CHARACTERIZATION OF A MUTANT STRAIN OF MURINE CYTOMEGALOVIRUS WHICH FAILS TO GROW IN SALIVARY GLANDS

by

JESSICA MIRIAM BONAME

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Department of Pathology

The University of British Columbia
Vancouver, Canada

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ABSTRACT

An understanding of the mechanisms of pathogenesis of cytomegaloviruses is essential for future work towards control and prevention of cytomegalovirus disease. Towards this end, a comparison of the Smith, Vancouver and K-181 strains of murine cytomegalovirus (MCMV), which differ in virulence, revealed differences in growth characteristics *in vivo* and *in vitro*. The Vancouver strain of MCMV grows to a limited extent in the spleen while it fails to grow in the salivary glands of inoculated mice (salivary gland growth mutant, Sgg^-). This mutation probably arose during multiple *in vitro* passage of the parental Smith strain. *In vitro*, the Vancouver strain replicated more quickly and produced a greater yield of virus per cycle which resulted in a larger plaque size relative to the Smith or K-181 strains. The Vancouver strain has a 9.4 kb deletion, which spans the XbaI *I/L* junction of the parental Smith strain (0.960 to 0.995 map units), and a 0.9 kb insertion which maps to the EcoRI *K* fragment (0.37 to 0.47 map units). Recombinant MCMV was generated by co-transfection of cells with whole Vancouver strain DNA and cloned fragments of wild-type DNA. Three independent Sgg^+ recombinant viruses were selected by passage of the co-transfected virus through mouse salivary glands. The three recombinants had recovered the DNA deletion, while they maintained the insertion associated with the Vancouver strain. Thus, the genotypic location of the defect responsible for the Sgg^- phenotype of the Vancouver strain of MCMV maps to 0.960 to 0.995 map units of the Smith virus genome. In addition, the restoration of this region of the genome correlated with the detection in the recombinants of a 42 kDa protein present during E and L times post infection in Smith- and K-181-infected cells, but not in Vancouver strain-infected cells.

The K-181 strain was associated with higher salivary gland titres and a low LD_{50} titre consistent with the increased virulence of this strain compared with the Smith strain. Differences in the K-181 restriction endonuclease pattern relative to the Smith strain were

mapped, and were mainly confined to 0.0 to 0.06 map units of the genome without any large insertions or deletions detected. Minor differences in the proteins of the Smith and K-181 strains at early and late times post infection were also detected. The identification of a gene involved in the salivary gland tropism of MCMV and the characterization of differences between the Smith and K-181 strains of MCMV should increase our understanding of the virulence mechanisms of cytomegaloviruses.

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ABBREVIATIONS

α:	alpha (or immediate early) genes
Ab:	antibody
ActD:	actinomycin D
AIDS:	acquired immunodeficiency syndrome
ATCC:	American Type Culture Collection
β:	beta (or early) genes
Biorad:	Bio-Rad Laboratories
Boehringer Mannheim:	Boehringer Mannheim Canada Ltd.
bp:	base pairs
BRL:	Bethesda Research Laboratories, Life Technologies, Inc.
Calbiochem:	Calbiochem® Corporation
cAMP:	cyclic adenosine monophosphate
cGMP:	cyclic guanosine monophosphate
CH:	cycloheximide
CID:	cytomegalic inclusion disease
CHEF:	clamped homogeneous electric fields
CPE:	cytopathic effect (s)
CTL:	cytotoxic T lymphocyte (s)
DMEM:	Dulbecco's modified essential medium
DNA:	deoxyribonucleic acid
dsDNA:	double stranded DNA
dUTPase:	deoxyuridine triphosphatase
E:	early

ABBREVIATIONS CONTINUED

EBV:	Epstein Barr virus
EDTA:	ethylenediamine tetraacetic acid, disodium salt
eg.:	for example
et al.:	et alia; and others
FBS:	foetal bovine serum
γ:	gamma (or late) genes
Gibco:	Gibco® Laboratories, Life Technologies Inc.
gp:	glycoprotein
gc:	glycoprotein complex
HBSS:	Hank's balanced salt solution
HCMV:	human cytomegalovirus
HEPES:	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
Hoeffer:	Hoeffer Scientific Instruments
HSV-1, 2:	herpes simplex virus type I, and type 2
ICN:	ICN Biomedicals Canada Ltd.
ICP:	infected cell protein
IE:	immediate early
ie:	immediate early gene
Ig:	immunoglobulin
IgG:	immunoglobulin G
IgM:	immunoglobulin M
INF:	interferon

ABBREVIATIONS CONTINUED

IR_L:	internal repeat sequence flanking the long unique sequence of the HCMV or HSV genome
IR_S:	internal repeat sequence flanking the short unique sequence of the HCMV or HSV genome
kb:	kilobase pair (s)
kDa:	kilodalton (s)
Kodak:	Eastman Kodak Company
L:	late
LD_{50}:	50% lethal dose
MCMV:	murine cytomegalovirus
MEF:	mouse embryo fibroblasts
MHC:	major histocompatibility
MOI:	multiplicity of infection
mϕ:	macrophage
mRNA:	messanger RNA
NF1:	nuclear factor 1
NK:	natural killer
nm:	nanometer (s)
ori:	origin of DNA replication
PAA:	phosphonoacetic acid
PAGE:	polyacrylamide gel electrophoresis
PBS:	phosphate buffered saline
PFA:	phosphonoformic acid

ABBREVIATIONS CONTINUED

PFU:	plaque forming unit (s)
Pharmacia:	Pharmacia (Canada) Inc.
PMSF:	phenylmethylsulfonyl fluoride
pp:	phosphoprotein
PSI:	pounds per square inch
Rev.:	review
RNA:	ribonucleic acid
rpm:	revolutions per minute
SDS:	sodium dodecyl sulfate
Sigma:	Sigma® Chemical Co.
SP1:	cellular site-specific DNA-binding protein
SSC:	sodium chloride, sodium citrate
ssDNA:	single stranded DNA
TAE:	tris acetate EDTA buffer
TBE:	tris borate EDTA buffer
TEMED:	N,N,N'N'-tetramethylethylenediamine
T_H:	T helper cell
Tris:	tris(hydroxymethyl)aminomethane
TR_L:	terminal repeat sequence flanking the long unique sequence of the HCMV or HSV genome
TR_S:	terminal repeat sequence flanking the short unique sequence of the HCMV or HSV genome
U_L:	long unique sequence of HCMV or HSV genome

ABBREVIATIONS CONTINUED

U_S:	short unique sequence of HCMV or HSV genome
UV:	ultra violet
v/v:	volume to volume
VZV:	varicella zoster virus
w/v:	weight to volume

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INTRODUCTION

Cytomegaloviruses are ubiquitous agents which infect a number of animals, including man (Weller, 1971a; Plummer, 1973, Rev.). As a group, they are highly species specific and humans are thought to be the only reservoir of human cytomegalovirus (HCMV). HCMV is endemic in the population. Socio-economic factors other than hygiene alone are believed to influence the spread of the virus; crowded areas yield higher rates of infection. Indeed, rates of infection during childhood in developing countries are between 90 and 100%. Because HCMV is highly labile, it is believed that close or intimate contact is the major factor influencing the horizontal spread of this virus (Alford & Britt, 1990).

HCMV cannot be used in animal models because of the species specificity of cytomegaloviruses, and thus infection with murine cytomegalovirus (MCMV) in the mouse has become the most widely used *in vivo* model system (Griffiths & Grundy, 1987; Hudson, 1979; Plummer, 1973). The Smith strain of MCMV was isolated by Margaret Smith (Smith, 1954), and the isolation of HCMV by three groups followed: a salivary gland isolate from a dead infant (Smith, 1956), strain AD169 from the adenoids of infected children (Rowe et al., 1956), and the Davis, Kerr and Esp strains from live infants with cytomegalic inclusion disease (CID) (Weller et al., 1957). In general, a balance exists in the relationship between host and virus and infections normally are subclinical. However, problems occur when the immune system is immature (congenital and perinatal infection) and in the immunocompromised individual (transplant recipients, patients undergoing cancer therapy, and patients with AIDS). A great deal of information has been gained about the epidemiology and pathogenesis of cytomegalovirus. However the molecular mechanisms involved in virus infection, tissue tropism, and persistence, require elucidation if successful treatment or prevention of disease attributable to cytomegalovirus is to become a reality.

CLASSIFICATION AND GENERAL CHARACTERIZATION

Cytomegaloviruses are members of the family herpesviridae. The herpesviruses have single, linear, double-stranded DNA genomes of greater than 120 kb, which are replicated via concatemeric intermediates. Production of progeny genomes is accomplished by site-specific recombination/cleavage reactions. The genomes are packaged into icosahedral nucleocapsids of 100 nm diameter containing 162 capsomeres, which are then enclosed in a protein tegument and enveloped to yield their infectious extracellular forms (Honess, 1984).

The herpesviruses have been subdivided into sub-families based on species specificity, length of replication cycle, and site of latency *in vivo* (Roizman, 1982). The alphaherpesvirinae (HSV-1, HSV-2, VZV) are variable in their host specificity, have a relatively short replication cycle with rapid progression of cytopathic effects (CPE), and remain latent in nerve ganglia. The betaherpesvirinae are characterized by their high degree of species specificity, their relatively slow rate of replication and their ability to remain latent in numerous tissues *in vivo*. Although cytomegaloviruses are the major example of this group, human herpesvirus 6 (Salahuddin et al., 1986) is closely related to HCMV on the basis of DNA sequence analysis (Lawrence et al., 1990) and may therefore be grouped within the betaherpesvirinae. The gammaherpesvirinae (EBV) are limited to replication and latency within lymphoid tissues and demonstrate heterogeneity in the lengths of the replicative cycle and the extent of CPE.

GENOME ORGANIZATION

Two groups have published restriction endonuclease maps of the MCMV genome (Mercer et al., 1983; Ebeling et al., 1983). Neither group found evidence for the type of genome inversion that occurs with HCMV and HSV. Mercer et al. (1983) mapped the HindIII and EcoRI fragments and calculated that the genome size was approximately 240 kb. Ebeling et al. (1983) also constructed a restriction endonuclease map using the enzymes HindIII, EcoRI and

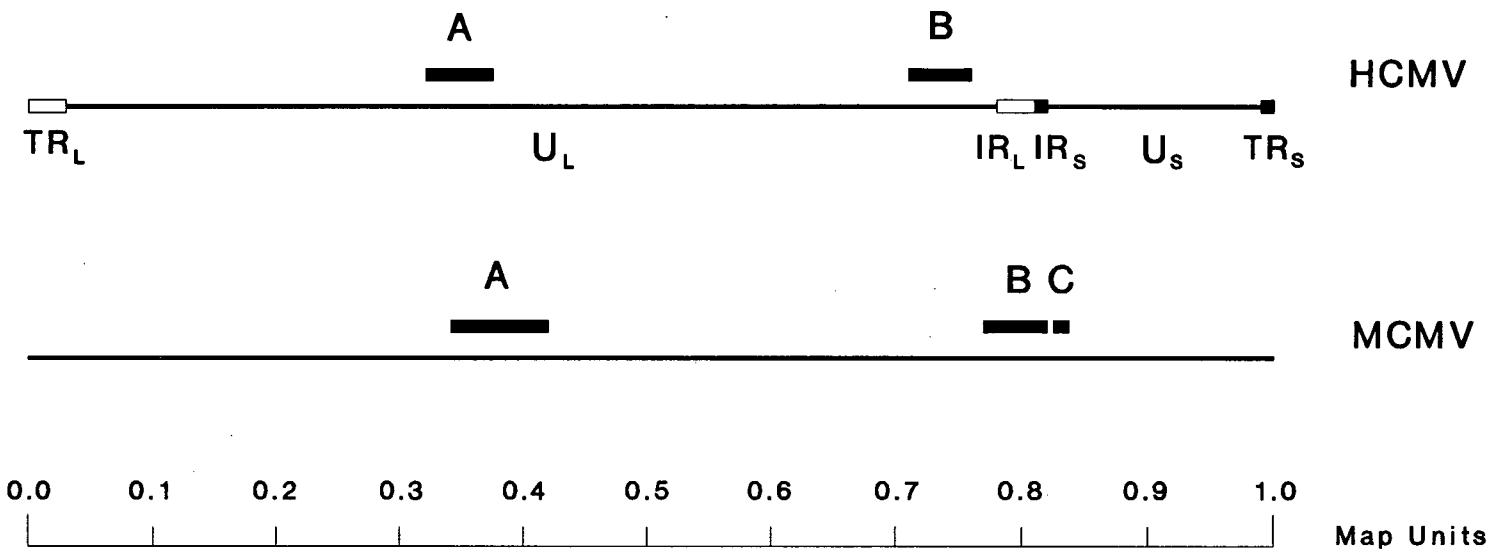
XbaI and found that the genome had a molecular mass of 155 x 10^6 kDa, or 235 kb, with equimolar amounts of each DNA fragment.

In the case of HCMV, Kilpatrick and Huang (Kilpatrick & Huang, 1977) found evidence for four isomeric forms of the AD169 genome, which could be explained by the inversion of two unique sequences, unique long (U_L) and unique short (U_S), flanked by two sets of redundant or repeat sequences. Thus terminal repeat long (TR_L) and internal repeat long (IR_L) delimit the U_L sequence while terminal repeat short (TR_S) and internal repeat short (IR_S) delimit the U_S sequence. This has been confirmed by restriction endonuclease mapping analysis (Westrate et al., 1980; Tamashiro et al., 1982), and genome sequence analysis (Chee et al., 1990a). Even though the isomeric structure of the HCMV genome is more complex than the non-isomerizing structure of the MCMV genome, the genome size, gene organization and regulatory sequences appear to be remarkably similar (Mocarski et al., 1990)[Fig. 1].

VIRUS LIFE CYCLE

All viruses are obligate intracellular parasites, dependent upon the host cell for their reproduction. During the replicative cycle, viruses go through the processes of attachment, entry, uncoating, transcription, translation, nucleic acid replication, assembly and release. Unlike less complex viruses, such as poliovirus, which encodes only virion components and proteins involved in RNA synthesis and cleavage, the herpesviruses are extremely complex. Herpesviruses undergo a three-stage "cascade" of transcription and translation termed alpha (α) or immediate early (IE), beta (β) or early (E), and gamma (γ) or late (L, Honess & Roizman, 1974). *De novo* protein synthesis is not required for transcription of the IE genes, and the products of these genes are generally regulatory. The IE gene products are required for transcription of the E genes. Many of these E gene products are required for DNA replication. Full expression of the γ or late gene products does not occur until after the onset of DNA

FIGURE 1: COMPARISON OF THE HCMV AND MCMV GENOMES



TR_L: terminal repeat, long
IR_L: internal repeat, long
U_S: short unique sequence
U_L: long unique sequence
IR_S: internal repeat, short
TR_S: terminal repeat, short

A: region which encodes gB, DNA polymerase, and the ICP8 homologue, and contains the *ori* region
B: region which encodes IE1 and IE2, and contains the major IE enhancer
C: region which encodes Sgg1

Sources: Manning, 1990; Mocarski et al., 1990; Chee et al., 1990a; Hamzeh et al., 1990; G. Keil, personal communication; M.J. Masse, personal communication

replication; however, some transcription from these genes does occur at early times post-infection. These genes are sometimes referred to as $\gamma 1$ genes while those genes only transcribed after the onset of DNA replication are termed $\gamma 2$ genes (Roizman & Sears, 1990). The late gene products are primarily viral structural proteins, or proteins required for virion assembly. Comparison of MCMV to HCMV will be made throughout the following sections as there is limited information available about some aspects of the MCMV life cycle.

Attachment, Entry and Uncoating: Infection is initiated by attachment of the virus to the host cell. This occurs by the interaction of the virus attachment protein, present on the surface of the virion, and a viral receptor on the cell surface. Viral receptors can be carbohydrates, lipids and proteins. Although the virus may be able to bind to a number of molecules on the cell surface, not all of the interactions are likely to be biologically relevant. Once the virus is in close contact with the cell surface, it enters either by direct fusion of the virus membrane with the cell membrane, or by receptor-mediated endocytosis (Lentz, 1990, Rev.).

The viral attachment protein and the virus receptor have not yet been definitely elucidated for either MCMV or HCMV, although there have been suggestions that class I MHC molecules may be involved (Price et al., 1987a). By analogy to HCMV and its ability to bind $\beta 2$-microglobulin, it has been suggested that $\beta 2$-microglobulin is the intermediate between the MCMV attachment protein and the cellular receptor (Hodgkin et al., 1988). A more recent study showed that tissue-culture-passaged MCMV binds to N-acetylglucosamine residues on the surface of mouse embryo fibroblasts (MEF), and that the shift to virulence, accomplished by salivary gland passage of virus, correlated with viral recognition of sialic acids (Ravindranath & Graves, 1990). These two hypotheses are not mutually exclusive, in that class I MHC contain complex carbohydrates (Bjorkman et al., 1987).

The situation with HCMV has been studied more thoroughly; however, there appear to be lines of evidence pointing to two different cellular receptors. Grundy et al. (Grundy et al., 1987) reported that HCMV binds to class I major histocompatibility complex (MHC) molecules. They showed that the addition of serum containing β2-microglobulin to extracellular medium increased the infectivity of HCMV in a specific manner, and that the affinity of HCMV for class I MHC was greater than that of soluble β2-microglobulin. They also showed that cells expressing class I MHC antigens were able to bind more β2-microglobulin-coated HCMV than cells not expressing class I MHC. Human cytomegalovirus has been shown to encode a protein that is similar to the class I MHC, and it has been proposed that this protein is responsible for the ability of the virus to bind β2-microglobulin (Beck & Barrell, 1988). There are two other groups which have identified the putative cellular receptor for HCMV as a membrane glycoprotein of between 30-34 kDa (Taylor & Cooper, 1990; Adlish et al., 1990). Specific, saturable, high-affinity binding of the virus to this cellular protein, which was too small to be a class I MHC molecule, was demonstrated. What these authors have failed to demonstrate is a biologically relevant event associated with virus binding to this protein. It is possible that HCMV uses more than one mechanism for entry into the cell. However, if β2-microglobulin binds to the virus via the class I MHC homologue present in the virion, as an intermediate between the virus attachment protein and the viral receptor on the cell surface, the class I MHC binding site on the β2-microglobulin would presumably not be available for binding to class I MHC on the surface of the cells, suggesting an alternative role for the interaction between β2-microglobulin and the HCMV class I MHC homologue.

Very little is known about the entry and uncoating of either MCMV or HCMV. Penetration is believed to occur by fusion of the viral envelope with the host cell plasma membrane and, like HSV, probably involves one or more viral glycoproteins (Tyler & Fields, 1990; Herold et al., 1991; Morgan, et al., 1968; Cai et al., 1988; Ligas & Johnson, 1988).

Thereafter, the capsids are probably transported to nuclear pores and the DNA released into the nucleoplasm of the cell (Batterson et al., 1983; Roizman & Sears, 1990).

Transcription and Translation: As described above, the herpesviruses undergo a cascade of transcription and translation with IE, E and L phases, which will be discussed separately below. The biochemical evidence defining each class was obtained by the use of cycloheximide (CH) to inhibit translation, actinomycin D (ActD) to inhibit transcription, and phosphonoformic acid (PFA) or phosphonoacetic acid (PAA) to inhibit viral DNA replication.

Immediate Early Transcription and Translation: During IE times post infection, most mRNA transcripts mapped to 0.769 to 0.817 map units on the MCMV genome. A minor area of transcription was also noted between 0.944 and 0.002 map units (Marks et al., 1983; Keil et al., 1984). The most abundant transcript during IE times encodes the major ie1 gene product, an 89 kDa phosphoprotein designated pp89 (Keil et al., 1985, 1987a, 1987b). In addition, pp89 has also been shown to be a transcriptional transactivator (Koszinowski et al., 1986) capable of stimulating c-*fos* expression (Schickedanz et al., 1988). Conflicting reports suggest that this major IE protein can bind directly to DNA or interact with DNA only in association with other cellular DNA-binding factors (Walker & Hudson, 1988b; Munch et al., 1988).

The major IE region of the genome also contains two other transcriptional units, ie2 and ie3. The ie2 gene gives rise to a 43 kDa protein product, while the ie3 gene probably initiates within ie1 and is spliced to give full-length ie3 transcripts (Keil et al., 1987a, Messerle et al., 1991). The ie2 gene product is dispensable for growth in tissue culture, but plays a role in reactivation from latency (Manning & Mocarski, 1988; Manning, 1990). Transcription in the major IE region is driven from an enhancer located between the ie1 and ie2 coding regions

(Dorsch-Häsler et al., 1985). No information about specific transcription or translation products from the ends of the genome has been reported.

Several groups have described between 3 and 10 MCMV IE proteins, ranging from 100 to 28 kDa in size (Chantler & Hudson, 1978; Moon et al., 1979; Walker & Hudson, 1987). Some were phosphorylated, some had ssDNA or dsDNA binding characteristics, and most were present both in the nucleus and the cytoplasm (Walker & Hudson, 1987).

Early Transcription and Translation: During E times post-infection, transcription occurs from most regions of the MCMV genome, with transcripts from 0.840-0.861 being the most abundant (Marks et al., 1983; Keil et al., 1984). These early transcripts require *de novo* protein synthesis and can be identified after the removal of a CH block. Transcription of these early genes is believed to require induction by the IE proteins, thus explaining the requirement for *de novo* protein synthesis. An early gene spanning the MCMV HindIII *I/J* junction (within the most abundantly transcribed E region) was cloned and sequenced (Vellani, 1991), and a protein of 135 kDa was predicted from the sequence analysis. In another detailed study of a single locus, Bühler et al. (1990) characterized an E gene which was located between 0.709-0.721 map units. Sequence analysis predicted a polypeptide of 36.4 kDa. In further studies which illustrated the dependence of E transcription on IE gene products, they showed that *in vitro* transfection of cells stably transfected with the ie1/ie3 complex produced three antigenically related proteins of 36, 37, and 38 kDa, and another 33 kDa protein from an unspliced transcript. The functions of these proteins have not yet been characterized.

Analysis of the early phase by characterization of MCMV proteins produced prior to DNA synthesis has also been carried out. Moon et al. (1979) detected 5 early proteins of between 33 to 58 kDa in size. Seven early proteins ranging in size from 36 to 91 kDa have been characterized: 3 nuclear phosphoproteins with DNA-binding characteristics, and 4

cytoplasmic proteins, one of which (60 kDa) was a DNA-binding protein (Walker & Hudson, 1987, 1988a, 1988b). It is not surprising that so few early proteins have been characterized since they are probably enzymes and regulatory proteins, and would be present in small amounts compared to the structural proteins seen during the late phase of viral replication.

Early functions of herpesviruses include two categories of proteins: those essential for viral DNA replication and those involved in nucleic acid metabolism. There are 7 genes essential for HSV-1 origin-dependent DNA amplification including a heterotrimer known as the helicase-primase complex, an origin binding protein, the major DNA binding protein ICP8, and a heterodimer which comprises the DNA polymerase (Challberg, 1986; McGeoch et al., 1988; Wu et al., 1988;). Apart from DNA replication, HSV enzymes involved in nucleic acid metabolism include thymidine kinase, ribonucleotide reductase, dUTPase, uracil-DNA glycosylase and alkaline exonuclease.

Very few of these enzymes have been identified for MCMV, however many have been identified in HCMV. The entire sequence of the HCMV AD169 strain genome was recently reported (Chee et al., 1990a), and the gene designations (eg. 54th open reading frame from the unique long region, UL54) which follow come from that paper. Of the 7 genes known to be essential for HSV replication, only the DNA polymerase and the major ssDNA binding protein, or ICP8 homolog, have been clearly identified in HCMV. The DNA polymerase is a 140 kDa protein encoded by the UL54 gene (Heilbronn et al., 1987; Kouzarides et al., 1987; Chee et al., 1990a). The ICP8 homolog, UL57, encodes a 135-140 kDa ssDNA binding protein which is localized in the nucleus of infected cells (Kemble et al., 1987; Anders & Gibson, 1988; Chee et al., 1990a). Interestingly, the MCMV DNA polymerase and ICP8 homologue have been mapped to the HindIII *D/H* region of the Smith and K-181 genomes, in a conserved gene block analogous to that found in HCMV (Mocarski, personal communication; Keil, personal communication). Other proteins thought to be involved in DNA replication and nucleic acid

metabolism have also been identified by sequence analysis as HCMV homologues to specific herpesvirus proteins (Chee et al., 1990a).

Some other early HCMV proteins have been characterized. A group of related phosphoproteins, pp84, pp50, pp43 and pp34, have been mapped to the UL112 and UL113 genes, and are associated primarily with the nuclei of infected cells (Wright et al., 1988; Chee et al., 1990a). An early phosphoprotein, pp68, is associated with the nucleus and cytoplasm of infected cells and maps to 0.510 to 0.525 map units. It has an associated protein kinase activity, and is also found in the virion (Britt & Vugler, 1987). The function of ICP22, an early nuclear protein released from infected cells, has not been determined but the gene has been mapped to between 0.92 to 0.93 map units of the HCMV genome (Mocarski et al., 1988). No virus-specific thymidine kinase activity has been found in either HCMV- or MCMV-infected cells (Zavada et al., 1976; Estes & Huang, 1977; Muller & Hudson, 1977a), nor has a functional ribonucleotide reductase been described. More of these virus-specific metabolic enzymes must be identified for both MCMV and HCMV so that a better understanding of the viral replicative process can be achieved.

Late Transcription and Translation: Transcription occurs from all areas of the MCMV genome during the late phase of virus replication (Marks et al., 1983; Keil et al., 1984). The late gene products are virion structural components and proteins required for viral assembly. Between 23 and 33 structural proteins, and up to 8 non-structural proteins, have been identified for MCMV ranging from 11.5 kDa to 261 kDa (Hudson et al., 1988; Chantler & Hudson, 1978; Kim et al., 1976). Five envelope glycoproteins were identified by surface iodination of MCMV (Loh & Qualtiere, 1988). Three of these were antigenically related glycoproteins, gp150, gp105 and gp52 and together they made up the major envelope glycoprotein complex. This disulphide-linked complex was present on the surface of the virion. A monoclonal antibody directed against

this complex also recognized glycoprotein complex I (gcI) of HCMV, suggesting a conservation of the reactive determinant (Loh et al., 1988). A fourth envelope glycoprotein, gp87, was probably present in monomeric form on the surface of the cells, while the fifth envelope glycoprotein, gp46, has not been further characterized (Loh & Qualtiere, 1988). As yet, no functions have been ascribed to these viral envelope constituents.

As with the E proteins, more information is available for the late proteins of HCMV than MCMV. Three glycoprotein complexes (gcI, gcII, gcIII) have been identified on the HCMV envelope (Gretch et al., 1988a). Glycoprotein complex I contains three disulphide-linked glycoproteins, gp55, gp93 and gp130. The gp55 component of HCMV is the HSV gB homologue (gB), and is encoded by the UL55 gene of HCMV (Cranage et al., 1986; Chee et al., 1990a). However, HCMV gp93 and gp130 have not been mapped. Recently, HCMV gB was implicated as the viral attachment protein (Nowlin et al., 1991). By analogy, the MCMV major envelope glycoprotein complex gp150/gp105/gp52, which is antigenically related to HCMV gcI may include the viral attachment protein. Glycoprotein complex II (gcII) of HCMV is made up of a family of glycoproteins known as gp47-52 which maps to US10 and US11 (Gretch et al., 1988b). Its function is unknown. Glycoprotein complex III (gcIII) of HCMV, approximately 240 kDa in size, is made up of gp86 (the HSV gH homologue) and a 145 kDa protein which has not yet been mapped. Cranage et al. (1988) mapped gp86 to UL75 of the HCMV genome, and Keay et al. (1989) suggested that it may be involved in penetration and the cell-to-cell spread of HCMV. An additional 45 kDa integral membrane envelope protein has been described which maps to the HCMV UL100 gene (Lehner et al., 1989). The sequence analysis of HCMV (Chee et al., 1990a) also identified some genes homologous to eukaryotic genes. They include a T-cell receptor homologue (UL20), the MHC class I homologue (UL18) discussed earlier (Beck & Barrell, 1988), and a family of G-protein coupled receptor homologues

(US27, US28, US33) which are transcribed during late times post infection (Chee et al., 1990b; Welch et al., 1991).

Constituents of the tegument or matrix of HCMV, also synthesized late in infection, have been described; however the precise function of these proteins in the virus structure or assembly is unknown. There are at least five phosphoproteins: the 150 kDa matrix protein, pp150 (Jahn et al., 1987), the upper matrix protein, pp71 (Yamauchi et al., 1985), the lower matrix protein, pp65 or ICP27 (Pande et al., 1984; Landini et al., 1987), pp67 (Davis & Huang, 1985), and pp28 (Landini et al., 1987). These matrix components map to UL32, UL82, UL83, and possibly UL65 and UL99 respectively (Chee et al., 1990a). The phosphoproteins pp65 and pp67 have associated protein kinase activity. Recently, an Fc receptor for human IgG was localized within the tegument of HCMV virions (Stannard & Hardie, 1991). The Fc-binding was associated with polypeptides of approximately 33 and 69 kDa in size which were indistinguishable from the HCMV proteins which bind to β2-microglobulin.

Two additional late structural proteins, the HCMV major capsid protein, a 150 kDa protein which maps to UL86 (Chee et al., 1989), and a 32 kDa minor capsid component, which maps between 0.62 and 0.64 map units (Pande et al., 1988), have been identified. In addition, two late non-structural proteins have been described. A 35-40 kDa capsid assembly protein found in the nucleus of HCMV-infected cells is a product of the UL80 gene (Gibson et al., 1990, Chee et al., 1990a). The 52 kDa UL44 gene product is a glycosylated phosphoprotein. It is a DNA binding protein which is transcribed at early times post infection, but not translated until the late phase of infection (Mocarski et al., 1985; Leach & Mocarski, 1989).

Although little is known about the MCMV late proteins, they can be presumed to have functions similar to those described for HCMV and HSV. Some, like the capsid proteins, may have a straightforward function in virion structure. Others, such as the matrix components and proteins associated with the viral DNA, may play a role in uncoating of the virus, IE gene

transactivation, regulation of host cell gene expression, or stability of the virion. Once the functions of more of these gene products have been established, one will have a better understanding of the nature of viral tropism and species specificity, and a starting point for designing a more effective vaccine and antiviral drug strategies.

Gene Regulation: A great deal of work has been carried out on the regulation of herpesvirus gene expression. In particular, IE gene regulation has been the most thoroughly studied for both HCMV and MCMV. There is a strong enhancer located between the ie1 and ie2 genes of MCMV, which is similar to that found in the major IE region of HCMV (Dorsch-Häsler et al., 1985; Boshart et al., 1985). Both enhancers contain repeated sequences responsive to both viral and host cellular factors (Simminger et al., 1990; Cherrington & Mocarski, 1991; Zhang et al., 1991). In addition, the HCMV enhancer was shown to be responsible for positive and negative regulation of the major IE genes in different cell types (Nelson et al., 1987).

In addition to the enhancer element, the ie1 genes of HCMV and MCMV have RNA polymerase II TATA and CAAT consensus sequences in the promoter region. These consensus sequences, also found 5' to most MCMV and HCMV E and L genes described thus far, enable transcription by the host cellular RNA polymerase II. The enhancer is downstream from the promoter and is preceded by a series of nuclear factor 1 (NF1) binding sites, and finally a modulator region which acts in a differentiation-dependent fashion (Nelson et al., 1990). The host factor binding sites found in the IE promoter-regulatory region are important for regulation of viral gene expression, and may be responsible for tissue specificity.

