Selected Cell Lines

In the preceding section it was demonstrated that a cell line (LMTK$^-$) which supported a persistent MHV infection differed from the fully permissive L-2 cell line in two characteristics, namely a

Figure 5: Demonstration of cell surface fusion activity in MHV-infected L-2 and LMTK$^-$ cells. To washed, sparse cultures of 10^5 infected L-2 (moi = 0.01; panels C and D) and LMTK$^-$ (moi = 10; panels A and B) cells were added 10^6 uninfected L-2 (panels A and C) or LMTK$^-$ (panels B and D) cells to complete the monolayer (10^6 cells per 35mm dish). Photographs of representative fields were taken 3 h after addition of the uninfected cells (8 h P.I.) using a Leitz inverted microscope.


A
C
B
D

relative resistance to initial MHV infection and to virus-induced cell fusion. Subsequently, in surveying the properties of MHV replication in other cell lines, it was apparent that persistent MHV infections were readily established in cells with similar characteristics. Therefore, a study was undertaken to determine if the documented characteristics of MHV persistence in LMTK$^-$ cells were applicable to other cell lines, and if so, to underscore the generality of such mechanisms of MHV persistence. Much of the data pertaining to MHV infection of LMTK$^-$ cells described herein is similar to that found in sections 3.1 to 3.4, but is included here as a reference point for comparison with the other cell lines.

Shown in Table 2 is an overview of selected cell lines, the outcome of MHV infection, and information regarding initial infection and fusion expression. Three of the cell lines (section 2.1) (LMTK$^-$, LM-ATCC and C-1300; moi = 10) were found to produce much fewer numbers of infected cells compared to fully permissive L-2 cells, when exposed to the same virus inoculum. Two of these cell lines (LMTK$^-$ and LM-ATCC) became persistently infected while the C-1300 cells were destroyed by cytocidal infection within 16 h in a manner approaching that observed in the L-2 infection (moi = 0.1).

3.7 Expression of Cell Fusion in the Semi-Permissive Cell Lines

A major difference between the C-1300 and the LMTK$^-$ or LM-ATCC cells was the expression of virus-induced cell fusion (Figure 6). The F.I. values (section 2.6) for L-2 cells (moi = 0.1 and 0.01) and LMTK$^-$ cells (moi = 10) are in close agreement with previous data

TABLE 2

Characteristics of MHV Infection of Cultured Cell Lines

Cell	moi[a]	Infectious Centers (%)[b]	Virus output per Infected Cell[c]	Type of Infection
L-2	10	99	61.2	acute, fusogenic
L-2	1.0	59	57.6	acute, fusogenic
L-2	0.1	9	72.1	acute, fusogenic
L-2	0.01	0.9	53.0	acute, fusogenic
C-1300	10	1.5	200.0	acute, fusogenic
LMTK⁻	10	1.5	615.0	persistent, weakly fusogenic
LM-ATCC	10	0.2	48.2	persistent, weakly fusogenic
Vero	10	0.0	0.0	refractory
C-6	10	0.0	0.0	refractory

[a] Based on MHV titered on L-2 cells by plaque assay (section 2.4.1).

[b] Percentage of cells infected by MHV as determined by infectious center assay (section 2.4.5).

[c] Ratio of total plaque-assayable virus in 12 h culture fluid divided by total number of infectious centers.

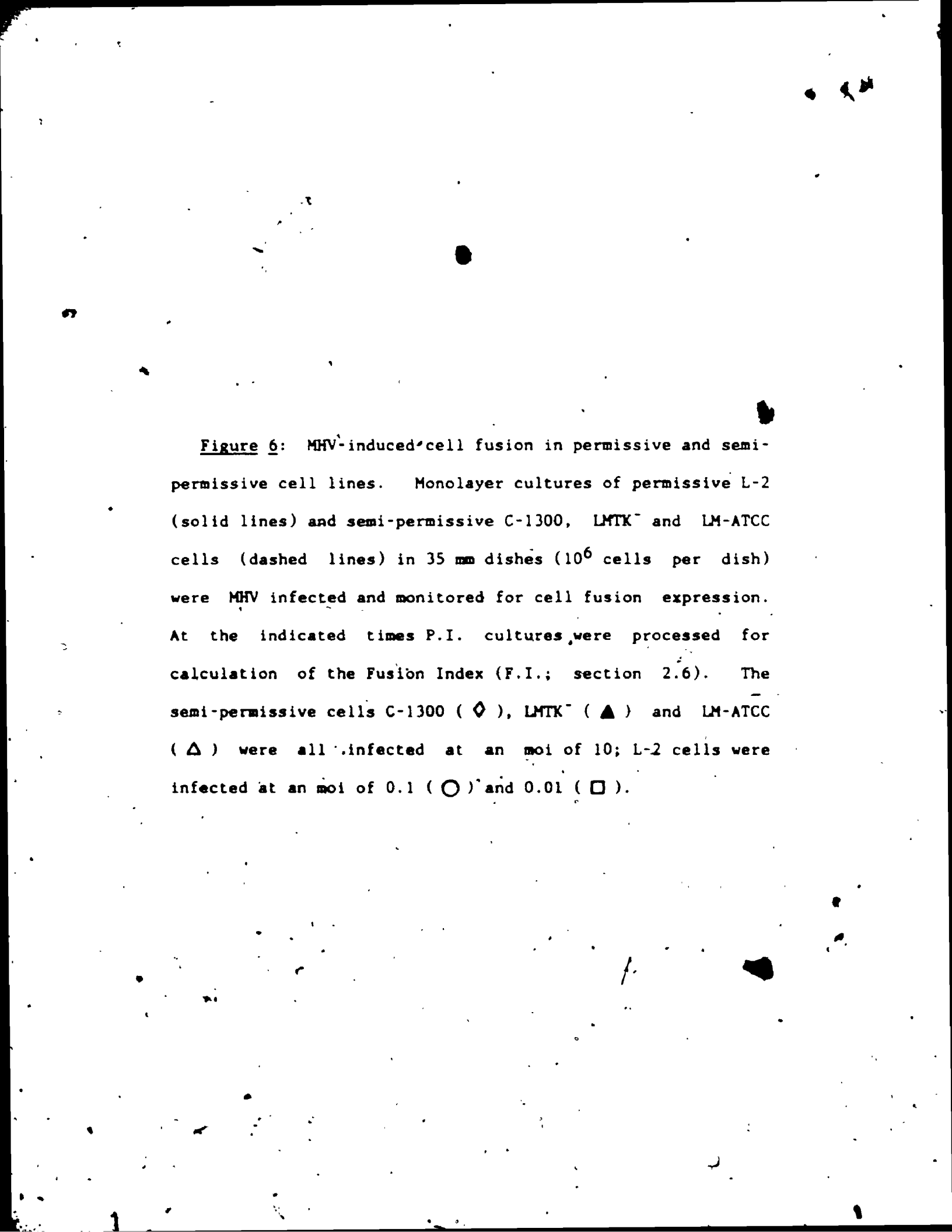
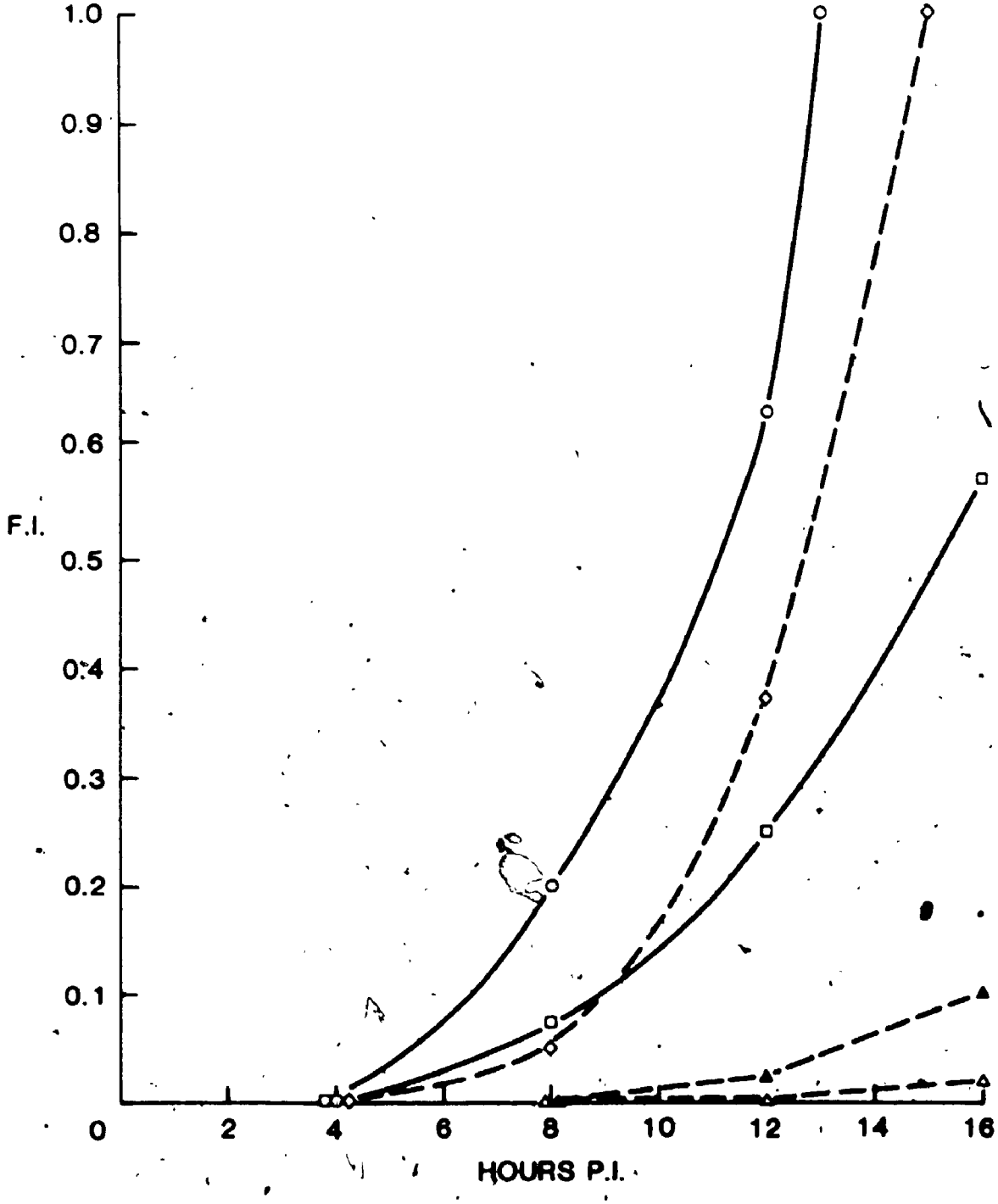


Figure 6: MHV-induced cell fusion in permissive and semi-permissive cell lines. Monolayer cultures of permissive L-2 (solid lines) and semi-permissive C-1300, LMTK$^-$ and LM-ATCC cells (dashed lines) in 35 mm dishes (10^6 cells per dish) were MHV infected and monitored for cell fusion expression. At the indicated times P.I. cultures were processed for calculation of the Fusion Index (F.I.; section 2.6). The semi-permissive cells C-1300 (◇), LMTK$^-$ (▲) and LM-ATCC (△) were all infected at an moi of 10; L-2 cells were infected at an moi of 0.1 (○) and 0.01 (□).


1.0
0.9
0.8
0.7
0.6
F.I.
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
HOURS P.I.

(Figure 2) indicating the relative fusion resistance of the LMTK⁻ cell line. The other two cell lines, C-1300 and LM-ATCC, were strongly contrasted by their expression of virus-induced cell fusion. The progression of cell fusion in C-1300 cells was similar to that observed in the fusion-permissive L-2 cell (moi = 0.1), whereas in LM-ATCC cells, cell fusion was reduced even more so than in the LMTK⁻ cell line. It would therefore appear that, while LMTK⁻ and LM-ATCC cells can support continued MHV infection by restricting virus spread to discrete pockets of infected cells, the C-1300 cells soon succumb to cytocidal infection through unrestricted virus dissemination via cell fusion.

3.8 Infectious Centre Assay of the Semi-Permissive Cell Lines

For purposes of subsequent analysis, a semi-permissive category of cells was defined with respect to MHV infection. Semi-permissive cells, in contrast to fully permissive L-2 cells, give rise to substantially lower (100-1000 fold) numbers of infected cells as indicated by infectious centre assay. In Table 2 are the results of infectious center assay for permissive (L-2), semi-permissive (LMTK⁻, LM-ATCC and C-1300) and non-permissive (C-6 and Vero) cells. Using L-2 cells as a reference, different moi's of MHV inoculum were employed and resultant numbers of infectious centers determined. As shown in Table 2 there are, with decreasing moi, fewer infected L-2 cells, approximating those expected from the Poisson distribution (Dulbecco and Vogt, 1954). In general, for a given moi, the semi-permissive cells give rise to fewer numbers of infectious centres than permissive L-2 cells. Using these data it was possible to

assess parameters of virus replication, on an infected cell basis, for the various cell lines under study.

All three semi-permissive cell lines, assayed at 12 h P.I., were found to replicate yields of progeny MHV, per infected cell, comparable to or greater than permissive L-2 cells (Table 2). The ability of these semi-permissive cell lines to serve as efficient virus "factories" is an interesting phenomenon and will be discussed later (section 4.2).

3.9 Viral RNA Synthesis in the Semi-Permissive Cell Lines

In order to rule out the possibility that viral RNA synthesis was deficient in the semi-permissive cell lines, a dot-blot analysis performed using a [^{32}P]-labeled virus-specific cDNA probe (Cheley et al., 1981a). Dot-blot analysis of MHV RNA from L-2 cells infected at decreasing moi's produces the pattern shown in Figure 7 A-D. At high moi (Figure 7 A) viral RNA synthesis peaked rapidly and was terminated by 8-9 h P.I. due to complete destruction of the cell monolayer. With decreasing moi's, the pattern of viral RNA synthesis was similar but shifted to later times P.I., associated with prolonged survival of the monolayer.

Viral RNA synthesis in the semi-permissive cell lines, LMTK$^-$ (Figure 7 E) and LM-ATCC (Figure 7 F), resembled both chronologically and quantitatively that observed in permissive L-2 cells, when relative differences in infectious centers (Table 2) are taken into account. A similar relation holds true for the semi-permissive C-1300 cells (Figure 7 G), with the exception that viral RNA was no

Figure 7: MHV-RNA synthesis in permissive, semi-permissive and non-permissive cells. RNA was extracted (section 2.17) from MHV-infected monolayer cultures (35 mm dishes, 10^6 cells) of L-2 (panels A to D), LMTK$^-$ (panel E), LM-ATCC (panel F), C-1300 (panel G), vero (panel H) and C-6 (panel I) cells at 0, 4, 8, 12 or 16 h P.I., and aliquots corresponding to that extracted from 10^4 or 10^3 cells were dot-blotted onto nitrocellulose (section 2.19). Synthesis of MHV-specific RNA was detected by hybridization with a [^{32}P]-labelled cDNA probe made against isolated nucleocapsid (N) protein mRNA (section 2.18 and 2.19) and subsequent autoradiography (section 2.23). Cells were inoculated with MHV at mois of 10 (panels A, E to I), 1.0 (Panel B), 0.1 (panel C) and 0.01 (panel D).

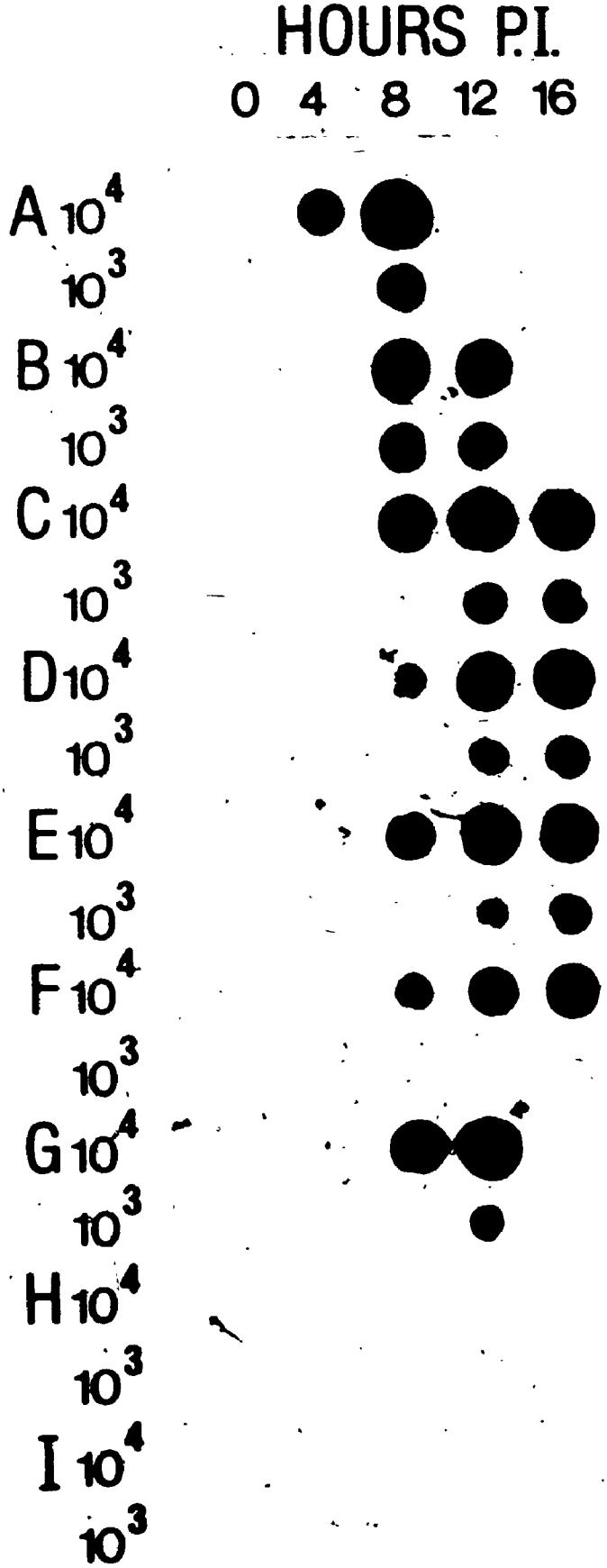
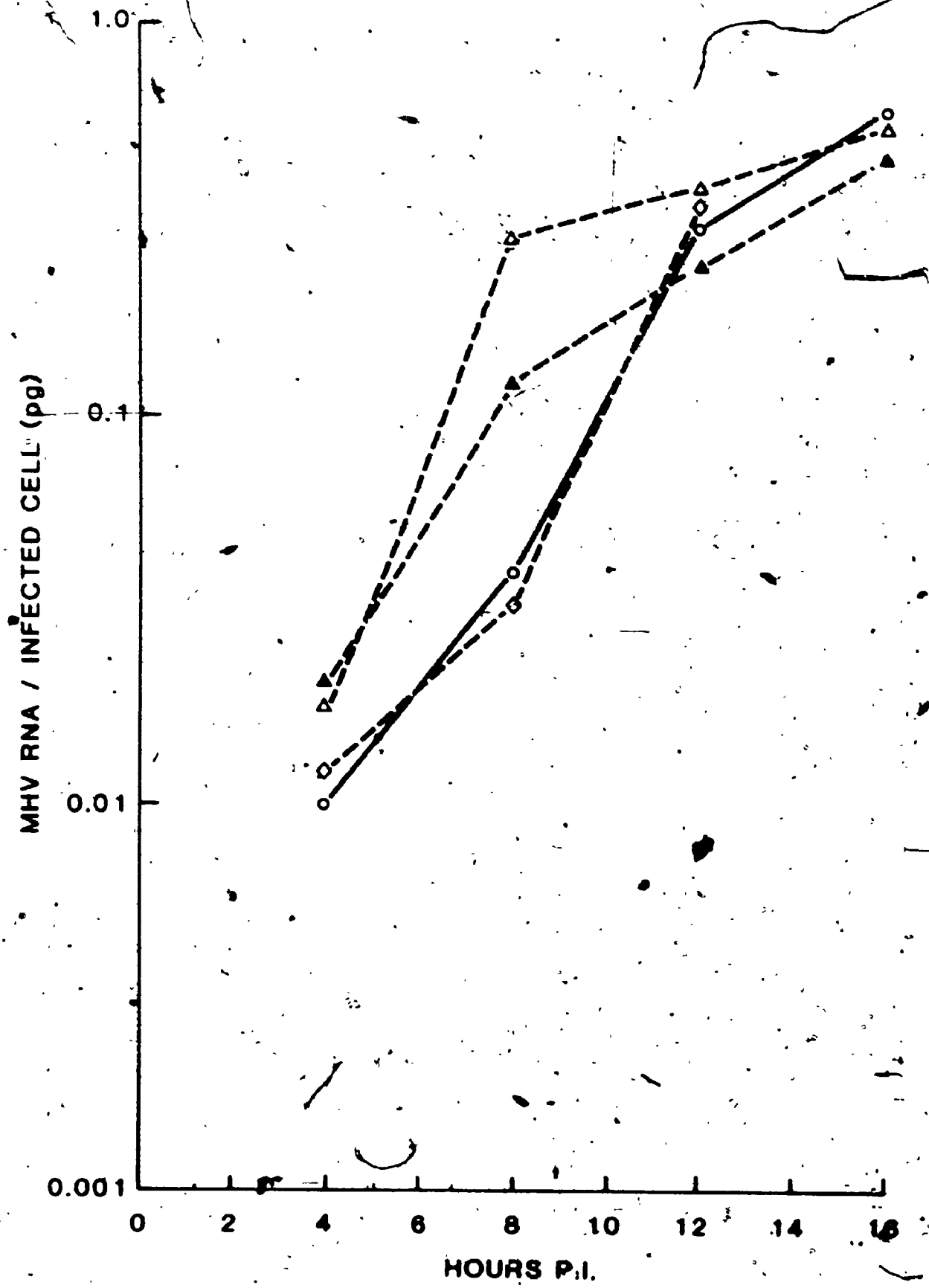
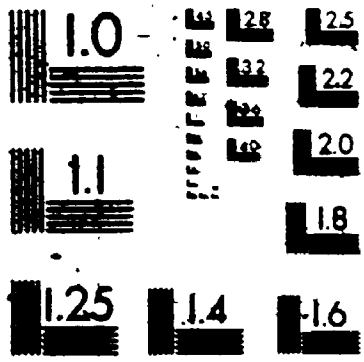

