ANALYSES OF IMMEDIATE EARLY AND EARLY TRANSCRIPTS AND MAJOR EARLY REGION, E10, OF MURINE CYTOMEGALOVIRUS

By

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ABSTRACT

Murine cytomegalovirus (MCMV) is used as a biological model for human cytomegalovirus (HCMV). Latency, persistence and reactivation are some of the important aspects of the murine model that share analogies with human CMV infections. In order to elucidate the molecular mechanisms leading to these events, in-depth analyses of the murine model are required at the transcriptional level. During the MCMV replication cycle, there is a sequential expression of different regions of the viral genome, hence the transcripts are divided into three kinetic classes; the immediate early (IE), early (E) and late (L). This study presents the analyses of MCMV (Smith strain) transcripts of the major IE and E transcriptional units, and a more detail analysis of one of the major E regions, E10.

The IE and E transcripts were studied by probing them with Complementary DNAs (cDNAs). The cDNAs were prepared from mRNA isolated from the IE and E phases of the viral replication cycle and cloned into the bacteriophage Lambda gt10. Ten E cDNAs were mapped to specific locations of the virus genome, and these represented transcripts from the major E regions in HindIII fragments A, B, E, F, and I-J.

Five E cDNAs, each representing a different major E region, and two IE cDNAs representing the major IE region, were applied as probes in one of the studies to determine the relative transcript levels during the course of infection of 3T3L1 fibroblast cells with MCMV.

The major E transcriptional units were investigated further in a study where Northern blots of RNAs, isolated from different phases of the viral replication cycle, were probed with the five E cDNAs. This study revealed transcripts that were temporally regulated since they were present only during the E and usually L phases of the viral replication cycle. In addition, the quantities of these transcripts varied depending on the phase.

However, all five cDNAs detected more than one transcript which indicates complex splicing events, overlapping genes, multiple initiation sites and/or the presence of gene(s) in the complementary DNA strand.

One of the E cDNAs, E10, corresponding to a transcript from a major E region of HindIII fragment I-J, was selected for further analysis. The E10 cDNA detected four transcripts of 9.5, 6.9, 4.7 and 2.1 kb in size, which were found to be transcribed from the same DNA strand. The DNA sequence of this E10 cDNA was determined and shown to contain 3223 nucleotides, however it lacked a polyadenylation signal and a poly A tract at the 3' end. The missing 3' terminus, designated as E10-A, was isolated using the polymerase chain reaction (PCR) method and its DNA sequence of 1422 nucleotides was also determined. The combined sequence of E10 and E10-A (total of 4606 nucleotides) was designated as E10-C and is presented in this thesis.

The E10-C cDNA (4.6kbp) most likely represents the 4.7 kb transcript. The E10-C cDNA sequence has one minor and one major open reading frame (ORF). The minor ORF is initiated by the first ATG triplet (nucleotide position 114) while the major ORF is initiated by the second triplet (nucleotide position 155). Since the sequence preceeding the second ATG triplet is in "good context" with regard to the translation initiation consensus sequence, it is most likely that the major ORF is translated. The major ORF (3600 bases) encodes a 1200 amino acid polypeptide, the putative E10 protein of approximately 135 kd in size. A protein close to that size was detected in one of the experiments in which RNAs, that were hybrid-selected by the E10 cDNA and eluted, were translated in vitro. The putative E10 protein lacks homology with any other protein in the data banks (SWISSPRT and GENPEPT). Portions of the viral genomic fragments HindIII I and J were also sequenced to reveal the orientation of the gene coding for the E10 cDNA and its related transcripts.

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Dedicated to Nosh Vellani

LIST OF ABBREVIATIONS

A	absorbance/wavelength
AIDS	acquired immune deficiency syndrome
A+T	adenine + thymine
ATP	adenosine 5'-triphosphate
bp	base pairs
C	cytosine
°C	degree centigrade
Cat	catalogue
cDNA	complementary DNA
CH	cycloheximide
CPE	cytopathic effect
cpm	counts per minute
Ci	curies
Da	daltons
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
dNTP	deoxyribonucleotide triphosphate mix
dTTP	deoxythimidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ds	double-stranded
DTT	dithiothreitol

E	early
E. coli	Escherichia coli
EDTA	ethylenediamine tetraacetic acid
EtBr	ethidium bromide
FBS	fetal bovine serum
g	gram (s)
G+C	guanine + cytosine
GITC	guanidinium isothiocyanate
HCMV	Human Cytomegalovirus
hr	hour (s)
HSV	Herpes simplex virus
IE	immediate early
IFN	interferon
IPTG	isopropylthiogalactoside
IUdr	5-Iodo-2'-deoxyuridine
kb	kilobases
kbp	kilobasepairs
kd	kilodaltons
mM	millimole
M	moles/litre
MCMV	Murine cytomegalovirus
mg	milligram (s)
ml	millilitre (s)
mRNA	messenger ribonucleic acid
nm	nanometer
p	page number
ORF	open reading frame

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pol	polymerase
poly A^+	polyadenylated
poly A^-	non polyadenylated
RNA	ribonucleic acid
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
rDNA	ribosomal deoxyribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
T	Thymine
TEMED	N,N,N',N'-tetramethylethylene diamine aminomethane
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer ribonucleic acid
UV	ultraviolet
V	volts
VIP	virus induced protein
VISF	viral induced suppressive factor
VRC	vanadyl-ribonucleoside complex
VZV	Varicella Zoster
Xgal	5-bromo 4-chloro 3-indolylgalactoside

1.0 INTRODUCTION

Human cytomegalovirus (HCMV) plays an important etiologic role in human disease. Since cytomegaloviruses (CMVs) are species specific, it is necessary to study CMV pathogenecity in an animal. The most frequent animal model used is the murine CMV (MCMV) infection in mice because it has biological properties similar to those of human infection.

The objective of this project has been to investigate the molecular biology of MCMV infection at the transcriptional level. This section will discuss both HCMV and MCMV [and herpes simplex virus (HSV), where relevant] with the emphasis on the regulation of viral gene expression, pathogenesis and persistent infections.

1.1 HERPESVIRUSES

1.1.1 GENERAL DESCRIPTION:

The Herpesviridae are a family of enveloped viruses with a core containing double stranded (ds) linear DNA, enclosed by an icosahedral capsid of 162 capsomeres (Mathews, 1982). There are nearly 100 known herpesviruses, of which seven have been identified as human pathogens: HSV-1, HSV-2, HCMV, varicella-zoster (VZV), Epstein-Barr virus (EBV), Human B-lymphotropic virus and Human herpes virus-7 (HHV-7) (Salahuddin et al., 1986; Roizman, 1982 1990; Frenkel et al., 1990).

1.1.2 PROPERTIES AND CLASSIFICATION OF HERPESVIRUSES:

In spite of sharing a common morphology, herpesviruses vary in biological properties such as host range, duration of replication cycle, cytopathic effect (CPE), and latency. Therefore, on the basis of their biological properties, herpesviruses are classified into three groups or sub-families:

alphaherpesvirinae, betaherpesvirinae and gammaherpesvirinae (Mathews 1982; Roizman, 1982, 1990).

Alphaherpesvirinae:

Examples such as HSV-1 and HSV-2 are members of the alphaherpesvirinae sub-family. The membership criteria of this group includes a broad host range, relatively short replication cycle, extensive CPE during infection in culture and the capacity to produce latent infection in vivo primarily in sensory ganglia.

Betaherpesvirinae:

CMV belongs to the betaherpesvirinae sub-family. The most important criterion of this group is a limited host range. In vitro, these viruses replicate readily in fibroblasts, but their replication cycle is relatively slow. The term 'cytomegalovirus' reflects on the fact that the infected cells manifest a characteristic CPE with the formation of enlarged multinucleated cells (cytomegalia). Their infection can result in latency in tissues of secretory glands, kidneys, reproductive organs, etc.

Gammaherpesvirinae:

Ebstein-Barr virus is one of many members of the gammaherpesvirinae sub-family. This group of viruses is lymphotropic and has a limited host range. The duration of the replication cycle is variable and infection often results in latency in tissues that are lymphoid in origin.

1.2 ISOLATION OF HCMV AND MCMV

Murine cytomegalovirus (MCMV) was first isolated in 1954 by propagating the virus contained in filtrates of infected mouse salivary glands in cultures

of mouse embryonic fibroblast cells (Smith, 1954). Subsequently, three independent groups isolated HCMV (1956-1957) by employing tissue culture: Smith (1956) isolated the virus from infant salivary gland and infant kidney; Rowe et al. (1956) isolated three strains from human adenoid explant tissue; and Weller et al. (1957) isolated the virus from infant urine and infant liver biopsy.

1.3 PATHOGENESIS OF HCMV AND MCMV

1.3.1 INFECTION AND EPIDEMIOLOGY OF HCMV

The prevalence of HCMV in the general population is high, and it varies with the geographic region, age, socioeconomic status and perhaps host genetic factors. In children and adults HCMV infection is usually asymptomatic, although some adults develop symptoms of mononucleosis (Alford and Britt, 1985). In immunocompromised patients such as AIDS victims, recipients of organ transplants, and patients undergoing chemotherapy, the infection results in severe disease associated with high mortality (Osborn, 1981, Hackman et al.; 1985, Nelson et al., 1988). In addition, congenital infection with HCMV can cause a serious disease referred to as cytomegalic inclusion disease (CID). Classical CID may involve infection of multiple organs, the reticuloendothelial system and the central nervous system (Alford and Britt, 1985).

The incidence of HCMV infection in the general population is high because the spread of the virus occurs either vertically (in utero) or horizontally by the excretion of the virus in secretory fluids such as urine, saliva, breast milk, vaginal secretions, cervical secretions, and semen (Alford et al., 1981; Osborn, 1981; Ho, 1982; Hunter et al., 1985). Other modes of transmission relate to procedures such as blood transfusion (Prince et al.,

1971), leucocyte transfusion (Winston et al., 1980), bone marrow transplant (Hackman et al., 1985) and organ transplants particularly kidney (Naraqi et al., 1977, Chatterjee et al., 1978).

An average of 1% of live infants born in the United States are congenitally infected with HCMV and of these, 5-10% show symptoms of CID (HCMV infection) (studies summarized in Alford and Britt, 1990; Stagno et al., 1982a; Kinney et al., 1985). Many of these congenital infections are believed to have resulted from primary infections acquired during the gestation period. Statistics show that the percentage of seronegative women in childbearing years in the United States varies widely and appears to depend on race and socioeconomic status (Stern et al., 1973; Grant et al., 1981; Stagno et al., 1982b; Kinney et al., 1985). The infection rate is highest amongst the people of lower socioeconomic status in United States and the people of underdeveloped countries and therefore, these groups acquire the infection at an earlier age. Due to its mode of transmission (natal, breastmilk, saliva and urine), those that escape the congenital infection may usually acquire the infection during infancy or childhood (Alford et al., 1981; studies summarized in Alford and Britt, 1985, 1990). Frequently, CMV infects ductal epithelial cells in the renal system and therefore viruria is common. Also, in up to a third of infected infants and children, salivary glands and often the parotid gland are involved. This often leads to a chronic infection as well as to the excretion of the virus in saliva. In general, the spread of the virus may be prevented by good hygiene.

1.3.2 EXPERIMENTAL INFECTION OF MICE WITH MCMV

The pathogenesis of MCMV in mice depends on a variety of factors such as age of the host, infective dose, route of infection, host genetics (mouse

strain), history of virus passage, and the virus strain.

Newborn mice are highly susceptible to lethal infection by MCMV but this susceptibility decreases with age. Neonates infected intraperitoneally show infection in multiple organs such as spleen, liver, pancreas, lungs, kidneys, ovaries and adrenals (Osborn, 1982). In normal adult mice, infected intraperitoneally, the common features of MCMV pathogenesis in the acute phase include general immunosuppression, viremia, thrombocytopenia and inclusion bodies in organs such as the liver, kidney, spleen, ovary, pancreas, and salivary glands (Hudson, 1979; Osborn, 1982). The acute phase is followed by the chronic phase and this transition is marked by a decrease in detectable levels of infectious virus. During this phase, the salivary glands continue to shed a significant amount of virus, followed by lower amounts over a long period of time; however, the duration of shedding depends on the mouse strain (Gonczol et al., 1985; Mercer and Spector, 1986).

There is a significant increase in the degree of resistence to MCMV infection as the animal matures from newborn to weanling. Studies by Hayashi et al. (1985) supports the notion that the ability to resist MCMV infection depends on the protection provided by the natural killer (NK) cells. It is speculated that as the animal matures, the population of the NK cells increase and this reflects on the degree of increased resistance. Furthermore, a study by Ebihara and Minamishima (1984) has shown that the activators (OK-432 and PS-K) of NK cells in susceptible mouse strains, increase resistance to MCMV infection.

In susceptible mice, the size of inoculum can also determine the pattern of

pathogenesis. For example in Balb\c mice infected intraperitoneally, $2X10^6$ pfu is a lethal dose, while $2X10^5$pfu and $2X10^4$pfu are nonlethal doses that produce an acute and asymptomatic infection respectively (Leung et al., 1986). Another important contributing factor to the degree of pathogenesis in susceptible mice is the route of inoculation. The most common route of inoculation in the laboratory is intraperitoneal which usually results in the infection of multiple organs. However, studies have shown that subcutaneous inoculation of adult mice with a low dose of MCMV results in an infection which is limited to the submaxillary gland only (Brody and Craighead, 1974). Also, a lower dose of MCMV inoculation is required to establish a pathologic effect by the intravenous route compared to that required by the intraperitoneal route (Mannini and Medearis, 1961).

Susceptibility to MCMV infection amongst adult mice depends on the host strain type. Two H-2 linked genes $H\text{-}2^k$ and $H\text{-}2^d$ of the major histocompatibility complex, and another set of undefined non-H-2 linked genes are responsible for determining the degree of susceptibility to infection of a particular mouse strain (Chalmer et al., 1977; Grundy et al.,1981; Allen and Shellam, 1984; Quinnan and Manichewitz, 1987). The two distinct traits associated with the $H\text{-}2^k$ haplotype and the non-H-2 linked genes are the resistant genotypes, while the trait associated with $H\text{-}2^d$ haplotype is the susceptible genotype. The $H\text{-}2^d$ susceptible trait is dominant over the $H\text{-}2^k$ resistant trait, while the $H\text{-}2^d$ and non-H-2 linked traits are independent of each other (Allan and Shellam, 1984; Quinnan and Manichewitz, 1987). Studies have also shown that the resistance offered by $H\text{-}2^k$ trait is interferon (INF) dependent while the non-H-2 linked trait is INF independent (Quinnan and Manichewitz, 1987).

Currently, two MCMV strains, the Smith strain and the K-181 strain are used in our laboratory. For reasons unknown, the K-181 strain is more virulent in vivo than the Smith strain. There are also significant differences in their restriction endonuclease digestion patterns (Misra and Hudson, 1980; Hudson et al., 1988). In addition to the virus strain, the passage history is important in the determination of virulence and pathogenesis. MCMV isolated from salivary glands at 2-3 weeks post infection is highly virulent in new born mice; however, the virus becomes attenuated by a single passage in vitro in mouse embryo fibroblasts (Osborn and Walker, 1970; Selgrade et al., 1981). The attenuation is easily reversed to virulence by one passage of the virus through mouse salivary gland in vivo (Osborn and Walker, 1970). The precise mechanism responsible for the rapid attenuation and its reversion to virulence has not been identified. However, a recent study by Ravindranath and Graves (1990) suggests that the shift to virulence in MCMV may be related to the viruses ability to recognize sialic acid residues on a cell receptor.

There are conflicting reports about the possibility of vertical transmission of MCMV in mice. Johnson (1969) suggested that vertical transmission of CMV in mice did not occur, and this was attributed to the presence of three trophoblastic layers in mouse placenta which provides efficient protection to the mouse fetus. However Chantler et al. (1979) indicated that vertical transmission is possible, and that the presence of the virus in offspring may be latent. Horizontal spread of MCMV in mice has also been shown, although close contact is required for transmission as it occurs by the oral route in body secretions such as breastmilk, urine and saliva (Mannini and Medearis, 1961).

MCMV is an immunosuppressive virus and during infection the mouse becomes susceptible to opportunistic infections. Preliminary studies have shown that MCMV appears to infect macrophage-like cells (Loh and Hudson, 1979, 1982) and as a result these mice respond poorly to foreign antigens, mitogens and INF inducers (Hudson, J. B., 1979; Osborn, J. E., 1982). An MCMV-induced suppressive factor (VISF), which suppresses concanavalin A stimulated mitogenesis in spleen cells, appears to be responsible for this immunosuppression phenomenon (Whyte et al., 1987). This factor needs to be characterized further and its precise mode of action determined.

1.4 MOLECULAR BIOLOGY OF HCMV AND MCMV

1.4.1 REPLICATION OF HCMV AND MCMV IN CELL CULTURE

Infection is initiated by attachment of the virus to the cell receptor. The molecules involved in this process in MCMV and HCMV infections are still under investigation. However, evidence suggests that either a class I HLA antigen (Grundy et al., 1987) or another glycoprotein of molecular weight 30kd may be the cell receptor for HCMV (Taylor et al., 1990). As for MCMV, a preliminary study by Ravindranath and Graves (1990) indicates that the virus attaches to the cell by binding to N-acetylglucosamine on the cell surface. Following attachment, virus enters the cytoplasm by fusion of the viral envelope with the plasma membrane and subsequently, the nucleocapsid is transported to the nucleus, where viral transcription, DNA replication and assembly of capsids takes place (Morgan etal., 1968; Kohn, 1985). One of the important characteristics of the herpesviruses family is the mode of replication which occurs in a temporally regulated manner (Mathews, 1982). Accordingly, replication may be divided into three kinetic phases: immediate early (IE), early (E), and late (L).

1.4.1.1 REPLICATION OF HCMV

The replication cycle for HCMV in diploid human fibroblast cultures takes 48 to 72 hr before the release of progeny virus (Stinski, 1977). During the course of replication, there is sequential expression of different areas of the viral genome. The first kinetic class genes, IE, are expressed in the absence of de novo protein synthesis, and are detected at 2 to 4 hr post infection (p.i.) (Stinski, 1978; DeMarchi et al., 1980). IE proteins are regulatory in function and are required for the expression of genes in the next kinetic phase, E, which begins at 2 hr p.i. and proceeds up to 24 hr p.i. (DeMarchi et al., 1980). The E proteins of herpesviruses are mainly involved in DNA synthesis and nucleotide metabolism, and many of these proteins whose functions have been identified are described in reviews by Roizman et al. (1990) for HSV, and Spector et al. (1990) for HCMV.

Viral DNA synthesis commences during the E phase and although it can be detected by 15 to 16 hr p.i., it does not peak until 72 to 96 hr p.i. (St. Jeor and Hutt, 1977; Stinski, 1978). Viral DNA synthesis is thought to occur by a rolling circle mechanism. This notion is supported by the fact that during infection, the HCMV genome termini fuse to form a circle (Lafemina and Hayward, 1983). Furthermore, HCMV DNA sequence has been shown to contain conserved sequence elements analogous to those found in HSV-1 and MCMV (Spate and Mocarski, 1985; Marks and Spector, 1988) which appear to contain sites of DNA cleavage and signal for packaging of herpes viral DNA. Both the E phase and viral DNA synthesis are prerequisites for the expression of L phase, which begins at approximately 24 hr p.i. (Stinski, 1977, 1978; DeMarchi et al., 1980). Late proteins are usually structural in nature and are therefore involved in the assembly process (Honess and Roizman, 1974, 1975). The capsids are assembled in the nucleus, but the

steps involved in this process for herpesviruses have not been defined. Finally, the assembled virions are released from the cell and during this event, they acquire their envelope either from the cell or nuclear membrane (Mathews et al., 1982; Kohr, 1985).

1.4.1.2 REPLICATION OF MCMV

The IE phase of the replicative cycle of MCMV lasts from 0 to 4 hr p.i. and as for other herpes viruses, it is independent of de novo protein synthesis (Chantler and Hudson, 1978). The IE proteins are regulatory in function and are required for the expression of the next kinetic phase, E, which may be detected as early as 2 hr p.i. (Misra et al., 1978; Marks et al., 1983; Keil et al., 1984; Buhler et al., 1990).

Viral DNA synthesis commences later, during the E phase, between 8 to 12 hr p.i. and is thought to occur by the rolling circle mechanism as described for HCMV and HSV (Misra et al., 1978; Chantler and Hudson, 1978; Marks et al., 1983). Evidence similar to that described for HCMV supports this notion: circular viral genomes have been identified in infected cells and the viral DNA termini have been shown to contain the conserved sequence elements common to both HSV and HCMV, which appear to be the sites of cleavage of concatemers and contain the signal for packaging of viral genome (Marks and Spector, 1983; 1988).

The final kinetic phase, L, may be detected by 10 hr p.i. The majority of the L proteins are structural in nature and involved in the packaging and assembly of virions (Chantler and Hudson, 1978). Finally, the progeny virus (Smith strain) begin to be released at approximately 20 hr p.i. (Mossmann & Hudson, 1973). Like other herpesviruses, MCMV acquires its envelope during

the release process either from the nuclear or cellular membrane (Weiland et al., 1986). In general, the pattern of the replication cycle of MCMV closely resembles that of HCMV, although, HCMV replication is relatively slower.

Contrary to HCMV replication which is independent of the cell cycle, MCMV replication is dependent upon the S phase of the cell cycle (Muller and Hudson, 1977a); cells in Go phase do not support viral DNA replication. Despite the presence of a virus induced-DNA polymerase in infected Go phase cells, the viral DNA is unable to replicate, perhaps due to the requirement of a cellular factor which is present only during the S phase (Muller and Hudson, 1978).

1.4.2 STRUCTURES OF THE HCMV AND MCMV GENOMES

1.4.2.1 THE HCMV GENOME

The HCMV genome is a double stranded linear DNA (150 x 10^6 daltons) equivalent to 240 kilobasepairs (kbp) in length and has a G+C content of 57% (Kilpatrick and Huang, 1977; DeMarchi et al., 1978; Geelen et al., 1978; Lakeman and Osborn, 1979; Stinski et al., 1979). Various strains of HCMV show polymorphisms in their restriction endonuclease sites and this may be due to genetic variation resulting from different passage history (Westrate et al., 1980; Oram et al., 1982; Spector et al., 1982). The complete nucleotide sequence of HCMV (AD169) has been determined and upon analysis has revealed over 200 ORFs, nearly three times the number of genes in HSV (Chee et al., 1990). However, less than 50 HCMV gene products have been analysed and mapped (summary of studies in Spector et al., 1990). Some regions of the HCMV DNA contain nucleotide sequences homologous to some of

those of human, murine, sea urchin and 28S rRNA gene (Penden et al., 1982; Reger et al., 1984; Shaw et al., 1985), which were presumably acquired by the virus during the course of evolution.

Although similar in structure, the HCMV genome is 1.5 times the size of the HSV genome. The HCMV genome consists of 2 unique sequences, the unique long (U_L) and the unique short (U_S) sequences, which represent 82% and 18% of the genome, respectively (Westrate et al., 1980; DeMarchi, 1981; Oram et al., 1982; Spector et al., 1982). The unique sequences are bound by terminal and internal repeat regions; U_L is bound by TR_L and IR_L, and U_S is bound by TR_S and IR_S (Figure 1a). Since the unique sequences can invert relative to each other, there are 4 genome isomers which are found in equimolar concentrations (Figure 1b) (Westrate et al., 1980; DeMarchi, 1981). The significance of such a complex DNA structure has not been elucidated.

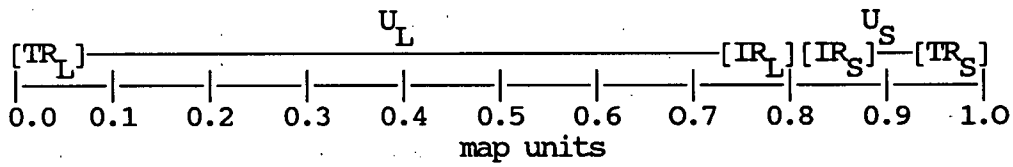
1.4.2.2 THE MCMV GENOME

The MCMV genome is linear double stranded DNA with a molecular weight of 132 x 10^6 daltons equivalent to 240 kbp in length (Mossman and Hudson, 1973; Lakeman and Osborn, 1979; Mercer et al., 1983). As shown in Figure 1c, the MCMV genome, unlike HCMV, is a single, long and unique sequence lacking terminal or internal repeat sequences (Ebeling et al., 1983; Mercer et al., 1983). The overall G+C content of the MCMV genome is 59%, but variation in this content exists in specific regions of the genome (Mossman and Hudson, 1973, 1974).

Genetics maps for the MCMV genome have been constructed using restriction

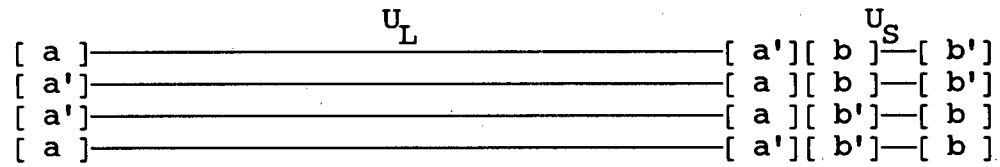
Figure 1: Organization of Human and Murine CMV genomes

a. Organization of HCMV genome



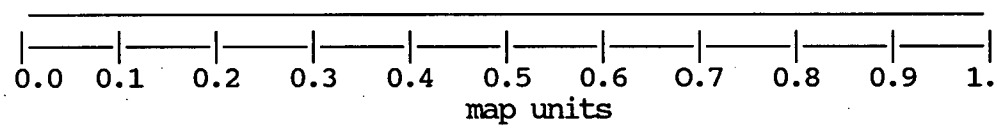
The HCMV genome consists of two unique sequences, the unique long (U_L) and unique short (U_S) sequences. The unique sequences are bound by the terminal and internal repeats. The U_L is bound by TR_L and IR_L, and U_S is bound by TR_S and IR_S [data taken from Mach et al. (1990)].

b. Representation of isomeric forms of HCMV genome



The unique sequences invert in relation to each other at the junction where the internal repeats meet. The inversion results in four possible isomeric forms as illustrated above [data taken from Mach et al. (1990)].

c. Organization of MCMV genome



MCMV genome is a single, long and unique sequence lacking terminal and internal repeat sequences (Ebeling et al., 1983; Mercer et al., 1983).

enzyme digests, by two research groups. Mercer et al. (1983) determined the map for restriction enzymes HindIII and EcoR1, while Ebeling et al. (1983) determined the map for restriction enzymes HindIII, EcoR1 and XbaI (Figure 2). These two maps differ slightly, and this may be attributed to genetic variation resulting from continuous passage of the virus in different laboratories. The genomes of two commonly used strains in our laboratory, the Smith and K181 also differ slightly in their endonuclease digestion patterns (Misra et al., 1980; Hudson et al., 1988). The mapping of the precise differences in their genomes is still in progress (Boname, unpublished data).

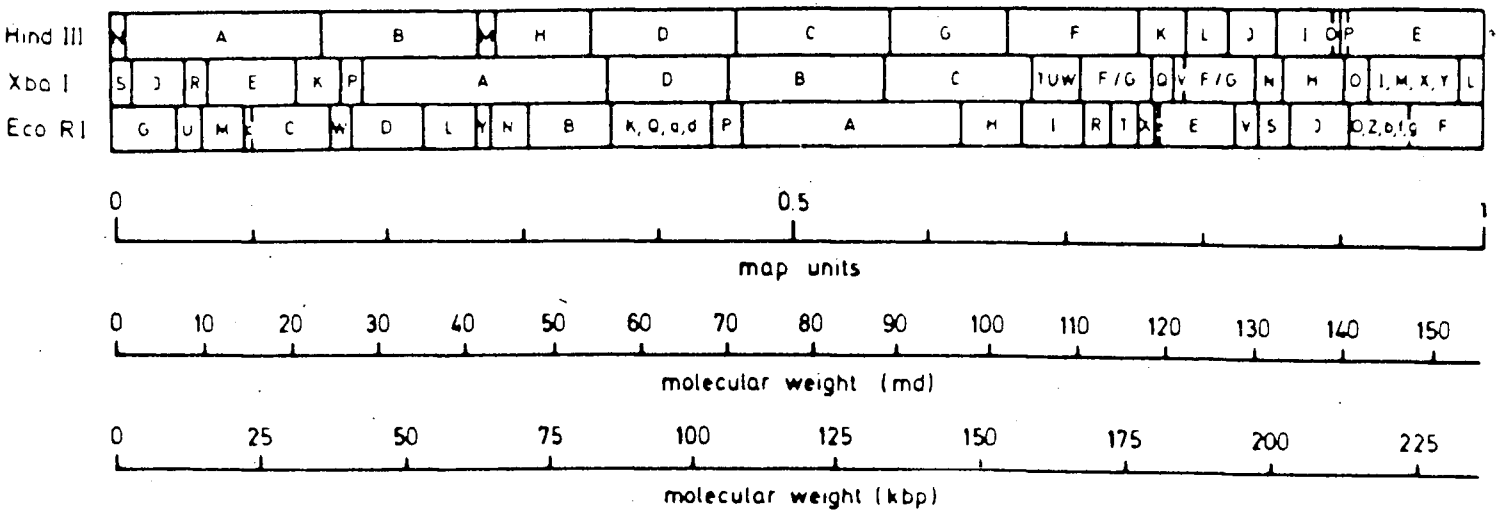
Due to the large size of the genome and the complexity of its gene expression, much of the virus remains to be analysed. The complete DNA nucleotide sequence of the MCMV genome is not known. The few documented DNA sequences to have been elucidated are confined to the major IE gene (ie1), IE enhancer element and a major E gene (e1) (Keil et al., 1987; Buhler et al., 1990; Koszinowski et al., 1990). The results reported in this thesis add the nucleotide sequence of an E cDNA, E10 (4606 bases), which belongs to another major E transcribed region.

1.4.3 HCMV AND MCMV GENE PRODUCTS

1.4.3.1 HCMV GENE PRODUCTS

A major IE region confined to a small fraction of the genome exists for all herpes viruses and is transcribed by host RNA polymerase (Spate and Mocarski, 1985b; Stinski and Roehr, 1985). For HCMV, this locus has been defined between 0.66 and 0.77 map units in the long unique region (DeMarchi, 1981; Wanthen and Stinski, 1982; Jahn et al., 1984). As illustrated in Figure 3a, the IE region comprises four transcription units: IE1, IE2, IE3

Fig 2: Restriction enzyme map of MCMV Smith strain DNA.
Physical map of MCMV Smith Strain genome for enzymes HindIII, EcoR1 and XbaI.
The map was orignally constructed and presented in Ebeling et al. (1983).

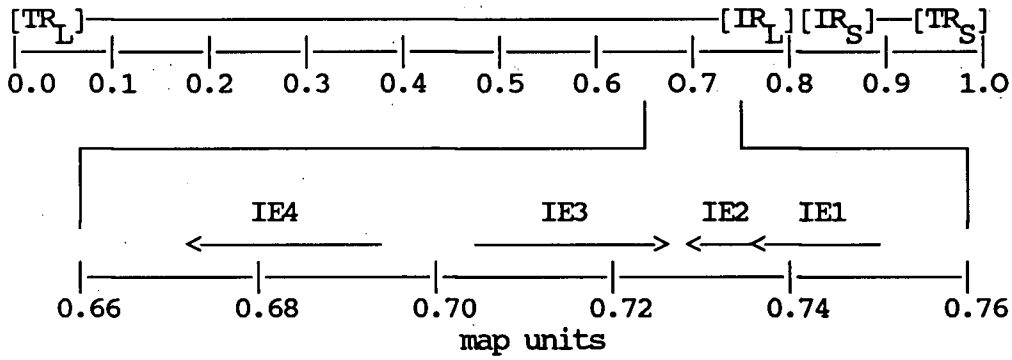


and IE4 (Stinski et al., 1983; Stenberg et al., 1984; Jahn et al., 1984; Plachter et al., 1988). The most abundant transcript of 1.95kb is transcribed from a 2.8 kb genome region IE1. This transcript is spliced and comprises four exons. It codes for a phosphoprotein with a molecular weight ranging from 68 to 79 kd depending on the strain type (Michelson et al., 1979; Gibson, 1981; Cameron and Preston, 1981; Stinski et al., 1983). The IE1 unit also codes for another protein of 39kd. The IE2 gene is transcribed in the same direction as IE1, and codes for mRNAs ranging in size from 1.10 to 2.25 kb which have been translated in vitro into four minor proteins (16.5 to 56 kd) (Stinski et al., 1983; Stenberg et al., 1985; Akrigg et al., 1985). The IE3 gene codes for a less abundant mRNA of 1.95 kb which is translated into a minor protein of 68kd, and the IE4 unit is transcribed into an unspliced, noncoding RNA of 5kb (Stinski et al., 1983; Plachter et al., 1988). The major IE region encodes both positive and negative functions. The IE2 gene products repress the IE promoter, while the IE1 and IE2 gene products together function as transactivating factors for E gene transcription (Pizzorno et al., 1988; Staprons et al., 1988).

In addition to the promoter sequences, a cis-acting element or a strong enhancer sequence exists upstream of IE1 region (Weber et al., 1984; Boshart et al., 1985; Stinski and Roehr, 1985). This enhancer element has demonstrated to contain binding sites for factors and has shown to comprise several sets of repeat regions of which the 18 and 19 bp repeats contribute significantly to the strength of the promoter. The 18bp repeat has sequences homologous to that of the 18bp repeats also found in the enhancers of HIV, SV40 and MCMV iel promoter (Dorsh-Hasler et al., 1985; Davidson et al., 1986; Nabel and Baltimore, 1987) and is a binding site for a cellular transcription factor, NFkB (Sambucetti et al., 1989). In addition, the

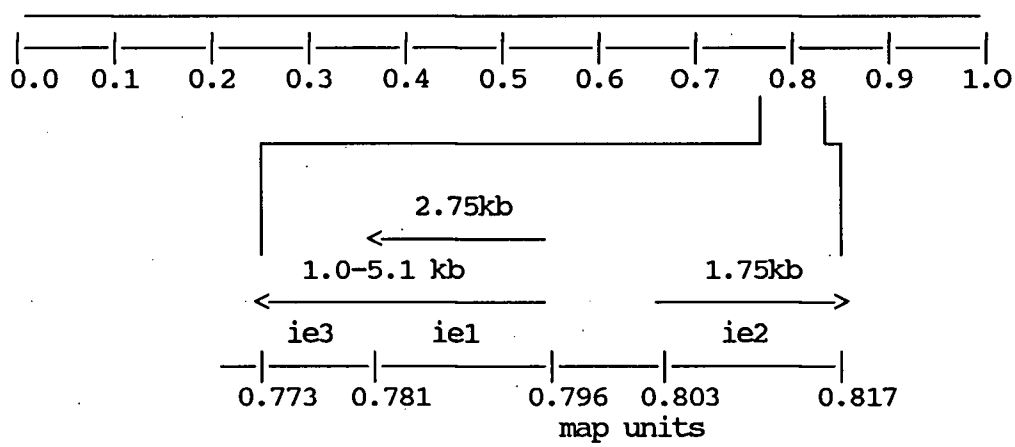
Figure 3: Organization of IE genes in Human and Murine CMV genomes

a. Organization of IE genes in HCMV



This figure illustrates transcripts and orientations of four IE genes in the major IE region (data taken from Mach et al. (1990).

b. Organization of IE genes in MCMV



This figure illustrates organization and transcripts of the major ie region in MCMV genome [data taken from Keil et al. (1987)].

enhancer element has demonstrated binding sites for a nuclear factor, NF-1 (Henninghausen and Fleckenstein, 1986; Jeang et al., 1987). The 19bp repeat element has sequences identical to that of cyclic AMP response element and appears to be a binding site for another cellular transcription factor (Lee et al., 1987; Hunninghake et al., 1989). The enhancer element has been discussed in great detail in a recent review by Stanninger and Flekeinstein (1990).

The IE region is transcribed begining 2-4 hr p.i. and continues throughout the replication cycle, particularly during the L phase (Stinski, 1978; DeMarchi et al., 1980; Sternberg et al., 1989). However, as a result of some post-transcriptional control, only some of the IE RNAs are translated (Stenberg et al., 1989). Overall, the IE gene expression is complex and requires many cellular and virally coded factors. The putative IE proteins of less predominant species and precise functions of some known IE proteins have yet to be identified.

Unlike IE, E transcripts map to many regions of the genome and their translational products are usually involved in viral DNA synthesis and nucleotide metabolism, and the regulation of L gene expression (DeMarchi, 1981; Wathen and Stinski, 1982; McDonough and Spector, 1983; Chang et al., 1989; Spector et al., 1990). Among the E proteins are the 140kd nonphosphorylated DNA binding protein and the DNA polymerase (Anders et al., 1986; 1987; Heilbournn et al., 1987; Anders and Gibson, 1988). The 140kd protein is a single stranded DNA binding protein and a homologue of the HSV-1 major DNA binding protein ICP8 (Anders and Gibson, 1988). The function of ICP8 relates to viral DNA synthesis (Wu et al., 1988) and by

analogy, the 140kd protein may have a similar function.

Although several E transcription units have been identified, only two are mentioned here as their gene products are relatively abundant and they have been extensively characterized. Also these E transcription units are similar in their transcription pattern to some of the MCMV E transcription units studied in this thesis. In AD169 and Town strains, the first transcription unit maps within the long inverted repeat and contains three major E genes (McDonogh et al., 1985; Huchison et al., 1986). These genes code for 2.7, 2.0 and 1.2 kb transcripts, of which the 2.7kb transcript is the most abundant. To date, the protein coded by the abundant 2.7kb transcript has not been identified.

The second E transcription unit belongs to EcoR1 fragments R and d (Staprons and Spector, 1986, Spector et al., 1990). The transcripts from this unit are spliced and of sizes 2.1, 2.2, 2.5 and 2.65kb, and these encode proteins of sizes 50, 43, 84 and 34kd respectively (Staprons and Spector, 1986; Wright et al., 1988; Wright and Spector, 1989). These proteins are DNA binding proteins and therefore, are likely to be involved in the regulation of viral gene expression and DNA synthesis (Spector et al., 1990). The expression of this transcription unit is regulated at the transcriptional level. Initially, only two transcripts (2.1 and 2.2kb) originate from this unit, but as the replication cycle progresses, the splicing pattern changes resulting in two additional transcripts. In general, many of the E gene products have not been identified or fully characterized. Therefore, much of the E phase remains to be analyzed.

During the L phase, all regions of the genome are transcribed, although the

abundant transcription occurs only in the unique long region (Wathen and Stinski, 1982; McDonough and Spector, 1983). Most of the late proteins are structural proteins and up to forty proteins have been associated with the HCMV virion (Stinski, 1976, 1977; Fiala et al., 1976; Kim et al., 1976a; Gupta et al., 1977; Mach et al., 1990). Approximately eight glycoproteins have been detected, of which a glycosylated protein VP17 of molecular weight 66kd is reported to be the major structural virion protein (Stinski, 1976; Kim et al., 1976a; Fiala et al., 1976; Gibson, 1983; Farrar and Oram, 1984; Nowak et al., 1984). Once again, like the E phase, most of the L phase has yet to be analysed.

Some transcripts of HCMV are differentially spliced, but the mechanism responsible for it has not been defined. Splicing events allow genomic economy and in viruses, these events are also important in the regulation of gene expression. In addition to differential splicing, there is further control of HCMV gene expression by regulation of mRNA transport to the cytoplasm, transcript stability and accumulation, and association of transcripts with polyribosomes (Wathen and Stinski, 1982; DeMarchi, 1983a; Geballe et al., 1986; Stenberg et al., 1989; Wright and Spector, 1989). Twenty, seventy five, and ninety percent of the HCMV genomic sequence is transcribed in IE, E and L phases respectively, but many of the transcripts synthesised do not become ribosome-associated and therefore are not translated (Wathen and Stinski, 1982; DeMarchi et al, 1983a; Geballe et al., 1988; Wright and Spector, 1989; Stenberg et al., 1989).). Therefore, while whole viral RNA, isolated from different times of the infection, maps to many restriction fragments, polysome associated mRNA maps to highly specific regions of the viral genome (Chua et al., 1981; Wathen and Stinski, 1982;

DeMarchi, 1983a, Gaballe et al., 1988). In summary, HCMV gene expression is a complex event and many of the mechanisms involved have not been elucidated. However it is clear that there exists temporal, quantitative and post transcriptional control of HCMV gene expression during the replication cycle.

1.4.3.2 MCMV GENE PRODUCTS

Preliminary studies conducted by Misra et al. (1978) have shown that only 50% of the MCMV transcripts are transported to the cytoplasm. Like HCMV, these and other results (presented below) indicate post-transcriptional and temporal regulation during the MCMV replication cycle, but the mechanisms involved have not been fully characterized.

As illustrated in Figure 3b, the main IE transcripts originate from a small portion of the genome, 0.769-0.817 map units (Marks et al., 1983; Keil et al., 1984). This region contains part of the HindIII fragment K and the entire HindIII fragment L (Keil et al., 1984). Six poly A^+ IE transcripts have been detected from this region of the genome: 5.1, 2.75, 2.0, 1.75, 1.65 and 1.05 kb. The organization of the major IE region is similar to that of HCMV, that is, it is divided into 3 units; ie1, ie2 and ie3 (Keil et al., 1987a). The ie1 gene codes for the abundant 89kd IE phosphonuclear protein, pp89, which is translated from the abundant IE 2.75kb transcript. This transcript is spliced and composed of four exons (Keil et al., 1987b). A strong enhancer of approximately 700bp has been identified within the ie1 promoter region (Dorsch-Hasler et al., 1985). The enhancer element comprises several sets of repeats of which the 18bp repeat sequence is similar to that found in the HCMV IE1 enhancer element. The next gene, ie2, lies in the opposite orientation to ie1 and ie3 genes, and is transcribed

into a 1.75kb mRNA (Keil et al., 1984). This transcript codes for a 43kd protein, which has not been identified in infected cell extracts, but has been translated in vitro (Keil et al., 1985). Although the ie2 gene has been shown to be dispensable for viral growth in fibroblast cell cultures, it is required for establishing latency in mouse spleen cells in vivo (Mocarski et al., 1990). The ie3 gene is responsible for a group of less abundant RNAs, ranging in size from 1.0 to 5.1kb. The only information known about these transcripts is that they use the ie1 transcription start site and terminate in the ie3 region (Keil et al., 1984; 1987a). The pp89 and other proteins of the major IE region bear very little sequence homology to the IE proteins of HCMV (Keil et al., 1985; Buhler et al., 1990). However, recent studies indicate that products of ie1 (pp89) and ie3 cofunction as transactivators and therefore bear functional homologies to the IE gene products of HCMV (Buhler et al., 1990). IE transcription in regions other than HindIII K and L have also been shown to occur (Keil et al., 1984). These transcripts map to the two DNA terminal fragments HindIII E and N, but their gene products remain unidentified.

