CHARACTERIZATION OF IMMEDIATE-EARLY AND EARLY PROTEINS OF MURINE CYTOMEGALOVIRUS SYNTHESIZED IN PERMISSIVE AND NONPERMISSIVE CELLS.

by

DOUGLAS GORDON WALKER

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Department of PATHOLOGY

The University of British Columbia
1956 Main Mall
Vancouver, Canada
V6T 1Y3

Date 2 Sept 85

ABSTRACT.

The gene products produced by murine cytomegalovirus (MCMV) in infected cells prior to viral DNA synthesis are believed to control the interaction of the virus with the cells, determining whether a permissive infection results, with virus replication, or whether further virus gene expression is inhibited, resulting in a latent or abortive infection. The aim of this study was to characterize the early viral gene products that are produced in permissive and nonpermissive cells.

The proteins produced in 3T3-L1 cells, permissively infected with MCMV, during the first six hours of infection (the period prior to viral DNA replication) were characterized by polyacrylamide gel electrophoresis. Ten of the proteins were classified as immediate-early (IE) and seven as early according to their time of synthesis and also according to their synthesis in the presence of actinomycin D following the reversal of a cycloheximide mediated block in protein synthesis. The estimated molecular weights ranged from 28K - 100K. The synthesis of a dominant IE protein of 100K was significantly increased, after the reversal of a cycloheximide block, compared to unenhanced conditions. The synthesis of two other major IE proteins of 96K and 89K were also significantly enhanced by this treatment. The 100K and 89K proteins partitioned with the nuclear, cytoplasmic and cytoskeletal fractions, while the 96K protein partitioned more strongly with the nuclei. These proteins were phosphorylated. The other IE proteins were synthesized in lesser amounts. The major early proteins, which had molecular weights of 39K and 36K, were also phosphorylated and were exclusively nucleus-associated. A number of the IE and early proteins had affinity for native and denatured DNA-cellulose.

The same major IE and early proteins were identified in nonpermissively

infected J774A.1 macrophage cells. Although 0.6% of these cells became permissively infected with MCMV and the rest appeared to be nonpermissively infected, viral DNA and late protein synthesis was not detected. The major difference between the proteins produced in 3T3-L1 cells and J774A.1 cells was the affinity of the 96K protein for denatured DNA-cellulose, which was only observed when the protein was synthesized in J774A.1 cells.

The main IE and early MCMV induced proteins were also synthesized in nonpermissively infected human fibroblast cells. The only difference between the proteins produced in these cells and 3T3-L1 cells was that the 100K IE protein appeared to have a greater nuclear-affinity, when produced in the human fibroblasts, than was found when synthesized in infected 3T3-L1 cells.

In conclusion, a larger number of IE and early MCMV-induced proteins were identified in infected cells than had been previously characterized. There was no evidence of restricted MCMV gene expression occurring in two different cell types that were nonpermissively infected. This appeared to indicate that, in the nonpermissive experiments described, MCMV replication was inhibited at the stage of viral DNA synthesis.

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INTRODUCTION

The human cytomegalovirus (HCMV) is ubiquitous in human populations. It has been shown that most people acquire HCMV antibodies during their lifetime but at varying ages according to locale (Alford et al., 1981). In developing countries, HCMV infection is usually acquired during the first 2-3 years of life, while in industrialized countries primary HCMV infection is more common between 14-35 years of age.

While the vast majority of cases of HCMV infections are subclinical, this virus can be a serious pathogen in hosts who are compromised immunologically. HCMV is now known to be responsible for a wide spectrum of diseases that can be considered in three categories; 1) congenital infections, 2) postnatal infections and 3) iatrogenic infections.

In the first 10-15 years of HCMV study, it had been widely characterized in its role as the causative agent for congenital cases of cytomegalic inclusion disease (Weller, 1971). Since 1971, the role of HCMV infection has been recognized in less severe but serious congenital complications, such as auditory defects and diminished intelligence quotients (Reynolds et al., 1974). Also it is now recognized as the primary determinant in success or failure of organ transplantation procedures (Rubin et al., 1977).

Another characteristic with primary HCMV infection has been the observed profound transient immunosuppressive effect it has on its host. In cases of HCMV mononucleosis, lymphocyte proliferative responses to T and B cells are depressed (Rinaldo et al., 1980). This is believed to be a predisposing factor for the observed increased incidence of opportunistic bacterial and fungal infections that are associated with primary HCMV

infection, particularly in transplant patients who are immunosuppressed (Chatterjee et al., 1978).

With this range of effects of HCMV on its human host, much emphasis has been placed on understanding the mechanisms of HCMV infections. In many cases, investigations have been done with the use of animal models. As the cytomegaloviruses have evolved in a species specific manner, model systems have involved the use of homologous viruses as well as animal host systems.

The mouse cytomegalovirus (MCMV) model has been used over the years by many investigators and has proven to be a good model system for many of the disease syndromes that are associated with HCMV infections (Hudson, 1979).

The aim of this thesis has been to understand some aspects of the biology and molecular biology of latent MCMV infections. In vivo and in vitro systems were established to investigate aspects of latent (or nonpermissive) infections by MCMV of animals and cultured cells. The main aspect of the work was a study of the MCMV-induced proteins that are synthesized prior to virus DNA replication and that are believed to function in controlling the outcome of virus-cell interactions.

The following literature review will deal mainly with studies of HCMV and MCMV (and other herpesviruses, where relevant) that involved the biology and significance of latency, in vivo, and the regulation of virus replication, in vitro, in particular to emphasize any similarities and differences between MCMV and HCMV.

LITERATURE REVIEW

Classification. Human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV) were classified as members of the herpes group of viruses, on the basis of similar morphology, as revealed by electron microscopy of negatively stained specimens (Wright et al., 1964). The HCMV and MCMV are grouped taxonomically as members of the family HERPESVIRIDAE, subfamily Betaherpesvirinae. HCMV is considered as type species Human (beta) herpesvirus 5 and MCMV as Murid (beta) herpesvirus 1 (Matthews, 1978).

Historical aspects. HCMV was first isolated by three different groups at around the same time. The isolation of the HCMV agent, as with most other human viruses, was not possible until human cells could be grown routinely in culture. HCMV was first isolated independently from salivary gland, kidney and urine and also grew out from explanted human adenoid tissue (Smith, 1956,; Weller et al., 1957; Rowe et al., 1956).

MCMV was first isolated in vitro two years before HCMV (Smith, 1954). Smith showed that mouse embryonic explant tissue cultures developed the characteristic intranuclear inclusions following inoculation with salivary gland homogenates from mice with intranuclear inclusions in these organs.

HCMV infections: significance of reactivation. The role of HCMV latency and reactivation as a cause of significant morbidity can be considered under two categories: Disease that is observed in a previous seropositive patient, as a result of reactivation of endogenous HCMV and disease that is produced in a seronegative patient as a result of reactivation of HCMV from transplanted cellular material (eg. from blood or organs carrying latent HCMV).

Congenital reactivated infections: Epidemiological studies, in a highly immune population, showed that congenitally infected babies (as demonstrated by virus excretion) were born to 3.4% of mothers who had been shown to have HCMV antibodies before or during the pregnancy (Stagno et al., 1977). Restriction enzyme analysis of DNA of HCMV isolates showed that most infants had been infected with the same HCMV strain as the mother (Huang et al., 1980). This indicated that an endogenously reactivated HCMV strain is able to cause intrauterine infection.

Iatrogenic reactivated infections: HCMV infections following renal transplantation occurred in 71% of patients (results of studies summarized by Ho, 1982) with 53% being primary infections and 85% being reactivated infections. The severity of reactivated infections appeared to be related to the severity of the immunosuppressive regimen used to prevent graft rejection, in particular the use of antilymphocyte or antithymocyte globulin (Pass et al., 1978; Marker et al., 1981). A summary of observations from four groups (Ho, 1982) showed that symptomatic reactivated infections occurred in 44% of cases, while symptomatic primary infections occurred in 83% of cases.

Forty-four percent of transplant patients receiving organs from antibody positive donors developed lethal, severe or moderate HCMV related diseases, while transplant patients receiving organs from antibody negative donors only developed moderate infections (Peterson et al., 1980). It is possible that HCMV infections in some antibody positive recipients are manifestations of endogenous reactivation of HCMV and in the more severe cases represent the reinfection with a different strain of virus from the transplanted kidney. Implication of the transplanted kidney as a source of virus came from a compilation of results (Ho, 1982), which showed that 57%

of seronegative recipients had primary HCMV infections when transplanted with kidneys from positive donors, while an 8% infection rate occurred in seronegative recipients receiving kidneys from seronegative donors.

A higher percentage of bone marrow transplant patients who had low levels of HCMV antibodies before transplantation developed HCMV-related lethal pneumonitis than those who were HCMV antibody negative or who had HCMV complement fixation antibody titers of more that 1:64 (Neiman et al., 1977).

A. Biology of HCMV and MCMV

1. In vitro replication cycles. The cytomegaloviruses are characterized by their slow replication cycle compared to herpes simplex virus (HSV). With HSV, infectious virus was first released from cells within 8 hours of infection and peaked at 14 hours after infection. With HCMV, virus was first detected approximately 4 days after infection with a peak release of infectious virus at approximately 5 days (Smith and DeHarven, 1973).

The replication cycle of MCMV is shorter than that of HCMV. Viral DNA synthesis can be detected by 8 hours after infection in synchronized cell cultures (Misra et al., 1978). It peaks at approximately 24-28 hours after infection and remains constant up to 36-38 hours (Moon et al., 1976). The initiation of HCMV strain Towne DNA synthesis was detected by DNA hybridization analysis by approximately 12 hours after infection (Stinski, 1978), but maximum viral DNA replication does not occur until 72-96 hours after infection.

Murine CMV replication was shown to be cell cycle dependent (Muller and Hudson, 1977a). MCMV DNA synthesis appeared to require factors associated

with host cell DNA synthesis. Viral DNA synthesis could be detected in asynchronously infected cells at 10-12 hours, but in cells infected late in the G_1 period (11 hours after subculture) virus DNA replication was detected after 8 hours (Muller and Hudson, 1977a; Misra et al., 1978). In comparison, HCMV did not require the cell to enter S phase for successful replication of the viral genome (DeMarchi and Kaplan, 1976). At 96 hours after infection, only 0.7% of fluorouracil treated cells showed evidence of cellular DNA synthesis, while 20.2% of these cells showed evidence of viral DNA synthesis. However they also showed that the physiological state of the cell affected its susceptibility to HCMV. At 48 hours after infection, less than 50% of confluent cells showed the presence of HCMV antigens, while 90% of subconfluent cells at that time were positive for HCMV antigens.

HCMV and MCMV do not code for their own thymidine kinase enzymes (Zavada et al., 1976; Muller and Hudson, 1977b). However, MCMV has been shown to multiply in TK^- 3T3 cells, while HCMV can stimulate the levels of cellular thymidine kinase (Muller and Hudson, 1977b, Estes and Huang, 1977).

2. Infection of cultured cells A number of researchers have studied HCMV and MCMV infections of different types of cells in vitro as models for the events in vivo. In vitro studies have shown a number of differences in the behaviour of HCMV compared with MCMV.

HCMV will only replicate fully in human diploid fibroblasts in vitro. The species specificity observed for this virus is almost absolute, though in one case (Waner and Weller, 1974), HCMV was reported to replicate in Vero (monkey) cells and to produce infectious, cell-associated virus. In these infected cells, herpes-like virus capsids were observed in the nuclei, 14 days after infection, but the titers of released virus did not exceed 100

infectious units/ml. In comparison, stringent species specificity of MCMV replication *in vitro* is not observed. MCMV replicated permissively in the following non-murine cells: BSC-1 (African green monkey kidney), BHK-21 (baby hamster kidney), RK-13 (rabbit kidney) lines and primary rabbit kidney and fetal sheep brain. No MCMV replication occurred after infection of HeLa (human epithelial carcinoma), WI-38 (human fibroblasts) or human embryonic brain and kidney cells (Kim and Carp, 1971).

The interaction of MCMV with human diploid fibroblasts (WI-38) was further studied (Kim and Carp, 1972). Although no infectious virus was produced in these cells, early morphological effects of infection were observed within 12 hours of infection. A rounding of the cells occurred with the formation of intranuclear inclusions. These effects were inhibited if actinomycin D was included in the medium at the time of infection. It indicated that some viral gene expression occurred in the nonpermissively infected cells. A similar cytopathic effect was seen after abortive infection of guinea pig (Fioretti *et al.*, 1973) and bovine embryonic fibroblasts (Waner and Weller, 1974) by HCMV. In both cases, HCMV specific antigens were detected in these cells by immunofluorescence.

Further investigations indicated that the HCMV genome could persist in abortively infected cells. At 28 days after infection, HCMV-induced antigens could still be detected in rabbit lung fibroblasts and infectious HCMV could be rescued if the rabbit cells were cocultivated with human fibroblasts (Farber *et al.*, 1979). Similarly, infection of mouse fibroblasts with HCMV induced virus specific antigens. These antigens could be detected as long as 2 weeks after infection. Fusion of infected mouse cells with human fibroblasts, 20, 30, 40 and 50 days after infection, lead to the reinduction of virus-specific antigens by 12 hours. If the mouse

cells were pretreated for 3 days with 5-Iodo 2'-deoxyuridine (IUdR) prior to fusion or for 3 days subsequent to fusion, infectious virus was recovered (Boldogh et al., 1977). This effect occurred even if the heterokaryons formed were grown for 40 days before IUdR treatment. It was suggested that IUdR treatment may cause repression of a cellular product in cells, which normally would interfere or inhibit virus replication (StJeor and Rapp, 1973).

In other studies, HCMV and MCMV infection of epithelial cells has been shown to follow a prolonged chronic infectious cycle. HCMV infection of BAMB epithelial cells (derived from human amnion) resulted in the formation of syncytia, with virus titers rising slowly and peaking at 25 days after infection (Vonka et al., 1976). MCMV infection of tracheal organ cultures infected mainly epithelial type cells and also resulted in a protracted replication cycle (Nedrud et al., 1982). With this system, virus was shown to be produced for up to 180 days.

A persistent form of infection resulted from HCMV infection of lymphoblastoid cells. Infection with HCMV of a line of B lymphocyte cells, that had originated from a leukemic patient, resulted in a chronic persistent infection (Furukawa, 1979). Infected cultures persistently yielded HCMV for over a year. Infectious center assays showed that 1-10% of the cells were infected. In this case, persistent infection arose as a result of a balance between release of virus and growth of uninfected cells. A similar study, using several human lymphoblastoid cell lines (of B and T cell origin) infected with HCMV, also showed that a persistent infection occurred (Tocci and St Jeor, 1979). T cell lines appeared less susceptible to infection than B cell lines. The percentage of infectious centers ranged from 0.06%-0.5%, though infectious virus was released for up

to 28 days after infection, although at very low titers. HCMV early antigens (expected in nonpermissively infected cells) were not detected in any of the infected cell lines.