HOURS P.I.
0 4 8 12 16
A 10^4
10^3
B 10^4
10^3
C 10^4
10^3
D 10^4
10^3
E 10^4
10^3
F 10^4
10^3
G 10^4
10^3
H 10^4
10^3
I 10^4
10^3

Figure 8: Quantitation of MHV RNA synthesis in permissive (solid line) and semi-permissive cells (dashed lines). Densitometric scanning of the dot-blot autoradiogram shown in Figure 7 was performed (using purified MHV $mRNA_N$ as internal standard; section 2.19) and in conjunction with infectious centre results (Table 2) this data was plotted as pg of MHV RNA per infected cell. Cultures were inoculated at moi's of 10 (C-1300 (◇), LMTK$^-$ (▲) and LM-ATCC (△)) and 0.01 (L-2 (○)).


1.0
0.1
0.01
0.001
MHV RNA / INFECTED CELL (pg)
0
2
4
6
8
10
12
14
16
HOURS P.I.



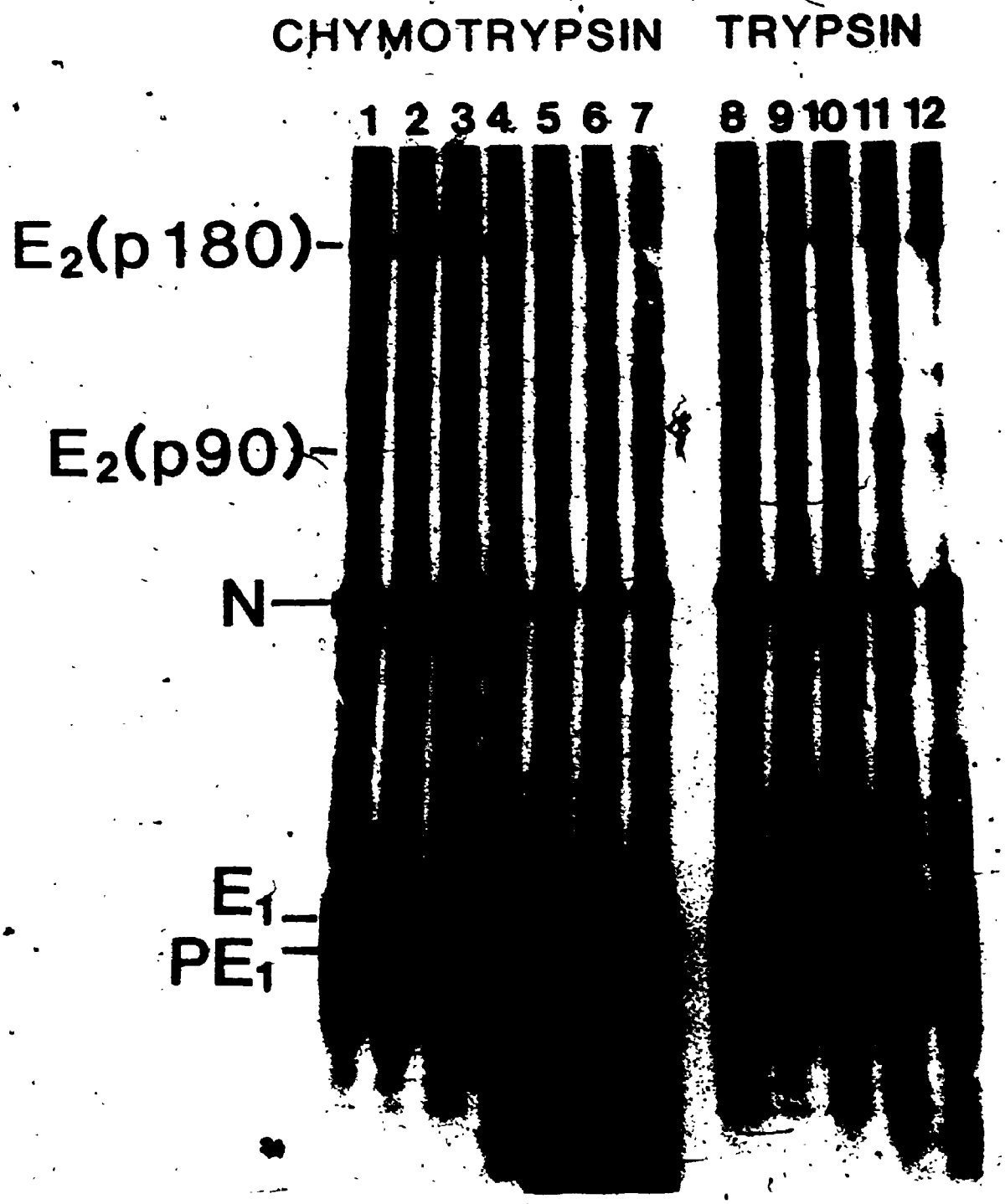
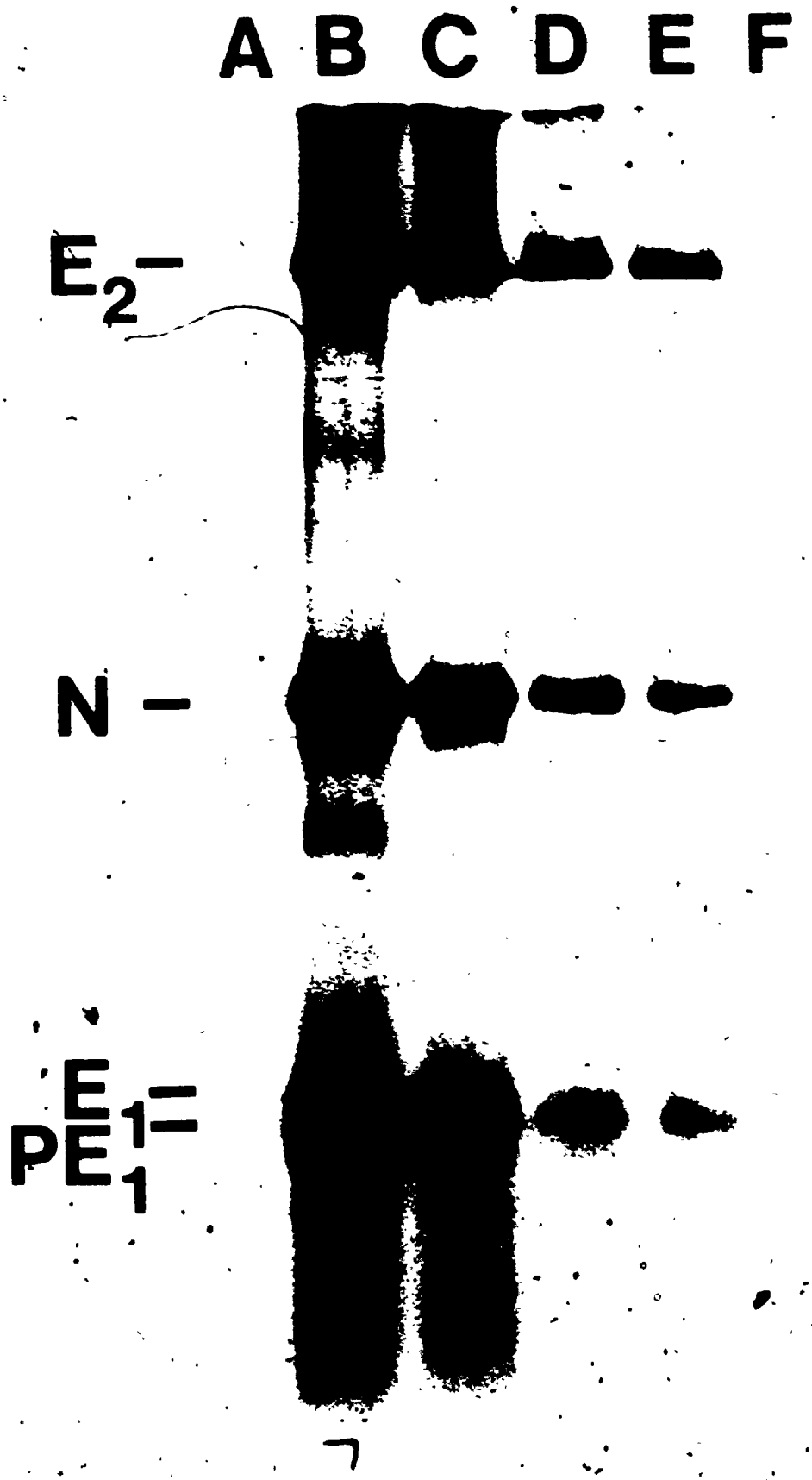

CHYMOTRYPSIN
TRYPSIN
1 2 3 4 5 6 7
8 9 10 11 12
E2(p180)
E2(p90)
N
E1
PE1

Figure 9: Demonstration of MHV structural polypeptides in permissive and semi-permissive cells. Cultures of L-2 (lanes A and B), C-1300 (lane C), LMTK$^-$ (lane D), LM-ATCC (lane E) and Vero (lane F) cells in multiwell trays (4×10^5 cells per well) were either uninfected lane (A) or MHV-infected at an moi of 10 (lanes B to F). At 6 h (lane B) and 12 h (lanes C to F) P.I., cells were labelled with [^{35}S]-methionine for 1 h, harvested for immunoprecipitation (section 2.15) and subjected to SDS-PAGE (section 2.13) and fluorography (section 2.23). Equal aliquots were used in lanes C to F whereas lane B received a 1/10th aliquot of the sample. The positions of the MHV structural proteins are indicated.


A B C D E F
E_2
N
E_1
PE_1

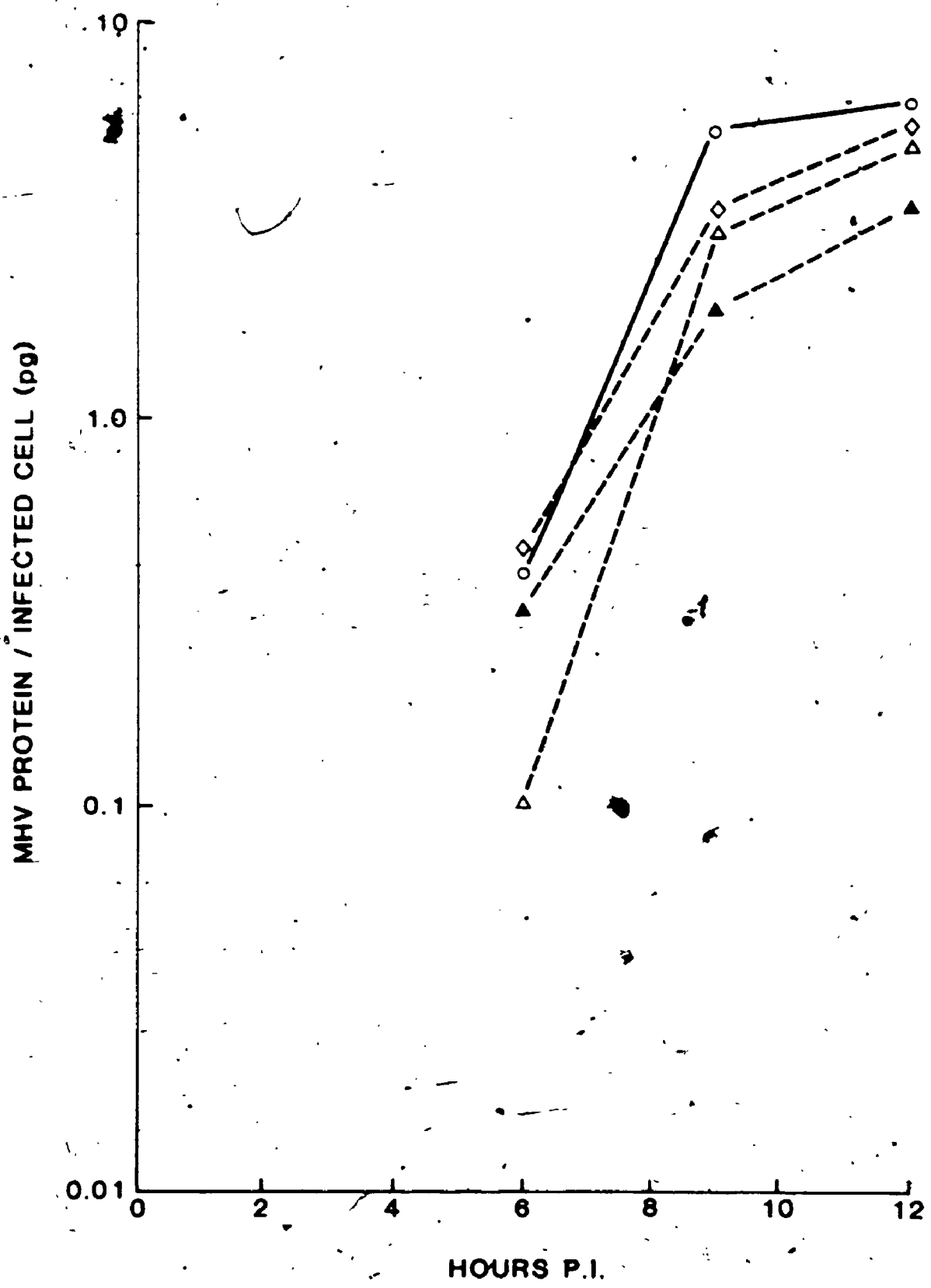
1982). Although not quantitative, the above SDS-PAGE analysis demonstrates the presence of this glycoprotein in its fully mature 180K MW form in MHV-infected L-2, LMTK$^-$, LM-ATCC and C-1300 cells.

3.11 Quantitation of MHV Polypeptides in the Semi-Permissive Cell Lines

Having established that all MHV structural polypeptides are present in the infected semi-permissive cell lines, an ELISA (section 2.16) was developed to quantitate the amount of total MHV protein synthesized in the permissive and semi-permissive cell lines.

The ELISA was performed on MHV-infected L-2, LMTK$^-$, LM-ATCC and C-1300 cultures grown in 96-well microtitre trays using differential moi to standardize numbers of infected cells. In order to correlate the absorbance values obtained by ELISA with amounts of viral protein, a separate experiment was performed (section 2.16) to determine the total and MHV-specific protein contents of L-2 cells inoculated at moi's of 0.1 and 0.01 (to approximate the number of infected cells obtained in the semi-permissive infections). Therefore, the relationship between the ELISA absorbance values and corresponding amounts of MHV protein was determined for infected L-2 cells and applied to the semi-permissive cell lines. Having quantitated MHV protein synthesis in this manner, this data was then expressed in terms of μg MHV protein synthesized per infected cell (Figure 10) using the infectious centre data in Table 2. From Figure 10, it is evident that the semi-permissive cell lines synthesize comparable levels of MHV protein, per infected cell, as permissive L-

Figure 10: Quantitation of MHV protein synthesis in permissive and semi-permissive cells. Cultures of permissive (solid line) and semi-permissive (dashed lines) cells in 96 well microtitre trays (approx. 6×10^4 cells per well) were MHV infected at various moi's to standardize equal numbers of infected cells (Table 2). Cultures were inoculated at moi's of 10 (C-1300 (◇), LMTK$^-$ (▲) and LM-ATCC (△)) and 0.01 (L-2 (○)) and at 6, 9 and 12 h P.I. the monolayers were processed for ELISA (section 2.16). In conjunction with the ELISA, parallel experiments were performed on infected L-2 cells (moi = 0.01) to determine total and MHV-specific protein contents at 6, 9 and 12 h P.I. (section 2.16). Using the infectious center results (Table 2), and the above ELISA and MHV protein content data, the calculation of pg MHV protein per infected cell was performed and plotted as shown.


10
1.0
0.1
0.01
MHV PROTEIN / INFECTED CELL (pg)
0
2
4
6
8
10
12
HOURS P.I.

2 cells, with maximum accumulation (4-7 pg/cell) occurring by 12 h P.I.

3.12 Assessment of Membrane Fusion Activity in Permissive and Semi-Permissive Cell Lines

Even though the semi-permissive cell lines, on an infected cell basis, display no deficiencies with respect to most aspects of MHV replication (protein, RNA synthesis and virion production), two of these cell lines, LMTK$^-$ and LM-ATCC, support a persistent infection in which there is a marked reduction in cell fusion (Figure 6).

To further characterize the reduced fusion capacity of these cells, a contact fusion assay was performed in which uninfected LMTK$^-$, LM-ATCC or C-1300 cells were seeded together with infected L-2 cells (Table 3). Contact fusion (within 3 h) was observed only with C-1300 cells. However, in the reciprocal assay (infected LMTK$^-$, LM-ATCC or C-1300 cells seeded with uninfected L-2 cells), fusion occurred in each case, demonstrating the presence of fusion-active protein at the surface of the semi-permissive cells.