Most of the work on the regulation of specific IE, E and L genes of cytomegaloviruses has been done for HCMV, but is probably also applicable to MCMV. The IE genes are regulated by *cis* sequence elements located in or near their promoters. As well as host regulatory factors, a virion protein and the IE-gene products have been implicated as *trans*-acting

factors (Stinski & Roehr, 1985; Spaete & Mocarski, 1985b; Pizzorno et al., 1988; Cherrington & Mocarski, 1989). Early and L gene expression is controlled, in part, by transactivation by IE gene products -- ie1 and ie2 in HCMV, and ie1 and ie3 in MCMV (Staprans et al., 1988; Depto & Stenberg, 1989; Klucher et al., 1989; Bühler et al., 1990). In addition, multiple, differentially-regulated promoters can be used for temporal expression of certain genes (Leach & Mocarski, 1989). Finally, post transcriptional modification may be responsible for the delay in expression of certain genes. *Cis*-acting sequences in E transcripts can delay translation until L times post infection (Geballe et al., 1986a; 1986b; Geballe & Mocarski, 1988), while 3'-end processing can delay transport of E transcripts to the cytoplasm (Goins & Stinski, 1986).

Viral DNA Replication: Replication of MCMV DNA begins between 10 to 12 hours post infection in MEF or 3T3 cells (Moon et al., 1976; Muller & Hudson, 1977b). After infection, there is a decrease in host cell DNA synthesis, even if UV-irradiated or heat-inactivated virus is used, suggesting that a viral structural protein mediates this effect (Moon et al., 1976). MCMV is a cell-cycle dependent virus, having a requirement for an S-phase component of the host cell in order to replicate; cells held in G_0-phase have limited protein expression and fail to undergo viral DNA replication (Muller & Hudson, 1977b; Muller et al., 1978).

The linear genome of herpesviruses becomes circularized early after infection (Stinski, 1990). Fusion of the termini of the MCMV genome was detected as early as 2 hours post infection, and the number of intracellular fusion fragments, relative to free terminal fragments, increased until late in infection. Fusion of termini does not require *de novo* protein synthesis, and is thus mediated by virion and/or host cell protein(s) (Marks & Spector, 1984). Characterization of the structure of the termini of virion DNA and the fusion fragment was consistent with end-to-end ligation of the termini (Marks & Spector, 1988). It is believed that genomes with fused termini are replicative intermediates. Replicative forms of HCMV were

found in permissively infected cells of human origin, but not in non-permissive mouse cells (Lafemina & Hayward, 1983), suggesting that circularization was integral to viral replication. Pac-1 and pac-2, *cis*-acting signals for cleavage and packaging conserved in other herpesviruses including HCMV and HSV (Spaete & Mocarski, 1985a), were found near the presumed site of cleavage of replicative intermediates (Marks & Spector, 1988). An 89 kDa host cell protein binds to the pac-2 signal of the HCMV genome, but the role of this pac-2 DNA binding protein has not yet been determined (Kemble & Mocarski, 1989). Interestingly, this pac-2 DNA binding protein was only found in cells of human origin; perhaps it is a determinant of species specificity.

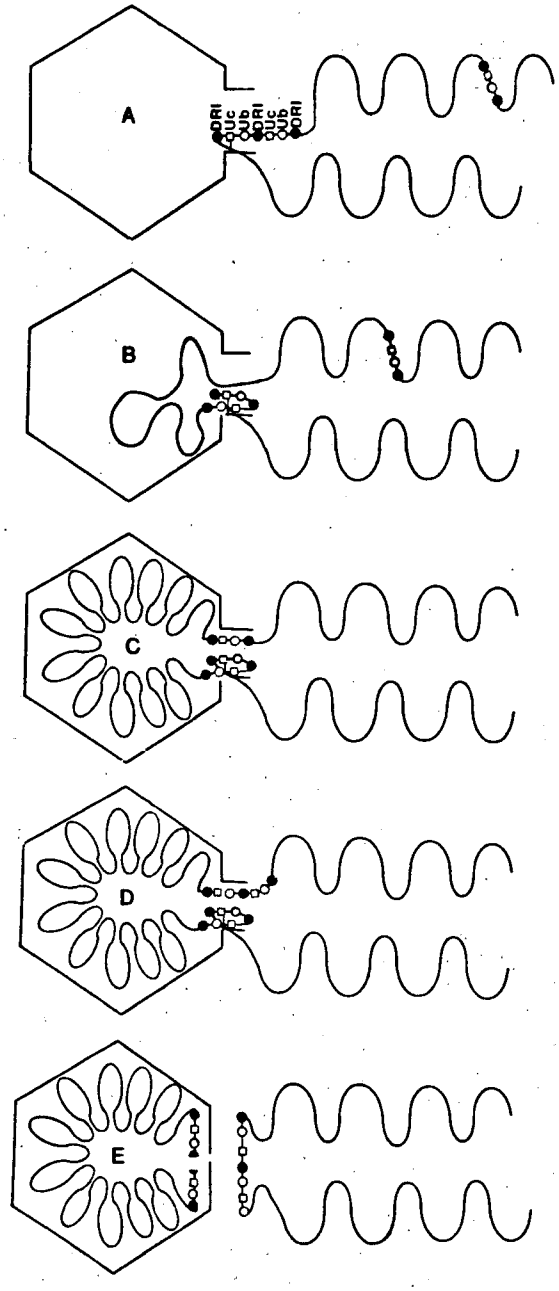
Once the viral genome has entered the cell nucleus and circularized, DNA replication begins at a specific site, referred to as the origin of DNA replication, or *ori*. The HCMV *ori* was identified just upstream of the gene encoding the major DNA binding protein, ICP8, between 0.36 and 0.4 map units (Hamzeh et al., 1990; Anders & Punturieri, 1990). This region of the genome seems to be conserved in MCMV and there is evidence that the MCMV *ori* is located in the HindIII *D* fragment, which also encodes an ICP8 homologue (M.J. Masse and G. Keil, personal communication). As described in the section dealing with early proteins, there are a number of virally-encoded proteins required for origin-dependent DNA synthesis -- seven have been described for HSV including DNA binding proteins, DNA polymerase and a helicase/-primase complex. The *ori*-binding protein interacts with the *ori* site (Hernandez et al., 1991) and DNA replication proceeds in combination with both viral and cellular factors. Cellular topoisomerase II is required for replication of HCMV, and correspondingly, its expression is induced in HCMV infected cells (Bensen & Huang, 1988; 1990). Replication is believed to proceed bi-directionally by a rolling circle mechanism and viral and/or cellular proteins act in *trans* to mediate cleavage and packaging of genome-length DNA (Stinski, 1990).

Packaging and Release: Capsids are assembled in the nucleus, however little is known about how the capsomeres aggregate, or how the newly-replicated DNA becomes associated with the capsids. At late times post infection, three successive stages of intranuclear capsid formation were observed in MCMV-infected 3T3 cells: capsids with electrolucent cores, followed by capsids which were associated with filamentous material believed to be deoxyribonucleoprotein, and eventually, electron dense cores inside capsids, which were believed to be the mature nucleocapsids (Weiland et al., 1986; Papadimitriou et al., 1984).

The process by which the DNA is packaged has not been elucidated, however the cleavage and packaging of DNA are probably linked. The sequence at the ends of herpesvirus genomes, containing all the signals required for fusion of termini, and cleavage and packaging of new viral genomes, is referred to as the *a* sequence. Deiss and Frenkel (Deiss & Frenkel, 1986) examined the cleavage and packaging of HSV-1 amplicons (plasmids containing the origin of replication and the *a* sequence). They proposed a packaging model based on the following results: that the cleavage/packaging signal is contained within the terminal *a* sequence, that cleavage of viral DNA concatamers is coupled to packaging, and that the *a* sequence is amplified during this process [Fig. 2].

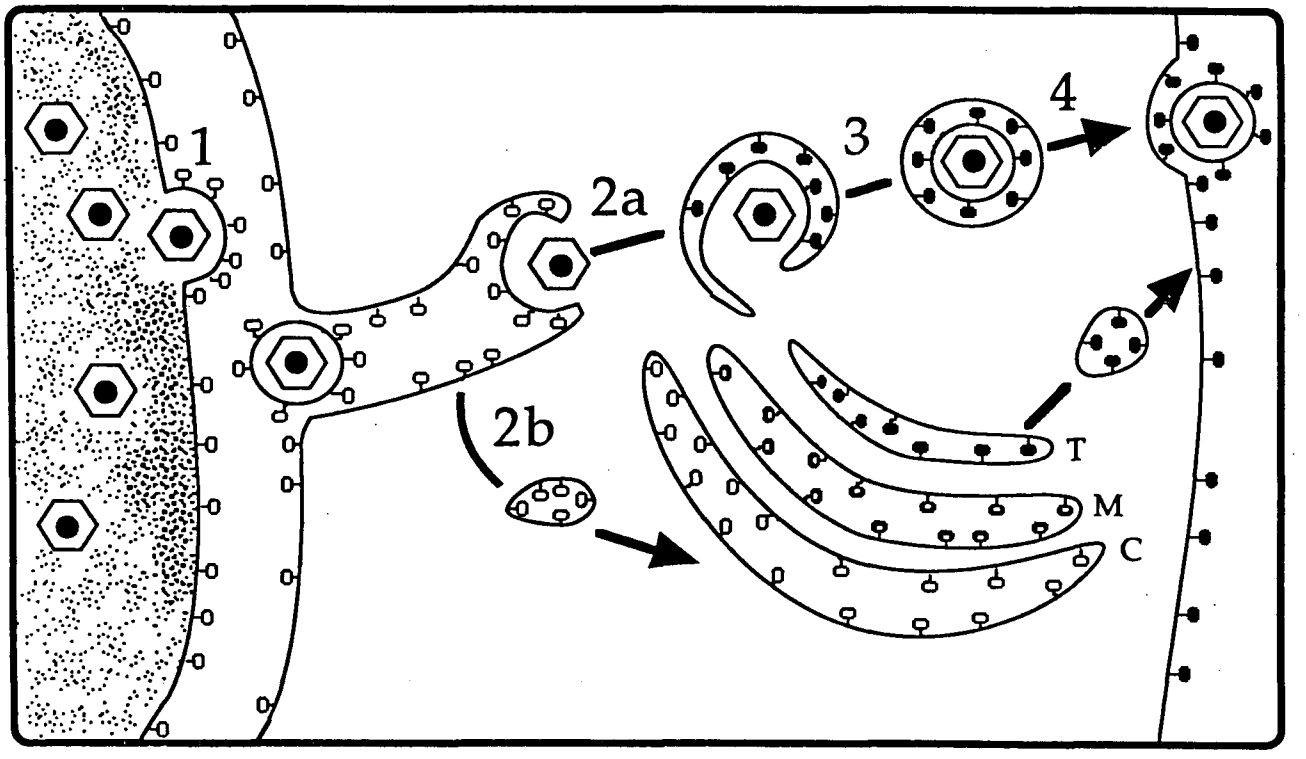
Following nucleocapsid assembly, envelopment and release of infectious virus must occur. Any mechanism of envelopment must take into account the glycosylation of the viral membrane proteins, and the packaging of tegument proteins. Studies on the morphogenesis of MCMV (Weiland et al., 1986; Papadimitriou et al., 1984) are consistent with a model of membrane protein glycosylation and egress in alphaherpesviruses as proposed by Whealy et al. (Whealy et al., 1991)[Fig. 3]. Envelopment of a group of nucleocapsids would explain the formation of multicapsid virions which is associated with MCMV infection (Hudson et al., 1976).

FIGURE 2: HERPESVIRUS PACKAGING MODEL



Model of packaging of HSV-1 DNA (Deiss & Frenkel, 1986; Deiss et al., 1986; Roizman & Sears, 1990). The empty capsid "scans" the concatameric DNA until it encounters an *a* sequence **[A]**. DNA from that *a* sequence forward is packaged **[B]** until the next *a* (in the same orientation) is encountered **[C]**, at which time the *a* sequences are amplified **[D]** and cleavage occurs **[E]**. This results in one genome length of DNA packaged in the capsid.

FIGURE 3: VIRION ENVELOPMENT AND RELEASE



Model of alphaherpesvirus virion assembly and egress (Whealy et al., 1991). Nucleocapsids initially acquire a membrane by budding into the perinuclear space **[1]**, and are then transported through the endoplasmic reticulum, only to be de-enveloped and released into the cytoplasm in proximity to the *trans*-Golgi **[2a]**. Viral glycoproteins mature independently, via the normal exocytic pathway **[2b]**. The nucleocapsids are enveloped by membrane derived from the *trans*-Golgi which contains mature, fully processed viral glycoproteins **[3]**. The resulting enveloped virus, is transported to the cell plasma membrane in a vesicle **[4]**, where fusion releases a mature, enveloped virus particle from the cell.

INTRACELLULAR RESPONSE TO VIRAL INFECTION

Little is known about the cellular events which follow MCMV infection. However, Albrecht et al. (1989, Rev.) liken the attachment of the virus to the binding of growth factors to their cellular receptors with the subsequent triggering of cell activation. The hydrolysis of inositol lipids to yield inositol triphosphate and diacylglycerol was detected within 20 minutes of virus infection, and may have been an effect of virus binding (Valyi-Nagy et al., 1988). Arachidonic acid release was also observed, probably associated with the activation of phospholipase A_2 and protein kinase C (AbuBakar et al., 1990). The release of these products led to a cascade of physiological responses including an increase in the concentration of cytosolic free Ca^{2+}, cAMP, cGMP, Na^+ and a triggering of G_1 and S to G_2 events in the cell replicative cycle (Albrecht et al., 1989). An 8-13 fold increase in the production of cytomegalovirus was observed following the addition of the phorbol diester 12-0-tetradecanoyl phorbol-13-acetate (TPA) to infected cells, thus supporting the hypothesis that increased cAMP levels favour viral replication (Forbes et al., 1990). Cytomegalovirus may induce the cell to enter the cell cycle by the induction of the proto-oncogenes *c-fos, c-jun,* and *c-myc* in HCMV infected cells (Boldogh et al., 1990) and the induction of *c-fos* by the IE protein pp89 in MCMV infected cells (Schickedanz et al., 1988). These proto-oncogenes are thought to be involved in cellular signal transduction and regulation of cell proliferation.

High cGMP levels corresponded to the morphological contraction seen in the initial stages of infection, and as the cGMP levels decreased, and the level of Na^+ increased, cell enlargement or cytomegalia ensued. The cell enlargement was concomitant with viral DNA replication and progeny virion assembly (Albrecht et al., 1990). The morphological changes seen in cytomegalovirus-infected cells were probably due to alterations of the cytoskeleton, involving both actin and fibronectin complexes. A rapid progressive disruption of the host cell cyto-skeleton correlated with depolymerization of actin during the first few hours of HCMV infection

(Jones et al., 1986), while a progressive loss of cellular fibronectin was a result of a decrease in fibronectin RNA (Pande et al., 1990).

HOST RESISTANCE AND THE IMMUNE RESPONSE

Many groups have studied the role of the host immune response in the pathogenesis of cytomegalovirus infections. Both HCMV and MCMV have been shown to infect a wide variety of cell types, and in both systems the interaction of virus and host plays a major role in the outcome of disease. Host factors involved include genetically-determined resistance, humoral and cell mediated immunity, and cytokine induction, while cytomegaloviruses have been shown to induce non-specific immunosuppression, perhaps via a virus-induced suppressive factor.

Host Genetics: The mouse model has been very useful for looking at the genetic resistance to MCMV infection. Both MHC-linked and non-MHC-linked genes are involved. The $H2^k$ allele of the MHC conferred a 10-fold higher resistance to MCMV lethality than the susceptible $H2^d$ or $H2^b$ alleles, with susceptibility being the dominant trait (Chalmer et al., 1977; Grundy et al., 1981). More recently, Quinnan and Manischewitz (1987) found that the genetically-determined resistance to MCMV infection was associated with two distinct resistance traits -- one interferon-dependent (INF-dependent) and one INF-independent. The INF-dependent trait was linked to the $H2^k$ phenotype, and was termed the H2-linked INF-response gene. The INF-independent trait was non-H2-linked and associated with the C57BL mouse strain. Scalzo et al. (1990) mapped a gene (*Cmv-1*) associated with the control of splenic replication of MCMV in C57BL mice to chromosome 6, and they showed that *Cmv-1* segregated as an autosomal dominant trait. The genetically-determined resistance to MCMV infection was the same in adults and newborns, with both H2 and non-H2 genes contributing to resistance (Grundy et al., 1981; Shellam & Flexman, 1986).

Natural Killer Cells: It has been suggested that natural killer (NK) cells are responsible for the control of the acute, viremic phase of infection, and correspondingly, that an increase in NK cell activity occurrs in the first 3 to 6 days post infection with MCMV (Quinnan & Manischewitz, 1979). Selective depletion of NK cells in mice with drugs or antibody treatment early in infection led to a substantial (1000X) increase in MCMV replication and lethality, even when T-cell, antibody, and INF levels remained normal (Quinnan et al., 1982; Bukowski et al., 1984; Ebihara & Minamishima et al, 1984). Beige mice have low NK activity even in the presence of high INF activity, and again, these mice were more susceptible to lethal infection with MCMV (Grundy et al., 1982). Depletion of NK cell activity later during an acute infection had no adverse effect. However, in persistently-infected mice, decreased NK cell activity led to increased secretion of MCMV from the salivary glands (Bukowski et al, 1984). Thus NK cells limit the severity, extent, and duration of acute MCMV infection, and may be involved in regulating persistent infection. It is possible that the *CMV-1* gene is involved in the induction of NK cell activity early during infection (Scalzo et al., 1990).

Macrophages: The role of the macrophage (mϕ) in MCMV infection is complex. Selgrade and Osborn (1974) showed that if mϕ function was blocked, mortality due to MCMV infection was increased in adult mice, and that adoptive transfer of adult mϕ to suckling mice increased resistance to subsequent infection with MCMV. Generalized immunosuppression due to infection with MCMV correlated with the peak of infected adherent cells isolated from spleen, and may have been caused indirectly by infection of these cells (Loh & Hudson, 1981). In addition, a virus-induced suppressive factor specific for MCMV-infected animals was isolated. This factor was characterized as a small peptide of between 1-1.4 kDa in size, which appeared to be produced by adherent spleen cells, probably mϕ (White et al., 1987). In further studies, *in vitro* infection of peritoneal mϕ by MCMV was found to be solely dependent on H2

phenotype. Less than 10% of $H2^k$ mϕ became infected as compared to 80-90% of $H2^b$ or $H2^d$ mϕ. Macrophage activation was dependent on $H2^d$-determined sensitivity to infection in BALB/c mice and these mϕ showed impaired accessory cell function (Price et al., 1987a; 1987b). In addition to these effects on mϕ, neutrophils infected *in vitro* with MCMV also showed evidence of impaired function -- decreased chemotactic activity and decreased engulfment of latex spheres (Bale et al., 1985).

Human CMV infection of mϕ caused increased expression of interleukin 1 (Lewis et al., 1990) and infected mϕ also exhibited impaired stimulation of antigen-specific cytotoxic T lymphocytes (CTL)(Campbell et al., 1990). Moreover, transient immunosuppression seen in conjunction with either HCMV or MCMV infection may relate to the generation of suppressive mϕ in susceptible individuals.

T Cells: The T-cell response to MCMV infection also plays an important role in the progression of disease. Nude mice (athymic) are susceptible to MCMV infection; infection with as few as 10 plaque forming units (PFU) led to death. In addition, adoptive transfer of immune T-cells protected against lethal challenge in these animals (Starr & Allison, 1977). Depletion of helper T-cells (T_H) led to a decreased antibody response and chronic replication of virus in spleen, lungs and salivary glands. However, a higher challenge dose of MCMV was required to kill the animals, suggesting that the T_H cell population contributed to disease.

Limiting-dilution analysis of lymphocytes primed against MCMV revealed 2 groups of CTL precursors; one specific for a membrane protein, and one against an IE antigen (Reddehase et al., 1984). CTL specific for the major IE protein, pp89, which was expressed on the cell surface at both IE and late times post infection, mediated protection in MCMV infected mice. Furthermore, recombinant vaccinia virus expressing pp89 induced cellular immunity in mice that were challenged subsequently with MCMV (Koszinowski, et al., 1987a; 1987b; Reddehase et

al., 1986). As with all CTL recognition responses, pp89 must be expressed in conjunction with an MHC antigen (class I) for recognition by specific CTL to occur (Reddehase et al., 1987). More recently, the epitope on pp89 responsible for the CTL recognition was mapped to amino acid residues 161-179 (Del Val et al., 1988; Reddehase et al., 1989).

Antibody Response: Adoptive transfer of immune Ig gives protection from disease on subsequent challenge with MCMV. However, it does not prevent the establishment of latency (Shanley et al., 1981). Antiviral IgM was detected in MCMV infected mice by 3-5 days post infection, while virus-specific IgG was detected by 5-7 days, and peak IgM and IgG titres were detected at 10 and 20 days post infection respectively. Neutralizing antibody, correlating with the induction of the IgM response, was detected as early as 3 days post infection. Unlike the cell mediated immune response, the antibody response was not related to H2 phenotype (Lawson et al., 1988).

The immunological control of MCMV infection is very complex. During the acute phase of primary infection, NK cell activity is important in the initial containment of the infection and the non-MHC linked resistance phenotype seen in MCMV-infected C57BL mice may be related to this NK cell activity. A generalized immunosuppression may be caused by the infection of mϕ with impairment of their accessory function, while neutrophil function is effectively blocked by infection with MCMV. The MHC-linked resistance may be associated with the infection of mϕ. Subsequent induction of specific CTL and antibody responses is important for clearing the acute infection, and for controlling reinfection and reactivation. A delicate balance between productive replication and latency ensues, such that immunosuppression can lead to reactivation and severe disease. The interaction of all these different cell types, both infected and uninfected,

will have to be more thoroughly investigated before a complete understanding of the immune response to MCMV infection is obtained.

PATHOGENESIS

The study of the pathogenesis of infection involves an examination of the mechanisms by which cells in particular organs produce signs and symptoms of disease. As with infection of the cell, there are several stages in viral pathogenesis including: entry into the host, site of primary replication, dissemination of infection, cell and tissue tropism, site of secondary replication, cell injury, interaction with the host immune response, and persistence or clearing of the infection. The host immune system plays an important role in the pathogenesis of cytomegalovirus infection, and as such pathogenesis in the immunocompromised host and the host with an immature immune system (fetus and newborn) will be considered separately. Because the relevance of MCMV as a model system is to gain a better understanding of infection with HCMV, the pathogenesis of cytomegalovirus in both mice and humans will be examined.

Pathogenesis in the Immunocompetent Host: The major portal of entry into the host is through the gastrointestinal tract following intimate contact with saliva containing infectious cytomegalovirus (Alford & Britt, 1990; Mannini & Medearis, 1961). Blood transfusion in humans is also associated with an increased risk of HCMV infection (Alford & Britt, 1990). Injection of MCMV-infected blood products into mice has been used to mimic this type of infection (Cheung & Lang, 1977a). In addition, intranasal inoculation of MCMV has been effective as a model of pneumonitis in mice (Shanley et al., 1982).

The epithelial cells of the oropharynx, the endothelial cells lining blood vessels, and the white blood cells found in blood transfusions probably represent the site of primary replication of cytomegalovirus in both the human and the mouse (Alford & Britt, 1990; Mercer et al.,

1988). Following primary infection in humans, a 4-8 week incubation period ensues prior to detection of viremia which can last from a few weeks to a few months. Virus was found in association with cells of the "buffy coat" including polymorphonuclear leukocytes, monocytes and occassionally T lymphocytes (Alford & Britt, 1990). Viremia occured 3-5 days following intraperitoneal injection of mice, this method of infection with MCMV being the most widely used experimentally. In both instances, viremia is the mechanism by which virus is disseminated throughout the host. In MCMV-infected mice, blood-borne virus was isolated both from infected mϕ (Tegtmeyer & Craighead, 1968) and from leukocytes (Bale & O'Neil, 1989). Bale & O'Neil recovered infectious MCMV from a small percentage (0.1% to 0.001%) of circulating leukocytes from susceptible mice, but were unable to isolate infectious virus from leukocytes of resistant mice. They speculated that replication of MCMV in leukocytes may be an important factor in the dissemination of the virus in susceptible mice.

Tissue Tropism: The site of secondary replication of virus is dependent upon dissemination, cell and tissue tropism, and interaction with the host immune system. In the case of a low inoculum of virus, the yield from the site of primary replication may be lower, and dissemination may take longer and may be less widespread. When a low inoculum is encountered, some tissues may not be exposed to the virus, and the host immune response may be able to contain the infection. If, however, the inoculum is relatively large, the site of secondary replication is only limited by tissue tropism and access to permissive cell types until the onset of a specific antiviral host immune response.

Both HCMV and MCMV show a widespread tissue distribution. In the immunocompetent host, the major organs affected in humans include: salivary glands, spleen, liver, kidney, and lymph nodes, with less involvement of the lungs, adrenals, ovaries, bones, and pancreas (Ho, 1982). Tissues of the mouse which show evidence of MCMV infection included: throat,

salivary glands, kidney, liver, spleen, lymph nodes, adrenals, ovaries, heart, testes, prostate, gastrointestinal tract, fat, connective tissue, lung, pancreas and CNS (McCordock & Smith, 1936; Medearis, 1964; Cheung & Lang, 1977b; Mims & Gould, 1979; Baskar et al., 1983). MCMV infection in the spleen and salivary glands of mice has been most widely studied in the adult mouse. Immunohistochemistry, electron microscopy and *in situ* cytohybridization, have identified spleen cells infected with MCMV during an acute primary infection as predominantly sinusoidal-lining cells (epithelial/endothelial in nature)(Mercer et al., 1988).

MCMV infection of the salivary glands has also been widely studied. The phenomenon of rapid attenuation of virulent MCMV by passage *in vitro*, and subsequent restoration of virulence by a single passage of virus *in vivo* was first reported by Osborn and Walker (1970). Virus isolated from tissue culture or from any organ other than the salivary glands is less virulent than salivary gland-passaged virus. A comparison of virus pools from the salivary glands of susceptible and resistant strains of mice showed that genetic differences between mouse strains had little effect on the virulence of virus produced in the salivary glands (Selgrade et al., 1981). MCMV replicated in the acinar cells of the salivary gland (McCordock & Smith, 1936; Mercer & Spector, 1986), in contrast to HCMV which replicated in the ductal epithelial cells of human salivary glands (Ho, 1982). The significance of these findings has not been established.

The ability of cytomegaloviruses to infect and multiply productively in discrete tissues or populations of cells within a tissue is not solely dependent upon attachment and entry into cells. The use of cell lines, or separated populations of cells in tissue culture has demonstrated that both MCMV and HCMV can infect cells which are nonpermissive for viral replication, and that the state of differentiation of cells is also important for productive infection. As noted earlier, replication of MCMV is cell-cycle dependent. Providing cells have not entered G_0-phase, the virus can permissively infect and replicate in primary cultures including MEF,

activated peritoneal macrophages, and primary rabbit kidney cells (Smith, 1954; Tegtmeyer & Craighead, 1968; Brautigam et al., 1979; Kim & Carp, 1971). Cell lines permissive for MCMV replication include 3T3 (mouse fibroblast), BSC-1 (african green monkey kidney), BHK-21 (baby hamster kidney), and Y-1 (murine continuous epithelial)(Muller et al., 1978; Kim & Carp, 1971; Shanley et al., 1979).

Although entry into cells can also be a block to permissive infection, this block can be overcome. Nedrud et al. (1979) found that tracheal organ cultures from susceptible mice were permissive for MCMV replication; however, primary tracheal epithelial cells were non-permissive unless co-cultivated with MEF (Nedrud & Wu, 1984). Cell-cell contact or fusion of infected MEF with the tracheal epithelial cells was required for productive infection (Nedrud & Wu, 1984). In a related study, Kim & Carp (1971) found that fetal sheep brain cells were permissive for MCMV infection only following the passage of cells by trypsinization. This suggested that a potential receptor may be rendered more accessible to the virus following typsinization.

Cells nonpermissive to MCMV infection include human embryonic brain, human embryonic kidney, WI-38 (human lung fibroblast), HeLa, Hep2, HFF (primary human fibroblast), HTC (rat hepatoma), and a murine macrophage cell line J774A.1 (Kim & Carp, 1971; Shanley et al., 1979; Walker & Hudson, 1987). The block to viral replication in these cells is not necessarily at the level of entry. As was the case in G_0-phase cells, the block to infection in HFF and J774A.1 cells occurs prior to viral DNA replication, but both IE and E MCMV proteins were detected in these cells (Walker & Hudson, 1987). In the case of MCMV-infected Schwann cells, de-differentiation (loss of cytochemical markers and a cessation of myelin production) in culture was associated with reactivation of virus (Abols-Mantyh et al., 1987). Thus, tissue tropism in animals infected with MCMV can reflect the inability of the virus to enter a cell, or the lack of an early function required for viral DNA replication. This

function may be supplied subsequently by stimulation of the cell replication cycle, co-cultivation with permissive cell types, activation of cells such as macrophages, or a change in the state of differentiation of an infected cell.

In the same way that murine fibroblasts are permissive to MCMV infection *in vitro*, primary human fibroblasts, or human fibroblast cell lines such as WI-38 are fully permissive to HCMV *in vitro*. No permissive non-human cell line has been described for HCMV but the virus can abortively infect cells of guinea pig, mouse, rabbit, bovine and simian origin. The block to permissive infection in these cells is prior to DNA replication (Ho, 1982).

Cell Injury: The degree of cell injury caused by cytomegalovirus is highly dependent upon the integrity of the host immune response. The inflammatory response itself contributes to organ dysfunction in cases of severe disease. The number and distribution of cytomegalic cells does not often reflect the degree of functional disturbances seen, suggesting that mechanisms other than specific viral cytolysis contribute to organ dysfunction (Alford & Britt, 1990). The contribution of the host immune response to protection against cytomegalovirus infection is manifest in the low degree of re-infection seen in the immunocompetent host; secondary infection with HCMV is due usually to reactivation rather than re-infection. In addition, vaccination with HCMV or MCMV can protect against disease following subsequent challenge with virulent virus, but cannot prevent re-infection (Plotkin et al., 1990; Del Val et al., 1991).

Latency: The frequent occurence of cytomegalovirus reactivation indicates that persistence normally occurs following primary infection in both humans and mice. Such persistence of virus includes both a true latent state, in which no infectious virus can be demonstrated, and a low level of chronic shedding of virus from tissues such as the salivary glands. A consideration of latency must take into account both the site of persistence and the mechanism of persistence.

Studies of MCMV infected mice have shown that latent virus can be reactivated from explanted spleen, kidney, lymph nodes, prostate, salivary gland, the peripheral nervous system, and lymphocytes (Henson et al., 1972; Cheung & Lang, 1977b; Davis et al., 1979; Jordan & Mar, 1982; Porter et al., 1985; Abols-Metyh et al., 1987; Mercer et al., 1988). Latency in the spleen of MCMV-infected animals has been more thoroughly studied than in other organs. Latent MCMV was demonstrated in spleen explants, especially from B-cell enriched fractions of lymphocytes. Although virus replicated primarily in mϕ from these spleen explant cultures, the virus was never found to be latent in these cells (Jordan & Mar, 1982). A closer analysis of the stromal elements from explanted spleens of latently infected mice revealed that mature T-cells, mature B-cells, mϕ, and dendritic cells were <u>not</u> harbouring latent MCMV. These studies could not exclude B lymphocyte progenitors, granulocytes, granulocyte-monocyte colony forming cells, or fibroblasts as the site of latent MCMV but concluded that the latent virus was probably reactivated from sinusoidal lining cells (Mercer et al., 1988). The situation in humans has not been documented in this manner; however, the reactivation of cytomegalovirus during immunosuppressive treatment demonstrates that HCMV does remain latent in some cells (Rubin, 1990; Winston et al., 1990).

Based on these data, one can start to speculate as to the mechanism of persistence of cytomegaloviruses. The ability to regulate lytic potential exists, as demonstrated by the ability of the virus to become latent under nonpermissive conditions and then reactivate when conditions have changed. Whether or not that ability is controlled by viral functions or based purely on host cellular functions remains to be seen. Cytomegaloviruses are also able to avoid detection by the host's immune system. Co-expression of the HCMV-coded MHC class I homologue and β2-microglobulin leads to surface expression of the complex. It is postulated that the function of the MHC class I homologue was to sequester β2-microglobulin thereby preventing the surface expression of mature class I molecules (Browne et al., 1990). The T-cell receptor homologue

(Chee et al., 1990a) may also interfere with immune-mediated cytolysis. Finally, the ability of the virus to infect and replicate in cells of the immune system has been shown to impair their function and may be responsible for transient immunosuppression.