3. Macrophage infections by MCMV. Studies of the role of macrophages in the persistence of MCMV in the mouse have produced some contradictory reports; some indicated the macrophage to be a permissive cell and others a non-permissive cell. Tegtmeyer and Craighead (1968) infected adult peritoneal macrophages in vitro with tissue culture adapted MCMV. Although the infectious cycle was slow, by 16 days the macrophages had produced equivalent amounts of virus to infected mouse embryo fibroblast cells.

A comparative in vivo study of the effects of early splenic viral replication of MCMV on disease pathogenesis also showed spleen macrophages to be relatively permissive to infection (Katzenstein et al., 1983). Infection of mice with virulent MCMV led to high titers of detectable virus in the spleen at 96 hours after infection. Electron microscopic studies of spleen, after administration of markers to detect phagocytic cells, showed that MCMV replication was only detected in macrophage-like cells.

In comparison, Selgrade and Osborn (1974) found that only a small percentage of macrophages from susceptible and resistant strains of mice formed infectious centers upon infection by MCMV, and only small amounts of infectious virus were released from the cells of the cultures. Similar results were obtained using virulent or attenuated MCMV.

Mims and Gould (1978) also found that the efficiency of MCMV infection of peritoneal macrophages was low. Less than 5% of the cells became infected with salivary gland passaged virus, while virus that had been attenuated by passage in tissue culture was able to infect macrophages at a

5-15 fold greater efficiency. Yields of virus were also reported to be low, whether virulent or attenuated virus had been used. Macrophages that became infected were able to prevent the spread of virus, either in an *in vitro* or *in vivo* assay, while macrophages that did not become infected were less successful in preventing spread of virus to underlying cells.

Loh and Hudson (1979) infected separated spleen cell populations with MCMV. Most of the virus was taken up by the macrophages. They formed infectious centers but were relatively restrictive to virus growth. Only a small percentage of macrophages formed infectious centers. The splenic macrophages were not all permissive for virus uptake; even with infection at 100 PFU/cell, only 7.7% of total spleen cells were positive, while macrophages constitute 10-15% of total spleen cell population.

Brautigam *et al*. (1979) compared thioglycollate-stimulated with unstimulated peritoneal macrophages for permissiveness to MCMV infections. Virus was able to replicate in stimulated macrophages from different strains of mice, forming infectious centers in 45-56% of cells. Unstimulated macrophages could form infectious centers in 12-18% of cells. *In situ* hybridization showed genomes to be present in approximately 90% of both unstimulated and stimulated cells. Thus it appears that, depending on the state of activation of the cells, macrophages may be important for disseminating virus within the infected host and also have the potential to harbor the viral genome in a latent fashion.

4. *Latent in vivo infections by HCMV and MCMV*. The establishment of latent infection in tissues of the host is a hallmark of infection with herpesviruses. In contrast to herpes simplex virus (Stevens and Cook, 1971) and Epstein Barr virus (Pattengale *et al*., 1974), the site(s) of latency of

both HCMV and MCMV remain a mystery (Jordan, 1983). In addition, the true state of the persistent infection in the host has not been elucidated. It is assumed that the CMV genome is held in a static state and the virus is stimulated to replicate by some mechanism. This has been the approach in the design of most experiments, but the fact that the infection may be a low level chronic type can not be ruled out.

Most of the evidence that HCMV can persist in humans in a latent form comes mostly from indirect evidence. Epidemiological evidence has indicated that HCMV can persist and be transmitted in the blood. It was shown that there was approximately a 7% risk of infection following transfusion of a single unit of blood (Prince et al., 1971). There is also epidemiological evidence that HCMV can be transmitted through leucocyte transfusions (Winston et al., 1980) and can be transmitted by the kidney of a HCMV seropositive donor (Naraqi et al., 1977). HCMV was shown by direct methods to be latent in fibroblasts from some patients with Charcot-Marie-Tooth disease (Williams et al., 1980a). Virus was reactivated from a fibroblast cell line from one of these patients, and two other cell lines were shown to contain HCMV DNA (Williams et al., 1980b).

Several cell and tissue types have been implicated as sites of latency of the MCMV. These include B lymphocytes, peritoneal macrophages, salivary gland, prostate, ovaries and testes.

MCMV was isolated, by cocultivation, from B lymphocyte populations from spleens of several strains of mice that had been infected (in utero or within 24 hours of birth) with tissue culture passaged (attenuated) MCMV (Olding et al., 1975). MCMV was only recovered by cocultivation of isolated B lymphocytes with fibroblasts from allogeneic strains of mice. Two factors indicated that virus was being reactivated from a latent infection.

Firstly, virus could not be recovered from sonicated lymphoid cells, and secondly, the time taken to detect virus cytopathic effects (2-5 weeks) was much longer than if free virus had been present in the spleen cells. These investigators also showed that 'activation' of the B lymphocytes was necessary for reactivation of the virus to occur. It was noted that virus did not reactivate if spleen cells were cocultivated on fibroblasts from mice of the same haplotype as the spleen cells. This observation has never been confirmed by other investigators.

B lymphocytes were also implicated by Jordan and Mar (1982) as being the cells from which virus was reactivated. Spleen cells from 33 of 34 mice, cultured using an explant technique, produced MCMV spontaneously after 12-18 days. The mice used had been infected at least 4 months previously. Once virus had been reactivated, a prolonged infectious cycle occurred in the cultures. Electron microscopic evidence showed that MCMV replicated in splenic macrophages (nucleocapsids were identified in macrophages but not lymphocytes). Separated macrophage and lymphocyte populations were cocultivated with fibroblasts and MCMV was isolated in 14/19 cases from lymphocyte cultures, but in no case was latent MCMV activated from the macrophages. In addition, after separation of lymphocytes into B and T cell enriched populations, virus was only activated from B cell enriched cultures.

A separate system for investigating MCMV latency was developed that involved infection of animals by subcutaneous inoculation. This system has been used to mimic the clinical situation with HCMV that is found in organ transplant patients (Jordan et al., 1977). After 4 months, when most of the animals were shown to be free of infectious virus, animals were immunosuppressed with antilymphocyte serum (ALS). Within 2 weeks, 45.8% developed active MCMV infections in salivary glands and 25% developed MCMV

infections in spleens. With more potent immunosuppression (ALS and cortisone), the rate of salivary gland reactivation increased to 96%. Virus was rarely isolated from salivary gland homogenates of mice given no immunosuppression (6.6%).

To correlate the findings from some of the systems for MCMV latency, several of the variable parameters were investigated (Jordan et al., 1982). Latent MCMV infections were established with virulent and attenuated strains of the virus. The effect of subcutaneous (sc) versus intraperitoneal (ip) modes of infection on the ability to reactivate virus by cocultivation and immunosuppression, as well as the strain of mice, the age at time of infection and the need to use allogeneic fibroblasts for cocultivation was examined. In most cases, MCMV could be reactivated and disseminated following ALS and cortisone treatment in all strains of mice, whether inoculated sc or ip with virulent or attenuated virus. However 46-62% of mice inoculated before 1 week of age developed long term chronic salivary gland infection. Virus was only reactivated, by cocultivation, from spleen cells of animals that had been inoculated ip and never from animals inoculated sc. After ip inoculation, virulent virus was reactivated more often than attenuated MCMV (69 vs 20% of cultures respectively). The ability to detect MCMV by cocultivation of spleen cells seemed to be related to the amount of virus replication that had occurred in the spleen during the acute stage of the infection.

In a comparative study, Olding et al. (1976) were able to recover MCMV from viable spleen cells but not from cocultivated kidney, liver, salivary gland and brain tissue. MCMV DNA was present at 4 genome equivalents per 100 cells in spleen cells from these mice and at 2 genomes per 100 cells in salivary glands. The same group (Brautigam et al., 1979) also showed that

peritoneal macrophages may harbor latent MCMV for prolonged periods after infection. Virus could be quickly reactivated if macrophages were stimulated by thioglycollate treatment, but was rarely reactivated from unstimulated macrophages.

MCMV was apparently reactivated from explants of salivary glands and prostate tissue (and cell lines derived from them) taken from mice latently infected with MCMV (Cheung and Lang, 1977). A subsequent report indicated that MCMV DNA could be detected in these organs by DNA hybridization analysis, even when virus could not be activated by growth in vitro (Cheung et al., 1980). MCMV DNA was shown to be present in testes and ovaries of 5-6 month old mice that had been infected at birth (Brautigam and Oldstone, 1980).

In summary, the results from a number of investigations indicate that CMVs can become latent in several different cell types.

B. Molecular Biology of HCMV and MCMV

A comparison of the similarities and differences in the molecular biology of HCMV and MCMV will aid in comparing the two viruses in any model experimental systems. In addition, certain experiments with HSV will be considered, where no equivalent experiments have been performed with the cytomegaloviruses.

1. Structure of DNA. Recent analyses of the genomes of HCMV and MCMV, by restriction enzyme digestion and separation by agarose gel electrophoresis, have shown them to be almost identical in size. HCMV strain AD169 has a genome of 240 kilobase pairs (kbp), corresponding to a molecular weight of

158 Megadaltons (Md)(Tamashiro et al., 1982). MCMV has a genome of 235 kbp, corresponding to a molecular weight of 155 Md (Ebeling et al., 1983). Similar results have been obtained for the other strains of HCMV used in most research laboratories (LaFemina and Hayward, 1980; Demarchi, 1981). The CMV genomes are 50% larger than those of herpes simplex virus (HSV-1,HSV-2), however the genomes of HCMV and HSV share the same structure, which is different from that of MCMV. The HCMV and HSV genomes consist of a long (L) unique region and a short (S) unique region flanked by different repeat sequences that are inverted relative to each other (LaFemina and Hayward, 1980; Sheldrick and Berthelot, 1974). As the L and S regions can invert relative to each other, four genome arrangements are possible for HSV and HCMV DNA (Locker and Frenkel, 1979; Spector et al., 1982). In comparison, the MCMV genome consists of a single long unique sequence without detectable terminal or internal repeat sequences (Ebeling et al., 1983; Mercer et al., 1983), thus only one arrangement of the genome is possible. The significance of these differences, as they relate to the replication of the viruses, is not known.

The commonly used laboratory strains of HCMV were shown by DNA reassociation kinetics to share approximately 80% genome homology (Huang et al., 1976), while HCMV (Towne) and HCMV (AD169) share approximately 90% of their nucleotide sequences (Pritchett, 1980). No detectable homology was found between HCMV DNA and HSV-1, HSV-2, Epstein-Barr virus, simian and murine cytomegalovirus DNA (Huang and Pagano, 1974).

The restriction endonuclease pattern of DNA from a virulent isolate of MCMV (K181) was shown to differ slightly from that of the Smith strain of MCMV (Misra and Hudson, 1980). Although DNA from the two strains of MCMV exhibited the same reassociation kinetics, alterations of some of the

restriction fragments indicated a possible genetic basis for the enhanced virulence of the K181 strain.

2. Virion protein composition: Analysis of purified HCMV virions have been complicated as preparations by most purification methods are contaminated with dense bodies (Stinski, 1976). In contrast, dense bodies are not seen in preparations of MCMV virions, but instead, multicapsid virions are present (Hudson et al., 1976). Analysis of virions and dense bodies of HCMV (Towne), purified on D-sorbitol gradients, identified 35 distinct proteins (Stinski, 1976), of which 9 could be identified as glycoproteins. Various other studies have estimated that 33-35 proteins are virion-associated (Fiala et al., 1976; Kim et al., 1976a; Gupta et al., 1977). Comparison of virion proteins of a number of strains of HCMV have shown them to be similar (Gupta et al., 1977)

Two studies have been done on the virion proteins of MCMV (Kim et al., 1976b; Chantler and Hudson, 1978). In both cases, the virions were purified by centrifugation through gradients of potassium tartrate. Kim et al. (1976b) found 33 proteins associated with the virion, six of which were identified as glycoproteins. Chantler and Hudson (1978) identified 29 virion proteins.

Identification of phosphorylated virion proteins in a number of CMV strains (human and simian) showed that there were four main proteins in each case, two of which were more highly labeled than the others. The two highly phosphorylated proteins had been identified as tegument proteins for Colburn strain CMV (Gibson, 1983).

3. Early viral effects on infected cells: Several non-structural proteins

of herpes simplex virus have been identified with known functions. The thymidine kinase was identified as a 44K early protein (Summers et al., 1975), the viral DNA polymerase as a 150K early protein (Powell and Purifoy, 1977) and ICP 22 as the alkaline exonuclease (Banks et al., 1983)

In comparison, for HCMV, only the virus-induced DNA polymerase has been identified (Huang, 1975). However, a number of studies have provided information about effects of various virus-induced early functions on the infected cell. Stimulation of host cell DNA synthesis occurs after infection, but variable results have been obtained as to whether this function is active in u.v. inactivated virus. StJeor et al. (1974) found no stimulation occurred using u.v. inactivated HCMV. In comparison, Furukawa et al (1975), Boldogh et al. (1978) and DeMarchi and Kaplan (1977b) showed that u.v. inactivated HCMV could still stimulate cellular DNA synthesis and in the latter case, stimulation was greater with the use of u.v. irradiated virus. These results would seem to indicate that the stimulation was caused by a virion component. A protein induced by MCMV early in the replication cycle was shown to cause the inhibition of cellular DNA synthesis by 12 hours after infection (Moon et al., 1976).

Tanaka et al. (1975) showed that stimulation of host cell RNA synthesis in HCMV infected cells was dependent on a protein synthesised early in the infectious cycle. Further characterization of this, showed that endogenous RNA polymerase activity was enhanced 3-fold at 48 hours after infection in cells infected with HCMV (Tanaka et al., 1978). Enhancement was abolished if infected cells were treated during the first 6 hours with actinomycin D or cycloheximide. This indicated that a HCMV immediate-early or early function was responsible for the stimulation. In addition, this group (Kamata et al., 1978; 1979) characterized two HCMV-induced chromatin factors,

that are responsible for changes in chromatin template transcription activity and changes in the structure of infected cell chromatin, as HCMV early gene products. These factors were separated from each other on DNA-cellulose columns. Chromatin factor (1) was responsible for a rapid increase in chromatin template activity, while chromatin factor (2) was responsible for the chromatin conformation change. Synthesis of both factors, whose appearance correlated with the appearance of immediate-early nuclear antigens, was sensitive to u.v. inactivation of virus or treatment of cells with cycloheximide (Kamata et al., 1979). The role that these factors have in virus replication is not known.