In our laboratory, 10 IE proteins (Smith strain) have been identified, of which eight have demonstrated the ability to bind to DNA and therefore are perhaps regulatory in function (Walker and Hudson, 1987; 1988a). Three DNA binding proteins of sizes 89, 96 and 100kd have been identified as the major IE proteins and these may correspond to proteins 76, 84 and 89kd (pp89) also described by Keil et al. (1985) for Smith strain of MCMV. The molecular weight differences may be attributed to different experimental procedures, different cell lines (phosphorylation patterns may differ), and/or viral strain differences. Of the 10 IE proteins, some of the less predominant species may belong to the IE genes other than those of the major IE region,

although further analysis is required to verify this. As mentioned for HCMV, the factors essential for the IE phase have not been defined, some putative IE proteins remain unidentified and many of the known IE proteins have not been substantially characterized.

During the E period, transcription from the IE region is down regulated, but many regions of the genome are transcribed (Misra et al., 1978; Marks et al., 1983). Two individual groups have identified regions that are transcriptionally active during the E and L times of infection, although details on individual transcription units within these regions are lacking. Marks et al. (1983) have shown that the E transcripts of intermediate quantity originate from the HindIII fragments A, B, G, F and E, and of high quantity originate from the termini of the genome and map units 0.824 to 0.861, a region within the HindIII I-J fragment. One of the E cDNA clones, E10, studied extensively in this thesis maps to the actively transcribed E region, HindIII I-J. In addition, Keil et al. (1984) have shown that regions of active E transcription map to HindIII fragments B, F, K and J. Five of the E cDNAs selected and analysed in this thesis map to HindIII fragments E, B, F, J-I, and A. Therefore, the cDNAs chosen for this study represent transcripts from actively transcribed E regions.

In our laboratory, seven E proteins (91, 60, 54, 51.5, 51, 39 and 36kd) have been identified of which three (91, 39 and 36 kd) are abundant (Walker and Hudson, 1987). These three E proteins are likely to be regulatory in function because they have shown to localize in the nucleus and in addition, have the ability to bind to DNA (Walker and Hudson, 1988a). To date, only one MCMV E transcription unit or gene, e1, has been extensively analysed and documented (Buhler et al., 1990). The e1 gene maps to HindIII F region and

one of the E cDNAs studied in this thesis appears to correspond to this gene. The e1 transcript is a spliced product and codes for proteins (36, 37 and 38kd) of unknown function. At this point further analysis is required to clarify whether the 39 and 36 kd proteins identified in our laboratory correspond to the e1 gene products described by Buhler et al. (1990). Although several other transcriptionally active E regions have been mapped, details on individual transcription units within these regions are lacking. In this thesis, five E transcription units, and in particular one (E10) which represents an E region of intense transcription (HindIII I-J), were subjected to extensive analysis.

During the L phase, transcription occurs from most regions of the genome, but transcription from the major E regions decrease two to five fold (Marks et al., 1983; Keil et al., 1984). At the peak of the L phase (20-24 hr p.i.), certain regions are transcribed actively and these map to HindIII fragments B, C, D, F, G, H and M (Keil et al., 1984). Up to 33 structural or L proteins have been identified, of which six are glycosylated (Kim et al., 1976b; Chantler et al., 1978). A 172 kd protein (VP2 or VP7) has been identified as the major L protein. Information on the L phase is limited, therefore, much of the phase gene expression remains to be analysed. In general, gene expression in MCMV has not been examined as extensively as in HCMV and HSV. Further studies are required to reveal and characterize individual transcription units, especially those that belong to the E and/or L phase, and this thesis fulfills that in part.

1.5 LATENCY, PERSISTENCE AND REACTIVATION OF HCMV AND MCMV IN VIVO

1.5.1 LATENCY, PERSISTENCE AND REACTIVATION OF HCMV

All herpesviruses including cytomegaloviruses have the ability to persist as undetectable or latent infections for the lifetime of their hosts. Molecular mechanisms responsible for latency, persistence and reactivation of herperviruses including HCMV have not been defined. However, from a number of studies, it is apparent that, in-vivo, the host immune system plays an important part in the maintenance of the HCMV in its latent state. In a healthy individual, there appears to be a balance maintained between the host and the latent HCMV, and conditions favouring immunosuppression reactivate the virus. Sometimes the host's depressed immune system cannot eliminate the infected cell and this perhaps contributes to the efficient replication of the virus thus resulting in reactivation (Oldstone, 1989).

In renal or bone marrow transplant recipients there is a high incidence of acute HCMV infection. Iatrogenic immunosuppression and possibly the immunological response to foreign antigens appear to encourage CMV infection. The source of infection may be reactivated virus from the recipient or the donor tissue (Naraqi et al., 1977, Hackman et al., 1985). Also, due to the immunosuppressive nature of HCMV infection, opportunistic infections in these individuals are common (Chatterjee et al., 1978).

Acquired immunodeficiency syndrome (AIDS) patients are often victims of reactivated HCMV as a result of their immunosuppressed condition, and this reactivation of virus is potentially fatal. Recent studies have indicated that HCMV infection may be a cofactor in the progression of the AIDS disease because herpesviruses have been shown to encourage HIV-1 (Human immunodeficiency virus type 1) replication in vitro (Nelson et al., 1988).

In addition to that, HCMV itself may cause further immunosuppression by interacting with the cells of the immune system such as lymphocytes and NK cells (Starr et al., 1979; Levin et al., 1979). The exact mechanism involved in this virus-induced immunosuppression phenomenon is still under investigation and the subject is reviewed in great detail in Waner et al. (1989).

One of the common modes of transmitting HCMV is through blood and leucocyte transfusions (Prince et al., 1971, Winston et al., 1980). HCMV appears to persist in leucocytes, especially in the peripheral blood mononuclear cells, and the virus may be reactivated, perhaps by allogeneic responses in the transfused recipient (Winston et al., 1980; Schrier et al., 1985; Nelson et al., 1990).

In addition to the host immune surveillance, latency and reactivation depend on the ability of the virus to down-regulate viral gene expression (Oldstone, 1989). When the virus limits its gene expression, the host immune system is unable to recognize and clear the infected cell and thus the virus may persist. The down-regulation of herpesviral gene expression during latency appears to be a virus coded property, and the prime candidates in HSV and HCMV are the IE class genes and other genes (in HSV) that are expressed during latency (Schrier er al., 1985; results summarized in Stevens, 1989). Unlike for HCMV, significant progress has been made in the analysis of HSV latent infections. However, to date, the factors that are responsible for HSV latency have not been fully characterized. Although an IE gene, ICP0, (Russell et al., 1987) and a unique transcript termed as 'latency associated transcript' (LAT) appear to be associated with HSV

latency. Studies have shown that the ICP0 gene is required for reactivation and the LAT transcripts are found to be present in latently infected neurons (Wagner et al., 1988). LAT RNA is transcribed from the DNA strand opposite that encoding ICP0 (Stevens, 1989) and has been suggested to function as an antisense RNA to the ICP0 transcript, perhaps inhibiting the ICP0 function during the latent state.

In HCMV, gene products that are analogous to ICP0 and LAT have not been identified. However, studies involving detection of latent HCMV in blood cells such as peripheral blood mononuclear cells have revealed the presence of the major IE transcripts (Schrier et al., 1985; Nelson et al., 1990). Thus, these transcripts may be associated with latency. Most of the details on gene expression involved in HCMV latency remain unknown. To summarize, evidence to date cannot identify any cell type as the definite reservoir for latent HCMV. Also, the molecular mechanisms responsible for latency, persistence and reactivation of HCMV remain largely unclear. Since it is not possible to study these events in humans, an animal model such as mice infected with MCMV is best suited for dissection at the transcriptional level to provide insight into the molecular mechanisms leading to these events.

1.5.2 LATENCY AND REACTIVATION OF MCMV

One of the most important characteristics of MCMV is the ability to produce latent infections, a function which makes it an important animal model for HCMV. MCMV genes involved in latency and reactivation have not been fully defined but studies to date have shown that the IE class gene, ie2, is required for establishing MCMV latency in spleen cells (Mocarski et al.,

1990). Further studies are required to identify other genes and virus-coded functions that are involved in maintenance of the latent state and reactivation of MCMV. Like HCMV, host immune surveillance also appears to play an important role in latency and reactivation of MCMV. Several investigators have demonstrated reactivation of MCMV in latently infected mice by inducing conditions that promote immunosuppression by administering drugs such as cyclophosphamide or corticosteroid with anti-lymphocyte serum (Mayo et al., 1977; Jordan et al., 1977; Gonczol et al., 1985). Provoking allogeneic T cell responses in latently infected mice can also reactivate the virus as can infusion of blood from latently infected mice into uninfected mice, or vice versa (Cheung and Lang, 1977b).

Investigations so far indicate that numerous types of cells and tissues have the potential to become sites for MCMV latency. This broad tissue range for MCMV persistence complicates the study of the mechanisms involved. Several groups have reported that latent MCMV persists in spleen cells, especially in the B cell population (Wise et al., 1979; Olding et al., 1975, 1976; Wu and Ho, 1979). Observations that confirm the occurrence of latency in spleen cells include reactivation of the virus either by cocultivation with allogeneic uninfected mouse embryo cells or cultivation in the presence of lipopolysaccharide (B cell mitogen), with detection of the viral genomes by hybridization to MCMV DNA probes. More recently, a conflicting report from Mercer et al. (1987) suggests that stromal cells are the predominant reservoir of latent MCMV in the spleen rather than B cells, T cells or monocytes. Peritoneal macrophages have also been shown to harbour latent virus, and there are reports of MCMV reactivation from stimulated macrophages or detection of MCMV genome in macrophages (Brautigam et al.,

1979).

Salivary glands are another site of MCMV persistence. The virus can be reactivated from this organ by administering to the mice cyclophosphamide or anti-lymphocyte serum (Mayo et al. 1977; Jordan et al., 1977; Gonczol et al. 1985). Other sites in mice that may harbour latent virus, include ovaries, testes, prostate glands, kidney and even the sciatic nerve (Brautigam and Oldstone, 1980; Cheung and Lang, 1977a; Porter et al., 1985; Abols-Mantyh et al., 1987; Nelson et al., 1990). In summary, evidence so far predicts several potential sites for MCMV latency and conditions that promote immunosuppression or immunological responses reactivate the virus from its latent state. Details on viral functions that control persistence and latency are lacking. Therefore, further investigations especially at the molecular level need to be pursued in order to provide insight into these events.

1.6 NONPRODUCTIVE INFECTIONS IN VITRO

1.6.1 HCMV

HCMV displays a limited host range and its replication is best demonstrated in human fibroblast cells (Vonka et al., 1976). HCMV infection in nonpermissive cells results in limited viral expression and no viral DNA replication. Rodent cells such as guinea pig fibroblast, rabbit kidney cells (RK) and Balb/c-3T3 mouse cells are nonpermissive for HCMV replication (Fioretti et al., 1973; Stinski, 1978; DeMarchi, 1983b; Lafemina and Hayward, 1983, 1988). Although human fibroblast cells are permissive for HCMV, addition of immune serum or interferon during infection causes a switch to restricted viral expression or nonproductive infection (Mocarski

and Stinski, 1979; Rodrigueze et al., 1983). The outcome may be reversed by simply eliminating the antiserum or interferon. These observations support the notion that the immune system and down-regulation of viral gene expression may be involved in the maintenance of persistence and latency of HCMV.

Like rodent cells, human peripheral blood mononuclear cells are also nonpermissive to HCMV, but investigations involving blood transfusions have indicated that the virus can infect and persist in these cells in a latent state (Prince et al., 1971; Winston et al., 1980). With the aid of monoclonal antibodies, the presence of the major IE protein has been detected in these persistently infected mononuclear cells (Rice et al., 1984).

The undifferentiated human teratocarcinoma stem line is another cell line nonpermissive for HCMV. The restriction in this cell line lies at the level of IE transcription. These cells become permissive when cell differentiation is induced by retinoic acid (Gonczol et al., 1984; LaFemina and Hayward, 1986, 1988; Nelson and Groudine, 1986). The IE1 gene appears to be inactive in undifferentiated cells due to the absence of unidentified cellular factors (Nelson et al., 1990). These factors, when present, interact with the viral DNA to bring about a conformational change which is required for the expression of IE genes. In conclusion, observations to date indicate that immune responses and nonpermissive infections may help maintain HCMV latency and persistence, and certain changes in conditions which may induce cellular and/or viral factors may be the cause of reactivation.

1.6.2 MCMV

Productive infection of MCMV is best demonstrated in primary mouse embryonic fibroblasts, and in 3T3 and 3T6 cell lines (Hudson, 1984). Other mouse cells such as Y-1 (adrenal cells), primary kidney, primary spleen, primary liver, several macrophage lines, T lymphocytes and L5178Y (leukaemic T) cells are permissive for MCMV, but at a very low level (results summarized in Hudson, 1984). Unlike HCMV, MCMV has a more diverse species range and has been shown to replicate at a low level in monkey (BSC-1), hamster (BHK-21), rabbit (primary kidney and RK-13) and fetal sheep brain cells (Kim and Carp, 1971). Human and simian fibroblast are nonpermissive for MCMV and the viral gene expression in these cells is limited to IE genes (Kim and Carp, 1972; Hudson and Walker, 1987; LaFemina and Hayward, 1988).

In tracheal epithelial cells in culture, a productive infection of MCMV is dependent on cell-cell contact or fusion with infected fibroblast cells (Nedrud and Wu, 1984). This suggests that the infected fibroblast may contain a factor that is essential to support viral replication. This factor may be required for DNA synthesis as although MCMV replication in mouse fibroblast cells occurs, it is dependent on the S-phase of the cell cycle. (Muller et al., 1978; Hudson et al., 1979). Cells in Go phase do not support viral replication, but stimulation of cells to enter the cell cycle induces viral replication. This phenomenon is perhaps involved in MCMV latency and reactivation, but further analysis would be required to verify this statement.

Reports from numerous investigators are contradictory as to whether macrophages are permissive to MCMV (Selgrade and Osborn, 1974; Mims and Gould, 1978; Loh and Hudson, 1979; Tetgmeyer and Craighead, 1988;

Katzenstein et al., 1983; Walker and Hudson, 1987). Studies have indicated that macrophages may harbour latent virus, and factors present in the stimulated cells may cause its reactivation (Brautigam et al., 1979; Yamaguchi et al., 1988). According to one report, infection of macrophages is dependent upon the H-2 phenotype, but viral replication in the majority of susceptible macrophages is blocked at day 3 p.i., perhaps due to the presence of interferon (Price et al., 1987).

MCMV infection in the undifferentiated mouse cell lines such as OTT6050AF1 BrdU, F9 and PCC4 is nonproductive, and like HCMV, the block is evidently at the level of IE transcription (Dutko and Oldstone, 1981). However, as mentioned for HCMV, some of these cell lines do become permissive to MCMV when induced to differentiate (Dutko and Oldstone, 1981; LaFemina and Hayward, 1988).

To date, the molecular basis for latency and reactivation of MCMV and HCMV in vivo remain unclear. As speculated for HCMV, MCMV may remain latent in nonproductive and/or undifferentiated cells and perhaps some cellular and/or viral factors, induced at some time or during differentiation, may reactivate the virus. Nonpermissive system such as an undifferentiated cell line may serve as a model for in vivo latency for both MCMV and HCMV, but the challenge to establish more in vitro systems that would more closely mimick the in vivo system still remains. Until then, we also depend on the in vivo studies of the murine model for information on CMV latency which is rather limited and technically difficult at the molecular level. The best approach in this case would be to use probes that have been fully defined in order to detect specific transcripts. Some of the studies performed in this thesis involve the use of cDNAs as defined probes to investigate the gene

expression at the transcriptional level in a permissive cell line (3T3L1 mouse fibroblast). The results of this study may be extended in the future to investigate gene expression in vivo and in nonpermissive cell lines in vitro during latency.

1.7 MCMV AS A BIOLOGICAL MODEL FOR HCMV

HCMV is extremely host specific and therefore cannot be used to infect animals other than humans. An alternative is to study an animal CMV in its natural host and for many reasons MCMV is an excellent choice. Both HCMV and MCMV are morphologically alike. They belong to the same sub-family (betaherpesvirinae), are immunosuppressive, and are capable of causing persistent and latent infections in their natural hosts (Mathews et al., 1982; Whyte et al., 1987; Waner et al. 1989). They are lethal for young animals and immunosuppressed adults. Although their DNA genomes differ in structure, they are approximately the same length, contain one major IE region, and exhibit temporal gene expression (Misra et al., 1978; Demarchi, 1981; Mercer et al., 1983; Griffith and Grundy, 1987; Osborn, 1982). Due to these similarities, MCMV is an excellent choice as the biological model for HCMV.

1.8 RATIONALE AND OBJECTIVES

The ultimate goal of the murine model is to help define the molecular mechanisms leading to acute or persistent cytomegalovirus infection. To achieve this, gene expression during a permissive infection with MCMV has to be fully characterized and understood especially at the transcriptional level. When we embarked upon this project, the transcriptionally active regions of IE, E and L had been mapped in the MCMV genome, but the individual transcription units and details such as the size, number and pattern of temporal expression of individual transcripts had not been

defined for the E phase. As a detailed analyses of the IE region was being carried out by another group (Keil et al., 1984; 1987), E transcription became the major focus of this project. The importance of this phase in the replication cycle can be deduced from the fact that many of the virus coded functions that regulate herpesvirus expression, DNA synthesis and pathogenesis are expressed at this time. Moreover, several MCMV E proteins are known to be DNA-binding proteins and to be found exclusively in the nucleus, clearly indicating their potential as regulators of gene expression or involvement in DNA synthesis (Walker and Hudson, 1987a).

There are at least two indications that the E phase expression of MCMV regulates pathogenesis. The first indication comes from the study involving an E gene, sgg-1. This gene has been proven to be essential for the virus ability to replicate in the salivary gland tissue of mice (Mocarski et al., 1990). The second indication comes from Val et al. (1989). Their study suggests that unidentified factors produced during the E phase, aid in the survival of the infected cell because they appear to interfere with the presentation of the antigen, pp89, the primary target of the cellular immune response. Thus, there is clear evidence of the contribution of the E phase genes in determining MCMV pathogenesis. Furthermore, understanding of both IE and E gene expression is essential in order to elucidate the underlying mechanisms involved in establishment, maintenance and reactivation of latent infections.

The purpose of this project was to investigate individual patterns of MCMV gene transcription in genomic locations previously identified as IE and E regions by other investigators. The approach taken was to prepare an IE and E cDNA library from MCMV infected cells, and characterize the cDNAs by

mapping them to specific restriction fragments (HindIII, Eco R1 and XbaI) on the MCMV genome. These were then used as defined probes to study expression from individual transcription units. In this way a detailed picture of MCMV transcription during acute infection was obtained. These results will provide the basis for a future detailed analysis of MCMV expression during latency. In addition, an in-depth study of one particular E cDNA, E10 has been carried out. This cDNA was chosen because it mapped to a highly transcribed E region of the MCMV genome, HindIII I-J. Two independent groups have identified this region as one of the major regions expressed during E phase (Marks et al., 1983; Keil et al., 1984). Therefore, with the view that the E10 cDNA may code for an important function and will provide novel information on the structure of the corresponding transcript and gene, its DNA sequence was determined, and this sequence used to predict the properties of the putative E10 protein.

This study represents the first detailed analysis of E region gene expresssion, and the E10 cDNA is the second E gene to have been mapped and sequenced. The cDNAs prepared will provide useful probes in future work for the analysis of MCMV expression during persistence and latency in vivo.

1.9 SUMMARY OF THESIS PROJECT

Initially, the transcription pattern during the course of a permissive infection with MCMV was analysed using the cDNAs of RNA transcripts present at IE and E times The first section of the 'Results and Discussion' describes the detailed characterization of these IE and E cDNAs. The study involved the preparation of the cDNAs from poly A^+ RNAs isolated at IE and E times of infection, cloning of the cDNAs at the EcoR1 site in the Lambda gt10 system, and screening for viral inserts with MCMV DNA probes. Subsequently, some of these cDNAs were characterized by estimating their

sizes and mapping them to specific locations of the genome.

Five E cDNAs, each representing a different and actively transcribed E region (HindIII A, B, G, E, F, and I-J) of the genome, and four IE cDNAs mapping to the major IE region were chosen as probes to investigate the degree of expression displayed by their corresponding gene during a course of permissive infection. This task was accomplished by isolating RNA from infected cells at different times, binding the RNA to a nylon membrane and hybridizing the RNA to the defined cDNA probes. All five E cDNAs and two of the four IE cDNAs detected transcript levels that displayed the typical E and IE expression respectively. Furthermore, these experiments also verified the viral origin of most of the cDNAs, with the exception of two of the four IE cDNAs, which were found to be cellular in origin.

The main focus of the thesis was to investigate the expression of individual E transcription units. Northern blot analyses were performed in which the five E cDNAs were chosen as probes, and details such as the size and number of the respective transcripts, and the pattern of complex temporal expression of individual RNA in a permissive system were revealed. These results provide a good basis for comparison of viral expression during latency, at the molecular level in the future.

The final portion of the thesis focuses on one E cDNA, E10. The criterion for selecting that particular cDNA for extensive analysis was the fact that it mapped to a region (HindIII I-J) known to be heavily transcribed during E phase. The E10 cDNA lacked a polyadenylation tract. This missing 3' fragment, designated as E10-A, was isolated using the PCR method (illustrated in Figure 31). Both the E10 cDNA and its 3' end (E10-A) were

sequenced as illustrated in Figures 30 and 32. A combined DNA sequence, designated as E10-C, contained a total of 4606 bases (E10-C) and had a major ORF of 1200 amino acids with the potential to encode a 135 kd polypeptide. Details and further analysis on both the DNA sequence and deduced protein sequence are presented in this thesis. This includes the search for homologous DNA and protein sequences in the data banks (Genbank, European Molecular Biology Labs, Genpept, Swissprt) and HCMV (AD169). In addition, the hypothetical properties of the encoded 135 kd protein were also determined using available computer programs [PC gene (Intelligenetics) and Seqnce (Delaney software Ltd, Universion and version 2.1)].

Further experiments to identify the orientation of the major gene coding for the E10 cDNA were carried out. The restriction enzyme map of E10-C cDNA sequence was matched to the restriction enzyme map of Hind III I-J fragment in order to determine the orientation of the gene. In addition, portions of the HindIII fragments I and J were also sequenced and portions of DNA with sequences identical to those present on the cDNA identified, to confirm the orientation of the gene.

Finally, attempts to map the 5' end of the E10 cDNA and to identify the protein coded by the cDNA were conducted. Due to the protocol for construction of the cDNA library, the 5' end of the transcript would not be included in this clone, hence primer extension of the E mRNAs was used in an attempt to map the 5' end. Attempts to identify the protein of 135 kd coded by the major ORF of E10 cDNA involved hybrid selection of RNA by E10 cDNA and its translation in a in-vitro rabbit reticulocyte system. A virally induced protein of approximately 135 kd, and of E and L origin was detected in one of these experiments.

2.0 MATERIALS AND METHODS

2.1 3T3L1 CELLS

3T3L1 cells (ATCC CCL 92.1) are a continuous line of Mouse embryonic fibroblast cells. The cells were grown and passaged in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco), 0.37% sodium bicarbonate and 50 ug/ml gentamicin sulfate (Sigma) in a 37°C incubator supplied with 5% CO_2 and 95% air. For virus propagation, cells were grown in roller bottles (Falcon) in DMEM containing 10% FBS, 0.15% sodium bicarbonate and 50 ug/ml gentamicin sulfate at 37°C.

2.2 PREPARATION OF MURINE CYTOMEGALOVIRUS (MCMV) STOCK

Murine cytomegalovirus, Smith strain was originally obtained from American Type Culture Collection (ATCC). Concentrated stocks of the virus were prepared by infecting subconfluent 3T3L1 cells in roller bottles at a multiplicity of infection (MOI) of 0.01 plaque forming unit (pfu)/cell. The medium from infected cell cultures was centrifuged at 6000 rpm in a Sorvall GSA rotor for 20 minutes to remove cellular debris. The supernatant was recentrifuged at 12000 rpm in the Sorvall GSA rotor for 4 hours to pellet the virus. The pellet was resuspended in phosphate buffered saline (PBS; 8 g NaCl/l, 0.2 g KCl/l, 1.15 g Na_2HPO_4/l, 0.2 g KH_2PO4/l, 0.1 g $CaCl_2$/l, 0.1 g $MgCl_2$ /l), recentrifuged at 19,000 rpm in a Sorvall rotor (SS-34) for 1.5 hours to recover the virus, which was resuspended in a small volume of PBS, and stored at -70°C, in aliquots.

2.3 TITRATION OF MCMV

Subconfluent 3T3L1 cells equivalent to $8x10^6$ cells per plate (35x100mm, Nunc) were infected with 1.0 ml of medium containing appropriately diluted virus. For a standard titration, the infected cells were incubated at 37°C

for 30 minutes with virus to allow adsorption. For centrifugal titration, the infected cells were centrifuged with 2.0 ml virus inoculum in their dishes at a speed of 2000 rpm in large buckets of an IEC centrifuge. The medium was replaced with 2 ml of overlay medium (DMEM, 5% FBS, 0.5% agarose, 0.37% sodium bicarbonate, and 50 ug/ml gentamicin sulfate). Infected cells were incubated at 37°C in 5% CO_2 atmosphere for approximately five days for the plaques to appear. The titre was expressed as either standard pfu/ml or as centrifugal pfu/ml depending on the method used. Centrifugal titre is 20 to 50 fold higher than the standard titre (Hudson et al., 1976, 1988).

2.4 PREPARATION OF MCMV DNA

The MCMV pellet was obtained from cell free supernatant by the procedure described in Section 2.2. Virus from 5 roller bottles was treated with 1 ml of PEST solution [100 ug Proteinase K (Beckman)/ml, 0.01 M EDTA, 1% SDS, 0.1 M Tris-Cl pH 8.0] at 65°C for 2 hr, followed by overnight incubation at 37°C. Sterile water was added to a final volume of 7.0 ml, then 9.1 g of cesium chloride (CsCl) were added to give a refractive index of 1.402 g/cc to 1.403 g/cc. The DNA was centrifuged to equilibrium in a quick seal tube (Beckman) at 35,000 rpm in Ti75 rotor for 65 to 72 hr at 20°C. Fractions of 0.5 ml were collected from the bottom of the tube. Those that coincided with the first Absorbance peak (A_{260}), a region of high viscosity and refractive index of 1.401 g/cc, were selected (Mosmann and Hudson, 1973) and dialysed against TE (10 mM Tris-Cl pH 7.4, 1 mM EDTA) buffer.

2.5 PLASMID ISOLATION

E. coli strains JM109 [recA1, endoA1, gyrA96, thi-, hsdR17, supE44, relA1 (lac, pro) F'traD36 pro AB $lacI^q$, lacZ M15] and DH5-α (supE44 lac hsdR17

recA1 gyrA96, thi-1 relA1) were transformed with plasmid DNA (Section 2.18.3) containing an ampicillin resistant gene (Sambrook et al., 1989). Bacterial cultures were grown in the presence of ampicillin at a final concentration of 50 ug/ml in YT medium (8.0 g Bacto-Tryptone/l, 5.0 g yeast extract/l, 5.0 g NaCl/l) (Maniatis et al., 1982).

2.5.1 SMALL SCALE PLASMID ISOLATION

The method used was that described in Maniatis et al. (1982) with minor modifications. The alkaline lysis method was used to isolate plasmid DNA from 1.5 ml overnight cultures of E. coli. The plasmid DNA was dissolved in TE buffer containing 20 ug/ml RNAse A and 20 units/ml RNAse T1, and incubated at 37°C for 30 minutes. The DNA preparation was extracted with phenol/chloroform (1:1) as in Section 2.7. DNA was precipitated with ethanol, washed, dried and finally dissolved in 50 ul of TE buffer as in Section 2.7.

2.5.2 LARGE SCALE PLASMID ISOLATION

The method used was essentially that described in Maniatis et al. (1982). Bacterial cells were subjected to alkaline lysis. The crude plasmid DNA was purified by banding in a CsCl gradient containing ethidium bromide (EtBr; 600 ug/ml). EtBr was removed by performing extractions with 1-butanol and CsCl was removed by dialysing against TE buffer.

2.6 RESTRICTION ENDONUCLEASE DIGESTION OF DNA

All restriction enzymes were purchased from Besthesda Research Laboratory (BRL). Approximately 1 ug of DNA was digested with 1 to 5 units of restriction enzyme using conditions recommended by the manufacturer.

2.7 QUANTIFICATION, PURIFICATION AND PRECIPITATION OF NUCLEIC ACIDS

Concentrations of both purified DNA and RNA were quantified by UV spectrophotometry. Absorbance was read at wavelengths 260 nm and 280 nm; for DNA, 1.0 A_{260} was assumed to equal 50 ug/ml and for RNA, 1.0 A_{260} was assumed to equal 40 ug/ml. The ratio A_{260}/A_{280} of purified DNA was approximately 1.8, and that of purified RNA was approximately 2.0.

Purification and precipitation of DNA and RNA were performed as described in Maniatis et al. (1982). For purification and deproteinization, aqueous solutions of DNA and RNA were extracted with organic solvents such as phenol, chloroform (24 parts chloroform:1 part isoamylalcohol) and phenol/chloroform (1:1). Nucleic acids were precipitated by addition of recommended volumes of salt solutions and ethanol. The precipitate was washed in 70% ethanol, dried in a desiccator, and finally resuspended in an appropriate buffer.

2.8 GEL ELECTROPHORESIS AND TRANSFER OF DNA TO FILTERS

The method was based on the procedure described in Maniatis et al. (1982) with some modifications. Restriction enzyme digested DNA fragments were separated by electrophoresing through an agarose (Ultrapure, BRL) gel in TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.0) or TAE (40 mM tris acetate, 0.2 mM EDTA pH 8.0). DNA fragments were viewed with the aid of a UV transilluminator (260 nm) and EtBr (0.5 ug/ml) present in both gel and running buffer. Before transfer, the gel was shaken gently in depurinating acid (0.25 M HCl) for 15 minutes, twice in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes and finally in neutralizing solution (1.5 M NaCl, 1.0 M Tris-Cl pH 8.0) for 30 minutes. DNA was transferred to Hybond-N membrane (Amersham) in 10X SSPE (1X SSPE = 10 mM sodium phosphate pH 7.4,

150 mM NaCl, 1 mM EDTA). The filter was air dried and UV irradiated as recommended by the manufacturer (Amersham). Sizes of DNA fragments were estimated by the presence of DNA markers, normally HindIII digested bacteriophage Lambda DNA, in the gel. The sizes were calculated as described in Maniatis et al. (1982).

2.9 SLOT BLOTTING OF DNA

DNA was blotted using a Schleicher and Schuell minifold II apparatus. Samples were prepared and blotted according to the manufacturer's instructions. DNA was denatured in 0.3 M sodium hydroxide at 70°C for 1 hr, followed by neutralization with the addition of 1 volume of 2 M ammonium acetate (pH 7.0). Samples were slot blotted onto Hybond N that had been pre-wetted with 1 M ammonium acetate. The membrane was air dried, placed directly on the transilluminator and irradiated with UV light (260 nm) for 3-5 minutes.

2.10 ISOLATION OF DNA FRAGMENTS FROM AGAROSE GEL

DNA fragments were separated by agarose gel electrophoresis in TAE buffer. The band of interest was cut out and the fragment isolated using Gene Clean^{R} (Biolabs), according to the manufacturer's instructions.

2.11 PREPARATION OF RADIOACTIVE DNA PROBES FOR HYBRIDIZATION

DNA probes labelled with [α-^{32}P] dCTP (New England Nuclear, 3000Ci/mmol) were prepared with the BRL random primer DNA labelling system (Cat# 8187SA), according to the manufacturer's instructions.

2.12 HYBRIDIZATION: DNA PROBES

This procedure was utilized for both DNA-DNA and RNA-DNA hybridization in which a DNA probe was used. The procedure was based on the method described by Singh and John (1984) with minor modifications. Filters from plaque lifts and gel transfers were submerged in hot water for 10 minutes to remove residual agarose or agar, followed by equilibration in 4x SSPE for 30 minutes at 20-25°C. The filters were prehybridized in a sealed bag (BRL hybridization bags) containing a solution of 50% (V/V) formamide, 4x SSPE, 0.2% SDS, 50 ug/ml Heparin (Sigma, sodium salt grade II), and 0.05% sodium pyrophosphate at 42°C for 30 minutes. Filters were then hybridized overnight at 42°C with the same solution containing the denatured DNA probe ($2-5x10^6$ cpm/ml). The specific activities of the probes used were approximately 10^8-10^9 cpm/ug DNA. The filters were washed twice in 2x SSPE, 0.1% SDS for 15 minutes at RT, twice in 0.1x SSPE, 0.1% SDS for 15 minutes at RT, and finally once in 0.1x SSPE, 0.1% SDS for 1 hr at 50°C for DNA-DNA hybridization, or at 55°C for RNA-DNA hybridization. Damp filters were wrapped with saran wrap and exposed to X-ray film (XRP-1, Agfa).

The removal of DNA probes for reprobing of blots:

Success with removing the probe was achieved by keeping the filter damp at all times. The probe was stripped off by submerging the blot in a solution of 0.1% SDS. The solution was brought to 100°C and the filter was kept submerged for 30 to 60 minutes. The procedure was repeated until the membrane was free of the probe. The blot was autoradiographed overnight to confirm complete removal of the probe.

2.13 RADIOACTIVE END-LABELLING OF DNA

DNA restriction fragments were end labelled with [α-^{32}P] dATP (NEN; 3000

Ci/mmole) using T4 DNA polymerase (BRL), essentially as described in Maniatis et al. (1982).

2.14 PREPARATION OF IE AND E cDNA MCMV LIBRARY

2.14.1 ISOLATION OF TOTAL RNA

Guanidinium isothiocyanate (GITC)/CsCl method:

Subconfluent monolayers of 3T3L1 cells were infected with MCMV at MOI of 20-30 centrifugal pfu/cell; the infected cells were centrifuged at 2000 rpm to enhance infection. For the isolation of Immediate Early (IE) RNA, the cells were maintained in medium containing cycloheximide (CH, Gibco) 2 hours prior to infection at a concentration of 100 ug/ml, and during infection at a concentration of 50 ug/ml. The cells were harvested at 4 hours post infection. CH is a protein synthesis inhibitor, and since IE proteins are required for the Early (E) phase, the presence of CH blocks the transition of IE to E phase (Walker and Hudson, 1987). For the isolation of E phase RNA, the infected cells were treated with CH 4 hours post infection at a concentration of 100 ug/ml and harvested at 7 hours post infection. The procedure for RNA isolation is based on that of Chirgwin et al. (1979). Infected cells were washed 3 times with ice cold PBS and scraped into 1 ml PBS per plate with a rubber policeman. The cells were pelleted by centrifugation in a Sorvall GSA rotor at 5,000 rpm for 5 minutes at 4°C. Cells were lysed in 8 volumes of GITC solution (4.0 M GITC, 50 mM sodium citrate, 0.5 % Na-N-lauroyl- Sarkosine pH 6.5, 10 mM EDTA pH 8.0, 0.1 M ß-mercaptoethanol (Biorad)). CsCl was added to the lysed mixture (2.0 g/ml of lysate), which was then layered onto a 4.5 ml cushion of 5.7 M CsCl solution in a quick seal tube (Beckman). The tube was centrifuged in a Ti75 rotor at 44.5 K rpm for 24 hours at 20°C to pellet the RNA. The RNA pellet was dissolved in sterile diethyl pyrocarbonate (DEPC; Sigma) treated water

and precipitated with 0.1 volume of 2 M K-acetate (pH 5.5) & 2 volumes of 95% ethanol at -20°C. The RNA precipitate was dissolved in sterile DEPC treated water and stored at -70°C.

2.14.2 ISOLATION OF POLY A^+ RNA

Poly A^+ RNAs were obtained by fractionating total RNA in a buffer (0.5 M NaCl, 10 mM Li-citrate, 5 mM EDTA pH 8.0) through an oligo-dT cellulose (Pharmacia) column according to the procedure of Aviv and Leder (1972), except that the Poly A^+ RNA was eluted from the column using DEPC treated water. The poly A^+ RNA was precipitated with 0.1 volume of 2 M K-acetate (pH 5.5) and 2 volumes of 95% ethanol. The RNA was collected by centrifugation, redissolved in DEPC treated water and stored at -70°C.

2.14.3 PREPARATION OF cDNA AND CLONING IN BACTERIOPHAGE LAMBDA gt10

IE and E cDNA were synthesised using the Amersham cDNA synthesis kit (cat # RPN.1256). cDNAs were ligated to EcoR1 linkers and cloned into Lambda gt10 at the EcoR1 site using the Amersham cDNA cloning system kit (cat. # RPN 1257). In order to protect the internal EcoR1 sites, the cDNAs were subjected to a methylation (EcoR1 methylase) step before ligation to EcoR1 linkers. Preparation and cloning procedures were those described by the manufacturer.

2.14.4 SCREENING OF IE AND E cDNA LIBRARIES: PLAQUE LIFT METHOD

The procedure was essentially that recommended by the manufacturer (Amersham) of the cDNA cloning kit.

Preparation of Phage plating cells:

Cells were prepared by inoculating 50 ml Luria-Bertani medium (LB; 10 g

Bacto-Tryptone/l, 5 g NaCl/l, 10 g Bactoyeast/l) that was supplemented with 0.4% maltose, with 1 ml of overnight grown LB culture of E. coli strain NM514 (hfl^+). The culture was shaken vigorously at 37°C for approximately 3 hr until the A_{600} reached 0.5. The cells were cooled on ice, centrifuged at 3000 rpm at 4°C and resuspended in 15 ml of cold 10 mM $MgSO_4$.

Phage plating and plaque lifts:

Lambda gt10 phage in 100 ul SM buffer [5.8 g NaCl/l, 2 g $MgSO_4.7H_2O$/l, 5 ml 1 M Tris-Cl (pH7.5)/l, 5 ml of 2% gelatin/l] was added to 100 ul of phage plating cells and incubated at 37°C for 15 minutes. Approximately 4 ml of liquid top agar (1 g Bacto-Tryptone/l, 0.5 g bacto-yeast extract/l, 0.5 g NaCl/l, 0.25 g $MgSO_4$/l, 1 g bacto-agar/l) at 45°C was added, mixed quickly and poured over a plate of LB agar (15 g Bacto-agar /litre LB). The plate was allowed to set and incubate overnight at 37°C. The plate was cooled to 4°C before a Hybond-N (Amersham) membrane was placed carefully over the surface of the cold agar for 30 seconds. The membrane was peeled off, placed plaque side up on filter papers (Whatman 3MM) soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 minutes. The membrane was then placed on filter papers soaked in neutralizing solution (0.5 M Tris-Cl pH 7.0, 1.5 M NaCl) for 5 minutes, and finally rinsed in 2x SSPE. The membrane was air dried, placed on a transilluminator and irradiated with UV light (260 nm) for 3 to 5 minutes.

2.15 PREPARATION OF BACTERIOPHAGE LAMBDA gt10 PHAGE STOCK

The procedure was that recommended by the manufacturer of the cDNA cloning kit, Amersham. A plaque of interest was picked with a sterile pasteur pipette and added to 100 ul of phage plating cells (cells prepared as

described in Section 2.13). Adsorption was allowed to proceed at RT for 15 minutes followed by addition of 5 ml of LB medium containing 5 mM $CaCl_2$. The culture was then shaken at 37°C for 4.5 hr. A few drops of chloroform were added and the culture was allowed to shake for an additional 5 minutes. Bacterial debris was removed by spinning at 3000 rpm in a bench centrifuge for 10 minutes. The supernatant was stored over a few drops of chloroform at 4°C.

2.16 ISOLATION OF BACTERIOPHAGE LAMBDA gt10 PHAGE DNA

2.16.1 SMALL SCALE ISOLATION OF PHAGE DNA

The method is based on that of Maniatis et al. (1982) with some modifications. Phage were plated (10^5/80mm plate) to give confluent lysis; 5 ml of SM buffer were added to the plate and left at RT for 2-3 hr. The SM buffer was transferred to a 15 ml Corex tube and left at RT for 15 minutes with 2-3 drops of chloroform. Bacterial debris was removed by centrifugation at 10,000 rpm in a Sorvall GSA rotor for 10 minutes at 4°C. One volume of SM buffer containing 2.5 M NaCl and 20% (w/v) polyethylene glycol (PEG 8000; BDH) was added, and chilled on ice for 1 hr. Phage were recovered by centrifuging at 11,000 rpm in a Sorvall GSA rotor at 4°C for 20 minutes. The pellet was resuspended in DNase I buffer (10 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, BSA 100 ug/ml) with addition of RNase A and DNase I to a final concentration of 1 ug/ml each. The mixture was incubated at 37°C for 30 minutes, followed by centrifugation at 10,000 rpm in a Sorvall SS-34 rotor for 10 minutes at 4°C. To the supernatant, 5 ul each of 10% SDS and 0.5 M EDTA pH 8.0 were added and incubated at 68°C for 15-60 minutes. The solution was extracted with phenol/chloroform, followed by chloroform. One volume of isopropanol was added and stored at -20°C overnight to precipitate

DNA. The DNA precipitate was recovered by centrifugation in a Sorvall SS-34 rotor at 12,000 rpm for 20 mins, washed in 70% ethanol, dried and resuspended in 25 ul TE containing 20 ug/ml RNase A.

2.16.2 LARGE SCALE ISOLATION OF PHAGE DNA

The procedures were a combination of methods from Maniatis et al. (1982) and Davis et al. (1986) with some modifications.

Propagation of phage:

Two methods were used for the propagation of phage for DNA isolation.

Method I: Phage propagation in dishes

Phage were plated in ten 82 mm dishes to give confluent lysis (10^5 pfu per dish); 5 ml of SM buffer was added to each plate and gently shaken for 2 hr at RT. The SM buffer from the dishes was combined and centrifuged at 15,000 rpm in a Sorvall SS-34 rotor for 10 minutes to remove the bacterial debris. To the supernatant, 0.15 volume of 5 M NaCl and 0.3 volume of 50% PEG 8000 were added, and the solution was stored on ice for 2 hr to precipitate the phage.

Method II: High titre lysate

NM514 cells were grown at 37°C in 1 litre of LB medium supplemented with 2 mM $MgSO_4$, 4 uM $FeSO_4$, 0.1 M $CaCl_2$, 0.15% glucose with vigorous shaking to an A_{600} of 0.2. Cells were inoculated with $2x10^9$ pfu and shaken vigorously for 4-6 hr, followed by addition of 20 ml chloroform and 120 ml of 5 M NaCl. The culture was shaken for an additional 5 minutes at 37°C. Bacterial debris was removed by centrifuging at 15,000 rpm in a Sorvall SS-34 rotor for 10 minutes at 4°C. Molar $MgSO_4$ (10 ml) and PEG 8000 (120

g) were added and the phage allowed to precipitate on ice for 2 hr.

Isolation of phage DNA:

Phage were recovered by centrifuging at 10,000 rpm in a Sorvall GSA rotor for 15 minutes at 4°C. The pellet was resuspended in 5 ml of DNase I buffer (10 mM Tris-Cl pH 7.5, 5 mM $MgCl_2$, 100 ug BSA/ml). DNase I and RNase A were added to a final concentration of 20 ug/ml and 200 ug/ml respectively and the phage were incubated at 37°C for 30 minutes. The preparation was extracted twice with equal volume of chloroform, followed by the addition of 0.75 g of CsCl/ml to give a refractive index of 1.45-1.5 g/cc. The phage were centrifuged in a quick seal tube in Beckman Ti75 rotor at 60,000 rpm for at least 16 hr. Phage which appeared as a bluish band were isolated by puncturing the side of the tube with an 18 gauge needle. CsCl was removed by dialysing against TE pH 7.4 at 4°C. The dialysed phage were treated with 1% SDS, 20 mM EDTA and 100 ug Proteinase K/ml at 65°C for 1 hr, followed by extractions with phenol/chloroform and chloroform. Finally, the DNA in solution was dialysed against TE pH 7.4 at 4°C.