Pathogenesis in the Immunocompromised host: Reactivation of latent cytomegalovirus is the most common concern. For example, reactivation of HCMV in seropositive transplant recipients can be as high as 100% (Ho, 1990). However, primary infection following transplantation, which occurs less often depending on exposure to HCMV, appears to be more serious than reactivation (Ho, 1990). Loss of immune function often leads to disease if reactivation of cytomegalovirus occurs; such immunosuppression occurs as part of a disease such as AIDS, or it can be induced in organ transplantation or cancer chemotherapy.

In AIDS patients, severe colitis and progressive retinitis are the most serious consequences of cytomegalovirus reactivation or infection. Although cytomegalovirus is often isolated from the lungs of AIDS patients with pneumonia, it was thought to be a secondary pathogen (Merigan & Resta, 1990).

In transplant recipients, symptoms associated with HCMV disease are usually manifest within 3-4 months of transplantation, and generally consist of mononucleosis, leukopenia, pneumonia and occasionally hepatitis, while retinitis and encephalitis could be later sequelae. These symptoms are never seen in organ transplantation between identical twins where no immunosuppressive measures were required (Ho, 1982; Merigan & Resta, 1990). During immunosuppression of transplant recipients, circulating antibody afforded some degree of protection (Alford & Britt, 1990). The source of infection can be saliva or the transplanted organ itself. Therefore an effort is made to match a seronegative recipient with a seronegative donor. Other risk factors associated with cytomegalovirus disease during transplantation include

the use of whole blood products before or after transplantation in a seronegative recipient (Ho, 1982).

In the mouse model, immunosuppression of mice by treatment with anti-lymphocyte serum and cortisone leads to reactivation and dissemination of virus (Shanley et al., 1979). In humans, the use of cytotoxic drugs (azolthioprine, methotrexate, cyclophosphamide) or anti-thymocyte globulin is associated with an increased risk of reactivation, while the use of cyclosporin has profound effects on the host response to replicating virus during cytomegalovirus infection in transplant recipients (Rubin, 1990). It is interesting to note that whereas graft-versus-host reactions are often associated with HCMV infection, host-versus-graft reactions are not (Ho, 1982). Graft-versus-host disease in MCMV-infected, immunocompromised mice "transplanted" with spleen cells, leads to severe diffuse pneumonia not seen during MCMV infection or graft-versus-host disease alone (Shanley et al., 1987).

Thus in the immunocompromised individual, HCMV can produce severe disease if infection is not controlled. Primary infection can be minimized by screening organ and blood donors for previous cytomegalovirus infection, while measures to prevent reactivation of latent cytomegalovirus will also decrease the risk of disease.

Pathogenesis in the absence of a mature immune system: CID in the newborn is the number one viral cause of mental retardation in developed countries. Approximately 1% of infants are congenitally infected; 10% of these babies show clinically apparent infection, while the majority remain asymptomatic. The symptoms of CID include: hepatosplenomegaly, jaundice, petechiae, microcephaly, hydocephalus, periventricular calcification, and intrauterine growth retardation, and are usually associated with permanent and severe brain damage. The mortality rate of severely affected infants can be as high as 30%, and sequelae for those who survive include seizures, blindness, or paraparesis. It is becoming apparent, however, that even in cases of

asymptomatic infection *in utero*, HCMV infection is associated with developmental abnormalities including sensorineural hearing loss, microcephaly, motor defects, mental retardation, chorioretinitis and dental defects (Alford et al., 1990).

Perinatal infection, either during the course of delivery or through infected breast milk, occurs in about 50% of infants exposed to cytomegalovirus in the birth canal and/or breast-fed by seropositive mothers. The majority of these infants remain asymptomatic, although they may develop pneumonia, and chronic shedding of virus can last for years. Primary infection of the mother during the perinatal period is associated with high mortality of the infant which suggests that maternal antibody can decrease the severity of disease, although it is not protective against infection. In addition, most cases of generalized CID are a result of primary maternal infection; however, not all primary infections result in intrauterine transmission.

The immaturity of the immune response is believed to be the major virulence determinant in congenitally and perinatally acquired HCMV infection. An understanding of the mechanisms of transmission, and pathogenesis will lead to better prevention of infection and control of reactivation, as well as more effective treatment and prophylaxis of cytomegalovirus disease.

TREATMENT AND PROPHYLAXIS

The antiviral agent, ganciclovir (9-[(1,3-dihydroxy-2-popoxy)methyl]guanine), is a nucleoside analogue which is the most effective agent in the treatment of HCMV disease, especially chorioretinitis and gastrointestinal infection (Alford et al., 1990). Maintenance therapy is required, however, and has been limited by the drug's toxicity. Foscarnet (phosphonoformate) is useful *in vitro* but its associated nephrotoxicity will limit its usefulness *in vivo*. Oral administration of acyclovir was found to be effective in mice with disseminated MCMV (Glasgow, et al., 1982) and indeed prophylactic use of acyclovir in transplant recipients has been correlated with significantly less extensive cytomegalovirus disease as compared to

controls (Dummer, 1990). The efficacy of intravenous CMV-specific Ig is still not resolved; however, administration in combination with ganciclovir has led to significantly higher survival rates as compared to the use of these agents alone (Winston et al., 1990). Interestingly, the prophylactic use of cyclosporin against graft-versus-host disease in transplant recipients was protective against cytomegalovirus pneumonitis (Wingard et al., 1990).

Vaccination has historically been used as prophylaxis against viral disease. Two attenuated live HCMV vaccines have been developed (Towne & AD169) and while the protection they afford is not complete, they have been shown to decrease the severity of symptomatic illness following infection with wild-type virus (Merigan & Resta, 1990). Nevertheless, results of vaccination with the Towne vaccine showed that it induced humoral and cellular immunity without viral excretion or demonstrable latency, and that it was partially protective against severe disease although it did not prevent re-infection (Plotkin et al., 1990). The use of live vaccines, however, remains controversial, and the following concerns need to be addressed. How long-lasting is vaccine-elicited immunity? Will the latency of HCMV become a problem? Does a vaccine containing cytomegalovirus DNA have oncogenic potential? Would a live vaccine made from a single strain of HCMV be protective against re-infection with other strains? Finally, would a subunit vaccine be a viable consideration (Alford et al., 1990)? The use of the mouse model can help to answer these questions. A subunit vaccine (pp89) protects against lethal challenge with MCMV (Jonjic et al., 1988, Del Val et al., 1991), demonstrating that a subunit vaccine is protective in mice.

Thus, the pathology of cytomegalovirus is quite complex. The ability of the virus to infect a wide variety of organs does not lead to disseminated disease in the immunocompetent individual. Indeed, most infections of the immunocompetent host remain asymptomatic, as virus and host maintain a delicate balance between severe disease and complete clearance of the infection. It is only when that balance is lost that the potential of cytomegalovirus as a truly

pathogenic organism is realized. The absence of pre-existing immunity can lead to severe cytomegalovirus disease, thus vaccination may prove to be useful for the prevention of disease. For example, vaccination of a seronegative mother may help to prevent HCMV disease in the fetus/newborn, while immunization of patients receiving organ transplants may lessen the severity of subsequent HCMV infection.

MUTATIONAL ANALYSIS

Temperature sensitive mutants of MCMV have been used to study the pathogenesis of avirulent virus (Tonari & Minimishima, 1983; Sandford & Burns, 1988; Sammons & Sweet, 1989). Disadvantages to this approach are that the identity of the mutated gene(s) is unknown, and that the temperature sensitivity of the mutant may not be related to its avirulence (Kumura et al., 1990). When the location of a gene of interest is known, recombination can be used to engineer mutations in the genome. This technique was used to construct a recombinant MCMV containing a *lacZ* insertion which inactivated the transcription of ie2 (Manning, 1990).

Co-transfection and *in vivo* selection has been used previously to isolate virulent recombinants, and to map the location of the viral gene function(s) involved in the neurovirulence of HSV (Thompson et al., 1983; Thompson et al., 1986; Javier et al., 1987; Thompson & Wagner, 1988). Restriction endonuclease fragments from a phenotypically wild-type strain of HSV-1 were used to locate the HSV-1 gene(s) which restored neurovirulence to a non-neurovirulent intertypic (Hsv-1/Hsv-2) recombinant (Thompson et al., 1983). Selection of recombinants which had regained neurovirulence was accomplished by *in vivo* passage of the recombinant virus through mouse brains. Subsequent co-transfection of the DNA from the non-neurovirulent virus with an electrophoretically-purified DNA fragment yielded neurovirulent recombinant virus. This confirmed that an HSV-1 gene function(s) mapping to 0.71-0.83 map units of the HSV-1 genome was associated with the increased neurovirulence of this virus

compared to the intertypic recombinant. This analysis was the first to employ *in vivo* enrichment for recombinant virus based on biologically selectable differences between strains, enabling a molecular approach to mapping viral genes involved in pathogenesis (Thompson et al., 1983).

RATIONALE FOR THESIS RESEARCH

An understanding of the mechanisms of pathogenesis of cytomegalovirus is essential for future work towards control and prevention of cytomegalovirus disease. The salivary glands are believed to be a site in which HCMV remains latent, and are often associated with the shedding of infectious virus and thus dissemination into the population. The identification of a gene involved in the salivary gland tropism of MCMV would provide an initial step towards understanding the spread of this virus, and could lead to the identification of a similar function in HCMV.

It was hypothesized that a comparison of different strains of MCMV at the molecular level would reveal alterations that could be correlated with changes in disease expression. Three strains were utilized. The Smith strain of MCMV was obtained from the American Type Culture Collection (ATCC) and is the wild-type reference strain for this investigation. The K-181 strain was isolated from mouse salivary glands by Dr. J. Osborn following repeated passage of the Smith strain through mice and has been shown to be more virulent than the Smith strain. In addition, these two strains differ in their restriction endonuclease digestion pattern (Misra & Hudson, 1980). During the course of my project, it was determined that the "Smith" strain being used had altered during tissue-culture passage and had become severely limited in its ability to grow in mice. It was renamed the "Vancouver" strain to distinguish it from the original Smith strain. Further characterization showed that the major pathogenic difference between the Vancouver strain and the parental Smith strain virus was the inability of the Vancouver strain to productively infect salivary glands *in vivo*. Thus, the major aims of the study were:

A. To identify and characterize differences between the Smith, K-181 and Vancouver strains of MCMV.

B. To identify the viral gene(s) regulating salivary gland tropism.

MATERIALS AND METHODS

Cells and Virus: A mouse embryo fibroblast cell line, 3T3-L1, was obtained from the American Type Culture Collection (ATCC CCL 92.1) and routinely grown in DMEM (Gibco) containing D-glucose, L-glutamine, 1 mg/ml streptomycin, 1 unit/ml penicillin, 1 μg/ml of the antifungal agent Econazole (Cilag, Switzerland) (complete medium) and 10% fetal bovine serum (FBS), in a humidified incubator at 37°C and 5% CO_2. Infected cells were maintained in complete medium containing 5% FBS. The Smith strain of MCMV was purchased from the ATCC (VR-194) in 1988. The Vancouver strain was isolated after multiple passage of the Smith strain in tissue culture. The K-181 strain was obtained from June Osborn's laboratory. It originated as a virulent strain of MCMV isolated after multiple passage of virus through mice. Stock virus was prepared by infecting 3T3-L1 cells with a low multiplicity of infection (MOI) of 0.01 plaque forming units per cell (PFU/cell), and harvesting the cells 24 hours after 100% cytopathic effect (CPE) was observed. The cells were sonicated to release infectious virus, and stored at -70°C in 1 ml aliquots.

Infection of Mice: Adult female CD-1 mice between 4-6 weeks of age were infected intraperitoneally with MCMV as noted. Mice were sacrificed at various times post inoculation by asphyxiation with CO_2. Organs were removed, homogenized and resuspended as a 10% w/v homogenate in complete medium containing 5% FBS. Homogenates were stored in aliquots at -70°C and standard plaque assays were used to determine infectious virus titres.

Calculation of LD_{50}: The LD_{50} for salivary gland passaged MCMV was determined by intraperitoneal inoculation of virus into 4 to 6 day old mice. 3 mice per group were used and the groups consisted of dilutions of MCMV in PBS. The death or survival of the mice was

recorded; all mice which died did so within 5 days of inoculation. The dose at which 50% of the mice died (LD_{50}) was then calculated by the Reed Meunch method (Cruickshank et al., 1975).

Plaque Assay: The plaque assay was used to determine the titre of infectious virus in stock preparations and as part of the viral growth curve analysis. 3T3-L1 cells were infected with sample virus ranging from the undiluted supernatants of growth curve experiments, denoted as a dilution of 10^0, to dilutions as high as 10^{-8} of stock virus preparations. Dilutions were carried out in complete medium containing 5% FBS. After 2 hours the inoculum was removed and replaced with 0.5% agarose in complete medium plus 5% FBS. After 4 to 7 days, the infected cells were fixed in 5% formyl saline and the agar overlay was removed. The cell monolayer was then stained with 0.05% methylene blue for 5 minutes, and then washed with tap water to remove the excess stain. Plaques were visible as dark blue spots on a lighter blue background. The plaques were counted and the titre of infectious virus was determined by calculating the average number of plaques from duplicate plates and serial dilutions.

Morphology in Tissue Culture: 3T3-L1 cells were infected with virus at a low MOI (0.1 PFU/cell) or a high MOI (5 PFU/cell) 12-16 hours following sub-culture. Cytopathic effects were documented at various times post infection by photography with a Nikon FE2 camera attached to a Nikon phase contrast microscope.

Comparison of Plaque Morphology: 3T3-L1 cells were infected 12-16 hours following sub-culture with 10-100 PFU of each virus strain. After a 2 hour adsorption period, the inoculum was removed and the cells were overlaid with 0.5% agarose in complete medium containing 5% FBS. The infected cells were fixed and stained as for plaque assays 7 days post infection.

Growth in Tissue Culture: 3T3-L1 cells were infected with MCMV and the production of infectious virus was monitored over time. In the standard growth curve, cells were infected with virus 12 to 16 hours following sub-culture. The inoculum was removed 2 hours post infection and the cells were washed twice with HBSS, and then once with medium at 37°C for 5 minutes in order to remove reversibly bound infectious virus (Hodgkin et.al, 1988). This medium was then removed and replaced with 2 ml of complete medium containing 5% FBS. At various times post infection, the supernatant was removed and reserved in order to assay for infectious virus. The cells were washed 3 times in PBS, harvested by scraping into fresh medium and sonicated in order to release intracellular virus. The samples collected at the various time points were subjected to a standard plaque assay to test for the production of infectious virus.

In order to examine the growth of MCMV in G_0 phase cells, 3T3-L1 cells were incubated for 5 days after reaching confluency without changing the medium. This depleted medium was used to dilute the virus for infection and to maintain the cells once the inoculum was removed, to minimize cell activation (Muller et al., 1978). Otherwise, the assay was carried out under the same conditions as the standard growth curve. In order to examine the growth of MCMV during other phases of the cell cycle, 3T3-L1 cells were infected at various times following sub-culture, in the same manner as for the standard growth curve.

Electron Microscopy: K-181 and Vancouver stock virus preparations were examined using the electron microscope to determine the total number of virus particles present, and to compare the relative amount of multicapsid virions and the number of capsids enclosed in a single viral envelope. Stock virus was mixed with a known concentration of uniform polystyrene beads which served to normalize the virus particle counts between different preparations. Samples containing an equivalent titre of infectious virus and polystyrene beads were centrifuged in a Beckman airfuge at 30 PSI of air pressure for 30 minutes onto formvar coated grids. The grids

were then negatively stained with 2% phosphotungstic acid pH 6.3 for 30 seconds, blotted dry and examined under a Philips CM10 electron microscope at 46,000x enlargement. 20 random fields per specimen were scored for the number of polystyrene particles, the number of total virus particles, the number of unenveloped particles, the number of single capsid particles and the number of multicapsid particles with 2, 3, 4, 5, or greater than 5 particles per single envelope.

Viral DNA Replication: 3T3-L1 cells were infected with MCMV at various MOI. The cells were lysed at various times post infection in DNA lysis buffer. The lysate was incubated at 65°C for 2 hours and then subjected to phenol/chloroform extraction, followed by ethanol precipitation at -20°C. The samples were then centrifuged in an Eppendorf 5415C microcentrifuge at 14,000 rpm for 30 minutes to pellet the DNA. The DNA pellet was washed three times with 70% ethanol, dried briefly in a Savant vacuum desiccator, and then resuspended in dH_2O. Equivalent concentrations of total infected cell DNA were then prepared for application to nitrocellulose (Schleicher & Schüll), or nylon (Hybond, Amersham) membranes using a slot blot apparatus and conditions suggested by the manufacturer (Wahl, Schleicher & Schüll). Briefly, the appropriate amount of DNA (as noted in the Results section) was diluted to a total volume of 50 μl in TE buffer pH 7.4. Three volumes of 2 M NaOH (150 μl) were added to each sample, and the samples were incubated at 70°C for 30 minutes. The samples were then transferred to ice, and 1 volume (200 μl) of 2 M ammonium acetate was added before the samples were transferred to the appropriate membrane using the slot blot apparatus. Following application of the samples, the membranes were dried, and the DNA was covalently attached by baking for 2 hours at 80°C. The membranes were then hybridized to labelled DNA probes (see Southern Blotting and Hybridization).

Restriction Endonuclease Digestion and Agarose Gel Electrophoresis: Restriction endonucleases were purchased from a variety of sources (Pharmacia, BRL, Boehringer Mannheim) and all gave similar results. High molecular weight DNA markers were purchased from BRL, while lambda DNA used to generate molecular weight markers was purchased from Pharmacia. Electrophoresis grade agarose (Biorad) was used for conventional flat bed agarose gel electrophoresis and "clamped homogeneous electric fields" (CHEF) electrophoresis using a Bio-rad CHEF-DRII® system. Conventional electrophoresis was carried out at room temperature, in 1X TAE buffer at 5 V/cm. Gels were stained with ethidium bromide and visualized using UV light. In some experiments, DNA was end-labelled with [^{32}P]dCTP (ICN) and the Klenow fragment of DNA polymerase I (BRL) after cleavage with restriction endonuclease. Small end-labelled restriction endonuclease cleavage products were separated in polyacrylamide (Biorad) gels. Gels containing end-labelled DNA were fixed in 5% methanol/5% acetic acid for one-half hour before being dried onto Whatmann 3mm paper in a Bio-rad gel drier and then subjected to autoradiography. CHEF electrophoresis was carried out in 1% agarose gels with circulating, cooled 0.5X TBE buffer for 24h at 150V, with pulse times as noted. The gels were then stained with ethidium bromide and visualized with UV light.

DNA Probe Production: HindIII restriction fragments from the Vancouver and K-181 strains of MCMV were cloned into the plasmid pACYC184 by standard methods (Maniatis et al., 1980). In addition, cloned HindIII *D* and *E'* fragments of MCMV were generously provided by Dr. D. Spector. The *E'* designation represents the fusion of the terminal HindIII fragments *E* and *Q* (Mercer et al., 1983). The cloned plasmids were transfected into E. coli and bacteria harbouring recombinant plasmids were selected on the basis of chloramphenicol resistance and tetracycline susceptibility. The positive bacterial colonies were then screened by agarose gel electrophoresis following small scale plasmid isolation. Large scale plasmid isolation was used

to prepare greater quantities of plasmid DNA for use as probes (Maniatis et al., 1980). On some occasions, restriction fragments of MCMV DNA to be used as probes were isolated from agarose gels. This was accomplished by using a Geneclean kit™ (Biocan) according to the manufacturer's instructions, or by binding to and recovery from DEAE membranes (NA45, Schleicher & Schüell) as follows: the band of interest was electrophoretically transferred to the DEAE membrane, the membrane was rinsed with TE buffer pH 8.0, and the DNA was eluted from the membrane in 1 M NaCl/0.05 M arginine-free base at 68°C (2X 30 minutes). The DNA was then extracted with phenol/chloroform and precipitated with 2 volumes of ethanol at -20°C (Pauline Stasiak, personal communication).

Southern Blotting and Hybridization: DNA fragments separated on agarose gels were depurinated in 0.25 M HCl, cleaved and denatured in 0.5 M NaOH/1 M NaCl, neutralized in 1 M Tris-Cl pH7.4/1.5 M NaCl and then transferred to nitrocellulose (Schleicher & Schüll) or Hybond (Amersham) overnight in 20X SSC by the method of Southern (1979a). The filters were baked at 80°C for 2h, to covalently bind the DNA to the membrane. Prehybridization was carried out at 42°C for 2h in a solution containing 50% formamide, 5X Denhardt's solution, 5X SSC, 25 mM sodium phosphate buffer pH 7.0, and 100 μg/ml of sheared, single-stranded salmon testes DNA. Hybridization was allowed to proceed for 16 to 24h at 42°C in a solution similar to the prehybridization buffer, in which the Denhardt's solution was reduced to 1X and heat denatured, labelled DNA probe was added. The probes were labelled with [^{32}P]dCTP (ICN) using the Random Primers DNA Labelling System (BRL), or a ^{T7}QuickPrime™ kit (Pharmacia) or with the digoxygenin-based nonradioactive DNA labelling kit (Boehringer Mannheim), as noted in the Results section. After hybridization, the filters were washed twice in 2X SSC/0.1% SDS for 5 min at room temperature, twice in 0.2X SSC/0.1% SDS for 5 min at room temperature, twice in 0.16X SSC/0.1% SDS for 15 min at 55°C and once in 2X SSC

for 5 min at room temperature. The filters were then air dried, and subjected to autoradiography for ^{32}P-labelled probes, or developed according to manufacturer's instructions for the non-radioactively labelled probes. The Hutchison cross-blot hybridization was conducted by Dr. L. Loh, as previously published (Loh et al., 1981). Briefly, a restriction endonuclease-digested unlabelled DNA was loaded across the entire width of a square slab gel using 10-15μg of viral DNA, and electrophoresed through a 0.8% agarose gel. The DNA was then transferred to nitrocellulose. A second preparation of viral DNA was digested with restriction endonuclease, end-labelled with [^{32}P]-dCTP, and subjected to agarose gel electrophoresis. The nitrocellulose sheet with the unlabelled, immobilized DNA was placed on top of the gel containing the radioactive sample such that the original orientation was perpendicular to that of the labelled bands. The labelled bands were then transferred to the nitrocellulose and hybridized to the unlabelled DNA in a buffer containing 6XSSC/0.1% SDS at 65°C for 8h in a humidified oven. The filter was then washed, blotted dry and exposed to film; spots of developed silver grains marking the intersection points of homologous DNA sequences.

Size Determination of DNA and Proteins: The size of the DNA in kilobase pairs (kb) was determined in relation to standard markers of known size by comparing the reciprocal of their electrophoretic mobility with the fragment length (Southern, 1979b) using a computer program which incorporates a least-squares analysis (Schaffer & Sederoff, 1981, see Appendix, page 139). The average values from a number of different gels run under various conditions were used to calculate the final values. Calculation of protein size was also determined relative to protein standards of known molecular mass by plotting a standard curve of the $\log_{10} M_r$ against the $\log_{10}$ of the polyacrylamide concentration at that part of the gel, and was expressed as kilodaltons (kDa)(Poduslo & Robard, 1980).

[^{35}S]Methionine Labelling of Infected Cell Proteins: 3T3-L1 cells were infected with MCMV at a high MOI (2-10 pfu/cell) under various conditions appropriate for harvesting infected cell proteins at immediate early (IE), early (E) and late (L) stages of infection. In order to enhance the incorporation of [^{35}S]methionine into the infected cell proteins, cysteine/methionine free medium was added to the cells 30 minutes prior to the addition of labelling medium; the labelling medium itself contained methionine-free medium with 5% FBS and 50 μCi/ml of [^{35}S]methionine:[^{35}S]cysteine 85:15 (ICN, Tran^{35}S-label™). Immediate early protein production was enhanced by the addition of 50 μg/ml of cycloheximide (Sigma) 30 minutes prior to infection and during infection until the addition of labelling medium. Prior to the addition of the labelling medium, the cells were washed 3 times in HBSS to remove the cycloheximide. In order to distinguish between IE proteins and E proteins, 10 μg/ml of actinomycin D (Sigma) was included in the labelling medium used during the IE labelling experiments. In order to enhance the production of E proteins, cycloheximide was added 2 to 5 hours post infection for 4 to 6 hours. The cycloheximide block was then reversed by washing with HBSS prior to the addition of labelling medium. The E proteins were labelled for 2 hours, in the presence of 50 μg/ml of phosphonoformic acid (PFA, Sigma) a specific inhibitor of MCMV DNA replication, and thus also an inhibitor of the late phase of protein expression. Late proteins were labelled for various time intervals from 12 to 28 hours post infection, as noted in the results section.

Antibody Production: All antisera were raised in mice. Adult female CD-1 mice were inoculated intraperitoneally with $1x10^5$ PFU of infectious MCMV. The mice were then boosted at 30 and 60 days post inoculation with an intraperitoneal injection of $1X10^5$ PFU of infectious MCMV. Two weeks following the second antigen injection, the mice were bled, the blood was allowed to clot overnight, and the immune serum was then removed and stored in aliquots at -20°C until use.

Immune Precipitation: Labelled infected cells were lysed in IP buffer. 5 mM phenylmethylsulfonyle floride (PMSF, BRL) was added to inhibit serine proteases. The infected cell lysate was then centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 30 minutes. The supernatant was removed and used for immune precipitation experiments. One to 5 μl of immune mouse serum was added to each supernatant, and allowed to react for 1 hour on ice. Fifty μl of Staphylococcus aureus cells (Pansorbin© Cells, Calbiochem) were then added and allowed to react for 1 hour on ice. The antibody/antigen/cell complexes were pelleted by centrifugation in an Eppendorf microcentrifuge at 12,000 rpm for 3 minutes, washed twice with IP buffer, and once with wash buffer. The pellets were then resuspended in 40 to 60 μl of PAGE loading buffer, boiled for 2 minutes, and centrifuged for 3 minutes in an Eppendorf microcentrifuge at 12,000 rpm before loading onto polyacrylamide gels.

Polyacrylamide Gel Electrophoresis of Proteins: Infected cell proteins were separated under reducing conditions on 5-20% gradient polyacrylamide gels in a Hoeffer SE600 gel apparatus. The gradient gels were made in a BRL gradient former from two solutions containing 20% and 5% acrylamide. The gel was allowed to polymerize for 2 to 6 hours before the stacking gel was added. The stacking gel was allowed to polymerize for 30 minutes before the well-forming comb was removed and the samples were added. Between 5 and 15 μl of sample were loaded per well, and the samples were electrophoresed in a discontinuous buffer system (Laemmli, 1970) under a constant current of 30 mA per gel until the bromophenol blue dye front reached the bottom of the gel. Protein size standards (BRL, Protein Molecular Weight Standards, High Range) were included on each gel. The gels were stained with 0.1% (w/v) Coomassie brilliant blue (Sigma) in a fixative solution of 25% methanol/7% acetic acid, destained in 25% methanol/7% acetic acid/2% glycerol, and then soaked in 25% (v/v) methanol/2% (w/v) sodium salicylate before being dried in a Biorad gel drier and subjected to autoradiography.

Autoradiagraphy: The dried polyacrylamide or agarose gels, or the dried hybridization filters were placed under a sheet of Kodak X-OMAT™ RP film within a film holder. The film holder was then wrapped in foil and sandwiched between 2 glass plates held together with metal clamps, and the whole unit was placed at -70°C for 2 hours to 14 days, depending on the intensity of the signal. The film package was then thawed, and the film was developed in a Kodak X-OMAT™ RP automated film developer.

Recombinant Virus Production: Two methods were used for the production of recombinant viruses. The first was that used Dr. E. Mocarski's group at Stanford University (Mocarski, personal communication), while the second is based on the method of Chen and Okayama (1988). The method used by Mocarski's group is detailed as follows. The appropriate DNA mixture was diluted in sterile dH_2O to a final volume of 250 μl. An equal volume of 2X HEPES buffer was added, followed by 30 μl of 2.2 M $CaCl_2$ (Malincroft). The mixture was mixed gently and allowed to sit at room temperature for 20 to 30 min. Meanwhile the cells were washed twice with HBSS and overlaid with 200 μg/ml of DEAE dextran in HBSS for 3 min at room temperature. The dextran was removed by aspiration and the cells were washed once with 1X HEPES buffer. The precipitated DNA was mixed gently and slowly added to the cells. Complete media containing 10% FBS was added to cover the cells, and the culture was returned to the incubator for 4 to 6 hours. The DNA mixture was then removed, the cells washed once with HBSS, and then subjected to osmotic shock with 15% (v/v) glycerol in 1X HEPES buffer for 5 minutes at room temperature. Finally the cells were washed twice with HBSS before fresh complete medium containing 10% FBS was added and the cells returned to the incubator. To identify the recombinants prepared by this method, they were given the prefix "**B/G**" in the Results section.

The method of Chen and Okayama is very similar, and was used with slight modification as follows. $5x10^5$ cells were seeded onto 100 mm tissue culture dishes the day before the co-transfection experiment. The appropriate DNA mixture was added to 0.5 ml of 0.25M $CaCl_2$. An equal volume of 2X HBS buffer was added, and the mixture incubated at room temperature for 10 to 20 minutes. The calcium phosphate/DNA mixture was then added dropwise to the cell medium while gently swirling the plate. The cells were incubated overnight at 37°C and 5% $C0_2$. The next morning the medium was removed, the cells washed twice in PBS, and refed with complete medium containing 10% FBS and returned to the incubator. The recombinants generated by the method of Chen and Okayama were designated with the prefix "**J**".

Prior to the co-transfection experiments, the whole Vancouver viral DNA was titrated to determine the amount of DNA which produced the optimal virus yield. An optimal concentration of 0.5 μg of target Vancouver strain DNA was determined using the first (**B/G**) method, while 5 μg of target DNA per well was required for optimal virus yield using the second method (**J**). For each co-transfection experiment the optimal amount of whole Vancouver viral DNA was mixed with varying amounts of effector DNA as noted in the Results section. Co-transfection experiments were designated by method as **B/G** or **J** linked by a hyphen (-) to the target/effector combination (**V/Hind**, **V/Eco**, **V/D**, **V/E**, **V/D+E**). The cultures were scored for viral CPE for two weeks following the co-transfection and any cultures yielding virus were harvested. Virus produced from the same effector DNA group was pooled and served as the inoculum used for the selection procedure. When Smith virus DNA cleaved with restriction endonuclease was used as effector DNA, complete cleavage was monitored by agarose gel electrophoresis. In addition, this DNA was used to transfect cells; complete cleavage was confirmed if no viral CPE was detected in these transfected cells.

In Vivo Selection of Recombinant Virus: Pooled virus isolated from the transfected tissue culture cells was inoculated intraperitoneally into 4-6 week old female CD-1 mice. The mice were sacrificed 13 days post inoculation, the salivary glands were removed and resuspended (10% w/v) in complete medium containing 10% FBS, and disrupted by sonication. The salivary gland preparation was then assayed for infectious virus in the same manner as a standard plaque assay, but without the addition of agarose.

Plaque Purification of Recombinants: Recombinant virus was plaque purified twice to ensure that the virus studied had arisen from a single clone. Serial dilutions of the recombinant viruses were plated as for a standard plaque assay. A plug of agarose was removed from above an isolated virus plaque using a pasteur pipette, and resuspended by sonication in 1 ml of complete medium containing 5% FBS, thus yielding plaque pure virus. This procedure was repeated, and the resulting virus suspension used as the inoculum for the preparation of recombinant virus stocks. The purity of the isolate was monitored by restriction endonuclease digestion of plaque purified viral DNA.