HCMV early functions have also been shown to cause suppression of fibronectin synthesis in cells (Ihara et al., 1982). This seems to be a specific effect as most macromolecular synthesis is enhanced during the early stages of HCMV infection. This effect occurred if cells were infected with DNA replication deficient temperature sensitive mutants (at the non-permissive temperature) but not with u.v.-irradiated virus. This feature may be responsible for the early cytopathic effect seen in infected cells.

4. Herpesvirus gene expression in permissive infections.

The concept of coordinated regulation of three phases of gene expression for herpesviruses was originated by Honess and Roizman (1974) working with HSV-1. The first viral gene products synthesized in infected cells are termed immediate-early proteins. These are defined operationally as those encoded by genes which are transcribed and translated in the absence of prior viral protein synthesis. The transcription of early genes requires de-novo viral protein synthesis and occurs prior to viral DNA

synthesis. Late genes are transcribed subsequent to viral DNA synthesis. This coordinated regulation of viral gene expression has been shown to occur in a number of herpesviruses; HSV (Clements et al., 1977; Honess and Roizman, 1974) HCMV (Stinski, 1978; Wathen and Stinski, 1982), pseudorabies virus (Feldman et al., 1979) and infectious bovine rhinotracheitis virus (Misra et al., 1981).

a) HCMV transcription: A number of studies of HCMV transcription involved the immediate-early (IE) genes. An understanding of how IE genes regulate subsequent viral gene expression is necessary for understanding the underlying mechanisms of latency and reactivation.

Differences are noticed in the location and orientation of IE class RNAs on the HSV genome as compared to HCMV (Wathen and Stinski, 1982). Four of the six HSV IE transcripts originate from the long and short repeat sequences and adjacent sequences (Clements et al., 1977), whereas for HCMV (Towne), the transcripts originate mainly from the long unique region. 88% of whole cell RNA hybridized to the region of 0.660-0.770 map units, while only 6% hybridized to the long repeat sequences (Wathen et al., 1981).

A characteristic of IE transcription of HCMV (observed in all strains studied) is the presence of a predominant IE transcription site. The strong IE transcription site of HCMV (AD169) at 0.061-0.110 map units (McDonough and Spector, 1983) is analogous to the IE transcription region of HCMV (Towne) at 0.660-0.770 map units (Wathen and Stinski, 1982) and to the region of HCMV (Davis) at 0.686-0.733 map units (De Marchi, 1981).

Analysis of the transcripts of different strains of HCMV produced at IE times showed similar features. Three predominant poly A^+ transcripts of HCMV (AD169) (2.3, 2.2 and 1.9 kb) were detected that hybridized to the area

stated, with the 1.9kb transcript present in most abundance (Jahn et al., 1984). These appear analogous to the 1.95 and 2.25 kb IE transcripts of HCMV (Towne)(Stinski et al., 1983).

After a 2 hr period of de-novo protein synthesis in HCMV-infected cells, a switch to early transcription occurred with transcripts originating from more extensive regions of the genome. Wathen and Stinski (1982) found that a large amount of the whole cell RNA extracted at early times after infection, that hybridized to areas of the long unique section (Xbal B 0.324-0.460 map units), was preferentially retained in the nucleus.

In contrast, the most abundant polysome-associated RNA contained five sizes of RNA that hybridized to the long terminal and internal repeat sequences of the genome. After the initiation of viral DNA synthesis, the most abundant polysome-associated RNA size classes originated from the long repeat sequences (Wathen and Stinski, 1982).

A certain level of post-transcriptional control is exerted by HCMV on gene expression. DeMarchi (1983a) showed that post-transcriptional control in HCMV-infected cells existed at the level of transcript accumulation, retention of transcripts in the nucleus, preferential association of transcripts with polysomes and stability of RNAs.

b) MCMV transcription. Analysis of MCMV transcription has indicated that it is under the same temporal, abundance and post-transcriptional controls as other herpesviruses. Twenty-five percent of the MCMV DNA was transcribed prior to viral DNA replication, as shown by reassociation kinetic analysis with whole genome DNA probes, and 38% of the genome subsequent to DNA replication (Misra et al., 1978). In both cases, slightly less than half of the total transcripts were transported to the cytoplasm.

Recent transcription analyses have used cloned subgenomic pieces of MCMV DNA. Similar to that found with all strains of HCMV, transcripts found at IE times originated from a narrow portion of the genome (0.770-0.816 map units)(Marks et al., 1983; Keil et al., 1984). This location is in close proximity to the consensus site for IE transcription of other strains of CMV. The switch to early transcription resulted in the detection of RNA corresponding to all areas of the genome except 0.278-0.305 map units. The most abundant transcription occurred in two small areas of the genome, 0.824-0.861 map units and the two terminal pieces of the genome that are detected fused during DNA replication (Marks and Spector, 1984). Sequential regulation of transcription can be observed in that RNA, that originated from the area of most abundant IE transcription (0.770-0.816 map units), has decreased 10-fold at early times. Similarly, RNA from the abundant transcription site at early times was reduced 2-5 fold at the late time period. MCMV transcription was also studied by Northern blot analysis (Keil et al., 1984). Six poly A^+ IE transcripts (5.1, 2.75, 2.0, 1.75, 1.65 and 1.05 kb) were detected. Similar to HCMV (Stinski et al., 1983), one MCMV IE transcript (2.75 kb) was present in greater abundance than the others. Low amounts of IE transcription were also detectable from the end fragments of the genome, similar to that seen for HCMV (Wathen and Stinski, 1982).

Changes in transcription patterns were studied by analysis of transcripts at various times after infection and were related to changes in proteins synthesized (Keil et al., 1984). In vitro translation of RNA, isolated at IE times, gave three proteins. New species of RNA were detected at 2 hours after infection and these correlated with the appearance of three new virus specific proteins. No further new transcripts were detected until

16 hours after infection. This corresponded to the onset of viral DNA replication in this system. The appearance of new transcripts correlated with the synthesis of new species of proteins.

c) Virus protein synthesis in permissive infections. Most understanding of herpesvirus protein synthesis regulation has come from experiments with herpes simplex virus. The roles of viral proteins synthesized early in the infectious cycle have been elucidated through the use of a number of HSV mutants with temperature sensitive (ts) lesions in various genes. Similar experiments have not been performed with HCMV or MCMV due to the unavailability of such mutants. Some of the findings with HSV can be applied to the CMVs, but any comparisons have to be interpreted with caution.

i) Herpes simplex virus: Honess and Roizman (1974) identified three classes of HSV-1 proteins produced at various times in HSV-1 infected cells. The nomenclature of immediate-early, early and late will be used here to refer to the classes of HSV proteins (instead of α, β and γ) to conform to the nomenclature most commonly used to describe CMV proteins. A high level of IE protein synthesis was seen after the removal of a protein synthesis inhibitor, but the synthesis of a further class of proteins (early) was inhibited if the transcription inhibitor actinomycin D was added to the cultures at this stage. If early protein synthesis was allowed to occur, the levels of IE protein synthesis declined. This indicated a negative regulatory role of the early protein(s) on IE mRNA translation or RNA stability (Honess and Roizman, 1974). The regulatory role of the early proteins was also apparent as prolonged synthesis of IE proteins occurred if early protein synthesis was inhibited. The presence of early proteins was

necessary for late protein synthesis to occur. Honess and Roizman (1974) found that late protein synthesis could still occur in the presence of viral DNA synthesis inhibitors, even though at reduced amounts.

The transition from IE to early gene expression required functioning IE proteins. Addition of the amino acid analogues canavanine or azetadine to medium of HSV-1 infected cells resulted in prolonged synthesis of IE proteins, with only a limited amount of early protein synthesis (Honess and Roizman, 1975). Also, a functional early gene product was required to shut off the synthesis of IE proteins. Similarly, the cessation of early protein synthesis required the presence of functional late proteins (Honess and Roizman, 1975). The IE proteins produced in the presence of the analogues still underwent normal patterns of post-translational modifications and were also able to cause inhibition of host cell protein synthesis.

The role of the IE protein of molecular weight of 175,000 (designated ICP 4 or VP 175, Honess and Roizman, 1974; Courtney and Benyesh-Melnick, 1974) in the regulation of early transcription was identified by the use of a class of ts mutants. Preston (1979a) showed that, at the non-permissive temperature (38.5°C), the ts mutant tsK failed to produce the early class mRNA that codes for the thymidine kinase enzyme. A further study identified ICP 4 as the protein that was affected by this temperature sensitive lesion (Preston, 1979b). Two properties of ICP 4 synthesized at 38.5°C were abnormal. Analysis of the intracellular distribution of the mutant ICP 4 showed that most was found in the cytoplasm of the cell compared to a normal nuclear location. In addition, the mutant protein was not processed normally into the higher molecular weight forms found in the nuclei of wild type HSV-infected cells. Shift from 38.5 to 31°C resulted in this protein being processed and transported normally. Analysis of two other IE proteins

of the tsK mutant showed that they behaved in the same manner at 31°C as at 38.5°C.

Various studies have been made on properties of ICP 4 as well as the four other HSV IE proteins. ICP 4 has been shown to exist in 3 or 4 forms. The molecular weight increases after synthesis from 169,000 to 177,000 (Bookout and Levy, 1980). Pulse-chase analysis of this protein showed that the lower molecular weight form was more abundant in the cytoplasm of infected cells and the higher molecular weight forms appeared in the nuclei after a chase period. All forms of this protein labeled with ^{32}P but labeling was more intense in proteins localized in the nuclei (Pereira et al., 1977). Two studies analyzed DNA-binding properties of HSV IE proteins. ICP 4, 6, 0 and 27 had affinity for double-stranded DNA in vitro (Hay and Hay, 1980). The same result was obtained with columns of single-stranded DNA-cellulose (Bookout and Levy, 1980). ICP 0, 22 and 27 were found to partition more strongly with nuclei, while ICP 6 partitioned more strongly with the cytoplasm (Hay and Hay, 1980). However, some workers consider ICP 6 to be an early protein (Wilcox et al., 1980).

A detailed study was made of the effects of phosphorylation of HSV-1 and HSV-2 proteins on their cellular localization and affinity for double-stranded DNA (Wilcox et al., 1980). It was found that the bound phosphate of many proteins cycled on and off at subsequent periods after synthesis. The phosphate cycled on and off the lower and higher molecular weight forms of ICP 4 (termed ICP 4a and 4c), while it remained stably attached to ICP 4b, the intermediate molecular weight form. It was noticed that ICP 4a and 4c could be phosphorylated as late as 24 hours after infection, which indicated that this protein has a function throughout the infectious cycle. The site of localization of proteins was not affected by

their phosphorylation, while it had a varying effect on their affinity for double-stranded DNA. Of the seven virus-specific proteins studied (ICP 5, 6, 11, 19, 20 and 29) (ICP 4 was not solubilized by the extraction procedure used), DNA-binding properties were observed for them whether they were isolated from nuclear or cytoplasmic fractions of infected cells. Phosphorylated forms of ICP 6, 11 and 20 were preferentially found in non-bound fractions of material applied to a DNA-cellulose column, while phosphorylated forms of ICP 19 and 29 were preferentially bound (Wilcox et al., 1980).

Characterization of ICP 4, 0 and 27 by monoclonal antibodies and two-dimensional gel electrophoresis showed that multiple forms of each of these proteins exist (Ackermann et al., 1984). ICP 4, ICP 0 and 27 formed a series of five spots that differed in charge rather than molecular weight. All three proteins were poorly soluble in two-dimensional lysis buffer and ICP 4 precipitated completely when run from acid to base on an electrofocusing gel.

Another well characterized HSV regulatory protein is the major DNA-binding protein, designated ICP 8 for HSV-1 (Knipe et al., 1982). This protein is a member of the early class of virus-specific proteins and has a multifunctional role in regulating virus DNA synthesis and gene expression. This protein binds to single-stranded DNA more tightly than double-stranded DNA (Purifoy and Powell, 1976) and mutants defective for ICP 8 can not replicate viral DNA (Powell et al., 1981). Precipitation of ICP 8 from sonicated nuclear preparations (Lee and Knipe, 1983) or from isolated deoxyribonucleoprotein complexes (Leinbach and Casto, 1983), showed that the protein was associated with virus DNA during permissive infections. Evidence indicated that ICP 8 bound to the complete viral genome and not to

specific sequences.

ICP 8 also has a role in regulating viral gene expression that appears to be independent of its viral DNA replication function (Godowski and Knipe, 1983). Higher levels of ICP 4 (IE), ICP 8 (early) and ICP 5 (late) were present in cells infected with an ICP 8 ts mutant, at the non-permissive temperature, than in cells infected with wild type HSV-1 and treated with PAA. The defective ICP 8 protein was not able to regulate gene expression. In particular, the ICP 8 mutants did not control late gene expression even though viral DNA replication was blocked. This would seem to indicate that these two events were under independent levels of control. The proposed mechanism of action of this protein was through binding to viral DNA. In cells infected with ICP 8 ts mutants, release of this binding could lead to simultaneous expression of several classes of genes (Godowski and Knipe, 1983).

ii) Human cytomegalovirus: Analysis of the sequence of protein synthesis of HCMV (Towne) showed that the same classes could be identified as were found for HSV-1 (Stinski, 1978). Within six hours of infection, 3 IE and 7 early proteins were identified. Subsequent work, that utilized in vitro translation and immunoprecipitation techniques, added some other proteins to the two groups (Wathen et al., 1981; Stinski et al., 1983). The major IE protein of HCMV (Towne) in infected cells is a protein of 72K, which is synthesized in vitro from hybrid-selected RNA as a 75K protein. It can be detected in vivo as a broad band of 75K to 68K but mainly of 72K. This indicated that some post-translational modification occurred (Stinski et al., 1983). It appeared that most of the early proteins observed in the earlier paper (Stinski, 1978) may be of the IE class as they were synthesized by in vitro translation of hybrid-selected IE RNA. Also, with

the exception of the early protein of 27K, all proteins synthesized *in vitro* from early RNA (Wathen *et al.*, 1981) were noticeably different in mobility from those previously classified as being early class.

In confirmation of the results of Honess and Roizman (1975) with HSV, it was shown that functional early proteins were required for viral DNA synthesis. The addition of canavanine and azetadine to the medium early in the infectious cycle suppressed the levels of viral DNA synthesis (Stinski, 1978).