Preliminary experiments indicated that crude lysed cell preparations from MHV-infected L-2 cells would rapidly (within 1 h) induce fusion when added to uninfected L-2 cell monolayers (ie. "fusion from without"). However, cellular membranes (freed of cytosoluble components) prepared from such lysates failed to induce fusion. These findings suggested that a soluble factor was required for the fusion process. Since Sturman et al. (1985) had reported that purified trypsin-treated MHV virions could induce "fusion from without", experiments were performed to investigate the possibility

TABLE 3

Demonstration of Cell Surface Fusion Activity in MHV-Infected Permissive and Semi-Permissive Cells by Contact Fusion Assay

		Infected cell type[a]			
	moi[c]	0.01	1	10	10
		L-2	C-1300	LMTK$^-$	LM-ATCC
Uninfected "Indicator" cell type[b]	L-2	+	+	+	+
	C-1300	+	ND	ND	ND
	LMTK$^-$	-	ND	ND	ND
	LM-ATCC	-	ND	ND	ND

a 10^5 cells seeded in a 35 mm dish were MHV infected at the moi's indicated to produce equal numbers of infected cells (Table 2).

b 10^6 uninfected cells were added to the sparsely-seeded infected cells to complete the monolayers and after 3 h incubation at 37°, syncytia formation scored as + or -. (ND; not determined).

c As determined by plaque assay (section 2.4.1) on L-2 cells.

that an intracellular protease was involved in MHV-induced L-2 cell fusion. Purified membranes isolated from infected L-2 cells (section 2.8) were monitored for their ability to induce fusion in permissive (L-2), semi-permissive (LM-ATCC) and non-permissive (Vero) cells in a membrane fusion assay (section 2.9). As shown in Figure 11 A, C, E, none of these combinations resulted in manifestation of fusion. However, when the membranes were pre-incubated with trypsin (or, in other experiments, chymotrypsin; 10 μg/ml for 1 h at 37°), there was rapid fusion with L-2 cells (Figure 11 B) but not with LM-ATCC or Vero cells (Figure 11 D and F). In control experiments with protease-treated uninfected L-2 cell membranes, no fusion activity could be demonstrated (data not shown). This suggests that fusion expression is a consequence of proteolytic activation of viral, rather than cellular polypeptide(s).

To identify biochemical changes which correlated with the protease-dependent activities of the membranes described above, parallel preparations were obtained from MHV-infected L-2 cells which had been labeled with [^{35}S]-methionine from 5-6 h P.I. The purified radiolabeled membranes were subjected to protease digestion (section 2.10) with either trypsin or chymotrypsin at a range of concentrations including that used in the membrane fusion assay. Following protease treatment, the distribution of radiolabel among the membrane polypeptides was examined by fluorographic SDS-PAGE (Figure 12). Of interest is the observed reduction in radiolabeled E_2(p180) with increasing concentrations of either trypsin or chymotrypsin. In addition, beginning at a chymotrypsin concentration

Figure 11: Light micrograph of membrane fusion assay performed with permissive (L-2), semi-permissive (LM-ATCC) and non-permissive (vero) cells. To uninfected 35 mm dish cultures (10^6 cells) of L-2 (panels A and B), LM-ATCC (panels C and D) and Vero (panels E and F) cells was added purified MHV-infected cell membranes (section 2.8 and 2.9). In panels B, D and F, the membranes were pre-incubated with 10 µg/ml trypsin for 1 h at 37° prior to addition to the monolayers. After 1 h incubation at 37°, the membranes were washed off the monolayers and the cultures fixed, Giemsa stained (section 2.6) and photographed using a Leitz Dialux 20 microscope. In panel B (L-2 cells plus protease-treated membranes) numerous areas of cell fusion are evident (arrow indicates syncytium).

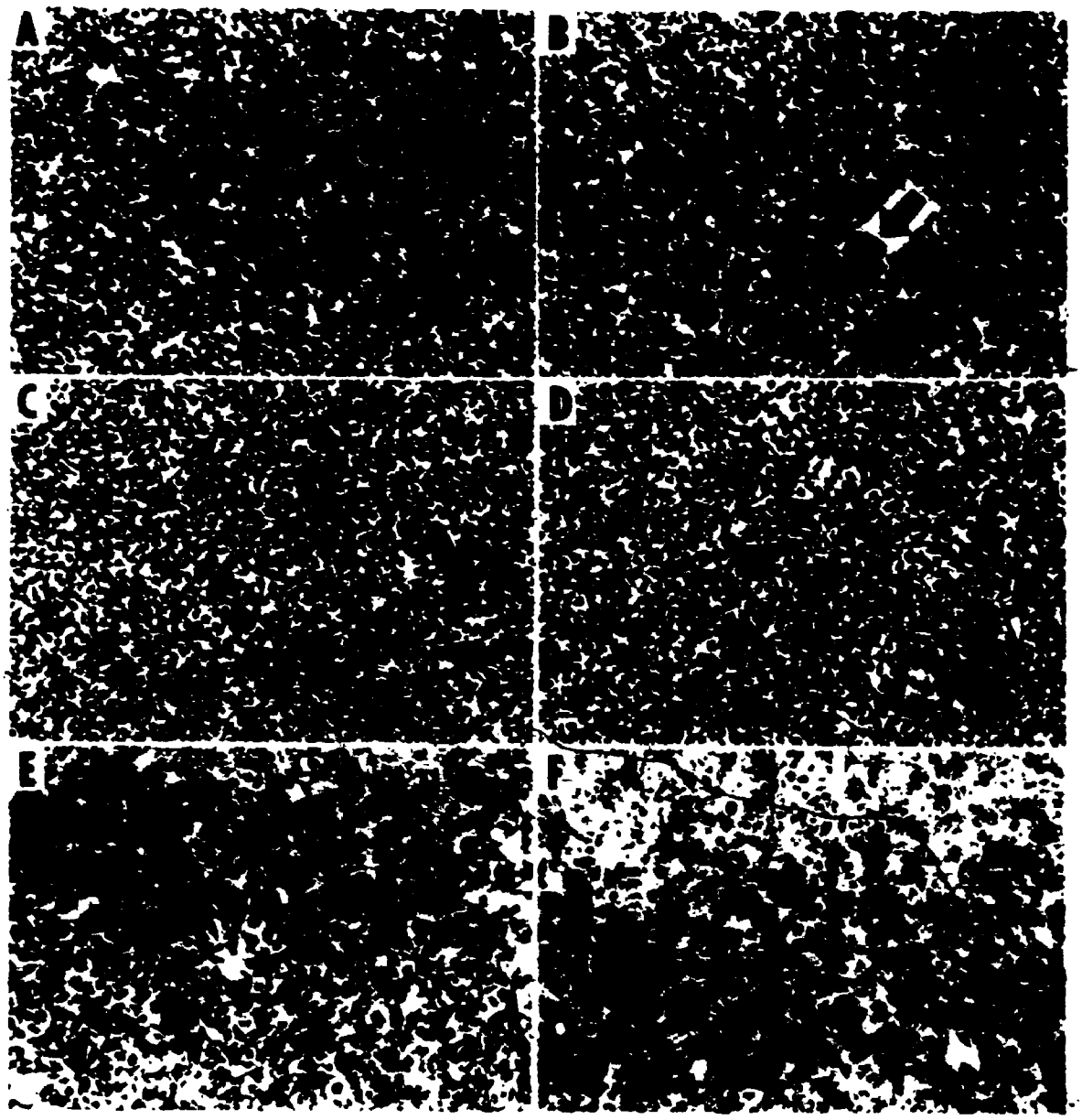
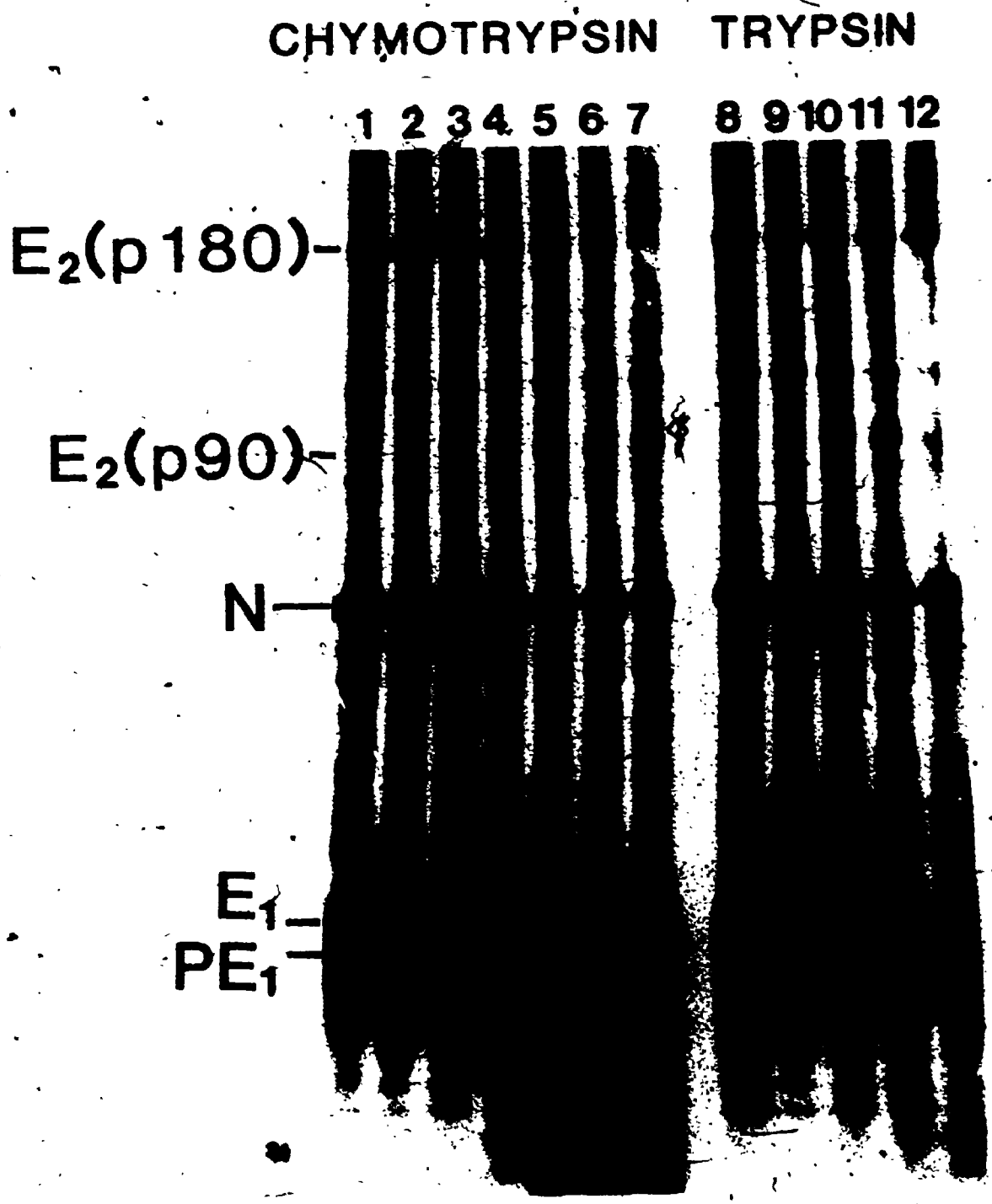

A
B
C
D
E
F

Figure 12: Protease treatment of membranes prepared from [^{35}S]-methionine-labelled MHV-infected L-2 cells. MHV-infected L-2 cells in a 100 mm dish (10^7 cells) were labelled with [^{35}S]-methionine from 5-6 h P.I. (section 2.10) and the cellular membranes harvested as described (section 2.8). Aliquots were incubated for 1 h at 37° with trypsin or chymotrypsin at concentrations of 0 µg/ml (lane 1), 0.5 µg/ml (lane 2), 1.0 µg/ml (lanes 3 and 8), 5.0 µg/ml (lanes 4 and 9), 10 µg/ml (lanes 5 and 10), 50 µg/ml (lanes 6 and 11) and 100 µg/ml (lanes 7 and 12). Protease digestion was then terminated with TLCK (for trypsin) and TPCK (for chymotrypsin), the samples lyophilized, and analyzed by fluorographic SDS-PAGE (sections 2.12, 2.13 and 2.23). The positions of the MHV structural proteins (E_2(p180), N, E_1 and PE_1) are indicated as well as the product of E_2(p180) proteolytic digestion, E_2(p90).


CHYMOTRYPSIN
TRYPSIN
1 2 3 4 5 6 7
8 9 10 11 12
E2(p180)
E2(p90)
N
E1
PE1

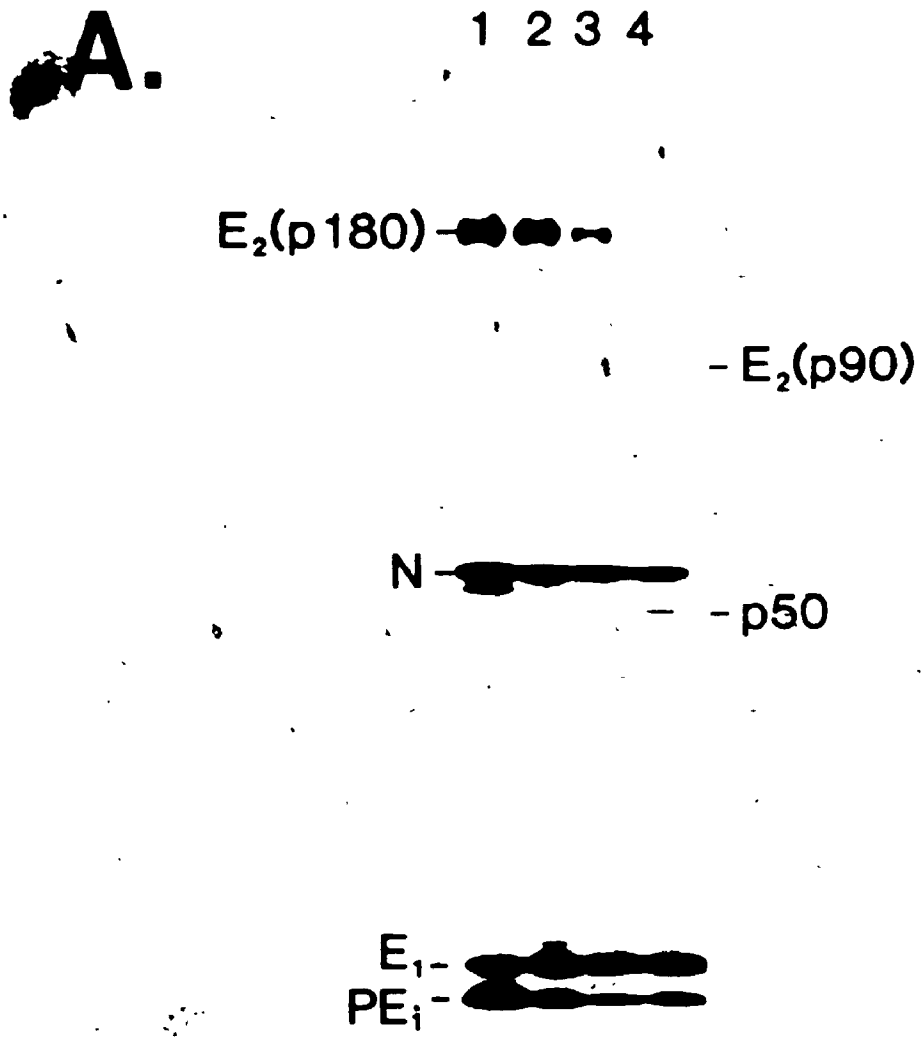
of 5 µg/ml or a trypsin concentration of 10 µg/ml (Figure 12 lane 4 or 10), a polypeptide with an apparent molecular weight of 90K (by comparision with molecular weight protein standards) was detected. This polypeptide was related to E_2(p180), as indicated by peptide mapping (data not shown) and is likely related to the 90K MW form of the E_2 which is found in varying amounts in MHV virions (Sturman and Holmes, 1977; Sturman et al., 1985). The extent of proteolysis of E_2(p180) and appearance of the 90K MW form, E_2 (p90), do not correlate quantitatively, suggesting that additional proteolytic events may be taking place.

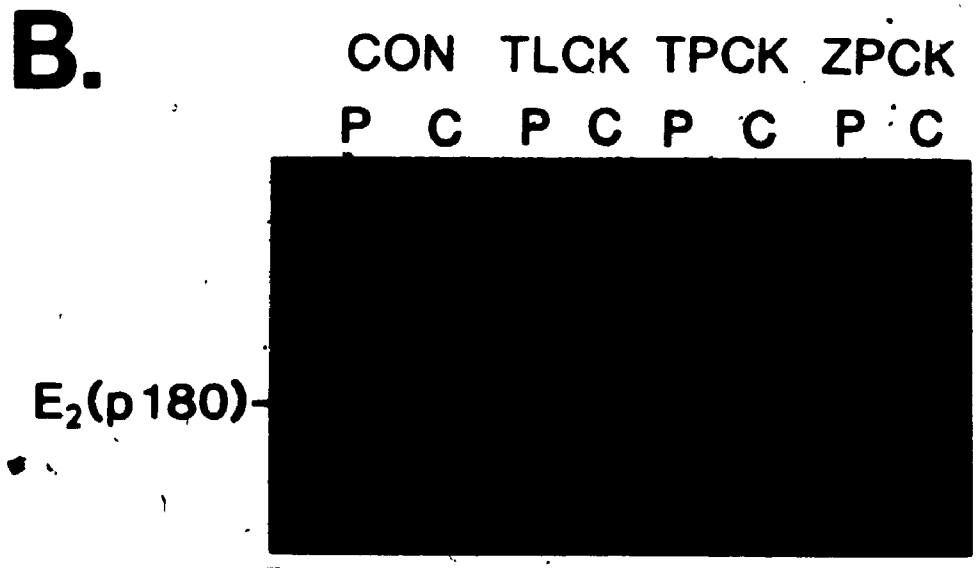
3.13 The Nature of the E_2 Glycoprotein in MHV-Infected L-2 Cells

In MHV virions, the glycoprotein E_2 may exist in either a 180K or 90K MW form (Sturman and Holmes, 1977) and the latter form may be responsible for "fusion from without" induced by trypsin-treated virions (Sturman et al., 1985). Since L-2 cells are extremely susceptible to "fusion from within" (Locas et al., 1977; Mizzen et. al, 1983) it is of interest to note that the 180K MW form of E_2 is by far the predominating species labeled in infected L-2 cells (Cheley and Anderson, 1981).

In a pulse-chase study of infected L-2 cultures, pulse-labeled E_2(p180) (section 2.11) declined rapidly and with prolonged "chase" periods (90 min. and 120 min.) gave rise to small amounts of E_2(p90) (Figure 13 A, lanes 3 and 4). The distribution of radiolabel in the other MHV polypeptides under these conditions was similar to that previously published (Cheley and Anderson, 1981). Note that whether

Figure 13: Behaviour of pulse-labelled E_2(p180) in the absence or presence of protease inhibitors. Cultures of MHV-infected L-2 cells (moi = 10) in 35 mm dishes (10^6 cells) were pulse-labelled with [^{35}S]-methionine (section 2.11) for 15 min. at 5 h P.I. and "chased" either in the absence (Figure 13A) or presence (Figure 13B) of protease inhibitors. In Figure 13A, the chase periods were 0 (lane 1), 45 (lane 2), 90 (lane 3) and 120 min.(lane 4). The positions of the MHV structural polypeptides (E_2(p180), N; E_1 and PE_1) or related forms (E_2(p90) and p50) are indicated. In Figure 13B, pulse-labelled (P) E_2(p180) was chased (C) for 120 min. in medium without (CON) or with the protease inhibitors TLCK, TPCK or ZPCK at a final concentration of 10^{-4}M.


A.
1 2 3 4
E2(p180)
E2(p90)
N
p50
E1
PE1


B.
CON TLCK TPCK ZPCK
P C P C P C P C
E2(p180)

the conversion of E_2(p180) to E_2(p90) is achieved by exogenous protease treatment of membranes isolated from infected cells (membrane fusion assay) or by pulse-labeling of infected monolayers, the amount of E_2(p90) generated is much less than expected if this event is simply cleavage of parental E_2(p180) to E_2(p90) species. Therefore, within the infected cell, E_2(p180) may be susceptible to extensive intracellular proteolysis.