SOLUTIONS

Formyl Saline

5% v/v formaldehyde (30%)

150 mM NaCl

Complete medium

DMEM containing D-glucose, L-glutamine (Gibco)

1 mg/ml streptomycin

1 unit/ml penicillin

1 μg/ml of Econazole (Cilag, Switzerland)

Hanks Balanced Salt Solution

5.4 mM KCl

0.44 mM KH_2Po_4

140 mM NaCl

4.2 mM $NaHCO_3$

0.33 mM Na_2HPO_4

5.5 mM D-glucose

Phosphate Buffered Saline

150 mM NaCl

7.5 mM Na_2HPO_4

2.5 mM NaH_2PO_4

Lysis Buffer for DNA Extraction

10 mM Tris·Cl pH 8.0

100 mM NaCl

10 mM EDTA

1% SDS

100 μg/ml Proteinase K

TE Buffer

10 mM Tris·Cl pH 7.4

1 mM EDTA

1X TAE Buffer

40 mM Tris·acetate pH 7.8

1 mM EDTA

1X TBE Buffer

89 mM Tris·borate pH 7.8

89 mM boric acid

2 mM EDTA

20X SSC

3 M NaCl

0.3 M sodium citrate

100X Denhardt's Solution

1% w/v polyvinylpyrrolidone

1% w/v bovine serum albumin (fraction V)

1% w/v ficoll 400

Prehybridization Buffer

50% formamide

5X Denhardt's solution

5X SSC

25 mM sodium phosphate buffer pH 7.0

100 μg/ml of sheared, single stranded salmon testes DNA

Hybridization Buffer

50% formamide

1X Denhardt's solution

5X SSC

25 mM sodium phosphate buffer pH 7.0

100 μg/ml of sheared, single stranded salmon testes DNA

10^6 cpm/ml [^{32}P]dCTP-labelled single stranded DNA probe

Immune Precipitation (IP) Buffer

10 mM Tris.Cl pH 7.4

1 mM EDTA

150 mM NaCl

0.5% w/v sodium deoxycholate

1% v/v Nonidet P40

Wash Buffer (immune precipitation)

50 mM Tris.Cl pH7.4

100 mM NaCl

60% Acrylamide Stock Solution

60% w/v acrylamide (Biorad)

0.3% w/v bisacrylamide (Biorad)

5% Acrylamide Solution

5% acrylamide/bis from 60% stock solution

569 mM Tris·Cl pH 8.8 (Biorad)

0.1% w/v SDS

0.0132 % w/v ammomium persulfate (Biorad)

0.132% v/v TEMED (Biorad)

20% Acrylamide Solution

20% acrylamide/bis from 60% stock solution

568 mM Tris·Cl pH 8.8 (Biorad)

0.1% w/v SDS

8% v/v glycerol

0.0066% w/v ammonium persulfate (Biorad)

0.132% v/v TEMED (Biorad)

Acrylamide Stacking Gel

6% w/v acrylamide/bis from 60% stock solution

0.1% w/v SDS

0.7% w/v ammonium persulfate (Biorad)

0.1% v/v TEMED (Biorad)

PAGE Running Buffer

100 mM Tris pH 8.4 (Biorad)

380 mM glycine (Biorad)

0.1% w/v SDS

PAGE Staining/Fixing Solution

0.1% w/v coomassie brilliant blue

25% v/v methanol

7% v/v acetic acid

PAGE Destaining Solution

25% v/v methanol

7% v/v acetic acid

2% v/v glycerol

2X HEPES Buffer (co-transfection)

274 mM NaCl

10 mM KCl

1.4 mM Na_2HPO_4

0.2% w/v dextrose

42 mM HEPES pH 7.05

HBS Buffer (co-transfection)

50 mM HEPES pH 6.95

280 mM NaCl

1.5 mM Na_2HPO_4

PAGE Loading Buffer

45 mM Tris·Cl pH 8.8

2.8% β-mercaptoethanol

1.9% SDS

16% glycerol

bromophenol blue

RESULTS AND DISCUSSION

The results are presented in two parts: Section I is a comparison of the Smith, Vancouver and K-181 strains of MCMV, while Section II details the rescue of the Sgg^+ phenotype of the Vancouver strain of MCMV and a characterization of the recombinants. In Section I, the Vancouver and K-181 and/or the Smith strains of MCMV are compared in terms of their *in vivo* and *in vitro* biological properties, and on a molecular basis by analysis of DNA and protein production. The initial studies were carried out using the Vancouver and K-181 strains, and if no differences were observed these results were not duplicated with the Smith strain. In Section II, an *in vivo* selection technique enabled the identification of recombinant viruses that had regained the ability to grow in the salivary glands of inoculated mice (Sgg^+ phenotype). The positive recombinants were then plaque purified and characterized for their DNA restriction endonuclease patterns to map the Sgg trait, and their protein profiles to determine whether there was a correlation between growth in the salivary glands and MCMV protein expression.

SECTION I

COMPARISON OF THE SMITH, K-181 AND VANCOUVER STRAINS OF MCMV

GROWTH *IN VIVO*

Mice were infected with MCMV to characterize biological differences between the Smith, Vancouver and K-181 strains. Adult female CD-1 mice (susceptible strain), 4-6 weeks of age, were injected intraperitoneally with Smith, Vancouver or K-181. The animals were sacrificed at various times post-infection, and the liver, spleen, kidneys, and salivary glands were removed and assayed for infectious virus. Virus was never recovered from mock-infected animals

(n=10). The results of the infection in the spleen and salivary glands of the infected mice following a typical experiment are presented in Figure 4. The Smith and K-181 mice were injected with $1X10^4$ PFU of virus, while $1X10^6$ PFU of the Vancouver strain virus was required before infectious virus could be detected in the visceral organs of infected mice. The peak virus titre in the spleen was detected at 3 days post-inoculation in all three strains [Fig. 4A]. Viral replication in the spleen of infected mice was less for the K-181 strain than the Smith strain, while replication of the Vancouver strain (with the 100-fold higher inoculum) was comparable to that of the Smith strain.

The most dramatic difference in the ability of the 3 MCMV strains to replicate *in vivo* was manifest in the salivary glands of inoculated mice [Fig. 4B]. The replication of the K-181 strain in the salivary glands produced higher viral titres than that of the Smith strain. Conversely, no virus was isolated from the salivary glands of mice infected with the Vancouver strain despite a 100X higher inoculum. Additional experiments, in which adult or suckling mice were inoculated with up to 10^6 PFU of the Vancouver strain, failed to yield infectious virus from the salivary glands (data not shown). Moreover, serial passage of homogenized salivary glands from Vancouver-infected mice also failed to yield infectious salivary gland virus. Additional *in vivo* differences were found in the virulence of the three MCMV strains. The LD_{50} of the K-181 strain in suckling mice was determined to be $3X10^3$ PFU, while intraperitoneal injection of stock concentrations of Vancouver strain virus ($1X10^6$ PFU) were unable to kill suckling mice.

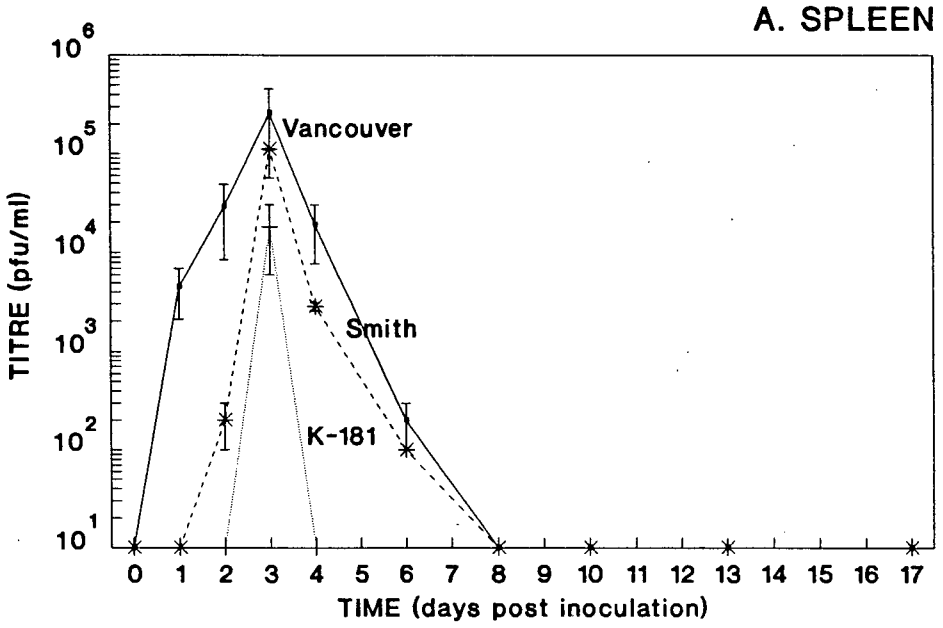
The results of the *in vivo* analyses confirmed that K-181 grows to higher titres than the Smith strain in the salivary glands of adult mice and exhibits increased virulence, as measured by LD_{50} (Misra & Hudson; 1980). A comparison of the K-181 and Smith strains at the molecular level was initiated to clarify the differences between these two biologically-distinct strains. Furthermore, the failure of the Vancouver strain to exhibit the *in vivo* virulence normally associated with the Smith strain warranted a comparison of these two virus isolates.

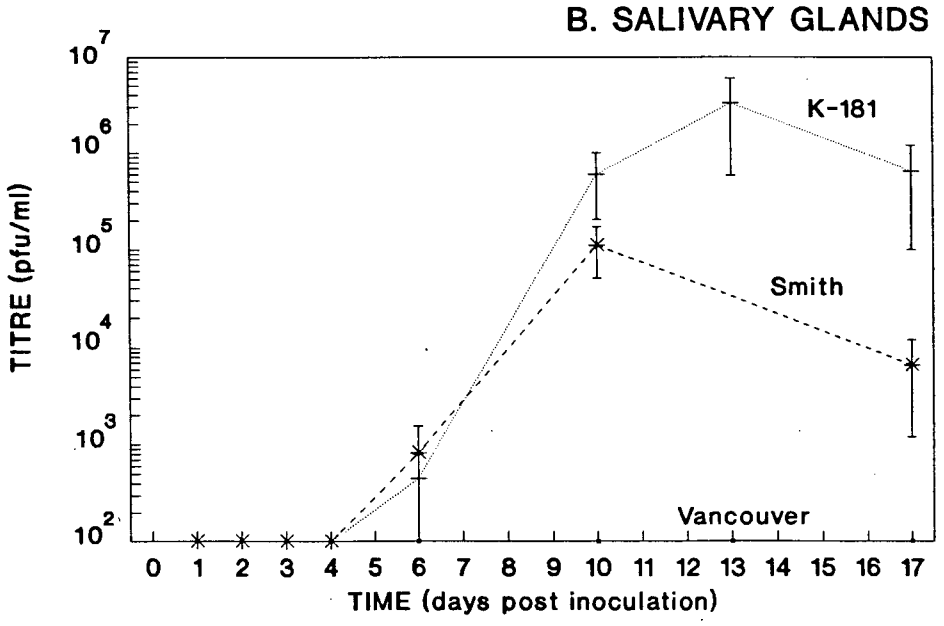
FIGURE 4: GROWTH *IN VIVO* OF SMITH, VANCOUVER AND K-181

Adult female CD-1 mice, 4-6 weeks of age, were injected intraperitoneally with $1X10^6$ PFU of Vancouver strain virus or $1X10^4$ PFU of Smith or K-181 strain virus. 3 mice per group were sacrificed at 1, 2, 3, 4, 6, 10, 13 and 17 days post inoculation, and the organs were removed and homogenized (10% w/v) in complete medium containing 5% FBS. The titre of infectious virus present in each organ homogenate was determined by standard plaque assay. Each point represents the average of 3 mice, with duplicate plaque assays for each sample, and the bars represent the highest and lowest values obtained at each time point. ■ =Vancouver; ✱ =Smith; + =K-181. No virus was ever isolated from mock-infected animals (n=10).

A. Infectious virus isolated from the spleen of inoculated mice.

B. Infectious virus isolated from the salivary glands of inoculated mice.


A. SPLEEN
TITRE (pfu/ml)
10^6
10^5
10^4
10^3
10^2
10^1
Vancouver
Smith
K-181
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TIME (days post inoculation)


B. SALIVARY GLANDS
TITRE (pfu/ml)
10^7
10^6
10^5
10^4
10^3
10^2
K-181
Smith
Vancouver
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
TIME (days post inoculation)

MORPHOLOGY IN TISSUE CULTURE

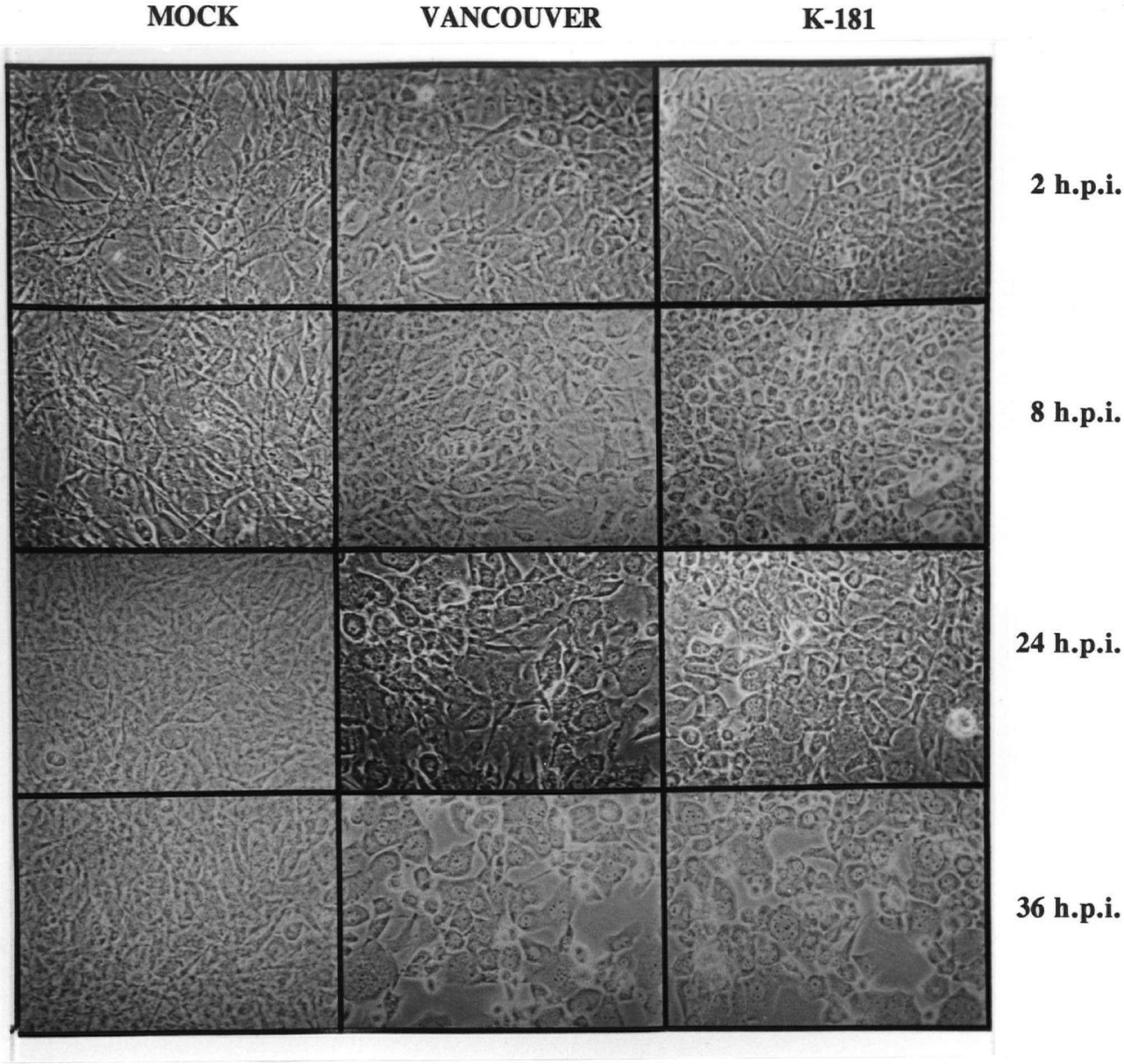
The morphology of 3T3-L1 cells infected with the Vancouver and K-181 strains of MCMV was compared to detect differences in the type or progression of CPE within an infected cell, or in the spread of the virus through the culture. The progression of CPE was monitored by photographing the infected cells at various times post-infection. An MOI of 1.0 PFU/cell was used to look at infection of the majority of cells through a single growth cycle, while a low MOI (0.01 PFU/cell) was used to assess the cell-to-cell spread of virus during several cycles of virus replication. At the higher MOI [Fig. 5A], CPE was seen as early as 2 hours post-infection and the spread and extent of CPE was indistinguishable in cells infected with either the Vancouver or K-181 strains through 36 hours post-infection. Both viruses induced syncytia and rounding up of the cells as evidenced by the birefringence of the cells at 36 hours post-infection. Thus, no reproducibile differences were seen at the level of *in vitro* infection of individual cells.

Similarly, no difference in the morphology of cells infected with either strain was observed when a low MOI was used to infect cells *in vitro* [Fig. 5B]. Although CPE was not detectable in either culture at 24 hours post-infection, foci of infected cells were observed by 47 hours post-infection. By 76 hours post-infection the CPE had progressed to encompass the entire monolayer. Moreover, the extent of syncytial formation in cells infected at a low MOI was less than that observed when cells were infected at a high MOI.

COMPARISON OF PLAQUE MORPHOLOGY

During routine titration of virus stocks, it was observed that the plaques formed by the Vancouver strain of MCMV were often larger than those of the K-181 strain. To assess this more carefully, subconfluent monolayers of 3T3-L1 cells were infected with the Smith, Vancouver and K-181 viral strains and overlaid with agarose. These infected cells were fixed and stained at 7 days post-infection. Plaques formed by the Vancouver strain were considerably

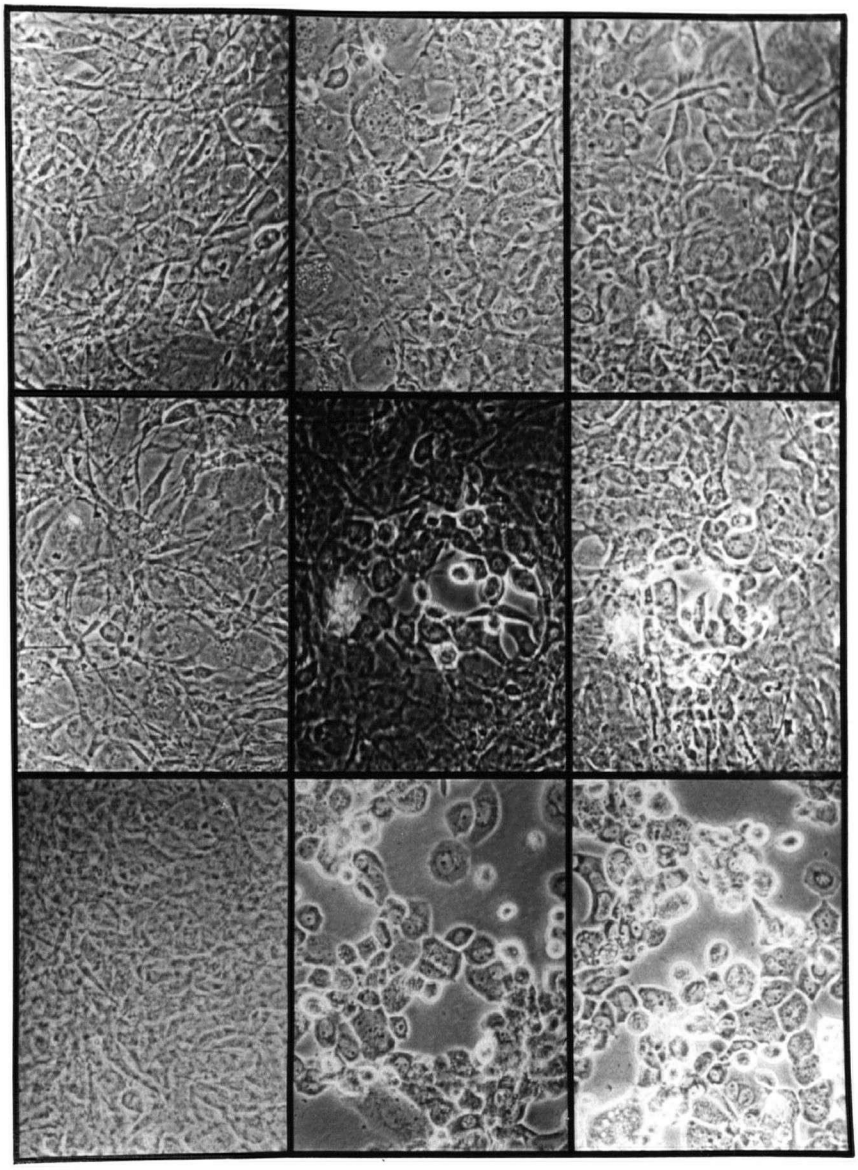
FIGURE 5A: MORPHOLOGY OF MCMV-INFECTED CELLS



3T3-L1 cells were infected with K-181 and Vancouver strains of MCMV and monitored for the production of CPE.

A. High input MOI (1 pfu/cell) photographed at 2, 8, 24, 36 hours post infection **(h.p.i.)**.

FIGURE 5B: MORPHOLOGY OF MCMV-INFECTED CELLS CONT'D



B. Low input MOI (0.01 pfu/cell) photographed at 24, 47, 76 hours post infection (**h.p.i.**).

larger than those formed by the K-181 strain, while those of the Smith strain were intermediate in size [Fig. 6]. As cell-to-cell spread and formation of syncytia [Fig. 5] was similar in both Vancouver and K-181-infected cells, it was concluded that release of virus was probably not responsible for the smaller plaque size seen with the K-181-infected cells [Fig. 6].

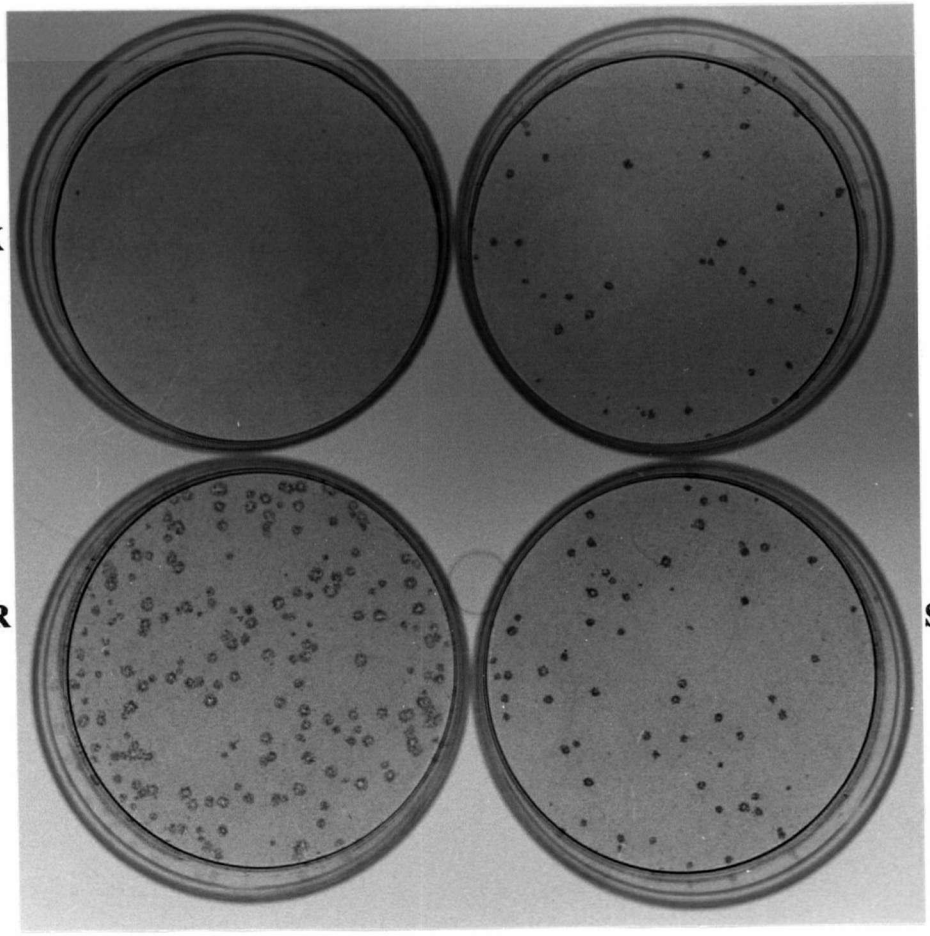
It was hypothesized that larger plaques would form if more copies of viral DNA were taken up in cells infected with the Vancouver strain versus the K-181 strain following infection with an equivalent MOI. This could occur if the stock preparation of the Vancouver strain virus had more multicapsid virions than the stock K-181 virus preparation. However, no differences were found in the proportion of multicapsid virions in either strain (data not shown). It was more likely that the difference in plaque size was due to a faster replication cycle or more infectious virus released per cycle, both of which would result in a faster rate of cell-to-cell spread and ultimately a larger plaque size.

GROWTH IN TISSUE CULTURE

To determine whether the difference in plaque size was a reflection of a faster replication cycle and/or a greater yield of infectious virus per cycle, a one-step growth curve for each of the three viruses was generated [Fig. 7]. The release of progeny Vancouver strain virus preceded that of the K-181 and Smith strains by 1 to 3 hours, and the yield of Vancouver strain virus by 32 hours post-infection was ten-fold higher than for the K-181 and Smith strains [Fig. 7A]. An examination of intracellular virus [Fig. 7B] revealed a similar lag in viral production of the Smith and K-181 strains, suggesting that an event prior to the release of virus was responsible for the differences seen in Figure 7A.

It is known that MCMV is cell-cycle dependent (Muller & Hudson, 1977b). To see if the Vancouver strain had overcome this restriction in non-dividing cells, the growth of virus in G_0-phase cells was monitored. Neither the K-181 strain nor the Vancouver strain replicated in

FIGURE 6: COMPARISON OF PLAQUE MORPHOLOGY OF SMITH, VANCOUVER AND K-181



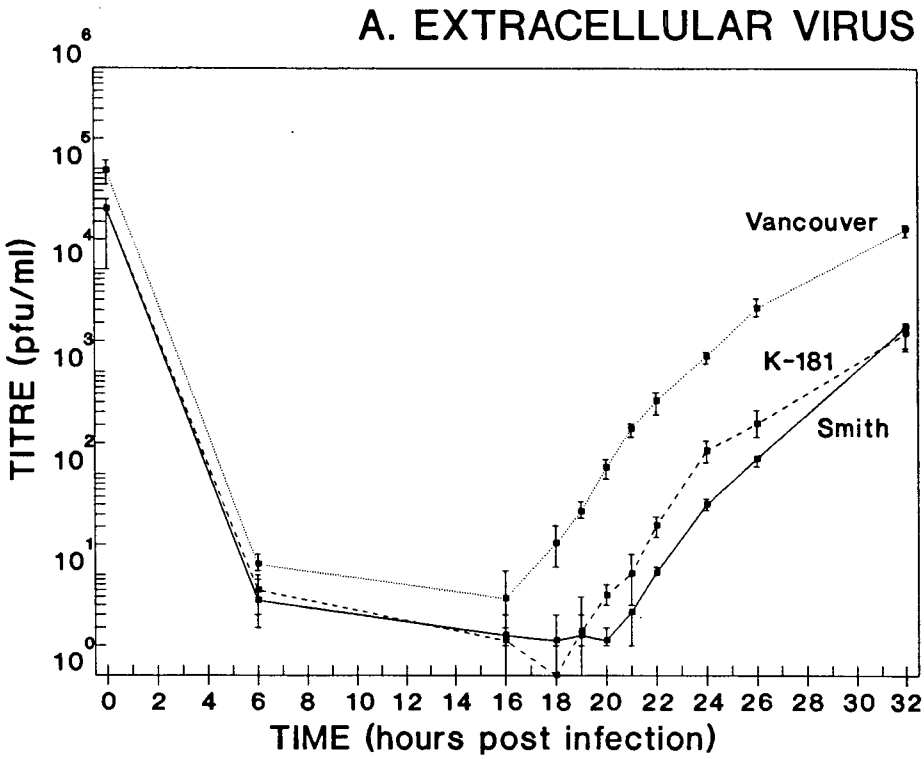
3T3-L1 cells were infected 12-16 hours following subculture with 1 X 10^1 PFU of MCMV as noted, under standard conditions for 2 hours. The inoculum was then removed, and the infected cells were overlaid with 0.5% agarose in complete media containing 5% FBS. Seven days post infection, the cells were fixed and stained with methylene blue.

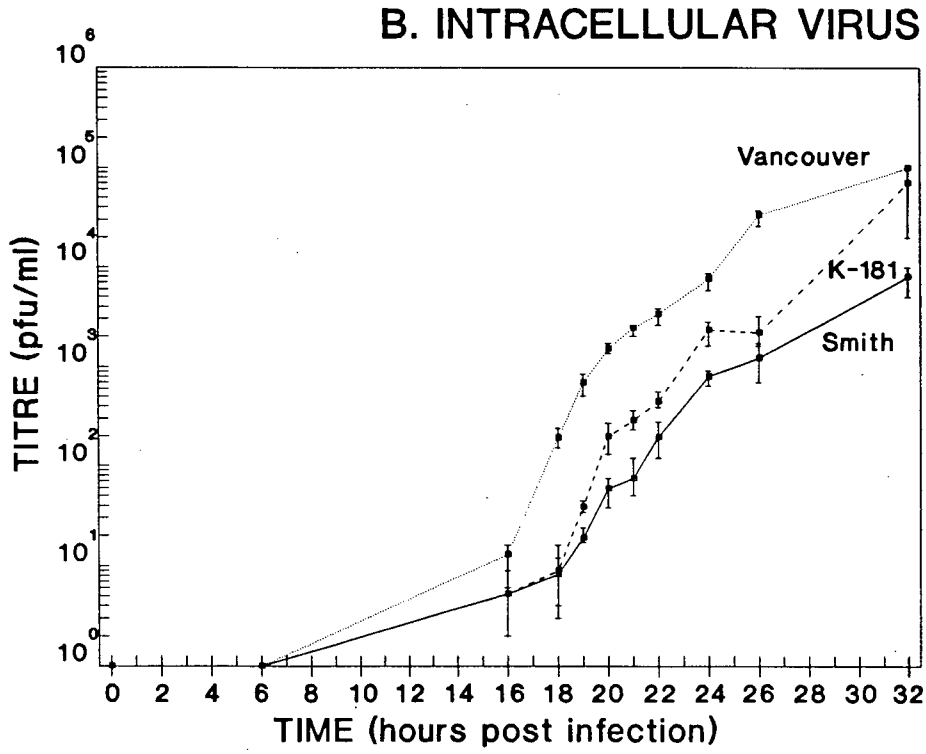
FIGURE 7: VIRAL GROWTH CURVE

3T3-L1 cells were infected 12-16 hours following subculture with an MOI of 1 PFU/cell under standard conditions, for two hours. The inoculum was removed, and the cells were washed twice with HBSS, and then once with medium for 5 minutes at 37°C, before being replaced with complete medium containing 5% FBS. Duplicate samples were removed at 0, 6, 16, 18, 19, 20, 21, 22, 24, 26 and 32 hours post infection. The titre of each sample was then determined by standard plaque assay. Each point represents the average of duplicate samples and duplicate plaque assays of each sample, and the bars represent the highest and lowest values obtained at each time point.

A. Extracellular virus -- samples consisted of supernatant from infected cells.

B. Intracellular virus -- samples consisted of cells which had been washed three times in PBS, scraped into 2 ml of complete media containing 5% FCS, and then sonicated to release intracellular virus.