Michelson *et al.* (1979) identified two major IE proteins (of 76K and 82K) in HCMV-infected cells by immunoprecipitation. The appearance of these proteins correlated with appearance of nuclear antigens in infected cells. The 76K protein was found in nuclear and cytoplasmic fractions of partitioned cells, while the 82K protein was only found in nuclear fractions.

Twenty HCMV (AD169)-induced IE and early proteins were identified during the first 6 hours of infection by immunoprecipitation (Blanton and Tevethia, 1981). Four of these bands were identified as IE proteins by their synthesis in the presence of actinomycin D, following the reversal of a cycloheximide block.

Detailed analyses of the major IE proteins of several strains of CMVs have been made (Jeang and Gibson, 1980; Gibson, 1981). The major protein synthesized following cycloheximide treatment of CMV (Colburn)-infected cells was one of 94K. Two-dimensional analysis showed it to have a distinctly acidic net charge. The level of this protein was considerably elevated by the cycloheximide pretreatment, but the same protein was detected in 'unenhanced' infected cells. Under enhanced conditions of synthesis, this protein was found to partition about equally between nuclear and cytoplasmic fractions of NP40 solubilized cells. Under different

experimental conditions, Tween40/deoxycholate treatment of NP40 released nuclei removed all the IE 94K protein from the nuclei. This was taken to indicate that nucleus-associated protein was in fact associated with the cytoskeletal framework. Pulse/chase studies indicated this protein to be relatively stable in infected cells.

The dominant IE proteins of HCMV strains AD169, Davis and Towne had molecular weights of 79K, 78K and 76K respectively and were distinctly different from the IE 94K of CMV (Colburn). The main IE proteins of AD169 and Towne migrated as broad heterogeneous bands on the gel while those of strains Davis and Colburn appeared as narrow bands. Translation of mRNA in vitro indicated that differences in size of the proteins were due to inherent genetic differences rather than differences in post-translational modification. Phosphorylation was observed in the 76K IE protein of HCMV (Towne) and 94K IE protein of CMV (Colburn). Other IE proteins were observed by Gibson (1981) in HCMV-infected cells. Minor bands of 57K, 53K and 38K were observed in HCMV (Towne)-infected cells.

While studying a late CMV (Colburn) protein with DNA-binding properties, Gibson et al. (1981) observed that the IE 94K protein did not have this property. This is in comparison to initial reports that HCMV strains Towne and AD169 IE proteins had DNA-binding properties (Stinski et al., 1981; Gergely et al., 1980; Musiani et al., 1979).

iii) Murine cytomegalovirus: Studies of protein synthesis of MCMV indicated a clear delineation existed between early and late protein synthesis (Chantler and Hudson, 1978). Three early proteins were detected by 4 hours after infection. This was followed by a lag period until after initiation of viral DNA synthesis, when structural proteins were synthesized. The identification of only three early proteins was confirmed as an

underestimation. In a further study, a total of 8 virus-induced proteins were detected during the first three hours of infection with the use of hypertonic labeling conditions (Chantler, 1978). However, due to the design of the experiments, it was not possible to delineate these proteins into IE and early classes. A similar study identified 11 MCMV-induced proteins during the first 4 hours of infection, of which six were present in relative abundance (Moon et al., 1979). The three major MCMV IE proteins were recently characterized (Keil et al., 1985). The three proteins (89K, 84K and 76K) were precipitated by the same monoclonal antibody. The 84K and 76K proteins represented post-translational processed forms of the 89K protein. Hybrid selection of IE RNA followed by in vitro translation produced only the 89K protein.

5. Herpesvirus gene expression in nonpermissive infections.

Several nonpermissive culture systems for HSV have been investigated but the controlling factors seem to be specific for each case. For example, HSV-1 infection of XC cells resulted in an abortive infection (Jacquemont et al., 1984). A small amount of HSV gene expression occurred with the production of some proteins of all three classes, but viral DNA synthesis was not detected (Epstein and Jacquemont, 1983). The translation of the RNA for the IE protein ICP 22 did not happen even though the RNA for ICP 22 was detected in the nuclei and cytoplasm of infected cells. The other IE proteins were detected in infected XC cells.

Nonpermissive infections by HCMV of cultured cells of non human origin have all been characterized by a restriction of early viral gene expression, absence of viral DNA synthesis and absence of late protein synthesis.

Infection of guinea pig fibroblasts with HCMV (Towne) resulted in the

synthesis of the same IE proteins as permissively infected cells except that two of the early designated proteins were absent (59K and 21K) (Stinski, 1978). HCMV structural proteins were not detected. A higher degree of restriction of early gene expression occurred following CMV infection of Balb/c-3T3 cells or Rat-1 cells (Jeang et al., 1982). CMV infection of these cells did not produce a cytopathic effect, in contrast to HCMV infection of guinea pig cells (Fioretti et al., 1973). CMV (Colburn) infection of Rat-1 cells did not result in viral DNA replication and only a 94K IE protein was synthesized. This protein was phosphorylated, similar to that produced in permissively infected cells (Jeang and Gibson, 1980).

Further investigation of this system (HCMV strain Towne infection of Balb/c-3T3 cells) confirmed that virus gene expression was restricted to a major IE protein (in this case IE 68K) and no viral DNA synthesis occurred (LaFemina and Hayward, 1983). In addition, the viral DNA in the infected Balb/c-3T3 cells did not appear to form the circular or concatomeric replicative forms that are seen in permissively infected cells.

The effect of nonpermissive infection of rabbit kidney (RK) cells, by HCMV (Davis), on host cell DNA synthesis was compared to infection of permissive human (HEL) cells (DeMarchi, 1983b). Infection of both cells by HCMV resulted in a stimulation of host cell DNA synthesis, but this had previously been categorized as the result of an abortive infection (DeMarchi and Kaplan, 1977b). In HEL cells, this effect was seen in non-productively infected cells when virus antigen expression did not occur. In RK cells, which were all nonpermissively infected, host cell DNA stimulation and virus antigens were detected in the same cells. Permissive infection of HEL cells, with antigen expression, resulted in an inhibition of host cell DNA synthesis even when the infected cells were treated with phosphonoacetic acid

This 'early' function was thus not expressed in infected RK cells. The virus-induced DNA polymerase was also not expressed in RK cells.

Analysis of the transcription of early HCMV genes in infected RK cells showed that some had been restricted in their expression (DeMarchi, 1983c). Transcripts present in infected HEL cells that hybridized to three EcoR1 areas of the genome were not detected in infected RK cells. In addition, two other transcripts were underrepresented on the polysomes from infected RK cells compared to those from HEL cells.

Rice et al. (1984) reported that HCMV infections of human peripheral blood mononuclear cells were nonpermissive. This is the only reported investigation of a nonpermissive infection where the reactivity of the antibody used was restricted to a single class of proteins. The cells showed positive fluorescence with a monoclonal antibody against the major HCMV IE protein (72K) but no reactivity was detected with a monoclonal antibody against a late protein. The highest percentage of cells showing evidence of infection were monocytes (10%), although B and T lymphocytes and NK cells were also infected. However, it still can not be determined from these results at which stage a block to gene expression happened, as it was not determined if there was synthesis of any of the early proteins. This was the first in vitro system that might be closely modelling the events that occur after nonpermissive HCMV infections in vivo.

6. Persistent infections of HCMV and MCMV: In vitro models.

The molecular events that lead to latency of CMVs in cells are poorly understood because of the lack of cell culture systems that can be used to mimic the events in vivo. Several have been developed to investigate certain aspects of latent infections.

A continuous cell line of human fibroblasts, derived by Mocarski and Stinski (1979), that was persistently infected with HCMV, contained a mixture of cells that were permissively and nonpermissively infected with HCMV. Separation of the two types revealed that nonpermissive cells harbored HCMV genomes and expressed early antigens only. Analysis of the proteins suggested that there was a restriction in the number of early proteins. Prolonged treatment of the cells with HCMV antiserum resulted in elimination of infectious virus, repression of early antigens and also, the cells resumed a normal fibroblastic shape. However, even though gene expression was completely inhibited, the HCMV genome persisted at the level of 45 copies/cell. Removal of antiserum after a further 3 week period resulted in the immediate reappearance of virus.

The replication of MCMV was inhibited when infection was carried out in non-replicating, serum starved, G_0 phase 3T3 cells (Muller et al., 1978). In cells maintained under these conditions, no viral DNA replication occurred and the amount of transcription at early and late times was reduced. A comparison of protein synthesis in G_0 cells with that found in productively infected cells, showed that 5 of 6 early proteins were synthesized in G_0 cells but no structural proteins were detected. The virus remained in a viable state while the cells were being held in the G_0 phase, since the numbers of infectious centers remained constant. Viral DNA synthesis occurred and virus could be 'reactivated' if the cells were exposed to serum and fresh medium.

Two other systems have involved the establishment of latency of MCMV and HCMV in undifferentiated embryonal carcinoma cells (Dutko and Oldstone, 1981: Gonczol et al., 1984). MCMV could not replicate in undifferentiated cells. There was no significant amount of infectious virus replication and

no MCMV-specific mRNA was produced. However, the viral DNA content remained constant and there was restricted amounts of nuclear RNA production. Upon differentiation of the cells, viral DNA concentration increased and infectious virus was produced. In a similar system, MCMV transcripts produced in undifferentiated cells were shown to come from the IE region of the genome (Fibi et al., 1984). Preliminary studies also showed that HCMV did not replicate in undifferentiated human embryonal carcinoma cells, and no viral antigens were expressed. However, cells that were treated with retinoic acid to induce differentiation became permissive for virus production (Gonczol et al., 1984).

Another potential mechanism of the latency of HCMV was demonstrated in vitro using interferon treated cells (Rodriguez et al., 1983). Cells pretreated with interferon, infected at a low multiplicity of infection with HCMV and maintained in the presence of interferon over 16 days, did not produce infectious virus. Removal of interferon resulted in growth of HCMV in the cultures. Thus interferon treatment inhibited the replication of HCMV, but the genome was able to remain in a latent state with reactivation occurring once interferon was removed.

SECTION 1

CHARACTERIZATION OF IMMEDIATE-EARLY AND EARLY PROTEINS OF MURINE CYTOMEGALOVIRUS SYNTHESIZED IN PERMISSIVE AND NONPERMISSIVE CELLS.

INTRODUCTION.

General consensus has been that an understanding of how immediate-early and early gene products regulate subsequent viral gene expression is necessary in order to understand the underlying mechanisms of viral latency (Stenberg et al., 1984).

Nonpermissive interactions of CMVs with cells, mainly of a different species to the virus, have all been characterized by some restriction of immediate-early or early gene expression (discussed in literature review). The degree of restriction has varied between different cell types. This indicated that CMV replication can be controlled at a number of stages during the early phase of the replication cycle.

The aim of the work described in this section was to analyse the replication and 'early' protein synthesis of MCMV in nonpermissively infected cells, in order to identify any restriction in early gene expression, with the aim of identifying and characterizing the important regulatory protein(s). This also necessitated the identification and characterization of proteins induced during the immediate-early (IE) and early stages of permissive MCMV infections. Previous published work (Chantler and Hudson, 1978; Chantler, 1978; Moon et al., 1979) had not fully defined the 'early' proteins identified into classes and, as 17-20 IE and early proteins have been identified for two strains of HCMV (Wathen et

al., 1981; Blanton and Tevethia, 1981), it seemed that previous estimations may have underrepresented the true numbers of MCMV 'early' proteins.

In addition, it was considered that the results would be of greater relevance to in vivo latency if nonpermissive infections were studied in murine cells and in a cell type that has been implicated as a possible site of latency in the mouse. A murine macrophage cell line (J774A.1) was used in most experiments and the results were also compared with those from nonpermissively infected human fibroblast cells.

The experiments described can be considered in two parts. Firstly, the parameters describing nonpermissive infections (yields of infectious virus, infectious centers, viral DNA synthesis and the presence of structural proteins) were investigated to determine their similarities to previous observations with HCMV. Secondly, the MCMV-induced proteins synthesized during the first six hours of infection, in permissive and nonpermissive cells were identified, using different forms of gel electrophoresis. In addition, properties of these proteins were characterized in terms of their locations within infected cells, affinity for double and single stranded-DNA cellulose and their phosphorylation characteristics.

MATERIALS AND METHODS.

Virus. The Smith strain of murine cytomegalovirus (MCMV) used in this study was originally obtained from the American Type Culture Collection, Rockville, Md. Stocks of virus were prepared by low multiplicity passage (0.01 plaque forming unit (PFU)/cell) in roller bottle cultures of 3T3-L1 cells. High titered stocks of MCMV were prepared by concentrating virus from the tissue culture media by centrifugation at 11,000 r.p.m for 3.5 hr (GSA rotor, Sorvall centrifuge).

Cells. 3T3-L1 cells (ATCC CCL 92.1), a continuous line of 3T3 (Swiss albino) mouse embryo fibroblast, J774A.1 cells (ATCC TIB 67), a transformed macrophage type cell line derived from Balb/c mice and P388D.1 cells (ATCC TIB 63), a transformed macrophage type cell line derived from DBA/2 mice, were obtained from the American Type Culture Collection. Human fibroblasts were propagated from collagenase dissociated human foreskins and used between passages 5 and 15. NS-1 cells, a Balb/c derived B cell myeloma line, were obtained from Dr. J.K. Chantler, University of British Columbia. All cells (except P388D.1) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% (vol/vol) fetal bovine serum (FBS, GIBCO), 0.37% sodium bicarbonate and gentamicin sulfate (50 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). P388D.1 cells were grown in RPMI 1640 medium (GIBCO) with 10% (vol/vol) FBS. Cells were grown in plastic petri dishes (Nunc) in a humidified 5% CO_2 atmosphere at 37°C.

Assays of infectivity of MCMV in 3T3-L1, J774A.1 and human fibroblast cells. Confluent cultures of 3T3-L1, J774A.1 and human foreskin fibroblast (HFF) cells were infected, in sets of 6 cultures, with MCMV at a multiplicity of infection (m.o.i.) of 1 PFU/cell. Virus, in 1ml of DMEM with 5% FBS, was added to the cells for 1 hr at 37°C. This was removed, cells were rinsed once and refed with fresh maintenance medium (DMEM with 5% FBS). Maintenance medium was changed at 48 and 96 hr after infection. Cells were harvested, at 4, 24, 48, 72, 96 and 120 hr after infection, by scraping into the medium and both were collected and stored at -70°C until assayed.