To examine this possibility, a pulse-chase experiment was performed in the presence or absence of specific protease inhibitors. Cultures of L-2 cells, inoculated with MHV, were maintained in medium containing either no inhibitor, or the inhibitors TLCK, TPCK or ZPCK at a concentration of 10^{-4}M. At 4 h P.I. the cultures were pulsed with [^{35}S]-methionine and subsequently chased. As shown in Figure 13 B, pulse-labeled E_2(p180) disappeared rapidly during the chase period in normal medium, as well as in medium plus TLCK. However, the protease inhibitors TPCK and ZPCK markedly prolonged the survival time of E_2(p180), suggesting inhibition of proteolysis. The pulse-chase relationships for the remaining radiolabeled polypeptides not shown in Figure 12 B were not affected by any of the three protease inhibitors.

In addition, TPCK and ZPCK, but not TLCK, were also effective in inhibiting MHV-induced cell fusion <u>in vitro</u>. At 7 h P.I., at which time MHV-infected L-2 cells (moi = 10) were completely fused into a syncytial sheet, parallel cultures treated with TPCK or ZPCK (starting at 3 h P.I.) contained only occasional syncytial foci. Given the demonstrated activities of TLCK against trypsin (Shaw <u>et</u>

al., 1965) and TPCK, ZPCK against chymotrypsin (Schoellmann and Shaw, 1962; Segal et al., 1971) the above results implicate a chymotrypsin-like enzyme in intracellular processing of E_2(p180) and in activating MHV-induced cell fusion in L-2 cells.

Therefore, when viewed in light of MHV replication in permissive and semi-permissive cell lines, differences in host-cell proteolytic processing of E_2(p180) and/or the ability of the cell plasma membrane to undergo fusion when presented with fusogenic E_2 ultimately determine whether a cell will experience acute or persistent MHV infection.

Subsequent to completion of this work, a publication (Frana et. al., 1985) confirmed many of these results on host-cell specific proteolytic processing of MHV E_2(p180).

3.14 Effect of Ammonium Chloride on the Production of Progeny MHV

Having established that in MHV infection of a variety of cell lines in vitro, viral persistence is regulated by host cell determinants governing both early (initial infection) and late (expression of cell fusion) events in the replication cycle, moderation of these membrane fusion events in MHV replication in a fully permissive host cell could conceivably lead to a state of viral persistence.

Ammonium chloride had been reported in the literature to inhibit both early events such as viral uncoating (e.g. Helenius et al., 1982) and late events in viral replication, such as induction of cell fusion (Holland and Person, 1977).

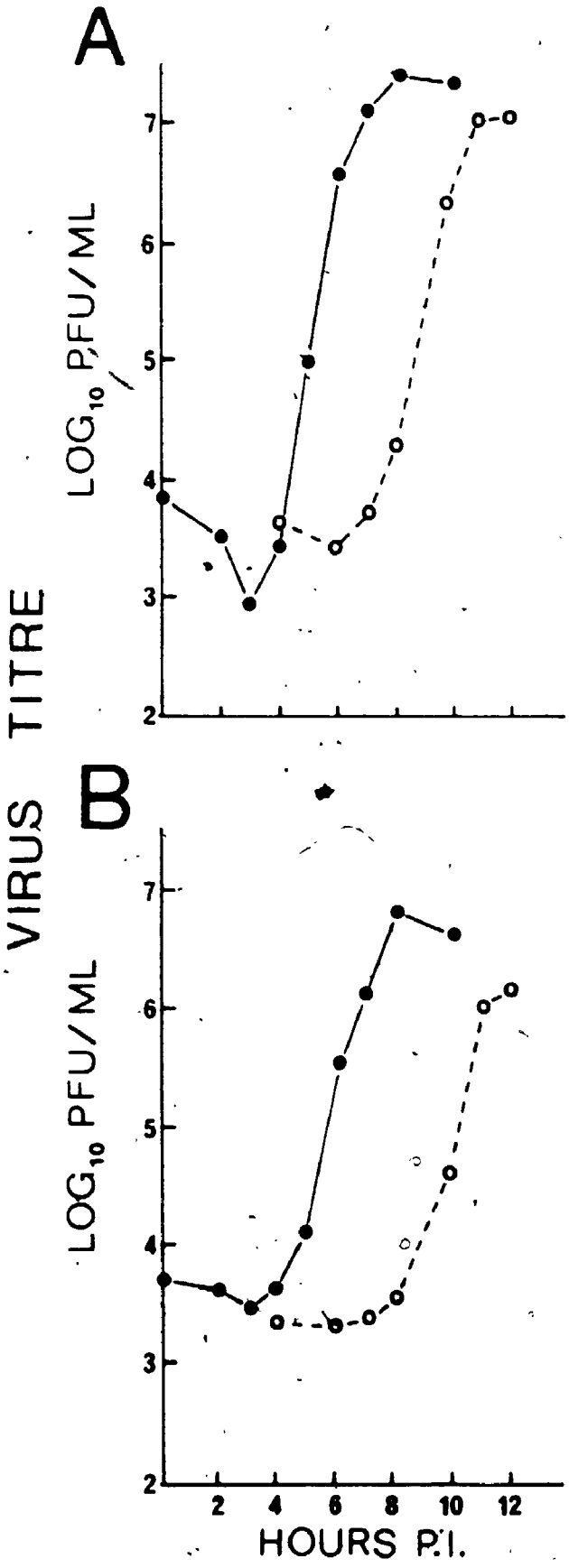
At the time this work was undertaken, the effect(s) of ammonium chloride on coronavirus replication were not known. Therefore, the

rationale for using ammonium chloride was two-fold: first, to document its effects on the replication cycle of MHV and, second, on the basis of its reported effects on viral replication, to employ ammonium chloride in an attempt to convert the normally acute MHV infection of L-2 cells to a state of viral persistence.

The presence of ammonium chloride in the culture medium at a concentration of 20 mM delayed the production of progeny MHV by roughly 4 h in MHV-inoculated L-2 cells (Figure 14). As shown in Figure 14 A, intracellular MHV progeny (section 2.4.2) is first detectable under normal culture conditions at approximately 4-5 h P.I. In contrast, intracellular MHV progeny does not appear until approximately 8 h P.I. in the presence of ammonium chloride. From the times of initial appearance of progeny virus, however, the replication profiles, either in the presence or absence of ammonium chloride, are very similar. Moreover, the final titers of MHV produced in either case differ only by a fraction of a log.

A similar delaying effect of ammonium chloride was observed on the production of extracellular virus (section 2.4.1). Under normal conditions, MHV-infected L-2 cells first produce detectable extracellular virus at approximately 5 h P.I. (Figure 14 B). In the presence of ammonium chloride, however, extracellular virus is not seen until 9-10 h P.I. Again, as in the case of intracellular virus, the subsequent replication curves and final virus titers are very similar, whether monitored from control or ammonium chloride-treated cultures.

Figure 14: Production of intracellular (panel A) and extracellular (panel B) virus from MHV-infected L-2 cells maintained at 37° in the absence (●——●) or presence (○——○) of 20 mM ammonium chloride. MHV-infected cultures of L-2 cells (moi = 20) in 35 mm dishes (10^6 cells) were assayed for intracellular virus (section 2.4.2) and extracellular virus (section 2.4.1) at the indicated times P.I.


A
B
VIRUS TITRE
LOG10 PFU/ML
LOG10 PFU/ML
2
3
4
5
6
7
2
4
6
8
10
12
HOURS P.I.

3.15 Ammonium Chloride Delays MHV-Induced Cell Fusion

The above results suggest that the action of ammonium chloride is to attenuate a step in MHV replication, which once overcome, permits normal virus production. If this step is an early one, the normal chronology for the majority of the events associated with MHV replication will be displaced by a 4 to 5 h time interval similar to that observed with virus replication. In agreement with this prediction, the manifestation of cell fusion, as indicated by the F.I. data (section 2.6), was found to be delayed by ammonium chloride to an extent similar (4-5 h) to that observed with the production of virus progeny (Figure 15). Furthermore, like the replication curves, the F.I. data from ammonium chloride-treated and control cultures are virtually superimposable once this 4-5 h delay is taken into account.

There would, therefore, appear to be no specifically inhibitory effect of ammonium chloride on MHV-mediated cell fusion per se.

3.16 Number of Cells Infected by MHV in the Presence of Ammonium Chloride

In order to confirm that the effect of ammonium chloride on MHV replication was one of attenuation rather than suppression, the number of productively infected L-2 cells was monitored by an infectious center assay. Cultures of MHV-inoculated L-2 cells were incubated for 2 h at 37°C in the presence or absence of ammonium chloride, then trypsinized and plated on uninfected L-2 cell monolayers in order to quantitatively screen for numbers of productively infected cells. As shown in Table 4, ammonium chloride-

Figure 15: MHV-induced cell fusion in the absence and presence of ammonium chloride. MHV-infected cultures of L-2 cells (moi = 20) in 35 mm dishes (10^6 cells) were incubated in the absence (●——●) or presence (○——○) of 20 mM ammonium chloride at 37°. Cultures were processed for calculation of the Fusion Index (F.I.; section 2.6) at the indicated times P.I.

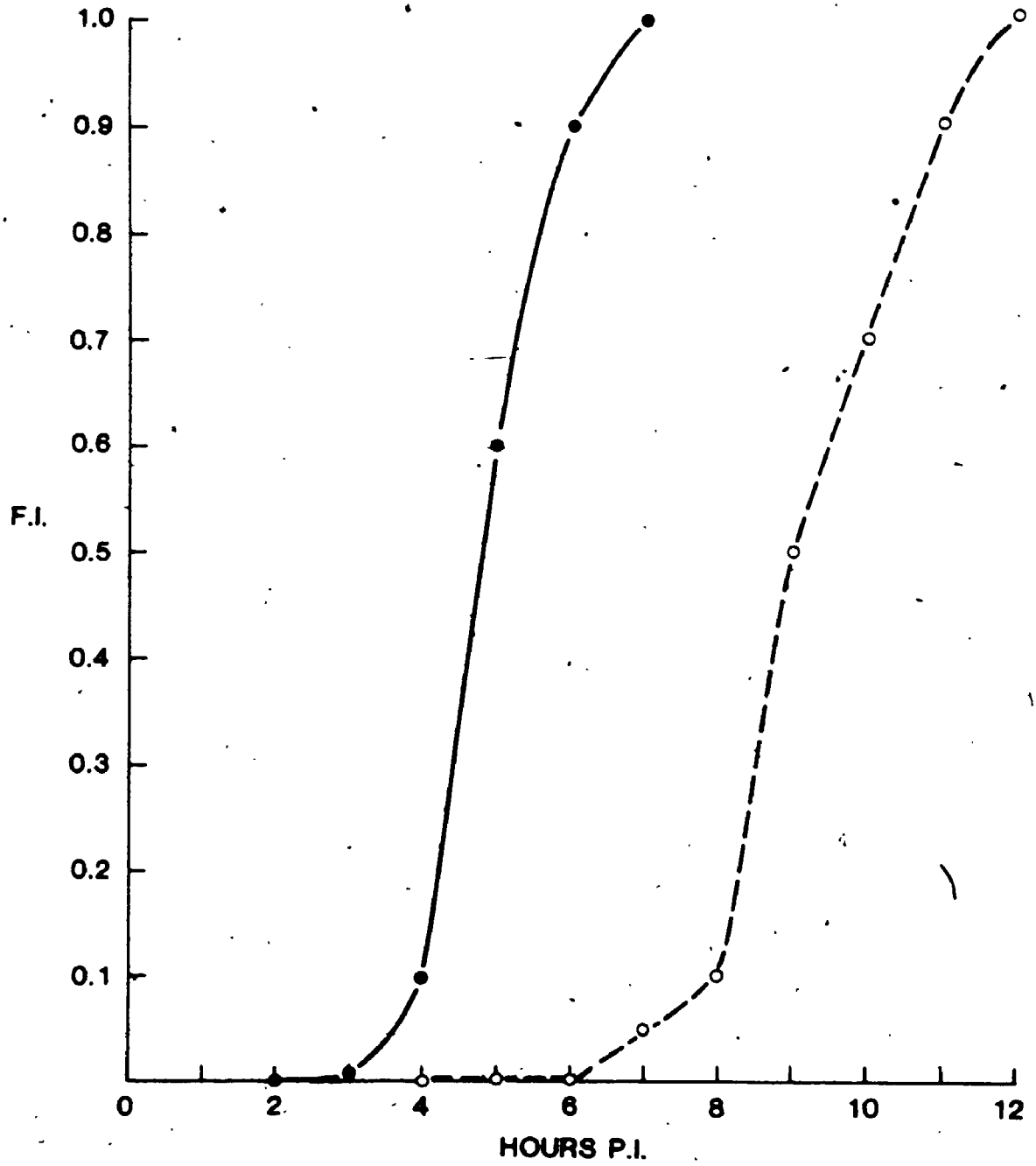

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
F.I.
0
2
4
6
8
10
12
HOURS P.I.

TABLE 4

Ammonium Chloride Does Not Markedly Reduce the Number of Cells Infected with MHV

Initial Medium (0-3 h P.I.)	Number of cells infected[a] Assay Medium: Control	Assay Medium: NH_4Cl
Control	6.8×10^3	6.1×10^3
NH_4Cl	5.2×10^3	4.9×10^3

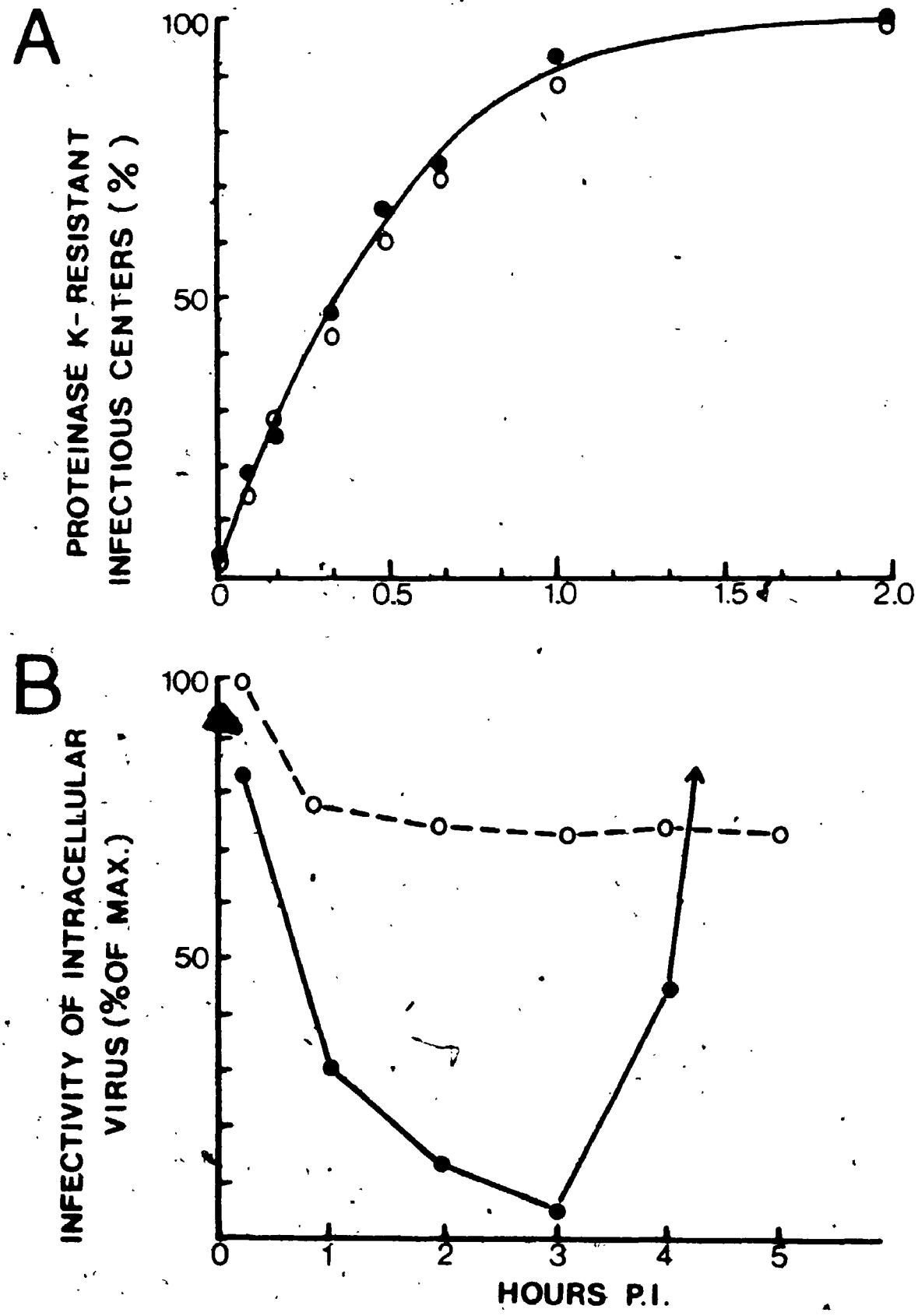
[a] Cultures of L-2 cells in 35 mm dishes (10^6 cells) were inoculated with MHV at an moi of 0.01, adsorbed 30 min. at 4° and then incubated 2 h in the absence (control) or presence (NH_4Cl) of 20 mM ammonium chloride. Cells were recovered intact by trypsinization and aliquots were plated out for infections center assay on monolayers of L-2 cells (section 2.4.5). Assays were performed for 24 h in the absence (control) or presence (NH_4Cl) of 20 mM ammonium chloride in the medium.

treated cultures had similar numbers of infected cells as untreated cultures. Moreover, little difference was observed even when ammonium chloride was included in the medium used for the infectious center assay incubation. The size of the plaque produced by each infectious center, however, was considerably smaller in the case of the cells which were cultured in the presence of ammonium chloride. This result is entirely consistent with the idea that ammonium chloride attenuates or delays MHV replication, without imposing an absolute block.

3.17 Effects of Ammonium Chloride on Early Stages of MHV Infection

In agreement with the studies by Krzystyniak and Dupuy (1984), our preliminary expetiments had shown that MHV adsorption to L-2 cells was unaffected by the presence of ammonium chloride. Accordingly, all studies reported here were performed with the addition of ammonium chloride immediately following the adsorption period. The entry of MHV into the host cell appears to be one involving viropexis (David-Ferreira and Manaker, 1965) in which virus inoculum is internalized within a cytoplasmic vesicle or endosome. In order to investigate whether ammonium chloride affected the process of virus internalization (section 2.4.3), MHV-adsorbed cells were warmed to 37°C for various intervals in the presence or absence of ammonium chloride, then stripped of external virus with proteinase K, and assayed for internalized virus by infectious center assay. The results shown in Figure 16 A demonstrate that virus internalization occurred with equal efficiency, whether or not ammonium chloride was present. In either case, uptake of MHV into

Figure 16: Effect of ammonium chloride on virus internalization (A) and infectivity of internalized virus (B). In (A), 35mm dish cultures (10^6 cells) of MHV-adsorbed L-2 cells (performed at 4°) (moi = 20) were warmed to 37° for various times in the absence (●——●) or presence (○——○) of 20 mM ammonium chloride. After treatment with proteinase K to remove external virus (section 2.4.3), cells were assayed for internalized virus by infectious centre assay (section 2.4.5). In (B), a second set of MHV-inoculated cultures (same conditions as (A)) were warmed to 37° for 20 min. in the absence (●——●) or presence (○——○) of 20 mM ammonium chloride. Cells were harvested and lysed for assay of infectivity of intracellular virus at the indicated times P.I. (section 2.4.4). Results are expressed as percentage of the maximal titer obtained (20 min., ammonium chloride treated).