A. EXTRACELLULAR VIRUS
TITRE (pfu/ml)
TIME (hours post infection)
Vancouver
K-181
Smith


B. INTRACELLULAR VIRUS
TITRE (pfu/ml)
TIME (hours post infection)
Vancouver
K-181
Smith

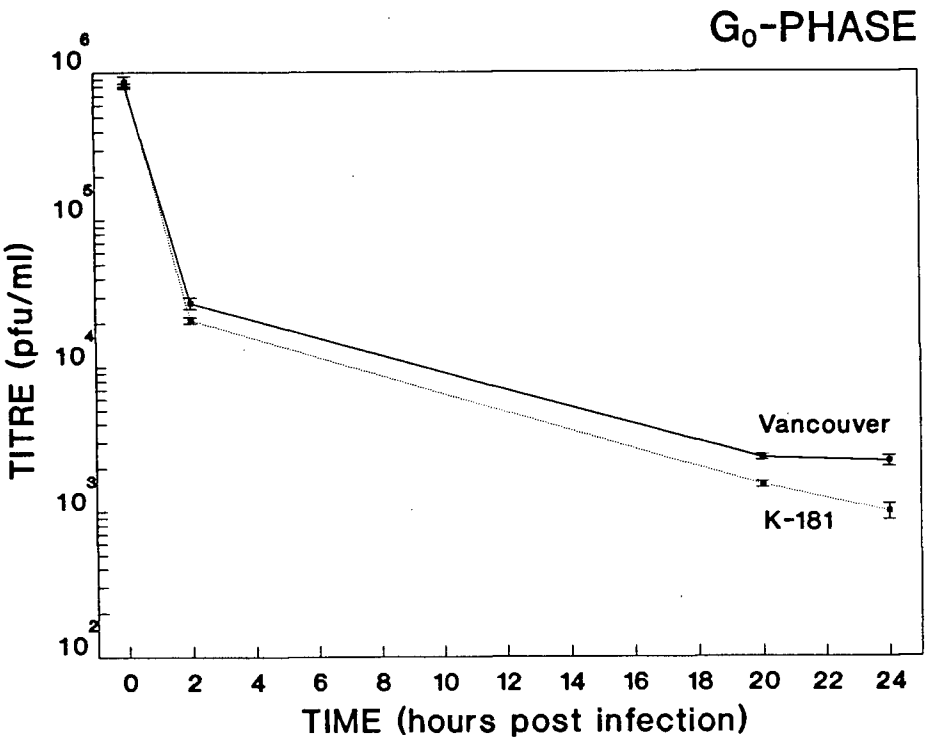
these growth-arrested cells [Fig. 8]. In addition, neither strain had a growth advantage in cells infected at various times following subculture. Thus, the differences observed in the growth curve were not caused by a change in the cell-cycle dependence of either strain.

VIRAL DNA REPLICATION

In some semi-permissive cell types, although IE and E proteins are produced, virus production is blocked at the level of DNA replication (Walker & Hudson, 1987). To determine whether the ability of the Vancouver strain to produce progeny virus more quickly than the Smith or K-181 strains was related to DNA replication, the onset of viral DNA replication for each strain was compared. Infected-cell DNA isolated at various times post-infection was applied to nitrocellulose filters and incubated with a [^{32}P]dCTP-labelled HindIII *D* fragment, which encompassed the putative MCMV origin of replication (M.J. Masse, personal communication). Experiments using 3 different MOI (0.5, 1.0 and 5.0) showed no consistent difference in the onset of viral DNA replication in the three strains (data not shown). In other experiments, the total amount of Vancouver strain DNA at 20 hours post-infection was more than double that of the K-181 strain (data not shown), correlating with the higher yield of infectious virus from Vancouver infected cells.

DNA replication in non-dividing cells would be a growth advantage for virus reproduction *in vivo*. To determine whether the Vancouver strain had overcome the cell-cycle dependence of MCMV, DNA replication in growth-arrested (G_0-phase) cells and cells infected at different times following subculture was also analyzed. DNA replication of the Vancouver and K-181 strains was minimal by 24 hours post-infection and was attributed to a minority of cells in the cultures which were not in G_0-phase (data not shown). No difference in viral DNA replication was detected in cells infected at different times following subculture (data not shown).

FIGURE 8: GROWTH OF MCMV IN G_0-PHASE CELLS



Growth arrested 3T3-L1 cells were infected under standard conditions, for two hours. The inoculum was removed and replaced with depleted medium. The supernatant of duplicate plates was removed at 0, 2, 20 and 24 hours post infection. The titre of each sample (cell supernatant) was then determined by standard plaque assay. Each point represents the average of duplicate samples and duplicate plaque assays of each sample, and the bars represent the highest and lowest values obtained at each time point.

Thus, as was the case with the production of progeny virus, the differences observed in viral DNA replication were not caused by a change in the cell-cycle dependence of either strain. Furthermore, the onset of viral DNA replication appeared similar in all three strains, thus it is likely that a more rapid accumulation of viral DNA was responsible for the detection of infectious Vancouver virus at earlier times post-infection in the growth curve experiments.

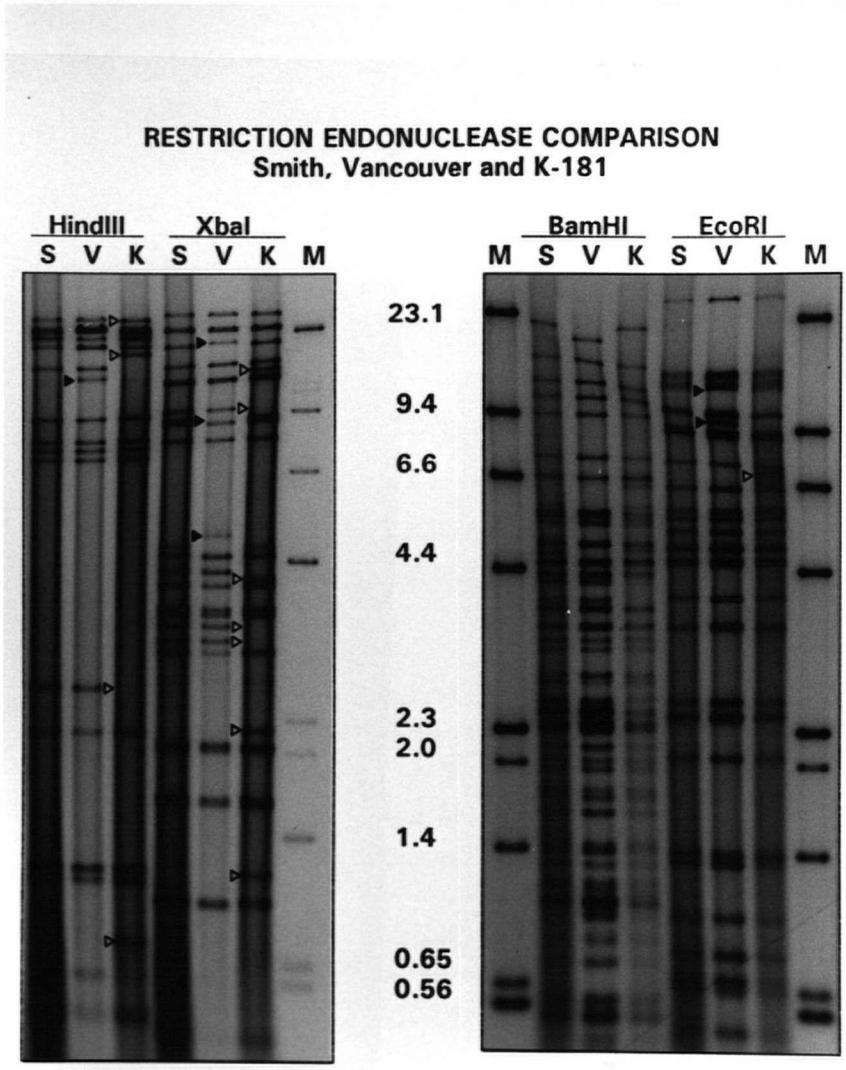
VIRAL GENOME ANALYSIS

A comparison of the restriction endonuclease profiles of the Smith, Vancouver and K-181 strains of MCMV after cleavage with HindIII, XbaI, BamHI and EcoRI [Fig. 9] revealed a number of differences between all three strains as summarized in Table I. Analysis of small fragments by electrophoresis through 3.5% polyacrylamide [Fig. 10] revealed two additional fragments generated by cleavage with XbaI, and one additional fragment generated by cleavage with EcoRI, which were not seen in the previous figure, are shown in Figure 10 (arrows; bands in the K-181-digested samples were visible after longer exposure of the gel). These fragments were, however, present in all three strains and no further attempt to analyze or map them was made.

CHEF analysis of the high molecular weight fragments generated by cleavage of viral DNA with HindIII [Fig. 11] demonstrated that the HindIII *D* and *E* fragments detected in the Smith and K-181 strains were not seen in the Vancouver strain of MCMV. There was one novel fragment generated by cleavage of the Vancouver strain DNA with HindIII which migrated between the HindIII *H* and *I* fragments of the Smith strain, but it was too small to compensate for a deletion of both the HindIII *D* and *E* fragments and still correlate with the EcoRI and XbaI restriction endonuclease cleavage results.

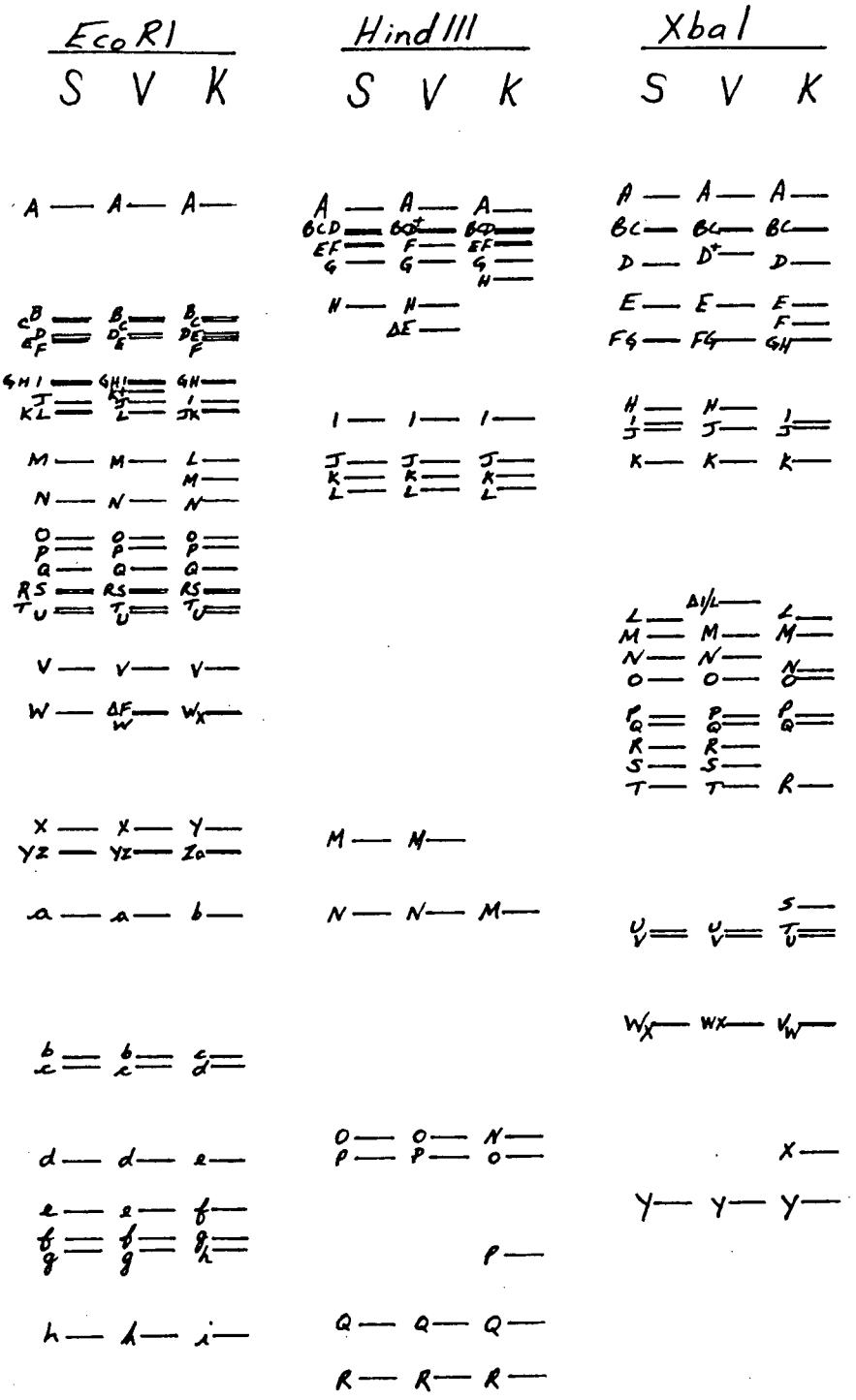
CHEF electrophoresis of the whole viral genomes of the Vancouver and K-181 strains revealed that the Vancouver genome is significantly smaller than the K-181 genome [Fig. 12].

FIGURE 9A: RESTRICTION ENDONUCLEASE COMPARISON OF SMITH, VANCOUVER AND K-181 DNA



A. Viral DNA was digested with restriction endonuclease, end-labelled with [^{32}P]dATP and subjected to gel electrophoresis through 0.8% agarose. **S**=Smith, **V**=Vancouver, **K**=K-181, **M**=HindIII/XbaI-digested lambda DNA as markers (size in kb).

FIGURE 9B: RESTRICTION ENDONUCLEASE COMPARISON OF SMITH, VANCOUVER AND K-181 DNA



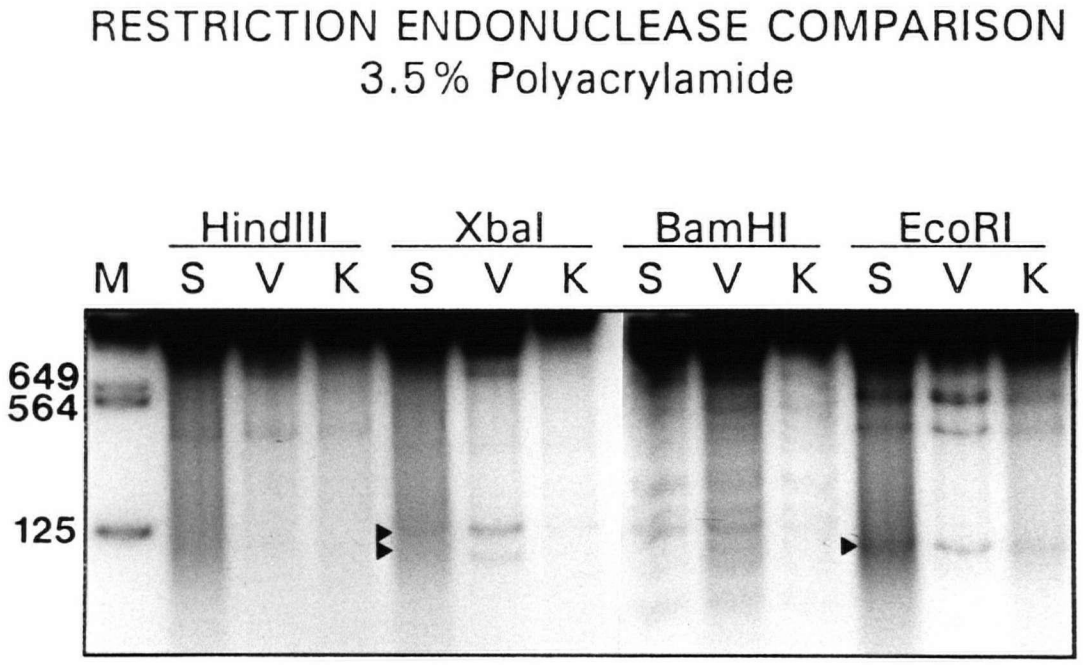
B. Schematic representation of Fig. 9A including fragment designations. S=Smith, V=Vancouver, K=K-181

TABLE I: RESTRICTION ENDONUCLEASE ANALYSIS
DIFFERENCES BETWEEN THE SMITH, VANCOUVER AND K-181 STRAINS OF MCMV

Vancouver*			K-181*		
HindIII	XbaI	EcoRI	HindIII	XbaI	EcoRI
extra fragment between *H* and *I*	larger *D* fragment	loss of the *F* fragment	smaller *A* fragment	extra fragment between *E* and *F*	extra fragment between *M* and *N*
	loss of the *I* fragment	extra fragment between *I* and *J*	larger *H* fragment	loss of the *H* fragment	
	larger *L* fragment		loss of the *M* fragment	smaller *N* fragment	
			extra fragment between *P* and *Q*	loss of *R* and *S* fragments	
				extra fragment between *T* and *U*	
				extra fragment between *X* and *Y*	

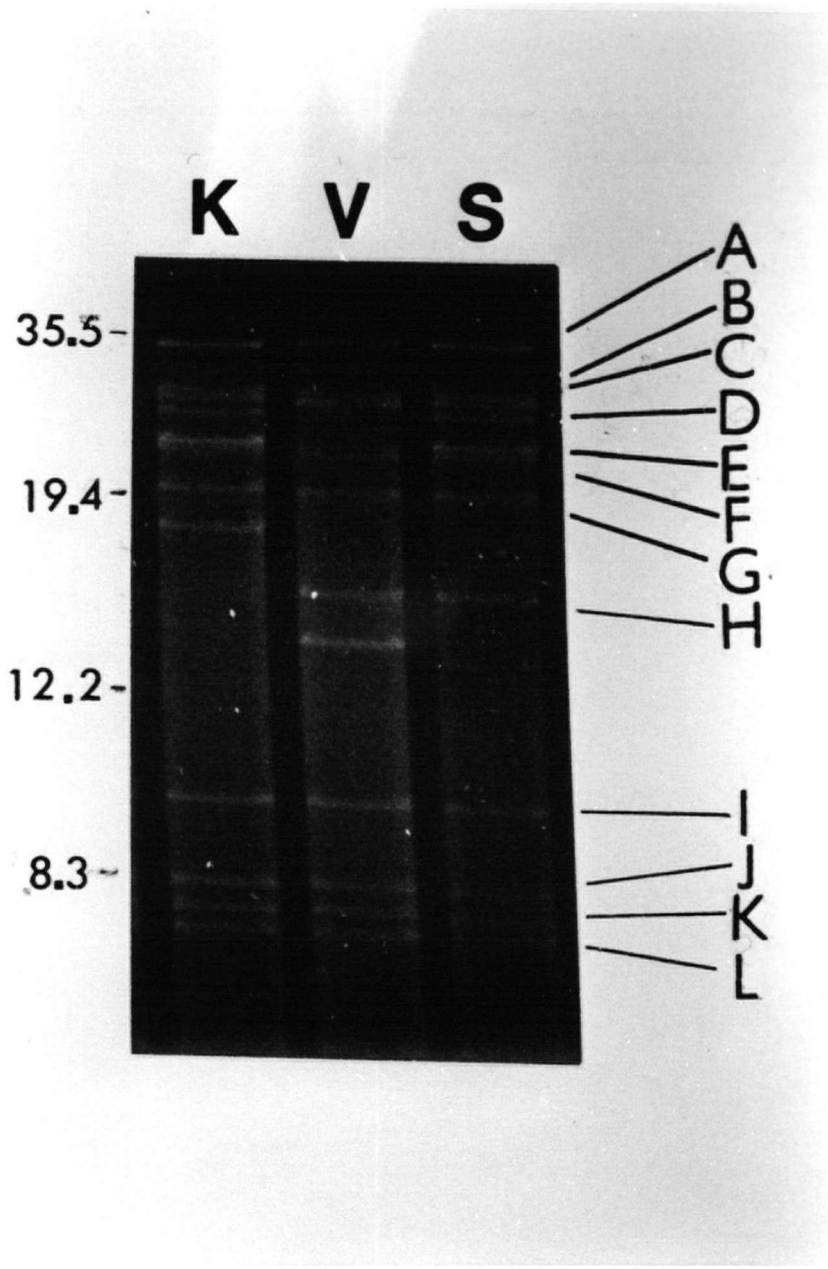
* The comparisons for the Vancouver and K-181 strain genome fragments are made relative to the Smith strain restriction endonuclease profile depicted in Figure 11.

FIGURE 10: RESTRICTION ENDONUCLEASE COMPARISON OF MCMV DNA IN 3.5% POLYACRYLAMIDE GELS



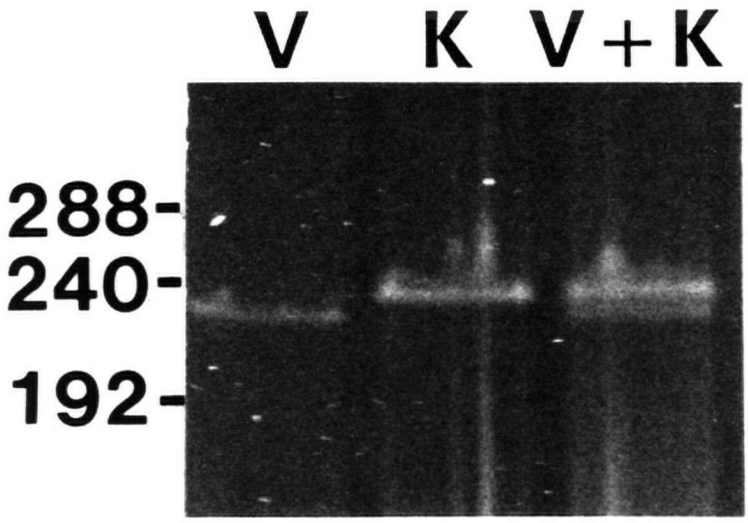
Viral DNA was digested with restriction endonuclease, end-labelled with [^{32}P]dATP and subjected to gel electrophoresis through 3.5% polyacrylamide. **S**=Smith, **V**=Vancouver, **K**=K-181, **M**=HindIII/XbaI-digested lambda DNA as markers (size in bp). ▸ = additional fragments which were not detected in Fig. 9.

FIGURE 11: RESTRICTION ENDONUCLEASE COMPARISON OF HINDIII FRAGMENTS OF MCMV DNA SUBJECTED TO CHEF ELECTROPHORESIS



Viral DNA was digested with HindIII and subjected to CHEF electrophoresis through 1% agarose. The intial pulse time was 0.5 seconds and the final pulse time was 0.9 seconds, with a total run time of 24 hours. **S**=Smith, **V**=Vancouver, **K**=K-181. Letters **A** - **L** designate HindIII fragments; DNA standards (size in kb) are indicated to the left of the figure.

FIGURE 12: SIZE COMPARISON OF WHOLE VIRAL DNA



Viral genomic DNA from the Vancouver (**V**) and K-181 (**K**) strains of MCMV were subjected to CHEF electrophoresis through 1% agarose. 0.5 μg of each viral DNA was loaded per well, and electrophoresed with an initial pulse time of 50 seconds and a final pulse time of 90 seconds, for a total time of 24 hours. DNA standards (λ concatamers, size in kb) are indicated to the left of the figure.

A determination of the size of the genomes of the three strains (total size of the restriction endonuclease fragments generated by cleavage of the genome with EcoRI and XbaI, Table II) precluded the possibility of a deletion involving the entire HindIII *D* and *E* fragments of the Smith strain. The total size of the Vancouver strain genome was calculated at approximately 227 kb, as opposed to 235 kb for the Smith and K-181 strains. A genome size of 207 kb rather than 227 kb would be expected if both the HindIII *D* and *E* fragments of the Vancouver strain had been deleted.

Southern blot analysis was used to identify the "missing" HindIII *D* and *E* fragments identified above. MCMV was cleaved with HindIII, subjected to CHEF electrophoresis and transferred to nitrocellulose. Initially the filter was probed with the cloned [^{32}P]dCTP-labelled HindIII *E'* insert [Fig. 13]. The probe hybridized to the HindIII *E* fragment of the Smith and K-181 strains, while in the Vancouver strain it hybridized with a fragment migrating between the HindIII *H* and *I* fragments of the Smith strain [see Fig. 11, page 73], designated ΔE. The filter was stripped of this probe and rehybridized to [^{32}P]dCTP-labelled ΔE [Fig 13]. The results were the same; the probe hybridized to the HindIII *E* fragment of the Smith and K-181 strains and the HindIII ΔE fragment of the Vancouver strain.

DNA cleaved with restriction endonucleases and subjected to conventional agarose gel electrophoresis followed by Southern blotting is depicted in Figure 14. The identification of fragments was made by comparison to an ethidium bromide-stained gel. Hybridization to the HindIII *E'* probe revealed a loss of the EcoRI *F* and the HindIII *E* fragments coupled with the appearance of an additional smaller fragment in each profile compared to the Smith strain, and a loss of the XbaI *I* and *L* fragments to yield an additional fragment of intermediate size. Taken together, these results indicated that there was a deletion in the Vancouver strain compared to the Smith strain, which included part of the EcoRI *F* and HindIII *E* fragments and included the restriction endonuclease site which separated the XbaI *I* and *L* fragments (XbaI *I/L* junction).

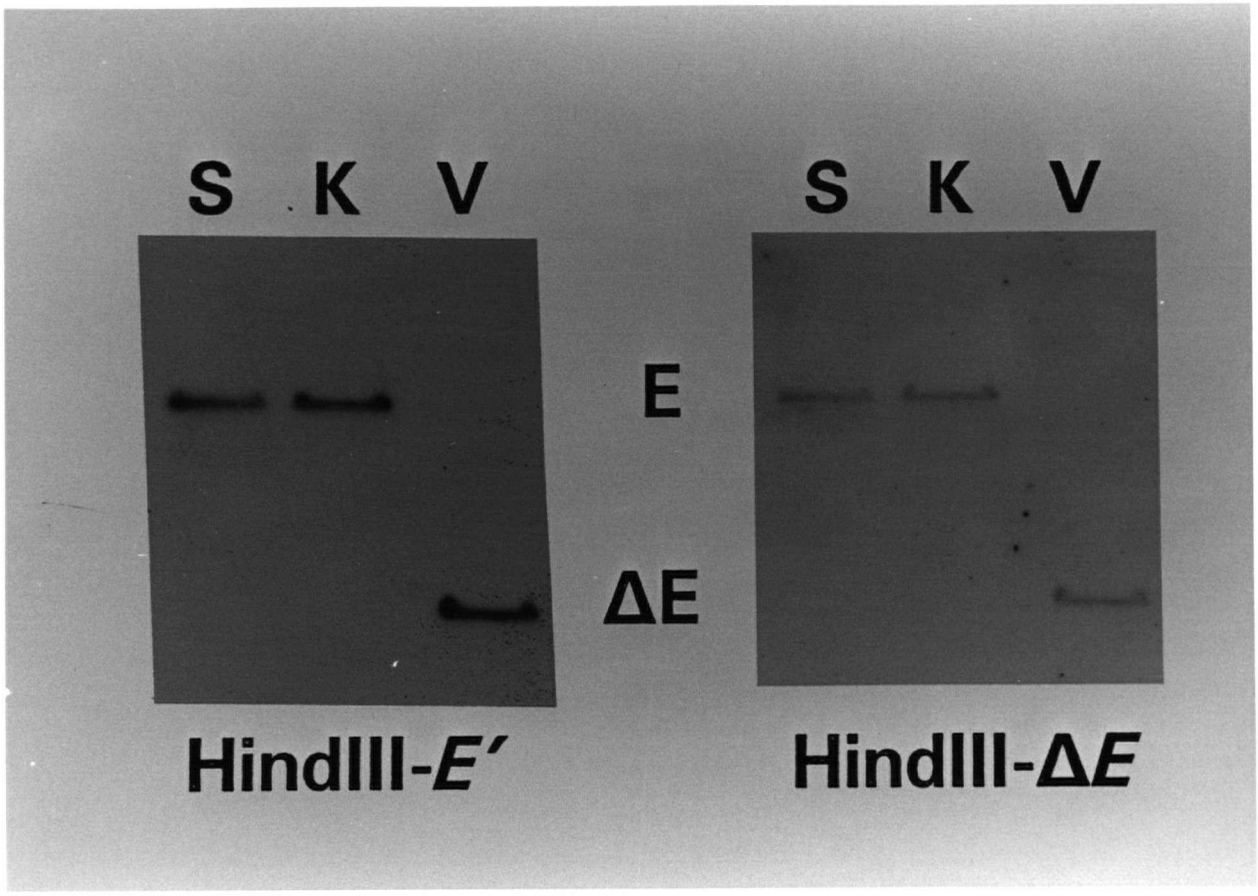
TABLE II: RESTRICTION ENDONUCLEASE FRAGMENT SIZE (kb)

	HindIII				XbaI		
	Smith	Vanc.	K-181		Smith	Vanc.	K-181
A	33.6	33.6	33.2	A	40.1	40.1	40.1
B	26.9	26.9	26.9	B	25.6	25.6	25.6
C	26.4	26.4	26.4	C	25.6	25.6	25.6
D	25.0	26.4	25.0	D	20.6	21.0	20.6
E	23.1		23.1	E	16.2	16.2	16.2
F	22.4	22.4	22.4	F	13.6	13.6	14.9
G	20.2	20.2	20.2	G	13.6	13.6	13.6
H	16.3	16.3	18.4	H	10.2	10.2	13.6
ΔE		13.9		I	9.6		9.6
I	10.1	10.1	10.1	J	9.2	9.2	9.2
J	8.3	8.3	8.3	K	8.1	8.1	8.0
K	7.8	7.8	7.8	ΔI/L		4.8	
L	7.3	7.3	7.3	L	4.5		4.5
M	2.5	2.5	2.1	M	4.4	4.4	4.4
N	2.1	2.1	1.1	N	4.1	4.1	4.0
O	1.1	1.1	1.1	O	3.8	3.8	3.8
P	1.1	1.1	0.72	P	3.6	3.6	3.6
Q	0.58	0.58	0.58	Q	3.5	3.5	3.5
R	0.42	0.42	0.42	R	3.3	3.3	2.9
				S	3.0	3.0	2.1
				T	2.9	2.9	2.0
				U	2.0	2.0	2.0
				V	2.0	2.0	1.6
				W	1.6	1.6	1.6
				X	1.6	1.6	1.1
				Y	0.98	0.98	0.98
				Z	0.3	0.3	0.3
				a	0.13	0.13	0.13
				b	0.1	0.1	0.1
Total	235.2	227.4	235.1	Total	234.2	225.2	235.6

Table II Cont'd

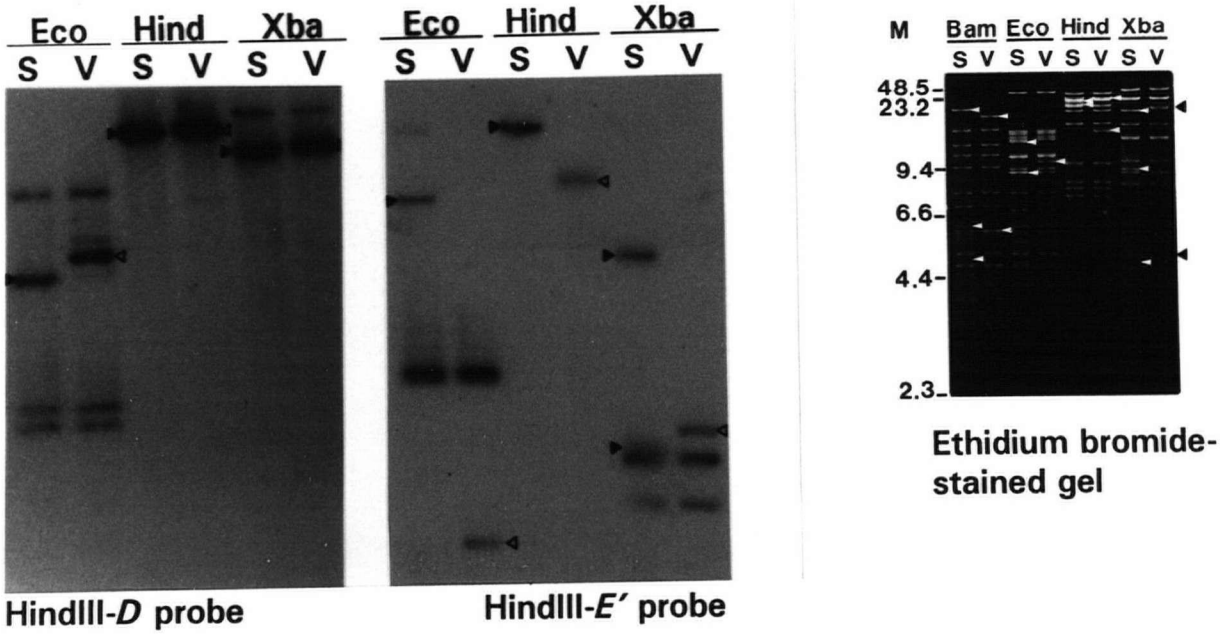
	EcoRI				EcoRI Cont'd		
	Smith	Vanc.	K-181		Smith	Vanc.	K-181
A	33.1	33.1	33.1	Z	2.4	2.4	2.4
B	15.2	15.2	15.2	a	2.0	2.0	2.4
C	14.7	14.7	14.7	b	1.4	1.4	2.0
D	14.1	14.1	14.1	c	1.3	1.3	1.4
E	13.4	13.4	13.4	d	1.0	1.0	1.3
F	13.1		13.1	e	0.83	0.83	1.0
G	10.9	10.9	10.9	f	0.72	0.72	0.83
H	10.9	10.9	10.9	g	0.67	0.67	0.72
I	10.9	10.9	9.4	h	0.51	0.51	0.67
J	9.4	9.4	9.1	i	0.1	0.1	0.51
K	9.1	10.1	9.1	j			0.1
L	9.1	9.1	7.4	Total	235.8	227.2	235.4
M	7.4	7.4	7.0				
N	6.4	6.4	6.4				
O	5.7	5.7	5.7				
P	5.5	5.5	5.5				
Q	5.2	5.2	5.2				
R	4.8	4.8	4.8				
S	4.8	4.8	4.8				
T	4.5	4.5	4.5				
U	4.5	4.5	4.5				
V	3.9	3.9	3.9				
W	3.5	3.5	3.5				
ΔF		3.5					
X	2.5	2.5	3.5				
Y	2.4	2.4	2.5				

FIGURE 13: SOUTHERN BLOT ANALYSIS OF CHEF-SEPARATED HINDIII FRAGMENTS OF MCMV DNA



Viral DNA was digested with HindIII and subjected to CHEF electrophoresis through 1% agarose. The intial pulse time was 0.5 seconds and the final pulse time was 0.9 seconds, with a total run time of 24 hours. The DNA was then transferred to a Hybond membrane by Southern blotting, and the filter was hybridized to a cloned **HindIII *E'*** fragment insert which was labelled with [^{32}P]dCTP (1X10^{6} cpm/ml). Following autoradiography, the membrane was stripped of probe, and then hybridized to a second [^{32}P]dCTP-labelled probe which consisted of the gel-purified **13.9** kb HindIII fragment (**HindIII Δ*E***) which migrated between the HindIII *H* and *I* fragments of the Smith strain of MCMV. **S**=Smith, **V**=Vancouver, **K**=K-181, **E**=HindIII *E* fragment of Smith and K-181, **ΔE**=deleted form of the HindIII *E* fragment found in the Vancouver strain.