Virus was titrated for PFU on monolayers of 3T3-L1 cells in 35 mm petri dishes. Cells and supernatant mixtures were frozen and thawed twice and then briefly sonicated. Each sample was serially diluted in maintenance medium and 1 ml of each dilution was added to a petri dish. Absorption was carried out at 37°C for 30 min. The inoculum was then decanted and 2 ml of overlay medium (DMEM containing 5% FBS and 0.5% agarose (Seakem, Marine Colloids, Rockland, Me.)) was added to each dish. Plaques were counted, without staining, after 4-7 days incubation at 37°C. Results were expressed as total PFU/10^6 cells.

Infectious center assays. Confluent cultures of J774A.1 and human fibroblast cells were infected with MCMV at a m.o.i. of 1, as described in the previous section. At 24, 48, 72 and 120 hr after infection, cells were harvested intact, by trypsinization of human fibroblasts, or by gentle scraping of J774A.1 cells. They were pelleted, washed once and counted. Their numbers were adjusted by dilution with media to 10^5 cells/ml and 10^4 cells/ml respectively and 2 ml of each dilution were added to

confluent cultures of 3T3-L1 cells. Each estimation was performed in duplicate. Petri dishes were incubated 2-4 hr at 37°C to allow cells to adhere, then the medium was decanted gently and replaced by an overlay of DMEM with 5% FBS and 0.5% agarose. The cells were incubated at 37°C until plaques could be counted (4-7 days).

Purification of MCMV DNA. MCMV DNA used in hybridization experiments was prepared from extracellular virions that had been partially purified from cell-free culture supernatant. Medium was centrifuged at 5000 r.p.m. in a Sorvall centrifuge (GSA rotor) for 10 min, to remove cellular debris, and virions were pelleted (11,000 r.p.m. for 3.5 hr using a GSA rotor). The pellet was resuspended in a small volume of PBS with 2% FBS and centrifuged at 18,000 r.p.m. for 90 min using the SS34 Sorvall rotor.

The pellet was suspended in 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, to which proteinase K (Bethesda Research Laboratories (B.R.L.), Gaithersburg, Md.) was added to 100 μg/ml and sodium dodecyl sulfate (SDS) to 1%. The mixture was digested at 65°C for 2 hr and then overnight at 37°C. Cesium chloride (optical grade: B.R.L.) and water was added to the solution to bring the volume to 11.5 ml and density to 1.71 g/ml of CsCl and this was spun in the Ti75 rotor (Beckman Instruments, Inc., Fullerton, Calif.)(35,000 r.p.m. for 65 hr at 20°C). Fractions were collected dropwise from the bottom of the spun gradients and fractions of 1.718 g/cc CsCl (containing viral DNA) were identified by refractometry. The DNA was dialysed against 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE, pH 8.0), precipitated with 2 vol of 95% ethanol, recovered by centrifugation and redissolved in sterile TE (pH 8.0). Concentration and purity was estimated by measuring absorbance at 260 and 280 nm.

Assay of viral DNA synthesis. Viral DNA synthesis was measured by dot hybridization. Cultures of 3T3-L1, J774A.1 and human fibroblast cells (5 x 10^5 cells) were mock and MCMV-infected in duplicates at a m.o.i. of 10. Cells were harvested at 2 hr pi. and 24 hr pi. and stored at -70°C as pellets. Cells from each petri dish were resuspended in 20 µl H_2O and half was spotted onto each of two strips of nitrocellulose (Schleicher and Schuell, Inc., Keene, N.H.) (each estimation performed in quadruplicate). Prior to use, nitrocellulose strips had been soaked in 10X standard saline citrate (SSC) solution (1X SSC is 150 mM NaCl, 15 mM sodium citrate, pH6.8) and rinsed in distilled water. Nitrocellulose strips were treated as described by Brandsma and Miller (1980), with modifications. The strips were soaked consecutively in 0.5 M NaOH for 7 min, twice in 0.6 M NaCl, 1 M Tris-HCl (pH 6.8) for 1 min and once in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.4) for 5 min. These operations were carried out by placing strips (cell side up) on pieces of 3MM filter paper (Whatman Ltd., England), that had been saturated with the appropriate solutions. Filters were then air dried and baked at 80°C for 2-4 hr.

Hybridization reactions were carried out in made to measure boilable bags (Sealobags, Sears). Prehybridization was performed in 50% formamide, 5X Denhardt's reagent (1X Denhardt's reagent is 0.02% w/v each of bovine serum albumin (Sigma), polyvinylpyrrolidone (Sigma) and Ficoll [M_w 400,000] (Pharmacia Fine Chemicals AB, Sweden), 5X SSC, 50 mM sodium phosphate (pH 6.8) and 0.1% SDS, with 250 µg/ml sonicated denatured salmon sperm DNA (Sigma), for 4 hr at 42°C. The same buffer was used for hybridization except 1X Denhardt's and 100 µg/ml salmon sperm DNA were used as substitutions. Hybridization was carried out with 10^6 c.p.m. of whole genome MCMV DNA for 18 hr. Viral DNA was nick translated with

(α^{-32}P) dATP (New England Nuclear Corp., Boston, Mass.) using a B.R.L. nick translation kit, according to the manufacturer's instructions. Strips were washed twice in 2X SSC/0.1% SDS (3 min at room temp.), twice in 0.2X SSC/0.1% SDS (3 min at room temp.), twice in 0.16X SSC/0.1% SDS (15 min at 50°C), and rinsed in 2X SSC and then exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, N.Y.). Each spot was then cut out and counted by liquid scintillation counting (LSC) using a Searle Isocap 300 machine.

Infection and labeling of cells. Confluent cultures of 3T3-L1 or J774A.1 cells were subcultured into 60 mm petri dishes, 16-20 hr before infection. Subconfluent cultures of cells were infected at a m.o.i. of 25 PFU/cell, in 2 ml of DMEM with 5% FBS, at 37°C for 30 min in the presence or absence of cycloheximide (GIBCO). Cells were labeled in minimal essential medium (MEM)(Select-amine kit with Earle's salts, GIBCO) lacking methionine, supplemented with 2% FBS and 25-50 μCi/ml (^{35}S) methionine. L-(^{35}S) methionine (specific activity 1000-1200 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass. Specific conditions of radiolabeling varied with experiments and are described in the text or figure legends. To detect phosphorylation of proteins, cells were labeled in phosphate-free MEM with 2% FBS and 200 μCi/ml (^{32}P) orthophosphate (carrier free) (New England Nuclear). To detect glycosylated proteins, cells were labeled in glucose-free MEM with 2% FBS and 20 μCi/ml (^{3}H) glucosamine hydrochloride (New England Nuclear).

Use of metabolic inhibitors. In certain experiments, cycloheximide was included in the tissue culture medium (50 μg/ml), at the time of infection (to detect immediate-early proteins) or at later times, to amplify levels of

viral-induced proteins. The cycloheximide-containing medium was removed and cells were rinsed three times in complete medium prior to radiolabeling. Actinomycin D (Sigma), in 95% ethanol, was stored at 5 mg/ml and used at a final concentration of 10 μg/ml. Viral DNA synthesis was inhibited with sodium phosphonoacetate (PAA: Abbott Laboratories, N. Chicago, Ill.) at 100 μg/ml.

Processing of cells for gel electrophoresis. After the labeling period, cells were rinsed with cold phosphate buffered saline (PBS), scraped from the petri dish and collected by brief centrifugation in an Eppendorf bench centrifuge. Whole cell pellets were resuspended in 100 μl buffer (10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$) and frozen at -70°C until processed for gel analysis. Cells to be fractionated were treated as described by Jeang and Gibson (1980), with minor modifications. To separate into nuclear and cytoplasmic fractions, cells were resuspended in 200 μl of 1% NP40 (in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 5 mM $MgCl_2$) and incubated at 4°C for 10 min. This method leaves the insoluble cytoskeletal framework adhering to the nuclei. Nuclear and cytoplasmic fractions were separated by brief centrifugation. Phenylmethylsulphonyl fluoride (PMSF)(Sigma) was added to all samples to 0.5 mM. In certain experiments, the cytoskeletal framework was removed from nuclear fractions by the method of Penman (1966). Each nuclear fraction, isolated as described above, was resuspended in 100 μl buffer (50 mM NaCl, 100 mM HEPES, 2.5 mM $MgCl_2$, 0.3 M sucrose, pH 7.4) to which was added 10 μl of a mixture containing 10% Tween 40 and 5% sodium deoxycholate (DOC) (v/v and w/v respectively). The nuclear pellet was vigorously vortexed for 30 s and then the fractions were separated after brief centrifugation.

Prior to electrophoresis in the presence of SDS, whole cell extracts and nuclear preparations were sonicated to shear nuclear DNA and then all samples were diluted with equal volumes of 2X SDS-sample buffer (4.6% (w/v) SDS, 10% (v/v) 2-mercaptoethanol, 20% (w/v) glycerol, 0.1% Bromophenol blue in 0.125 M Tris-HCl pH 6.8), and heated for 5 min at 100°C.

Immunoprecipitation procedure. Cell extracts were prepared and immunoprecipitations carried out according to the procedure of Blanton and Tevethia (1981), with minor modifications. MCMV antisera used were derived from pooled serum samples taken from a) acutely infected mice (28 days after infection), or b) chronically infected mice (6-8 months after infection).

Cultures of 3T3-L1 cells (1.5 - 2 x 10^6 cells) were infected and labeled as described above. At the end of the labeling period, cells were rinsed with cold PBS and lysed *in situ* with 1 ml of cold extraction buffer (EB). EB consisted of 0.5% NP40 (v/v) in 20 mM Tris, pH 9.0, 0.3 M NaCl, 10% glycerol (v/v), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2 mM EDTA with 300 µg/ml PMSF. Cultures were incubated in EB for 20 min on ice and lysates harvested for immunoprecipitation.

Extracts were spun at 25,000 r.p.m. for 30 min in a Beckman SW27 rotor and preabsorption was carried out on them to reduce nonspecific reactions: Clarified supernatants were mixed with 5% MCMV-negative mouse serum for 1 hr at 4°C and then each was transferred to another tube that contained the pellet of 400 µl of a 10% suspension (w/v) of protein A containing *Staphylococcus aureus* cells (Pansorbin: Calbiochem-Behring Corp., La Jolla, Calif.). The cells were resuspended, agitated for 10 min and centrifuged in an Eppendorf centrifuge. The supernatants were used as starting material for immunoprecipitation studies.

500 µl of each extract were incubated with 10-20 µl of MCMV antibody positive or negative serum for 18 hr at 4°C. 100 µl of a 10% (w/v) suspension of S. aureus cells were added to each sample and they were incubated for 30 min at room temp. Cells with trapped immune complexes were washed five times in washing buffer (50 mM Tris-HCl, pH7.4, 0.5 M LiCl, 0.1 M NaCl and 1% NP40), by pelleting and resuspension. Immune complexes were freed by boiling the cells in SDS-sample buffer at 100°C for 5 min and pelleting out the cells. The supernatants were analysed by SDS-PAGE and autoradiography.

Analysis of proteins by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. SDS-PAGE was performed according to the procedure of Laemmli (1970) in a discontinuous buffer system in slab gels, in either gradient gels of 10-30% acrylamide, using a model 221 gel electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.) or in linear gels of 7.5% or 10% acrylamide, using a Bio-Rad Protean Multiple gel apparatus. The linear gels were used in later experiments as they gave more reproducible migration patterns than the gradient gels. All electrophoresis chemicals were obtained from Bio-Rad Laboratories. In the former case, the resolving gel was a linear acrylamide gradient (10-30%) cross-linked with 0.05-0.15% N,N'-methylene-bis-acrylamide (Bis), and 0.1% SDS, 0.375 M Tris-HCl pH 8.6; the gel length was 30 cm. Linear gels consisted of 7.5% acrylamide, 0.2% Bis or 10% acrylamide, 0.26% Bis and 0.1% SDS, 0.375 M Tris-HCl pH 8.8; the gel length was 16 cm. Stacking gels consisted of 4.5% acrylamide, 0.12% Bis, 0.1% SDS and 0.125 M Tris-HCl pH 6.8. Polymerization was initiated by addition of ammonium persulphate (APS) to 0.0003% and N,N,N',N'-tetra-methylethylenediamine (TEMED) to 0.025%. Running buffer

contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS, pH 8.3-8.5. Samples were electrophoresed through the stacking gel at 20 mA constant current and through the separating gel at 30 mA constant current. Gels were fixed and stained for 30 min in water:acetic acid:methanol (5:1:4, by volume) containing 0.2% Coomassie brilliant blue, destained in water:acetic acid:methanol (7:1:2, by volume) and dried onto filter paper under vacuum. In certain cases, gels were prepared for fluorography at this stage by impregnation with En^{3}Hance or Enlightning (New England Nuclear), according to the manufacturer's instructions. Gels were exposed for various times to Kodak X-OMAT AR film and these were developed in a Kodak automated developer. High and low molecular weight standard markers (Bio-Rad) were coelectrophoresed on each gel. The identities and molecular weights of the standard markers were as follows: lysozyme, 14,400; soybean trypsin inhibitor, 21,500; carbonic anhydrase, 31,000; ovalbumin, 45,000; bovine serum albumin, 66,200; phosphorylase B, 92,500; β galactosidase, 116,250; myosin, 200,000. Molecular weights of radioactive bands were determined using a linear regression program for the Hewlett-Packard 25C calculator, after comparing relative mobilities (Rf values) of these bands with Rf values of the markers, according to the method of Weber & Osborn (1969) for linear gels or the method of Poduslo & Rodbard (1980) for gradient gels.

Two-dimensional gel electrophoresis. Charge-size separations in polyacrylamide gels were performed essentially as described by O'Farrell et al (1977). Non-equilibrium pH gel electrophoresis charge separations (the first dimension) were done in 12.5 cm x 1 mm glass tubes cut from disposable 1 ml serological pipettes. The gel solution consisted of 9 M Urea, 2% NP40 (BDH Chemicals, Toronto, Ont.), 4% acrylamide cross-linked with 0.17% Bis

and 2% pH 3-10 ampholyte (Bio-lyte: Bio-Rad Laboratories). Polymerization was initiated by addition of 0.014% APS and 0.08% TEMED. Whole cell pellets and nuclear pellets were sonicated to shear DNA, samples were incubated with DNase and RNase (Sigma: 50 µg/ml each) for 20 min on ice and then components of the sample buffer were added separately to each sample (9 M Urea, 2% NP40 (w/v), 2% ampholytes mixed in the ratio 4:1 pH 5-7:pH 3-10 and 5% 2-mercaptoethanol). After polymerization, gels were overlaid with 20 µl of sample buffer and left overnight. Before samples were run, the overlay was removed, samples were loaded on the gels and overlaid with 10 µl of overlay solution (9 M urea, 1% ampholytes (0.5% pH 5-7 and 0.2% pH 3-10)). Upper electrode buffer was 0.01 M phosphoric acid and lower electrode buffer was 0.02 M NaOH. Gels were run in the first dimension for 6 hr at 500 V and equilibrated in two changes of SDS-sample buffer for 1 hr before being embedded horizontally in 1% agarose along the tops of 10% SDS polyacrylamide gels. Samples were run in the second dimension, stained, dried and exposed to X ray film as described above.