A
PROTEINASE K-RESISTANT INFECTIOUS CENTERS (%)
100
50
0
0.5
1.0
1.5
2.0
B
INFECTIVITY OF INTRACELLULAR VIRUS (%OF MAX.)
100
50
0
1
2
3
4
5
HOURS P.I.

the cell occurred rapidly upon exposure to 37°C and was essentially complete after 2 h.

Events subsequent to virus uptake, which result in the liberation of viral genome into the cytosol have not been characterized in coronavirus replication. One possibility, analogous to that observed with many enveloped viruses, is that the viral envelope undergoes fusion with the membrane of the surrounding vesicle (e.g., Marsh et al, 1983). Since this latter fusion event is known to be inhibited by lysosomotropic amines in the case of infection with certain viruses, such as the enveloped togaviruses (Helenius et al., 1982), we monitored the effect of ammonium chloride on the eclipse phase of MHV infection. Virus uncoating, as well as any other degradative processes occurring during the eclipse phase, can be monitored by following the decline in infectivity of internalized MHV inoculum. In order to allow a reasonable degree of virus internalization to occur, MHV-adsorbed cells were warmed to 37°C for 20 min. in medium with and without ammonium chloride. Under these conditions, approximately 50% of cell-bound virus is internalized (Figure 16 A). Cells were then treated with proteinase K to remove external virus, incubated at 37°C in the presence or absence of ammonium chloride, then subsequently lysed and assayed for infectivity of internalized inoculum (section 2.4.4). While this procedure does not permit analysis of events occurring during the first 20 min., it is evident from the results shown in Figure 16 B that the eclipse phase in MHV infection is strikingly affected by the presence of ammonium chloride. At the first time point examined (20

min.), virus eclipse was seen to occur more rapidly in the absence of ammonium chloride, as judged by a 17% decrease in relative infectivity of intracellular inoculum. The effect of ammonium chloride on virus eclipse became more pronounced with time. Thus, while in normal MHV infection, virus eclipse was complete by 3 h P.I., the process occurred with clearly less efficiency in the presence of ammonium chloride (Figure 16 B), so that even as late as 5 h P.I. considerable infectivity was associated with the internalized virus inoculum. Taken together, this data on the eclipse stage of MHV infection suggests an inhibitory effect of ammonium chloride on one or more processes involved in the uncoating of MHV. It is not possible, however, with this data to distinguish which proportion of the eclipsed virus represents legitimate endosomal uncoating as opposed to a possible "dead-end" pathway of lysosomal degradation. Indeed, in light of the infectious center data (Table 4), it is apparent that, even in the presence of ammonium chloride, MHV must eventually uncoat to a similar degree as that attained in untreated cells. Given the evidence that ammonium chloride inhibits the activities of both lysosomes (Ohkuma and Poole, 1978) and endosomes (Maxfield, 1982) the results of Figure 16 B are best explained on the basis of combined inhibitory actions on lysosomal/endosomal processing of internalized MHV.

3.18 Effects of Ammonium Chloride on MHV-RNA Synthesis

If the eclipse phase is the primary site of action of ammonium chloride during MHV replication, then attenuation or delay of

infection at this early stage would be expected to give rise to similar delays in subsequent virus-encoded functions. Since the first easily assayable function following eclipse is viral RNA synthesis, we examined levels of MHV RNA produced in infected cells maintained in the presence or absence of ammonium chloride. [5-^{3}H]-uridine-labeled RNA (section 2.20) extracted at 6 h P.I. from MHV-infected L-2 cells (section 2.17), maintained in the presence or absence of ammonium chloride, was subjected to agarose gel electrophoresis (section 2.21) and fluorography (section 2.23). The normal pattern of MHV-encoded RNAs (Cheley et al., 1981) numbered according to the convention of Siddell et al. (1983) was obtained (Figure 17 lane C). When ammonium chloride was present immediately following adsorption, the level of radiolabeled MHV RNA was reduced (Figure 17 lane 0) to 8% of the control level, as determined by comparative densitometric scanning of lanes 0 and C. When the addition of ammonium chloride was delayed by 1, 2, or 3 h following adsorption, thus permitting progressively more virus uncoating, the levels of radiolabeled MHV RNA were seen to increase (Figure 17 lanes 1, 2, and 3) to 34, 52 and 85%, respectively of that obtained in the normal MHV infection (lane C). Furthermore, the normal electrophoretic pattern of MHV RNA was obtained in the presence of ammonium chloride.

To investigate the effect of ammonium chloride on the kinetics of viral RNA synthesis, a quantitative time-course analysis of viral RNA levels was performed on MHV-infected cells maintained in the presence or absence of ammonium chloride. Shown in Figure 18 A and B

Figure 17: Effect of ammonium chloride on the production of MHV-RNAs. Cultures of L-2 cells in 100 mm dishes were adsorbed with MHV (moi = 20) at 4° and incubated in the absence (C) or presence of 20 mM ammonium chloride, added either immediately after adsorption (0), 1 h P.I. (1), 2 h P.I. (2) or 3 h P.I. (3). Viral RNA was radiolabelled by culturing the infected cells with [5-^{3}H]-uridine in the presence of actinomycin D (section 2.20). Cultures were harvested at 6 h P.I., and the RNA extracted by guanidine-HCl (section 2.17) and analyzed by agarose gel electrophoresis (section 2.21). An autoradiograph of the dried, EN^{3}HANCE (NEN) - treated gel is shown. Numbering of the MHV-specific RNA species follows the convention of Siddell et al:, (1983).

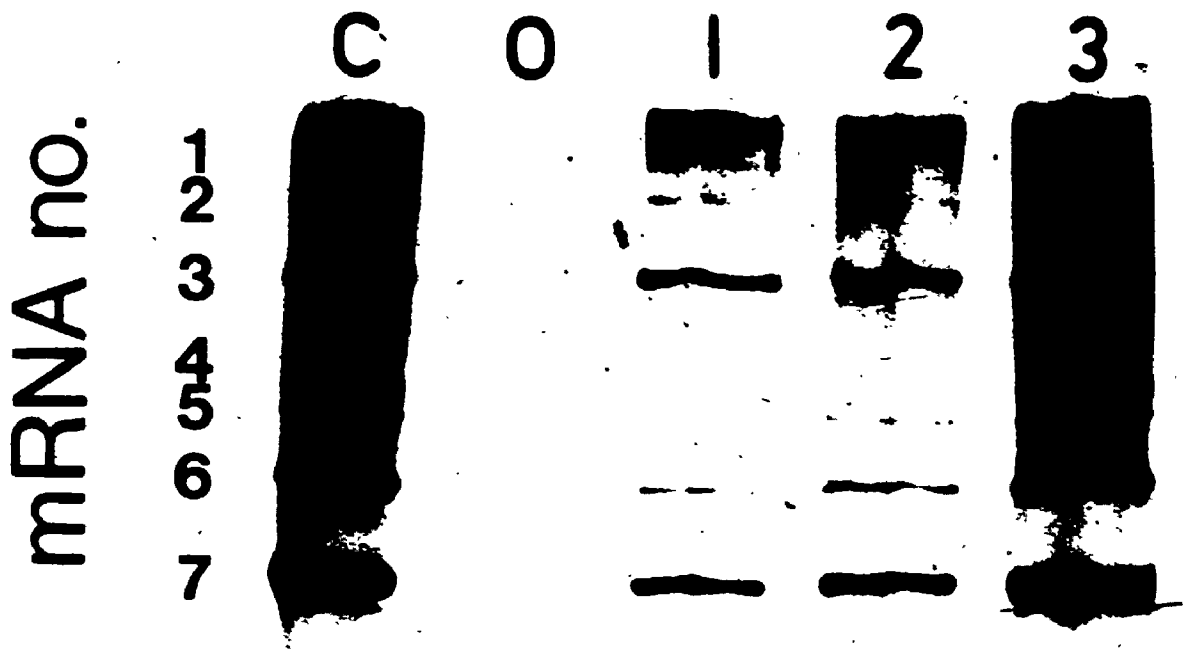
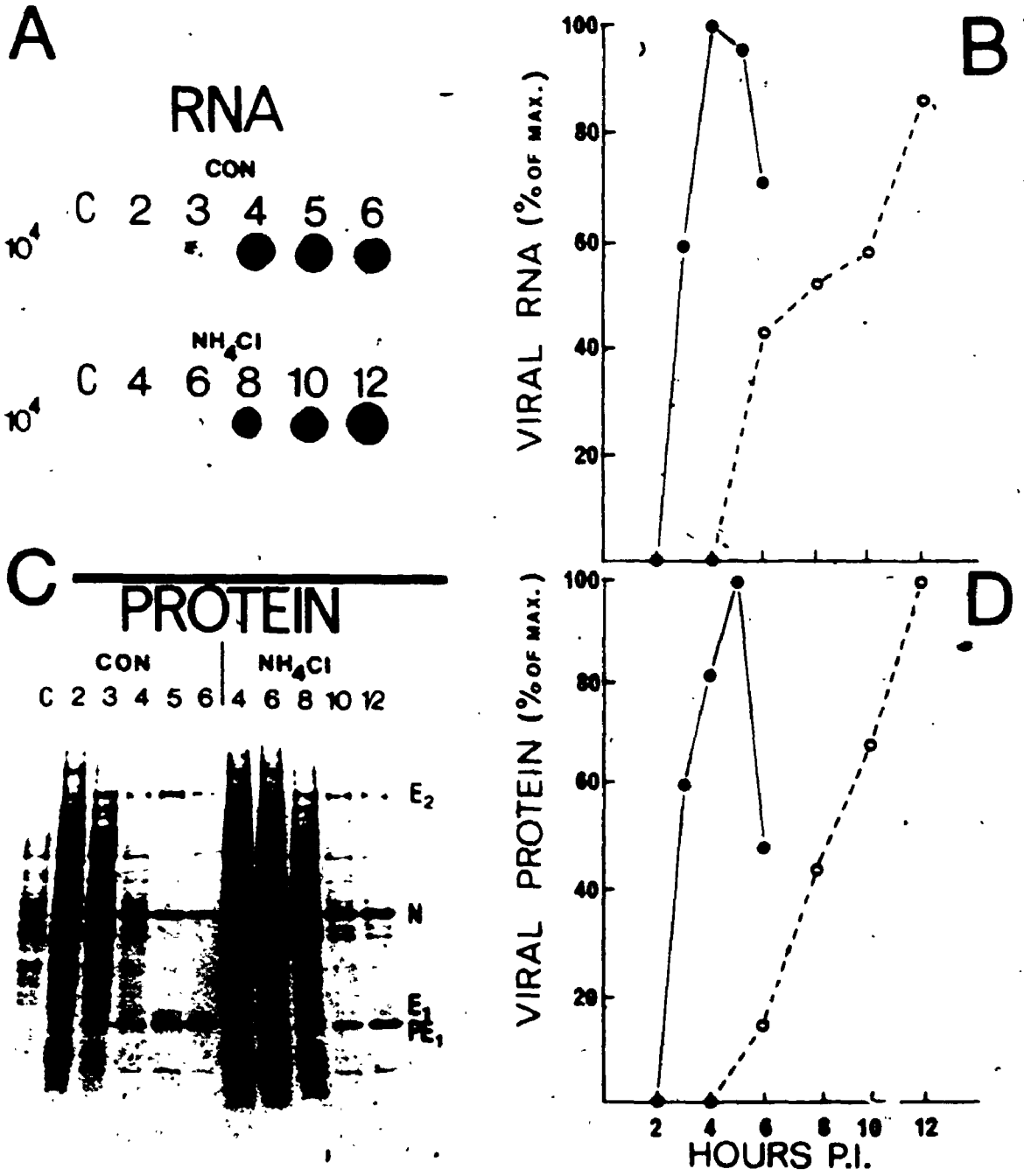

C
0
1
2
3
mRNA no.
1
2
3
4
5
6
7

Figure 18: Time-course study of MHV-RNA and polypeptide synthesis in the absence or presence of 20 mM ammonium chloride. Replicate 35 mm cultures (10^6 cells) of MHV infected L-2 cells (moi = 20) maintained in the absence (CON) or presence (NH_4Cl) of 20 mM ammonium chloride were processed at the indicated times P.I. for dot-blot analysis of viral RNA using a [^{32}P]-MHV specific cDNA probe (sections 2.17, 2.18 and 2.19; panel A), or were [^{35}S]-methionine labelled for analysis of MHV proteins by SDS-PAGE (sections 2.11 and 2.13; panel C). Relative quantitation of viral RNA (B) and protein (D) was performed by densitometric scanning of the autoradiograms shown in (A) and (C), respectively. Symbols in B and D represent data from cultures maintained in the absence (●——●) or presence (○— —○) of 20 mM ammonium chloride.


A
RNA
CON
C 2 3 4 5 6
10⁴
NH₄Cl
C 4 6 8 10 12
10⁴
B
VIRAL RNA (% OF MAX.)
100
80
60
40
20
C
PROTEIN
CON
NH₄Cl
C 2 3 4 5 6 4 6 8 10 12
E₂
N
E₁
PE₁
D
VIRAL PROTEIN (% OF MAX.)
100
80
60
40
20
2 4 6 8 10 12
HOURS P.I.

is the autoradiographic dot-blot analysis of MHV RNA extracted from control and treated cultures, and corresponding densitometric scan. MHV-infected L-2 cells, maintained in normal medium, first showed detectable levels of viral RNA at 3 h P.I.; this was delayed until approximately 6 h P.I. when ammonium chloride was present. Nevertheless, the ammonium chloride-treated culture eventually (at 12 h P.I.) produced levels of viral RNA very similar to the maximum levels produced in the untreated culture. Although the kinetics of RNA synthesis were noticeably slower in the presence of ammonium chloride, this is likely due to a certain degree of desynchronization in the initiation of RNA transcription, as a consequence of the protracted eclipse (uncoating) stage (Figure 16 B). Thus, it would appear that ammonium chloride does not inhibit viral RNA synthesis in a quantitative manner, but rather delays its onset.

3.19 Effects of Ammonium Chloride on MHV Protein Synthesis

In a time-course manner analogous to that used for viral RNA quantitation, viral protein synthesis was monitored in infected L-2 cells maintained in the presence or absence of ammonium chloride. Shown in Figure 18 C and D is the fluorographic SDS-PAGE analysis of [^{35}S]-methionine-labeled MHV proteins from control and treated cultures, and corresponding densitometric scan. Intracellular synthesis of viral polypeptides is delayed by some 4-6 h in the presence of ammonium chloride. Nevertheless, all three structural polypeptide classes (E_2, N, and E_1) are synthesized in normal proportions (Figure 18C) and in eventual levels (Figure 18D) similar to those seen in untreated cells. Therefore, these results on the

effects of ammonium chloride on MHV replication indicate that viral uncoating is the primary site of inhibition, and that subsequent replication events are accordingly delayed, but not otherwise inhibited. Furthermore, this data provides evidence for endosomal uncoating of MHV, as reported for other enveloped viruses (Helenius et al., 1982; Schlegel et al., 1982). However, unlike its reported effects on the cytopathic activity of other viruses (eg. Holland and Person, 1977), ammonium chloride did not inhibit the progression of MHV-induced cell fusion, and therefore, despite attenuation of early events in virus replication, was insufficient to prevent the lytic infection produced in permissive L-2 cells.

Restriction of virus dissemination via cell fusion, therefore, is of critical importance in regulating the acute vs. persistent state in MHV infected cells.

3.20 Characterization of Plasma Membrane Permeability During MHV Infection

Since the expression of MHV-induced cell fusion, mediated by the host cell plasma membrane, determines to a great extent the outcome of infection (ie. acute vs persistent), it was of interest to study the function of the plasma membrane during expression of cytopathic activity by MHV. For these studies, the lytic, fusogenic MHV infection of L-2 cells was used as a model since the expression of cytopathic effect (cell fusion) is maximal compared to other cell lines (see Figures 1 and 6).

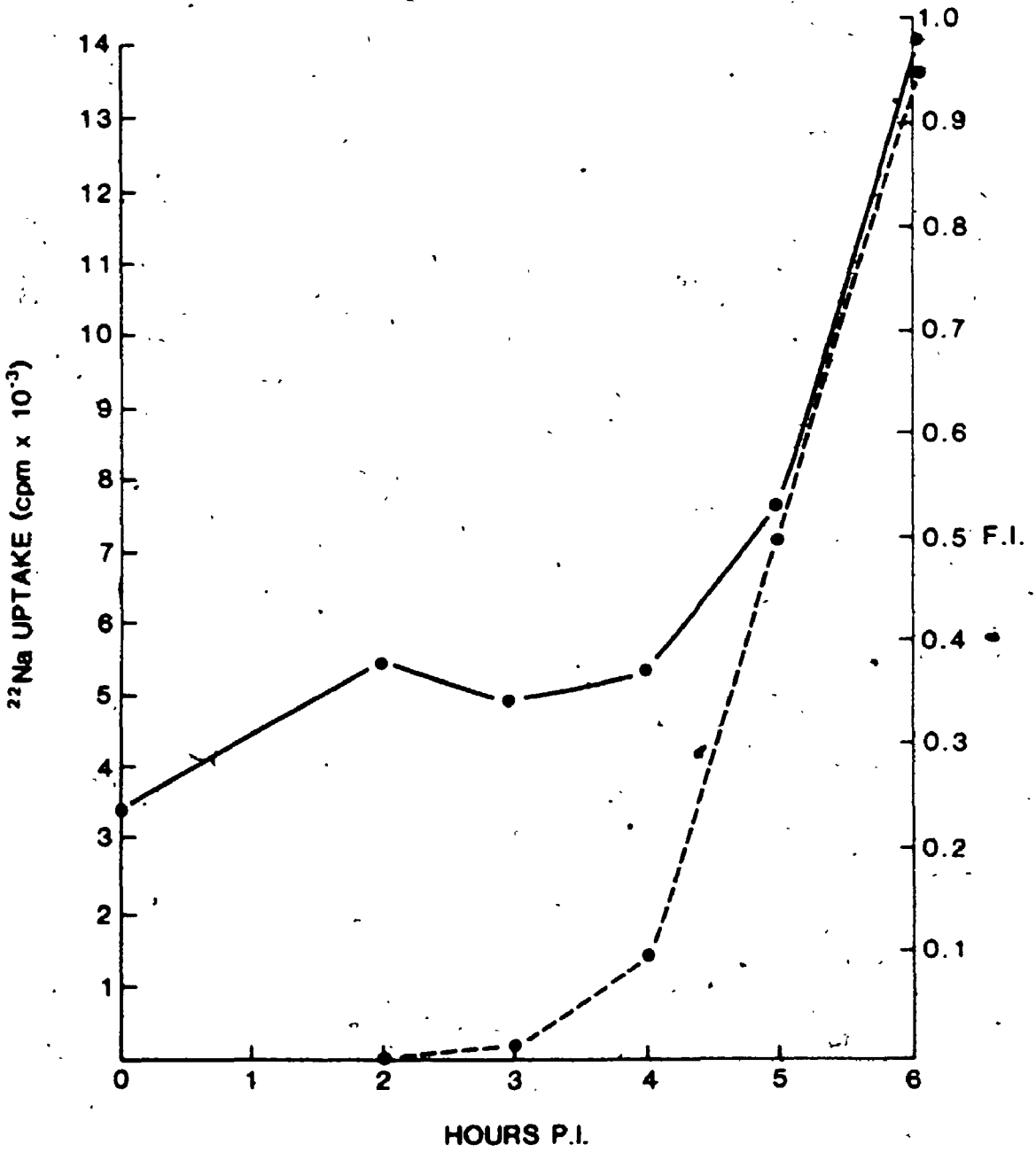
The alteration of intracellular monovalent cation levels

(particularly Na^+ and K^+) is a common result of lytic infection by several animal viruses (Carrasco and Smith, 1976; Hackstadt and Mallavia, 1982; Nair, 1984; Ulug et al., 1984) and is presumed to contribute to the cytopathic effect seen in the infected cells. It has been proposed that alterations in membrane permeability during virus infection would account for the altered intracellular monovalent cation levels (Carrasco, 1977; Pasternak and Micklem, 1981).