FIGURE 14: SOUTHERN BLOT ANALYSIS OF MULTIPLE RESTRICTION ENDONUCLEASE-DIGESTED MCMV DNA



Viral DNA was digested with restriction endonuclease and subjected to gel electrophoresis through 0.8% agarose, and stained with ethidium bromide. The DNA was then transferred to a Hybond membrane by Southern blotting, and the filter was hybridized to cloned **HindIII *D*** fragment insert or cloned **HindIII *E'*** fragment insert which was labelled with [^{32}P]dCTP (1X10^{6} cpm/ml). **S**=Smith, **V**=Vancouver, **Eco**=EcoRI, **Hind**=HindIII, **Xba**=XbaI, ▸=Smith fragment, ◂=Vancouver fragment containing an insertion (**HindIII *D* probe**) or a deletion (**HindIII *E'*** probe).

Hybridization to the HindIII *D* fragment probe [Fig. 14] revealed an insertion in the EcoRI *K*, HindIII *D*, and XbaI *D* fragments of the Vancouver strain compared to the Smith strain. Thus, during the CHEF analysis of the HindIII cleavage fragments [Fig. 11, page 75], the missing HindIII *D* fragment of the Vancouver strain co-migrated with a higher molecular weight fragment.

The size of each cleavage fragment for each virus strain was determined, and is presented in Table II (pages 76/77). This information was used to calculate the size of the insertion into the HindIII *D* fragment and the deletion in the HindIII ΔE fragment of the Vancouver strain, and to map the restriction endonuclease cleavage fragments. The size of the insertion was calculated by averaging the difference between the Vancouver fragment containing the insertion and the corresponding Smith fragment:

EcoRI:	$VK^{+} - SK = 10.1 - 9.1 = 1.0$ kb
HindIII:	$VD^{+} - SD = 26.4 - 25.0 = 1.4$ kb
XbaI:	$VD^{+} - SD = 21.0 - 20.6 = 0.4$ kb
Average:	$(1.0 + 1.4 + 0.4)/3 =$ **0.9 kb insertion**

The size of the deletion was calculated in a similar fashion by averaging the difference between the Smith fragment(s) and the corresponding Vancouver fragment:

EcoRI: $SF - V\Delta F = 13.1 - 3.5 = 9.6$ kb

HindIII: $SE - V\Delta E = 23.1 - 13.9 = 9.2$ kb

XbaI: $SI{+}L - V\Delta I/L = 14.1 - 4.8 = 9.3$ kb

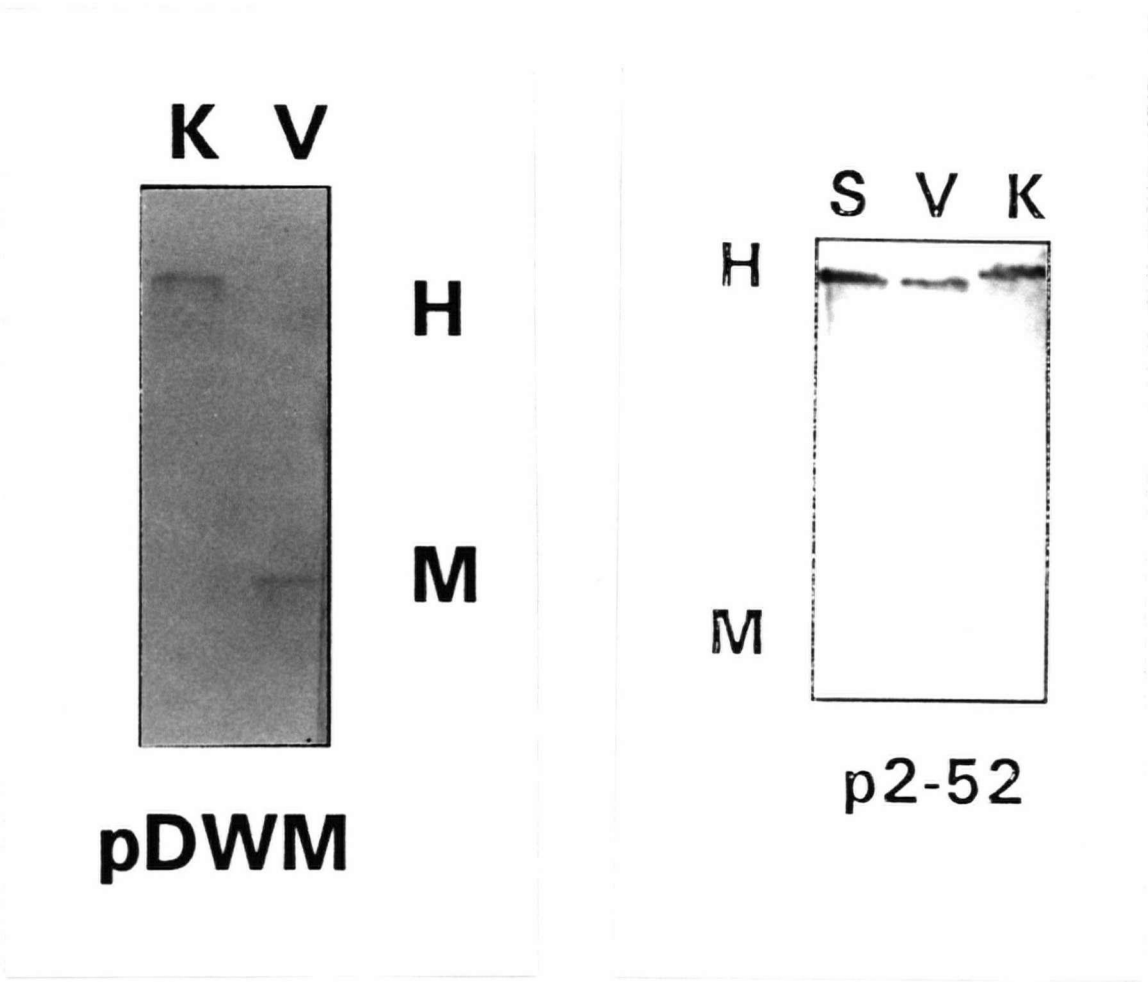
Average: $(9.6 + 9.2 + 9.3)/3 =$ **9.4 kb deletion**

Taken together, the 0.9 kb insertion and the 9.4 kb deletion resulted in an overall reduction of 8.5 kb in the Vancouver strain genome.

One of the features used to distinguish the Smith and the K-181 strains of MCMV is the presence in the Smith strain (and the Vancouver strain) of two HindIII fragments designated *M* and *N*, and the absence in the K-181 strain of the HindIII *M* fragment (Mocarski, personal communication). Southern blot analysis using a cloned HindIII *M* fragment [Fig. 15] to probe HindIII-digested K-181 and Vancouver DNA revealed hybridization of the probe to the HindIII *M* fragment of the Vancouver strain, as expected, and hybridization to the slightly larger *H* fragment of the K-181 strain. When a cloned HindIII *H* fragment of the K-181 strain (p2-52) was used as a probe during hybridization analysis of HindIII-digested Smith, Vancouver and K-181 DNA, hybridization to the HindIII *H* fragment of K-181, Smith and Vancouver, and hybridization to the HindIII *M* fragment of Smith and Vancouver was detected [Fig. 15]. The higher molecular weight of the HindIII *H* fragment and the absence of a fragment equivalent in size to the Hind III *M* fragment in the K-181 compared with the Smith and Vancouver strains, can be explained by the loss of a HindIII cleavage site between the HindIII *H* and *M* fragments in the K-181 genome.

Cross-blot hybridization of the Smith, Vancouver and K-181 strains was carried out by Dr. Lambert Loh, and autoradiograms were made available to me for analysis (Table III). The order of EcoRI fragments in the region between 0.37 to 0.47 map units could not be determined using these data. Because this area contained the EcoRI *K* fragment insertion in the Vancouver

FIGURE 15: SOUTHERN BLOT ANALYSIS
VANCOUVER HINDIII *M* PROBE AND K-181 HINDIII *H* PROBE



Viral DNA was digested with HindIII and subjected to gel electrophoresis through 0.7% agarose. The DNA was then transferred to filters by Southern blotting. The filters were hybridized to biotinylated plasmid **pDWM** (Vancouver HindIII ***M*** fragment insert) or digoxygenin-labelled plasmid **p2-52** (K-181 HindIII ***H*** fragment insert) and hybridization was detected using alkaline phosphatase amplification/detection systems. **S**=Smith, **V**=Vancouver, **K**=K-181.

TABLE III: CROSS-BLOT HYBRIDIZATION

A: CROSS-BLOT HYBRIDIZATION OF VANCOUVER VS. K-181

XbaI

K-181↓ \ Vancouver→	A	B	C	D	E	F	G	H	J	K	I/L	M	N	O	P	Q	R	S	T	U	V	W	X	Y
A	+																							
B		+																						
C			+																					
D				+																				
E					+																			
F								+					+											
G							+																	
H								+																
I											+													
J									+															
K										+														
L											+													
M												+												
N																		+						+
O														+										
P															+									
Q																+								
R																			+					
S																	+							
T																				+				
U																					+			
V																						+		
W																							+	
X																	+							

Hutchison cross-blot hybridization was carried out by Dr. Lambert Loh, and his autoradiograms were made available to me for analysis. + =cross hybridization between fragments.

A. A sample cross-blot hybridization analysis of Vancouver strain DNA cleaved with XbaI vesus K-181 strain DNA also cleaved with XbaI. Hybridization of fragments on the diagonal signifies homologous fragment lengths in the two strains, while hybridization of fragments off the diagonal signifies an additional restriction endonuclease cleavage site in the DNA of one of the strains.

B. A summary of cross-blot hybridization results for the Smith strain of MCMV using the HindIII, EcoRI and XbaI enzymes. BglII, and BamHI results were used to clarify fragment order when possible.

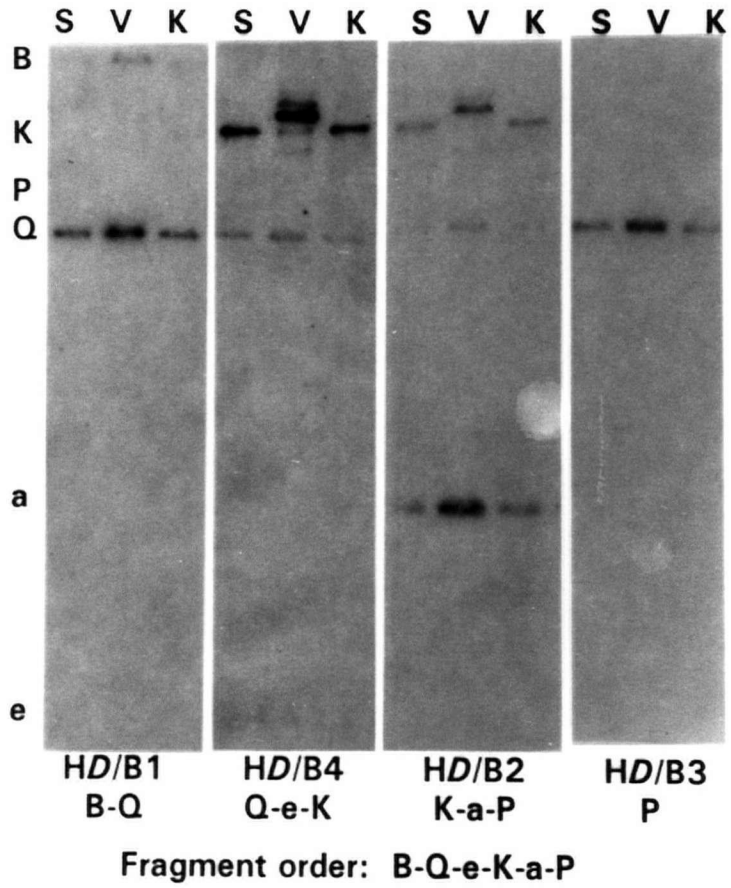
B: SUMMARY OF CROSS HYBRIDIZATION RESULTS
SMITH STRAIN

EcoRI↓	HindIII→																	↓XbaI	
	N	A	B	M	H	D	C	G	F	K	L	J	I	O	P	E			
G	+																S		linked in BglII digest with each
G		+															Y		other and also with XbaI L
G		+															J		
U		+															J		
U		+															R		
M		+															R		
M		+															E		
d		+															E		
C		+															E		
C		+	+														K		
W			+														K		
W			+														P		
D			+														P		
D			+														A		
L			+														A		
Y			+	+													A		
N					+												A		
B					+	+											A		
B						+											D		
(e						+											D		
(K	order					+											D		
(a	unknown					+											D		
(Q						+											D		
P						+											D		
P						+	+										B		
A							+										B		
A							+	+									C		
I									+								C		
H									+								U)		
H									+								T)		
H									+								W)		
H									+								F)		linked in BglII digest
S									+								F		
T									+	+							F		
g										+							F		
X										+							F		
X										+							Q		
E										+							Q		
E										+							V		
E										+	+	+					G		
V												+					G		
V												+					N		
R												+	+				N		
R													+				H		
J													+	+	+		H		
J																+	O		
Z																+	O		
O																+	O)		
O																+	X)		order derived from BglII digest
O																+	M)		
b/c																+	M		
f																+	I		
F																+	I		
F																+	L		
b/c																+	L		
	N	A	B	M	H	D	C	G	F	K	L	J	I	O	P	E			
	HindIII→																		

strain, an attempt was made to map this region. Viral DNA was cleaved with EcoRI and subjected to agarose gel electrophoresis, followed by Southern blotting and hybridization. Probes generated by cleavage of the cloned HindIII *D* fragment insert with BamHI were used to establish linkage between the EcoRI cleavage fragments. The results [Fig. 16] established an EcoRI fragment order of *BQeKaP* in the Smith and Vancouver strains (or *BQfJbP* in the K-181 strain).

The results of the cross-hybridization analyses were used to construct HindIII, XbaI and EcoRI maps for the Smith, Vancouver and K-181 strains of MCMV. Figure 17A groups the three restriction endonuclease cleavage maps together for each strain, while Figure 17B groups the three virus strains together for each enzyme. Comparison of the K-181 restriction endonuclease cleavage map with previously published maps (Mercer et al., 1983; Ebeling et al., 1983) indicated that our K-181 strain was identical to that of Mercer et al. (Mercer et al., 1983) for the HindIII enzyme, while only slightly different (order of the EcoRI *h* and *Y* fragments) for the EcoRI enzyme. The HindIII, EcoRI and XbaI maps reported by Ebeling et al. (Ebeling et al., 1983) matched those of our Smith strain, with slight discrepancies. A closer examination of Figure 17B revealed that the Smith and Vancouver strains of MCMV were identical except for an insertion in the EcoRI *K* fragment of the Vancouver strain, and a deletion spanning the XbaI *I/L* junction. The K-181 strain, however, shows differences in the restriction endonuclease map but there are no discernable insertions or deletions relative to the Smith strain. With the exception of the loss of the HindIII cleavage site between the *M* and *H* fragments, these differences between the Smith and K-181 strains were confined to 0.0 to 0.06 map units of the genome.

FIGURE 16: SOUTHERN BLOT ANALYSIS ECORI *B(KQae)P* FRAGMENT ORDER



Viral DNA was digested with EcoRI and subjected to DNA electrophoresis through 0.8% agarose. The DNA was then transferred to a Hybond filter by Southern blotting. The probes used for hybridization were BamHI subfragments (**B1-B4**) of a cloned HindIII *D* fragment which were purified from a 1% agarose gel and then labelled with [^{32}P]dCTP. **S**=Smith, **V**=Vancouver, **K**=K-181, **B**=EcoRI *B* fragment, **Q**=EcoRI *Q* fragment, **e**=EcoRI *e* fragment, **K**=EcoRI *K* fragment, **a**=EcoRI *a* fragment, **P**=EcoRI *P* fragment. Fragment designations refer to the Smith genome.

FIGURE 17: RESTRICTION ENDONUCLEASE MAP COMPARISON OF MCMV

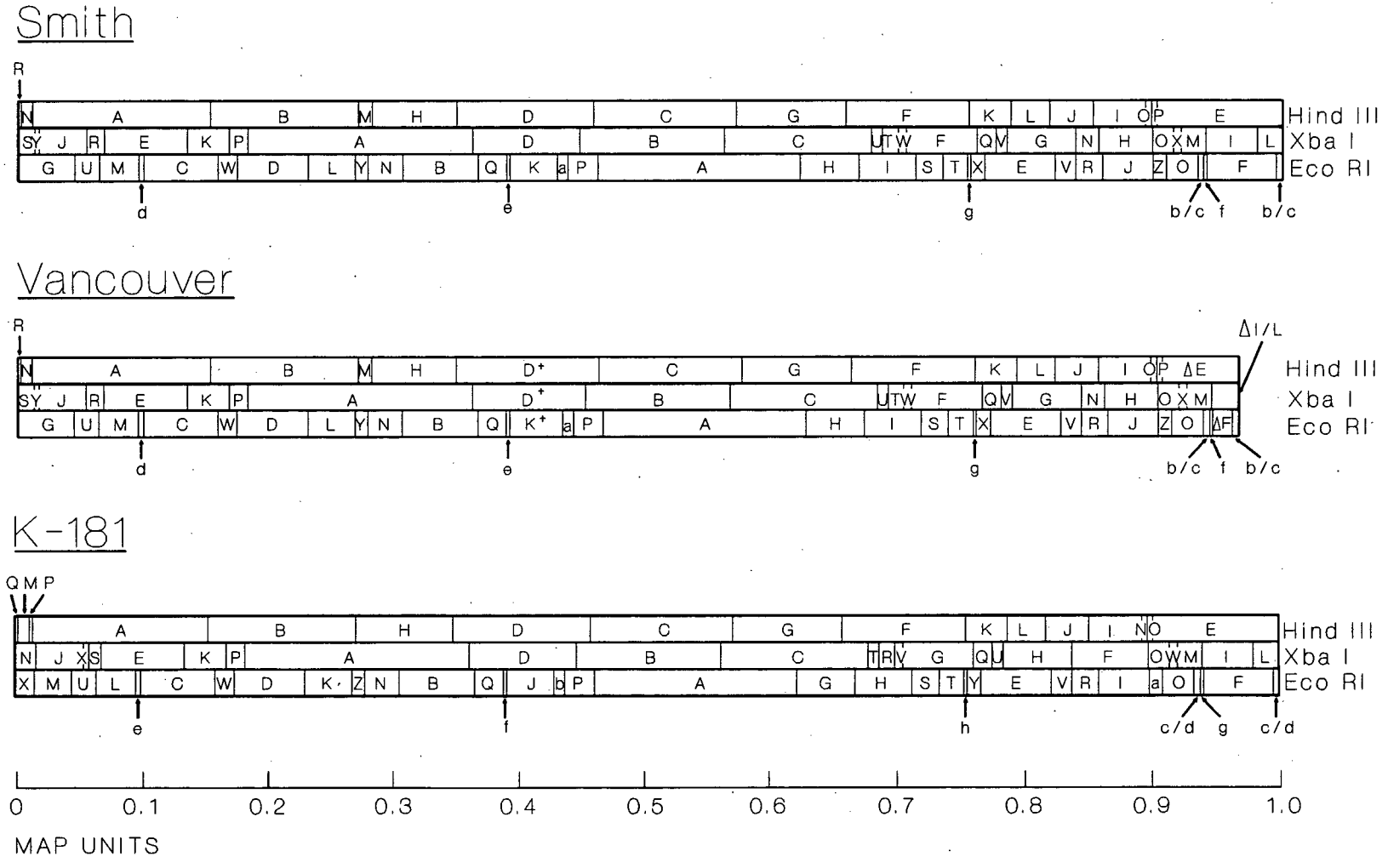
The data compiled from the previous figures were used to construct restriction endonuclease maps of the Smith, Vancouver and K-181 strains of MCMV.

A. **Smith** -- map of the HindIII, XbaI and EcoRI restriction endonuclease cleavage sites for the Smith strain of MCMV. **Vancouver** -- map of the HindIII, XbaI and EcoRI restriction endonuclease cleavage sites for the Vancouver strain of MCMV. **K-181** -- map of the HindIII, XbaI and EcoRI restriction endonuclease cleavage sites for the K-181 strain of MCMV.

B. **HindIII** -- comparison of the HindIII restriction endonuclease cleavage sites for the K-181, Smith and Vancouver strains of MCMV. **XbaI** -- comparison of the XbaI restriction endonuclease cleavage sites for the K-181, Smith and Vancouver strains of MCMV. **EcoRI** -- comparison of the restriction endonuclease cleavage sites for the K-181, Smith and Vancouver strains of MCMV.

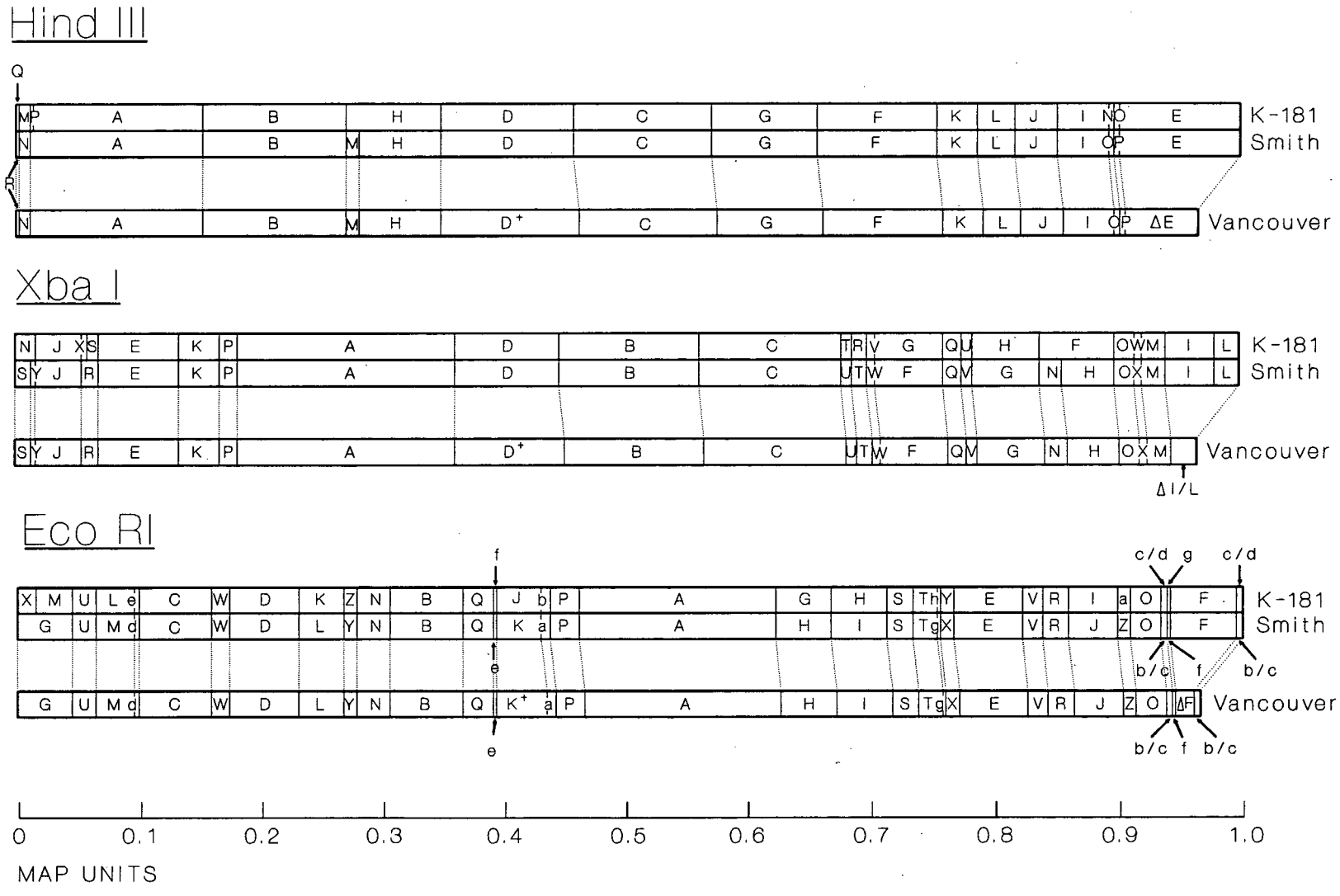
A. RESTRICTION ENDONUCLEASE MAP COMPARISON

Smith, Vancouver, K-181



B. RESTRICTION ENDONUCLEASE MAP COMPARISON

HindIII, XbaI, EcoRI

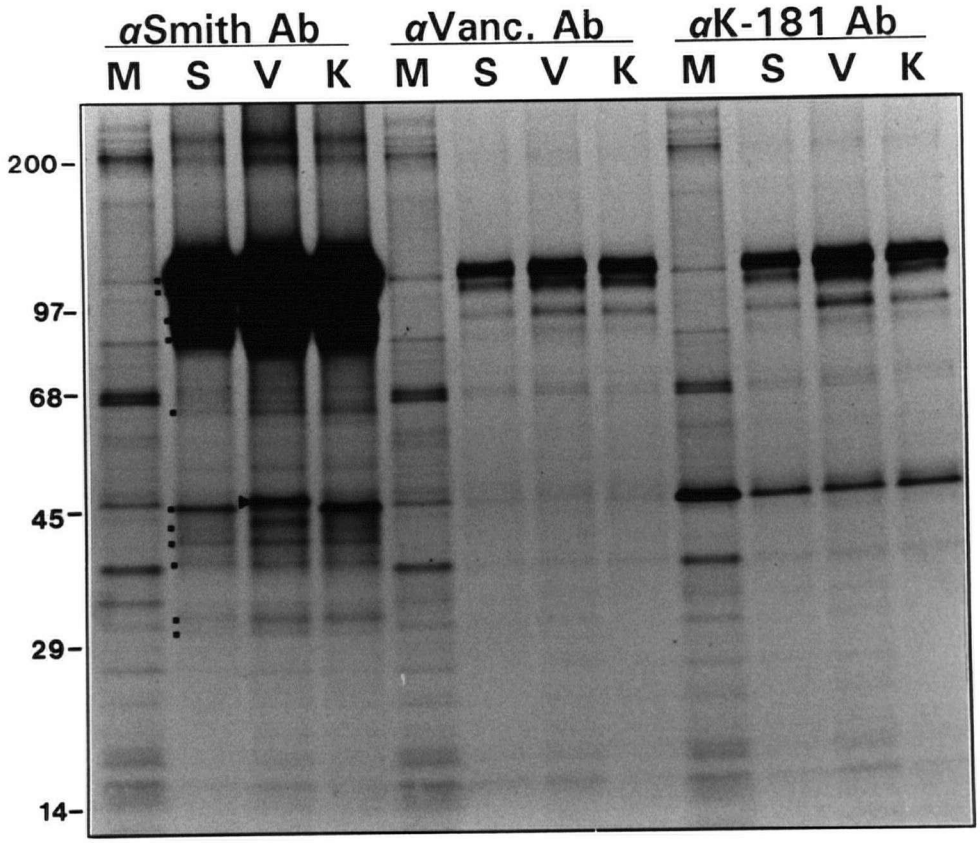


VIRAL PROTEIN ANALYSIS

Experiments were conducted to determine whether the deletion of 9.4 kb of DNA in the Vancouver strain of MCMV included genes expressed in the parental Smith strain, and also whether the differences in the restriction endonuclease cleavage profile of the Smith and K-181 strains were translated into differences in protein production. Immune precipitation of [^{35}S]-labelled proteins with serum from MCMV-infected mice was used to reduce the background of host proteins following PAGE. The three phases of MCMV replication, immediate early, early, and late, were analyzed separately. In addition, the infected cell lysates used for each experiment were divided into four aliquots and precipitated with anti-Smith, anti-Vancouver or anti-K-181 serum and with antiserum from mice immunised with sonicated control cell.

Immediate Early Proteins: PAGE of immune precipitated IE proteins is shown in Figure 18. It is evident that the anti-Smith mouse serum precipitated more virus-specific proteins than the anti-Vancouver or anti-K-181 serum [Fig. 18, overloaded S V K lanes -- αSmith Ab]. The results of *in vivo* growth of the Smith, Vancouver and K-181 strains demonstrated that the Smith strain was able to replicate in the visceral organs to a higher titre than either the Vancouver or K-181 strains, and the higher titre of anti-MCMV antibody from Smith-infected mice was probably a reflection of this. Eleven IE virus-specific proteins, ranging in size from 108 to 30 kDa were detected [Fig. 18, ▪; Table IV] including the 3 major immediate early proteins (approximately 108, 106 and 94 kDa in size). Forty-nine and 46 kDa IE proteins were detected in Vancouver strain-infected cells compared to 47 and 45 kDa in the Smith and K-181 strains [Fig. 18, ▸; Table V]. No differences in Smith and K-181 IE infected cell proteins were detected.

FIGURE 18: IMMEDIATE EARLY PROTEIN EXPRESSION OF MCMV



3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell in the presence of 50 μg/ml of CH. At 6 hours post infection the cells were washed, and then labelled with 50 μCi/ml of [^{35}S]methionine for 2 hours in the presence of 10 μg/ml of ActD. The cells were then lysed and the insoluble material was pelleted by centrifugation while the supernatant was used for immune precipitation analysis. The supernatant was divided into four aliquots and subjected to immune precipitation with serum from mock-infected mice (not shown), Smith-infected mice, Vancouver-infected mice or K-181-infected mice. The immune precipitated proteins were then subjected to PAGE analysis using 8-20% gradient gels. **M**=Mock, **S**=Smith, **V**=Vancouver, **K**=K-181; **αSmith Ab**= antiserum raised in mice infected with Smith virus; **αVanc. Ab**=antiserum raised in mice infected with Vancouver virus; **αK-181 Ab**=antiserum raised in mice infected with K-181 virus; ▪=position of proteins from Smith-infected cells; ▸=Vancouver protein with altered mobility. Protein size standards (kDa) are indicated to the left of the figure.