Preparation of proteins for DNA-affinity chromatography. Cell extracts were prepared for DNA-cellulose chromatography and samples run as described by Purifoy and Powell (1976). Nuclear extracts were prepared as described by Blair and Honess (1983).

3T3-L1 or J774A.1 cells, grown in 100 mm petri dishes, were mock or MCMV-infected (m.o.i. of 25) and maintained in cycloheximide containing medium (50 µg/ml) for 3 hr. Cells were rinsed three times and then labeled 6 hr with 25 µCi/ml (^{35}S) methionine in methionine-free MEM with 2% FBS. Cells were disrupted by sonication in low salt buffer (20 mM Tris-HCl, pH 7.4, bovine serum albumin (BSA) 500 µg/ml, 2 mM 2

mercaptoethanol, 0.5 mM PMSF) at 2 x 10^7 cells/ml. An equal volume of high salt buffer (20 mM Tris-HCl, pH 7.4, 3.4 M NaCl 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5 mM PMSF) was added, samples incubated on ice 1 hr and then spun at 30,000g for 30 min. The supernatants were dialysed against three changes of column buffer (20 mM Tris-HCl, pH 7.4, 10% (w/v) glycerol, 50 mM NaCl, 1 mM EDTA, 2 mM 2-mercaptoethanol, 0.5 mM PMSF), spun at 100,000g for 1 hr to remove insoluble material and applied to DNA-cellulose columns.

To prepare proteins from nuclear extracts, cells were infected and labeled as described above. Cells were lysed by suspension in buffer containing 1% NP40 in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 mM PMSF. Samples were held on ice with occasional vortexing for 10 min and nuclei were sedimented by low speed centrifugation. Nuclear fractions were resuspended in buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM PMSF, 5 mM EDTA, 2 mM 2-mercaptoethanol and 20% glycerol and lysed by addition of solid NaCl to 2.0 M. Samples were sonicated to shear DNA and held on ice 1 hr. Insoluble material was removed by centrifugation (100,000g for 1 hr) and supernatants made to 5 mM spermine and dialysed against buffer containing 5 mM spermine (Sigma) in 10 mM Tris-HCl, pH7.4, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM 2-mercaptoethanol and 10% glycerol. The precipitates were sedimented by centrifugation (11,000g for 15 min) and the supernatants were dialysed against 10 mM Tris-HCl, pH7.4, 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 1 mM 2-mercaptoethanol and 10% glycerol. This represented the starting material for DNA-cellulose chromatography.

Preparation of DNA-cellulose. DNA was adsorbed to cellulose according to the methods of Alberts and Herrick (1971). Prior to use, cellulose (Cellex 410, Bio-Rad) was washed three times (1 hr each) in 95% ethanol at

80°C. It was then filter washed successively in 0.1 M NaOH, 1 mM EDTA and 10 mM HCl and several changes of distilled water. The cellulose was then lyophilized and stored as powder.

Native DNA-cellulose was prepared by mixing cellulose with calf thymus DNA (Sigma)(2 mg/ml solution) in the ratio of cellulose:DNA solution at 1 g: 3 ml. The mixture was spread over an evaporating dish and left overnight to dry at 37°C. It was then ground into a powder and lyophilized to complete dryness. This powder was resuspended and washed three times in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA and then stored at -20°C in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA.

Denatured (single-stranded) DNA-cellulose was prepared by mixing cellulose with calf thymus DNA in the stated ratios. This time the DNA (2 mg/ml solution in 10 mM K_2HPO_4, 1 mM EDTA) was first denatured by treatment for 20 min at 100°C. It was then rapidly cooled, made 20 mM in Tris-HCl, pH 7.4, and mixed with cellulose. It was then processed as stated.

DNA-cellulose chromatography. DNA-cellulose was poured into columns (1 x 4 cm, 1.5 ml of DNA-cellulose) and equilibrated 1 hr with column buffer before samples were applied. All procedures were carried out at 4°C. Each sample was washed through the column with 50 ml of column buffer and DNA-binding proteins were eluted stepwise with the same buffer containing 0.1, 0.3, 0.5 and 1.0 M NaCl respectively. 50 µl aliquots of each fraction were precipitated with 10% trichloroacetic acid (TCA) and counted by LSC. Fractions that contained the peak counts of acid-precipitable (^{35}S) labeled proteins, for each concentration of elution buffer, were precipitated with 10% TCA, washed in 95% ethanol, dissolved in SDS-sample buffer and separated by electrophoresis on 10-30% gradient SDS-

SDS-polyacrylamide gels or 7.5% linear gels.

Detection of DNA-binding proteins by protein blotting. To detect DNA-binding of proteins that may be insoluble by the previous method, the protein blotting method of Bowen et al. (1980) was used with modifications. Proteins were electroblotted according to the method of Towbin et al. (1979).

Unlabeled proteins were separated by SDS-PAGE, as described before. Labeled samples, that had been prepared in the same manner, were run in parallel on the same electrophoresis apparatus. After the completion of electrophoresis, the polyacrylamide gel was mounted next to a sheet of nitrocellulose in a Bio-Rad Transblot cell. The samples were electroblotted for 4 hr at 300 mA in 20 mM Tris, pH 8.3, 0.192 M glycine and 20% methanol.

Filters were soaked in 1X SSC solution, containing 1X Denhardt's reagent, for 20 min at room temp. and then incubated for 1 hr with ^{32}P labelled denatured MCMV-DNA in 1X SSC solution, containing 1X Denhardt's reagent, with constant agitation. MCMV-DNA was nick translated with ($\alpha^{-32}P$) dATP, using a B.R.L. nick translation kit. 10^6 - 2 X 10^6 c.p.m. of DNA, denatured by incubation at 100°C for 20 min and then rapidly chilled, was used for each filter. Filters were washed for 1 hr in four changes of 1X SSC, dried and autoradiographed.

RESULTS.

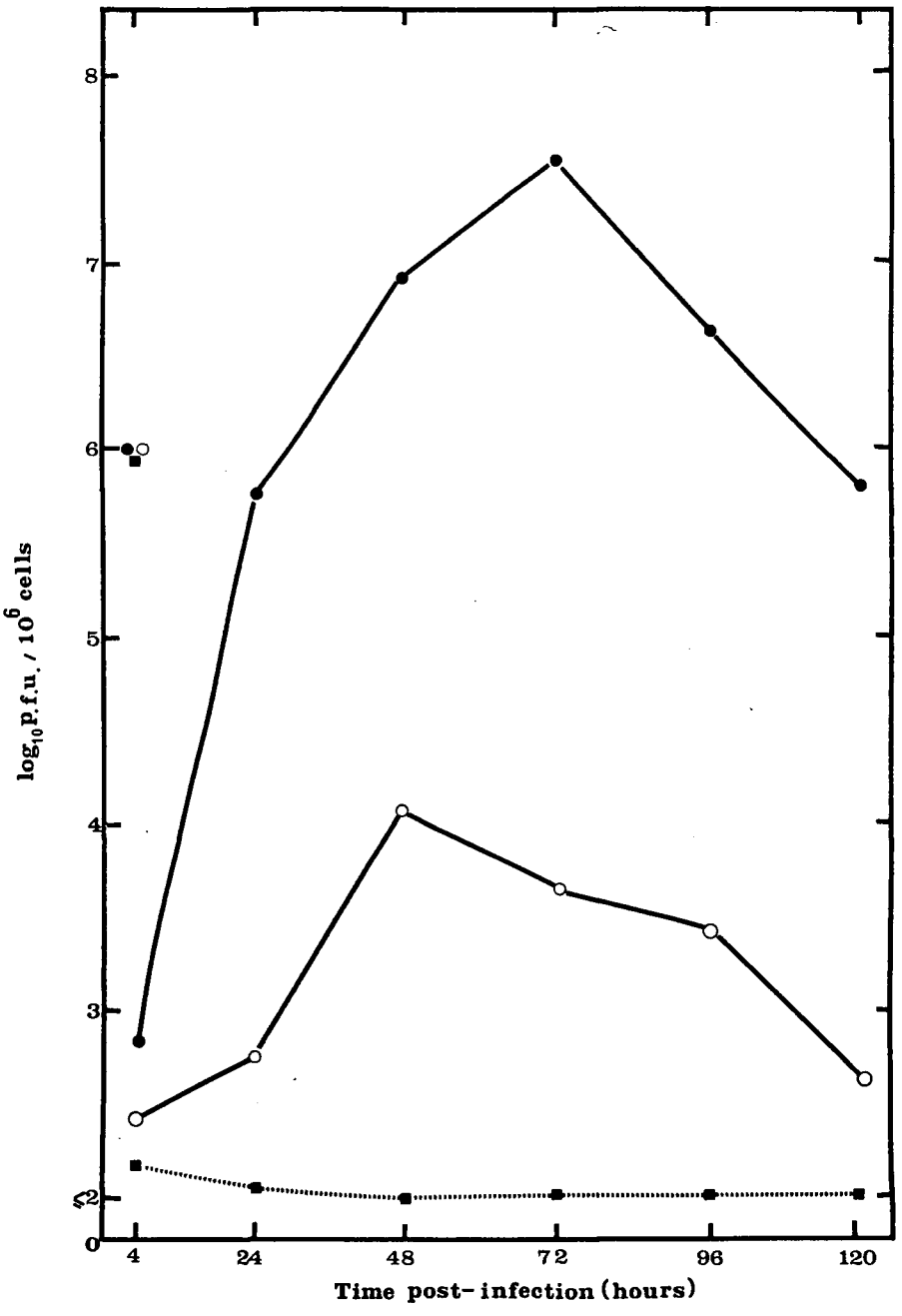
1. Characterization of nonpermissive infections of MCMV. Preliminary studies in our laboratory had shown that MCMV did not replicate fully in J774A.1 cells compared to 3T3-L1 cells (data not shown). These experiments also showed that MCMV did not replicate at all in human fibroblast cells.

The initial experiments were designed to compare the yields of virus from MCMV-infected 3T3-L1, J774A.1 and human fibroblast cells. Further to that, it was necessary to determine, by infectious center assay, the type of infection that occurred in J774A.1 cells. Namely, if most of the cells were producing low amounts of virus (semipermissive infection) or if virus was being produced by a small percentage of cells and the rest were nonproductive (nonpermissive infection). It had been decided that these cells would only be suitable for gene expression experiments if most were nonpermissively infected. This would mean that they were behaving in a similar manner to HCMV infection of nonhuman cells.

2. Growth of MCMV in 3T3-L1, J774A.1 and human fibroblast cells. The curves of MCMV replication in 3T3-L1, J774A.1 and human fibroblast cells are shown in Fig. 1. A comparison of the maximum titers obtained, showed that there was over a 2000-fold difference in the yields of virus between the two types of cells. The limited amount of replication of MCMV in J774A.1 cells represented a yield of approximately 1 PFU per 100 cells, compared to 20 PFU per infected 3T3-L1 cell. These results also confirmed that no replication of MCMV occurred in human fibroblast cells.

Fig. 1:

Growth curves of murine cytomegalovirus (MCMV) replication in 3T3-L1 cells (●), J774A.1 cells (○) and human fibroblasts (■). Titers of cell-associated plus supernatant virus were assayed on 3T3-L1 cells. Each point represents the mean of three estimations.



3. Detection of infectious centers. Cultures of J774A.1 and human fibroblast cells (HFF) were infected as described and the number of virus producing cells (infectious centers) was assayed. Table I shows that approximately 0.5% of the J774A.1 cells were permissive to virus replication. The growth of MCMV in only a small percentage of J774A.1 cells followed the same pattern that was seen with the infection of macrophages that had been isolated from mouse spleens (Hudson et al., 1978). Because there was an absence of a growth phase (as seen in Fig. 1), the low numbers of infectious centers for HFF cells probably represent residual virus from the inoculum. This shows the limitations of using HFF cells for studying latency, as an abortive type of infection occurs.

4. MCMV DNA synthesis in 3T3-L1, J774A.1 and human fibroblast cells. To determine if MCMV DNA replication in J774A.1 and HFF cells was inhibited during these nonpermissive infections, similar to that seen in all other nonpermissive CMV infections, increase in viral DNA concentration in infected cells was measured by autoradiography and liquid scintillation counting (LSC) of (^{32}P) labeled MCMV-DNA, that had hybridized to mock and MCMV-infected cell DNA. The autoradiogram (Fig. 2) shows a considerable increase in viral DNA at 24 hr pi. in infected 3T3-L1 cells. In comparison, there appeared to be no significant increase in the amount of viral DNA in infected J774A.1 cells at 24 hr pi.. A similar finding was seen for infected HFF cells. These results were quantified by LSC of each separate spot (Table II). There was a 30-fold increase in viral DNA content at 24 hr pi. in infected 3T3-L1 cells, while the increase observed for the other two cell types was less than 1.5-fold. This was calculated by dividing the mean c.p.m. hybridized at 24 hr pi. by the mean c.p.m. hybridized at 2 hr pi.,

Table I:

Infectious centers/10^5 cells (%) produced in MCMV-infected J774A.1 and HFF cells.

Cell Type		Time post-infection cells harvested (days)			
		1	2	3	5
J774A.1		508	642	198	49
	%	(0.508)	(0.642)	(0.198)	(0.049)
HFF		5	3	0	0
	%	(0.005)	(0.003)	(0)	(0)

Each result represents the mean of four estimations.

Fig. 2:

MCMV DNA replication in 3T3-L1, J774A.1 and human fibroblast (HFF) cells. Autoradiographic image of (^{32}P) labeled MCMV DNA hybridized to cell dots. Cells were mock or MCMV-infected at 10 PFU/cell. They were harvested at 2 and 24 hr pi., spotted onto nitrocellulose, denatured *in situ* and hybridized with 10^6 c.p.m. (^{32}P) labeled MCMV DNA.

2 - cells harvested 2 hr pi.

m - mock-infected cells.

24 - cells harvested 24 hr pi.

Fig. 2: MCMV DNA replication in 3T3-L1, J774A.1 and human fibroblast cells. Autoradiographic image of (^{32}P) labeled MCMV DNA hybridized to cell dots.