The integrity of the L-2 cell plasma membrane was examined during the course of high multiplicity MHV infection (moi = 20) by measuring permeability to $[^{22}Na]^+$ (section 2.7). Shown in Figure 19 is the influx of $[^{22}Na]^+$ into infected L-2 cells and corresponding F.I. values at various times P.I.

By 2 h P.I., when no cell fusion is evident (F.I. = 0) there was a small amount of $[^{22}Na]^+$ influx into the infected cells, which is suggestive of reported early membrane permeability changes due to virus binding and penetration (Carrasco and Smith, 1980; Kohn, 1979). Note that Figure 16 A indicates that MHV internalization occurs over the first 2 h P.I. The $[^{22}Na]^+$ influx over the period of 2 to 4 h P.I. was relatively unchanged from the initial increase seen early in infection but increased dramatically 4 to 6 h P.I. The expression of cell fusion, as given by the F.I. values, closely paralleled this large $[^{22}Na]^+$ influx late in infection. Therefore, this data suggests a correlation between expression of cell fusion and modification of plasma membrane permeability to $[^{22}Na]^+$.

Figure 19: Correlation of $[^{22}Na]^+$ uptake (●——●) and extent of cell fusion (●- - -●) in MHV-infected L-2 cells. Duplicate MHV-infected L-2 cultures (moi = 20) in 35 mm dishes (10^6 cells) were assayed for permeability to extracellular $[^{22}Na]^+$ (section 2.7) or expression of cell fusion (by calculation of the Fusion Index (F.I.; section 2.6) at the indicated times P.I.


^{22}Na UPTAKE (cpm x 10^{-3})
F.I.
HOURS P.I.
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
5
6

3.21 Virus-induced Inhibition of Cellular Protein Synthesis

Associated with many lytic virus infections is the rapid inhibition of cellular protein synthesis, which in some cases, has been correlated with changes in membrane permeability (Benedatto et al., 1980; Garry et al., 1979). Therefore, cellular protein synthesis was studied in MHV-infected L-2 cells to determine the temporal relationship between membrane leakiness and inhibition of host cell protein synthesis. [^{35}S]-methionine labeled proteins from MHV-infected L-2 cell cultures (moi = 20) harvested at various times P.I. were analyzed by SDS-PAGE and fluorography. The levels of total, cellular and viral protein synthesis at each time point were determined by densitometric scanning and plotted as shown in Figure 20. Several features are worthy of note. First, total protein synthesis declined considerably, starting at 3 h P.I., eventually reaching only 7% of its original level by 6 h P.I.. Second, the decline in total protein synthesis was due essentially to diminished translation of host cell polypeptides, seen most clearly by the abrupt decline in synthesis between 2 and 4 h P.I. Third, the synthesis of virus specific polypeptides, which was first evident at 3 h P.I., proceeded rapidly until (by 5 and 6 h P.I.) it accounted for virtually all protein synthesis in the infected cell.

By comparing Figure 19 and 20 it is clear that the inhibition of cellular protein synthesis by MHV begins well before any significant [^{22}Na]$^{+}$ influx into the cell. Indeed, by 4 h P.I., when cellular protein synthesis had declined by 90%, the [^{22}Na]$^{+}$ influx associated with cell fusion was minimal. Therefore, in this system, there is no

CHAPTER 4

DISCUSSION

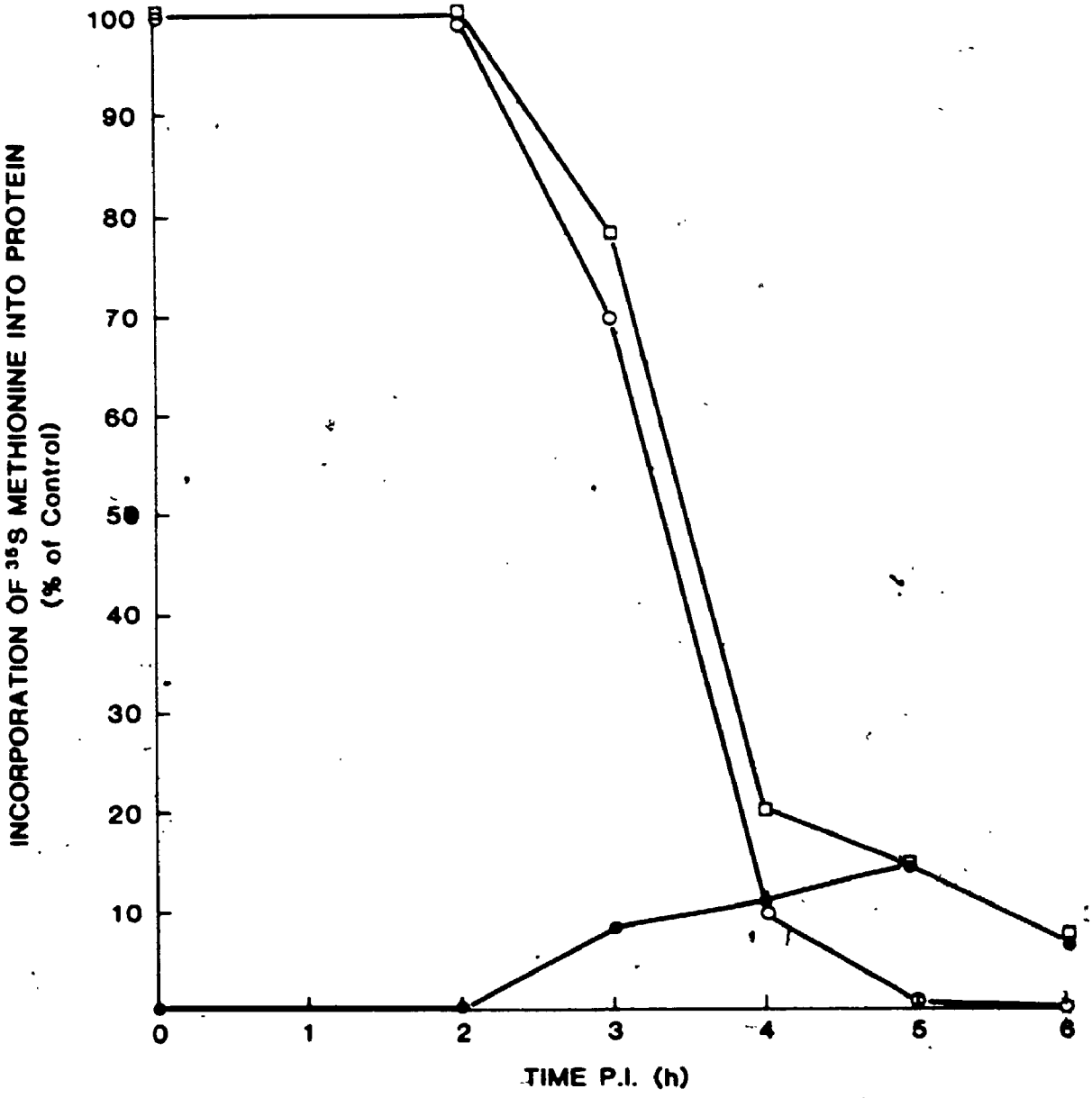
4.1 Establishment of MHV persistence in LMTK$^-$ cells

A number of strains of MHV have received much interest as a result of their demonstrated ability to become persistent under certain conditions, both *in vivo* and *in vitro* (Leibowitz *et al.*, 1984; Lucas *et al.*, 1977, 1978; Robb and Bond, 1979; Sorensen *et. al.*, 1980). A variety of mechanisms implicated in *in vitro* MHV persistence have been reported in the literature, involving the generation of DI particles (Makino et al., 1984, 1985) and temperature-sensitive (Holmes and Behnke, 1981), cold-sensitive (Stohlman *et al.*, 1979) and small plaque (Hirano *et al.*, 1981) virus mutants. Studies by Lucas *et al.*, (1977, 1978) however, demonstrated that MHV persistence in a number of cultured cell lines did not entail the production of detectable virus mutants, suggesting that these were not mandatory for establishment of the persistent state.

From the study of MHV replication in LMTK$^-$ cells (section 3.1 to 3.5), it is apparent that in this system, virus persistence is host cell-regulated and does not involve any apparent change in the input virus.

LMTK$^-$ cells possess two fundamental characteristics which allow cultures to support a persistent infection of MHV: a reduced level of infectability and a resistance to undergoing syncytial formation, when in contact with MHV-infected cell neighbors.

From the initial studies on the relative infectability of L-2


INCORPORATION OF ^{35}S METHIONINE INTO PROTEIN
(% of Control)
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
TIME P.I. (h)

direct correlation between $[^{22}Na]^+$ influx and inhibition of cellular protein synthesis.

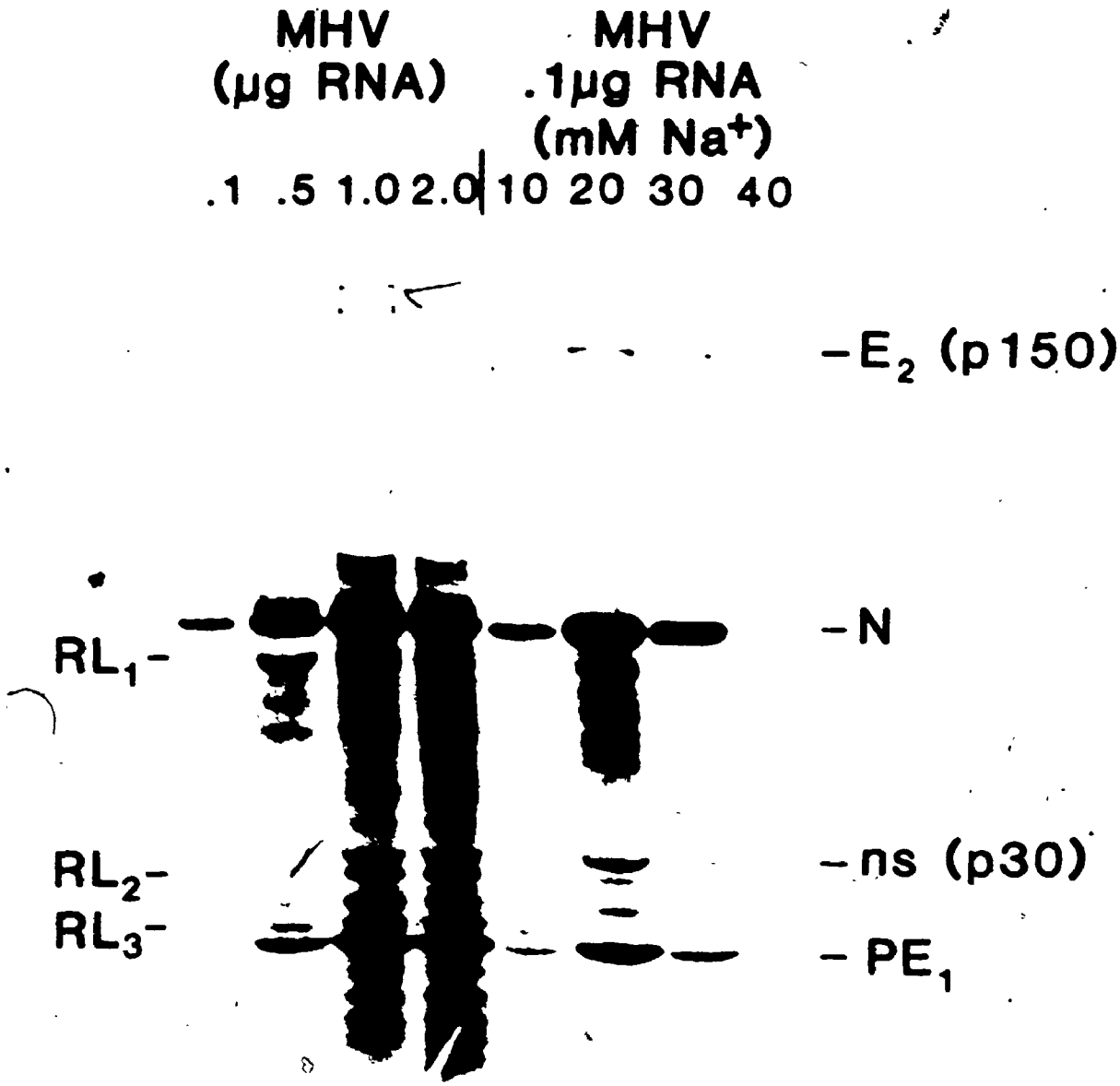
3.22 Na^+ Enhancement of In Vitro Translation of MHV mRNA

While modification of membrane permeability to sodium ions appears not to have initiated host inhibition, it is possible that such changes can promote viral gene expression and host shut off. Indeed, the messages specified by both DNA and RNA viruses (eg. Cherney and Wilhelm, 1979; Saborio et al., 1974) are efficiently translated under altered ionic conditions which block cellular protein synthesis.

Therefore, the translation of cellular and MHV mRNAs was studied in an in vitro translation system (section 2.22) in response to varying concentrations of sodium ions. Shown in Figure 21 is the SDS-PAGE fluorogram of $[^{35}S]$-methionine-labeled proteins translated from RNA extracted from MHV-infected L-2 cells, under standard conditions (optimized for K^+ and Mg^{++} concentration) and with addition of NaCl (10 to 40 mM) to the reaction mixture.

Both viral and cellular translation responded in a similar fashion to increasing amounts of input RNA (left half of Figure 21), reaching a maximum at 1.0 µg RNA per reaction. Under these conditions, and beginning at 0.5 µg RNA per reaction, the MHV polypeptides were detected against a complex background of cellular translation products. The pattern of MHV proteins synthesized in this cell-free system were similar to those previously reported (Leibowitz et al.,1982; Rottier et al.,1981) and included a partially glycosylated form of $E_2(E_2(p150))$, a non-structural protein, p30 as

Figure 21: Na^+ enhancement of *in vitro* translation of MHV mRNAs. Total RNA, prepared from MHV-infected L-2 cells in 100 mm dishes (10^7 cells per dish) by guanidine-HCl extraction (section 2.17) was quantitated by spectrophotometry (section 2.17), lyophilized and translated *in vitro* in a cell-free rabbit reticulocyte system (BRL) under standard conditions (section 2.22). The left half of Figure 21 shows the translation products made in response to 0.1, 0.5, 1.0 and 2.0 μg RNA per reaction. In the right half of Figure 21 are the translation products made with 0.1 μg RNA per reaction in response to the addition of 10, 20, 30 and 40 mM NaCl to the standard reaction mixture. The endogenous reticulocyte lysate bands RL_1, RL_2, and RL_3 are indicated, as well as positions of the typical *in vitro* translation products of MHV RNAs (Rottier *et al.*, 1981; Leibowitz *et al.*, 1982).


MHV
(µg RNA)
.1 .5 1.0 2.0
MHV
.1µg RNA
(mM Na+)
10 20 30 40
-E2 (p150)
RL1-
-N
RL2-
-ns (p30)
RL3-
-PE1

well as N and PE_1.

However, addition of NaCl to the reaction mixtures (right half of Figure 21) profoundly altered the translational specificity in favour of viral mRNAs. Using 0.1 μg RNA per reaction, the addition of 20 and 30 mM NaCl specifically enhanced translation of MHV mRNAs (with 20 mM Na^+ being optimal), while cellular translation was inhibited. By comparing densitometric scans of the standard and 20 mM sodium-enhanced translations using 0.1 μg RNA, the synthesis of N and PE_1 were found to be enhanced by sodium approximately five-fold while synthesis of the largest viral polypeptide, E_2(p150) was enhanced even moreso (note that E_2(p150) is not normally detected using 0.1 μg RNA). However, the translation of all mRNAs was totally inhibited by inclusion of 40 mM NaCl in the reactions, suggesting that at this ionic strength, all mRNA-ribosome interactions are prevented.

In vivo, under physiological ionic conditions, increased cellular permeability to monovalent ions would result in net Na^+ influx (and K^+ efflux) because of the normal active maintenance of low Na^+ : high K^+ levels intracellularly (typically 20-30 mM Na^+ : 110-120 mM K^+; Quissell and Suttie, 1973).

Therefore the demonstration of Na^+ influx associated with MHV-induced cell fusion and the enhanced translation of viral message in the presence of elevated Na^+ levels provides evidence that like many other viruses, the cytopathic activity of MHV in L-2 cells may be associated with membrane leakiness and consequent perturbation of intracellular monovalent ion levels.

CHAPTER 4

DISCUSSION

4.1 Establishment of MHV persistence in LMTK⁻ cells

A number of strains of MHV have received much interest as a result of their demonstrated ability to become persistent under certain conditions, both *in vivo* and *in vitro* (Leibowitz *et al.*, 1984; Lucas *et al.*, 1977, 1978; Robb and Bond, 1979; Sorensen *et. al.*, 1980). A variety of mechanisms implicated in *in vitro* MHV persistence have been reported in the literature, involving the generation of DI particles (Makino et al., 1984, 1985) and temperature-sensitive (Holmes and Behnke, 1981), cold-sensitive (Stohlman *et al.*, 1979) and small plaque (Hirano *et al.*, 1981) virus mutants. Studies by Lucas *et al.*, (1977, 1978) however, demonstrated that MHV persistence in a number of cultured cell lines did not entail the production of detectable virus mutants, suggesting that these were not mandatory for establishment of the persistent state.

From the study of MHV replication in LMTK⁻ cells (section 3.1 to 3.5), it is apparent that in this system, virus persistence is host cell-regulated and does not involve any apparent change in the input virus.

LMTK⁻ cells possess two fundamental characteristics which allow cultures to support a persistent infection of MHV: a reduced level of infectability and a resistance to undergoing syncytial formation, when in contact with MHV-infected cell neighbors.

From the initial studies on the relative infectability of L-2

and LMTK$^-$ cells with MHV, infectious centre data (Table 1) indicated that LMTK$^-$ cells were approximately 500-1000-fold less infectable than correspondingly inoculated L-2 cells. Despite the documented differences in infectability, when standardized on the basis of equal numbers of infected cells (eg. moi = 0.01 on L-2 and moi = 10 on LMTK$^-$; Table 1), MHV-infected L-2 and LMTK$^-$ cultures were comparable with respect to production of infectious progeny virus and synthesis of viral RNA (section 3.2). Furthermore, immunoprecipitation of MHV structural polypeptides from infected L-2 and LMTK$^-$ cultures (Figure 4) demonstrated the presence of all three classes of viral protein with normal apparent MW's, including the protein responsible for cell fusion, E_2 (Collins et al., 1982). However, the expression of MHV-induced cell fusion, characteristic of the acute infection in L-2 cells (Figure 1), was markedly reduced in infected LMTK$^-$ cells. Both the kinetics of cell fusion (Figure 2) and morphology of syncytia (Figure 3) are indicative of the relative fusion resistance of infected LMTK$^-$ cultures. Despite reduced expression of cell fusion, it is apparent that adequate amounts of viral fusion protein are expressed at the surface of infected LMTK$^-$ cells from the results of the contact fusion assay (Figure 5). Thus, the reduced incidence of cell fusion in the LMTK$^-$ culture is likely due to inherent resistance of the LMTK$^-$ cell plasma membrane to participate in MHV-induced syncytia formation.

The reduced levels of cell fusion in MHV-infected LMTK$^-$ (as contrasted with L-2) cell cultures would act as a dissemination-limiting factor, thus contributing to the continued survival of

remaining uninfected cells. Survival of uninfected LMTK$^-$ cells is also assured as a result of their resistance to initial MHV infection. Results presented in Table 1 indicate that MHV, even at an moi of 10 can only infect about 1% of inoculated LMTK$^-$ cells. Levels of circulating virus (of the order of 10^7 pfu/ml) would, therefore, be inadequate to ensure infection of a substantial proportion of all LMTK$^-$ cells (ca. 10^6 cells/culture). It is apparent, then, that MHV persistence in LMTK$^-$ cells represents a "carrier-culture" mechanism, maintained by a dynamic balance between infected and uninfected cells. Persistence is, therefore, a feature of the entire culture and not of individual cells.