2.17 ANALYSIS OF RNA

Various precautions were taken during the RNA procedures to prevent any RNA degradation by the presence of ribonuclease. All glassware and solutions were treated with 0.2% DEPC as described in Maniatis et al. (1982). Glassware was baked at 300°C for at least 12 hr. All items such as tubes, tips and pipettes were sterile and disposable. Gloves were worn during the procedure to prevent contamination of RNA from finger ribonuclease. Eppendorf tubes and pipette tips were siliconized to prevent RNA from sticking.

2.17.1 SMALL SCALE ISOLATION OF TOTAL RNA: LITHIUM CHLORIDE (LiCl) METHOD

The method was based on that of Cathala et al. (1983). RNA isolated by this method was used for slot blotting and hybridization experiments to evaluate individual cDNAs as probes. Subconfluent 3T3L1 cells were infected with MCMV at a MOI of 20-30 centrifugal pfu/cell. The cells were scraped from the plates (60x15mm) at various time intervals after infection into 1 ml cold PBS/plate. Cells were recovered by centrifuging at 1,000 rpm in an IEC bench centrifuge (model# HN-FII) for 5 minutes and resuspended in 7 volumes (relative to the volume of the pellet) of homogenization buffer (5 M GITC, 10 mM EDTA, 50 mM Tris-Cl pH 7.5, 0.1 M ß-mercaptoethanol). Seven volumes (relative to the volume of the homogenate) of 4 M LiCl (BDH) were added to the homogenate and the solution was placed overnight at 4°C. Crude RNA was recovered by centrifugation at 10,000 rpm in an eppendorf centrifuge for 20 minutes. The pellet was resuspended in 1 ml 3 M LiCl and repelleted. The crude RNA was dissolved in 0.4 ml solubilizing buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.1% SDS), 0.05 volumes of 5 M NaCl was added, followed by phenol/chloroform extraction. RNA was precipitated with 2.2 volumes of ethanol and then dissolved in 100 ul of DNase I buffer (1 mM DTT, 10 mM MgCl, 10 mM Tris-Cl pH 7.4, 1 mM EDTA) containing 100 units of RNasin (Promega) and treated with 10 units RNase-free DNase I (Pharmacia) at 37°C for 30 minutes. The reaction was stopped by adding 10 ul of 0.5 M EDTA and 2 ul of 10% SDS. The RNA was extracted with phenol/chloroform and precipitated with 95% ethanol. The RNA was recovered by centrifugation, redissolved in DEPC treated water and stored at -20°C.

2.17.2 SLOT BLOTTING OF RNA

The Schleicher and Schuell minifold II apparatus was used to slot blot RNA onto Hybond-N that had been prewetted with 20x SSC (Thomas, 1980). RNA samples were prepared by a combination of procedures recommended by the manufacturers, Schleicher and Schuell and Amersham. RNA samples were denatured in 50% Formamide and 4.6 M Formaldehyde (Baker) at 65°C for 5 minutes. The samples were chilled on ice, followed by the addition of 1 volume 20x SSPE. RNA was blotted onto the membrane, which was then air dried and UV irradiated for 3-5 minutes.

2.17.3 LARGE SCALE ISOLATION OF TOTAL RNA: VANADYL RIBONUCLEOSIDE COMPLEX (VRC) METHOD

RNA was prepared essentially as described in Kaufman and Sharp (1982). The RNA isolated was used for formaldehyde gel electrophoresis. For IE and E RNA, 3T3L1 cells were infected and harvested in PBS as described in Section 2.13.1. In the case of Late (L) RNA, 3T3L1 cells were infected at a MOI of 20-30 centrifugal pfu, and were harvested by scraping into PBS at 16 hr post infection. The RNA samples were treated with RNase-free DNase I as described in Section 2.16.1.

2.17.4 ELECTROPHORESIS AND TRANSFER OF RNA TO FILTERS

Preparation of samples and electrophoresis procedure was as described in Ausubel et al. (1987). Total RNA and RNA markers (BRL) were denatured with formaldehyde/50% formamide at 55°C for 15 minutes, and electrophoresed through a formaldehyde agarose gel in MOPS running buffer (0.2 M Morpholinopropanesulfonic acid, 50 mM sodium acetate, 1 mM EDTA pH 8.0). Integrity of the RNA was confirmed by the presence of discrete, non smeared 28S and 18S rRNA bands in the EtBr stained gel. Before the transfer, the

gel was washed in several rinses of water to remove the formaldehyde. The gel was soaked in 10X SSPE for 30 minutes, and transferred to Hybond-N (Amersham) in 20X SSPE overnight (Thomas, 1980). The filter was air dried, placed on a transilluminator and exposed to UV light (260 nm) for 3 to 5 minutes to fix the RNA.

2.17.5 PREPARATION OF RNA PROBES

Radioactively labelled RNA probes were prepared by in vitro transcription of cDNA. The recombinant plasmid (1 ug), pGEM3Z (Promega) was linearized and the cDNA was transcribed in a 20 ul reaction volume containing 10 mM DTT, 10 ug BSA, 20 units RNasin (Promega), 5 mM ATP, 5 mM GTP, 5 mM CTP, 0.5 mM UTP, 2 ul [α-^{32}P] UTP (NEN; 10 mCi/ml), 10 units T7 RNA polymerase (BRL) and 1X transcription buffer (supplied by BRL). The reaction mix was incubated at 37°C for 1 hr. Following transcription the reaction was incubated with 10 units of RNase-free DNase I (Promega) at 37°C for 15 mins, extracted with phenol/chloroform, and the RNA was precipitated with 95% ethanol in the presence of carrier 10 ug yeast tRNA (BRL).

2.17.6 RNA-RNA HYBRIDIZATION

Hybond N filters (Amersham) from Northern blots were equilibrated in 5X SSPE for 30 mins followed by hybridization to [alpha-^{32}P] UTP labelled RNA probe in hybridization buffer [50% formamide (BRL), 5X SSPE, 1.0% SDS, 0.1% Tween 20 and 100 ug tRNA], at 43-45°C for 16 hr. The hybridized filters were washed as described in Section 2.12 except for the final wash, which was done at 57°C.

2.17.7 ANALYSIS OF RNA BY PRIMER EXTENSION

The primer extension procedure was carried out essentially as described in Sambrook et al. (1989). An 18-mer oligonucleotide (5'ATGTCCAGCGTGTAGATC3') was synthesized by Mr. T. Atkinson (UBC). The oligonucleotide was labelled at the 5' end using γ-^{32}P-ATP and T4 polynucleotide kinase (BRL). Endlabelled primer (18-mer) (10^5cpm) was annealed to 50 ug E RNA and extended using Moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL). The extended products were electrophoresed through a 6% acrylamide gel as described in Section 2.18.6.

2.18 DNA SEQUENCE ANALYSIS OF E cDNA E10

The E10 fragment was subcloned into plasmid pGEM3Z (Promega) in both orientations and deletion clones were prepared (Henikoff et al., 1984). Clones with overlapping regions were selected by digesting with EcoR1 and sizing their plasmid DNA on agarose gels. Clones of both orientations were sequenced using the sequenase enzymatic sequencing procedure (United States Biochemical Corporation; USB). Overlapping sequences were compiled to give a full length sequence of the E10 cDNA. The strategy for the E10 cDNA seqencing is presented in Figure 30 (Section 3.4).

2.18.1 SUBCLONING OF DNA FRAGMENT IN pGEM3Z

The cDNA fragment E10 was cloned into the EcoR1 site of pGEM3Z. The plasmid was linearized with EcoR1 (BRL), then ligated to E10 fragment in presence of T4 DNA ligase (BRL) in the ligation buffer provided by the manufacturer at 16°C for 16 hr. Transformation of E. coli strain JM109 was carried out as outlined in Section 2.18.3.

2.18.2 PREPARATION OF FROZEN COMPETENT CELLS

The method is based on that of Hanahan et al. (1983). E. coli strain JM109 was maintained on M9 minimal agar plates (Na_2HPO_4 5 g/l, KH_2PO_4 3 g/l, NH_4Cl 1 g/l, NaCl 0.5 g/l, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 mM thiamine-HCl, 0.2% glucose, agar 1.5 g/l). A culture was prepared by inoculating 100 ml of SOB medium (Bacto-Tryptone 20 g/l, yeast extract 5 g/l, 0.5 g NaCl/l, 20 mM $MgSO_4$) with 1 ml of an overnight culture of JM109 in YT medium. Inoculated medium was shaken at 250 rpm until A_{550} reached 0.6-0.7 (approximately 3.5 hr). The wavelength of 550 nm was used rather than 600 nm, since the sensitivity of measuring the absorbance increases as wavelength decreases (Hackett et al., 1984). The culture was chilled on ice, and centrifuged at 3,000 rpm in a Sorvall GSA rotor for 5 minutes at 4°C. The pellet was resuspended in 30 ml of transformation medium [45 mM $MnCl_2$, 10 mM $MgCl_2$, 100 mM RbCl, 3 mM hexammine cobalt(III) chloride] and stored on ice for 15 minutes. Cells were recovered by centrifuging at 3,000 rpm in a Sorvall GSA rotor at 4°C and resuspended in 8 ml of fresh transformation medium. An aliquot of 280 ul DMSO was added to the medium and incubated on ice for 5 minutes. A second aliquot (280 ul) of DMSO was added and the cell suspension was divided into aliquots (210 ul/tube) which were quick frozen in a dry ice/ethanol bath and then stored at -70°C. Before transformation, cells were thawed and placed on ice for 10 minutes.

2.18.3 TRANSFORMATION OF JM109 WITH pGEM3Z

A maximum of 10 ul of each plasmid preparation was added to an aliquot of freshly thawed competent JM109 cells. Cells were left on ice for 15 minutes and then heat shocked at 42°C for 2 minutes. One ml of SOC medium (10 mM $MgSO_4$, 10 mM $MgCl_2$, and 20 mM glucose in LB; BRL) was added and shaken gently for 1 hr. Transformed cells were selected by plating 10-100 ul on

50 ug/ml ampicillin YT plates(8 g/l Bacto-Tryptone, 5 g/l Bacto-yeast extract, 5 g/l NaCl, 1.5 g/l agar) with 50 ul X-gal/IPTG (BRL) for ß-galactosidase color selection. White colonies indicated transformants with recombinant plasmid and blue colonies indicated transformants with non recombinant plasmids.

2.18.4 CONSTRUCTION OF UNIDIRECTIONAL DELETION CLONES

The method is based on the procedure of Henikoff et al. (1984). Approximately 15 ug of pGEM3Z containing the E10 insert (at the EcoR1 site) were linearized with SalI to give a 5' overhang. The DNA was extracted with phenol/chloroform, precipitated with ethanol , washed in 70% ethanol and dried in a desiccator. The DNA was redissovled in appropriate buffer and digested with SphI to give a 3' overhang. This allows unidirectional digestion of E10 insert since exonuclease III (ExoIII) is specific only for the 5' overhang. The 3' overhang protects the remainder of the vector from the ExoIII attack. The dry DNA pellet was dissolved in ExoIII buffer (66 mM Tris-Cl pH 8.0, 0.66 mM $MgCl_2$). The tube was warmed in a 37°C waterbath, then 500 units of ExoIII enzyme (Promega) were added, mixed rapidly and returned to the waterbath. Samples of 2.5 ul were removed at 30 seconds intervals for 10 minutes and added directly to 7.5 ul S1 mix [40 mM potassium acetate pH 4.6, 333 mM NaCl, 1.3 mM $ZnSO_4$, 6.7% glycerol, 60 units S1 (BRL)/200 ul] in a tube on ice. All S1 samples were transferred to RT and incubated for 30 minutes. The S1 enzyme was inactivated by adding 1 ul of S1 stop buffer (0.3 M Tris base, 0.05 M EDTA pH 8.0) and heating to 70°C for 10 minutes. From each sample, 3 ul were digested with EcoR1 and electrophoresed through a 1% agarose gel. The rate of digestion was approximately 300bp/minute. The S1 treated samples were transferred to a 37°C water bath and 1 ul Klenow mix (20 mM Tris-Cl pH 8.0, 100 mM $MgCl_2$,

5 units Klenow/20 ul) was added, followed by addition of 1 ul dNTP mix (0.125 mM each of dATP, dTTP, dCTP and dGTP). Incubation was for 10 minutes at RT. Finally, 40 ul of ligase mix (50 mM Tris-Cl pH 7.6,10 mM $MgCl_2$, 1 mM ATP, 5% PEG, 1 mM DTT, 5units T4 DNA ligase/ml) were added to each tube and ligation was allowed to proceed at 16°C for 16 hr. JM109 cells were transformed as described in Section 2.18.2.

2.18.5 DOUBLE STRANDED DNA SEQUENCING

Sequencing of both strands of E10 insert were carried out by the dideoxy chain termination method. Plasmids were isolated using both small scale and large scale procedures (described under Section 2.5). DNA was denatured by boiling in 200 mM NaOH for 2 minutes. Three molar ammonium acetate (0.1 volume) was added and DNA was precipitated with ethanol. DNA sequencing reactions were performed using the Sequenase^R kit (USB; Cat # 70700). The SP6 promoter site in pGEM3Z was used as the priming site. DNA was labelled with [α-^{32}P] dATP (NEN; 3000 Ci/mmole).

For one of the orientations, isolation of overlapping clones to provide sequences in two places, each of approximately 50 bp, failed. Synthetic 17-mer oligonucleotides (5'CGCACGAGTGTGTACGT3' and 5'GCTCAGAGAGTAGTGAC3') complementary to the sequence preceding the gap were prepared by Mr. T. Atkinson, UBC (Applied Biosystems, model 380B), and used as primers to sequence the two regions. The 17-mer oligonucleotides were purified using a Sep-pak column (Millipore) according to the manufacturer's instructions. The oligonucleotide fraction was eluted in 20% acetonitrile (American Burdick and Jackson), dried in a speed-vac and suspended in sterile water.

2.18.6 ACRYLAMIDE GEL ELECTROPHORESIS OF SEQUENCING REACTIONS

The sequence reactions were electrophoresed through a 6% (29:1, acrylamide:bisacrylamide) acrylamide (Bio-Rad)/7 M Urea (BDH) gel, at 32 Watts/gel in TBE (50 mM Tris, 50 mM boric acid, 1 mM EDTA, pH 8.3) buffer. The gel was dried on filter paper (3MM Whatman) and exposed to XRP-1 (Agfa) film at RT for approximately 16 hours.

2.19 ISOLATION AND DNA SEQUENCING OF THE MISSING 3'TERMINUS OF E10 cDNA

The 3' end of the E10 cDNA was found to lack an in frame stop codon, polyadenylation signal and poly A tract. The 3' terminus was obtained by amplification of E10 mRNA using PCR (polymerase chain reaction) or RACE (rapid amplification of cDNA ends) method as described in Frohman et al. (1988), with some modifications, and cloned into appropriate plasmids. The E10-A cDNA isolation and sequencing strategies are presented in Figures 31 and 32 (Section 3.4) respectively.

2.19.1 FIRST STRAND cDNA SYNTHESIS

Total E RNA (10 ug) was denatured in the presence of methyl mercuric hydroxide at RT for 10 mins in a volume of 15 ul, followed by snap freezing on dry ice. To the frozen sample was added 35 ul of a solution to give a final concentration of 200 uM DTT, 40 units RNasin (Promega), 200 uM dNTPs (dATP, dTTP, dGTP and dCTP), and 2 ug oligo $d(T)_{17}$ primer and 300 units Moloney murine leukemia virus (M-MLV) reverse transcriptase (BRL) per 50 ul. The 50 ul mix was incubated at 37°C for 1 hr, followed by dilution to 200 ul with water.

2.19.2 AMPLIFICATION OF 3' TERMINUS OF E10 cDNA BY RACE OR PCR METHOD

The amplification step was carried out using two primers:

1. oligo $d(T)_{17}$ with SalI/SphI sites (5'CGAGCATGCGTCGACAGGTTTTTTTTTTTTTTT3');
2. a 29-mer containing a HindIII site (5' end) and 20 bases specific to a sequence that was 50 bases away from the 3' end of the incomplete E10 cDNA (5'ACAAAGCTTAGAAGCAGAGACTTTGTCCT3').

The PCR reaction was carried out in a Perkin Elmer Cetus DNA cycler in a total volume of 50 ul containing 2-5 ng ss cDNA (from Section 2.19.1), 67 mM Tris-Cl pH 8.5, 0.5 mM $MgSO_4$, 10 mM ß-ME, 16.6 mM $(NH_4)_2SO_4$, 100 uM dNTPs (dATP, dTTP, dGTP and dCTP), 2 units of Taq DNA polymerase (Ampli Taq, BRL) and 20pmoles of each primer. The sample was subjected to 35 cycles of amplification with each cycle consisting of three steps: denaturation at 93°C for 10 secs; annealing at 53°C for 30 secs; and extension at 72°C for 60 secs. An aliquot of the RACE products was analysed by electrophoresis on an agarose gel.

2.19.3 CLONING OF THE 3' RACE PRODUCT

The major RACE product (1.4kb) was designated as E10-A. This fragment was digested with HindIII and SphI, recovered from the gel and cloned into the HindIII-SphI sites of pGEM4Z and pGEM3Z. Recombinant plasmids were transformed into E. coli DH5-α as described in Section 2.18.

2.19.4 DNA SEQUENCING OF E10-A cDNA

The sequencing strategy for E10-A cDNA is presented in Figure 32 (Section 3.4). Restriction fragments HindIII-SalI, SalI-SalI and SalI-SphI of E10-A cDNA were subcloned into pGEM4Z and pGEM3Z, and both strands of these inserts were sequenced with the dideoxy method as described in Section

2.18. The junctions of these restricion sites in E10-A cDNA were sequenced with the aid of 17-mer specific primers.

2.20 HYBRID SELECTION BY E10 cDNA AND IN VITRO TRANSLATION OF RNA

The hybridization, washing and elution procedures were carried out essentially as described by Keil et al. (1985). Filters (hybond N; Amersham) carrying 10 ug of linearized plasmid or 3 ug of E10 cDNA was hybridized to 5 ug of total cytoplasmic RNA in 100 ul hybridization buffer. The eluted RNA was translated in vitro in Rabbit Reticulocyte Lysate (Promega) according to the manufacturer's instructions in the presence of ^{35}S-methionine (NEN). The translation products were electrophoresed in either a linear 12% or 5-20% gradient SDS-polyacrylamide gel, essentially as described in Ausubel et al. (1987). The gels were fixed in 10% acetic acid and 40% methanol, impregnated with En^{3}Hance (NEN) for fluorography, and exposed to XPP-1 film (Agfa) for various times. The standard molecular markers (BRL) used were as follows: 200 kd, myosin; 97.4 kd, phosphorylase; 68 kd, bovine serum albumin; 43 kd, ovalbumin. 29 kd, carbonic anhyrase.

3.0 RESULTS AND DISCUSSION

3.1 CLONING AND CHARACTERIZATION OF IE AND E cDNAs

RESULTS

The purpose of this project was to reveal details on transcription from the major IE and especially major E transcription units. This task was made possible by preparing IE and E cDNAs, followed by characterization of these cDNAs and then utilizing a few cDNAs as probes to obtain new information on their respective transcription units. The aim of the work presented in this section (3.1) was to characterize the cDNAs by estimating their sizes and mapping them to specific regions (endonuclease restriction fragments) of the viral genome.

3.1.1 PREPARATION AND SCREENING FOR IE AND E cDNAs

The IE and E cDNAs of Smith strain MCMV were prepared and cloned into Lambda gt10 at the EcoR1 site as described in Materials and Methods. A total of 50 potential IE and 198 potential E clones were identified by the plaque lift method and hybridization to a MCMV DNA probe. Of these positive clones, nine IE and ten E clones were selected randomly and subjected to rescreening and characterization.

3.1.2 CHARACTERIZATION OF E cDNAs

3.1.2.1 SIZE ESTIMATION

DNA from the 10 selected E cDNA-Lambda gt10 recombinants were subjected to EcoR1 digestion and separated on an agarose gel along with HindIII digested Lambda DNA fragments as molecular weight markers, HindIII digested MCMV DNA (positive control) and EcoR1 digested Lambda gt10 DNA (negative control). The gel was stained with ethidium bromide and viewed with a UV transilluminator. DNA fragments were transferred to a membrane and

hybridized to MCMV DNA probe to confirm the origin of E cDNA clones (Figure 4). MCMV HindIII fragments (positive control) and all the E cDNAs hybridized to the MCMV probe, while Lambda gt10 DNA (negative control) did not hybridize to MCMV DNA. None of the inserts gave more than one band and therefore none of the cDNAs had an internal EcoR1 site. It is most likely that the internal EcoR1 sites were not protected by the methylase during the cloning procedure. Table I summarizes the molecular weights of the 10 E cDNA inserts, which range from 1.15kb to 3.2kb.

3.1.2.2 MAPPING THE E cDNAs

Unlike IE transcription, E transcription occurs in most regions of the genome (Marks et al., 1983; Keil et al., 1984). Due to the large size of the viral genome, the mapping of the cDNAs to these regions became an elaborate procedure. The E cDNAs were mapped to specific restriction endonuclease fragments of the MCMV (Smith strain) genome either by the slot blot method or by the Southern blot method.

METHOD 1: THE SLOT BLOT METHOD

Only two of the ten E cDNAs, E2 and E10 were mapped by this method. DNAs from E cDNA-Lambda gt10 recombinants, Lambda gt10 (negative control), and MCMV were blotted onto the membrane and hybridized to various probes including MCMV total DNA (positive control) (Figure 5) and individually to HindIII fragments I (Figure 6), J (Figure 7), K (not shown) and L (not shown). None of the DNAs, except for MCMV, hybridized to HindIII K or L fragments. The results of probing these blots with HindIII I and J fragments, and MCMV DNA were as follows:

1. All cDNAs and MCMV DNA hybridized to MCMV DNA probe (Figure 5)
2. Lambda gt10 DNA did not hybridize to either the MCMV DNA or HindIII

Figure 4: Southern blot analysis of E cDNAs.
DNA (0.5 ug) from 10 selected E cDNA clones (as Lambda gt10 recombinants, E1-E10) were subjected to EcoR1 digestion and separated on a 1.2% agarose gel along with 0.5 ug Lambda HindIII DNA fragments as molecular weight markers, 0.5 ug/lane Smith HindIII DNA fragments (positive control) and 0.5 ug Lambda gt10 EcoR1 digested DNA (negative control). The DNA fragments were transferred to a membrane, hybridized to ^{32}P-labelled Smith DNA probe and autoradiographed. The sizes of the inserts were estimated as described in 'Materials and Methods'.

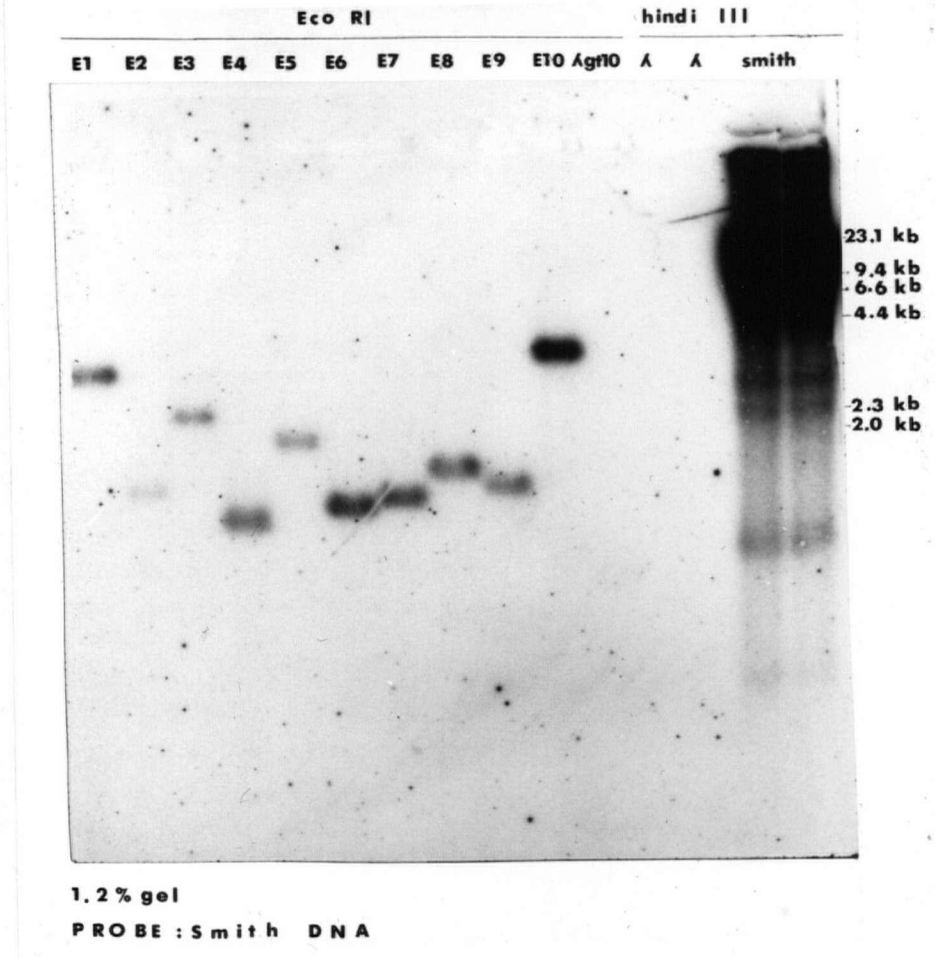


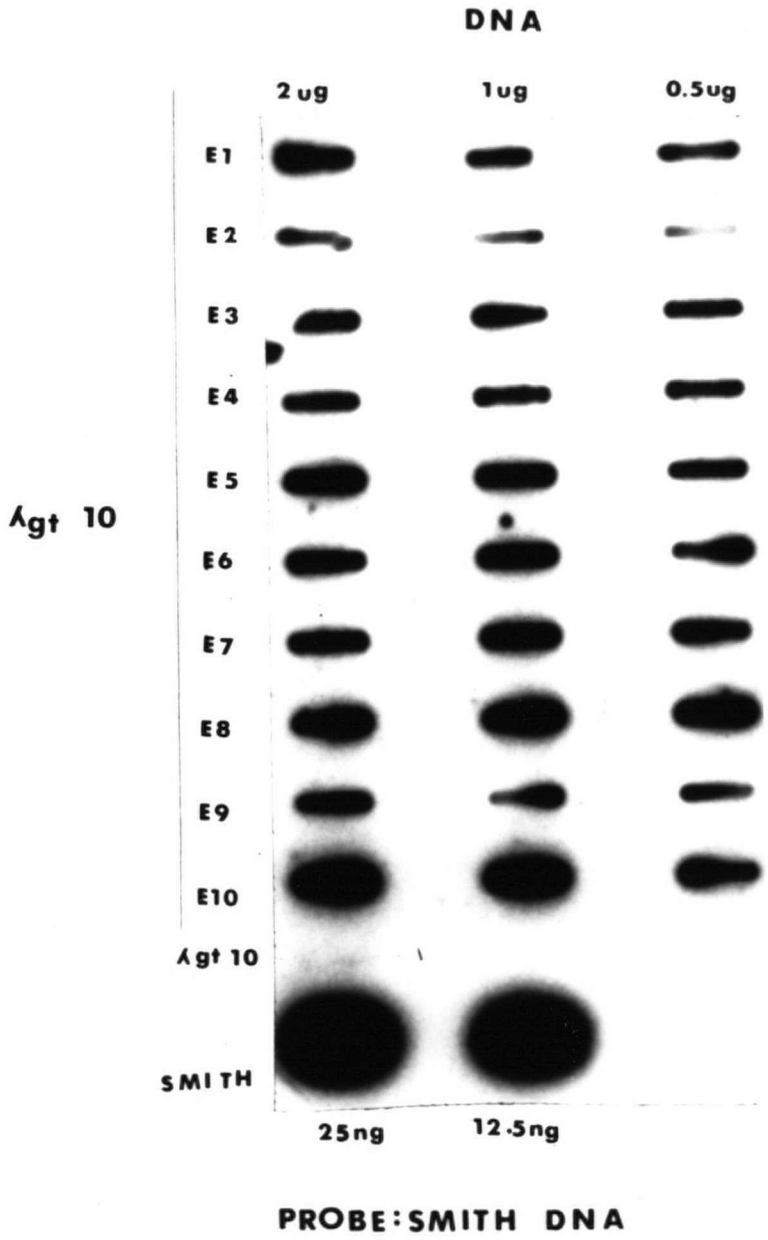
TABLE I

Summary of E cDNAs: insert sizes and hybridization properties with HindIII, XbaI AND EcoR1 fragments of Smith MCMV DNA.

E cDNA GROUPS	cDNA SIZE (kb)	HYBRIDIZATION TO SMITH DNA FRAGMENT		
		Hind III	Xba I	EcoR 1
GROUP 1				
E1	2.75	E	O,M,X/Y	O
E4	1.15	E	M	O
GROUP 2				
E8	1.5	E	L	-
GROUP 3				
E2	1.4	J	-	-
E10	3.2	I,J	-	-
GROUP 4				
E3	2.3	B	A	L
E5	1.6	B	A	L
GROUP 5				
E6	1.25	A	E	C
GROUP 6				
E7	1.30	F	F/G	R/S
GROUP 7				
E9	1.33	A	R	M

Note: Restriction fragments correspond to those on the physical map of MCMV constructed by Ebeling et al. (1983) (see Figure 2).

Figure 5: Hybridization of E cDNAs to MCMV DNA.
2 ug, 1 ug and 0.5 ug of E cDNAs (as Lambda gt10 recombinants E1-E10), Lambda gt10 DNA, and 25 ng and 12.5 ng of MCMV (Smith) DNA were blotted on a membrane. The membrane was hybridized to ^{32}P-labelled total Smith MCMV DNA and autoradiographed for 16 hr. Lambda gt10 and MCMV (Smith) DNA served as negative and positive controls, respectively.



fragments I and J as shown in Figures 5,6 and 7

3. The E2 cDNA insert hybridized with the HindIII J probe (Figure 7) and the E10 cDNA insert hybridized to both the HindIII J fragment (Figure 7) and I fragment (Figure 6)

In summary, the two E cDNA clones, E2 and E10 mapped to HindIII J and adjacent I-J fragments of the MCMV genome respectively. Preliminary studies have shown that this region is heavily transcribed during the E phase (Marks et al., 1983; Keil et al., 1984), therefore E2 and E10 are perhaps important cDNAs. Since none of the cDNAs mapped to the HindIII K-L region, none are of IE origin.

METHOD 2: THE SOUTHERN BLOT METHOD

The Southern blot method was used to map 8 E cDNAs to specific regions of the genome. Samples of MCMV (Smith) DNA were digested to completion with HindIII, XbaI or EcoR1 and the fragments separated in a 0.7% agarose gel. The digested samples were loaded at 0hr and 48hr, and the gel was electrophoresed at low voltage (30 volts) for a total of 72 hr. The 72 hr separation was to separate the cluster of large (33.8 to 14.3 kbp) and moderate (8.1 to 7.0 kbp) sized bands so that each band could be individually recognized. Since smaller bands run off the gel in 72hr, the same samples were loaded after 48 hr in order to visualize hybridization to smaller fragments. The Southern blot was hybridized to a specific cDNA probe and autoradiographed. The probe was subsequently stripped off the membrane (see Materials and Method) and in some cases rehybridized to another cDNA probe before finally being hybridized to MCMV DNA probe to identify the position of restriction fragments on the blot. The autoradiogram from the experiment with E cDNA probe was superimposed onto that from the experiment with the MCMV DNA probe and individual restriction

Figure 6:.Hybridization of E1-10 cDNAs to the MCMV HindIII I fragment.
250 ng and 125 ng of E cDNAs (as Lambda gt10 recombinants) were blotted onto a membrane, hybridized to the ^{32}P-labelled MCMV HindIII I probe and autoradiographed. 250 ng and 125 ng of Lambda gt10, and 25 ng and 12.5 ng of MCMV (Smith) DNA were present on the blot as negative and positive controls, respectively.

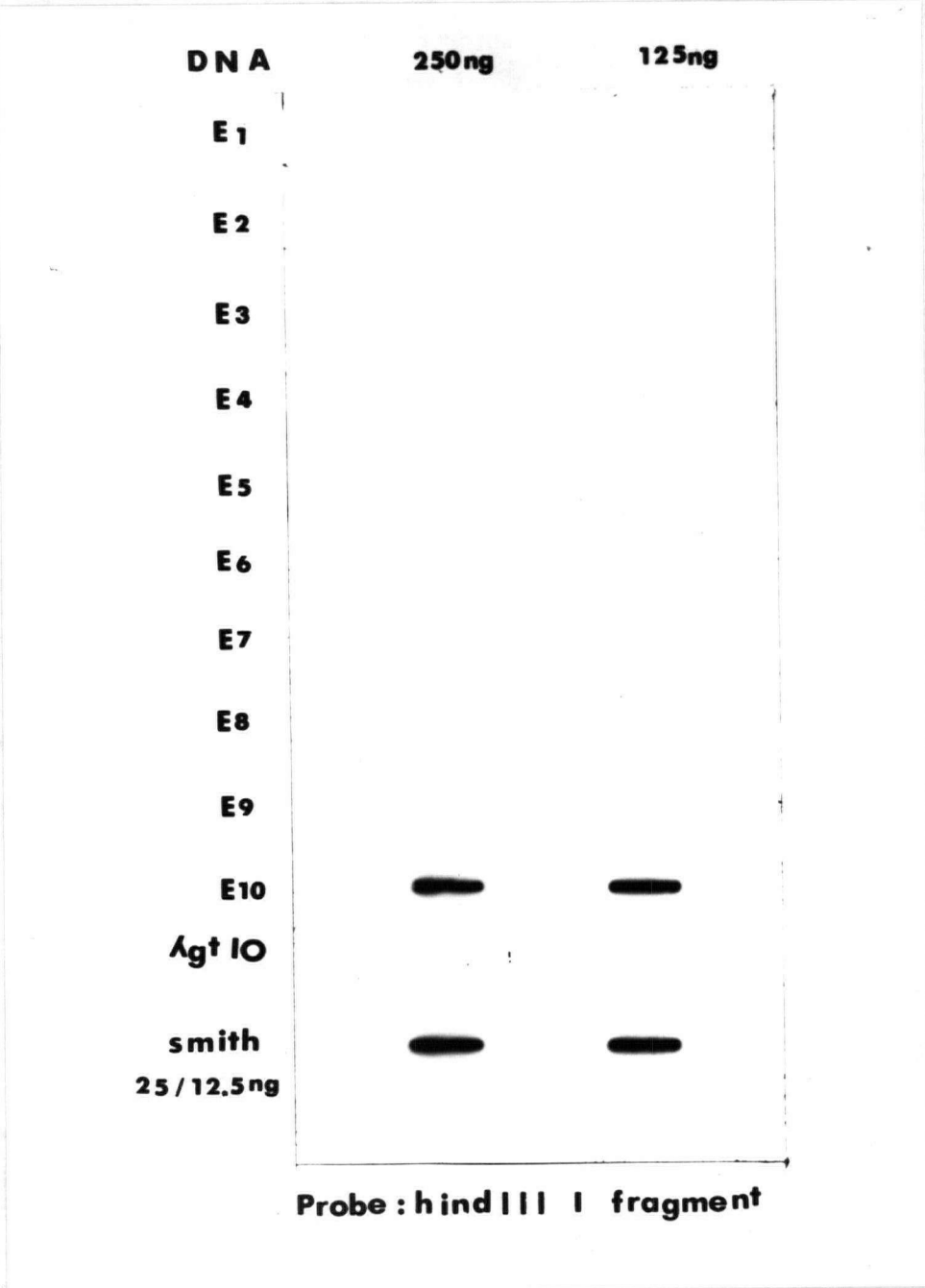
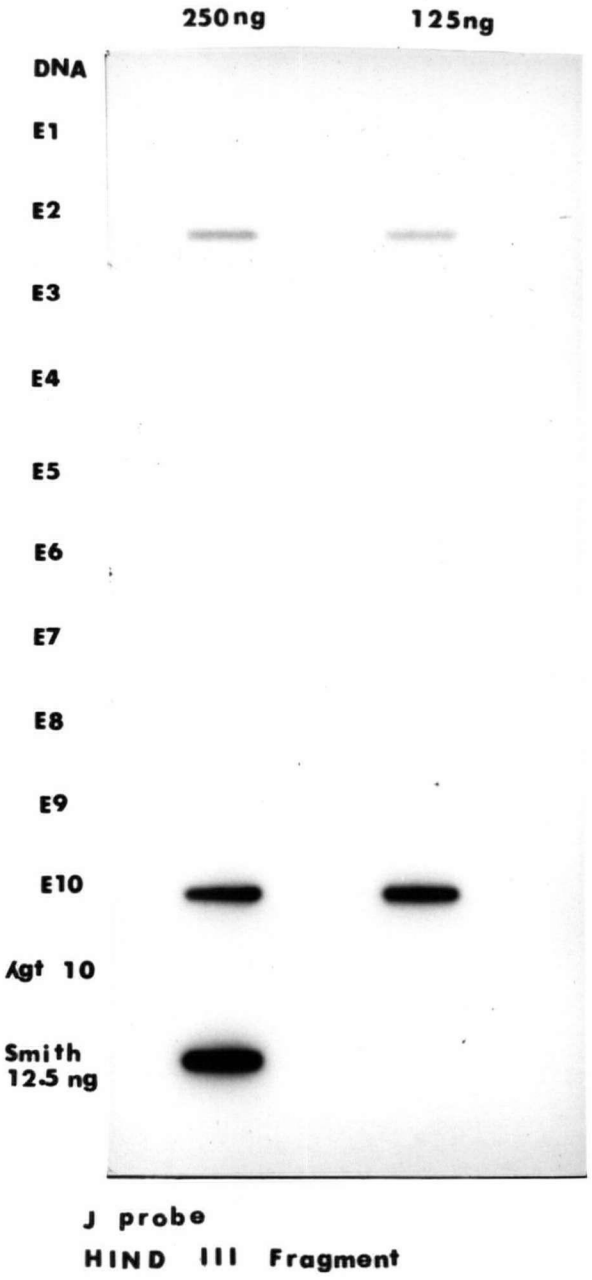


Figure 7:.Hybridization of E1-10 cDNAs to the MCMV HindIII J fragment.
250 ng and 125 ng of E cDNAs (as Lambda gt10 recombinants) and Lambda gt10 DNA were blotted onto a membrane, hybridized to the ^{32}P-labelled MCMV HindIII J probe and autoradiographed. 12.5 ng of MCMV (Smith) DNA was also present on the slot blot as a positive control.



digest fragments hybridizing to specific cDNA probe were identified (Figures 8 through 20). A summary of the E cDNAs mapped by this procedure to specific HindIII, XbaI and EcoR1 fragments on the MCMV physical map is presented in Figure 21. An important point to note is that the HindIII H fragment of the Smith strain of MCMV used in our laboratory represents the HindIII E fragment of MCMV in Ebeling et al. (1983) (unpublished data).

The ten E cDNAs were assigned to seven groups with respect to their genomic location, as summarized in Table I. Preliminary studies have indicated that the transcription during the E phase occurs in most regions of the genome, although abundant transcription occurs in HindIII fragments A, B, G, E, F, I and J. All of the seven groups map to one of these HindIII fragments and therefore may represent the important class of E genes.

Figure 8: Mapping of E1 cDNA to HindIII, XbaI and EcoR1 fragments of MCMV DNA.

MCMV (Smith) DNA samples were subjected to HindIII (H), XbaI (X) and EcoR1 (E) digestion, and the DNA fragments were separated on a 0.7% agarose gel and transferred to a membrane. The samples (250 ng DNA/well) were loaded at 0hr (total separation time 72 hr or 3 days) and at 48hr (total separation time 24 hr or 1 day). The first three lanes under 3d represent a three day run, and lanes 4, 5 and 6 under 1d represent a one day run. The last two lanes are molecular weight markers [Lambda HindIII fragments (250 ng/lane) separated for three days (3d) and one day (1d)]. The Southern blot was hybridized to E1 cDNA recombinant clone(^{32}P-labelled) and autoradiographed for 16 hr. The E1 cDNA probe was stripped off the blot and the blot was rehybridized to ^{32}P-labelled MCMV (Smith) DNA probe to identify all the restriction fragments (Figure 9). The Lambda HindIII markers are visible in the autoradiogram because the fragments hybridize to the Lambda gt10 DNA in the E1 cDNA recombinant clone. The sizes of the DNA fragments were estimated as described in 'Materials and Methods'.

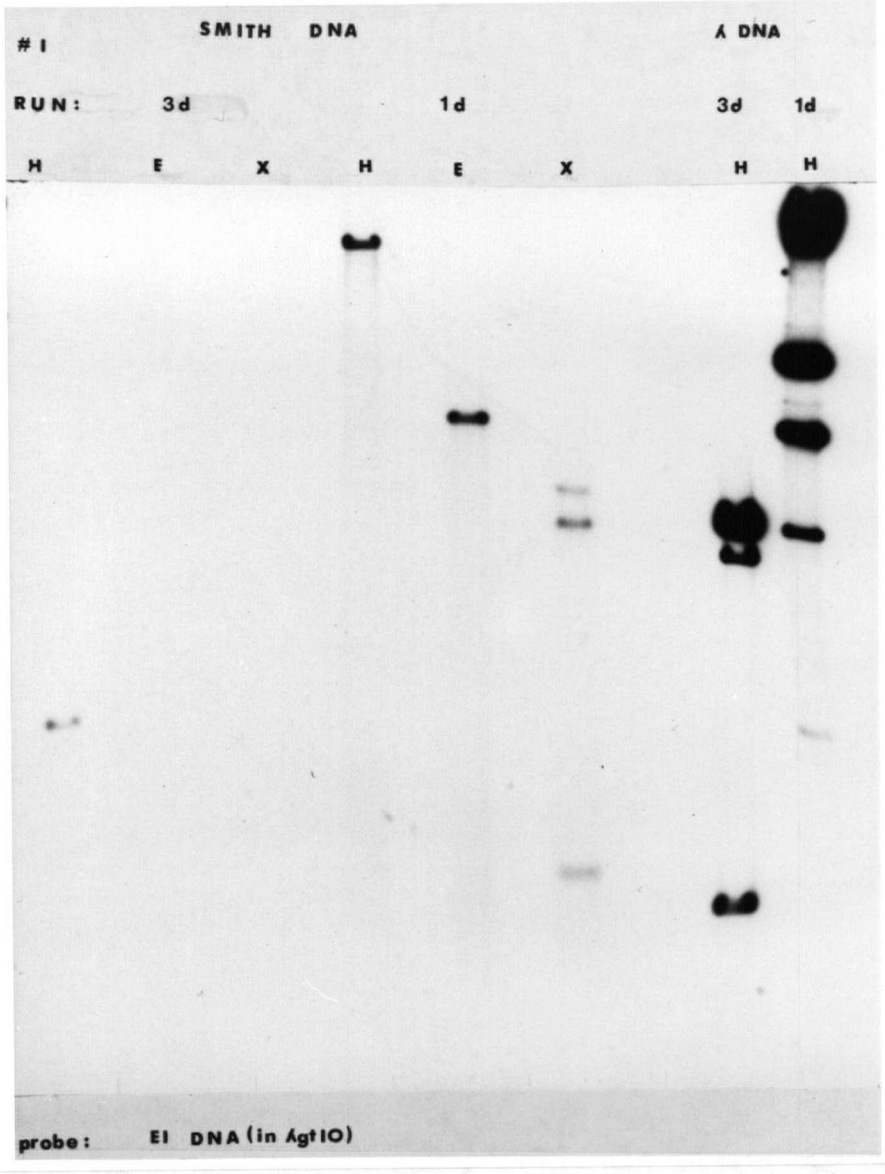


Figure 9: Hybridization of HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA with a labelled MCMV DNA probe.
Details of the separation are given in the Figure 8. The southern blot in Figure 8 was stripped of the E1 ^{32}P-labelled probes and hybridized with ^{32}P-labelled MCMV DNA to identify all HindIII, XbaI and EcoR1 fragments.