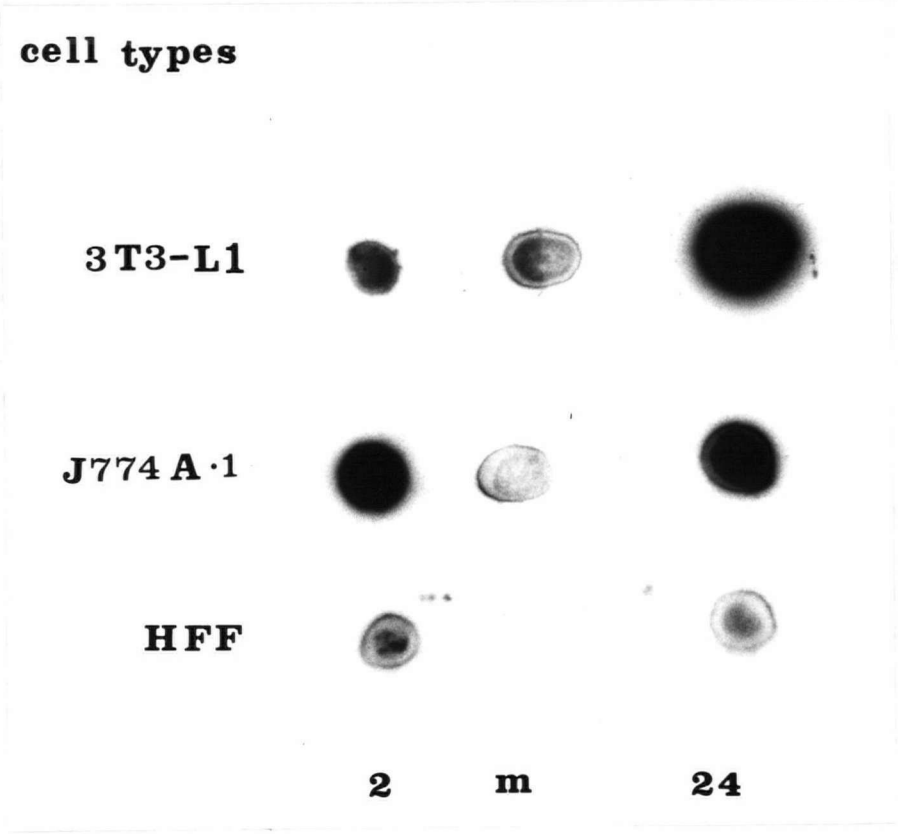


Table II:

CPM of ^{32}P labeled MCMV DNA hybridized to dots of mock, 2 and 24 hr MCMV-infected 3T3-L1, J774A.1 and human fibroblast cells.

Cell Type	Time (hr) post-infection cells harvested		
	M(2)	2	24
3T3-L1	1311	2093	24705
J774A.1	1584	4138	5519
HFF	854	1665	1797

Cell dots were hybridized with 10^6 cpm ^{32}P MCMV DNA. Counts were normalized to numbers of 3T3-L1 cells. Each result is a mean of four estimations.

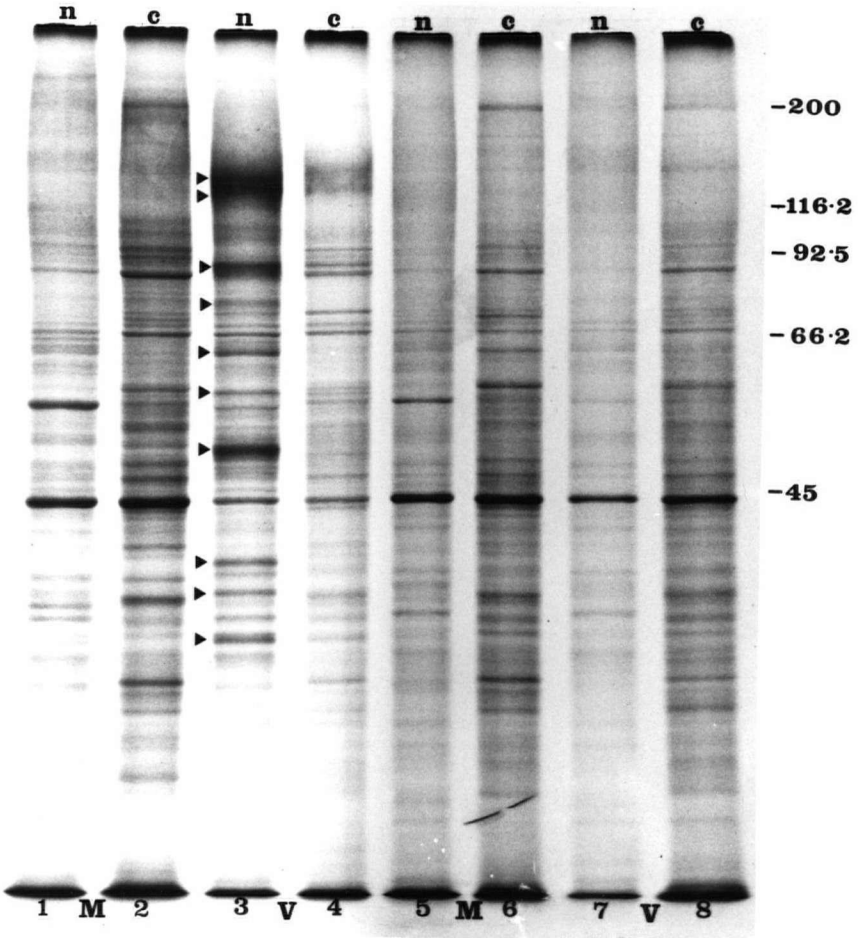
after the mean c.p.m. that hybridized to mock-infected cells had been subtracted from both figures. The positive reaction between mock-infected murine cells and MCMV DNA arose because there is cross hybridization between MCMV DNA sequences and murine cellular DNA (unpublished observations). This was not seen between MCMV DNA and human cellular DNA.

5. Identification of late infected cell specific proteins. To confirm the absence of significant amounts of late gene expression in J774A.1 cells, MCMV-infected 3T3-L1 and J774A.1 cell proteins were labeled from 28-32 hr pi., the period when peak amounts of structural proteins are known to be synthesized (Chantler and Hudson, 1978). The right side of the photograph of Fig.3, showing the proteins in J774A.1 cells, was overexposed compared to the left side, in order that the bands could be more clearly seen. Although the same numbers of cells were infected with the same amount of virus in both samples, the amount of (^{35}S) methionine incorporation was noticably less in the J774A.1 cells. On the gel, extracts from equivalent numbers of cells were electrophoresed in each lane. Fig. 3 shows that at least 10 distinct infected cell proteins were detected in nuclear fractions of infected 3T3-L1 cells (lane 3), while none of these bands were present in the equivalent sample from infected J774A.1 cells (lane 7).

Fig. 3:

Autoradiographic image of proteins from mock and MCMV-infected 3T3-L1 and J774A.1 cells, labeled to detect late proteins, and separated in a 10% polyacrylamide gel. Cultures of 3T3-L1 cells and J774A.1 cells were mock (M) and MCMV-infected (V) (m.o.i. 10) and labeled 28-32 hr pi. with (^{35}S) methionine (25 μCi/ml). Cells were separated into nuclear (n) and cytoplasmic (c) fractions. Lanes: 1,2: Mock-infected 3T3-L1 cell proteins. 3,4: Virus-infected 3T3-L1 cell proteins. 5,6: Mock-infected J774A.1 cell proteins. 7,8: Virus-infected J774A.1 cell proteins. Closed arrowheads indicate virus-induced proteins. Right hand margin - positions and mol. wt. (x 10^{-3}) of marker proteins: 200 - myosin; 116.2 - β galactosidase; 92.5 - phosphorylase B; 66.2 - bovine serum albumin; 45 - ovalbumin.

Fig. 3: Proteins in mock and infected 3T3-L1 and J774A.1 cells labeled 28-32 hr. pi. Autoradiographic image of mock and infected 3T3-L1 and J774A.1 cell proteins separated in a 10% polyacrylamide gel.



6. Early protein synthesis of murine cytomegalovirus. It had been previously shown that some viral genes were expressed during the first 4 hr of infection of murine fibroblasts by MCMV, even though virion proteins were not detected until approximately 12-16 hr after infection (Misra et al., 1978; Chantler and Hudson, 1978). As host protein synthesis continues at a high level during the period that MCMV 'early' proteins are produced, the study of these viral proteins has required a technique that increases their levels relative to the host proteins.

The technique of cycloheximide block reversal allowed enhanced levels of viral-induced proteins to be synthesized. Treatment of infected cells with this protein synthesis inhibitor is believed to cause selective enrichment of some classes of viral mRNA over host cell mRNA (Jeang and Gibson, 1980). Removal of cycloheximide leads to the translation of the mRNA with synthesis of higher amounts of certain viral proteins than would normally be found. This technique permitted detection of additional virus-induced proteins and avoided the disadvantages of immunoprecipitation, where there is a danger of not detecting some viral proteins. In addition, viral-induced proteins, synthesized up to 6 hr after infection, were characterized with the use of three different gel electrophoresis systems: 1) gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell lysates, 2) linear SDS-PAGE of fractionated cells and 3) non-equilibrium pH gel electrophoresis (NEPHGE)-SDS-PAGE (2D gel electrophoresis) of both whole cell and fractionated cell lysates.

7. Time course of synthesis of early infected cell proteins. Cultures of 3T3-L1, mock and MCMV-infected, were labeled with (^{35}S) methionine for 1 hr at 1 hr intervals following removal of a 3 hr cycloheximide block (with

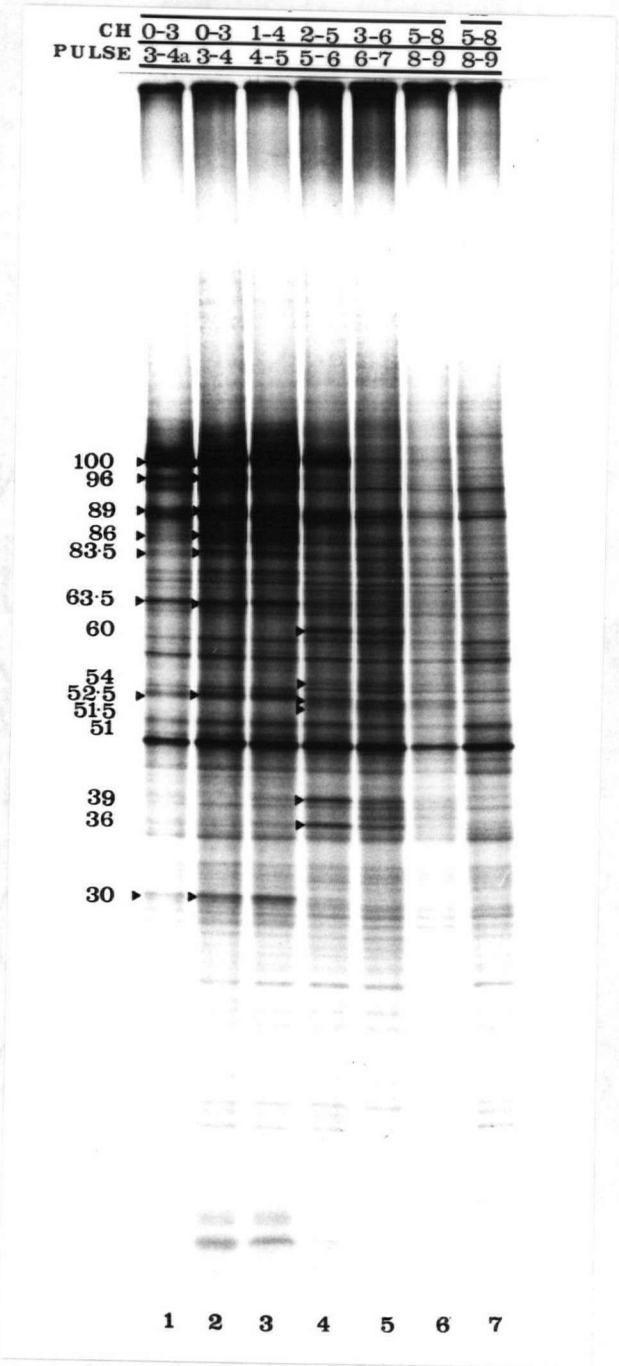
the exception of the 4-5 hr pi. period). The time period studied is considered to be equivalent to the first six hr after infection. This may be an oversimplification, but as protein synthesis during cycloheximide treatment was reduced to 0.3-0.7% of normal levels (as measured by (^{35}S) methionine incorporation, data not shown), the cycloheximide treatment was effective in inhibiting most protein synthesis. Fig. 4 shows that 14 viral-induced proteins were detected in 3T3-L1 cells over the period studied. With the one hr pulse labeling of cells, it can be seen that the viral-induced proteins were synthesized in two phases. The first group of proteins (preliminarily designated immediate-early (IE)) were all detected within 1 hr after the reversal of the cycloheximide block and had estimated mol. wts. of 100K, 96K, 89K, 86K, 83.5K, 63.5K, 52.5K and 30K. The 100K and 89K proteins were dramatically enhanced by the cycloheximide pretreatment, while the others were enhanced to a lesser extent. The peak period of synthesis of these proteins was during the first 2 hr of infection. A second group of proteins (preliminarily designated early) were detected following 1-2 hr of de-novo protein synthesis, with their peak period of synthesis being during the third hour of infection. These had estimated mol. wts. of 60K, 54K, 51.5K, 51K, 39K and 36K.

The initial designation of viral-induced proteins into IE and early classes on the basis of time of appearance could be considered somewhat arbitrary as the switch to early gene expression and protein synthesis may be occurring in the first hr of infection. However, the proteins in lanes 1 and 2 (Fig. 4), which show the same virus-induced proteins, were labeled at the same time and in the same manner, except that samples shown in lane 1 were labeled in the presence of 10 μg/ml of actinomycin D. This would inhibit early gene transcription from occurring during the labeling period.

Fig. 4:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV-infected 3T3-L1 cells separated by electrophoresis in a 10-30% gradient polyacrylamide gel. Replicate cultures of 3T3-L1 cells were infected with MCMV, incubated with cycloheximide for 3 hr at various times indicated after infection (CH) and then labeled for 1 hr with (^{35}S) methionine (50 μCi/ml) at the times indicated (PULSE). Closed arrowheads designate virus-induced proteins with their apparent mol. wt. ($x10^{-3}$) on the left side. Lane 1: proteins in MCMV-infected cells labeled in the presence of 10 μg/ml actinomycin D. Lanes 2. through 6: proteins in MCMV-infected cells labeled at the times indicated. Lane 7. proteins in mock-infected cells.