TABLE IV: SUMMARY OF PROTEINS PRECIPITATED WITH ANTI-SMITH SERUM SMITH, VANCOUVER AND K-181

Immediate Early			Early			Late		
Smith	Vanc.	K-181	Smith	Vanc.	K-181	Smith	Vanc.	K-181
108	108	108	208	208	208	208	208	208
106	106	106	156	156	156	149	149	149
94	94	94	143	143	146	130	130	130
87	87	87	138	138	141	126	126	126
64	64	64	108	108	108	110	110	110
47	49	47	93	93	93	89	89	89
45	46	45	85	85	85	82	82	82
44	44	44	69	69	69	78	78	78
39	39	39	64	64	64	64	64	64
33	33	33	56	56	55	62	62	62
30	30	30	53	53	54	56	56	55
			48	48	48	53	53	54
			46	46	46	48	48	48
			44	44	44	46	46	46
			42	-	42	42	-	42
			40	40	39	40	40	39
						32	32	32

Protein sizes (kDa) are approximate. Differences in size between strains represent differences in mobility detected following PAGE.

TABLE V: SUMMARY OF PROTEIN DIFFERENCES SMITH, VANCOUVER AND K-181

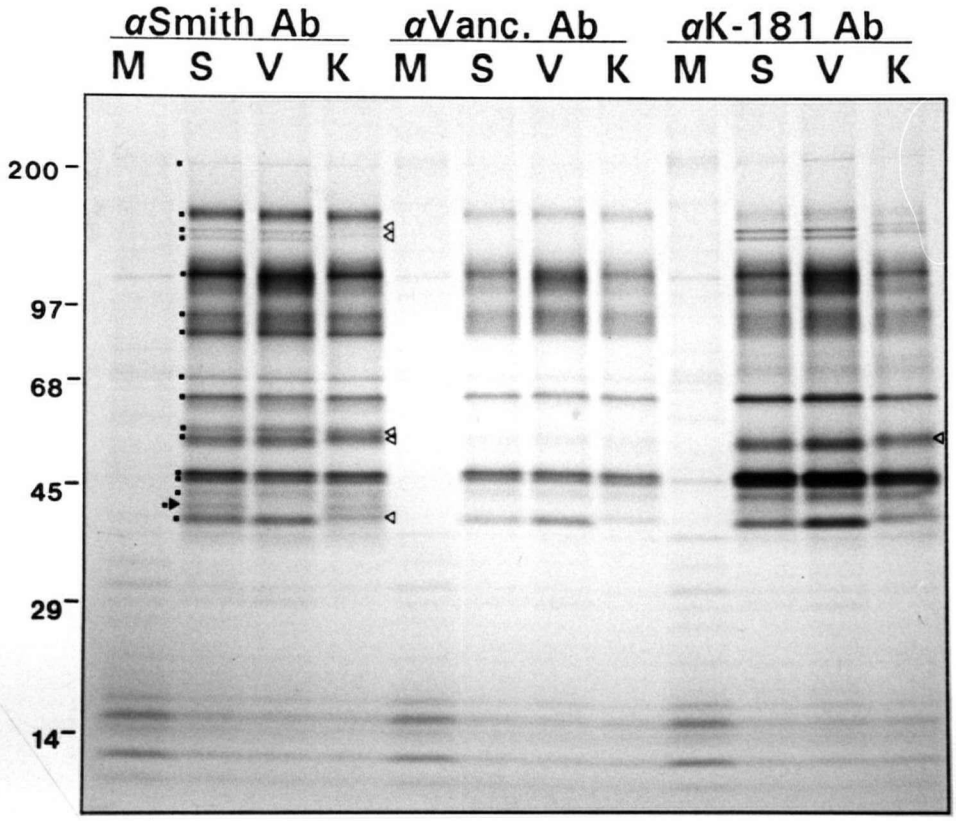
VANCOUVER	SMITH	K-181
Immediate Early		
49	47	47
46	45	45
Early		
-	43	42
143	143	146
138	138	141
56	56	55
53	53	54
39	39	40
Late		
-	42	42
56	56	55
53	53	54
39	39	40

Protein sizes (kDa) are approximate. Differences between strains reflect differences in mobility following PAGE.

Early Proteins: Analysis of immune precipitated E proteins [Fig. 19] revealed differences between all three strains. Sixteen proteins ranging in size from 208 - 40 kDa were detected in the Smith-infected cells [Fig. 19, ▪; Table IV]. The only difference between the Smith and Vancouver proteins was the absence in the Vancouver strain of a 42 kDa protein which was present in both the Smith and K-181 strains [Fig. 19, ▸; Table V]. The anti-Smith serum precipitated this protein in the Smith and K-181 strains, but not in the Vancouver strain. Moreover, the protein was also weakly detected in the Smith and K-181 samples precipitated with the anti-K-181 serum, but not in any of the samples precipitated with the anti-Vancouver serum. This suggests that the protein was not present in cells infected with the Vancouver strain; not only was it not precipitated from cells infected *in vitro* but it was not made *in vivo* as evidenced by a lack of antibody production against this protein in Vancouver-infected mice. There were a number of other E proteins which were not precipitated by the anti-Vancouver serum. It is possible that the lack of the 42 kDa protein in anti-Vancouver infected cells may be attributed to the same phenomenon which resulted in the lack of these other E proteins when the anti-Vancouver serum was used. However, this hypothesis is not favoured because these other proteins were detected in Vancouver-infected cells when the stronger anti-Smith serum was used.

A number of small differences between the Smith and K-181 E proteins could be seen following immune precipitation with anti-Smith serum [Fig. 19, ◃]. There was a shift in the migration of two high molecular weight proteins, 146 and 141 kDa, in the K-181 infected cells compared to the 143 and 138 kDa proteins in the Smith and Vancouver infected cells. In addition, there appeared to be a shift in the molecular mass of two proteins, 55 and 54 kDa in the K-181 sample, from a mass of 56 and 53 kDa in the Smith and Vancouver samples. Only the smaller of the two proteins was precipited by the anti-K-181 serum. Finally, there was an

FIGURE 19: EARLY PROTEIN EXPRESSION OF MCMV



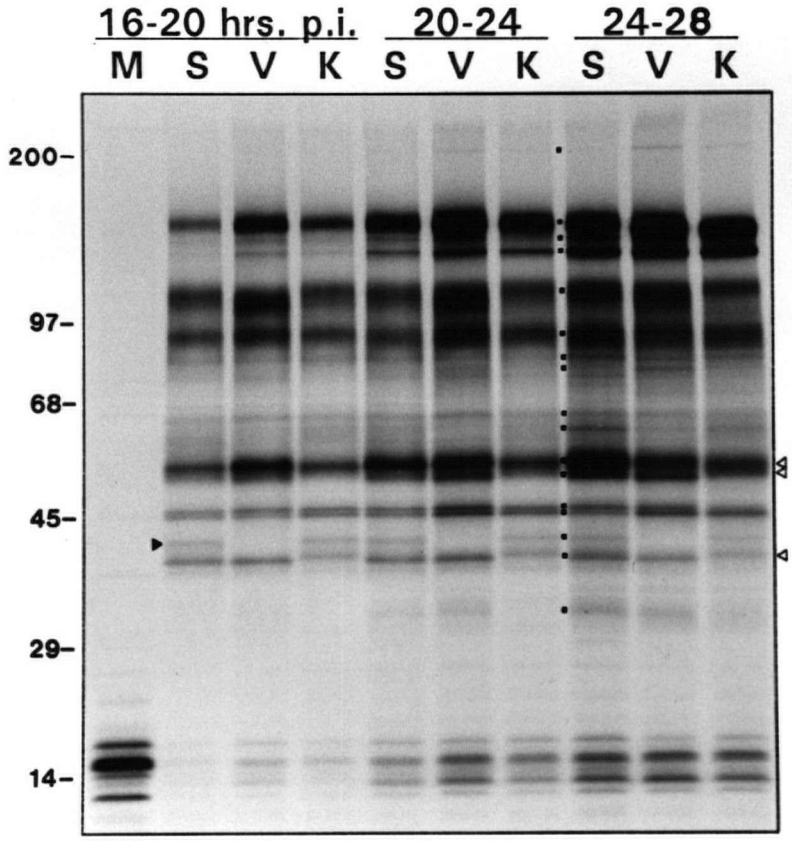
3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell. At 6 hours post infection, 50 μg/ml of cycloheximide was added to enhance early transcript accumulation. 4 hours following addition of CH, the cells were washed, and then labelled with 50 μCi/ml of [^{35}S]methionine for 2 hours in the presence of 50 μg/ml of PFA. The cells were then lysed and the insoluble material was pelleted by centrifugation while the supernatant was used for immune precipitation analysis. The supernatant was divided into four aliquots and subjected to immune precipitation with serum from Smith-infected mice, Vancouver-infected mice or K-181-infected mice. The immune precipitated proteins were subjected to PAGE analysis through 8-20% gradient gels. **M**=Mock, **S**=Smith, **V**=Vancouver, **K**=K-181, **αSmith Ab**= antiserum raised in mice infected with Smith virus; **αVanc. Ab**=antiserum raised in mice infected with Vancouver virus; **αK-181 Ab**=antiserum raised in mice infected with K-181 virus; ▪=position of proteins from Smith-infected cells; ▸=protein missing in the Vancouver strain; ◂=K-181 protein with altered mobility. Protein size standards (kDa) are indicated to the left of the figure.

apparent shift in the migration of a 39 kDa protein, found in Smith and Vancouver samples, to 40 kDa protein in the K-181-infected cells [Table V].

Late Proteins: Immune precipitation analysis of the L proteins was conducted with cells pulse-labelled from 16-20, 20-24 and 24-28 hours post-infection. Seventeen infected cell proteins ranging from 208 to 32 kDa in size were detected in the Smith-infected cells following immune precipitation with the anti-Smith serum and PAGE [Fig. 20A, ▪; Table IV]. The only difference between the Smith- and Vancouver-infected cells was the absence in the Vancouver strain of the 42 kDa protein present at E and L times post-infection in the Smith and K-181 infected cells [Fig. 20A, ▸]. As before, this protein was not detected in immune precipitation experiments using the anti-Vancouver serum [Fig. 20B, ▸].

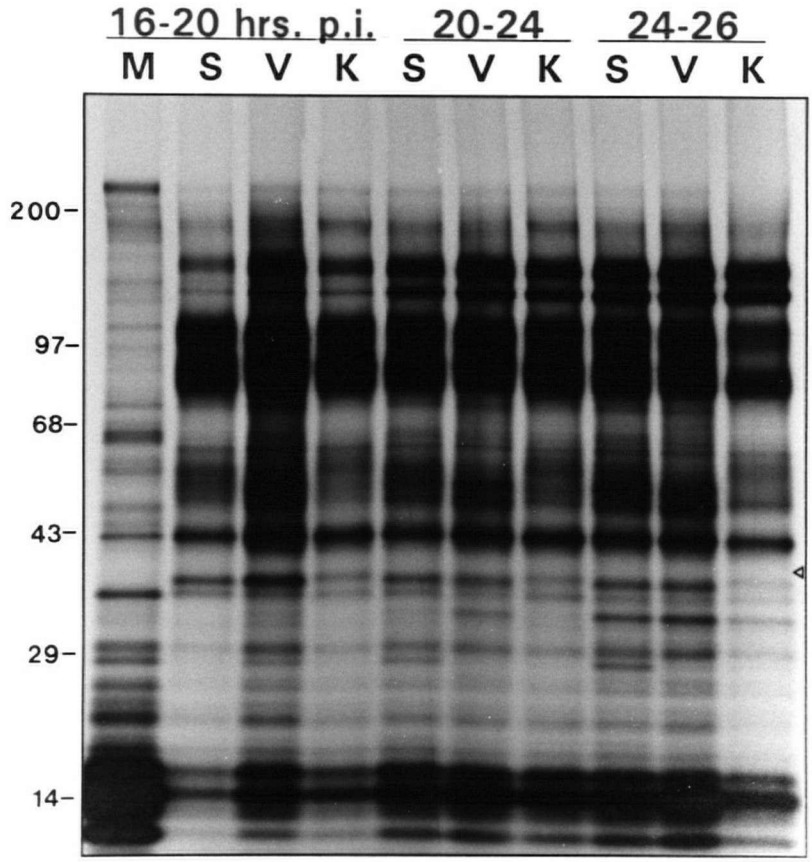
No additional differences between the Smith and K-181 infected cells were detected at late times post-infection. The 55 and 54 kDa proteins were still detected at late times post-infection in K-181 infected cells [Fig. 20A, ◃] as opposed to the 56 and 53 kDa proteins detected in Smith and Vancouver-infected cells. Similarly, the 40 kDa protein was still detected at late times post-infection in K-181 infected cells [Fig. 20A, ◃] as opposed to the 39 kDa protein detected in Smith and Vancouver infected cells [Table V].

FIGURE 20A: LATE PROTEIN EXPRESSION OF MCMV ANTI-SMITH SERUM



3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell. At 16, 20, and 24 hours post infection, cells were labelled with 50 μCi/ml of [^{35}S]methionine for 4 hours. The cells were then lysed and the insoluble material was pelleted by centrifugation while the supernatant was used for immune precipitation analysis. The supernatant was divided into four aliquots and subjected to immune precipitation with serum from Smith-infected mice [Fig. 21A], or Vancouver-infected mice [Fig. 21B]. The immune precipitated proteins were subjected to PAGE analysis through 8-20% gels. **M**=Mock, **S**=Smith, **V**=Vancouver, **K**=K-181; labelling times are indicated at the top of the lanes; ▪=position of proteins from Smith-infected cells; ▸=protein missing in the Vancouver strain; ◂=K-181 proteins with altered mobility. Protein size standards (kDa) are indicated to the left of the figure.

FIGURE 20B: LATE PROTEIN EXPRESSION OF MCMV ANTI-VANCOUVER SERUM



8-20% PAGE analysis of late protein samples immune precipitated with antiserum raised in Vancouver infected mice. **M**=Mock, **S**=Smith, **V**=Vancouver, **K**=K-181; labelling times are indicated at the top of the lanes; ▸ =position of missing Vancouver protein from [A]. Protein size standards (kDa) are indicated to the left of the figure.

SECTION II

RESCUE OF THE SGG PHENOTYPE IN THE VANCOUVER STRAIN OF MCMV

The ability of cytomegaloviruses to grow in the salivary glands is important as a mechanism of dissemination through the population, and as a reservoir of latent virus. As a step towards understanding the pathogenesis of MCMV in the salivary glands, recombinants were generated to rescue the missing gene function of the Vancouver strain required for the salivary gland growth (Sgg^+ phenotype) of MCMV in mice. The Sgg^+ recombinants were then enriched by passage *in vivo*, plaque purified, and analyzed for their restriction endonuclease profile and protein production.

GENERATION OF RECOMBINANT MCMV

Transfectable DNA was isolated and titrated in tissue culture to determine the optimum concentration for virus production. Recombinant Vancouver-strain virus was isolated following co-transfection of "target" whole Vancouver-strain genomic DNA with "effector" DNA comprising Smith-strain DNA fragments generated by restriction endonuclease cleavage. Alternatively, cloned MCMV fragments were used as effector DNA. HindIII and EcoRI were used to cleave the parental Smith DNA, facilitating random intracellular recombination events following co-transfection. These two enzymes were chosen because the region of the Smith strain which is deleted in the Vancouver genome does not contain a cleavage site for either one. Both were used in case the cleavage site of one of them intersected the gene of interest. Cloned MCMV HindIII *D* and *E* fragments were also used as effector DNA in co-transfection experiments, since the Vancouver strain of MCMV has an insertion in the HindIII *D* fragment and a deletion of part of the HindIII *E* fragment, both of which could disrupt/delete an Sgg gene

function. One co-transfection using both the HindIII *D* and *E* fragments was also included in case both areas of the genome were involved in the Sgg$^-$ phenotype.

Two methods of co-transfection were used, **B/G** (Mocarski, personal communication) and **J** (Chen & Okayama, 1988). A range of effector DNA concentrations yielded infectious virus for each co-transfection experiment (Table VI). Cleaved Smith strain DNA alone was transfected to control for incomplete digestion. The virus-infected cell cultures were harvested after CPE had spread to involve the entire monolayer, allowing for amplification of potential recombinants.

The cells from positive wells for each co-transfection experiment were pooled and injected intraperitoneally into mice. Cells from negative control wells were also pooled and injected as a further control for parental Smith-strain virus (Thompson et al., 1983). A negative control animal inoculated with virus from cells transfected with Vancouver-strain genomic DNA was also included to ensure that an artefact of the transfection process itself would not render the virus Sgg$^+$. Similarly, a positive control animal was injected with virus from cells transfected with Smith strain genomic DNA to ensure that the progeny virus was still able to replicate in mouse salivary glands. Salivary glands from 10 out of 13 animals were recovered following sacrifice at 13 days post-inoculation. Three animals died between 4-7 days post-inoculation and the salivary glands from 2 out of 3 of these animals were recovered. The salivary glands were homogenized and assayed for infectious virus (Table VII). No virus was detected in the mock (sonicated 3T3 cells), control or Vancouver-inoculated mice, while infectious virus was isolated from the animal inoculated with the Smith strain. Of the co-transfection groups, infectious virus was isolated from the salivary glands of 4 animals (designated by method-target/effector); **B/G-V/E', B/G-V/D+E', J-V/Eco, J-V/E'**. One isolate per mouse was designated as recombinant virus (R1 through R4 respectively) to avoid repeat isolation of the same recombinant (Thompson et al., 1983). The recombinant virus was

TABLE VI: *IN VITRO* CO-TRANSFECTION RESULTS

Method 1 (B/G)

Target DNA -- Vancouver Strain DNA at a concentration of 0.5μg/well

Effector* DNA Type\Conc	20μg	10μg	5μg	1μg	0.5μg	Control***
Hind	-**	-	+	+	+	-
Eco	-	-	+	+	+	-
	5μg	1μg	0.5μg	0.1μg	0.05μg	Control
D	+	+	+	+	+	-
E'	+	+	+	+	+	-
D+E'	-	-	+	+	+	-

Method 2 (J)

Target DNA -- Vancouver Strain DNA at a concentration of 5μg/well

Effector* DNA Type\Conc	20μg	10μg	5μg	1μg	0.5μg	Control***
Hind	+**	+	+	+	+	-
Eco	+	+	+	+	+	-
	5μg	1μg	0.5μg	0.1μg	0.05μg	Control
D	+	+	+	+	+	-
E	+	+	+	+	+	-

* Effector DNA type designations as follows:
Hind = HindIII-cut Smith DNA
Eco = EcoRI-cut Smith DNA
D = cloned HindIII-*D* fragment DNA
E' = cloned HindIII-*E'* fragment DNA

** Presence (+) or absence (-) of cytopathic effects after 10 days in tissue culture

*** Control consisted of transfection with the highest concentration of effector DNA alone

TABLE VII: RECOMBINANT VIRUS PRODUCTION *IN VIVO* SELECTION RESULTS

Co-transfection Pool	+/- Virus Isolation From Salivary Glands*	Recombinant Designation
Mock	-	
Control	-	
Vancouver	-	
Smith	+	
B/G-V/Hind	ND**	
B/G-V/Eco	-	
B/G-V/D	-	
B/G-V/E'	+	R1
B/G-V/D+E'	+	R2
J-V/Hind	-***	
J-V/Eco	+****	R3
J-V/D	-	
J-V/E'	+	R4

* Animals were sacrificed at 13 days post inoculation unless noted otherwise

** Salivary glands not recovered from this animal

*** This animal had been dead for at least 24 hrs. -- tissue was quite necrotic

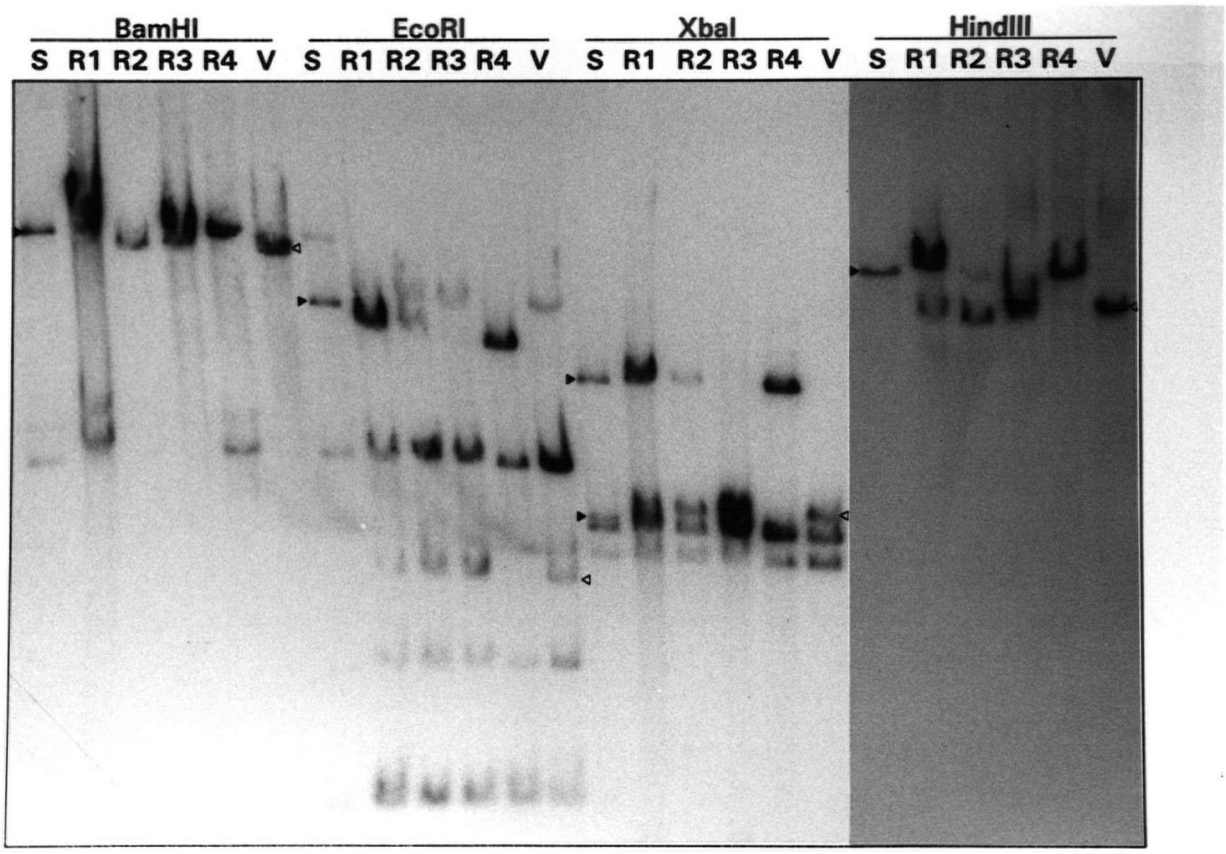
**** Virus was recovered from liver, kidney, spleen and salivary glands

amplified by replication in cells in tissue culture and the supernatant was harvested for infectious virus when CPE was maximal. The infected cells from these cultures were harvested for DNA analysis.

DNA ANALYSIS OF RECOMBINANT VIRUS

As 3 out of 4 of the recombinants involved the cloned HindIII *E* fragment, while the 4th had the potential to involve this region, viral DNA was screened for the HindIII *E* fragment. Whole cellular DNA was cleaved with restriction endonuclease and subjected to agarose gel electrophoresis followed by Southern blotting and hybridization to the radiolabelled HindIII *E* fragment [Fig. 21]. Smith and Vancouver DNA were included for comparison -- Smith-specific fragments are marked with ▸, while Vancouver-specific fragments are marked with ◂. R4 appeared to be a recombinant virus in which the deletion in the HindIII *E* fragment characteristic of the Vancouver strain was regained, and thus its enzyme cleavage profile matched that of the Smith strain. The size of the Smith-specific fragment in the R4 isolate digested with EcoRI was smaller than that seen in the Smith strain. However this was probably an artefact in that the migration of the two Smith-specific fragments of the R4 isolate cleaved with XbaI were identical to those in the Smith marker lane. R1 and R2 resembled a mixed culture of Smith and Vancouver strain MCMV, while R3 showed no evidence of Smith-specific fragments [Fig. 21, XbaI]. Southern blots for all four enzymes are included to show that the bands characteristic of both the Smith and Vancouver strains present in the R1 and R2 isolates were not a result of incomplete digestion of sample or other artefacts of the experiment. The R3 potential recombinant was isolated from the salivary glands of a mouse which died 5 days post-inoculation, and it is probable that the virus isolated was blood-borne rather than actually replicating in the salivary gland since at this stage of infection MCMV can be recovered from

FIGURE 21: SOUTHERN BLOT ANALYSIS OF POOLED RECOMBINANT VIRUS DNA



Salivary gland homogenates from mice inoculated with recombinant virus pools were used to infect 3T3-L1 cells. The DNA from infected cells was isolated once 100% CPE was observed. The DNA was then subjected to restriction endonuclease cleavage and electrophoresed through 0.8% agarose, before being transferred to a hybond membrane by Southern blotting. The filter was hybridized to a cloned **HindIII** ***E'*** fragment insert which was labelled with [^{32}P]dCTP ($1X10^6$ cpm/ml). **S**=Smith, **V**=Vancouver, **R1**=BGV/E', **R2**=BGV/D+E', **R3**= JV/Eco, **R4**=JV/E', ▸=Smith fragment, ◂=Vancouver fragment.

the blood but is not found in the salivary glands. No other evidence for recombination was ever detected in this isolate.

These results also indicate that while infectious virus was not recovered from salivary glands of Vancouver strain-infected mice, inoculation of the pooled recombinant virus resulted in replication of the Vancouver strain in the salivary glands. Replication of Sgg^+ recombinant virus may have provided some *trans*-acting factor which enabled the mutant Vancouver strain virus to replicate. Alternatively, a point mutation in some other region of the genome had restored the ability of the Vancouver strain to grow in the salivary glands of inoculated mice. The latter hypothesis seems unlikely, however, since three separate recombinants which regained the HindIII *E* fragment information regained the ability to grow in the salivary gland. Another alternative is that the Vancouver strain was unable to enter the cells of the salivary gland but during the generation of recombinants, multicapsid virions containing both Vancouver and recombinant DNA were produced. The Vancouver strain DNA was then carried into the salivary gland cells because it was enclosed within an envelope containing the required viral attachment proteins.

Recombinant viruses recovered from the salivary glands were plaque purified twice prior to further analysis. To aid in the selection of recombinants which had regained the full-length HindIII *E* fragment, a probe derived from BamHI-digested Smith DNA, which did not cross-hybridize with Vancouver strain DNA, was used. No such virus was recovered from the R3 recombinant pool.

It was not surprising that none of the unselected HindIII or EcoRI recombinant pools yielded Sgg^+ virus considering the small number of co-transfection plates analyzed (5 per group). Of the 65 transfection plates tested by Thompson et al. (1983), only 5 yielded recombinant virus as determined by neurovirulence. Given a larger sample size, it is likely that

unselected HindIII or EcoRI cleavage fragments generated from the Smith DNA would yield *Sgg*$^+$ recombinants following co-transfection with Vancouver DNA.

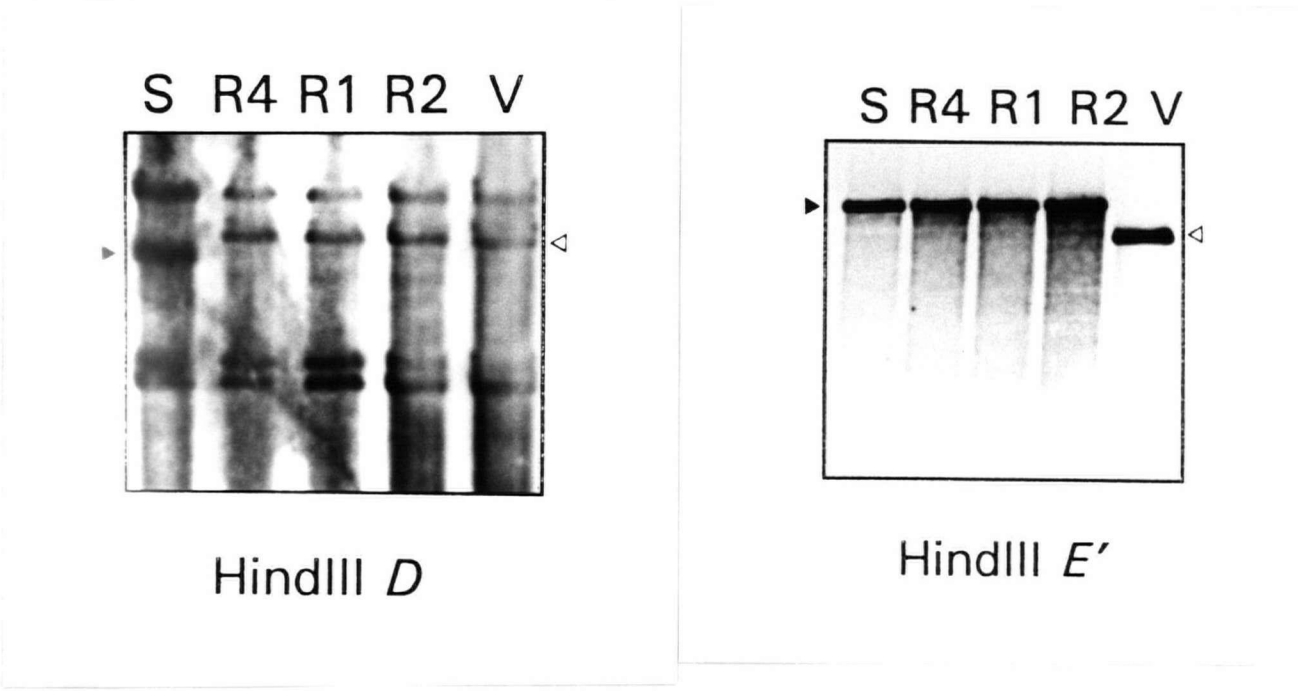
DNA ANALYSIS -- PLAQUE PURIFIED RECOMBINANTS

Viral DNA was isolated from cells infected with plaque-purified virus and subjected to restriction endonuclease digestion followed by agarose gel electrophoresis and Southern blotting. The filters were probed with the cloned HindIII *E'* fragment to establish that the designated recombinants did indeed contain the full-length HindIII *E* DNA typical of the Smith strain [Fig. 22B]. Hybridization to the cloned HindIII *D* fragment probe revealed that all the recombinants contained the insertion into the EcoRI *K* fragment characteristic of the Vancouver strain, thus confirming that they were Vancouver strain recombinants [Fig. 22A]. This also demonstrated that in the case of the co-transfection of Vancouver strain DNA and cloned HindIII *D* and *E'* fragments, it was the recombination of the HindIII *E'* fragment alone which was responsible for the reversion to Sgg$^+$ phenotype. No other changes in the genomes of the recombinants were detected.