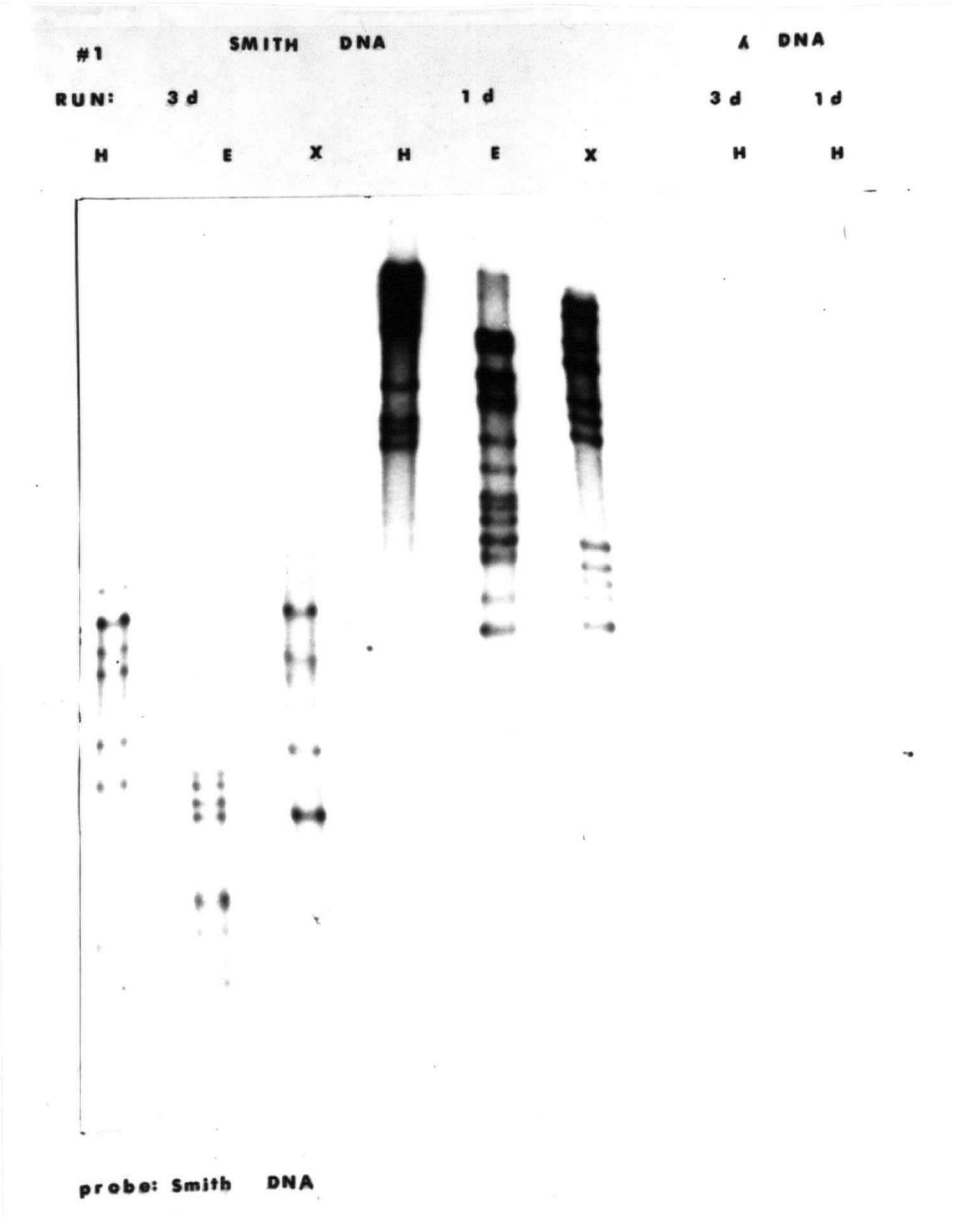


Figure 10: Mapping of cDNA E4 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
MCMV (Smith) DNA samples were digested with HindIII (H), XbaI (X) and EcoR1 (E) and separated on a 0.7% agarose gel and transferred to a membrane as described in Figure 8. The blot was hybridized first with ^{32}P-labelled E4 DNA (this figure) and then stripped of the probe and reprobed with E6 DNA (Figure 11) and finally MCMV DNA (Figure 12).

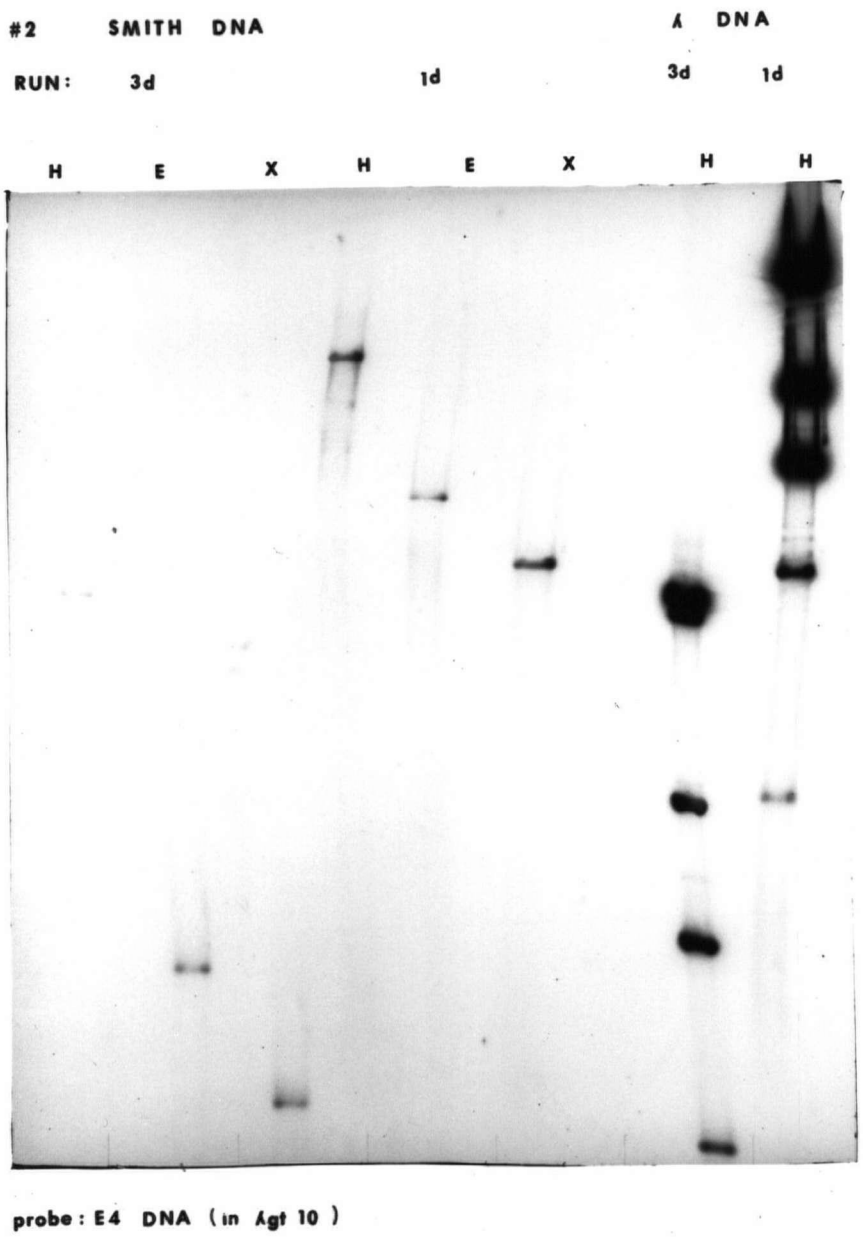


Figure 11: Mapping E6 cDNA to HindIII (H), XbaI (X) and EcoR1 (E) fragments of MCMV (Smith) DNA.
The blot from figure 10 was reprobed with ^{32}P-labelled E6 DNA. For Details see Figure 10.

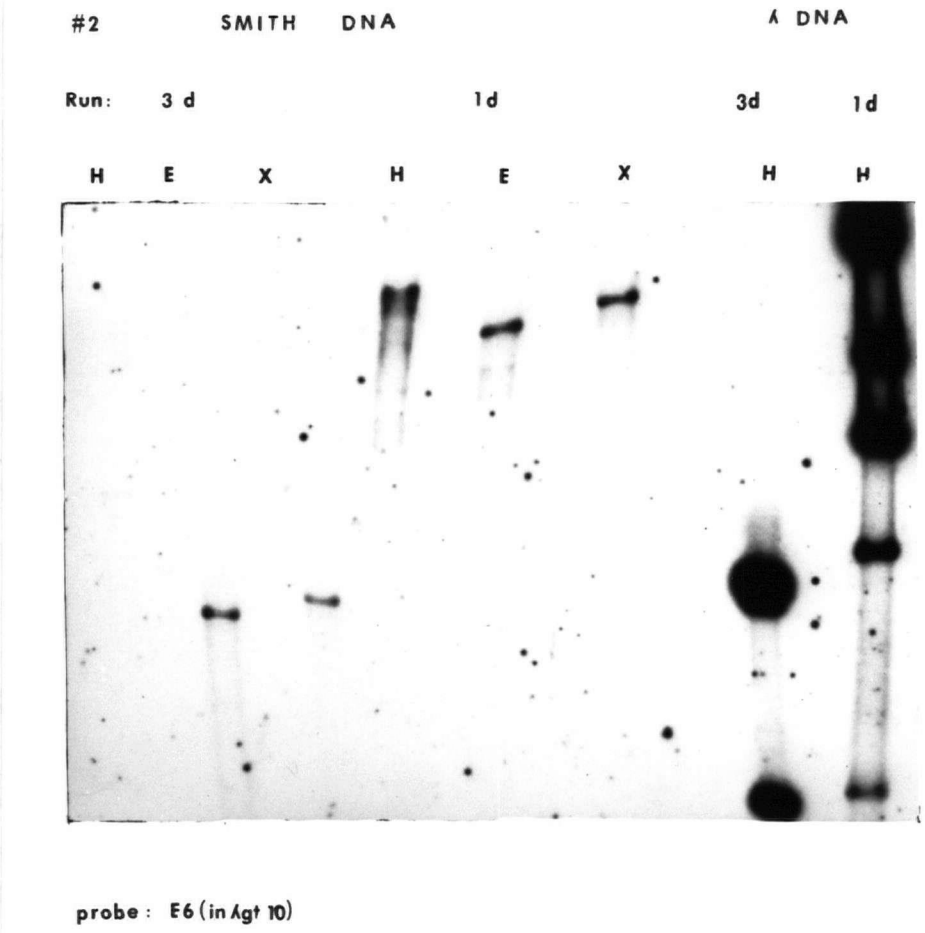


Figure 12: Hybridization of HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA with a labelled strain MCMV DNA probe. The blot from figure 10 was reprobed with ^{32}P-labelled MCMV DNA. For details see Figure 10.

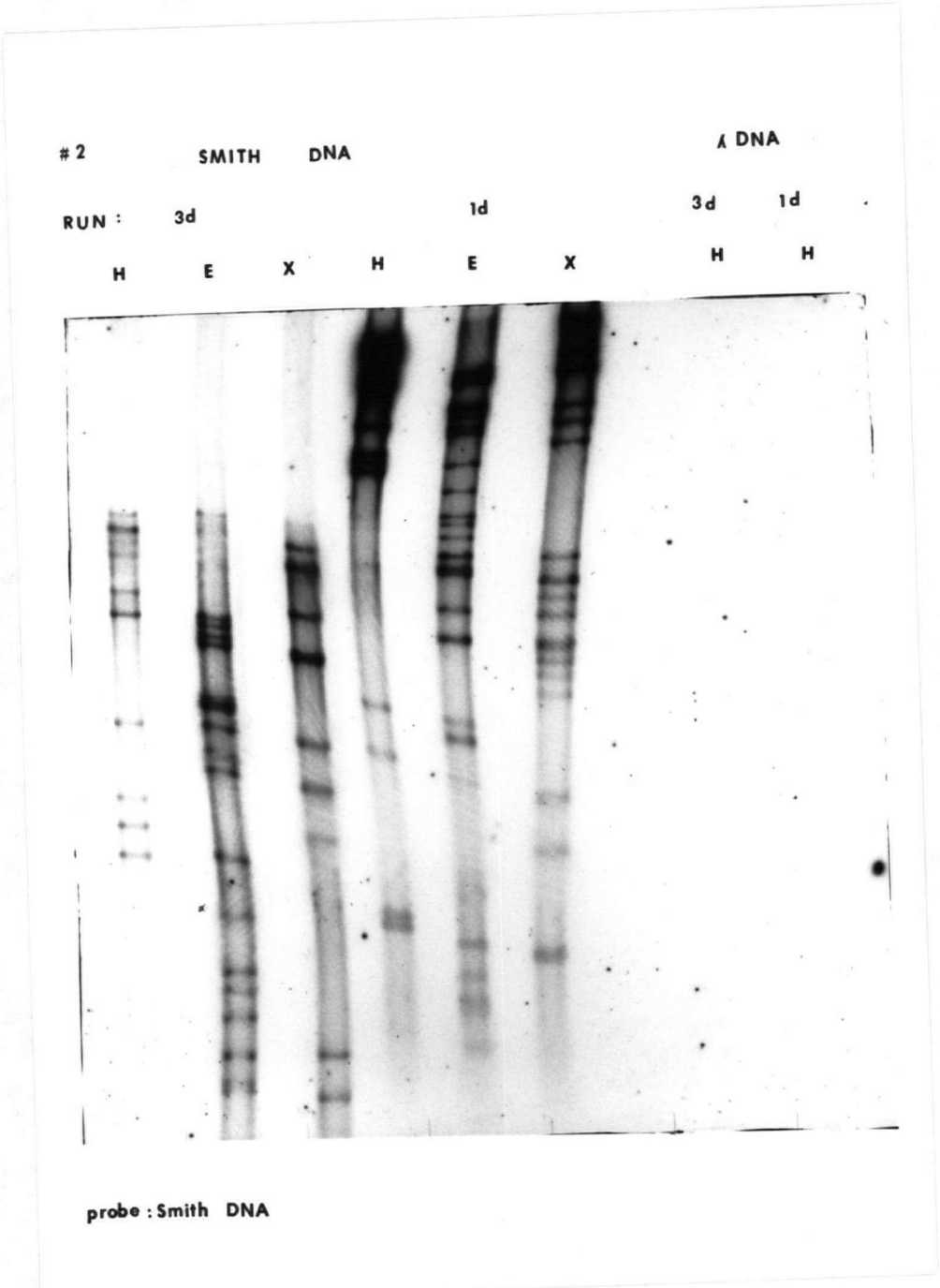


Figure 13: Mapping of cDNA E7 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
MCMV (Smith) DNA samples were digested with HindIII (H), XbaI (X) and EcoR1 (E) and separated on a 0.7% agarose gel and transferred to a membrane as described in Figure 8. The blot was hybridized first with ^{32}P-labelled E7 DNA (this Figure) and then stripped of the probe and reprobed with E8 DNA (Figure 14) and finally MCMV (Smith) DNA (Figure 15).

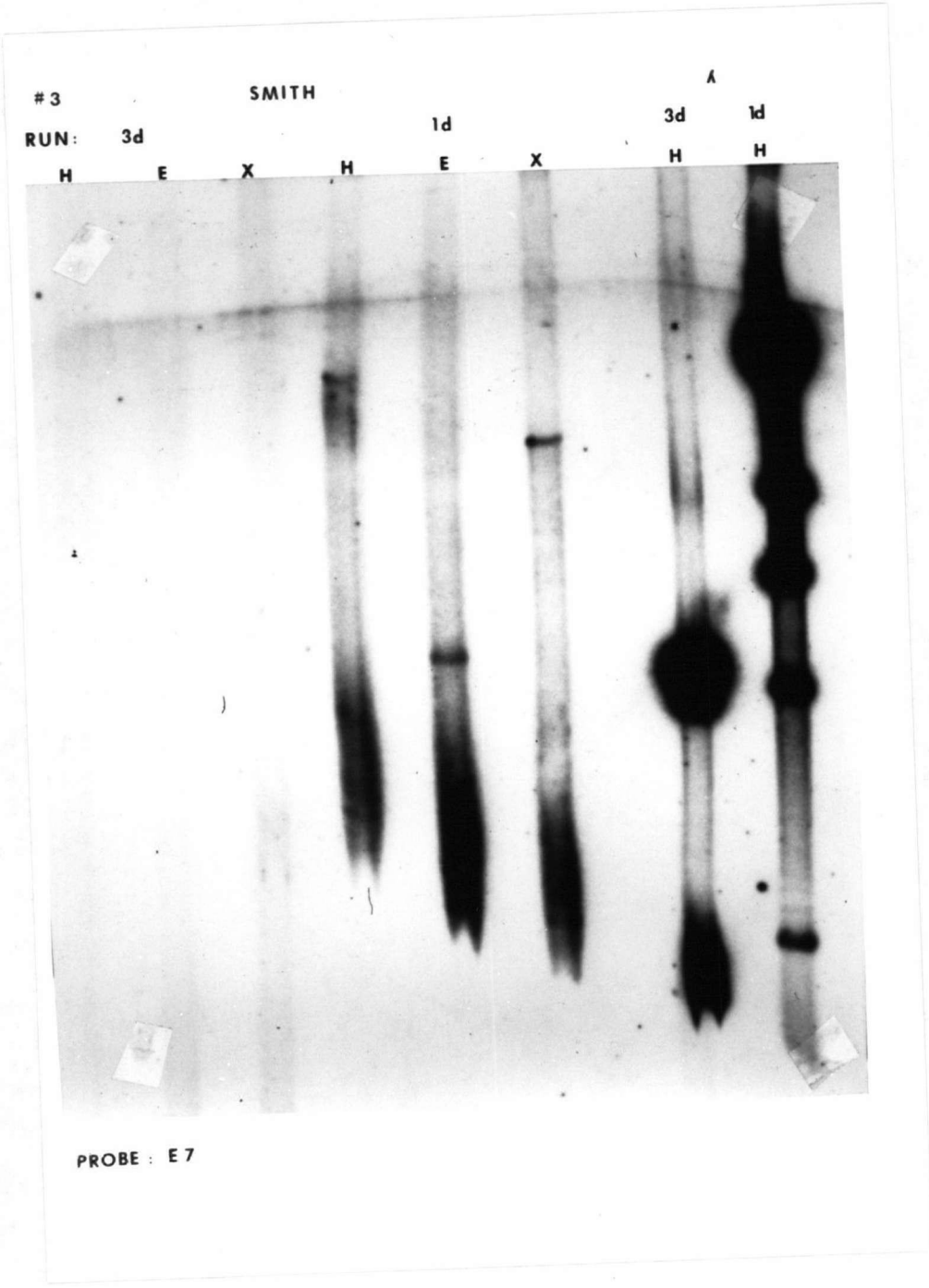


Figure 14: Mapping of cDNA E8 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
The blot from figure 13 was reprobed with ^{32}P-labelled E8 DNA. For details see Figure 13.

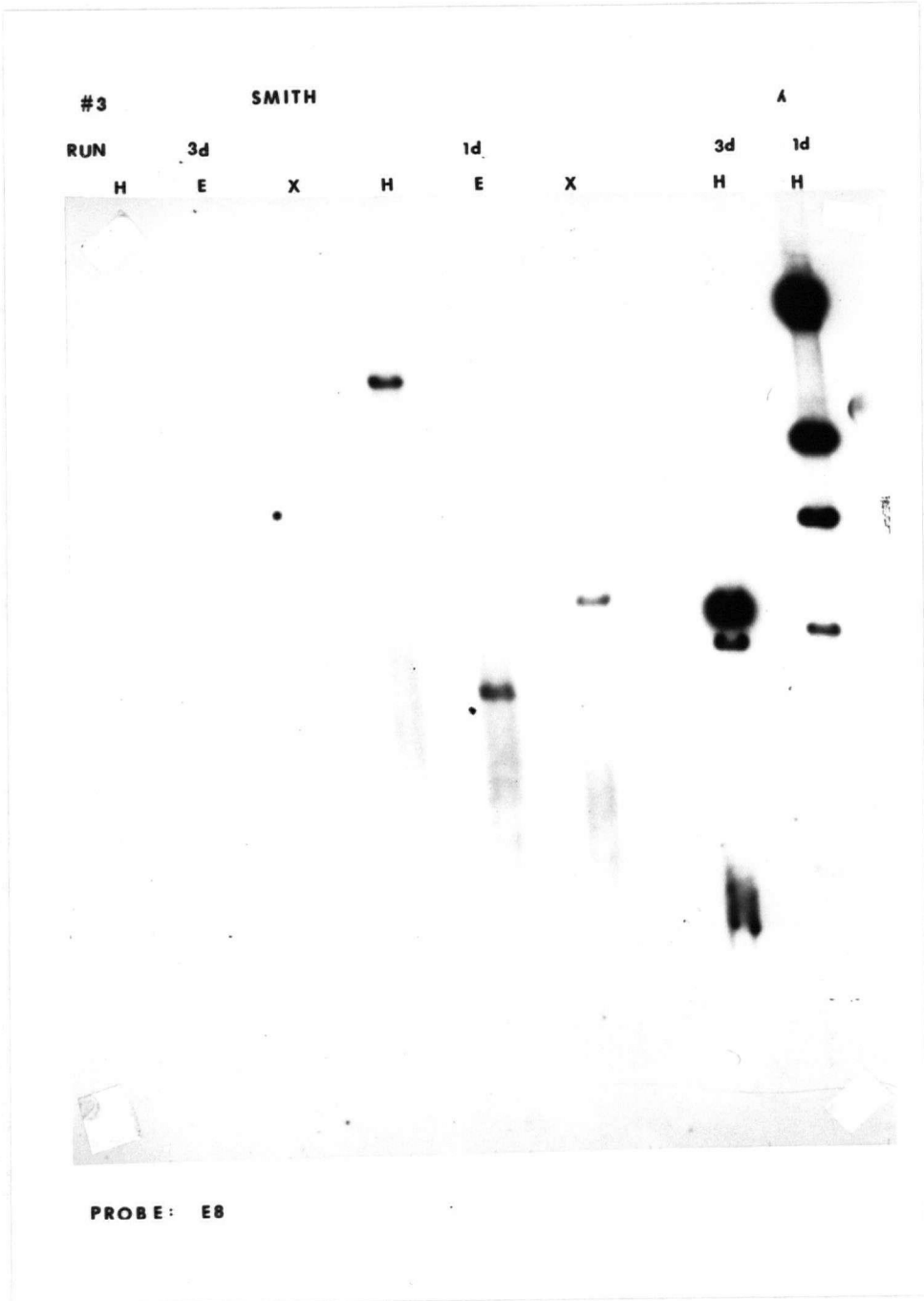


Figure 15: Hybridization of HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA with a labelled MCMV DNA probe.
The blot from figure 13 was reprobed with ^{32}P-labelled MCMV DNA. For details see Figure 13.

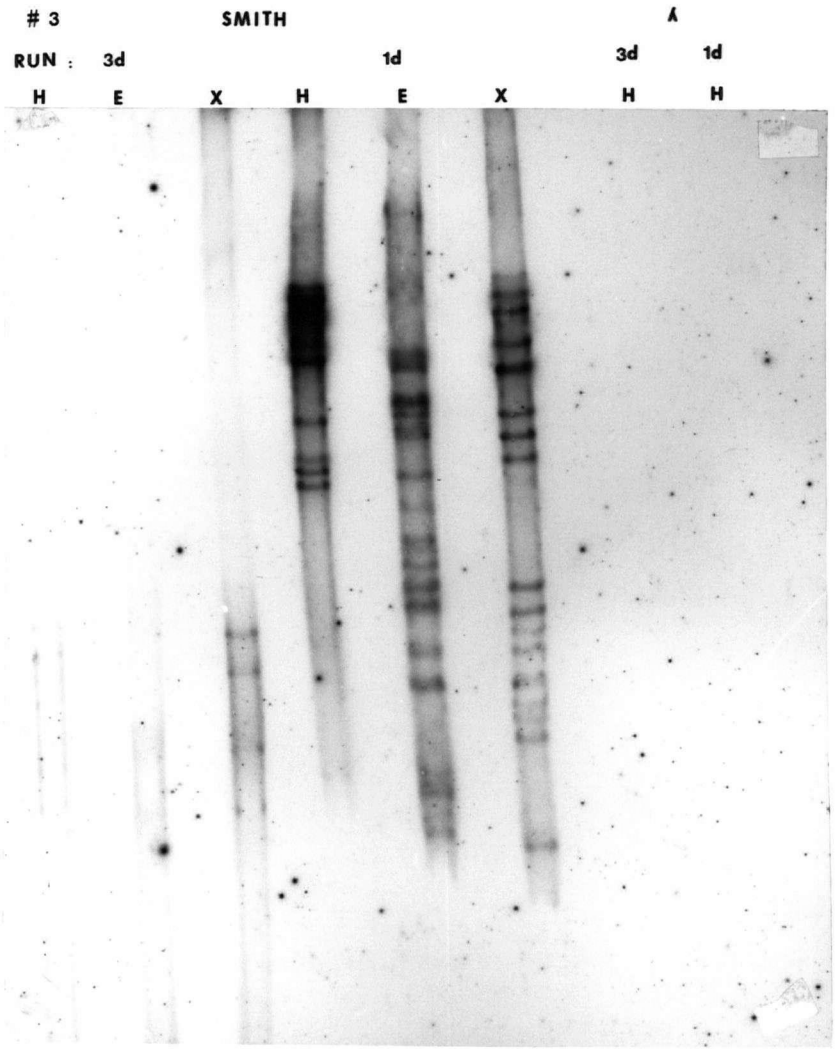


Figure 16: Mapping of cDNA E9 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
MCMV (Smith) DNA samples were digested with HindIII (H), XbaI (X) and EcoR1 (E) and separated on a 0.7% agarose gel and transferred to a membrane as described in Figure 8. The blot was hybridized first with ^{32}P-labelled E9 DNA (this Figure) and then stripped of the probe and reprobed with MCMV DNA (Figure 17).

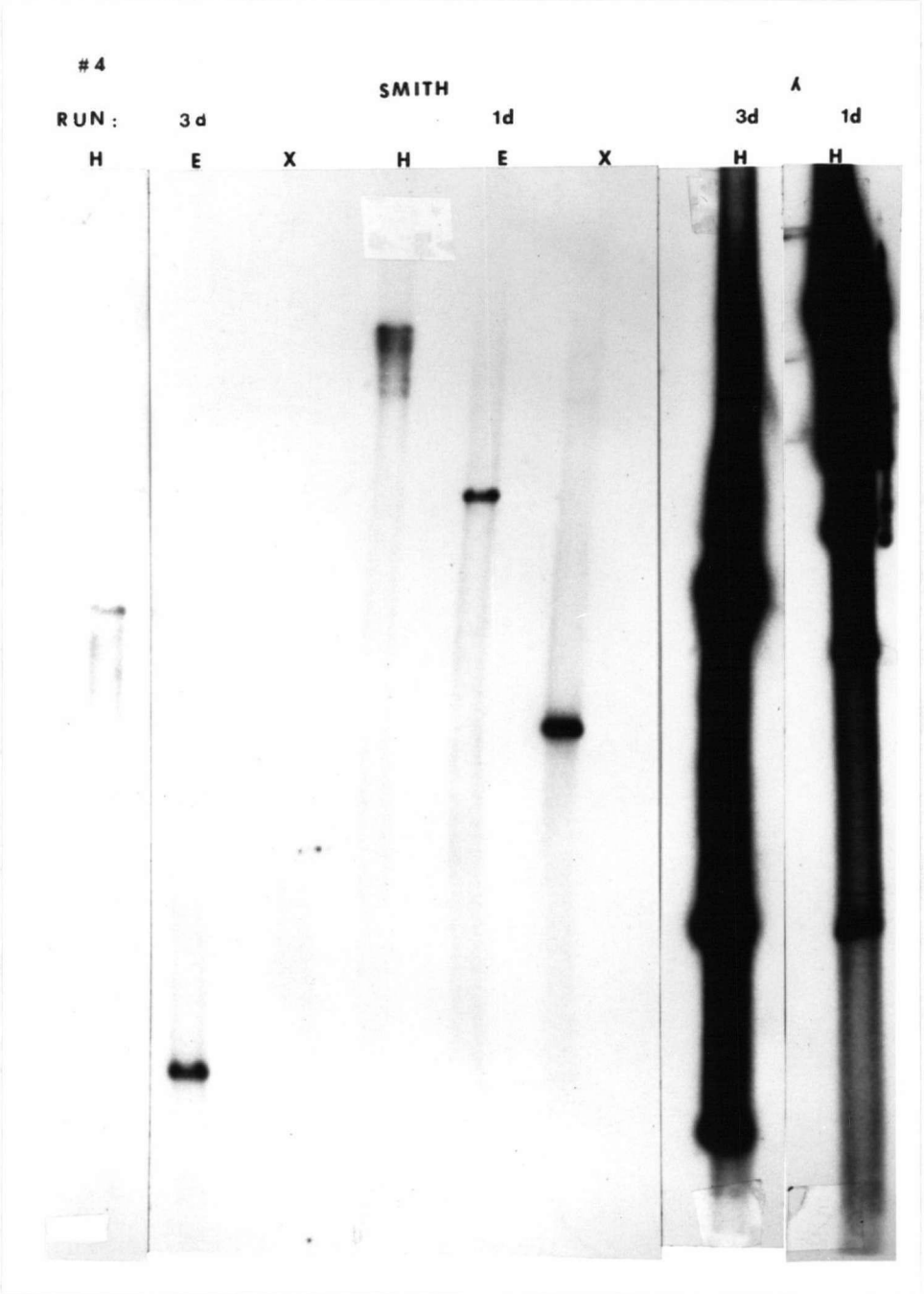


Figure 17: Hybridization of HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA with a labelled MCMV DNA probe. The blot from figure 16 was reprobed with ^{32}P-labelled MCMV DNA. For details see figure 16.

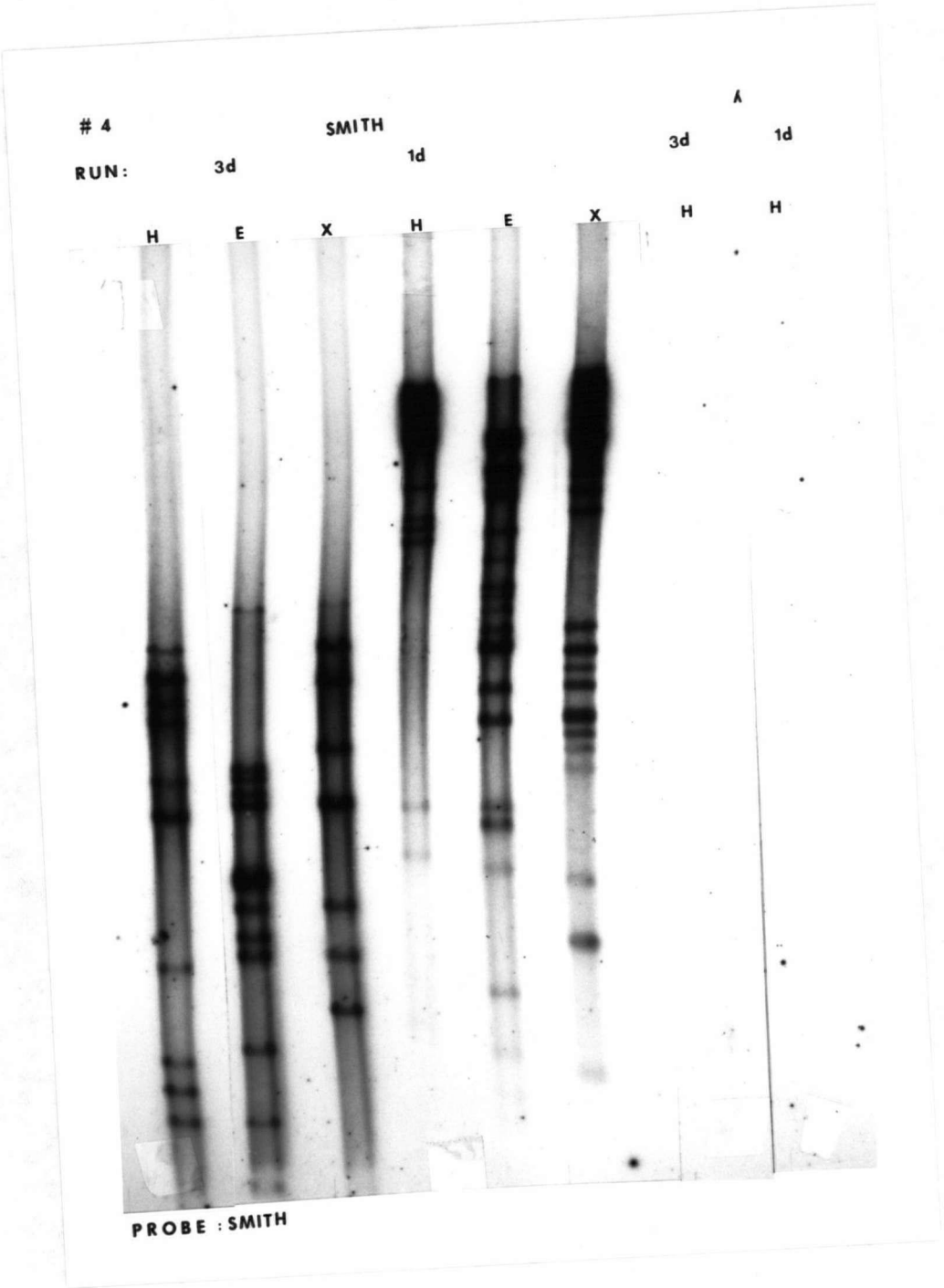


Figure 18: Mapping of cDNA E3 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
MCMV DNA samples were digested with HindIII (H), XbaI (X) and EcoR1 (E) and separated on a 0.7% agarose gel and transferred to a membrane as described in Figure 8. The blot was hybridized first with ^{32}P-labelled E3 DNA (this Figure) and then stripped of the probe and reprobed with E5 DNA (Figure 19) and finally MCMV DNA (Figure 20).

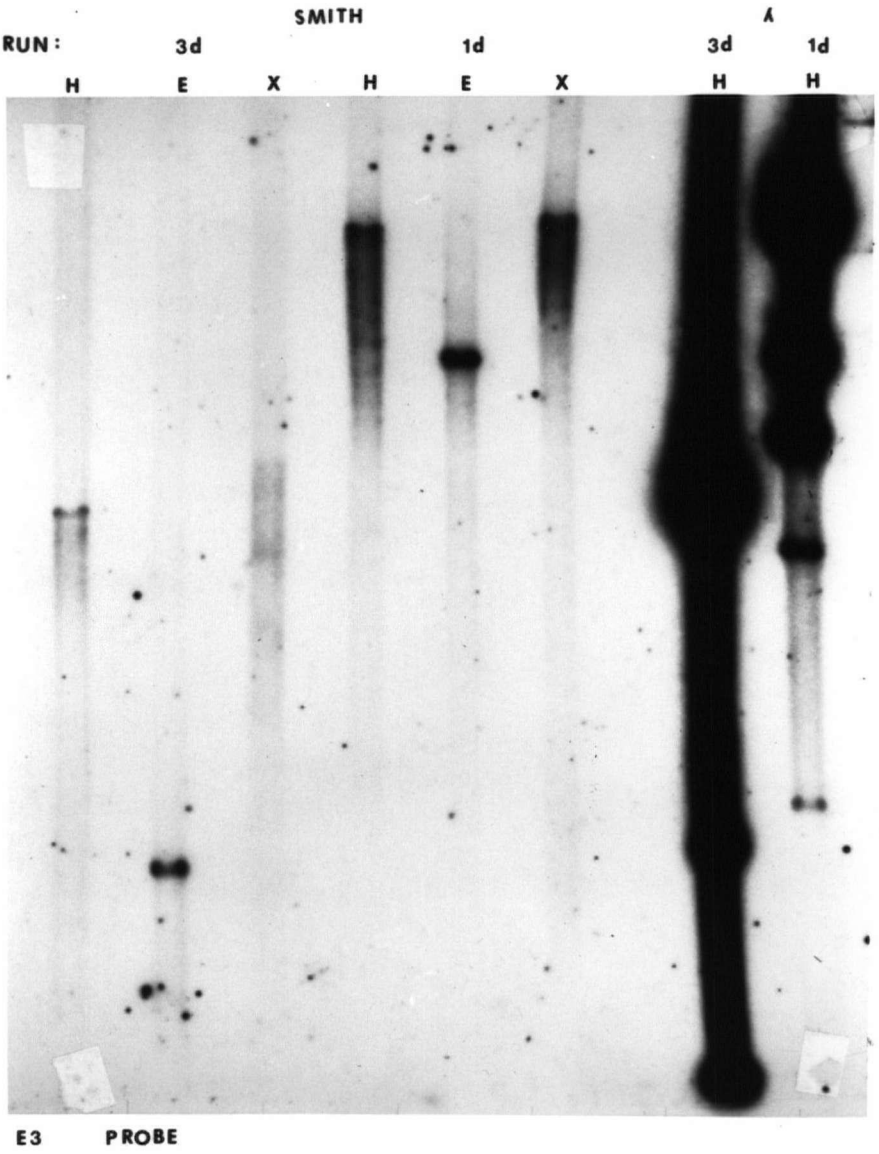


Figure 19: Mapping of cDNA E5 to HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA.
The blot from figure 18 was reprobed with ^{32}P-labelled E5 DNA. For details see Figure 18.

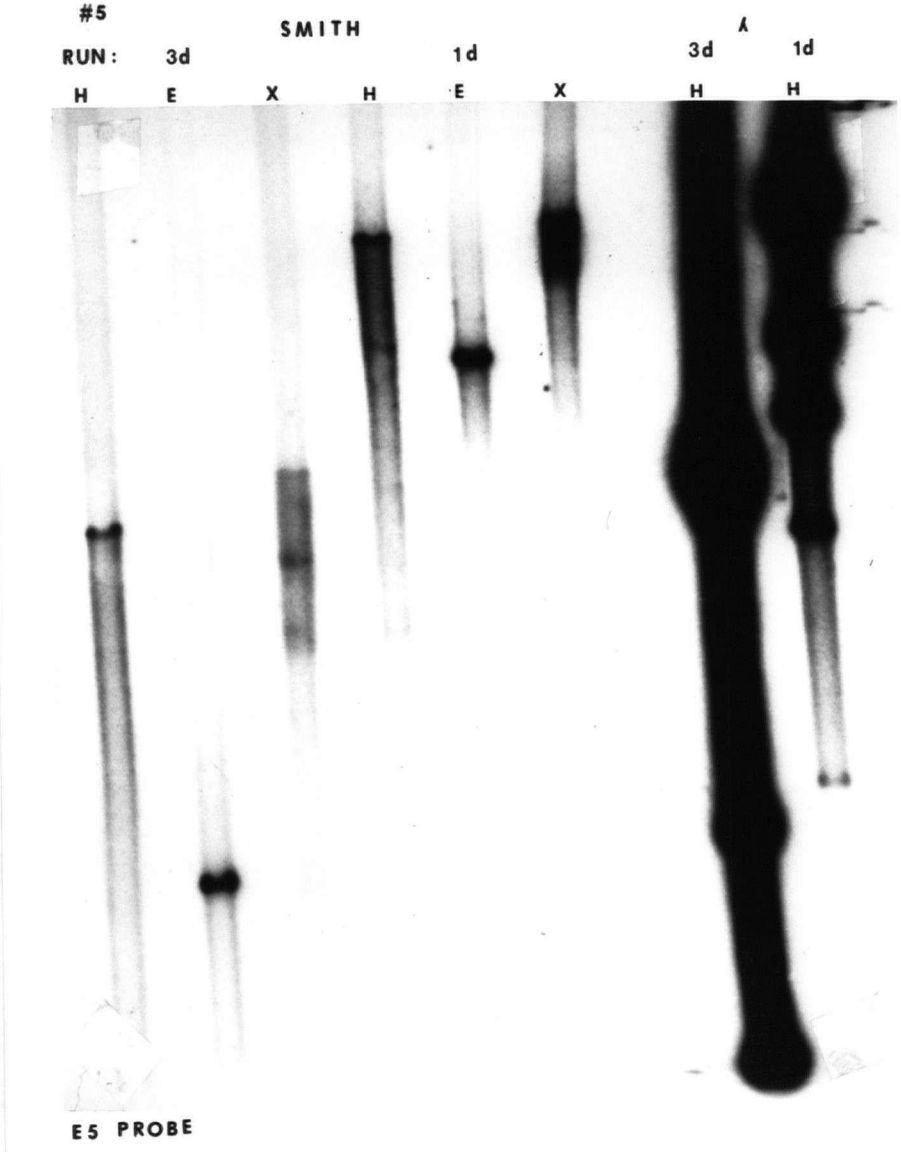


Figure 20: Hybridization of HindIII, XbaI and EcoR1 fragments of MCMV (Smith) DNA with a labelled MCMV DNA probe.
The blot from figure 18 was reprobed with ^{32}P-labelled MCMV DNA. For details see Figure 18.