Although it is conceivable that some genetically altered form of MHV is generated in LMTK$^-$ cells which accounts for persistence, it is unlikely for the following reasons. First, virus recovered from MHV-infected LMTK$^-$ cells is indistinguishable from input MHV in terms of non-ts replication and plaque morphology in L-2 cells. Second, persistence in LMTK$^-$ cells is already established within the first day of virus inoculation (c.f., complete lysis of L-2 cells within this time), arguing against appreciable genetic selection of a "persisting" virus variant. Furthermore, the presence of anti-viral substances (such as interferon) in persistently-infected LMTK$^-$ culture media could not be demonstrated.

Therefore, the commonly reported mechanisms of viral persistence in vitro which include production of DI particles (Holland et al., 1979), ts mutants (Preble and Younger, 1980) and induction of interferon (Sekellick and Marcus, 1978, 1979), do not appear to be

operative in the maintenance of MHV persistence in LMTK$^-$ cells.

Features displayed by the persistent MHV infection of LMTK$^-$ cells, such as cycling virus titres, low levels of infectious centers, little cytopathic effect and non-alteration of input virus are similar to those previously described for MHV infections of a variety of other cell lines (Lucas et al, 1978). It is possible, therefore, that dissemination-limiting mechanisms such as reduced infectability and fusion resistance, as described for LMTK$^-$ cells, may be operative in a wide spectrum of host cells persistently infected with MHV.

4.2 Replication of MHV in Permissive and Semi-Permissive Cell Lines

Despite extensive research efforts, the mechanisms underlying coronavirus persistence remain poorly understood. An attractive animal model for virus-mediated chronic neurological disease involves infection of rats or mice with the JHM strain of MHV (Cheever et. al., 1949); Haspel et al., 1978; Nagashima et al., 1978; Sorensen et al, 1980; Weiner, 1973). Typical of such infections is a state of prolonged virus persistence in the CNS in which discrete pockets of virus-infected cells coexist among areas of apparently normal, uninfected tissue. While the JHM strain of MHV has been the virus of choice in such experimental systems, there is evidence that other MHV strains, such as MHV-3 (Le Prevost et al, .1975) and A59 (Hirano et al., 1980), also readily undergo infections of a chronic nature in rodents.

In addition, however, to producing infections of a slowly progressive type, many MHV strains are also capable of inducing

dramatic, acute disease (reviewed by Wege, et al., 1982). Elucidating the conditions which favor persistent over acute infection is, accordingly, important to the understanding of MHV-induced disease.

In the preceding section, an in vitro model for MHV persistence in murine fibroblast cultures (LMTK$^-$) was discussed in which a steady state balance between virus replication and cell survival was maintained by host-imposed restriction of cell fusion. The results presented in sections 3.6 to 3.11 extend these findings to other cultured cell lines and suggest that intrinsic resistance to MHV-induced cell fusion may represent a general mechanism of in vitro MHV persistence.

A survey of the properties of MHV replication in permissive L-2 and semi-permissive LMTK$^-$, C-1300 and LM-ATCC cells (Table 2) indicates that the characteristics of virus replication in C-1300 and LM-ATCC cultures shares many similarities with that described previously for persistently infected LMTK$^-$ cultures (section 4.1).

Like LMTK$^-$ cells, the C-1300 and LM-ATCC cells displayed a reduced level of infectability by MHV, and as such, these three cell lines are defined as being semi-permissive for MHV replication. Under conditions where virtually 100% of permissive L-2 cells are infected by MHV (moi = 10), only 0.2 to 1.5% of the semi-permissive cells become infected (Table 2). Furthermore, like LMTK$^-$ cells, the C-1300 and LM-ATCC cultures, per infected cell, were not deficient in either the production of progeny virus (Table 2) or in the synthesis of viral RNA (Figures 7 and 8) and protein (Figures 9 and 10),

compared to permissive L-2 cells. Virus released from LM-ATCC and C-1300 cultures was also apparently wild-type, on the basis of its non-ts replication and normal plaque morphology on L-2 cells (data not shown).

The quantitation of viral RNA and protein during the course of MHV replication in the permissive and semi-permissive cultures (Figures 8 and 10) indicates that the synthesis of these macromolecules is not significantly delayed in onset nor markedly reduced in amount, per infected cell, in the semi-permissive cultures, compared to permissive L-2 cultures.

An interesting trend is observed in Figures 8 and 10 that would suggest similarities in MHV replication within L-2 and C-1300 cells, on the one hand, and LMTK$^-$ and LM-ATCC cells, on the other, on the basis of the temporal synthesis of viral RNA and protein. These similarities are reinforced by data regarding the expression of MHV-induced cell fusion (Figure 6 and Table 3). Although the C-1300 cell is considered semi-permissive for MHV replication on the basis of reduced infectability, it is clearly distinct from LMTK$^-$ and LM-ATCC cells on the basis of susceptibility to virus-induced cell fusion. Results presented in Figure 6 indicate that, per infected cell, the expression of cell fusion by C-1300 cells is comparable to that observed in fusogenic L-2 cells. In each case, cell fusion engulfs and terminates that monolayer. In contrast, cell fusion in LMTK$^-$ and LM-ATCC cultures was markedly reduced when examined on an infected cell basis. In these cultures, fusion was a relatively static process, restricted to infrequent, isolated syncytial foci.

The results of the contact fusion assay (Table 3) reinforce the distinction between fusion-permissive L-2 and C-1300 cells and fusion-resistant LMTK$^-$ and LM-ATCC cells. As demonstrated for LMTK$^-$ cells, LM-ATCC cells also possess an intrinsic resistance to MHV-induced cell fusion that functions at the level of the plasma membrane.

When these results are considered together, some general conclusions can be drawn regarding the replication of MHV in these cell lines.

First, reduced infectability to MHV is a feature common to all the semi-permissive cells. Second, resistance to MHV-induced cell fusion is characteristic of LMTK$^-$ and LM-ATCC cells only, with C-1300 cells being fusion permissive. Third, most aspects of MHV replication (virus release, RNA and protein synthesis), per infected cell, are normal in the semi-permissive cells. Fourth, the expression of MHV-induced cell fusion is the critical determinant influencing the outcome of MHV infection in these cultured cells. Intrinsic resistance to this fusion mediates the persistent, carrier-culture state, whereas fusion-permissive hosts are ultimately destroyed by unrestricted virus dissemination and cytopathic effect. Some features regarding the assembly and release of MHV from acutely and persistently cultures are noteworthy in light of these conclusions. In their study of MHV replication in 17Cl-1 and L-2 cells, Massalski et al. (1982) found that host cell type played a fundamental role in regulating the infectious process. These authors observed that the more efficient and prolonged release of virus from

17C1-1 cells was associated with a marked reduction in cell fusion. On the other hand, the rapid syncytiogenesis and cell killing caused in L-2 cells by MHV was believed to underlie the reduced efficiency in virus assembly, as witnessed by extensive accumulation of nucleocapsid cores within the cytoplasm. Similar results were observed for MHV replication in a variety of cell lines by Frana et. al (1985). Here, efficient assembly and release of virus was associated with cultures where cytopathology was reduced.

In the present study, an analogous situation exists, for example, with MHV infection of fusion-permissive L-2 and fusion-resistant LMTK⁻ cells. The output of virus, per infected cell, by LMTK⁻ cells is about 10-fold greater than L-2 cells (Table 2), and is maintained by persistently infected cultures long after termination of the L-2 cells. Therefore, persistently infected cultures such as the 17C1-1 cells mentioned above and the LMTK⁻ cells in the present study can serve as efficient "virus factories" since virus assembly and release is not prematurely terminated by cytopathic effect.

MHV persistence in vitro has been commonly associated with the production of mutant virus (Hirano et al., 1981; Holmes and Behnke, 1981; Leibowitz et al., 1984; Makino et al., 1984, 1985; Stohlman et. al., 1979). Furthermore, similar mechanisms are known to be operative in other persistent virus infections in vitro, involving, most notably, the rhabdovirus, vesicular stomatitis virus (VSV) (Holland et al., 1974, 1979; O'Hara et al, 1984; Youngner et al., 1978, 1981). As demonstrated in this study, persistence did not necessitate the production of virus variants; but rather, was

controlled at the level of the host cell.

Coronaviruses exhibit a high degree of host dependence in replication, cytopathology, tissue tropism and virulence (reviewed by Sturman and Holmes, 1983; Wege et al., 1982). There are important host controls over coronavirus replication at several levels. The immune response plays an important role in resistance to coronavirus-induced disease in vivo (Dupuy et al., 1975; Schindler et al., 1982; Sorensen et al., 1982; Virelizier, 1981). However, genetic factors are also important at the single-cell level. Resistance to different strains of murine coronaviruses is determined by a single host gene inherited as an autosomal recessive trait. For example, C3H mice are resistant to MHV-2 (Bang and Warwick, 1960), A/J mice are resistant to MHV-3 (Virelizier and Allison, 1976), and SJL/J mice are resistant to JHM (Stohlman and Frelinger, 1978; Knobler et. al., 1981). Such genetic factors governing resistance to MHV infection are also found in the rat (Sorensen et al., 1982). The applicability of studies conducted in vitro to understanding MHV pathogenesis in vivo is strengthened by the striking correlation between in vivo resistance to MHV infection and the characteristics of MHV replication in vitro.

The pattern of susceptibility and resistance observed in the mouse is reflected in the ability of MHV to replicate in primary cultures of peritoneal macrophages (Knobler et al., 1981; Krzystyniak and Dupuy, 1981; Stohlman et al., 1982; Virelizier and Allison, 1976), hepatocytes (Arnheiter et al., 1982) and neuronal cells (Dubois-Dalcq et al., 1982; Knobler et al., 1981). In these cultures, genetic resistance in vivo was associated with a reduction

in vitro, in both virus replication and cytopathic effect (lysis or cell fusion). These effects could not be correlated to immune mechanisms such as interferon induction, but appeared to represent some "intrinsic" feature(s) of the host cell. Similar studies involving primary cultures of endothelial and Kupffer liver cells (Pereira et. al., 1984) and embryonic fibroblast cells (Lamontagne and Dupuy, 1984) also demonstrated a marked reduction in cell fusion in cultures derived from "resistant" hosts. Lamontagne and Dupuy (1984) also demonstrated that the virus shed from the persistently infected embryonic fibroblast cultures was fully pathogenic in susceptible mice, arguing against the generation of virus variants.

Collectively, these results support the contention that in vitro MHV persistence can be maintained through host-cell modulation of virus-induced cell fusion, as demonstrated for the permissive and semi-permissive cell cultures in the present study.

The in vivo relevance of the present study is further strengthened by the importance of cell fusion in dissemination of virus in the animal. The JHM strain of MHV is well documented as an inducer of acute (Bailey et al., 1949; Lampert et.al., 1973) and chronic (Stohlman and Weiner, 1981) demyelination in mice, as well as subacute demyelination in rats (Nagashima et. al., 1978; Sorensen et al., 1980), due to a cytolytic infection of oligodendrocytes. However, replication of JHM in neurons, which reflects genetic susceptibility of the host, produces an acute, fatal necrotizing encephalomyelitis (Haspel et al., 1978; Knobler et al., 1981).

The type of disease produced by JHM (acute vs. chronic) is

directly related to the tropism for, and replication in, neurons and oligodendrocytes. Replication of JHM in neurons is an acute, lytic infection with extensive neuronal involvement as a consequence of extracellular virus spread. Replication of JHM in oligodendrocytes, however, is much less acute; transmission of virus by cell fusion is highly restricted, giving rise to characteristic foci of demyelination.

Persistent infection of the mouse CNS was demonstrated by Knobler et al. (1982) using a JHM mutant, ts 8, which displayed a unique tropism for oligodendrocytes, while sparing neurons. The chronic demyelination observed in the animals was correlated with persistent infection of oligodendrocytes. The importance of cell fusion in modulating disease is also indicated by in vivo studies demonstrating that during the course of chronic JHM disease, virus persists in the animal despite a continued neutralizing antibody response (Sorensen et al., 1984; Stohlman and Weiner, 1981) which would inhibit the extracellular (neuronal) mode of virus dissemination. Two different modes of spread in the animal were indicated by Buchmeier et al. (1984) in studies which showed that administration of neutralizing monoclonal antibodies blocked JHM replication in neurons but not oligodendrocytes. Buchmeier et al. (1984) and others (Wege et al., 1984), have shown that for antibody to be protective in vivo against acute JHM disease, it must both neutralize virus infectivity and inhibit cell fusion in vitro. Virus dissemination by cell fusion is also an important factor in paramyxovirus-induced disease. The expression of mumps virus fusing

activity *in vitro* correlated with neuropathogenicity *in vivo* (McCarthy *et al.*, 1980), and similar observations with respect to neurovirulence and cell fusion have been made for parainfluenza virus 3 (Shibuta *et al.*, 1982) and canine parainfluenza virus (Evermann *et al.*, 1980). In antibody protection studies with parainfluenza virus 3, Merz *et al.* (1980) also demonstrated that protection against disease required neutralization of extracellular virus as well as inhibition of cell fusion to prevent spread of virus.

Therefore, in many virus-host systems, manifestation of disease is correlated to dissemination of virus via cell fusion. With the demonstrated correlation between *in vivo* disease expression and properties of *in vitro* MHV replication, the present study of MHV replication within fusion-permissive and resistant cell lines represents a model system with applicability to MHV-induced disease.

4.3 Characterization of MHV-induced Cell Fusion

Proteolytic processing of viral proteins is an essential stage in the growth of viruses belonging to many different virus families. Such intracellular events are required for both the generation of discrete polypeptide species, as in, for example, the maturation of poliovirus (Jacobson and Baltimore, 1968) as well as in the activation of biological activity of proteins, such as the fusion proteins of paramyxo- and orthomyxo viruses (Lazarowitz and Choppin, 1975; Scheid and Choppin, 1974).

A role for protease has been implicated in enhancing infectivity (Sturman and Holmes, 1977), plaque production (Otsuki and Tsubokora,

1981; Storz et al., 1981) and fusion activity (Frana et al., 1985; Storz et al., 1981; Sturman et al., 1985; Toth, 1982; Yoshikura and Tejima, 1981) of coronaviruses. In the case of MHV, trypsin-activated whole virions caused rapid fusion ("fusion from without") with cell monolayers (Sturman et al., 1985); such trypsin activation was associated with a cleavage reaction in which the 180K MW form of E_2 was converted to two 90K MW forms.

The results presented in sections 3.12 and 3.13 provide biochemical evidence for protease activation of MHV-induced cell fusion in L-2 cells and demonstrates a host cell-specific response to such fusion. From the results of the contact fusion assay (Table 3) and radioimmunoprecipitation data (Figure 9) it is argued that while fusion-resistant LMTK$^-$ and LM-ATCC cells synthesize mature E_2 (180K MW) and express fusigenic protein at their surface, these cell lines are resistant to fusion induction by this protein. These findings are complemented by the results of the membrane fusion assay (Figure 11) which demonstrates that addition of trypsin (or chymotrypsin)-activated, plasma membrane-enriched fractions from MHV-infected L-2 cells to uninfected monolayers of LM-ATCC cells does not elicit cell fusion, while such preparations are fusion-active on uninfected L-2 cell monolayers. The results presented in Figure 12 identify the biochemical changes which correlated with the protease-dependent activities of such membrane preparations. Trypsin or chymotrypsin digestion of these membranes resulted in a reduction of radiolabelled E_2(p180) and appearance of a 90K MW form of E_2, designated E_2(p90). The relatedness of these two forms of E_2, as

demonstrated by others, (Sturman et al., 1985) was confirmed by one-dimensional peptide mapping (data not shown). The relationship between the generation of E_2(p90) and induction of cell fusion is inferred from the observation that membrane preparations which were not protease-digested were not fusogenic in this assay. Such samples contained no detectable radiolabelled E_2(p90), but only E_2(p180). It should be noted that while all three classes of MHV polypeptides are associated with these membrane preparations, only the E_2 undergoes significant protease-dependent cleavage under these conditions. Furthermore, the specific role of E_2 in the induction of cell fusion has been clearly defined by studies involving inhibition of cell fusion by monoclonal antibodies to E_2 (Collins et al., 1982).

Therefore, the fusogenic properties of these protease-treated membranes can be ascribed to generation of E_2(p90). This contention is further supported by the findings of Frana et al., (1985) who demonstrated that virions grown in sac(-) cells contain only the p90 form of E_2. Such virions mediated "fusion-from-without" when adsorbed to L-2 cell monolayers.

In MHV virions, the glycoprotein E_2 may exist in either the 180K or 90K MW form (Sturman and Holmes, 1977), the ratio of which is dependent upon the host cell used for virus propagation (Frana et al., 1985). Since the fusion permissive L-2 cells used in the present study are extremely susceptible to "fusion-from-within" (eg. Figure 1), the finding that the vast majority of labelled intracellular E_2 exists in the 180K MW form in these cells (Cheley and Anderson, 1981) is intriguing. The results presented in Figure

13A confirm these findings and further demonstrate that E_2(p90) can only be detected with prolonged chase periods (90 min or longer). However, evidence from this pulse-chase study plus that obtained from protease-treated membranes (Figure 12) indicate that the small amounts of E_2(p90) produced under such conditions are less than expected if conversion of E_2(p180) to E_2(p90) was quantitative. These results suggest that in the infected cell, E_2(p180) may undergo extensive proteolysis. Since the pulse-chase relationships of the other MHV structural polypeptides are in agreement with previous documentation (Cheley and Anderson, 1981), the absence of other labelled species that could be ascribed to E_2(p180) proteolysis in Figure 13A would suggest that a significant proportion of E_2 is digested into small peptide fragments not resolved by SDS-PAGE.

The significance of this finding, while at present unknown, could reflect the enhanced susceptibility of cleaved E_2(p90) to further proteolytic attack by cellular enzymes.

If cleavage of E_2 is required for cell fusion to occur during MHV infection, then inhibition of cellular protease activity might be expected to inhibit coronavirus-induced cell fusion. In the present study, the protease inhibitors TPCK and ZPCK, but not TLCK, were found to inhibit cell fusion in MHV-infected L-2 cells. The pulse-chase behaviour of E_2(p180) in such cultures (Figure 13 B) revealed that reduction of cell fusion was associated with specific inhibition of E_2(p180) proteolysis, which as shown in Figure 13 A, is necessary for generation of E_2(p90). The pulse-chase relationships of the other MHV proteins were not affected under these conditions (region

of gel not shown). Given the specificities of TLCK for trypsin (Shaw et al., 1965) and TPCK, ZPCK for chymotrypsin (Segal et al., 1971; Schoellmann and Shaw, 1962) the pattern of inhibition of cell fusion and E_2(p180) proteolysis by these synthetic protease inhibitors is highly suggestive that a cellular chymotrypsin-like enzyme is responsible for generation of E_2(p90). However, the finding that both trypsin and chymotrypsin treatment of MHV-infected cell membrane preparations activates the "fusion-from-without" phenomena (Figure 11) as well as generating E_2(p90) (Figure 12) indicates that extracellularly, both enzymes are capable of generating fusogenic E_2. The simplest interpretation of these results is that tryptic or chymotryptic cleavage of E_2(p180) can expose a putative "fusion-sequence" in the E_2(p90) species, perhaps through conformational change. Since these enzymes have very different substrate specificities (trypsin for arginine and lysine residues; chymotrypsin for aromatic and large nonpolar residues such as tyrosine, tryptophan,phenylalanine and leucine), the activation of fusogenic E_2 can be achieved by relatively non-specific means.