PROTEIN ANALYSIS -- PLAQUE PURIFIED RECOMBINANTS

The IE, E and L protein profiles of the recombinants R1, R2 and R4 were analyzed to determine whether or not the recovery of the HindIII *E* fragment was associated with any of the differences seen between the proteins of the Smith and Vancouver strain infected cells. Figure 23 shows the results of PAGE of IE proteins immune precipitated with anti-Smith mouse serum. The three recombinants retained the altered mobility of the 49 kDa protein found in the Vancouver strain-infected cells [Fig. 23, ◂] which was not present at this position in the Smith-infected cells [Fig. 23, ▹]. This indicated that the recombinants were derived from the Vancouver strain of MCMV. It also indicated that the altered mobility of this protein was not

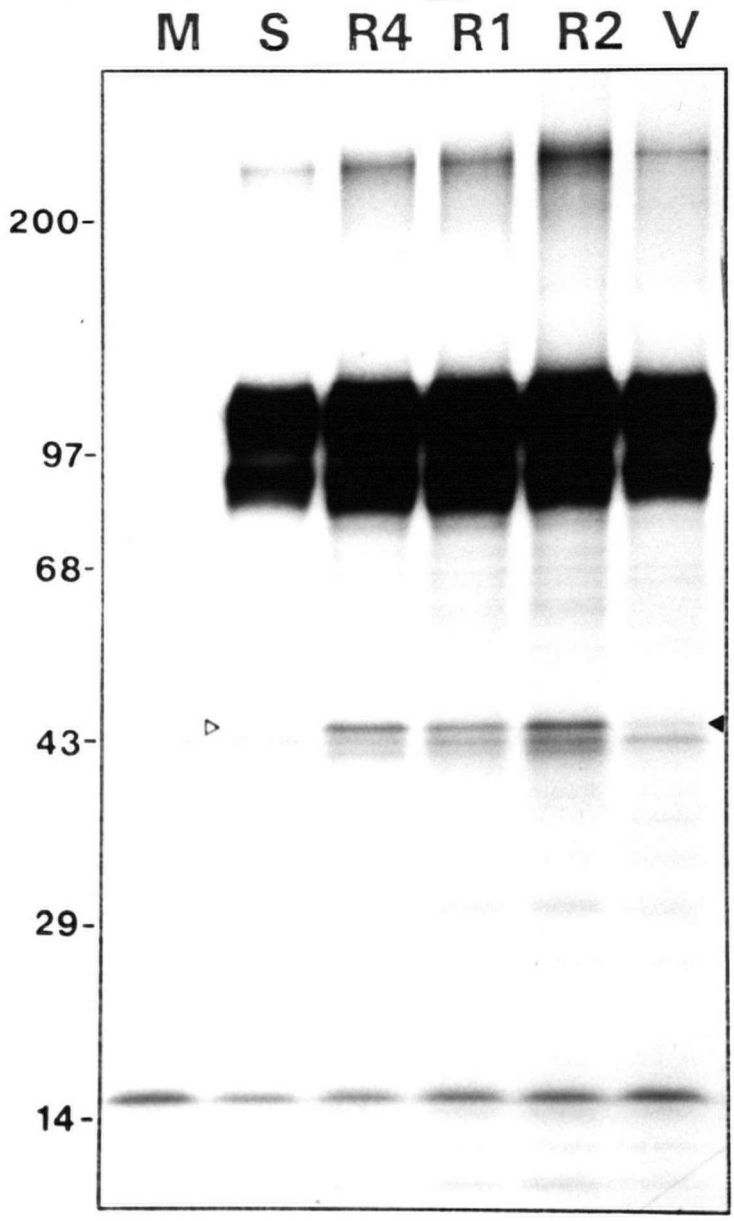
FIGURE 22: SOUTHERN BLOT ANALYSIS OF PLAQUE PURIFIED RECOMBINANT DNA



A **B**

DNA from plaque purified recombinants, and the Smith and Vancouver strains of MCMV, was subjected to restriction endonuclease digestion with EcoRI **[A]** or HindIII **[B]** and electrophoresis through 0.7% agarose. The DNA was transferred to a Hybond membrane by Southern blotting, and the filter was hybridized to a cloned HindIII *D* fragment insert **[A]** or a cloned HindIII *E'* fragment insert **[B]** which was labelled with digoxygenin and identified using a chemiluminescent detection system. **S**=Smith, **V**=Vancouver, **R1**=BGV/E', **R2**=BGV/D+E', **R4**=JV/E', ▸=Smith-specific fragment, ◂=Vancouver-specific fragment.

FIGURE 23: IMMEDIATE EARLY PROTEIN EXPRESSION OF PLAQUE PURIFIED RECOMBINANTS

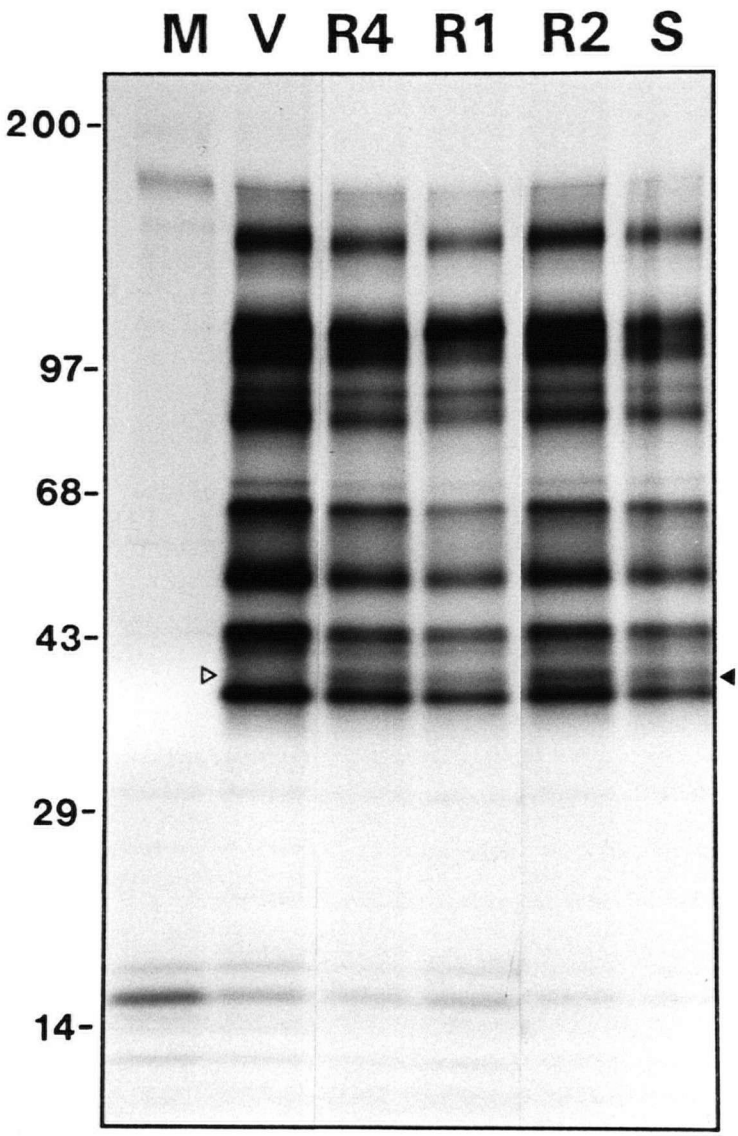


3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell in the presence of 50 μg/ml of CH. At 6 hours post infection, cells were washed and labelled with 50 μCi/ml of [^{35}S]methionine for 2 hours in the presence of 10 μg/ml of ActD. The cells were then lysed and the insoluble material pelleted by centrifugation. Supernatants were reserved for immune precipitation analysis using antiserum raised in Smith virus-infected mice. The immune precipitated proteins were subjected to PAGE through an 8-20% polyacrylamide gradient gel. **M**=Mock, **S**=Smith, **V**=Vancouver, **R1**=BGV/E', **R2**=BGV/D+E', **R4**=JV/E', ◂= Vancouver-specific IE protein, ▸= no Smith strain-specific protein at this position. Protein size standards (kDa) are indicated to the left of the figure.

a result of the deletion of 9.4 kb of DNA from the HindIII *E* region of Vancouver genome. Since HindIII *D* fragment recombinants were never isolated, further studies are required to determine whether or not the 0.9 kb insertion into this region was responsible for this difference between the Smith and Vancouver strains of MCMV.

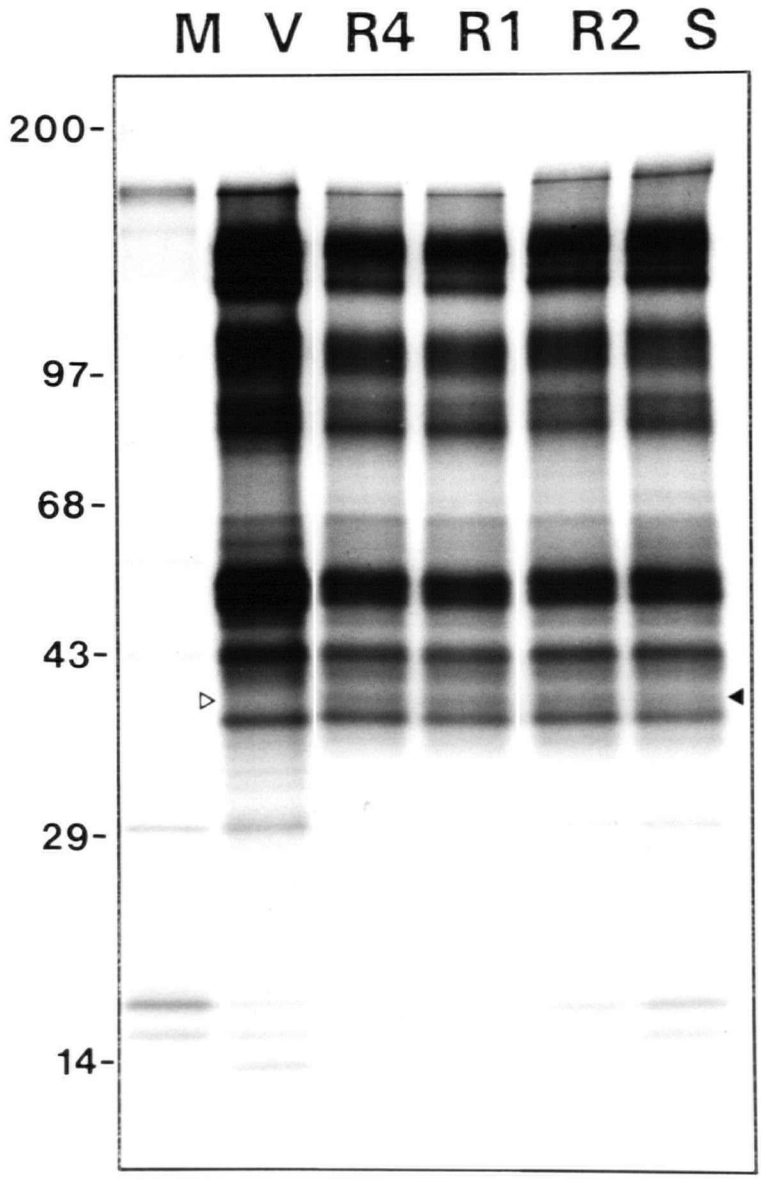
PAGE analysis of E protein expression in MCMV-infected cells revealed that the recombinants regained the expression of the 42 kDa protein missing in Vancouver-strain infected cells [Fig. 24]. Similarly, PAGE of proteins from cells isolated at late times post-infection showed that the recombinants once again expressed the 42 kDa protein not detected in Vancouver-infected cells [Fig. 25]. This suggested that the 42 kDa protein was encoded within the HindIII *E* fragment of MCMV.

FIGURE 24: EARLY PROTEIN EXPRESSION OF PLAQUE PURIFIED RECOMBINANTS



3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell. At 6 hours post infection, 50 μg/ml of cycloheximide was added to enhance early transcript accumulation. 4 hours following addition of CH, the cells were washed and then labelled with 50 μCi/ml of [^{35}S]methionine for 2 hours in the presence of 50 μg/ml of PFA. The cells were lysed and the insoluble material was pelleted by centrifugation. Supernatants were reserved for immune precipitation analysis using antiserum raised in Smith virus-infected mice. The immune precipitated proteins were subjected to PAGE through an 8-20% polyacrylamide gradient gel. **M**=Mock, **S**=Smith, **V**=Vancouver, **R1**=BGV/E', **R2**=BGV/D+E', **R4**=JV/E', ◂ = Smith-specific E protein, ▸ = no Vancouver strain-specific protein at this position. Protein size standards (kDa) are indicated to the left of the figure.

FIGURE 25: LATE PROTEIN EXPRESSION OF PLAQUE PURIFIED RECOMBINANTS



3T3-L1 cells were infected with MCMV at an MOI of 5 pfu/cell. At 20 hours post infection, cells were labelled with 50 μCi/ml of [^{35}S]methionine for 4 hours. The cells were then lysed and the insoluble material was pelleted by centrifugation. Supernatants were reserved for immune precipitation analysis using antiserum raised in Smith virus-infected mice. The immune precipitated proteins were subjected to PAGE through an 8-20% polyacrylamide gradient gel. **M**=Mock, **S**=Smith, **V**=Vancouver, **R1**=BGV/E', **R2**=BGV/D+E', **R4**=JV/E', ◂ = Smith-specific E protein, ▸ = no Vancouver strain-specific protein at this position. Protein size standards (kDa) are indicated to the left of the figure.

SUMMARY AND CONCLUSIONS

A comparison of the Smith, Vancouver and K-181 strains of MCMV revealed differences *in vivo* and *in vitro*. In this report, the Vancouver strain of MCMV was demonstrated to be a mutant which arose following multiple *in vitro* passage of the parental Smith strain of MCMV. The most striking biological difference between the two strains was the inability of the Vancouver strain to grow in the salivary glands of inoculated mice [Fig. 4B, page 58]. Coupled with this was a decrease in the ability of the Vancouver strain to grow in the spleen of mice [Fig. 4A, page 58]. In contrast, the Vancouver strain was able to grow better in tissue culture than the Smith and K-181 strains [Fig. 7, page 64]. The Vancouver strain's ability to replicate more quickly, and to produce a greater yield of virus per cycle, was responsible for the larger plaque size of the Vancouver versus the Smith strain of MCMV.

The onset of DNA replication was similar in cells infected with all three strains and viral replication remained cell-cycle dependent. The 5-15% increase in the rate of growth of the Vancouver strain compared to the Smith and K-181 strains was probably due, at least in part, to the 4% decrease in the overall size of the Vancouver genome. The Vancouver strain was shown to harbour a 9.4 kb deletion which spanned the XbaI *I/L* junction of the parental Smith strain, and a 0.9 kb insertion which mapped to the EcoRI *K* fragment. Since the HindIII *D* region of the genome is believed to contain an origin of DNA replication for MCMV (M.J. Masse, personal communication), the insertion into this region may somehow impart an advantage during replication in tissue culture. Additional studies are required to address this hypothesis.

Many interesting features of MCMV replication in salivary glands have been reported. Rapid attenuation of virulent MCMV by passage *in vitro*, and subsequent rapid restoration of virulence by passage of virus *in vivo* has been described (Osborn and Walker, 1970). Since

then, it has been determined that the salivary glands are the only source of virulent virus, and that virus isolated from any other organ is as avirulent as that passaged in tissue culture. Genetic changes in the virus are probably not responsible because the reversion to and from virulence is manifest in a single passage. Some feature of replication in the salivary glands themselves probably makes the virus more virulent. A feature unique to salivary glands is the production of primarily monocapsid virions as opposed to the multicapsid virions produced by cells of other organs, or tissue culture (Hudson et al., 1976). Attempts to purify monocapsid virions by filtration of tissue-culture-passaged MCMV did not render a preparation of virus equally virulent to that of salivary gland passaged MCMV indicating that other factor(s) are involved. *In vivo* passage of virus through salivary glands was found to increase binding of the virus to target cells through sialic acid residues in addition to the N-acetylglucosamine residues recognized by attenuated virus. In addition, neuraminidase treatment of the host cells prior to infection yielded equivalent binding of both virulent and attenuated virus (Ravindranath and Graves, 1990). The ability to recognize sialic acid residues on the host cell following passage through the salivary gland may be the mechanism of rapid reversion to virulence by salivary gland passaged MCMV.

The salivary glands are also a major site of persistence of cytomegaloviruses, and the site from which chronic shedding of virus can lead to dissemination through the population. Whether or not the ability to persist in salivary glands relates to host functions, such as a decrease in immune surveillance in the salivary glands, or virus-induced functions remains to be elucidated. Perhaps persistence in salivary glands is related to the increased virulence of the progeny virus compared to that produced following virus replication in other tissues.

Three recombinant viruses were recovered in which the Sgg^+ phenotype had been rescued relative to the Vancouver strain of MCMV. All three of the recombinants recovered the full-size HindIII *E* region of the genome and maintained the insertion into the HindIII *D* region of the

Vancouver genome. Thus the genotypic location of the defect responsible for the *Sgg*$^-$ phenotype of the Vancouver strain of MCMV maps to 0.960 to 0.995 map units of the Smith virus genome.

The *Sgg*$^-$ phenotype has been previously described by a number of investigators. Temperature-sensitive mutants of MCMV (Sandford and Burns, 1988) showed little or no ability to replicate in the salivary glands of inoculated mice; however, the defect resposible for this phenotype was not further characterized. In a previous study, a temperature-sensitive mutant lacking productive chronic infection in the salivary glands was described (Tonari and Minamishima, 1983). Rescue of the temperature sensitivity, but not the attenuation for salivary gland growth of the isolate, demonstrated that these phenotypes were caused by independent mutations. Once again, the defect responsible for the attenuation for growth in the salivary glands was not identified (Kumura et al., 1990). A deletion of 300 bp in an E gene (*Sgg1*) which maps to the HindIII *J* region of the MCMV genome resulted in a virus indistinguishable from wild-type for growth characteristics in tissue culture, or in the liver and spleen of inoculated mice (Manning, 1990). However, this mutant showed a greatly reduced ability to grow in the salivary glands and lungs of mice. These data, and those presented in this thesis, imply that more than one gene is responsible for controlling the growth of MCMV in the salivary glands of mice, although interaction between several genomic regions can not be ruled out. Further studies are needed to show whether or not the mutations responsible for the *Sgg*$^-$ phenotype in the mutants described by Sandford and Burns (1988) and Kumura et al. (1990) map to the HindIII *J* or *E* region of the genome, or whether there are other regions of the genome involved in MCMV pathogenesis in the salivary glands.

Two differences in the proteins of the Vancouver strain were detected when compared with the proteins of the Smith strain. A possible shift to 49 kDa in a 47 kDa IE protein detected in the parental strain, and the absence of a 42 kDa protein present during E and L times post-infection in the Smith strain (Table V). Restoration of the full-length HindIII *E* region of the

genome [Fig. 22, page 107] correlated with the appearance in the recombinants of a 42 kDa protein present during E and L times post-infection in Smith and K-181 infected cells, but not in the Vancouver-infected cells [Fig. 24, 25, pages 110, 111]. The larger 49 kDa IE protein characteristic of Vancouver-infected cells was still detectable in the recombinants [Fig. 23, page 108], suggesting that this protein was not involved in the *Sgg*$^-$ phenotype of the Vancouver strain. The synthesis of two proteins, 78 and 37 kDa in size, were linked to the *Sgg1* gene identified by Manning (Manning, 1990). The 37 kDa protein could be the 42 kDa protein identified in this report. This would suggest that the 37 kDa protein was encoded in the HindIII *E* fragment, while the 78 kDa protein was encoded in the HindIII *J* fragment and that both gene products were required for growth of MCMV in salivary glands, since inactivation of either gene resulted in reduced replication of MCMV in salivary glands.

There are at least three hypotheses which explain the ability of the Vancouver strain to replicate in the salivary glands following inoculation of mice with the pooled recombinant virus. A point mutation in a region other than that deleted in the Vancouver strain was dismissed since the likelihood of three identical point mutations occuring following three separate recombination events would be very small. It is possible that the Vancouver strain is unable to enter the cells of the salivary glands because it lacks the required viral attachment protein(s). Alternatively, it may not encode a protein required for DNA replication in the salivary glands. As the 42 kDa protein is expressed at early times post-infection, it is probable that it is involved in DNA replication or macromolecular synthesis, rather than virus structure. Further investigation to determine whether or not the Vancouver strain can enter the cells of the salivary glands would be required to test this hypothesis.

The results presented here have identified a region of the MCMV genome involved in the growth of MCMV in the salivary glands of mice. It will be important to determine whether this genomic region encodes the 42 kDa protein restored by generation of recombinants, and

whether this protein is involved in the replication of MCMV in the salivary glands of mice. Further studies could involve sequencing of this region to identify potential coding regions and expression of such proteins. Site-specific mutagenesis of genes in this region followed by recombination into the wild type Smith strain could be used to try to recreate the Sgg$^-$ phenotype of the Vancouver strain to investigate the gene(s) encoded by this region and their function.

There are also differences between the Smith and K-181 strains of MCMV. The increased virulence of the K-181 strain in comparison to the Smith strain has been previously documented (Misra & Hudson, 1980), and was confirmed here as an increased yield of virus from the salivary glands of infected mice, and a decrease in the LD_{50} from that reported for the Smith strain of MCMV. At the molecular level, the differences in the restriction endonuclease cleavage pattern between the Smith and K-181 strains were mapped [Fig. 17, page 87], and it was determined that while the map published by Ebeling et al. (1983) is that of the Smith strain, the map published by Mercer et al. (1983) is most likely that of the K-181 strain. While the differences in the restriction endonuclease cleavage sites were mainly confined to 0.0 to 0.6 map units of the genome without any large insertions or deletions being detected, this does not preclude more subtle differences in the genome which may be reflected in the increased virulence of the K-181 strain. Indeed, in contrast to the report of Hudson et al. (1988), a number of differences were detected in the proteins of the Smith and K-181 strains at early and late times post-infection (Table V, page 93). At early times, proteins of 146, 141, 55, 54 and 40 kDa were detected in K-181 infected cells, while proteins of 143, 138, 56, 53, and 39 kDa were detected in Smith or Vancouver infected cells. At late times post-infection, the apparent shift in the 54, 53 and 40 kDa K-181 proteins was still detected. Such subtle changes in the apparent molecular weight of the K-181 proteins compared to those of the Smith strain, could be a reflection of the differences in the restriction endonuclease cleavage sites between the two strains, or point mutations in other regions of the genome. In addition, the slight differences

in proteins could be responsible for the differences in the virulence of the Smith and K-181 strains. For example, it has been shown that a single point mutation in the rabies virus glycoprotein (G), which results in an amino acid substitution of glutamine or glycine for arginine at position 333, is responsible for the loss of neurovirulence of this virus (Seif et al., 1985).

The need to distinguish between the Smith and K-181 strains of MCMV is important, especially when an examination of biological properties is considered. A comparison of salivary gland-passaged MCMV with tissue-culture-passaged MCMV should not be further complicated by the use of different strains of virus (Sammons & Sweet, 1989). The Vancouver strain of MCMV has been used as the "Smith" strain for a number of years. For example, in a recent comparison of the "Smith" and K-181 strains of MCMV (Hudson et al., 1988), the restriction endonuclease cleavage fragments of the "Smith" strain (ie. the presence of the HindIII ΔE fragment) clearly identifies this virus as the Vancouver strain. However, the densitometer tracings comparing the HindIII restriction endonuclease cleavage fragments of the Smith and K-181 strains of MCMV first reported by Misra and Hudson (1980) do indeed match the fragments obtained in this report for the Smith and K-181 strains. Thus, at some point following the work published initially (Misra & Hudson, 1980), the Smith virus used in that laboratory became adapted to tissue culture and lost its *in vivo* virulence. It is also probable that work on the MCMV IE and E proteins (Walker & Hudson, 1987a; 1987b) was conducted using the Vancouver strain, and not the Smith strain of MCMV.

It is becoming increasingly clear that results based solely on experiments in tissue culture may not reflect the virulence of viruses *in vivo* (Manning, 1990; Cullen, 1991). For example, recent work on the *nef* gene of simian immunodifficiency virus (SIV) showed that a mutant containing a deletion in the *nef* gene replicated in a manner indistinguishable from wild type in primary monkey T cells and macrophages. However, monkeys infected with the *nef* deletion mutants gave rise to barely detectable levels of virus replication *in vivo*, and did not develop

AIDS, while monkeys infected with SIV containing an intact *nef* gene gave rise to high titres of virus and went on to develop AIDS. Thus a functional *nef* gene is an important virulence factor for SIV *in vivo*, and its function for maintaining replication *in vivo* is not reproduced by current assays of virus replication *in vitro* (Kestler et al., 1991).

Similarly, the investigation of the Vancouver strain of MCMV demonstrates that although the virus is able to grow efficiently in culture, it exhibits reduced virulence *in vivo*. The interaction of the virus with the host inflammatory and immune reactions are not represented in tissue culture, but they must play a role in the replication of virus *in vivo*. Because pathogenesis of recombinant HCMV can not be studied in humans, an alternative is justified. The similarity of HCMV and MCMV in terms of pathogenesis and gene co-linearity makes the MCMV model system an attractive alternative for the investigation of virulence mechanisms operative *in vivo*.

Characterization of the genes responsible for the replication of cytomegalovirus in the salivary glands would be useful for designing a live attenuated vaccine strain. The deletion of part of the HindIII *E* fragment of the Smith strain of MCMV resulted in a virus which not only failed to replicate in the salivary glands of infected animals, but also showed decreased replication in all other organs examined. Production of antiserum against the Vancouver strain revealed, however, that a significant immune response is initiated following inoculation. Most importantly, immune precipitation experiments using the anti-Vancouver serum demonstrated that a response to the major IE protein was illicited. This is significant since the immune response to this protein has been demonstrated to be sufficient to protect mice from lethal challenge with wild type virus (Koszinowski et al., 1987a; 1987b; Reddehase et al., 1986). The deletion of a gene involved in the *Sgg* phenotype of MCMV would also result in reduced dissemination of cytomegalovirus in the population. Further investigation of the immune response to such a virus strain is warranted.

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APPENDIX -- CALCULATION OF DNA RESTRICTION FRAGMENT SIZES

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10 DIM WT(50), DIST(50), PROD(50), DWT(50), DDIST(50), DPROD(50), C(50), D(50),
PREDWT(50), WTDEV(50), PERC(50), PREDLEN(50)
20 CLS
25 INPUT "Would you like the results printed";P$
26 CLS
30 INPUT "How many sets of data (weight and distance, 24 max)";N
40 IF N=0 GOTO 750
50 IF N>50 GOTO 26
60 GOSUB 5000
70 CLS
80 INPUT "How many predicted length calculations";NU
90 IF NU=0 GOTO 120
100 IF NU>50 GOTO 70
110 GOSUB 6000
120 SWT=0
130 SDIST=0
140 SPROD=0
150 FOR I=1 TO N
160 SWT=SWT+WT(I)
170 SDIST=SDIST+DIST(I)
180 PROD(I)=WT(I)*DIST(I)
190 SPROD=SPROD+PROD(I)
200 NEXT I
210 MWT=SWT/N
220 MDIST=SDIST/N
230 MPROD=SPROD/N
240 FOR I=1 TO N
250 DWT(I)=WT(I)-MWT
260 DDIST(I)=DIST(I)-MDIST
270 DPROD(I)=PROD(I)-MPROD
280 NEXT I
290 CSSL=0
300 CSSM=0
310 CSCPML=0
320 CSPMLL=0
330 CSPMLM=0
340 FOR I=1 TO N
350 CSSL=CSSL+DWT(I)^2
360 CSSM=CSSM+DDIST(I)^2
370 CSCPML=CSCPML+DWT(I)*DDIST(I)
380 CSPMLL=CSPMLL+DPROD(I)*DWT(I)
390 CSPMLM=CSPMLM+DPROD(I)*DDIST(I)
400 NEXT I
410 DET=CSSL*CSSM-CSCPML^2
420 M0=(CSSM*CSPMLL-CSCPML*CSPMLM)/DET
430 L0=(-CSCPML*CSPMLL+CSSL*CSPMLM)/DET
440 SC=0
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```
450 SSC=0
460 FOR I=1 TO N
470 C(I)=(WT(I)-L0)*(DIST(I)-M0)
480 SC=SC+(C(I)-C(1))
490 SSC=SSC+(C(I)-C(1))^2
500 NEXT I
510 CBAR=SC/N+C(1)
520 SDC=SQR((SSC-SC^2/N)/(N-1))
530 PRINT"STD LEN     DIST       PRED LEN    DEV          %D          C(I)"
540 PRINT
550 SD=0
560 SSD=0
570 FOR I=1 TO N
580 PREDWT(I)=CBAR/(DIST(I)-M0)+L0
590 WTDEV(I)=WT(I)-PREDWT(I)
600 PERC(I)=100*WTDEV(I)/WT(I)
610 SD=SD+WTDEV(I)
620 SSD=SSD+WTDEV(I)^2
630 PRINT WT(I) TAB(12) DIST(I) TAB(24) PREDWT(I) TAB(36) WTDEV(I) TAB(48)
PERC(I) TAB(62) C(I)
640 NEXT I
650 SDWT=SQR((SSD-SD^2/N)/(N-3))
660 PRINT
670 PRINT "M0 = ";M0 TAB(25) "L0 = ";L0 TAB(50) "CBAR = ";CBAR
680 PRINT
690 PRINT "SC = ";SDC TAB(25) "SD = ";SDWT
700 PRINT
710 FOR I=1 TO NU
720 PREDLEN(I)=CBAR/(D(I)-M0)+L0
730 PRINT "For a distance of  ";D(I); TAB(28);"predict a length of ";PREDLEN(I)
740 NEXT I
745 IF P$="Y" GOTO 8000
746 IF P$="y" GOTO 8000
750 GOTO 8140
5000 CLS
5010 FOR I=1 TO N
5020 LOCATE I,10
5030 PRINT I;"  Weight = ";
5040 INPUT WT(I)
5050 LOCATE I,40
5060 PRINT  I;"  Distance = ";
5070 INPUT DIST(I)
5080 NEXT I
5090 INPUT "Are the values correct";ANS$
5100 IF ANS$="Y" GOTO 5240
5101 IF ANS$="y" GOTO 5240
5110 INPUT "Which value is wrong";I
5120 PRINT "Re-enter weight ";I;
5130 INPUT WT(I)
```

```
5140 PRINT "Re-enter distance ";I;
5150 INPUT DIST(I)
5160 CLS
5170 FOR I=1 TO N
5180 LOCATE I,10
5190 PRINT I;"  Weight = ";WT(I)
5200 LOCATE I,40
5210 PRINT I;"  Distance = ";DIST(I)
5220 NEXT I
5230 GOTO 5090
5240 CLS
5250 RETURN
6000 CLS
6010 FOR I=1 TO NU
6020 LOCATE I,10
6030 PRINT I;"  Distance = ";
6040 INPUT D(I)
6050 NEXT I
6060 INPUT "Are the values correct";ANS$
6070 IF ANS$="Y" GOTO 6170
6071 IF ANS$="y" GOTO 6170
6080 INPUT "Which value is wrong";I
6090 PRINT "Re-enter distance ";I;
6100 INPUT D(I)
6110 CLS
6120 FOR I=1 TO NU
6130 LOCATE I,10
6140 PRINT I;"  Distance = ";D(I)
6150 NEXT I
6160 GOTO 6060
6170 CLS
6180 RETURN
8000 LPRINT "STD LEN     DIST        PRED LEN    DEV          %D          C(I)"
8010 LPRINT
8020 FOR I=1 TO N
8030 LPRINT WT(I) TAB(12) DIST(I) TAB(24) PREDWT(I) TAB(36) WTDEV(I) TAB(48)
PERC(I) TAB(62) C(I)
8040 NEXT I
8050 LPRINT
8060 LPRINT "M0 = ";M0 TAB(25) "L0 = ";L0 TAB(50) "CBAR = ";CBAR
8070 LPRINT
8080 LPRINT "SC = ";SDC TAB(25) "SD = ";SDWT
8090 LPRINT
8100 FOR I=1 TO NU
8105 PREDLEN(I)=CBAR/(D(I)-M0)+L0
8110 LPRINT "For a distance of  ";D(I); TAB(28);"predict a length of ";PREDLEN(I)
8120 NEXT I
8125 LPRINT CHR$(12)
8140 INPUT "Run the program again with the same standards";REP$
```

```
8150 IF REP$="Y" GOTO 70
8160 IF REP$="y" GOTO 70
8170 INPUT "Run the program again with new standards";REP$
8180 IF REP$="Y" GOTO 10
8190 IF REP$="y" GOTO 10
8200 CLS
8210 LOCATE 12, 38
8220 PRINT "BYE"
8225 FOR X=1 TO 10:PRINT:NEXT X
8230 END
```

From Schaffer & Sederoff, 1981; adapted by Jeff Bonnycastle