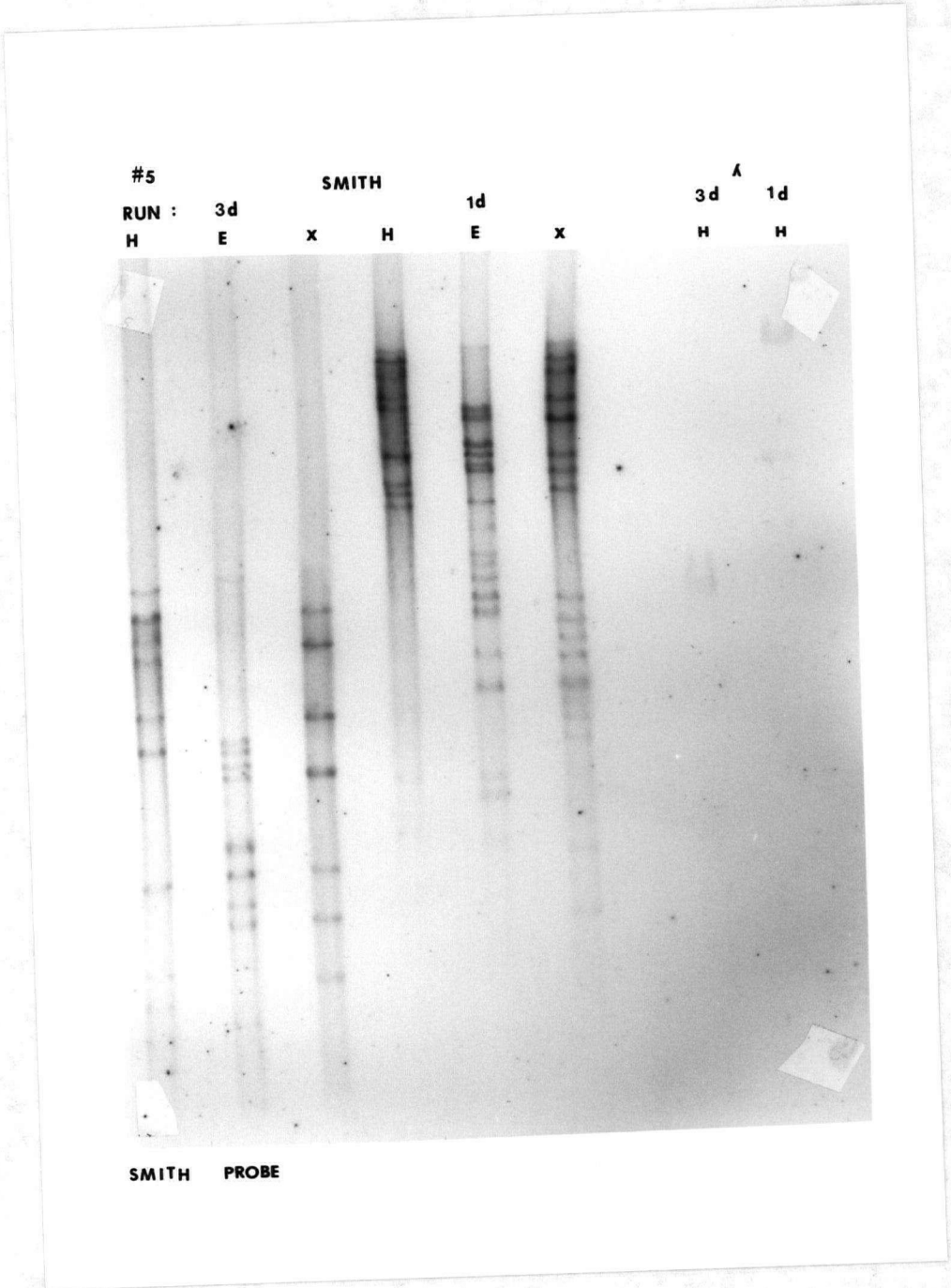
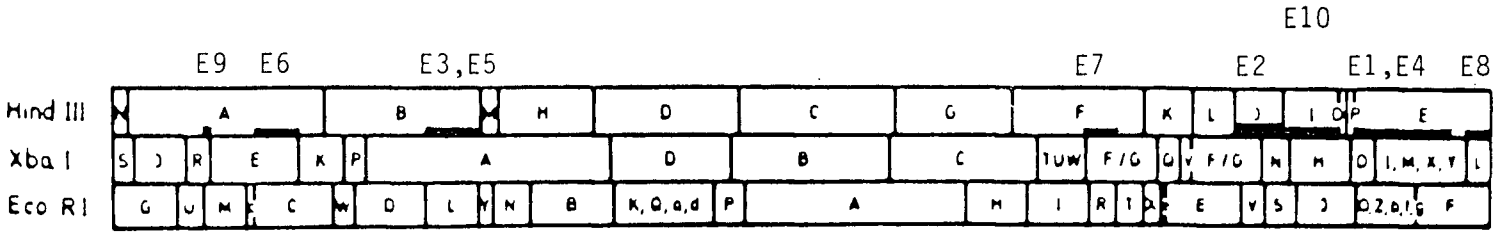


Figure 21: Summary of mapping studies of E1-10 cDNAs on the Smith strain MCMV genome. Locations of E cDNAs are assigned to the MCMV physical map. The horizontal bars represent the regions assigned to each cDNA clone. The restriction fragment map was originally constructed and presented in Ebeling et al., (1983).



3.1.3 CHARACTERIZATION OF IE cDNAs

The IE cDNAs were characterized by estimating their sizes and their hybridization properties to HindIII K-L region, the major IE region.

3.1.3.1 SIZE ESTIMATION

The insert sizes of the MCMV IE cDNAs were estimated by electrophoresing the EcoR1 digested recombinant phage DNAs on an agarose gel, along with HindIII digested MCMV DNA, EcoR1 digested Lambda gt10 DNA and HindIII digested lambda DNA fragments as molecular weight markers. The DNA fragments were transferred to a membrane and hybridized to MCMV DNA to confirm that the DNA fragments were of viral origin. Figure 22 shows that the MCMV (Smith) DNA probe hybridized to MCMV (Smith) HindIII fragments (positive control) and some of the IE cDNAs, but not to Lambda gt10 (negative control). Four of the nine IE cDNAs (IEa, IEb, IEh and IEi) did not contain a band hybridizing with MCMV DNA in this exposure however, examination of the ethidium bromide stained gel suggested this was likely due to insufficent DNA on the gel. Of these four, one cDNA insert (IEh) was visible on a longer exposure of the autoradiogram (not shown). Furthermore, evidence presented below (Figure 24) indicates that the IEi clone must also contain a cDNA sequence originating from MCMV sequences. Molecular weights of five of the IE cDNA inserts ranged from 1.10kb to 1.60kb (Figure 22 and Table III). All the inserts in the autoradiogram gave only one band. Hence there are no internal EcoR1 sites in these cDNAs and again it is likely that the methylation reaction to protect internal EcoR1 sites during the cloning procedures was ineffective.

Figure 22: Southern blot analysis of IE cDNA inserts.
DNA (0.5ug) of Lambda gt10-IE cDNA recombinants were subjected to EcoR1 digestion and separated on 1.2% agarose gel along with 250ng MCMV (Smith) HindIII DNA fragments as positive control, 0.5ug Lambda gt10 DNA digested with EcoR1 as negative control and 250ng Lambda HindIII fragments as molecular weight markers. The DNA fragments were transferred to a membrane and hybridized to ^{32}P-labelled MCMV (Smith) DNA probe and autoradiographed.

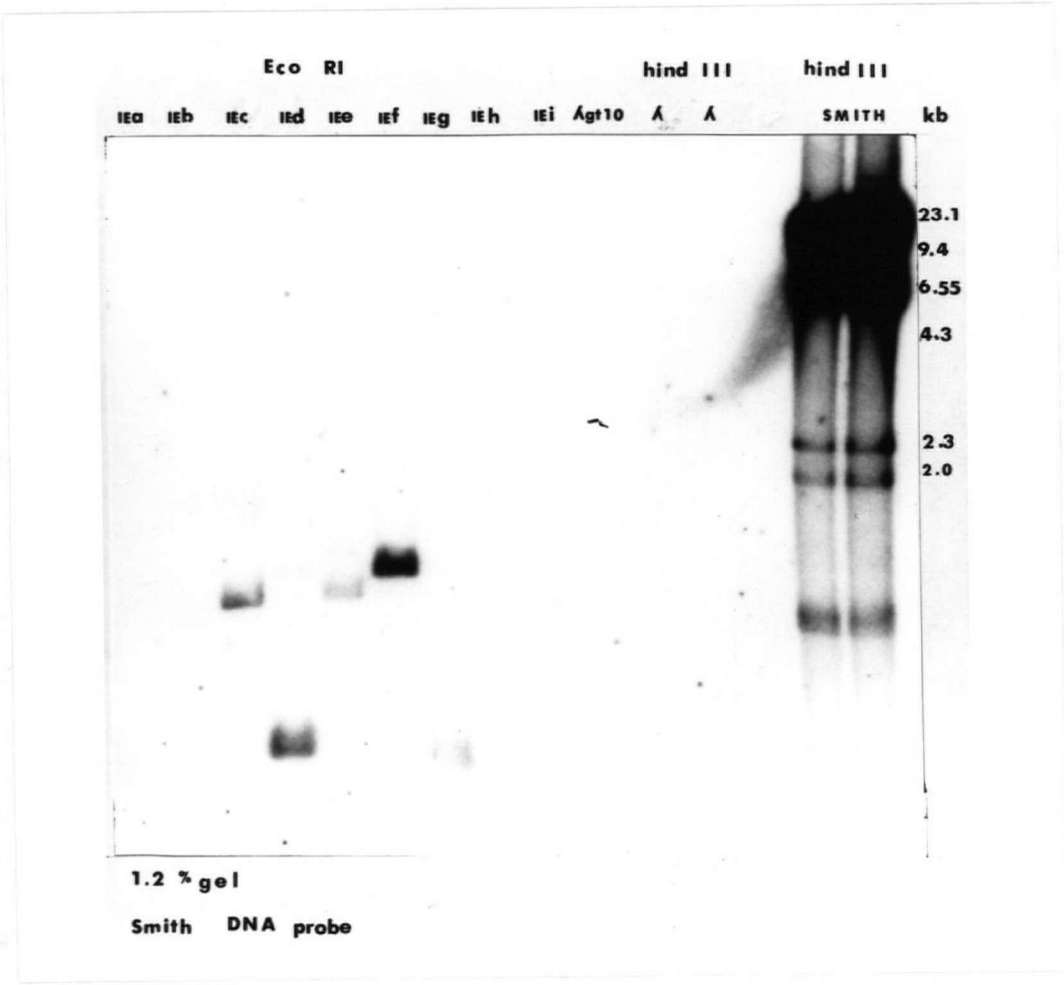


TABLE II

Summary of sizes of IE cDNA inserts and hybridization properties with the HindIII K and L fragments of MCMV (Smith) genome.

IE cDNAs	MOLECULAR WEIGHT (kb)	SMITH HIND III DNA FRAGMENTS	
		K	L
IEa	-	-	-
IEb	-	-	-
IEc	1.32	+	+
IEd	1.60	+	+++
IEe	1.40	-	-
IEf	1.60	++	-
IEg	1.10	-	(+++)
IEh	1.60	-	(++)
IEi	-	-	++

The relative intensities of hybridization are indicated by +, weak; ++, moderate; +++, strong. In two cases (IEh and IEg) the intensities of hybridization symbols are bracketed [(++) and (+++)] because later studies (Figure 24) indicated that these cDNAs are likely cellular in origin.

3.1.3.2 MAPPING THE IE cDNAs

To identify some of the IE cDNAs, the clones were rescreened with MCMV HindIII K and L probes since these fragments contain the major IE region (Keil et al., 1984). DNA from the nine IE cDNA-Lambda gt10 recombinants, MCMV DNA (positive control) and Lambda gt10 DNA (negative control) were blotted onto a membrane and hybridized to the HindIII K and L probes (Figures 23 and 24, respectively). The results showed that Lambda gt10 did not hybridize to either K or L HindIII probes, while MCMV DNA hybridized to both K and L HindIII probes. Three clones (IEc, IEd and IEf) hybridized to the HindIII K probe; the IEf clone gave the strongest signal (Figure 23). The weak bands in the IEe slots were disregarded as the level of hybridization was extremely low and of equal intensity in both 250ng and 125ng columns. Five clones (IEc, IEd, IEg, IEh and IEi) hybridized with the HindIII L probe. In this case the strongest signal was observed with the IEd clone. Of the nine clones, two (IEc and IEd) hybridized to both the HindIII L and K probes. Table II summarizes the sizes of the IE cDNA inserts and hybridization properties with respect to MCMV HindIII K and L restriction fragments. The IE clones (IEa, IEb and IEe), which did not hybridize to either HindIII K or L, may belong to IE regions other than the major IE region.

Figure 23: Slot blot analysis of IE cDNA clones hybridized with the MCMV HindIII K probe.
250 and 125ng IE cDNAs (as Lambda gt10 recombinant DNAs) and Lambda gt10 (negative control), and 25ng and 12.5ng of MCMV (Smith) DNA (positive control) were blotted onto a membrane and hybridized to the ^{32}P-labelled HindIII K DNA probe.

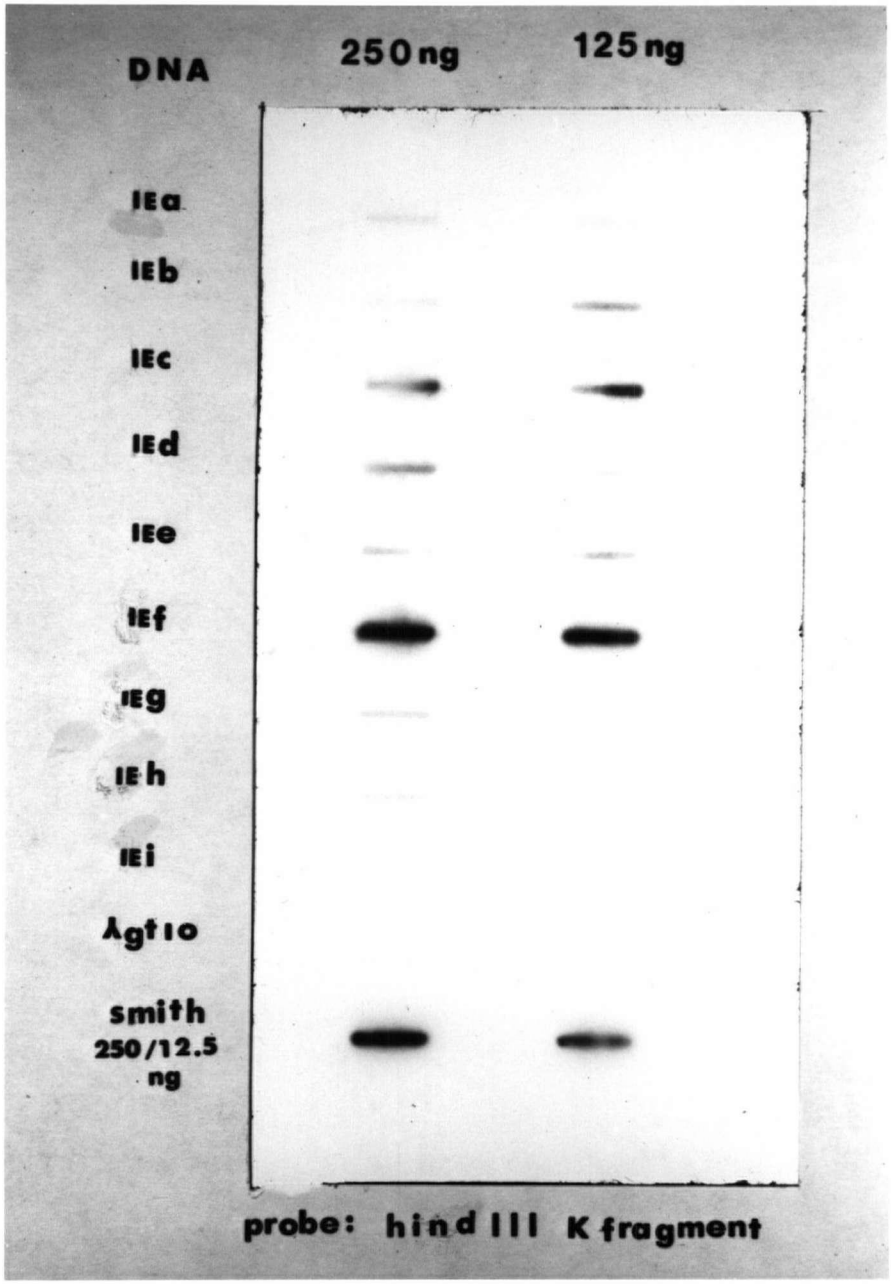
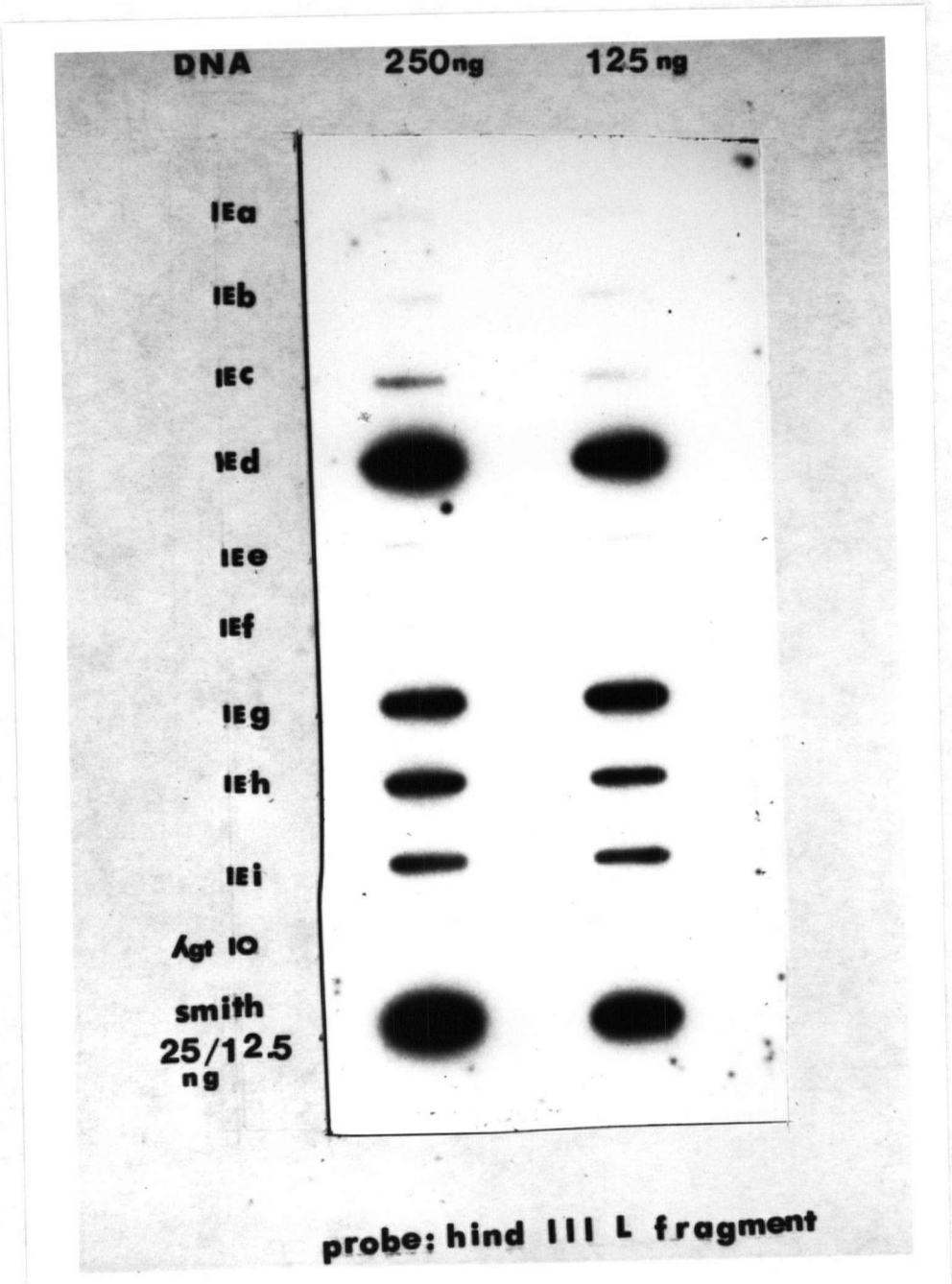


Figure 24: Slot blot analysis of IE cDNA clones hybridized with the MCMV HindIII L probe.
250 and 125ng of IE cDNAs (as Lambda gt10 recombinant DNAs) and Lambda gt10 (negative control), and 25ng and 12.5ng of MCMV (Smith) DNA (positive control) were blotted onto a membrane. The blot was hybridized to ^{32}P-labelled HindIII L DNA probe.



DISCUSSION

This study has established two MCMV cDNA libraries from infected cells, one for IE mRNAs and one for E mRNAs. Prior to this study, a cDNA library for MCMV had not been prepared. In this section of the study, the cDNAs were isolated and characterized in order to be utilized (in later sections of the project) as probes to obtain new information on transcription of their corresponding unit in detail. From a total of 198 E cDNA clones isolated, 10 were mapped in detail and categorized into seven groups based on their physical location within the MCMV genome. The results are summarized in Table I and Figure 21. Although the E transcripts of HCMV and MCMV originate from most regions of the genomes, the major E transcripts map to a few distinct regions of the genomes (Demarchi, 1981; Wanthen et al., 1981; Wanthen and Stinski, 1982; McDonough and Spector, 1983; Marks et al, 1983; Keil et al., 1984; Chang et al., 1988). For MCMV, these major E phase transcripts map to the HindIII fragments A, B, G, F, K, J, I, E and N (Marks et al., 1983; Keil et al., 1984). This study shows that all seven groups mapped within these major E regions: groups 1 and 2 mapped within the HindIII E region; group 3 mapped within the HindIII I-J region; group 4 mapped within the HindIII B region; groups 5 and 7 mapped within the HindIII A region; and group 6 mapped within the HindIII F region. Although the HindIII K region is a major region of transcription during the E phase, curiously none of the seven groups in this study mapped to the HindIII K or L fragments, the major IE region. Also, none of the seven groups mapped to the HindIII H-D region, which has been shown to be transcribed at a low level during the E phase (Marks et al., 1983; Keil et al., 1984). These results help to confirm that some of the major E transcription units are confined to previously identified HindIII fragments A, B, G, E, F and I-J,

since cDNA clones that were readily isolated mapped to these regions.

In this study nine of the 50 IE cDNA clones were analyzed further and six (IEc, IEd, IEe, IEf IEg and IEh) were found to hybridize with the HindIII K and L fragments (Table II and Figures 23 and 24). However, two of the clones, IEg and IEh were shown by RNA slot blot analysis, presented in a later Section (3.2), to occur at similar levels during all times of an infection cycle and to hybridize poorly with MCMV DNA (Figure 26). Hence the transcripts corresponding to these cDNAs are cellular in origin.

Previous studies have shown that the major IE region for herpesviruses is limited to a small region of the genome. This major IE region in the MCMV Smith genome maps within the two adjacent fragments, HindIII K and L, and is divided into three transcription units (ie1, ie2 and ie3) (Keil et al., 1984). The major IE region has been studied in great depth by the above group and the literature on the IE expression is presented in the 'Introduction'. The IEc and IEd clones (insert size of 1.32 and 1.15kb, respectively) mapped to both fragments K and L and the cDNA IEf (insert size approximately 1.6kb) mapped to fragment HindIII K only; therefore, these three cDNAs may be incomplete cDNAs corresponding to ie1 or ie3 genes, or one of the 2.75 and 1.0-5.1 kb transcripts which are encoded by ie1 and ie3 respectively, within the HindIII K and L region (Keil et al., 1984) (see Figure 3b for more details). The ie1 gene product has been shown to transactivate both viral and cellular promoters (Keil et al., 1987), and the ie1 and ie3 gene products have been shown to function together as co-transactivators of an E MCMV (e1) promoter (Buhler et al., 1990).

The IEe cDNA did not hybridize to either fragment HindIII K or L. It is possible that the cDNA was derived from an IE gene other than those present in the major IE region (HindIII K-L region) since low level of IE transcription has been reported in HindIII fragment D and the genome termini (HindIII E and N) (Marks et al., 1983; Keil et al., 1984). In addition, 10 IE proteins have been identified in our laboratory (Walker and Hudson, 1987a), of which only a few can be accounted for in the major IE region, while others may represent the minor IE regions (HindIII D, E and N). Two other IE cDNA clones (IEa and IEb) failed to hybridize with the HindIII K-L region. These clones may also be derived from IE genes other than those located in the major IE region.

The last clone, IEi, did hybridize to the HindIII L fragment and not to HindIII K, therefore it may represent the ie2 gene transcript that is encoded entirely within the HindIII L fragment. Recently, preliminary studies have shown that the ie2 gene is essential for latency in mouse spleen cells (Mocarski et al., 1990). Therefore, this clone (IEi) may serve as a valuable probe to investigate the expression of the ie2 gene in acute and latently infected cells.

In conclusion, the IE cDNA clones isolated appear to represent the IE genes of both the characterized major (HindIII K and L) and the uncharacterized minor regions. The E cDNAs that were isolated and characterized in this study were those that belonged to the known major E transcription regions, HindIII A, B, E, F and I-J.

3.2 TRANSCRIPT LEVELS PROBED WITH E AND IE cDNA CLONES DURING THE VIRAL REPLICATION CYCLE

RESULTS

Five E and four IE cDNAs that were characterized in the previous section (3.1) were selected as probes to investigate transcript levels from respective transcription units during a course of permissive infection. Each of the selected five E cDNAs, E1, E3, E6, E7 and E10, map to a different genomic location and represent the known actively transcribed E regions, HindIII E, B, A, F and I-J respectively. The four IE cDNAs selected and used in this analysis were those that mapped to the major IE region, HindIII K-L. Although the regions of IE and E transcription have been mapped, the expression pattern of individual transcription units during a course of infection had never been determined. Such a study also allowed further characterization and to confirm the viral origin of the cDNAs (as will be demonstrated below).

RNA was isolated at various times during the course of an infection of 3T3L1 cells with MCMV (Smith) and blotted onto a membrane. RNA-DNA hybridizations were performed to determine the relative levels of transcripts complementary to the respective cDNA (DNA isolated from IE and E Lambda gt10-cDNA recombinants). As controls, MCMV DNA and RNA from mock infected cells were also blotted onto the membrane. The autoradiograms of two experiments are presented in Figures 25 and 26 and the results are summarized in Table III. As expected, the 3T3L1 (cellular) DNA probe hybridized to RNA of all time intervals during and before (mock) the infection at approximately equal intensities (Figures 25 and 26). Another significant observation was that the 3T3L1 DNA probe hybridized to MCMV (Smith) DNA. This result has been previously noted in our laboratory and may be due to hybridization between

Figure 25: Use of cDNAs as probes to monitor transcript levels during the MCMV replication cycle.
RNAs were isolated at different time points [0 (mock infected), 1 through 8, 11 and 14 hr p.i.] during MCMV infection of 3T3L1 cells by means of the LiCl method (Materials and Method). The RNAs were blotted onto a membrane (3 ug/slot), hybridized to ^{32}P-labelled 3T3L1 DNA (positive control), Lambda gt10 DNA (negative control), MCMV (Smith) DNA and cDNAs, IEh, IEg and E10. As controls, mock infected RNA (0 hr) and 25 ng of MCMV DNA (S) were also present on each blot.

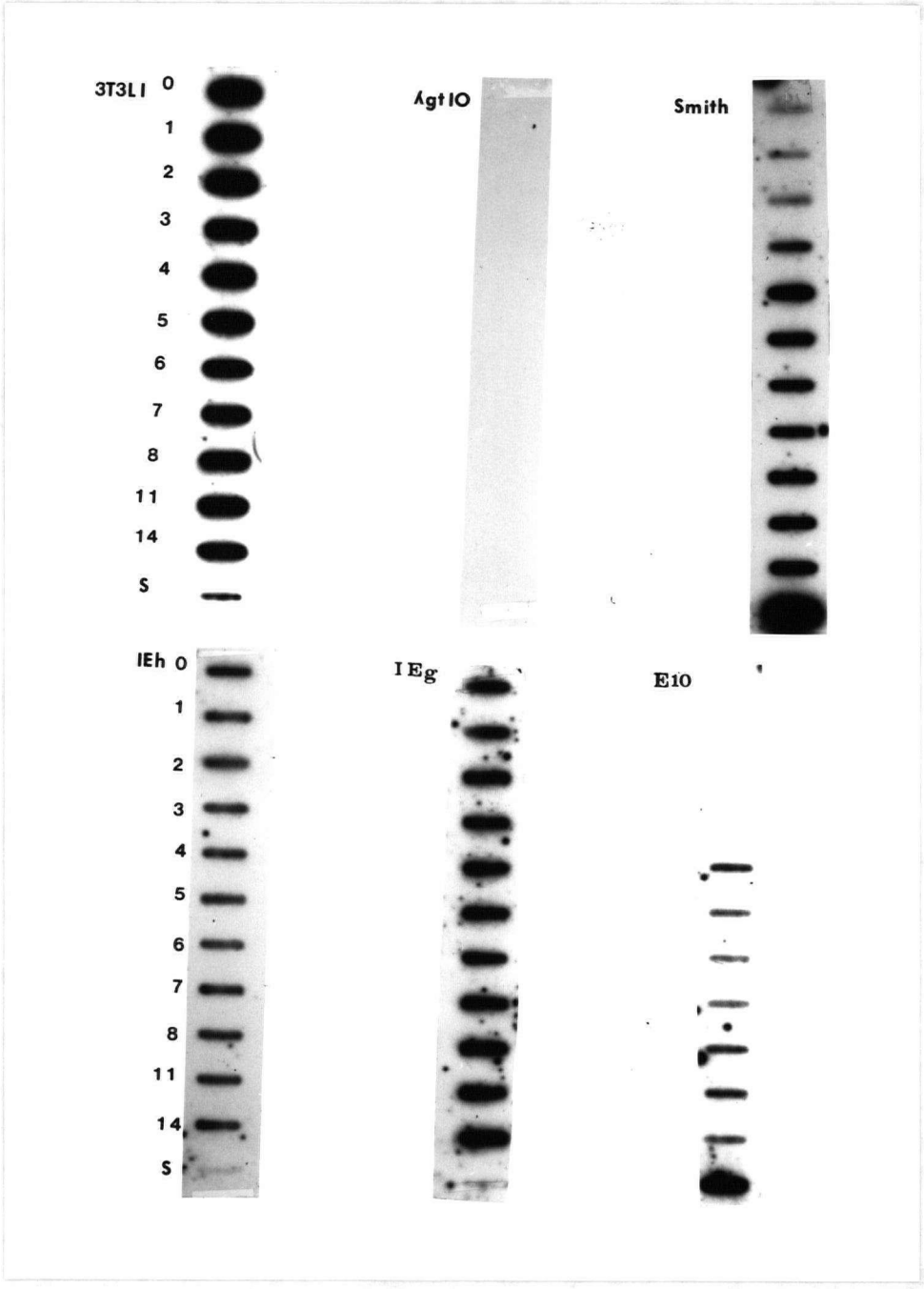


Figure 26: Use of cDNAs as probes to monitor transcript levels during the MCMV replication cycle.
The slot blot analysis was carried out as described in Figure 25. The ^{32}P-labelled probes used were 3T3L1 DNA (positive control), MCMV (Smith) DNA (positive control), cDNAs E1, E3, E6, E7, E10, IEd, IEf, IEg, IEh and Lambda gt10 (negative control). As controls, mock infected RNA (0 hr) and 25 ng of MCMV DNA (S) were also present on each blot.

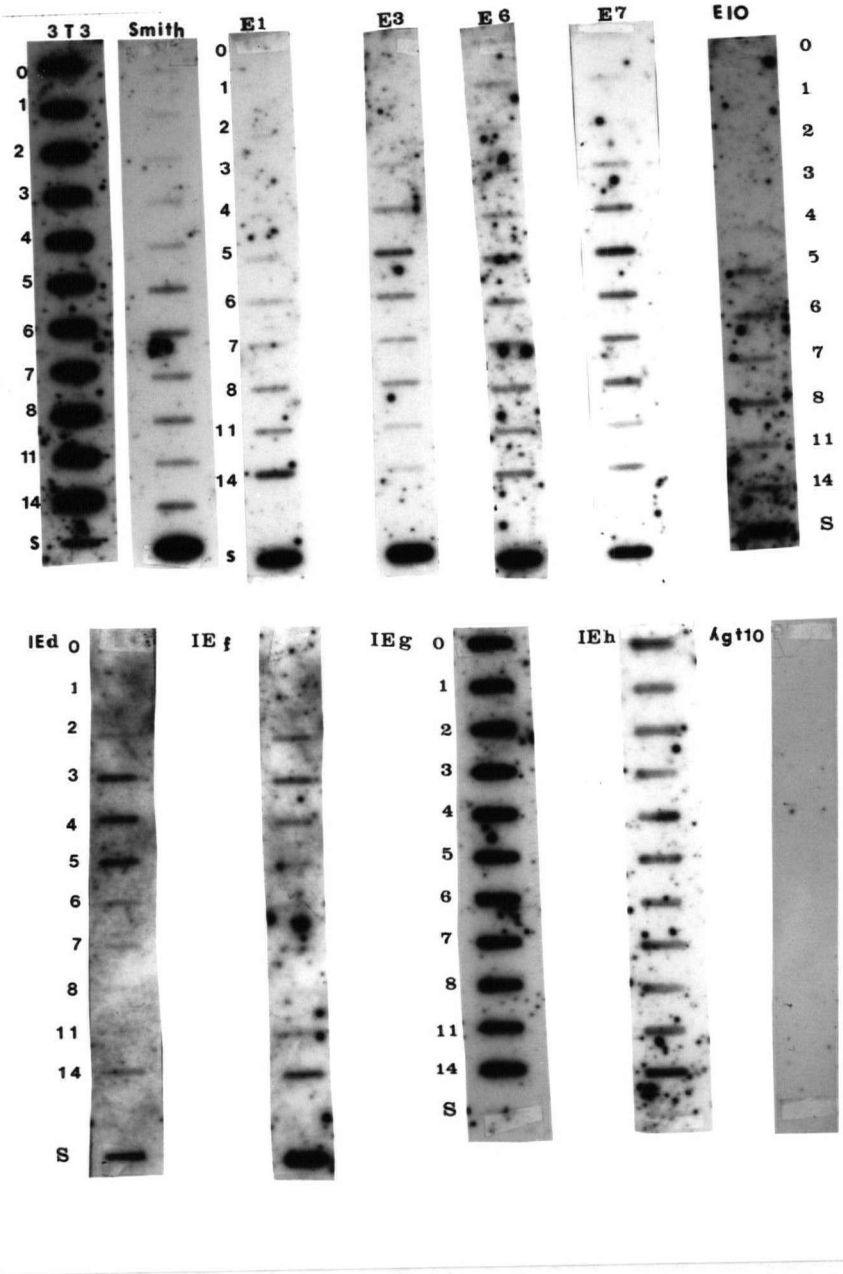


TABLE III

ANALYSIS OF TRANSCRIPT LEVELS PROBED WITH IE AND E cDNA CLONES

PROBES	MOCK	HOURS									Smith
	0	1	2	3	4	5	6	7	8	11	14	
3T3L1	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+
Lambda gt10	-	-	-	-	-	-	-	-	-	-	-	-
MCMV (Smith)	+	+	++	+++	+++	++	++	+++	+++	+++	+++	++++
IEd	-	-	+	+++	+++	+++	+	±	-	-	+	+++
IEf	-	-	++	+++	++	+	±	±	-	+	+++	++++
IEg	++	++	++	++	++	++	++	++	++	++	++	±
IEh	++	++	++	++	++	++	++	++	++	++	++	±
E1	-	-	-	-	±	+	+	+	++	++	+++	++++
E3	-	-	-	+	++	+++	++	++	++	+	+	++++
E6	±	±	±	±	+	++	+	+	+	+	+	+++
E7	±	±	-	+	++	+++	++	++	++	+	+	+++
E10	-	-	-	+	+++	++	++	++	+++	+++	++	+++

The intensity of hybridization (Figure 25 and 26) was determined visually and is indicated by ±, low; ++, moderate; +++, strong; and ++++, very strong. The hybridization pattern for clones IEg and IEh indicate these cDNAs are cellular in origin.

GC rich regions of the DNAs. The MCMV DNA probe hybridized to RNA in mock infected and 1 hr p.i. samples to some extent. This likely indicates binding to cellular RNAs present in the samples. The hybridization signal with MCMV DNA probe increased significantly from 2 hr p.i. onwards. As a positive control the MCMV DNA probe hybridized to MCMV DNA on each blot. The Lambda gt 10 DNA probe did not hybridize to any RNAs or MCMV DNA.

ANALYSIS WITH E cDNA PROBES:

Five E cDNAs representing transcripts from the known major E regions (HindIII B, A, F, E and J) were selected for the analysis (Marks et al., 1983; Keil et al., 1984). The E1 probe (Figure 26) first detected transcripts 5 hr p.i. The level increased gradually at each subsequent time point reaching a maximum at 14 hr p.i. (last time point). Transcripts complementary to the E3 probe (Figure 26) were detected at 3 hr p.i. These RNAs peaked at 5hr p.i. and subsequently decreased but were detectable up to 14 hr p.i. (last time point). The E6 probe (Figure 26) detected RNAs at 4 hr p.i. The transcript level peaked at 5 hr p.i. and remained steady at 6-8, 11 and 14 hr p.i. The E7 probe (Figure 26) hybridized to transcripts at 3 hr p.i. The signal increased to a peak level at 5 hr p.i. and decreased from 6 to 8 hr p.i. The transcripts were present at 11 and 14 hr p.i., but at still lower levels than at 6-8 hr.

The E10 probe (Figure 25) detected transcripts initially at 3 hr p.i. There was an apparent drop in the level of transcripts at 5-7 hr p.i. and then an increase again with peak expression at 8-11 hr p.i. Transcripts were present at 14 hr p.i., but at a lower level than at 8-11 hr p.i. In a second experiment with the E10 probe (Figure 26) quantitatively similar results were obtained although the background of this blot is higher. This

clone, E10, maps to a heavily transcribed E region, HindIII I-J (Marks et al., 1983; Keil et al., 1984), and therefore, was most extensively studied in the latter part of this thesis.

ANALYSIS WITH IE cDNA PROBES

The IEd probe began to detect transcripts at 2 hr p.i. (Figure 26). The transcript level peaked between 3 and 5 hr p.i. and dropped significantly at 7, 8 and 11 hr p.i. However it increased again at 14 hr p.i. The IEd probe detected RNAs that display the IE pattern of expression. The IEf cDNA probe (Figure 26) began to detect transcripts at 2 hr p.i. The transcript level peaked at 3 hr p.i. and decreased significantly at 6, 7 and 8 hr p.i. The transcripts increased again at 14 hr p.i. The RNAs detected by this cDNA probe also displayed the typical pattern of IE transcription.

The IEg and IEh probes (Figure 25 and 26) detected transcripts at all stages of infection, at approximately a constant level. In addition, the probes hybridized poorly to Smith MCMV DNA (positive control). Consequently the results obtained from the use of both IEg and IEh as probes, has confirmed that the transcripts of these cDNAs are almost certainly of cellular origin. An important point to note is that these experiments were conducted with higher stringency washes (0.1X SSPE, 0.1% SDS at 55°C instead of 50°C) than the previous experiments in which the IE cDNA phage clones were characterized. This suggests nonspecific binding of IEg and IEh cDNAs to MCMV DNA during previous experiments. However, in light of these results it would appear that the stringency of the washes should have included 0.1X SSPE, 0.1% SDS at atleast 65°C. The IEi probe was not analyzed in these RNA slot blot studies.

Mock infected RNA samples were isolated at 0 hr or before infection, and were used as negative controls. In all cases in which the cDNAs were of viral origin, hybridization to the RNA sample from infected cells (0 hr) was very low or not detectable. In two cases (IEg and IEh) the level of hybridization was high and as discussed above, it was concluded that the transcripts of these cDNAs were cellular in origin. In Figure 25 and 26 the mock infected RNA (0 hr) hybridized at low levels to the MCMV DNA probe indicating nonspecific or specific binding of cellular RNAs to MCMV DNA. The Mock infected RNAs did not hybridize to the Lambda gt10 DNA probe.

SUMMARY

A summary of results is presented in Table III.

1. E cDNAs:

   All the E cDNAs in these experiments detected transcript levels exhibiting the typical pattern for E genes. The observations confirmed their viral origin and expression during the E phase.

2. IE cDNAs:

   The patterns of transcript levels detected by IEd and IEf cDNAs confirms their viral origin and expression during the IE phase. Conversely, IEg and IEh were shown to be of cellular origin.

3. Binding of Smith DNA to 3T3L1 DNA:

   The hybridization experiments confirmed that there is some hybridization of MCMV DNA to cellular probes. These observations have been noted in previous studies in our laboratory (unpublished data). However, the exact location of the binding is unknown.

DISCUSSION

By the method of hybridization of RNA to a defined cDNA-probe, this study shows that it is possible to study the expression of an individual transcriptional unit. At the start of this project, E genes of MCMV had not been studied as extensively as the E genes of other herpesviruses. As stated earliar in the 'Rationale and Objectives' (Section 1.8), the E genes of herpesviruses are important because they are usually responsible for viral gene expression, DNA synthesis and pathogenesis. Many of the E MCMV proteins have demonstrated affinity for denatured DNA-cellulose and therefore are considered to be DNA binding (Walker and Hudson, 1987a). Some E gene products have been shown in preliminary studies to regulate pathogenesis (Val et al., 1990; Mocarski et al., 1989). For more details, see 'Rationale and Objectives' (Section 1.8). Furthermore, MCMV genes responsible for latency and reactivation have not been determined; the possible role of E genes in these events including pathogensis, viral gene expression and DNA synthesis need to be elucidated. The availability of the E cDNAs facilitated the study of their corresponding genes individually to provide insight into their expression during the replication cycle. Five E cDNAs, E1, E3, E6, E7 and E10, each mapping to a different and major E genomic region were included in this study. These E cDNAs detected transcript levels that all displayed a typical temporal pattern of E transcription (Figure 26). The cDNAs detected transcripts that began to appear during the E phase (3 to 6 hr p.i.) and the levels of these transcripts also fluctuated with time, confirming the viral origin of these cDNAs from the E phase of the replication cycle. During the later phase of the replication cycle, transcription from most regions of the MCMV Smith genome occurs and this accounts for the detection of trancripts at 14 hr

p.i. by all five E cDNAs.

It is important to note that in HCMV, some of the genes that are transcriptionally active at E times are also active at L times, and may code for L proteins (reviewed in Griffiths and Grundy, 1987). This delayed translation appears to be controlled post-transcriptionally by processes such as transcript transportation to the cytoplasm, transcript stability in the cytoplasm and transcript association with ribosomes. Therefore, mere detection of a transcript during both times E and L may not necessarily indicate that it codes for an E protein, and this may also apply to MCMV. The transcripts that hybridize to the E cDNAs in this study are present at both E and L times. By analogy to HCMV, these transcripts may be translated at both times (E and L) or at either E or L times. To elucidate these possibilities, further studies are required which would include the investigation of the individual MCMV E and L transcripts associated with the ribosomes.

Using IE cDNAs as probes, the study demonstrates the expression of the major IE genes at the level of transcription during the course of an infection. Only four of the nine IE cDNA clones were used to monitor transcript levels during the MCMV replication cycle. Two of these clones, IEd and IEf, originating from the major IE region (HindIII K-L and K fragments respectively) detected different groups of transcripts, as the levels of expression, although similar, showed slightly different patterns (Figure 26). In both cases there was a decrease in transcript levels during the E phase (5-11 hr p.i.). The transcripts were also detected at 14 hr p.i. These observations are analogous to those seen in previous studies (Marks et al., 1983; Keil et al., 1984). Although the HindIII K region is expressed

during the E phase, there is an overall tenfold decrease in transcription from the major IE region (HindIII K and L) (Marks et al., 1983; Keil et al., 1984). During the late phase, transcription occurs from most regions including HindIII K and L fragments of the MCMV genome, although the HindIII K is one of the major regions transcribed. This explains the presence of transcripts from the respective regions at 14 hr p.i., particularly in the case of IEf (HindIII K) where the transcript level is very high. The other two IE cDNA clones studied, IEg and IEh are likely cellular in origin.