It has been recently reported that trypsin treatment of MHV virions was associated with cleavage of E_2(p180) to E_2(p90) and subsequent activation of "fusion-from-without" by such virions (Sturman et al., 1985). These authors have demonstrated that two different 90K MW cleavage fragments, designated 90A and 90B are produced by tryptic digestion of E_2(p180). One of these 90K MW fragments, 90A, was found to be acylated, a feature common to the envelope glycoproteins of orthomyxo- paramyxo-, and alpha viruses

(Schmidt, 1982). Therefore, by analogy with the locations and proposed "functions" of fatty acids in these spike glycoproteins, it is presumed that the coronavirus E_2 is associated with the viral envelope through the 90A domain, aided by the presence of hydrophobic fatty-acid near the site of protein insertion into the lipid bilayer.

In the case of another coronavirus, infectious bronchitis virus (IBV), the large peplomeric glycoprotein (155K MW) is cleaved asymmetrically, giving rise to 90K and 84K MW products (Stern and Sefton, 1982). The significance of this cleavage is indicated by studies demonstrating IBV hemagglutinin activation by trypsin (Corbo and Cunningham, 1959), although this is not a universal feature of hemagglutinating coronaviruses (Bingham et al., 1975).

While it is clear from the present study and others (Frana et. al., 1985; Sturman et al., 1985) that cleavage of MHV-E_2 is associated with cell fusion, it is not known whether cleavage of E_2 is required for coronavirus infectivity, as there is yet no source of MHV with entirely uncleaved E_2.

Sturman et al. (1985) observed that partial cleavage of E_2 by 17Cl-1 cells was sufficient to activate infectivity but not rapid cell fusion and that further cleavage of E_2 by trypsin did not enhance infectivity but did activate rapid cell fusion. Similarly, partially cleaved influenza hemagglutinin is sufficient to activate infectivity (Klenk et al., 1975).

From such observations, it may be inferred that in the case of MHV, a higher local concentration of cleaved E_2 is required for rapid fusion than infectivity. However, as demonstrated in the present

study (eg. Figure 11), even when presented with highly fusogenic E_2, the expression of cell fusion is highly host-cell dependent.

Proteolytic cleavage of peplomeric glycoproteins has been demonstrated for several other enveloped RNA viruses and often plays a role in the function of the viral glycoprotein (reviewed by Klenk and Rott, 1980; White et al., 1983). The similarity in proteolytic activation of cell-fusing activity of coronaviruses, positive-strand RNA viruses, and negative strand orthomyxo- and paramyxoviruses is of considerable interest, as this host-controlled modification is an important determinant of virus pathogenicity (Bosch et al., 1979; Garten et al., 1980; Scheid and Choppin 1976).

In the case of the Sendai virus F protein and influenza virus HA protein, cellular proteolytic cleavage of the nascent precursor molecule is required for both virus infectivity and cell fusion activity (Huang et al., 1981; Lazarowitz and Choppin, 1975; Scheid and Choppin, 1974, 1976; White et al., 1981). Common to these activation mechanisms is the formation of a disulfide-linked, bipartite molecule in which a hydrophobic N-terminal sequence located in the envelope-anchored domain (F_1 for Sendai, HA_2 for influenza) is created. These N-terminal sequences (some 20-30 aa in length) mediate the membrane fusion events associated with virus penetration and cell fusion (Richardson et al., 1980; Richardson et. al., 1983).

While unknown at present, one might expect by analogy that the N-terminal sequences of the 90A domain of cleaved MHV E_2 possess a hydrophobic character to facilitate fusion with lipid bilayers.

Another important feature of proteolytic activation of the

paramyxo- and orthomyxoviruses is the enzymatic specificity required for fusion activation. Infectious, fusigenic virions were produced only in host cells which possessed enzymes capable of effecting proper cleavage of nascent F_o or HA_o. Treatment of inactive virions *in vitro* with trypsin, but not chymotrypsin restored biological activity (Lazarowitz and Choppin, 1975; Scheid and Choppin, 1974). Sequence analyses of the N and C terminii of *in vivo* and *in vitro* trypsin-activated HA revealed complete identity, implicating a cellular trypsin-like enzyme in fusion activation (Garten *et al.*, 1981).

These findings are in contrast to the activation of fusion activity *in vitro* by both tryptic and chymotryptic generation of E_2(p90) (Figures 11 and 12). However, *in vitro* proteolytic activation of some paramyxovirus glycoproteins, such as the Newcastle disease virus (NDV) neuraminidase can be accomplished by a wide variety of enzymes, including trypsin, chymotrypsin, elastase and thermolysin (Nagai and Klenk, 1977). Therefore, while *in vivo* activation of E_2 within MHV-infected L-2 cells appears to be a function of a chymotrypsin-like enzyme, *in vitro* activation may be relatively non-specific.

The virulence and pathogenicity of paramyxoviruses is closely related to host-cell dependent activation of the F protein (Nagai *et. al.*, 1976, 1979; Scheid and Choppin, 1976). These studies have shown that persistence of the inactive F_o form results in a limited, non-cytopathic infection. Such an infection is transformed to a cytopathic one (fusogenic) by addition of a protease capable of

cleaving F_o to active F protein. However, other studies have shown that F activation is not sufficient to guarantee fusion. One important factor which affects fusion is the susceptibility of the plasma membrane to the direct fusing effects of the cleaved fusion protein. The paramyxovirus SV5 causes rapid fusion and death of BHK cells but induces a persistent, productive, non-cytocidal infection of primary monkey kidney cells due to differences in the response of the cell membrane to the viral fusion glycoprotein (Holmes and Choppin, 1966).

Similar findings are of central importance to the present study of MHV replication in permissive and semi-permissive cell lines. Although Frana et al. (1985) have demonstrated host-cell dependent differences in the processing of E_2, the authors were not able to correlate these differences with the extent of cell fusion observed in four cell lines. Furthermore, in a related study (Sturman et. al., 1985), it was noted that some cell lines were more susceptible to immediate fusion by concentrated, trypsin-activated MHV than others.

Therefore, intrinsic resistance of the host-cell plasma membrane to MHV-induced fusion, as demonstrated in the present study to mediate in vitro virus persistence, represents a general mechanism for moderating MHV pathogenicity.

4.4 Effect of Ammonium Chloride on MHV Replication in L-2 Cells

From the foregoing discussion it is clear that the host cell can influence the outcome of virus infection. In vitro MHV persistence was associated with host cells which were less susceptible to virus-

induced cell fusion, due to an inherent property of their plasma membrane (as yet uncharacterized). The importance of membrane fusion in the replication of enveloped viruses is indicated by the mechanism of virus penetration, which must occur if infection is to proceed. This critical early step in virus replication is mediated through fusion of the viral envelope and a cellular membrane, resulting in the "uncoating" of the virus genome and its release into the cytosol. With the exception of the paramyxoviruses, the majority of enveloped viruses penetrate via endocytosis (to be discussed later). A relatively late event in the replication of viruses is the expression of cytopathic effect. For some of the enveloped animal viruses, this activity is manifested as induction of cell fusion ("fusion from within"), which represents a membrane fusion event involving the apposing plasma membranes of adjacent cells.

Given the reported inhibitory effects of ammonium chloride on viral penetration (Helenius, et al., 1982) and cell fusion (Holland and Persons, 1977), it seemed plausible that with this agent, acute MHV infection of L-2 cells could be moderated. In this regard, ammonium chloride had been reported to prevent the lytic growth of reovirus and establish a persistent infection in mouse L cells (Canning and Fields, 1983). Since in vitro MHV persistence was associated with decreased infectability and a resistance to cell fusion, MHV replication in the presence of ammonium chloride might become persistent within a normally permissive host.

The results of this study, presented in sections 3.14 to 3.19, indicate that ammonium chloride (20 mM) attenuated MHV replication in

L-2 cells but was not sufficient to prevent the infection from following a lytic, fusogenic course. All post-eclipse (uncoating) parameters examined, including progeny MHV production (Figure 14), cell fusion (Figure 15) and RNA and protein synthesis (Figures 17 and 18) were found to be chronologically displaced by about 4-7 h, but not otherwise inhibited, when the infection was performed in the presence of 20 mM ammonium chloride. These results demonstrate that ammonium chloride intervenes primarily at a stage prior to synthesis of viral RNA (positive-sense species) during the MHV replication cycle. The results presented in Figure 16 localize the inhibitory action of ammonium-chloride to virus penetration. It had been previously demonstrated that virus adsorption was not affected by ammonium chloride (Krzystiniak and Dupuy, 1984). The next step in endocytic entry, internalization, was shown to also be unaffected by ammonium chloride (Figure 16 A). However, the subsequent step, virus uncoating (virus-endosome fusion), was markedly inhibited by ammonium chloride. Since virus uncoating results in loss of infectivity due to disruption of the virion, the reduction in the loss of infectivity of internalized MHV in the presence of ammonium chloride suggests that the uncoating process is inhibited. Under the conditions of the present study, the inhibition of MHV uncoating by ammonium chloride, however, is not an absolute block, since virus infection, although delayed, eventually follows a productive course in a fashion similar to untreated cells.

The inhibition of MHV uncoating by ammonium chloride provides evidence that this virus effects entry into the cell by an endocytic

pathway. While a limited number of viruses, such as Sendai virus enter cells by a direct fusion process of viral envelope with host plasma membrane (Choppin and Compans, 1975), the vast majority of lipid-enveloped viruses are taken up by an endocytic mechanism (Dales, 1973; Fan and Sefton, 1978; Marsh and Helenius, 1980; Simpson et al., 1969). In the case of the rhabdovirus, vesicular stomatitis virus (Schlegel et al., 1982), and the togavirus, Semliki Forest virus (Helenius et al., 1980; Marsh et al., 1983), the endocytic process has been shown to involve uptake into clathrin-coated pits similar to those implicated in the receptor-dependent uptake of hormones and other proteins (Dickson et al., 1981; Goldstein et. al., 1979). Once inside the coated vesicle and subsequent endosome (Helenius et al., 1980) or receptosome (Willingham and Pastan, 1980), however, a low pH-dependent fusion event mediates final entry of the viral genome into the cytosol (Helenius et al., 1980; Maeda et al., 1981; White et al., 1981).

Lysosomotropic agents such as ammonium chloride and a variety of amines have been demonstrated to inhibit the replication of viruses from the toga- (Helenius et al., 1982), rhabdo- (Schlegel et al., 1982), orthomyxo- (Kato and Eggers, 1969), retro- (Pazmino et al., 1974) and herpes- (Shimuzu et al., 1972) virus families. A common characteristic of these agents is that they are weak bases and accumulate within acidic intracellular compartments, notably lysosomes, thereby elevating pH (Ohkuma and Poole, 1978). Perturbation of the pH within lysosomes and related vesicles has been shown (Helenius et al., 1980; Marsh et al., 1983) to inhibit the

fusion of the prelysosomal (or endosomal) membrane with the viral envelope of the togavirus, Semliki Forest virus. Such fusion inhibition thereby interferes with virion uncoating and liberation of the viral genome into the cytosol.

The role of lysosomes and related acidic compartments in the replication of coronaviruses is not well understood. Coronavirus infection frequently involves the intracellular accumulation of virus-filled, electron-dense structures which have been interpreted to be lysosomes (David-Fereira and Manaker, 1965; Ducatalle and Hoorens, 1984). Such structures are observed within 2-3 h P.I. and have, therefore, been suggested to be involved in virus uptake. Further evidence for a lysosomal role in coronavirus replication was provided by studies in which lysosomotropic agents were found to inhibit production of virus progeny and cytopathic effect in cells infected with MHV (Krzystyniak and Dupuy, 1984; Mallucci, 1966). There has to date, however, been no documentation, by electron microscopic or other means, of the uncoating process of intracellular coronaviruses. The present results provide evidence for pH-dependent endosomal uncoating of MHV in L-2 cells. This event is likely mediated by the E_2 glycoprotein since it is responsible for the cell fusion activity of the virus (Collins et al., 1982).

Endocytic uncoating of animal viruses represents a membrane fusion process that is fundamentally similar to the direct fusion-penetration of paramyxoviruses, and to a lesser extent, induction of cell fusion. The data available at present indicates that this fusion activity is the property of a single glycoprotein species

encoded by the viral genome and found in the virion as an external "spike" protein. For the purposes of discussion, the fusion proteins identified thus far can be tentatively classified into three groups (White et al., 1983): (1) fusion proteins with N-terminal hydrophobic sequences, (2) fusion proteins with internal fusion sequences and (3) fusion proteins without apparent hydrophobic fusion sequences.

Representatives of the first class include the paramyxovirus F proteins (Gething et al., 1978; Richardson, et al., 1980), the orthomyxovirus HAs (Dopheide and Ward, 1980; Porter et al., 1979) and the spike glycoproteins of murine retroviruses (Lenz et al., 1982; Schinnick et al., 1981). The N-termini are derived by proteolytic cleavage of an inactive precursor molecule. While orthomyxo- and retrovirus fusion is pH-dependent (Maeda et al., 1981; Pazmino et al., 1974), paramyxovirus fusion is relatively pH-independent (Lenard and Miller, 1981).

The E_1 glycoproteins of the alphaviruses Semliki Forest virus and Sindbis virus belong to the second class of fusion proteins. From sequencing data, a hydrophobic sequence close to the N-terminus was identified as a strong candidate for a fusion sequence (Kondor-Koch et al., 1982; Rice and Strauss, 1981). Fusion is pH-dependent (Dalrymple et al., 1976; Helenius et al., 1980) and does not require E_1 cleavage.

The third class of fusion proteins are represented by the rhabdovirus G-proteins of vesicular stomatitis virus and rabies virus. From sequencing data on both of these glycoproteins, no

hydrophobic domains or highly conserved regions such as those observed in the alpha-virus E_1 or influenza HA proteins are present (Rose et al., 1982). These proteins are not proteolytically activated and fusion is pH-dependent (White et al., 1981; Mifune et. al., 1982).

The molecular events involved in pH-dependent virus uncoating are best characterized in this case of influenza virus. Here pH-dependent fusion with the endosomal membrane is accompanied by a conformational change in the hemagglutinin glycoprotein resulting in exposure of a fusion-active, hydrophobic peptide sequence at the N-terminus of HA_2 (Skehel et al., 1982). This hydrophobic sequence is presumed to initiate the fusion event by insertion into the lipid bilayer of the apposing endosomal membrane.

At present, very little is known about the structure of the coronavirus E_2 fusion protein or its mechanism of action. However, on the basis that proteolytic cleavage of E_2 activates fusion activity (Sturman et al., 1985), the E_2 superficially resembles the fusion proteins common to the para- and orthomyxoviruses.

4.5 Cytopathic Effects Associated with MHV Infection

The modification of membrane permeability during virus infection is a widespread phenomenon (Carrasco and Smith, 1980; Kohn, 1979) and has been associated with alterations of intracellular monovalent cation levels (Garry et al., 1982; Pasternak and Micklem, 1981). Such permeability changes are observed during the late phase of infection by picornaviruses (Carrasco and Smith, 1976; Nair, 1984),

herpesviruses (Benedetto et al., 1980), paramyxoviruses (Benedetto et al., 1980) rhabdoviruses (Garry et al., 1979; Nair, 1984), papovaviruses (Contreras and Carrasco, 1979), and togaviruses (Garry et al., 1979; Ulug et al., 1984). Interestingly, the messages specified by a number of DNA and RNA viruses including vaccinia (Oppermann and Koch, 1976), adenovirus (Cherney and Wilhelm, 1979), poliovirus (Saborio et al., 1974), Sindbis virus (Garry et al., 1979), vesicular stomatitis virus and reovirus (Nuss et al., 1975) are efficiently translated under altered ion conditions which effectively block cellular protein synthesis.

The results presented in Figure 19 indicate that MHV infection of L-2 cells leads to induction of membrane permeability changes, as described above for a wide variety of viruses. Two features are of interest in Figure 19. First, there is a small but significant $[^{22}Na]^+$ influx within 2 h P.I. that is suggestive of altered membrane permeability during the binding and endocytic entry of MHV. The permeability of the plasma membrane becomes modified early during the adsorption-penetration phase of infection with a number of RNA and DNA viruses, both enveloped and non-enveloped (Carrasco, 1981; Foster et al., 1980; Impraim et al., 1980). These studies have demonstrated that such permeability changes involve the passage through the membrane of low MW as well as high MW compounds, although with different efficiencies. The disruption of the normal ionic gradient (ie Na^+/K^+) leads to a transient drop in the membrane potential (Impraim et al., 1980; Kohn, 1979).

That these early permeability changes are related to the pH-

dependent fusion event effecting entry of virus into cells was demonstrated for the haemolytic paramyxoviruses, Sendai virus and Newcastle disease virus (Foster et al., 1980; Klemperer, 1960). These permeability changes also underlie the ability of these viruses to mediate cell fusion (Knutton and Pasternak, 1979). It has been shown that the pH-dependent endocytic entry of influenza virus elicits similar permeability changes. Interestingly, these changes (Na^+ influx and/or K^+ efflux) are observed for Sendai and influenza virus only at the pH-optimum for virus-cell fusion (Patel and Pasternak, 1983).

A second feature of interest in Figure 19 is the striking correlation between the large $[^{22}Na]^+$ influx and expression of cell fusion observed late in MHV infection in L-2 cells. As mentioned earlier, alterations in membrane permeability are commonly observed late in infection, during expression of cytopathic effect by a number of viruses. Specifically, Na^+ influx has been demonstrated in cells infected with members of the picornavirus (Egberts et al., 1977; Nair, 1981, 1984), rhabdovirus (Nair et al., 1984) alpha virus (Garry et al., 1979; Gray et al., 1983) and poxvirus (Norrie et al., 1982) groups. However, there is at present no documentation of Na^+ influx during the course of virus infection where cell fusion is the primary cytopathic effect. The dramatic Na^+ influx observed during the progression of cell fusion in MHV-infected L-2 cells is not an unexpected result, considering the disruption of membrane structure on such a large scale. Polykaryon formation by the paramyxoviruses (fusion from without), initiated by virions with cleaved F protein is

known to result in similar Na^+ influx (Foster et al., 1980). By analogy with that defined system, MHV-induced L-2 cell fusion (fusion from within), while at present largely uncharacterized, would appear to perturb membrane integrity in a similar fashion.

The results presented in Figure 20 indicate that the inhibition of host protein synthesis in MHV-infected L-2 cells is not initiated by alterations in membrane permeability. Similar results were obtained for infections involving vesicular stomatitis virus (Francoeur and Stanners, 1978), poliovirus (Nair et al., 1981) and encephalomyocarditis virus and human rhinovirus (Nair, 1984). However, in one system, namely Sindbis virus infection of avian fibroblasts, a correlation between monovalent cation changes and host inhibition was reported (Garry et al., 1979).

In herpes-virus infected Vero cells, inhibition of host protein synthesis was observed in the absence of Na^+-K^+ alterations (Hackstadt and Mallavia, 1982). Thus, Na^+-K^+ changes in some systems are not required for host shut down, nor do they occur soon enough in several other virus infections to initiate host inhibition.

However, the alterations in monovalent ion levels observed late in infection with many viruses may contribute to cytopathic effect through preferential translation of viral mRNA. The results presented in Figure 21 indicate that MHV mRNAs are preferentially translated over cellular species under conditions of increased Na^+ concentration. Since sodium ions are known to be inhibitory for translation systems in general, these results suggest that MHV mRNAs may possess different ionic requirements and optima for maximal

translation than their cellular counterparts. A comparison of Figures 19 and 20 reveals that the bulk of viral protein synthesis, observed between 3-6 h P.I., occurs under conditions where there is significant Na^+ influx, particularly 4-6 h P.I. Therefore, in the MHV-infected cell, the induction of cell fusion and concomittant membrane permeability increase may lead to alterations of intracellular ion levels that favor the translation of viral messages.

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