Two IE and five E cDNAs clones were capable of identifying levels of specific viral transcripts characteristic of IE and E gene expression during the course of permissive infection. These experiments provided a conclusive test to prove the origins of the cDNAs, and have also shown variation in MCMV transcription with time, indicating temporal and quantitative regulation, as described by other investigators (Misra et al., 1978; Marks et al., 1983; Keil et al., 1984). The weakness of such a study is the inability to monitor levels of individual transcripts in the case of multiple transcripts originating from the same region. As shown in the Northern blot analysis in the next section (3.3), all five E cDNAs hybridize to more than one transcript and some show differential expression, therefore, the slot blot analysis method in such cases would only indicate the presence of transcripts from their respective regions.

The exact gene products associated with the establishment of latency in herpesviruses have not been defined. However, many investigators have reported detection of herpesviral transcripts in latently infected tissues (reviewed in Stevens, 1990). Schrier et al. (1985) and Galloway et al (1979, 1982) have reported detection of HCMV major IE mRNAs in peripheral

blood mononuclear cells and HSV mRNAs in human ganglia, respectively by hybridization techniques. Studies by Stevens (1990) have shown the presence of the unique HSV transcript, LAT (latency associated transcript), in latently infected murine neurons by in-situ hybridization. Therefore, the cDNAs may be useful as probes in similar studies to investigate the expression of different viral genes in latently and nonpermissively infected cells. The levels of transcripts present in these persistent infections may be compared to permissive infections to help identify the block in replication at the level of transcription. The significance of these observations may help reveal the critical role of individual gene(s) in latently and nonpermissively infected cells, although appropriate latency models and nonpermissive cell lines will need to be established in the laboratory prior to the application of the cDNA as probes.

In conclusion, all five E cDNAs of the major E regions detected typical transcription patterns and were also confirmed to be viral in origin. Of the four IE cDNAs used for the study, two (IEd and IEf) cDNAs of the major IE (ie1-ie3) region detected typical IE transcription patterns, although each detected a group of different transcripts. The transcripts of other two IE cDNAs (IEg and IEh) were shown to be cellular in origin. Therefore, the five E cDNAs and two IE (IEd and IEf) could serve as valuable probes in the future to investigate the expression of corresponding transcription units in different infection systems (permissive, latent, reactivated and nonpermissive infections).

3.3 ANALYSIS OF INDIVIDUAL TRANSCRIPTS MAPPING TO THE CORRESPONDING REGIONS OF E1, E3, E6, E7 AND E10 cDNAs

RESULTS

The aim of the study in this section (3.3) was to further investigate the five major E transcription units studied in the previous section. This investigation revealed the numbers and sizes of the transcripts, and temporal expression of individual RNA from the five actively transcribed E units of the HindIII fragments A, B, E, F and I-J. This task was accomplished by using the five E cDNAs (E1, E3, E6, E7 and E10) as specific probes in Northern blot analyses. The information that was obtained from this study provided additional insight into the transcription of the major E regions of the MCMV genome.

3.3.1 TRANSCRIPTS PROBED WITH EARLY cDNAs: E1, E3, E6, E7 AND E10

In order to examine the size, level and expression pattern of individual E transcripts, total cytoplasmic RNAs were isolated from mock infected 3T3L1 cells, and MCMV infected cells at IE, E and L phases of the replication cycle. The RNA isolation time point for IE was 4 hr p.i. with a cycloheximide block at 2 hr prior to infection, for E was 7 hr p.i. with a cycloheximide block at 4hr p.i., and for L was 16 hr p.i. without a cycloheximide block. Cycloheximide inhibits protein synthesis and therefore blocks progression into the next phase. For details, see 'Materials and Methods' (Sections 2.14.1 and 2.17.1). RNAs (10ug) were separated by gel electrophoresis (Materials and Methods), and transferred to a membrane, hybridized to E cDNA probe and autoradiographed. The E cDNAs selected for analysis were the same as those described in the previous Section (3.2). The quality of the total RNA was ensured by the presence of cellular rRNAs

Figure 27: Northern blot analysis of transcripts from MCMV infected 3T3L1 cells probed with E1, E3, E6 and E7 cDNAs

Total cytoplasmic RNA was isolated from mock infected 3T3L1 cells (C), IE, E and L (16hr p.i.) phases of MCMV replication using the vanadyl ribonucleoside complex method (Materials and Methods). RNA (10 ug/well) was electrophoresed through a 1% denaturing formaldehyde gel, transferred to a membrane and hybridized to ^{32}P-labelled E cDNAs: E1, E3, E6 and E7. The sizes of the transcripts were estimated by the presence of RNA markers (BRL; 9.5 to 0.24kb) in the gel.

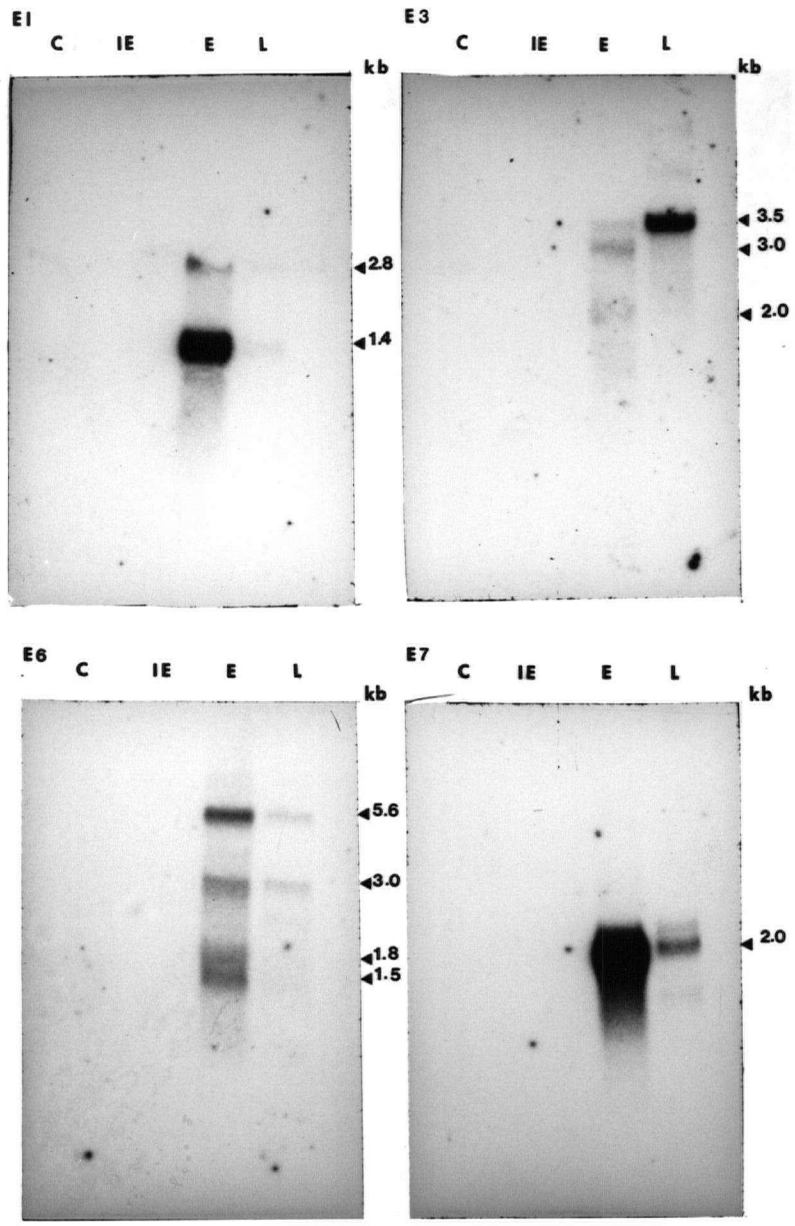
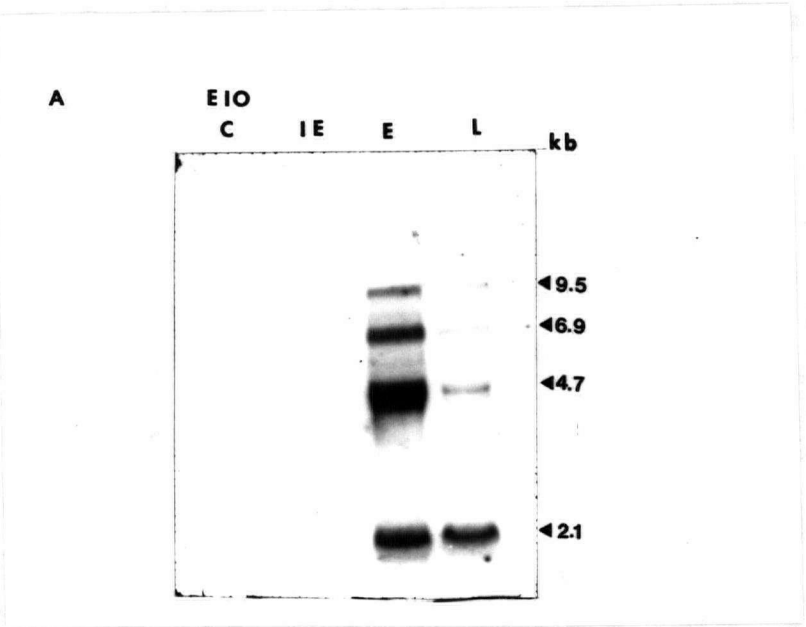


Figure 28: Northern blot analysis of transcripts which hybridized to E10 cDNA.
Northern blot analysis was carried out as in Figure 27. The blot was hybridized to ^{32}P-labelled E10 cDNA.



(18S and 28S) as discrete, non smeared bands in the duplicate portion of the gel which was stained with ethidium bromide. The E cDNAs hybridized to transcripts that began to appear during the E phase, and all E cDNAs hybridized to more than one transcript (Figure 27 and 28). Some of these transcripts were also detected during the late phase, usually at a different level.

These results showed that the E1 cDNA (Figure 27) detected three transcripts of sizes 2.8, 1.4 and 1.2kb that are present during the E phase. The 1.4kb transcript is by far the predominant RNA species. Both the 2.8 and 1.4kb transcripts are present at extremely low levels during the L phase and are absent during the IE phase. The E3 cDNA (Figure 27) detected three transcripts of sizes: 3.5, 3.0 and 2.0kb that are present at low levels during the E phase and absent during the IE phase. Of the three, the 3.5kb RNA appeared to accumulate and is present during the L phase, at a much higher level than during the E phase. The E6 cDNA (Figure 27) detected four transcripts of sizes 5.6, 3.0, 1.8 and 1.5kb that are present during the E phase. These transcripts are absent during the IE phase. Of the four RNAs, only two (5.6kb and 3.0kb) are present during the L phase, but at a lower level. The E7 cDNA (Figure 27) detected one predominant transcript of size 2.0 kb and a minor species at 2.1 kb. These transcripts are absent during the IE phase and present at a very high level during the E phase, while the levels are greatly reduced during the L phase.

The E10 cDNA (Figure 28) is the clone that was analyzed most extensively in this study because it mapped to the major E transcription region, HindIII I-J (Marks et al., 1983; Keil et al., 1984). This cDNA detected four transcripts of sizes 9.5, 6.9, 4.7 and 2.1kb that are present only during

the E and L phases. The major transcript that is present during the early phase is the 4.7kb species. During the L phase, the 9.5, 6.9 and 4.7kb transcripts decrease in amount, in contrast to the 2.1kb transcript, which remains at approximately the same level as in the E phase.

Since none of these cDNAs (E1, E3, E6, E7 and E10) hybridized to RNA present in the mock infected (C) lane (Figures 27 and 28), it was concluded that all of the transcripts detected were viral in origin. Sizes of some of the E cDNAs (E3, E7 and E1) do not correspond to the size of any of the transcripts they hybridize to. This may be be attributed to the failure to protect the internal EcoR1 sites (during cloning) and/or synthesis of incomplete cDNAs, hence resulting in shorter cDNAs A summary of these results is presented in Table IV. In general, this study showed that all the five E cDNAs hybridized to more than one transcript, indicating splicing and/or overlapping genes in the E regions of the genome. Also, these E units begin to transcribe from E phase onwards; none transcribed during the IE phase (confirming the results of the slot blot analysis in the previous section).

3.3.2 NORTHERN BLOT ANALYSIS OF TRANSCRIPTS PROBED WITH ANTISENSE AND SENSE E10 RNA:

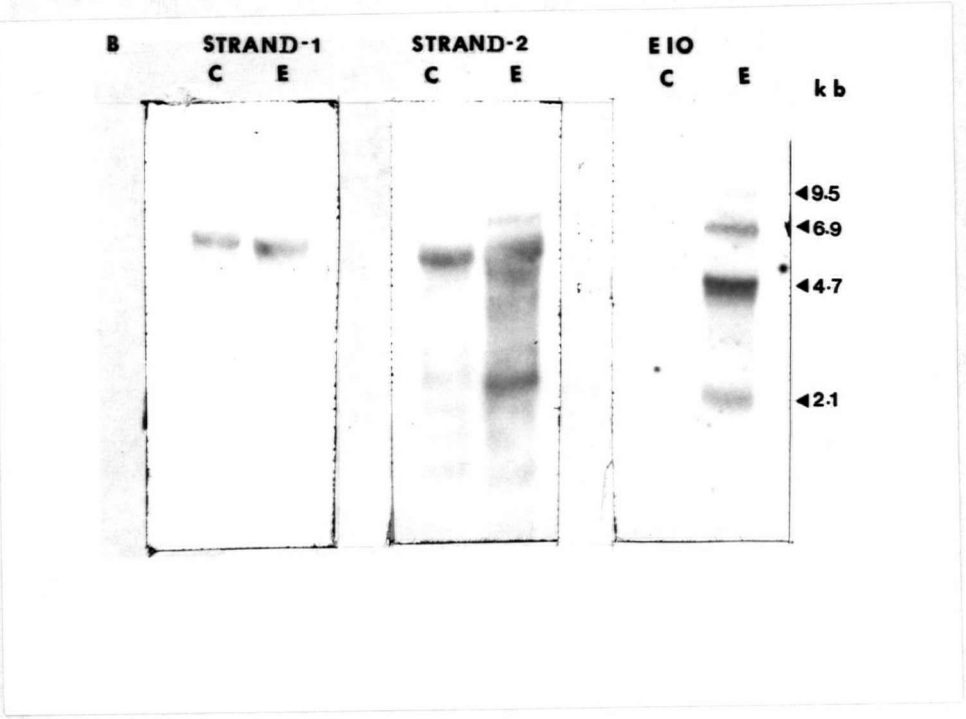
In further studies I focused my efforts on the E10 cDNA and the major E region to which it maps, HindIII fragments I-J. The transcripts of the E10 region were subjected to further Northern analysis in which sense and antisense E10 RNAs were used as probes. The purpose of this particular study was to determine whether the gene(s) coding for the four RNAs that hybridize to the E10 cDNA are transcribed in the same or opposite direction

TABLE IV

NORTHERN BLOT ANALYSIS OF TRANSCRIPTS PROBED WITH E cDNAs

E cDNA	HINDIII	E Phase	L Phase
E1	E	3 transcripts: 2.8 (high), 1.4 & 1.2kb	2 transcripts: 2.8 & 1.4 kb (lower levels)
E3	B	3 transcripts: 3.5, 3.0 & 2.0kb	1 transcript: 3.5kb (higher level)
E6	A	4 transcripts: 5.6, 3.0, 1.8 & 1.5kb	2 transcripts: 5.6 & 3.0.kb (both lower levels)
E7	F	2 transcripts: 2.0 (very high level) & 2.1kb	2 transcripts: 2.0 & 2.1kb (both lower levels)
E10	I & J	4 transcripts: 9.5, 6.9, 4.7 (high) & 2.1kb	4 transcripts: 9.5, 6.9, 4.7 (3-lower levels) & 2.1kb (same)

Figure 29: Northern blot analysis of transcripts which hybridized to E10 sense and antisense RNA.
Total cytoplasmic RNA, isolated from mock infected 3T3L1 cells (C) and E phase of MCMV replication cycle was analysed as described in Figure 27. The ^{32}P-labelled probes were antisense RNA (strand 1) and sense RNA (strand 2) prepared by in vitro transcription of the pGEM-E10 clones using T7 RNA polymerase (Materials and Methods). The third panel was probed with ^{32}P-labelled E10 cDNA (positive control).



within the E10 region.

Sense and antisense RNAs were transcribed in vitro from the E10 cDNA (see Materials and Methods) and designated as strand 1 and strand 2, respectively. Northern blots of mock infected (C) and E phase (E) RNA were hybridized to radioactive labelled E10 cDNA, strand 1 and strand 2. (Figure 29). Strand 1 did not detect RNAs in lane E other than those also detected in lane C (cell) as background. In addition to detecting some background RNAs that were common in both lanes C and E, strand 2 detected four transcripts (9.5, 6.9, 4.7 and 2.1kb) in lane E. As a positive control, the four E transcripts were also detected using the E10 cDNA probe. Since all four E transcripts hybridize to the same single stranded RNA probe, they must be encoded on the same DNA strand and are transcribed in the same direction.

DISCUSSION

Using E cDNAs as probes in Northern blot analysis has revealed details on the expression of individual transcripts from the major E transcription units of E1, E3, E6, E5 and E10 corresponding to HindIII regions E, B, A, F and I-J, respectively. Northern blot analysis of these five cDNAs have confirmed their origin from the E phase (Figures 27 and 28). The cDNAs hybridize to transcripts that are first detected during the E phase, some are also present during the L phase, but none was detected during the IE phase. The analysis also reveals some pattern of regulation in transcription involving the size and quantity of transcripts present during the E and L phases of infection. The presence of some of the E transcripts in the L phase is an observation that has also been made with HCMV (reviewed in Mach et al., 1989 and Spector et al., 1990), and this may be attributed to high stability of the E mRNAs in the cytoplasm and/or the continued expression of the gene. In general, all the observations mentioned above illustrate the temporal, quantitative, and perhaps post-transcriptional regulation of MCMV genome expression.

To study transcripts that map to the corresponding regions of the cDNAs, the Northern blot analysis method is more informative than the slot blot method (previous section). There are several advantages to performing a Northern blot analysis. This analysis reveals the size and number of transcripts originating from the corresponding region in each phase of the viral replication cycle. In addition, by using single stranded RNA probes corresponding to either strand, one is able to determine the direction of transcription. An important point to note relevant to these studies is that during the isolation of IE and E transcripts for Northern analysis, cycloheximide (CH) was used in order to block progression into the next

phase. Therefore, the time course analysed by the Northern blot may not necessarily correspond to the time course analysed by the slot blot. Another difference between the two analyses is that the late time point for the slot blot analysis was 14 hr p.i. while the time point for the isolation of late phase RNA for Northern blot analysis was 16 hr p.i. Therefore, differences in transcript levels between the last time point in the slot blot analysis and the late phase in the Northern blot analysis may not be comparable.

In this study, all five cDNAs (E1, E3, E6, E7 and E10) (Figures 27 and 28) showed hybridization to more than one transcript indicating a complex transcription pattern possibly similar to that of HCMV. Previous studies have shown that there are multiple transcripts originating from the IE major region of the MCMV (Keil et al., 1983, 1987) and HCMV genomes, and major E regions of HCMV genome (reviewed in Mach et al., 1989 and Spector et al., 1990) due to complicated splicing events and overlapping genes. It is likely that a similar expression system may exist for the major E genes of MCMV. To elaborate, there are many possible events that can lead to the detection of more than one transcript. The mRNAs may be spliced transcripts from a common gene and perhaps share a 5' untranslated region (leader sequence) and/or untranslated 3' region. Such events have been shown to occur in the E4 region of adenovirus (Virtanen et al., 1984). Sometimes, the leader sequence of one transcript may be a coding region for another transcript. Occasionally transcripts, although different in size, may code for the same protein. This event has been shown to occur in the major IE region of the MCMV Smith strain where the low abundant mRNAs of the ie3 gene, ranging from sizes 1.0 to 5.1 kb, code for a common polypeptide of size 15kd (Keil et al., 1984, 1987). Some transcripts may also share

sequences but code for different amino acid residues due to translation in a different frame. In adenovirus-2 infected cells, some viral transcripts contain complementary sequences to each other when encoded by genes in the opposite or complementary DNA strands (Sharp et al., 1980). This possibilty can be elucidated by probing the Northern blots with sense and antisense RNA as was done here for the E10 cDNA to reveal the coding strand. An interesting point to note is that, in HSV, only a small portion of the transcripts are derived by splicing in comparison to adenovirus (McLaughlan and Clements, 1982; Wagner, 1984). However, some HSV transcripts originate from genes that have been shown to contain multiple initiation sites (Watson et al., 1981; Zisper et al., 1981; Frink et al., 1981; Murchie and McGeoch, 1982; Sharp et al., 1983; Wagner et al., 1984). A similar organization in some of the major E genes in MCMV is possible and perhaps may be accounted for detection of more than one transcript. Further studies are required to precisely define the coding complexities of these multiple transcripts.

The E3 cDNA maps to the HindIII B fragment and detects three transcripts during the E phase, but only one transcript (3.5kb) is detected at a higher level, during the L phase. This sort of expression may be regulated at the transcriptional level or post-transcriptional level. Regulation at the transcriptional level may involve a change in the transcription initiation site, therefore favouring the transcription of the 3.5 kb transcript, and higher expression of the 3.5 kb transcript. The post-transcriptional regulation may involve changes in the splicing pattern, transportation of the transcripts to cytoplasm and stability of the transcripts in the cytoplasm, to favour the accumulation of the 3.5kb RNA. Further studies would be required to elucidate the above possibilities involved in this observation. Previous studies have shown that the HindIII B region is

abundantly transcribed during the E phase and a portion of this region is transcribed at a higher level during the L phase (Marks et al., 1983; Keil et al., 1987). Therefore, the 3.5kb transcript may represent this portion of the HindIII B fragment.

In previous studies, the HindIII F region has been shown to be transcribed abundantly during both E and L phases of the MCMV replication cycle (Marks et al., 1983; Keil et al., 1987). The first extensively studied and sequenced major E region, e1, maps within the HindIII F region, 0.709-0.721 mu (Buhler et al., 1990). The E7 cDNA also maps to this location and detects one predominant transcript of size 2.0kb that is present during the E phase at a considerably higher level than in the L phase. The presence of these transcripts during the L phase may be due to down-regulated expression or residual stable E transcripts in the cytoplasm. Similarly, Buhler et al. (1990) have detected one predominant transcript from the e1 gene which is present at a high level at E times. The level of this e1 transcript drops when the replication cycle is in the L phase, as also observed for the E7 transcript. Although the e1 mRNA comprises three exons that add up to a 2.1 kb transcript, a 2.6kb RNA is reported to be derived from this region and it has been speculated that the increase in the transcript size is due to post transcriptional modification such as polyadenylation (Buhler et al., 1990). The E7 cDNA appears to represent the e1 transcript. Differences in the transcript sizes detected by the E7 cDNA (2.0kb) (this study) and Buhler et al. (2.6kb) may be attributed to differences in post transcriptional modifications and/or genetic differences that may exist within the MCMV Smith strain. The e1 gene codes for three antigenically related proteins (36, 37 and 38 kd) presumably coded by the same transcript (Buhler et al., 1990). However the functions of these proteins have not been elucidated.

Since the e1 transcript peaks during the E phase, by analogy to other herpesviruses, the most likely function of these proteins may involve gene regulation, DNA synthesis or nucleotide metabolism [summary of review in Roizman (1990) for HSV and Spector et al., (1990) for HCMV]

An E gene, designated as the sgg-1 gene, maps within the HindIII J region and is transcribed into two RNAs of sizes 1.8kb and 1.5kb (unpublished data, Manning et al., Westcoast Herpesviruses Workshop, 1990). In the study reported here, the E10 cDNA maps to two adjacent fragments HindIII I and J and detects four transcripts of 9.5, 6.9, 4.7 and 2.1kb in size. Therefore, none of these four transcripts represent RNA from the sgg-1 gene. Earlier investigators have reported that the HindIII J fragment and a portion of HindIII I fragment are transcribed abundantly during both E and L phases of the MCMV replication cycle, but this transcription peaks during the E phase (Marks et al., 1983; Keil et al., 1984). This observation is analogous to that seen in the Northern blot analysis (Figure 28). During the L phase, levels of three of the four E transcripts decrease while one (2.1kb) remains the same. All four transcripts are encoded on the same DNA strand since the sense E10 RNA probe has been shown to hybridize to all four (Figure 29). It is interesting to note that there appears to be some sort of regulation that exists within this region resulting in decreased levels of three transcripts without affecting the levels of the 2.1 kb transcript. By analogy to HCMV, it is tempting to speculate that this regulation may be at the level of either transcription or post-transcription (Griffiths and Grundy, 1987; Spector et al., 1990). Regulation at the post-transcriptioal level would include low stability of the three transcripts in the cytoplasm during L phase or decreased transportation of the three transcripts to the cytoplasm. Regulation at the transcriptional level would include

down-regulated expression of the three transcripts, and this may be the most likely explanation since previous studies have indicate lower expression of the HindIII I-J region during the L phase (Marks et al., 1983; Keil et al., 1984).

E1 and E6 cDNAs map to the major E regions, E and A respectively. The transcripts from their respective regions have been shown to be present at lower levels during the L phase. These observations are similar to those documented in studies by Marks et al. (1983), although this study revealed significantly more details. The portion of the HindIII E region where E1 maps and the portion of the HindIII A region where E6 maps have been shown to be transcribed at a lower level during the L phase (Marks et al., 1983). Therefore, the presence of low levels of the corresponding E transcripts is most likely attributed to down-regulated expression of the (E1 and E6) transcription units during the L phase.

For future studies, similar Northern blot analysis may be performed with RNA isolated from nonpermissive and latently infected cells. Such a study would reveal individual transcripts present during infection and accordingly provide the basis for an in-depth comparison between permissive and nonpermissive or latent infections with MCMV at the transcription level.

In summary, the five E cDNAs (E1, E3, E6, E7 and E10) hybridized to more than one transcript and therefore, some of the major MCMV E gene expression consists of transcripts that may be spliced and/or originate from overlapping genes and/or from genes with multiple intiation sites. The major E genes corresponding to the five cDNAs begin to be express during the E phase and have been shown to be expressed in a temporally regulated

manner. In general, the temporal expression pattern of an individual transcription unit is very similar to the results of the major E regions previously identified in preliminary studies by Marks et al. (1983) and Keil et al. (1984), although this study provides significantly more detail on individual transcripts.

3.4 FURTHER ANALYSIS OF THE E10 cDNA

RESULTS

3.4.1 E10-C cDNA SEQUENCE

The E10 cDNA was subcloned from the Lambda gt10 recombinant phage into the plasmid pGEM3z at the EcoR1 site. Both strands of the E10 cDNA were sequenced as outlined in the 'Materials and Methods' and Figure 30. The majority of the sequence on both strands was obtained by generating a series of overlapping deletion clones (Henikoff et al., 1984) which were sequenced using the enzymatic procedure (Sanger et al., 1977). The E10 cDNA consists of 3223 bp, but on analysis it was observed that the clone was not full length. The E10 cDNA sequence revealed one major open reading frame (ORF) with no stop codon, and no polyadenylation signal or poly A tail at the 3' end. The missing 3' terminus of the E10 cDNA, designated as E10-A cDNA was successfully isolated using the polymerase chain reaction method as outlined in 'Materials and Methods' and Figure 31. This clone was sequenced as described in 'Materials and Methods' (Section 2.19) and Figure 32. The combined sequence of clones E10 and E10-A, designated as E10-C (C for complete) is presented in Figure 33. Bases 1 to 3223 represent the sequence from the E10 cDNA clone, and 3184 to 4606 represent the sequence from the E10-A cDNA clone. Analysis of the E10-C cDNA sequence and the putative protein it encodes were performed using available computer programs [PC gene (Intelligenetics) and Seqnce (Delaney Software Ltd, version 2.1 and Universion)].

3.4.1.1 ANALYSIS OF E10-C cDNA SEQUENCE CODING CAPACITY:

Analysis of the 4,606 base sequence illustrated in Figure 30 revealed one major ORF extending from nucleotide position 155 to 3754. A canonical poly adenylation signal (AATAAA) is present at nucleotide position 4569. The

first ATG triplet in the sequence is encountered at nucleotide position 114, but this ORF would code for a very short polypeptide of approximately 12kd ending at the TGA stop codon at position 380. However, the second ATG triplet (nucleotide position 155) initiates a major ORF. This ATG is preceded by the sequence TCACG which is close to the consensus sequence CCACC, and therefore may be the favourable start codon (Kozak, 1984). The cDNA in this case has an untranslated leader sequence of at least 154 bases [the full 5' leader has not been cloned (see Section 3.6)], and a major ORF (3600 bases) that has the potential to code for a 1,200 amino acid polypeptide of approximate molecular weight 135 kd, the putative E10 protein.

Figure 30: DNA sequencing strategy for E10 cDNA

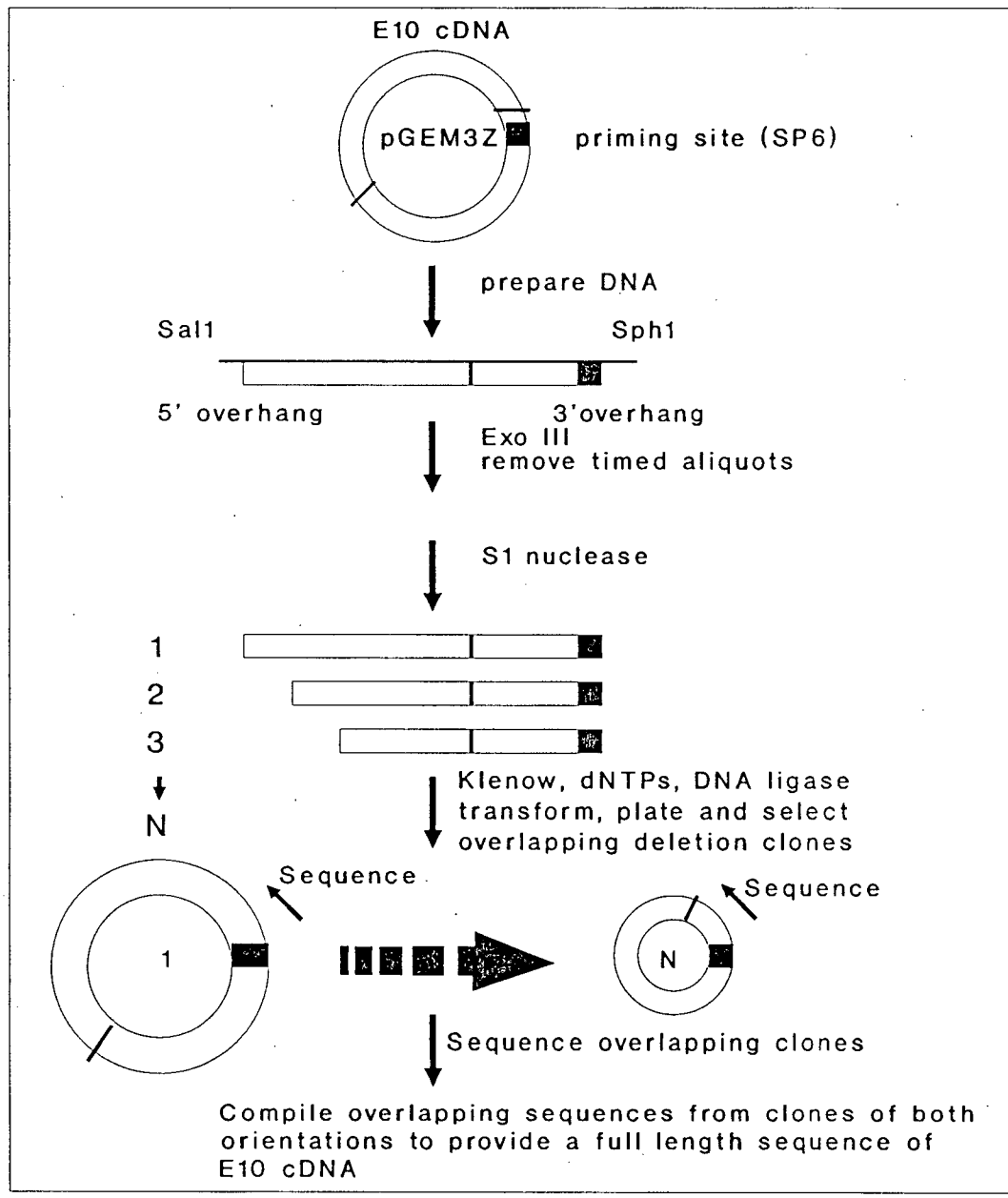


Figure 31: Isolation strategy for E10-A cDNA

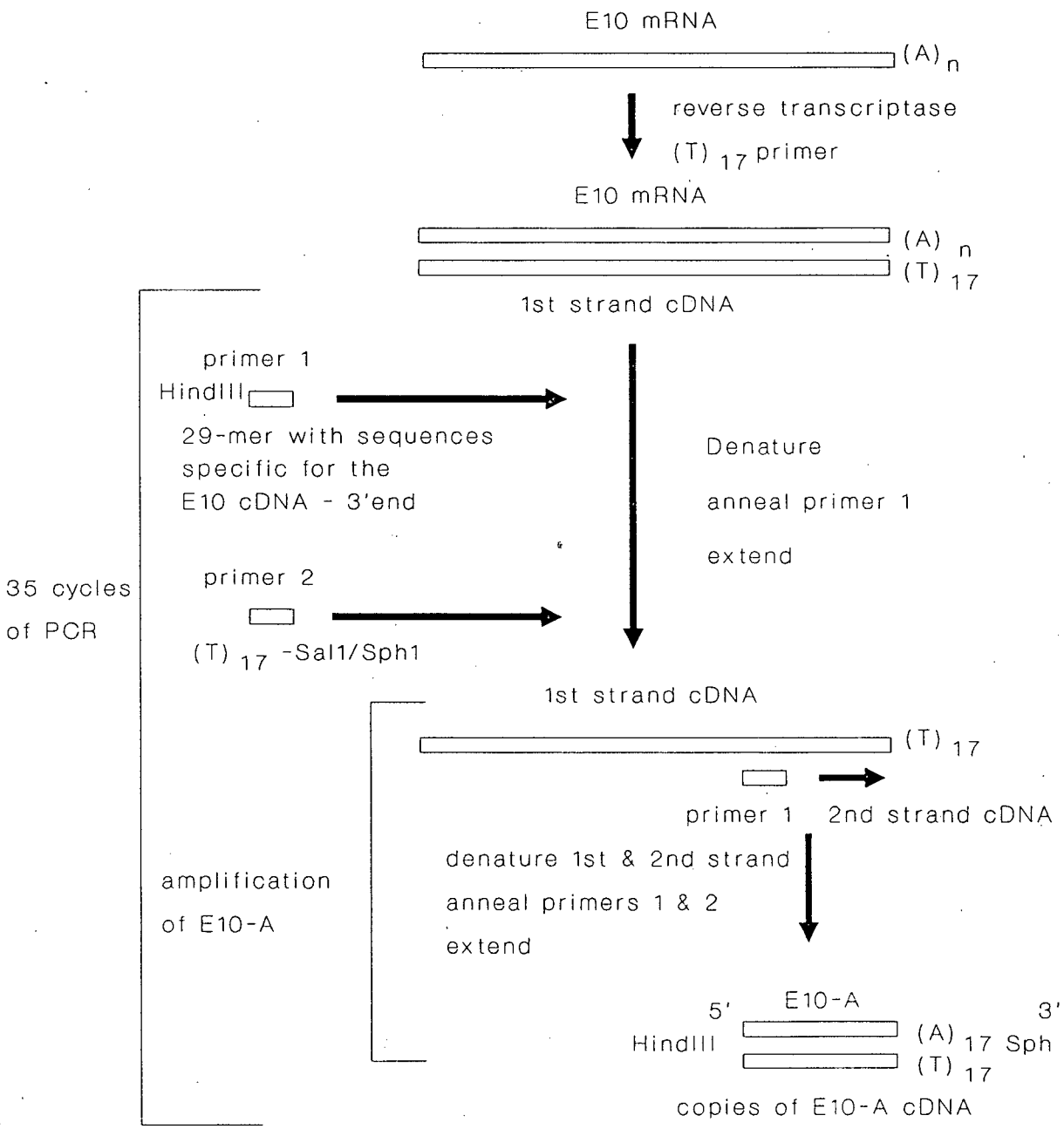


Figure 32: DNA sequencing strategy for E10-A cDNA

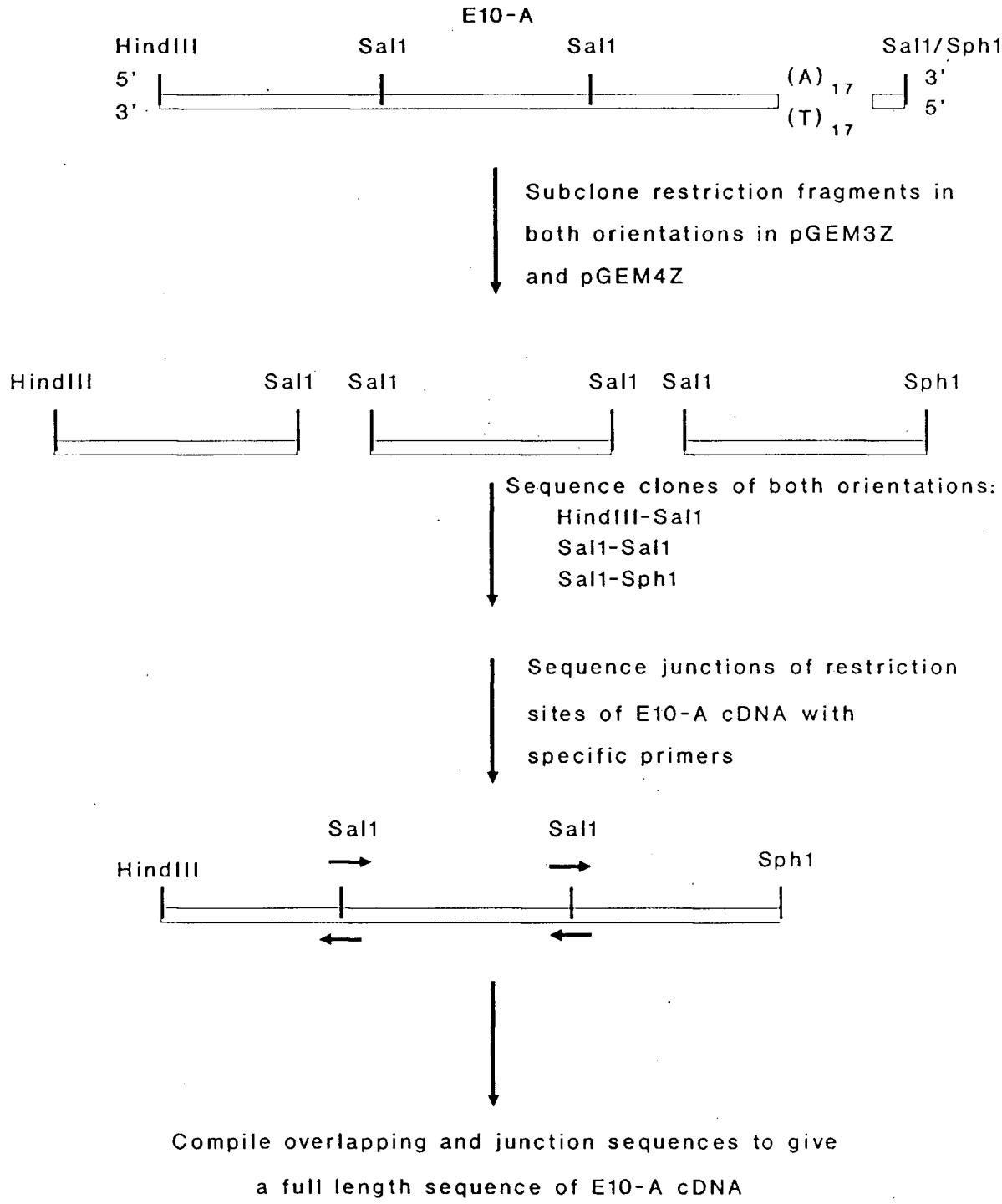


Figure 33: E10-C cDNA sequence

E10-C cDNA sequence consisting of 4,606 bases. The potential translation products of the minor and major ORF, extending from nucleotide positions 114 to 380 and 155 to 3754, respectively are indicated using the amino acid one letter symbols. The polyadenylation signal (AATAAA) is at position 4569 (underlined).

```
GGCGACTGTGCTGCTTCTGGCACGCGTTCGGCCGTGGGCGCCTACGCGGCAGGCTCTTCAGCATCCACCA
      10        20        30        40        50        60        70

                                                M  Q  R  E  K  F  Y  R  V
CCTGCTCGTCATCGACACGTTCGGCGCGATCTACACGCTGGACATGCAGCGCGAGAAGTTCTACCGCGTG
      80        90       100       110       120       130       140

 A  T  A  S  R
GCGACGGCATCACG
     150


 M  L  L  R  A  G  M  A  R  H  R  V  R  A  F  D  R  R  P  E  A  V  Q
 C  C  C  G  P  A  W  Q  G  I  A  F  G  P  S  T  V  G  R  R  P  C  R
ATGCTGCTGCGGGCCGGCATGGCAAGGCATCGCGTTCGGGCCTTCGACCGTCGGCCGGAGGCCGTGCAG
     164       174       184       194       204       214

 M  Q  S  S  V  H  C  R  S  R  N  S  S  A  R  V  D  Y  A  N  E  H  E
 C  S  H  L  S  T  A  D  H  A  T  R  A  P  G  S  I  T  P  T  N  T  S
ATGCAGTCATCTGTCCACTGCCGATCACGCAACTCGAGCGCCAGGGTCGATTACGCCAACGAACACGAG
     233       243       253       263       273       283

 W  L  C  R  R  D  R  F  R  P  D  M  R  T  W  D  D  A  D  K  L  A  I
 G  C  V  A  G  T  A  F  G  P  T  C  G  R  G  T  T  R  T  S  W  P  S
TGGCTGTGTCGCCGGGACCGCTTTCGGCCCGACATGCGGACGTGGGACGACGCGGACAAGCTGGCCATC
     302       312       322       332       342       352

 N  H  A  V  R  A  M  K  R  K  A  G  E  M  Y  T  N  E  E  E  D  W  T
 T  T  P  C  A  P
AACCACGCCGTGCGCGCCATGAAGAGAAAGGCCGGGGAGATGTACACGAACGAGGAGGAGGACTGGACG
     371       381       391       401       411       421

 D  T  N  R  V  G  T  G  G  R  R  Q  Q  G  I  R  R  T  R  D  G  H  H

GATACCAACAGAGTGGGAACAGGAGGACGACGACAACAGGGGATTCGACGGACACGCGATGGACATCAC
     440       450       460       470       480       490
```

G P R R W L A L M H V V A R E R A N D T P G S

GGGCCCCGACGATGGCTCGCGCTCATGCACGTGGTGGCCCGAGAGCGTGCTAACGACACGCCCGGATCG
509 519 529 539 549 559

Q P R H P N P V P L H V R E K G H V S R G G D

CAACCGAGACACCCGAACCCTGTTCCGTTACATGTCAGAGAAAAAGGTCACGTTTCAAGAGGTGGCGAC
578 588 598 608 618 628

Q K A L G E A R E S L L S E L F R S D S A D R

CAGAAGGCTCTTGGAGAGGCAAGAGAGTCACTACTCTCTGAGCTTTTCCGCTCAGACTCTGCAGACCGT
647 657 667 677 687 697

E L T A P A P D G A D R E R E G C G L S A A P

GAGTTGACGGCGCCGGCGCCAGACGGAGCAGACAGAGAGAGAGAAGGATGTGGTCTCTCCGCGGCGCCC
716 726 736 746 756 766

R D A T C V S M V R T T S G V W R T R I A W T

AGAGACGCGACCTGCGTTTCGATGGTCAGGACGACGAGTGGTGTTTGGCGTACGAGGATTGCATGGACC
785 795 805 815 825 835

R S S E N T A A W T R G A F P R S G H Q F T P

AGAAGTTCGGAGAATACGGCGGCGTGGACGAGAGGAGCCTTCCCCCGATCAGGTCACCAGTTTACCCCC
854 864 874 884 894 904

Q R S F I N D Q F F L R P A I G L H G S H G R

CAAAGAAGCTTTATAAATGACCAGTTTTTCCTTCGGCCTGCCATTGGACTCCACGGATCGCACGGCCGG
923 933 943 953 963 973

G R E S C G A M A E S D L F I D R A A L D G T

GGACGCGAATCCTGCGGCGCGATGGCGGAGTCTGACCTGTTCATAGATCGCGCCGCGCTGGACGGGACC
992 1002 1012 1022 1032 1042

G R S D A G S R D S E S D S D F D M E S D S D

GGGCGCTCTGACGCGGGCTCCCGCGACAGCGAGAGCGACTCAGACTTCGACATGGAGTCGGACTCGGAC
1061 1071 1081 1091 1101 1111

L S D D G D A A G A Y V Y G S P V E D L G R H

CTGTCCGACGACGGCGACGCGGCGGGAGCGTACGTGTACGGCTCGCCGGTGGAAGACCTCGGCCGTCAC
1130 1140 1150 1160 1170 1180

G V Q E G R R E I R E E E A G A R L Y S L T M

GGTGTCCAGGAAGGCCGTCGAGAAATACGAGAAGAAGAAGCTGGCGCACGACTCTACTCGCTGACGATG
1199 1209 1219 1229 1239 1249

R D F K S F H L A L S F R R A R S R P R G A L

CGCGACTTCAAGAGCTTCCACCTGGCCCTCAGTTTCCGCCGGGCCAGATCGCGGCCGCGTGGTGCACTA
1268 1278 1288 1298 1308 1318

P R Q A P R A P L P G E L V P D P A E T L R D

CCTCGGCAAGCCCCTCGTGCTCCGCTACCCGGAGAACTGGTACCTGATCCCGCAGAGACGCTCCGAGAT
1337 1347 1357 1367 1377 1387

P R R A R R G P V Q V S V L R R H R R R A D A

CCGCGTCGTGCGCGGCGTGGACCTGTCCAAGTATCTGTGCTGCGGCGACACCGCCGGCGAGCTGACGCC
1406 1416 1426 1436 1446 1456

P G G L A A R S P D L H F R E T P C A L V M D

CCTGGGGGTCTCGCCGCGCGCTCGCCCGACCTGCACTTCAGAGAGACGCCGTGCGCCCTGGTCATGGAC
1475 1485 1495 1505 1515 1525

E R G R F F L Y D A E S D G L Y Y A A R N I D

GAGCGCGGCCGCTTCTTCCTGTACGACGCCGAGTCCGACGGGCTGTACTACGCGGCCAGGAACATCGAC
1544 1554 1564 1574 1584 1594

Q L A R R G L S L C E P V Y R D G G A V V S M

CAGCTGGCGAGACGCGGGGCTGTCTCTGTGCGAGCCGGTGTATCGAGACGGCGGCGCCGTGGTCTCGATG
1613 1623 1633 1643 1653 1663

P K P K T L V R K I V S A A V V G L E K L R P

CCGAAGCCCAAGACCCTGGTCAGGAAGATCGTGTCCGCCGCCGTGGTCGGCCTGGAGAAGCTGCGGCCA
1682 1692 1702 1712 1722 1732

R H G V Q G L D D R P A R P G L G T A R D V S

CGCCACGGCGTTCAGGGGCTCGACGATCGCCCTGCGCGACCCGGTCTCGGGACGGCGCGAGACGTTTCA
1751 1761 1771 1781 1791 1801

G V R G V R P E E K P P F S R M D D T T Y T L

GGTGTTCGGGGCGTCAGACCTGAAGAGAAGCCGCCCTTCTCGCGGATGGACGACACGACGTACACACTC
1820 1830 1840 1850 1860 1870

V R E Y I T F R L A E A W T V I G A V G E Y R

GTGCGGGAGTACATCACCTTTCGGCTGGCCGAGGCGTGGACCGTCATCGGCGCCGTGGGAGAGTACCGG
1889 1899 1909 1919 1929 1939

D D G F V F E V S T V V L V G A R G T V Y G F

GACGACGGCTTCGTCTTCGAGGTGTCCACCGTCGTGCTGGTCGGAGCCCGCGGAACGGTGTACGGTTTC
1958 1968 1978 1988 1998 2008

```
 C  L  L  S  N  D  V  F  R  I  A  E  D  I  S  V  F  F  K  R  G  G  V

TGCCTGCTGAGTAACGACGTCTTCAGGATCGCCGAGGACATCTCGGTCTTCTTCAAGAGAGGAGGCGTG
      2027       2037       2047       2057       2067       2077

 S  G  R  R  R  A  Q  P  L  R  P  R  R  P  G  R  T  P  S  G  T  P  A

AGCGGCCGCCGCCGTGCCCAACCGCTTCGACCGAGGCGCCCGGGGAGAACTCCGTCTGGAACGCCCGCC
      2096       2106       2116       2126       2136       2146

 A  L  S  H  R  D  E  D  R  P  P  I  A  T  S  L  A  S  Q  E  I  A  R

GCTCTGTCCCACAGGGACGAAGACCGGCCGCCGATCGCCACGTCGCTCGCGAGCCAGGAGATCGCGCGT
      2165       2175       2185       2195       2205       2215

 R  D  L  E  D  W  Y  R  W  R  L  G  A  R  S  R  L  Q  D  G  V  A  L

CGCGACCTGGAAGACTGGTACCGCTGGCGACTCGGCGCCAGATCGCGGTTGCAGGACGGCGTGGCCCTG
      2234       2244       2254       2264       2274       2284

 S  N  V  S  E  A  K  R  Q  L  T  H  P  A  K  G  I  P  V  S  I  V  T

TCGAACGTGAGCGAGGCGAAGAGGCAGCTGACCCATCCCGCCAAAGGGATCCCCGTCTCCATCGTGACG
      2303       2313       2323       2333       2343       2353

 A  E  S  Y  H  P  R  G  I  P  R  K  P  S  N  P  V  G  T  D  E  A  V

GCGGAGTCTTACCACCCCCGGGGGATCCCGAGGAAGCCTTCGAATCCTGTCGGGACAGACGAGGCCGTA
      2372       2382       2392       2402       2412       2422

 P  I  P  A  G  L  R  R  L  P  V  V  L  R  P  R  L  L  S  S  L  R  P

CCGATACCCGCGGGTCTGCGACGCCTCCCTGTCGTGCTCCGACCGCGACTTCTATCGTCGTTACGTCCG
      2441       2451       2461       2471       2481       2491

 R  H  G  H  V  R  R  R  Q  G  E  E  R  A  R  D  Y  V  H  Q  G  R  E

CGCCATGGACATGTTCGACGGCGGCAAGGAGAAGAGCGAGCGCGAGATTATGTTCATCAGGGCCGAGAG
      2510       2520       2530       2540       2550       2560

 G  R  L  A  P  E  D  G  N  P  I  E  T  A  T  S  G  P  R  D  S  G  G

GGTCGGCTCGCTCCAGAAGACGGGAACCCCATTGAAACTGCCACCTCTGGTCCGCGCGACTCAGGCGGG
      2579       2589       2599       2609       2619       2629

 I  D  E  Q  Q  H  G  N  R  L  R  S  L  V  Q  R  R  C  V  S  R  S  R

ATCGACGAGCAACAACATGGAAACCGGCTCCGATCGCTAGTCCAGCGGCGCTGTGTCAGCCGCTCGCGG
      2648       2658       2668       2678       2688       2698

 G  A  R  T  N  H  Q  S  I  K  A  V  P  H  H  R  N  S  V  T  E  R  E

GGGGCGCGCACGAACCATCAGTCTATAAAAGCCGTGCCGCATCACAGAAACTCTGTCACAGAGCGCGAG
      2717       2727       2737       2747       2757       2767
```

L K S P S S L G R Q S R G D G A F D A D R T L

CTCAAGTCGCCATCATCTCTCGGTCGGCAGAGCCGAGGCGATGGCGCCTTCGACGCTGATCGCACTCTG
2786 2796 2806 2816 2826 2836

P P R G R D G A S I T C V P V A L A H R D L R

CCTCCTCGCGGCCGTGACGGCGCATCAATTACCTGCGTGCCAGTGGCGCTTGCCCACAGAGACCTCCGT
2855 2865 2875 2885 2895 2905

P V Q Y L L R P G E R L H G Q G L A R P L R R

CCCGTTCAATATCTCCTGCGACCTGGTGAACGGCTCCACGGTCAAGGTCTGGCGAGACCTCTGCGGCGA
2924 2934 2944 2954 2964 2974

R Q C Y R G M D R D G R R G D K L H F S A R W

AGGCAATGCTACCGAGGGATGGATCGGGATGGCCGGCGCGGAGACAAATTACATTTCTCCGCCAGGTGG
2993 3003 3013 3023 3033 3043

N Y S T A D I G V Y Y V N V T E R N L T T P L

AACTACTCGACCGCCGACATCGGGGTCTACTACGTCAACGTGACCGAGCGTAACCTGACGACTCCCCTG
3062 3072 3082 3092 3102 3112

T Q L I T P P M T I W G M R Q G G R S R D F V

ACGCAACTGATCACCCCACCGATGACGATCTGGGGCATGAGGCAGGGCGGCAGAAGCAGAGACTTTGTC
3131 3141 3151 3161 3171 3181

L C T V T G I F S K G E F T L E A N G A T V L

CTCTGCACCGTGACGGGCATCTTCTCGAAGGGTGAATTCACACTCGAGGCGAACGGCGCGACGGTTCTC
3200 3210 3220 3230 3240 3250

S V N F T K P G R Y A V K K A R V S L D L S H

TCTGTCAACTTCACAAAACCCGGACGATATGCAGTGAAAAAAGCGCGCGTCTCTCTAGACCTCTCACAT
3269 3279 3289 3299 3309 3319

R N G T M R F L V S I R G G P M T H V K Y Q C

CGCAACGGTACCATGCGATTTCTCGTCTCGATCAGAGGGGGGCCCATGACGCACGTGAAATATCAGTGC
3338 3348 3358 3368 3378 3388

I M T P L S V R S F C G Q Q A S D V G I R Q T

ATCATGACTCCTCTCTCTGTGCGTTCCTTTTGCGGTCAGCAAGCCTCTGATGTTGGAATACGACAGACC
3407 3417 3427 3437 3447 3457

M P S G V K G T P R T P P G L K S P T E S A V

ATGCCATCGGGAGTGAAAGGTACACCAAGGACTCCACCGGGACTGAAGTCGCCAACAGAGTCTGCTGTA
3476 3486 3496 3506 3516 3526

S S M I T I T G F A Q A R H R D Q N D R P V R

AGCTCGATGATTACCATAACTGGATTTGCCCAAGCGCGTCACCGTGACCAGAACGACCGTCCAGTGCGC
3545 3555 3565 3575 3585 3595

R T P V L V C L R H P A V H V L H S R G G K A

AGAACCCCAGTTCTTGTTTGCCTTCGACATCCCGCCGTACATGTTCTACATAGCCGCGGTGGAAAAGCC
3614 3624 3634 3644 3654 3664

A G I G L D A V R R R H M E L R F H L Q W R I

GCTGGAATCGGTCTTGATGCTGTACGACGTCGACACATGGAACTACGCTTTCACCTCCAGTGGCGTATC
3683 3693 3703 3713 3723 3733

D T N I

GATACAAACATCTAA
3752

AAAGACGGGTGTAGGCCTCCCCCCGGGTGAGTACCAGTGTATCTTCGAGAGAGAGAGTGGATCGTTATAC
3767 3777 3787 3797 3807 3817 3827

GGCCGGTCGCTGATGGTACACGGCAGCATAACGATCGAGGCGAAGACGACACACGACTTCGTGAGTAGAG
3837 3847 3857 3867 3877 3887 3897

TCGAGATCCAAATAACGTGTACCTTCGACGCGGTGTCCGACGGCGCGTTCAGCCTGGAGGCGCGTGGAAG
3907 3917 3927 3937 3947 3957 3967

ACGGACAGCCGGTGTATCAGAGCCGTGTCGGCACTAACGAGTCCTGTTCCTCCGACGACTCCATCTCGAT
3977 3987 3997 4007 4017 4027 4037

CAACGGCTCCGCGGCTGTCCGAGGGCAAGATCTCGGTGTCCGTGTACCTGAGAGAAGACGATGGCGTGATC
4047 4057 4067 4077 4087 4097 4107

GGCCGGTTTAGGTGCGTCCGCTCGGGATGCTTGAATCTGGTATCCCGAGCGATCACCGTGACGAAGCCCA
4117 4127 4137 4147 4157 4167 4177

CCAGGATGGAATACGCCCTCGTGACAGAGGAGCCCGTCACGTCCCCTCAGACCACCACCACTACTACTAC
4187 4197 4207 4217 4227 4237 4247

GACGACCACAACACCATCACCCCCCTCACCCGCCAAGAAACTAGAAGAGATCAGAATAAAGGTCGACGGA
4257 4267 4277 4287 4297 4307 4317

ACCGACGACGATGACTTTCTGTTGAAGACGCAAGAACCGCTCAAAAGCTACCCCTACTATCCTCTATTCG
4327 4337 4347 4357 4367 4377 4387

CTCGCGTCACGCGAAGCCGTCTGGCGGCCGTCGCTTGTTTCTTCCTAGCCGCGATCCTCGTGTTGGTCCA
4397 4407 4417 4427 4437 4447 4457

CATCTCGTTCTGGATCTCGTCGCCGCCGTGTAACTGCCGGGTTGCGTACGTCACACGTAACTCAAGTCAT
4467 4477 4487 4497 4507 4517 4527

CGACTACTAACACTTACTAATGACTGACCCCCAGCATATATAAATAAAAGCTCTCAAACTAGAAAAAAAA
4537 4547 4557 4567 4577 4587 4597

AAAAAAAAA

3.4.1.2 FURTHER ANALYSIS OF THE E10-C cDNA SEQUENCE AND PUTATIVE E10 PROTEIN:

The DNA sequence was analyzed for the presence of six base pair restriction endonuclease sites. These are summarized in Figure 34. Of note is an EcoR1 site at position 3224. During the construction of the Lambda gt10 recombinant phage library (Section 2.14.3), the cDNAs were methylated with EcoR1 methylase prior to addition of the EcoR1 linkers used to clone the cDNA into the Lambda gt10 phage. Clearly the methylation step was incomplete as this internal EcoR1 site was not protected from digestion at a later stage. Knowledge of the restriction endonuclease site map was used in determining the orientation of the gene coding for the E10-C cDNA (Section 3.5, below).

Other properties of the putative E10 protein are summarized in Figure 35 and Tables V & V1. Analysis of the hydrophilic and hydrophobic regions of the E10 protein is illustrated in Figure 35. The hydropathic index of -6.95 indicates that the protein is overall quite hydrophilic (basic) with a total of 242 positively charged residues (Arg, His, Lys) and 137 negatively charged residues (Asp, Glu) (Table V). There is one potential transmembrane segment (nt 601-624) (Table V). The amino acid sequence of this segment is GFVFEVSIVVLVGARGIVYGFCLL.

A computer search was carried out to determine if the E10 sequence is homologous with any of the sequences in the current data banks (European Molecular Biology Laboratory (EMBL), Oct 1990; Genbank, Oct 1990) and HCMV Ad169 (obtained from E. Mocarski, Stanford University). In addition, a computer search was carried out to determine if the putative E10 protein is homologous with any other protein present in the current SWISSPRT data base

Figure 34: Restriction map for DNA sequence E10-C cDNA.
The following 6 base pair restriction endonuclease sites were found within the E10-C cDNA sequence, BamHI, 2341, 2346; BglI, 47, 2863; EcoR1, 3224, HaeI, 357; HindIII, 920; PstI, 701; Sal, 3702, 4309; and XbaI, 3313.

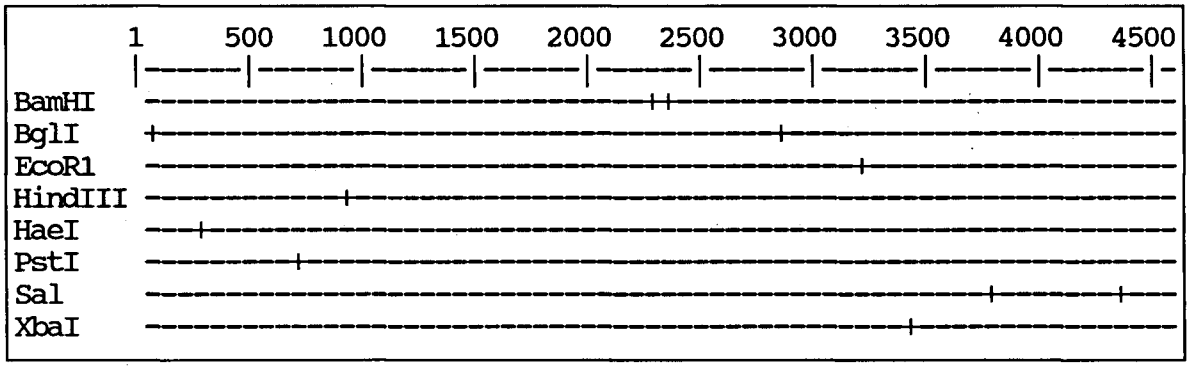
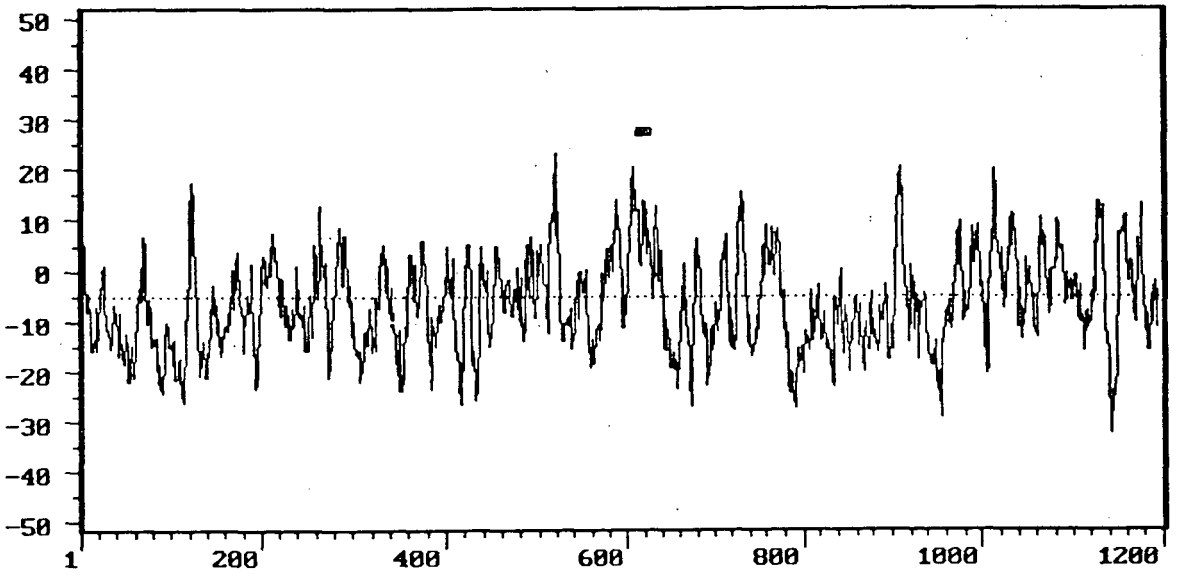


Figure 35: Hydropathy index of hypothetical protein E10.
Hydropathy index was prepared by a computer program, Soap [PC gene (Intelligenetics)]. The horizontal bar above the hydrophobic region represents a putative transmembrane segment with the following amino acid sequence: GFVFEVSIVVLVGARGIVYGFCLL.



Hydropathic index of E10PROT from amino acid 1 to amino acid 1200.
Computed using an interval of 9 amino acids. (GRAVY = -6.95).

TABLE V

PROPERTIES OF THE PUTATIVE E10 PROTEIN

Table V summarizes the different properties of the putative E10 protein. This protein is highly basic (by PC gene: Chargpro) and has several potential glycosylation and phosphorylation sites. A highly hydrophobic region qualifying as a transmembrane segment is predicted in the amino acid sequence position 601-624 residue and therefore, the hypothetical protein may be an integral membrane protein.

FEATURES	PROTEIN E10
Number of amino acid residues	1200
Molecular weight	135 kd
Nature of the protein	highly basic
Hydrophobic residues: Ala, Gly, Ile, Leu, Val	421
Hydrophilic residues: Asn, Gln, Ser, Thr	213
Positive residues: Arg, His, Lys	242
Negative residues: Asp, Glu	137
Number of potential trans-membrane segments	one
Location of potential trans-membrane segment in amino acid sequence	601-624
Amino acid sequence of potential trans-membrane segment	GFVFEVSIVVLVGARGIV YGFCLL

TABLE VI

SEGMENTS HOMOLOGOUS TO THE PUTATIVE E10 PROTEIN

A search for a homologous protein sequence in the data base (SWISSPRT; September 1990) revealed several regions of short homology, but small and perhaps insignificant to proteins of several herpesviruses (HSV, VZV and HCMV). The proteins are identified by the standard nomenclature system used in the SWISSPRT data base. The first four letters give the name of the protein or coding region, $ is a separator and the last five letters describe the organism or virus. Information on positions and sequences of these homologous segments is presented in the Table below.

PROTEIN TYPE	POSITION	POSITION IN E10	PROTEIN SEQUENCE SEGMENT
YHL7$HCMVA: HCMV HYPOTHETICAL PROTEIN	374-382	47-55	47 WLCRRDRFR |||| |||| 374 WLCRGDRFR
ICP3$HSV1F: VZV-1 ICP34.5 PROTEIN	20-34	647-661	647 RRRAQPLRPRRPGRT || | | ||| || | 20 RRHAGPRRPRPPGPT
EXON$VZVD: HSV EXONUCLEASE	266-280	397-411	397 RAPLPGELVPDPAET ||| | | | |||| 266 RDPLTGTLNPHPAET
US09$HSV11: HSV-1 TEGUMENT PHOSPHOPROTEIN US9	51-61	424-434	424 QVSVLRRHRRR | |||| ||| 51 QQSVLRRRRRR

(Sept, 1990) and ORFs of the HCMV AD169 (obtained from E. Mocarski, Stanford University). Several regions of short homology were detected to both known and hypothetical proteins from other herpesviruses (HSV, VZV and HCMV). These regions of short homology are summarized in Table VI. It is not known if any of these regions of apparent homology is significant.

3.4.2 ORIENTATION OF THE GENE CODING FOR E10-C cDNA

The orientation of the gene coding for the cDNA was determined initially by comparing the HindIII, EcoR1 and XbaI restriction enzyme map of E10-C cDNA (Figure 34) to that of the corresponding region on the Smith strain MCMV genome (HindIII I-J region) (Figure 2 in Introduction). Subsequently, a short region of the HindIII I or J region of the viral genome was sequenced to reveal sequences common to the 5' or 3' termini of the cDNA (Figure 33). The information for the restriction sites of the cDNA was obtained from the DNA sequence and the data indicated that the 5' and 3' termini of the E10-C cDNA mapped to HindIII fragments I and J respectively. This was confirmed by sequencing regions of HindIII I and J fragments with primers specific for the terminal sequence to reveal sequences common to the 5' and 3' termini of the cDNA respectively.

Figure 36 illustrates the restriction map of the E10-C DNA and the orientation of the gene relative to viral HindIII fragments I and J. Figures 34 and 35 represent DNA sequence present in HindIII fragments I and J respectively. The underlined sequences in Figures 37 and 38 show sequences common to the 5' and 3' termini respectively of the E10-C DNA sequence (Figure 33). The flanking sequence preceeding the underlined sequence in fragment I (Figure 37) does not appear to contain any consensus sequence common to a MCMV promoter region (Buhler et al., 1990). In fragment J (Figure 38), the sequence following the underlined segment is rich in T nucleotides. This is characteristic of a sequence downstream of a poly A signal in most genomic DNAs (Hames and Glover, 1988).

Figure 33: Orientation of the gene coding for E10 cDNA

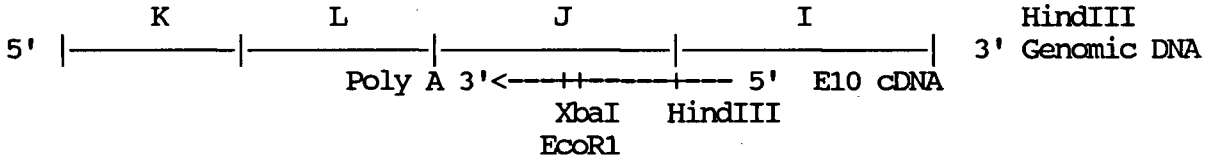


Figure 37: DNA sequence from HindIII I region of the MCMV (Smith) genome. The DNA sequence was obtained by sequencing the HindIII I fragment with a primer that was specific for the 5' end of E10-C cDNA. The underlined sequence represents sequence common to the 5' terminus of E10-C cDNA.

```
        10         20         30         40         50         60         70
CCCACGCCTC GTCGTGGGGC TGATCATGTG CCAGACGATC TCGACCGGGT GTCCGATTAC TGCCAGGAGA
        80         90        100        110        120        130        140
ACAGCGGGCA CGTCGCGCTG TACACCCCCG GATTCAAGTA CCAGCCGATG AAACTGCTGG GTGGCGTGAG
       150        160        170        180        190        200        210
AGACGCCGCG CGCTACTGGC CCCTGGATAT CATGAACCCG TCGAACCTGA AGGCCTGCCT GGACGAGATC
       220        230        240
ACGGGGCGAC TGTGCTGCTT CTGGCACGCG TTCG
```

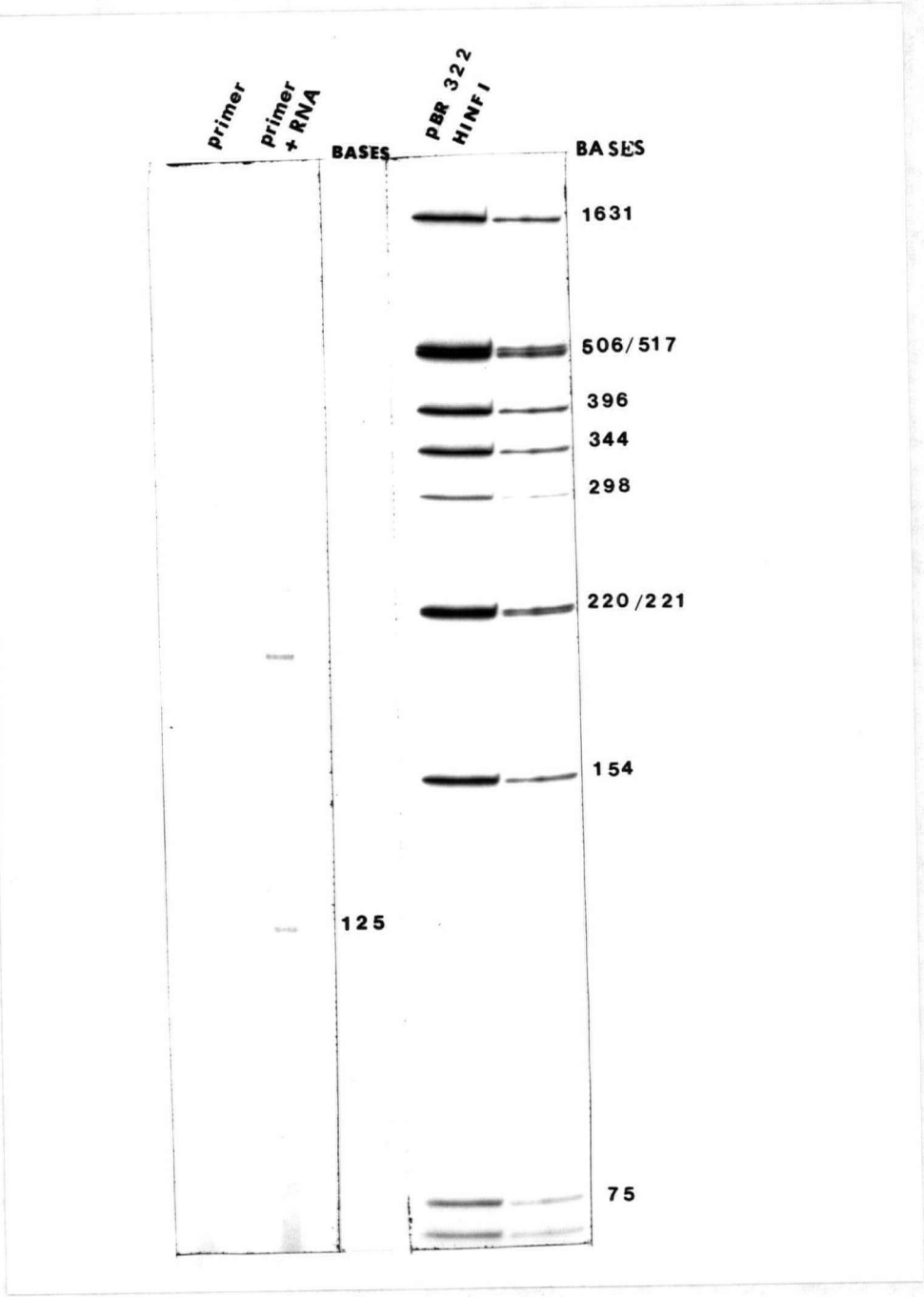
Figure 38: DNA sequence from HindIII J region of the MCMV (Smith) genome. The DNA sequence was obtained by sequencing the HindIII J fragment with a primer that was specific for the 3' terminus of E10-C cDNA. The underlined sequence represents sequence common to the 3' terminus of E10-C cDNA.

```
        10         20         30         40         50         60         70
GCTCTCAAAC TAGACGCGGT TCCTTCATTA CTCTGTTATT TTTCGGTCTC CGATGGGTCA CGGCTCTCTT
        80         90        100        110        120        130        140
GCCTCTCTCT CCATGTGGCG ATACTGGTAT CGGGGCGCCA TCGCGCGATA GTCGGCCATC ACCACGACGG
       150        160        170        180        190        200        210
CACTGATAGC CCCGGGGGGT GGGATCCCTC CGGCTTTGTC GTTCGAACAG CCCGAGTTCG CTGAGGGAAC
       220        230
ATGTACGACG ACCCAGCCCA CGTGTT
```

3.4.3 POSSIBLE IDENTIFICATION OF THE 5' START SITE OF THE E10 cDNA TRANSCRIPT

Due to the protocol used during the construction of the cDNA library, the 5' end of the cDNA would not necessarily be included. Therefore, primer extension experiments were carried out to determine the 5' end of the E10 cDNA; however, these studies were inconclusive. A γ-^{32}P-labelled primer (18-mer) (ATGTCCAGCGTGTAGATC) with DNA sequence specific to E10 cDNA and 98 bases away from the 5' end of the cDNA clone was annealed to E RNA and extended in the 5' direction using reverse transcriptase (Sambrook et al., 1989). As shown in Figure 39, several extended products were observed in the acrylamide gel, ranging in size from 125 to 600 bases. This may be attributed to the origin of multiple transcripts (9.5, 6.9, 4.7 and 2.0kb; Section 3.3) from the same genomic region (containing different exons), and/or pausing of the reverse transcriptase. The first major product of the extended primer was approximately 125 bases in length, only 9 bases more than the position of the 5' end of the cDNA clone. Since there are four transcripts hybridizing to E10 cDNA (Section 3.3), the interpretation of this observation would be subjective, although it is possible that the first extension product of 125 bases represents the 5' end of the cDNA clone characterized most extensively in this thesis.

Figure 39: Possible identification of the 5' start site of the E10 cDNA transcript using primer extension.
γ-^{32}P-labelled primer (ATGTCCAGCGTGTAGATC) specific for 5' end of the E10 cDNA was annealed to E RNA and extended in the 5' direction with reverse transcriptase. The extended products were electrophoresed through a 6% acrylamide denaturing gel (32 watts) along with γ-^{32}P-labelled unannealed primer (negative control) and denatured HinfI digested pBR322 markers.



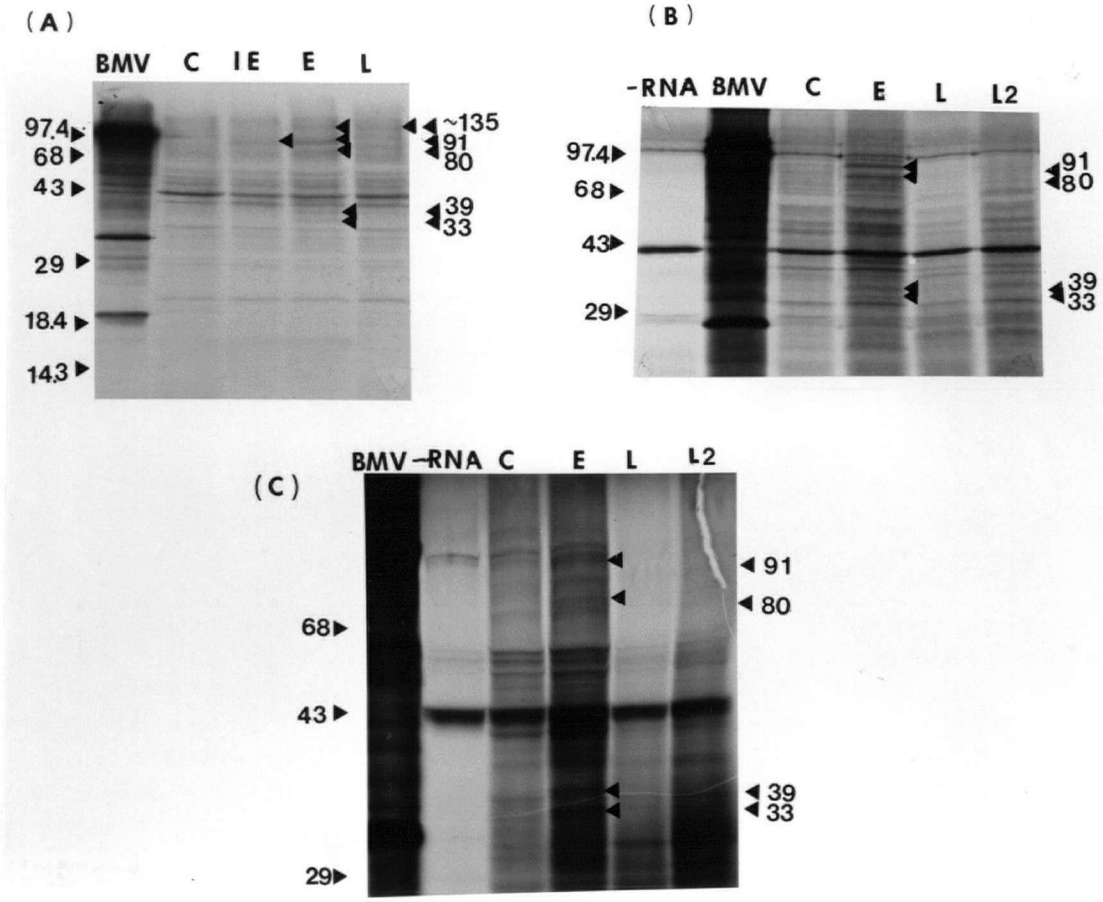
3.4.4 POSSIBLE IDENTIFICATION OF THE PROTEIN CODED BY E10 cDNA

Preliminary attempts were made to identify the proteins encoded by the E10 cDNA and its related transcripts. This procedure was made difficult by the fact that the E10 cDNA was incomplete. Also, the joining of E10 and E10-A cDNAs, to produce an uninterrupted continuous cDNA, E10-C, for in vitro transcription was technically difficult due to the presence of numerous endonuclease restriction sites in inappropriate places. Therefore, the protocol chosen to be the most likely to give positive results involved hybrid selection of RNAs using the E10 cDNA and subsequent translation of these RNA, in vitro, as described in 'Materials and Methods'. The results were difficult to interpret because of problems encountered during the procedures. These included nonspecific hybridization of cellular and viral RNAs to the E10 cDNA clone (probably due to the high G+C content) and therefore the presence of nonspecific high molecular weight cellular proteins which interfered with the identification of a 135 kd protein (the putative product of the major ORF of the E10 cDNA).

Autoradiograms of 12% acrylamide gels (A and B) and a 5-20% gradient gel (C) representing three experiments are presented in Figure 40. For gels B and C, hybrid selection for the appropriate transcripts was carried out using the E10 cDNA insert plus the plasmid DNA (pGEM3Z) bound to the nylon membrane. In the case of gel A, only the E10 cDNA insert (minus the plasmid DNA) was used to select for the RNA, and only in this gel, was a protein of approximately 135 kd detected. This protein was absent in the C (mock infection) and IE lanes, but was present in the E and L lanes.

Despite the fact that the nylon membranes following hybridization were washed at a high stringency (65°C, 0.1X SSPE) during the RNA enrichment

Figure 40: In vitro translation products of RNAs that hybridize to E10 cDNA. RNAs were isolated from the IE, E and L (lanes L and L2; L2 was loaded with two times the quantity of L) phases of infected and mock infected 3T3L1 cells (C). By the method of hybrid release, the RNAs that hybridized to the E10 cDNA (plus plasmid pGEM3Z DNA for gels B and C) were eluted and translated in a rabbit reticulocyte system in the presence of ^{35}S-methionine. The translation products were separated in either a linear 12% polyacrylamide gel (Figures A and B) or a gradient (5-20%) polyacrylamide gel (Figure C). Each gel represents a separate experiment. Arrows at the right side of the gel show the positions of molecular weight markers of virally induced proteins. Arrows at the left side of the gel show the molecular weights of the marker proteins. Lane 'BMV' represents a control in which Brome Mosaic virus RNA was translated. Lane '-RNA' represents endogeneously-labelled proteins which were present after translation when no exogenous RNA was added to the translation system.



procedure, many cellular transcripts bound nonspecifically to the blot. The presence of the proteins which resulted from the translation of these nonspecific transcripts makes these results difficult to interpret. Since a protein of approximately 135 kd can be identified only in one of the gels, gel A, in which the E10 cDNA insert (minus the plasmid) was used to enrich for the RNA, the selection procedure in this case appears to have worked better than that used in the experiments analysed on gels B and C where plasmid DNA was also present. In addition, the 135 kd protein may not be identified in gels B and C due to insufficient resolution of the high molecular weight proteins on these gels or inefficient translation of the corresponding transcript possibly due to the large size of the ORF of the E10 transcript.

Four virally induced proteins were detected consistently in all three gels and these were proteins of 33, 39, 80 and 91 kd. The first three proteins which appear clearly in the E lanes may correspond to the transcripts that were found to hybridize to the E10 cDNA on the Northern blot (Section 3.3, Figure 28) or may be the products of transcripts that have bound nonspecifically to the E10 cDNA (during hybrid selection). The 91 kd protein which is present in the IE and E lanes may correspond to the major IE protein that was previously detected in our laboratory (Walker and Hudson, 1987). Its presence is likely due to the nonspecific binding of the major IE transcript to the E10 cDNA. In addition, the hybridization experiments in Sections 3.1 and 3.2 have demonstrated that the HindIII K-L region (IE region) is particularly prone to bind nonspecifically to other DNA sequences, which may be due to its high GC rich regions.

In summary, a total of five virally induced proteins were identified by

translation of hybrid-selected RNAs in vitro. Of the five, one protein (91 kd in IE and E lanes) appears to represent the major IE protein and non-specific selection of this transcript by the E10 cDNA is presumed to have occurred. Only one E/L protein (approximately 135 kd) which was detected in one out of 3 gels (each of a different experiment) may represent the putative E10 protein. Detection of this protein in this case may be attributed to a better hybrid selection procedure (using only E10 insert) and better resolution of high molecular weight proteins in the gel. The presence of other E proteins (33, 39 and 80 kd) translated may correspond to the related transcripts that hybridized to E10 cDNA on the Northern blot, however, this observation would be subjective since there was a high degree of nonspecific binding of cellular transcripts as well.

DISCUSSION

Two independent groups have identified high levels of transcription from the HindIII I-J region during the early phase of the MCMV (Smith) replication cycle (Marks et al., 1983; Keil et al., 1984). This suggests that it is an important E region and therefore, the E10 cDNA clone was selected for extensive analysis since it maps to this location. This analysis included determining the DNA sequence and orientation of the E10 gene, attempts to identify the 5' end of the E10 transcript, and the in-vitro translation of the transcripts hybridizing to the E10 cDNA. The DNA sequence of the E10 cDNA has revealed two ORFs, a major ORF and a minor ORF. The first ATG triplet is encountered at nucleotide position 114 and has the potential to initiate the minor ORF that would code for a 12 kd protein. The second ATG triplet is encountered at nucleotide position 155 and has the potential to initiate the major ORF that codes for a 135 kd protein (1200 amino acid residues), the putative E10 protein. The preceding sequence to the first ATG triplet displays only two nucleotides common to the consensus sequence. Approximately 50% of the eukaryotic mRNA initiation codons have 3-4 nucleotides in the preceding sequence common to CCACC, with A as the highly conserved nucleotide (Kozak, 1984). The preceding sequence to the second ATG triplet displays TCACG, with 3 nucleotides including the A common to the consensus sequence and therefore, the second ATG triplet is more likely to be recognized by the ribosome as the initiation codon for the translation of the polypeptide (putative E10 protein). According to Kozak (1983, 1984), five to ten percent of the eukaryotic mRNA translation is not initiated at the first AUG, rather the ribosome recognizes the sequence closest to CCACCAUG to initiate translation. In addition, there have been reports on the presence of AUG triplets as part of short ORFs within the 5' leader sequence, upstream of a major ORF in transcripts of HCMV and the e1 gene of

MCMV (Jahn et al., 1987; Kouzarides et al., 1987; Geballe and Mocarski, 1988; Buhler et al., 1990). The RNA transcript of the E gene e1 of MCMV, which maps to HindIII F and is the first E MCMV gene studied, also has a minor and a major ORF (Buhler et al., 1990). The minor ORF is initiated by the first ATG triplet that is present upstream of the major ORF. It is speculated that the minor ORF regulates expression by delaying the translation of the major ORF (Buhler et al., 1990) and this may also be the case with the E10 transcript. Another possible reason for the existence of two start codons in the transcript may be due to its bicistronic function, similar to that found in the viral transcripts of HSV TK mRNA, simian virus 40 late 19S mRNA and adenovirus E1b mRNA (Preston and McGeoch, 1981; Kozak, 1983). The bicistronic function mRNAs code for two overlapping proteins and express both, therefore using the first and second initiation codons (Kozak, 1983). The possibility that the E10 transcript may be bicistronic in function is remote because we did not detect a 12 kd protein in the in-vitro translation experiments.

As stated earlier, herpes E proteins are usually involved in DNA synthesis, nucleotide metabolism and gene regulation [summary of review in Spector et al. (1990) and Roizman et al. (1990)]. Thus many herpes E proteins have been shown to be DNA binding proteins. Some of the MCMV E proteins identifed in our laboratory previously (Walker and Hudson, 1988a) are also DNA binding proteins, although this study did not reveal a protein that was close to 135 kd, the molecular weight of the putative E10 protein. The possible reasons for not detecting the putative E10 protein in the study by Walker and Hudson (1988a) are stated below. The putative E10 protein is highly basic, indicating the potential to be a DNA binding protein, since its high positive charge may allow electrostatic binding to DNA (Berg et

al., 1982) via charge-charge interaction between the DNA phosphate and basic amino acids. From the limited data available so far and the lack of homology to other DNA and protein sequences, the function of the protein coded by E10 cDNA can not be predicted. However, from information available from computer analysis of the putative E10 protein, it is tempting to speculate that the protein may play a role at the level of gene regulation or DNA synthesis because of its predicted DNA binding nature.

The hydropathicity profile of the putative E10 protein predicts a transmembrane segment within the 1200 amino acid sequence and therefore, the polypeptide has the potential to be an integral membrane protein. The amino acid sequence of integral membrane proteins have polar regions on both sides of the membrane with one or more transmembrane segments (hydrophobic) interacting directly with the hydrophobic core of the phospholipid bilayer (Wickner, 1979). Since only one transmembrane region has been predicted for the putative E10 protein, further studies are required to verify whether the protein could be a membrane protein.

From studies to date, the exact identification of the putative E10 protein is subjective. The identification of protein products of the E10 cDNA and its related transcripts in in-vitro translations experiment demonstrated at least four virally induced proteins of which, one that was of approximate molecular weight 135 kd, perhaps the putative E10 protein, was identified only in one out of three experiments. It is not known at this point if the other three proteins (33, 39 and 80kd) are encoded by the E10 cDNA or by any of the three related transcripts. In our laboratory, E proteins of sizes 36 and 39kd have been detected (Walker and Hudson, 1987, 1988a). These E proteins have been shown to be present in abundance and exclusively

in the nucleus, and therefore they may play a regulatory role in viral gene expression or DNA synthesis. At this point, it is premature to state that these proteins may represent the two proteins (33 and 39kd) detected in the in-vitro translation experiment. The results of the in-vitro translation experiment were difficult to interpret due to the high degree of nonspecific hybridization of transcripts to E10 cDNA. Further experiments would be required to confirm the identity of the proteins coded by the E10 and its related transcripts. One of the ways to overcome the problem would be to reisolate the E10 cDNA in a single and complete length of 4.6kb, and then subject the cDNA to in-vitro transcription and translation. This would result in a protein coded by the E10 cDNA and confirm the ORF translated by the ribosome.

In an investigation by Walker and Hudson (1987), in our laboratory, seven E proteins were detected and the highest molecular weight protein was 91kd. However, an important point to note is that the isolation time point for E proteins in Walker and Hudson (1987) was 6 hr p.i. Therefore the reasons for not detecting a higher molecular weight protein of approximately 135kd may be due to limited expression or no translation of the E10 transcript, at 6 hr p.i. in the E phase. Previous studies by Kim et al. (1976b) and Chantler and Hudson (1978) have detected a structural protein VP5 with a molecular weight of 132-137 kd. This protein was detected at E times, 10hr p.i. In addition to Kim et al. (1976b) and Chantler and Hudson (1978), recently Buhler et al. (1990) have detected an E protein of approximate size 130 kd along with other E proteins. This protein has been observed in 3T3 cells (permisssive system), but has not been ascribed any function or been characterized further. Therefore, three groups have identified a protein of approximate size 130-137 kd. It is premature to conclude that the proteins

identified by these three individual groups are the same and represent the putative E10 protein as well. In order to elucidate the possibility that VP5 or another E/L protein may represent the putative E10 protein, further studies are required. This would include reisolation of full length E10 cDNA, in-vitro transcription and translation of the full length E10 cDNA, and raising polyclonal antibodies to the entire in-vitro translated E10 polypeptide. Alternatively, polyclonal antibodies could be prepared against synthetic peptides determined from the DNA sequence. Finally, the protein coded by the E10 cDNA could be identified using these polyclonal antibodies on a western blot and determined if E10 is VP5.

MCMV like HCMV shows temporal and quantitative expression of their genome during infection. Many investigators have identified HCMV transcripts that are present during the E phase of the replication cycle, but are not translated until the L phase (Geballe et al., 1988; Wright and Spector, 1989). Differential expression through ribosome association may be another similarity of MCMV to HCMV and such a concept for the E10 transcript is possible. Since a long ORF exits within the E10 cDNA, it is very likely that the putative protein is translated, if not during the E phase, then at least during the L phase, and it may have an important function.

The 5' and the 3' termini of the E10-C cDNA maps to fragments HindIII I and J. The orientation of the gene(s) coding for the E10 transcript and the related transcripts is the same as for its neighbouring gene sgg-1 in the HindIII J region (unpublished data, Manning et al., Westcoast Herpesviruses Workshop, 1990). The restriction enzyme map of the E10 cDNA also helps to locate the precise location of the E10 cDNA. The T-rich sequence which is present downstream of the E10 poly A signal in the HindIII J fragment is a

requirement for efficient polyadenylation (Hames et al., 1988). Polyadenylation of mRNAs in eukaryotic cells is required in order to yield stable transcripts. Therefore, the presence of the additional T-rich signal flanking the poly A signal indirectly reflects on the stability of the transcript (Hames et al., 1988). Studies involving HSV have shown that the E transcripts are relatively stable compared to L transcripts (Frenkel et al., 1972, 1973; Siverstein et al., 1973; Wolf and Roizman, 1978) and thus, the E transcripts may remain in the cell at L times. This fact together with the above observation (T-rich region) indicates that the same may apply to the E10 transcript. Furthermore, E transcripts that hybridize to the E10 cDNA were found to be present at low levels at L times in the Northern blots.

The sequence upstream of the 5' end of the E10 cDNA in the HindIII I fragment is lacking MCMV promoter like (TATA) and CAAT sequences (Buhler et al., 1990). It is difficult to predict whether this sequence represents an intron or the uncloned 5' termini of the E10 cDNA since the results of the Primer extension experiment were complicated by the fact that four transcripts (9.5, 6.9, 4.7 and 2.1 kb) originated from this region. Although the primer extension experiment showed multiple extension products, the first major product was just nine bases longer than the position of the 5' end of the E10 cDNA. If the rest of the multiple extension products were due to other transcripts, then the E10 cDNA is only nine bases short of the 5' terminus. The E10-C cDNA is a 4606 nucleotide sequence and by allowing the addition of approximately 100 nucleotides for a poly A tail, this would result in a transcript of 4.7kb. Therefore, E10-C cDNA likely represents the 4.7kb transcript. Further analysis including sequencing of the HindIII I and J fragments are required to reveal the structure of the gene(s)

corresponding to the E10 and related transcripts. Also, the further sequence analysis of the HindIII I fragment coupled with assays for promoter function using a reporter gene such as the chloramphenicol acetyl transferase (CAT) could help to identify the promoter region. It is interesting to note that the size of the corresponding region coding for the four transcripts is large and is at least 9.5kb (the size of the longest transcript).

In conclusion, the E10-C cDNA is 4606bp in length and probably represents the 4.7kb transcript. The E10 cDNA most likely encodes a 1200 amino acid polypeptide of 135kd (putative E10 protein) from a major ORF of 3600 bases, which is initiated by the second ATG triplet (nucleotide position 156). The sequence of the putative E10 protein suggests that it may be a glycosylated, phosphorylated, DNA binding and/or integral membrane protein. The in-vitro translation experiment revealed inconclusive results, although an E/L protein of approximate 135 kd was identified in one of the gels which may represent the E10 putative protein. Also, three groups have identified an E protein with molecular weight of 130-137 kd. The 5' and 3' of the E10 cDNA map to fragments HindIII I and J, respectively and the orientation of the gene(s) coding for the four related transcripts is identical.

4.0 CONCLUSIONS

1. Ten E cDNAs were mapped to specific locations of the virus genome, and each of these represented transcripts from one of the major E regions, HindIII A, B, E, F and I-J.

2. Five E cDNAs (E1, E3, E6, E7 and E10), each representing a different major E region, and two IE cDNAs (IEd and IEf) representing the major IE region were successful as probes to study transcript levels from the corresponding transcriptional units in infected cells. The E and IE cDNA probes detected transcript levels that displayed the typical E and IE gene expressions, respectively.

3. The transcripts that correspond to the five E cDNAs begin to be transcribed during the E phase and are usually present during the L phase, although at a different level. This sort of expression pattern may involve regulation at the transcriptional or post-transcriptional level.

4. The five E cDNAs detected more than one transcript in the corresponding transcriptional units which indicates possible complex splicing events, overlapping genes, multiple initiation sites, and/or the presence of gene(s) in the complementary DNA strand.

5. There are four transcripts (9.5, 6.9, 4.7 and 2.1 kb in size) that correspond to the E10 region (in HindIII I-J). All of these are encoded on the same DNA strand, thus are transcribed in the same orientation. The E10-C cDNA (4.6kbp) most likely represents the 4.7 kb transcript.

6. The E10-C cDNA sequence (4606 bases) has one minor and one major open reading frame (ORF). The minor ORF is initiated by the first ATG triplet (nucleotide position 114) while the major ORF is intiated by the second triplet (nucleotide position 155). Since the sequence preceeding the second ATG triplet is in "good context" with regard to the translation initiation consensus sequence, it is most likely that the major ORF is translated. The major ORF encodes a 1200 amino acid polypeptide, the putative E10 protein of approximately 135 kd in size.

7. Using hybrid-release and in vitro-translation methods, a polypeptide of approximate 135 kd in size was detected in one of the experiments. This polypeptide may represent the putative E10 protein, although further studies are required to confirm the ORF translated in the E10-C cDNA.

8. The 5' and the 3' termini of the E10-C cDNA map to HindIII fragments I and J of the virus genome respectively, thus revealing the orientation of the gene coding for the E10 cDNA and its related transcripts. The size of the corresponding E region, E10, is at least 9.5kb (size of the longest transcript).

9. Finally, the study of the major E transcription units corresponding to E1, E3, E6, E7 and E10 cDNAs presented in this thesis sheds light on the kinetics of MCMV gene transcription and also provides us with an insight into the transcription pattern and regulation that exists in MCMV. The extensive study of the E10 cDNA and its corresponding transcription unit, E10, has revealed details such as the structure of the E10

transcript, putative E10 protein coded by E10 major ORF, potential properties of E10 putative protein, and the orientation of the gene coding for E10 and its related transcripts. This information will provide a good basis for future studies on the E10 region.

5.0 FUTURE EXPERIMENTS

This study has established two cDNA libraries, one for IE and one for E mRNAs. From the studies I have reported here, the E library appears to be a good source of authentic E cDNAs. In contrast, although the IE library does contain authentic IE cDNA clones, at least two clones proved to be cellular in origin, and selected apparently due to mispairing between these cellular clones with the MCMV HindIII K and L fragments. Regardless of this problem, both libraries should be a useful source of additional IE and E cDNA clones.

The most interesting experiments that should follow are additional analysis of transcripts hybridizing with the E10 cDNA clone and identification of the function of the putative protein. It should be possible to identify additional cDNA clones (from the E cDNA library of 198 clones) which hybridize with E10. In this way it should be possible to identify alternatively spliced and/or overlapping transcripts. Other studies that would be useful would be to use short restriction fragments from the E10 cDNA as hybridization probes for Northern analysis to determine if specific regions of the E10 transcript are contained within all four mRNAs (9.5, 6.9, 4.7 and 2.1kb). The putative E10 protein may be identified in MCMV infected cells by immunological techniques. An antibody to a specific peptide (corresponding to a hydrophilic region of the E10 amino acid sequence) could be prepared in rabbits and used in Western blots to identify the presence and size(s) of immunoreactive polypeptides. If a region of the E10 sequence was found to be unique for one of the four transcripts (see above), an antibody to this region would be expected to identify a single polypeptide.

In further studies, to elucidate the function of the E10 polypeptide, it should be possible to express the gene in COS cells (using an approriate

SV40 origin vector). This would allow one to use indirect immunofluoresence to determine the intracellular location of this protein. Again, if a region of this protein were shown to be unique for the E10 polypeptide, similar studies could be done in MCMV infected 3T3L1 cells. It would also be of interest to see if polyclonal antibody raised to the entire putative E10 protein could detect any polypeptides within HCMV infected cells or other Herpesvirus infected cells.

The functional importance of the E10 gene may be investigated by performing experiments such as insertional mutagenesis and marker rescue. A recombinant MCMV can be constructed by cotransfecting a stable cell line containing the E10 gene in an integrated form, with wild type MCMV Smith virus and a recombinant vector containing a lacZ gene within the E10 gene. The recombinant virus containing the mutated E10 gene can be identified by selecting for blue plaques (due to X-gal). The E10 gene may be proven essential by comparing the ability of the recombinant virus to grow in a normal cell line versus the cell line containing the integrated E10 gene. Finally, if the E10 gene appears to be essential, then this may be confirmed by a marker rescue experiment where a normal cell line may be cotransfected with the recombinant virus and a vector containing the wild type E10 gene. The rescued virus should resume its ability to grow in the normal cell line to the same extent as the wild type virus.

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7.0 APPENDIX

HOMOLOGY OF HSV-2 EcoR1 M FRAGMENT TO MOUSE HINDIII DNA FRAGMENT

The objective of this study was to isolate MCMV cDNAs coding for the viral DNA polymerase (pol). A restriction fragment (EcoR1 M) of the HSV-2 genome, known to contain the HSV DNA pol gene was selected as a probe to screen the MCMV DNA pol cDNAs. Although this investigation was unsuccessful, the results of the study were interesting and observations were noteworthy. (All the literature references in this appendix are included in Section 6.0, above).

6.1 RESULTS

6.1.1 HYBRIDIZATION OF E1 TO E10 cDNAs TO HSV-2 EcoR1 M FRAGMENT

Since MCMV DNA replication commences at 8-12 hrs p.i. (Misra et al., 1978; Chantler and Hudson, 1978; Marks et al., 1983), the E cDNAs were selected for the isolation of MCMV DNA pol cDNA. The ten characterized E cDNAs were slot blotted along with controls of Smith DNA and Lambda gt10 DNA. The blot was hybridized to the HSV-2 EcoR1 M probe. The probe hybridized nonspecifically to most of the cDNAs E1, E4, E5, E6, E7, E8 and E10, and to Smith DNA, but not to Lambda gt10 (not shown). The nonspecific binding was probably due to high GC regions present in either the HSV-2 M fragment, the cDNAs sequences or both. The next step was to make an attempt to isolate the pol cDNA directly from the unscreened E cDNAs using the HSV-2 EcoR1 M fragment as a probe.

6.1.2 SCREENING AND CHARACTERIZATION OF E cDNAs HYBRIDIZING TO HSV-2 EcoR1 M FRAGMENT

I. SCREENING WITH HSV-2 EcoR1 M FRAGMENT PROBE

The unscreened E cDNAs were selected for the DNA pol gene by hybridizing

the recombinant plaques to the HSV-2 EcoR1 M probe. Ten positives were picked of which the four (DP2, DP3, DP5 AND DP8) that hybridized strongly to both the HSV-2 EcoR1 M fragment and MCMV Smith strain DNA probes during the second screening, were selected for further analysis.

II. ESTIMATION OF SIZES AND SOUTHERN BLOT ANALYSIS OF DP cDNAs

The sizes of the cDNA inserts were estimated by separating the EcoR1 digested cDNA fragments on a 1% agarose gel along with Lambda HindIII fragments as molecular weight markers. As a control, MCMV Smith HindIII O fragment was also present in one of the lanes. The DNA fragments were transferred to a nylon membrane and hybridized to the Smith DNA probe to confirm the origin of the cDNAs. All the DP cDNAs and the Smith HindIII O fragment (positive probe) hybridized to Smith DNA (Figure 41). The blot was washed to strip off the probe and reused to hybridize to 3T3L1 DNA probe. Unfortunately, all the DP clones (DP2, DP3, DP5 and DP8) hybridized to 3T3L1 DNA probe while the Smith HindIII O fragment (negative control) did not hybridize to 3T3L1 DNA (Figure 42). The estimated sizes of the inserts were 1.0, 1.45, 3.16 and 3.2 kb for DP2, DP3, DP5 and DP8, respectively.

III. CHARACTERIZATION OF DP cDNAs

The DP cDNAs were mapped by the Southern blot method with stringent washes at 55°C (0.1X SSPE) rather than 50°C. None of the DP clones hybridized to Smith HindIII fragments. However, two of the four clones DP3 and DP5 hybridized to a 5.3kb HindIII fragment from 3T3L1 (mouse cell) DNA as shown in Figures 39 and 40, respectively and yet neither hybridized to HindIII fragments from Smith DNA. As a positive control, the blots were washed to strip off the probe and rehybridized to the Smith DNA probe (Figures 43 and

44). The Smith DNA probe detected the MCMV DNA HindIII fragments and showed no hybridization to 3T3L1 DNA HindIII fragments. The two clones DP2 and DP8 bound nonspecifically to most HindIII fragments of Smith and 3T3L1 DNA on the blots and this may be due to the presence of high GC region within the cDNAs (not shown). Therefore, DP3 and DP5 cDNAs are cellular (murine) in origin with probable homology to HSV-2 EcoR1 M fragment.

Figure 41: Southern blot analysis of DP2, DP3, DP5 and DP8 probed with Smith DNA.

The DNAs of Lambda gt10 recombinants with DP cDNA inserts were subjected to EcoR1 digestion and electrophoresed (500ng/well) through a 1% gel along with Smith Hind III O fragment (150ng) as a positive control. The gel was Southern blotted and hybridized to ^{32}P-labelled Smith DNA probe.

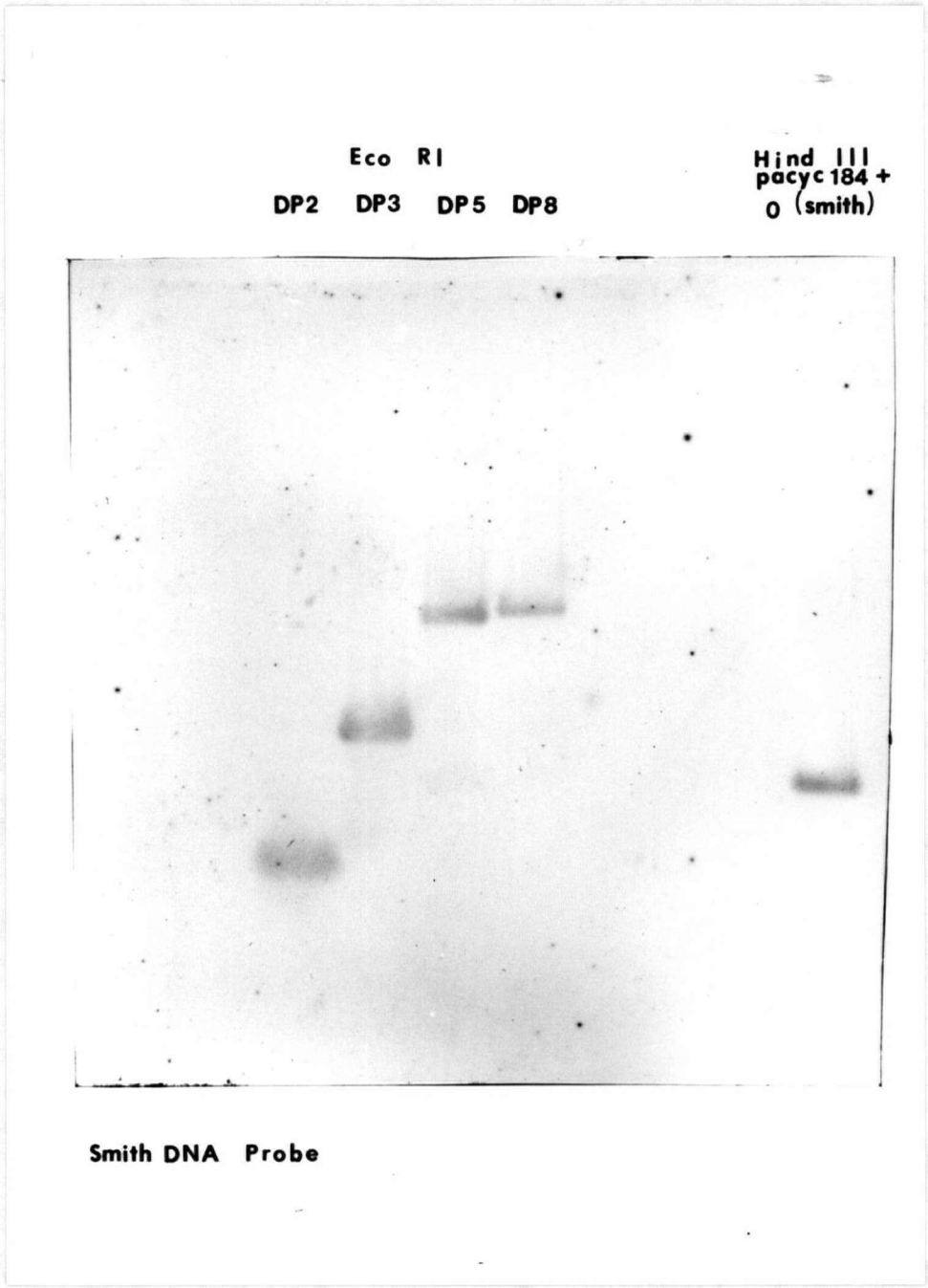


Figure 42: Southern blot analysis of DP2, DP3, DP5 and DP8 probed with 3T3L1 DNA.

The blot in the previous figure was washed to strip off the probe and rehybridized to ^{32}P-labelled 3T3L1 (mouse cell) DNA probe.

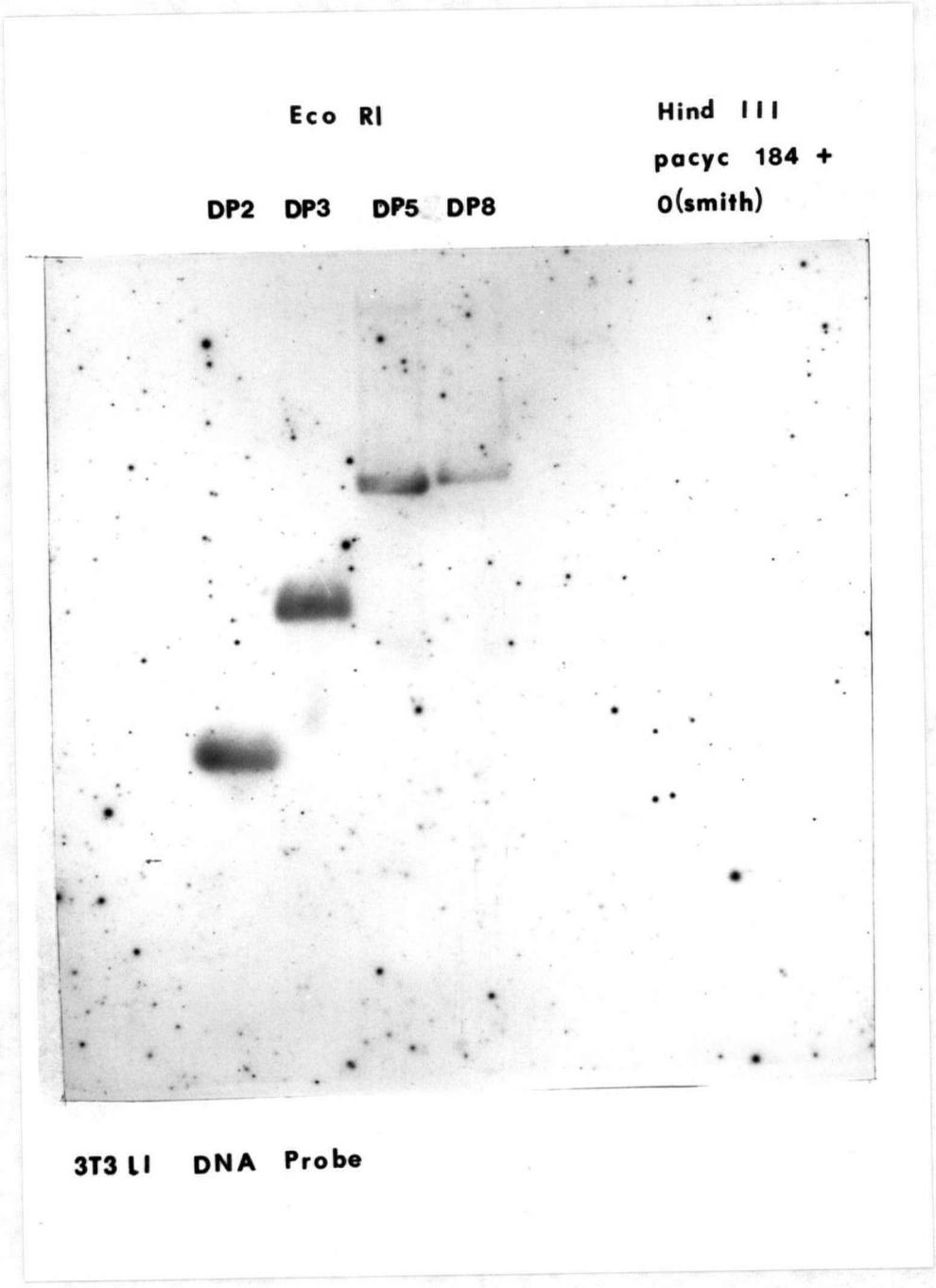


Figure 43: Mapping of cDNA DP3.

3T3L1 DNA (1ug), Smith DNA (S) (250ng) and Lambda DNA (250ng) were subjected to HindIII digestion, electrophoresed through a 0.7% agarose gel and transfered to a nylon membrane. As a positive control, the blot was hybridized to ^{32}P-labelled DP3 cDNA probe and autoradiographed. The blot was washed to strip off the probe and rehybridized to ^{32}P-labelled Smith DNA probe.

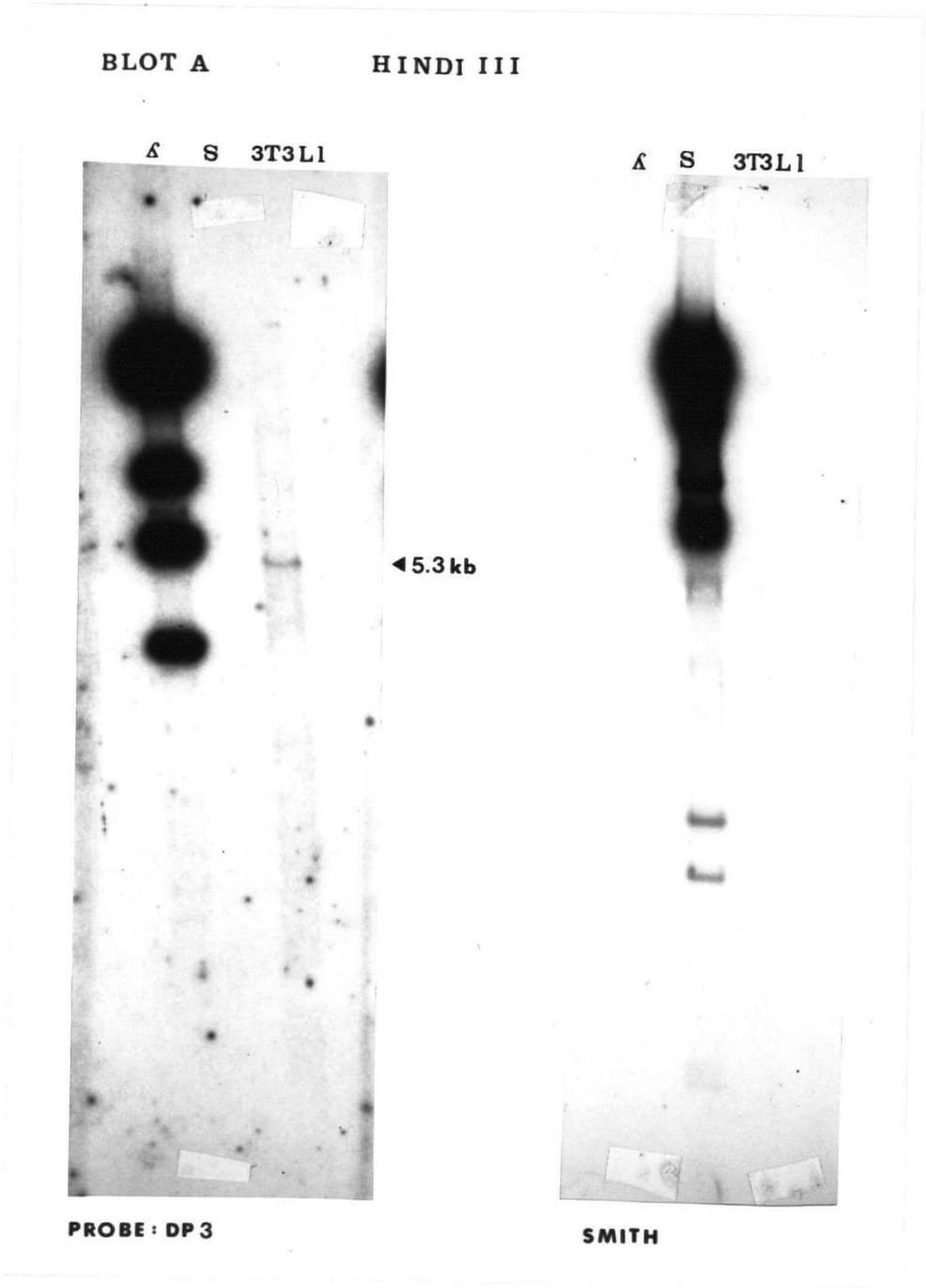
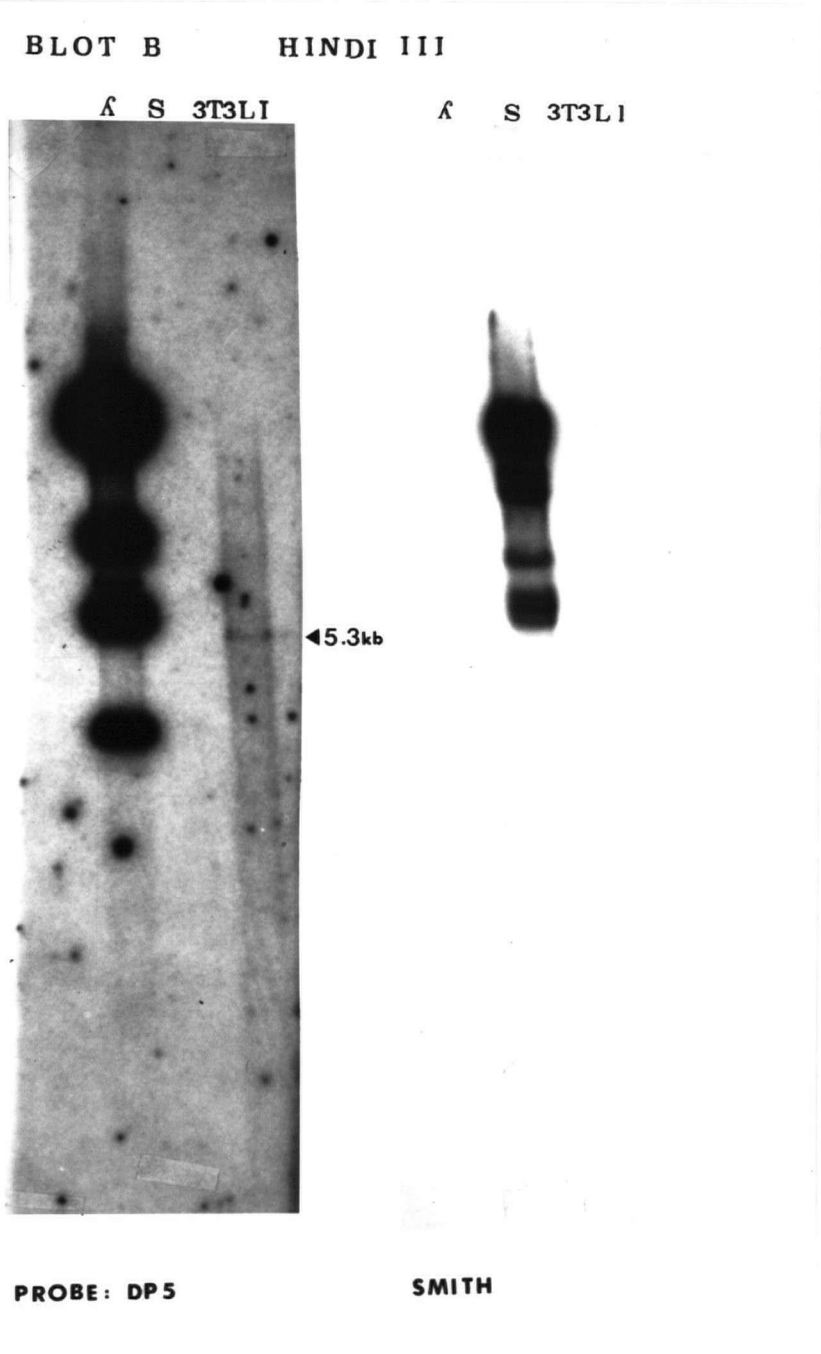


Figure 44: Mapping of cDNA DP5.

3T3L1 DNA (1ug), Smith DNA (S) (250ng) and Lambda DNA (250ng) were subjected to HindIII digestion, electrophoresed through a 0.7% agarose gel and transfered to a nylon membrane. As a positive control, the blot was hybridized to ^{32}P-labelled DP5 cDNA probe and autoradiographed. The blot was washed to strip off the probe and rehybridized to ^{32}P-labelled Smith DNA probe.



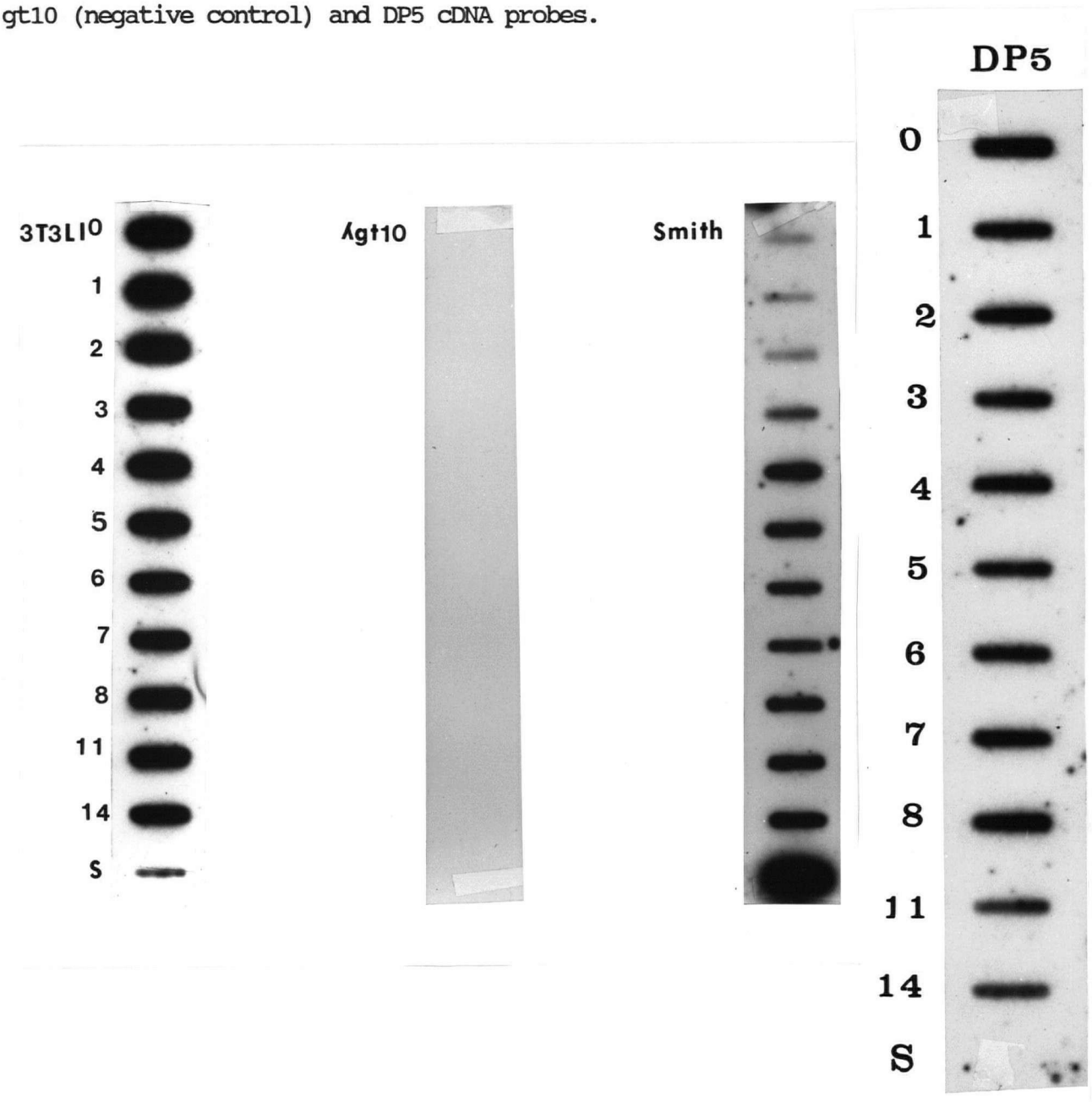
6.1.3 RNA SLOT BLOT ANALYSIS TO MEASURE LEVELS OF TRANSCRIPTS OF DP5 cDNA

Experiments were performed in which DP5 cDNA (Lambda gt10 recombinant) probe was used to monitor the levels of respective transcript(s) during the course of a 14 hr infection. The results (Figure 45) indicated that there is a significant and approximately similar amount of transcripts present before (mock infected) and during the infection. The DP5 DNA does not hybridize to Smith DNA in this experiment probably because the stringent washes were done at higher temperatures (55°C instead of 50°C). Since DP5 cDNA hybridized strongly to RNA at 0 hr (mock infected RNA) and did not hybridize to Smith DNA, these results confirmed its cellular origin. As a positive control, 3T3L1 DNA probe hybridized to transcripts before and during infection. It also hybridized to Smith DNA to some extent, confirming the probability of some sequence homology as previously suspected in our laboratory (unpublished data). The Smith DNA probe hybridized moderately to RNA at 0 hrs (mock infected RNA) perhaps due to specific or nonspecific binding to cell transcripts or rRNA. As the time elapsed, the Smith DNA probe hybridized to MCMV transcripts present on the blot. Lambda gt10 DNA probe (negative control) showed no hybridization to any transcripts or Smith DNA present on the blot.

In summary, the isolation of MCMV DNA pol cDNA failed for reasons discussed below. However, these experiments did result in the isolation of two definite cellular cDNAs mapping to a 5.3kb HindIII mouse DNA fragment that may have some homology to HSV-2 EcoR1 DNA fragment.

Figure 45: Analysis of transcription probed with DP5 cDNA.

RNA was isolated from infected 3T3L1 cells at different time intervals (0 hrs, 1 to 8 hrs p.i., 11 hrs p.i. and 14 hrs p.i.) and slot blotted (3ug RNA/slot). Mock infected RNA (0 hrs) and 25ng Smith DNA (S) were present on the blot as negative and positive controls, respectively. The blots were hybridized to ^{32}P-labelled 3T3L1 DNA (positive control), Smith DNA, Lambda gt10 (negative control) and DP5 cDNA probes.



6.2 DISCUSSION

HSV-2 EcoR1 M FRAGMENT HOMOLOGY TO MOUSE HINDIII DNA FRAGMENT

MCMV-induced DNA polymerase activity was first demonstrated by Muller and Hudson (1978) and its location within the viral genome has recently been mapped by Spencer et al. (unpublished data; Abstract. CMV workshop, 1989) to restriction fragments HindIII H-D and EcoR1 B. The investigators mapped the MCMV DNA pol gene by hybridizing the HincII subfragment of HCMV EcoR1 M fragment, known to contain the DNA pol gene, to MCMV Smith DNA. The transcription site and the termination site are in HindIII fragments D and H, respectively and the gene codes for an unspliced 3.7kb transcript (Spencer et al., 1989; CMV workshop).

The DNA restriction fragment EcoR1 M of HSV-2 contains a significant portion of the HSV DNA polymerase (pol) gene (Gibbs et al., 1985). The HSV DNA polymerase contains conserved sequences that are common to DNA polymerases of Human, HCMV, EBV and Vaccinia virus (Wang et al., 1989). HCMV and Bovine herpesvirus-1 DNA pol genes were mapped by hybridization to HSV DNA pol gene (Wang et al., 1989). In this study I have observed that the HSV-2 EcoR1 M fragment hybridized nonspecifically to E cDNAs E1 to E10 under normal stringent conditions and this may be attributed to a high G-C content of approximately 68% in the HSV-2 genome and G-C rich regions in MCMV genome (Honess, 1984). However, none of the ten E cDNAs originate from fragments HindIII D or H (Section 3.1) and therefore ,none encode the MCMV DNA polymerase.

Two cDNAs from my MCMV E library DP3 and DP5 cDNAs are of cellular origin, map to a common HindIII fragment (5.3kbp) and show nonspecific binding to

Smith DNA under normal stringent conditions (50°C). This study demonstrates the possible homology between the HSV-2 EcoR1 M fragment and a mouse DNA HindIII fragment. The HSV-2 EcoR1 M fragment may contain sequences other than the DNA pol gene and therefore, there is a possibility that the HSV-2 probe may have identified a cellular cDNA coding for a protein other than the DNA polymerase. One could determine if DP3 and DP5 are related by cross hybridization with each other. Also, DNA sequence analysis and a computer search of known sequences may identify the fuction of these cDNA clones. Finally, the DP5 cDNA probe detects transcripts that are present at approximately equal intensities during the course of the 14 hr MCMV infection, hence the cellular transcript levels of the corresponding region appears to be constant.

Recent studies have shown that the MCMV DNA pol transcripts are present during the E phase, but at a relatively low level (Elliot et al., 1990, Westcoast Herpesviruses workshop; unpublished data). These transcripts begin to accumulate as the replication cycle approaches the L phase. Therefore, the failure to isolate the MCMV DNA pol cDNA may be attributed to its absence or low level in the isolated E mRNA pool. Isolation of cDNA coding for MCMV DNA polymerase may still be possible by screening the late viral cDNAs with HindIII fragments H and D.