Fig. 4: Proteins synthesized 0-6 hr pi. in 3T3-L1 cells. Autoradiographic image of labeled proteins from mock and MCMV-infected 3T3-L1 cells separated in a 10-30% gradient polyacrylamide gel.



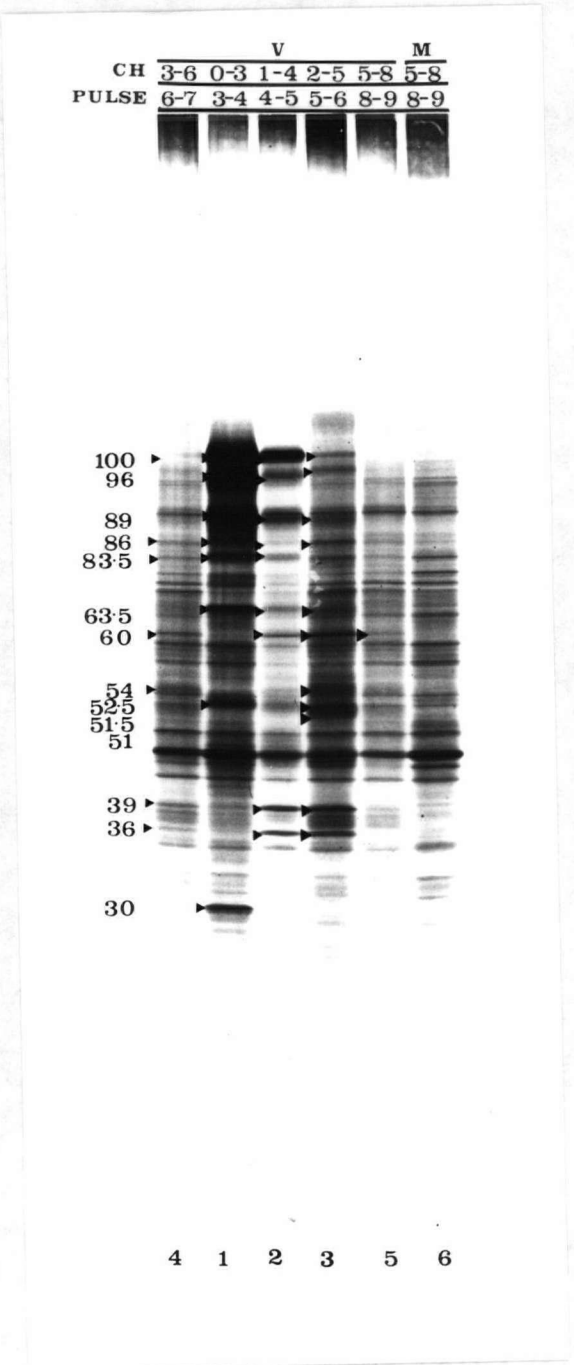
This indicated that there was not an immediate overlap between synthesis of the two groups of proteins and delineation into IE and early protein classes on the basis of time of appearance is possible. Similar to that observed with other CMVs (Gibson, 1981), large amounts of a dominant 'IE' protein (100K) were synthesized in MCMV-infected cells immediately after the reversal of a cycloheximide block that was present at the time of infection. However, the presence of a secondary major MCMV 'IE' protein (89K), separate from the other protein band, is a feature that has not been observed with the human and simian CMV strains. The presence of a protein intermediate between the major 'IE' proteins is also distinctly different from what has been observed with HCMV. Evidence will be shown that this protein has different properties from the 100K and 89K proteins. The presence of a number of minor 'IE' proteins (63.5K, 52.5K and 30K) was similar to that seen with HCMV (Gibson, 1981). The proteins of 86K and 83.5K may represent proteolytic breakdown products of the major 'IE' proteins, though these bands were detected consistently in a number of experiments, and in addition, labeled samples were processed in the presence of PMSF, an inhibitor of proteolytic enzymes.

Fig. 5 shows the proteins that were induced in MCMV-infected J774A.1 cells under the same experimental conditions as Fig. 4. It can be seen that the same number of 'IE' and 'early' proteins, with the same estimated mol. wts., were synthesized in these cells as in 3T3-L1 cells. The sequence of protein synthesis appeared to be the same in these cells, namely the presence of two phases of induced protein synthesis, except that the proteins designated as early could be detected during the second hr of infection. This is felt to represent an experimental variation rather than one of biological significance. The time taken to handle these cells,

Fig. 5:

Autoradiographic image of (^{35}S) labeled proteins from mock (M) and MCMV (V)-infected J774A.1 cells separated by electrophoresis in a 10-30% gradient polyacrylamide gel. Replicate cultures of J774A.1 cells were infected with MCMV, incubated with cycloheximide for 3 hr at various times indicated after infection (CH) and then labeled for 1 hr with (^{35}S) methionine (50 μCi/ml) at the times indicated (PULSE). Closed arrowheads indicate virus-induced proteins with their apparent mol. wt. ($x10^{-3}$) on the left side. Lanes 1 through 5: proteins in MCMV-infected cells labeled as indicated. Lane 6: proteins of mock-infected cells.

Fig. 5: Proteins synthesized 0-6 hr pi. in J774A.1 cells. Autoradiographic image of labeled proteins from mock (M) and MCMV (V)-infected 3T3-L1 cells separated in a 10-30% gradient polyacrylamide gel.



compared to the 3T3-L1 cells, when rinsing to remove the cycloheximide, was longer. This was because the infected cells did not adhere strongly to the petri dishes. They had to be rinsed slowly and the cells that had been dislodged had to be recovered by centrifugation. During the rinsing time (10-15 min), the cells will have restarted protein synthesis. This time was not accounted for in the labeling period. It can also be seen that the mol. wt. of the 96K protein increased slightly from lanes 1 to 3 in Fig. 5. This was also seen in some experiments with 3T3-L1 cells, although not evident in Fig. 4. The processing of this protein will be discussed in another section.

By 6 hr after infection, the total amount of virus-induced protein synthesis had declined to a low level. Fig. 6 shows the time course of synthesis of virus-induced proteins that were detected in both cell types, by gradient gel electrophoresis.

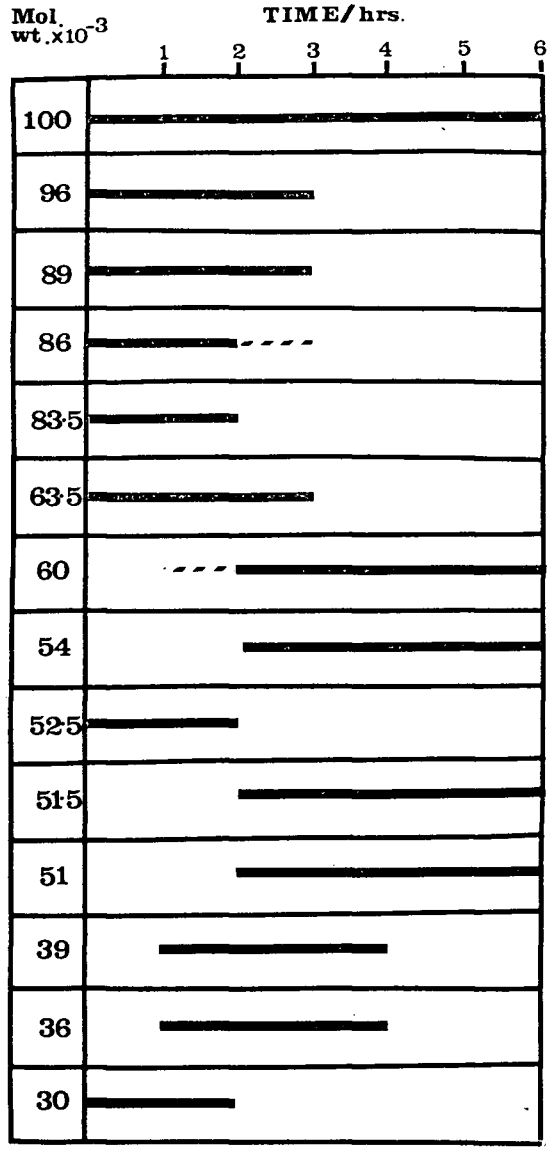
8. Definition of immediate-early and early proteins.

Herpesvirus-induced proteins have traditionally been classified into immediate-early and early classes according to their synthesis in the presence of certain metabolic inhibitors (Honess and Roizman, 1974). In these experiments, MCMV IE proteins were defined as those synthesized in cells that had been infected and maintained in the presence of cycloheximide and then labeled with (^{35}S) methionine in the presence of actinomycin D. Early proteins were defined as those synthesized in cells that had been infected and labeled from 2-5 hr pi. while maintained in the presence of the viral DNA synthesis inhibitor phosphonoacetic acid (100 μg/ml) and separate from those defined as IE proteins (this procedure also detected synthesis of some IE proteins).

Figs. 7 A and 7 B show the proteins induced under the conditions

Fig. 6:

Time course of synthesis of MCMV-induced proteins detected in infected 3T3-L1 and J774A.1 cells. (data taken from Fig. 4 and 5 respectively). The time of cycloheximide treatment is not included. Black bars designate time proteins were detected in both cell types; broken bars designate time proteins were detected in J774A.1 cells only.



described and separated on gels of two different acrylamide concentrations (Fig. 7 A - 7.5%; 7 B - 10%) (Not all the virus-induced bands are marked in each lane). The 100K and 96K IE proteins were only clearly resolved as separate bands by electrophoresis on the 7.5% polyacrylamide gel (Fig. 7 A). By these procedures, ten newly synthesized proteins with mol. wts. of 100K, 96K, 89K, 86K, 83.5K, 80K, 63.5K, 52.5K, 30K and 28K could be classified as IE and three with mol. wts. of 91K, 39K and 36K as early class. The early proteins of 60K, 54K, 51.5K and 51K, which had been detected on gradient gels, were not detected using these procedures. The IE proteins of mol. wts. of 80K and 28K and the early protein of 91K had not been detected in the previous experiments that had analyzed whole cell lysates by gradient PAGE. The experiments that indicate the 91K protein to be a separate early gene product will be discussed in a later section.

Initial observations can be made on the partitioning of these proteins within the infected cell. With the exception of the 80K protein, which was only found in cytoplasmic fractions, the other proteins were associated with the nuclear (and/or cytoskeletal framework) fractions. The efficiency with which they partitioned out of the soluble cytoplasmic fractions varied between proteins. The 91K, 39K and 36K proteins partitioned completely with the nuclear fractions and were not detected in the cytoplasmic fractions. The 100K, 89K, 83.5K, 63.5K, 52.5K, 30K and 28K were present in a higher proportion in the cytoplasmic fractions, while the 96K and 86K proteins were present in a higher proportion in the nuclear fractions.

It was noticed that MCMV-infected cells that had been treated with actinomycin D to prevent early gene expression did not exhibit a typical early cytopathic effect, even though the amounts of IE proteins were sufficiently elevated such that the 100K and 89K IE proteins could be

Fig. 7:

Autoradiographic images of (^{35}S) labeled proteins from mock and MCMV-infected 3T3-L1 cells separated in 7.5% (A) and 10% (B) polyacrylamide gels. To identify immediate-early (IE) proteins, replicate cultures of 3T3-L1 cells were infected with MCMV and treated with cycloheximide 0-3 hr pi. Cells were labeled 3 hr with (^{35}S) methionine (50 μCi/ml) in the presence of actinomycin D. To identify early (E) proteins, replicate cultures were infected with MCMV in the presence of phosphonoacetate and labeled 2-5 hr pi. with (^{35}S) methionine (50 μCi/ml) in the presence of phosphonoacetate. Lanes: 1-4: proteins in nuclear fractions of mock (m) and virus-infected (v) cells labeled under IE and E conditions. 5-8: proteins in cytoplasmic fractions of mock (m) and virus-infected (v) cells labeled under IE and E conditions. Virus-induced IE proteins are designated by closed arrowheads and early proteins by open arrowheads with their apparent mol. wt. ($x10^{-3}$) on the sides.

Fig. 7: Identification and location of IE and early MCMV proteins.

Proteins from mock and MCMV-infected 3T3-L1 cells separated in 7.5% (A) and 10% (B) polyacrylamide gels.

Fig. 7 (A).

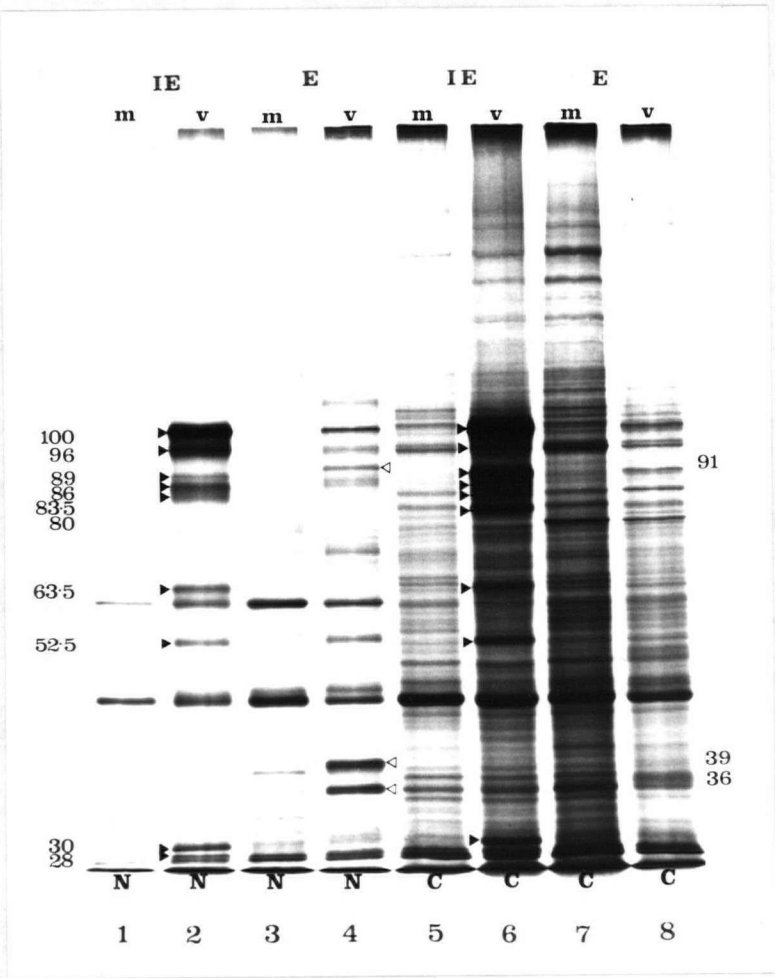
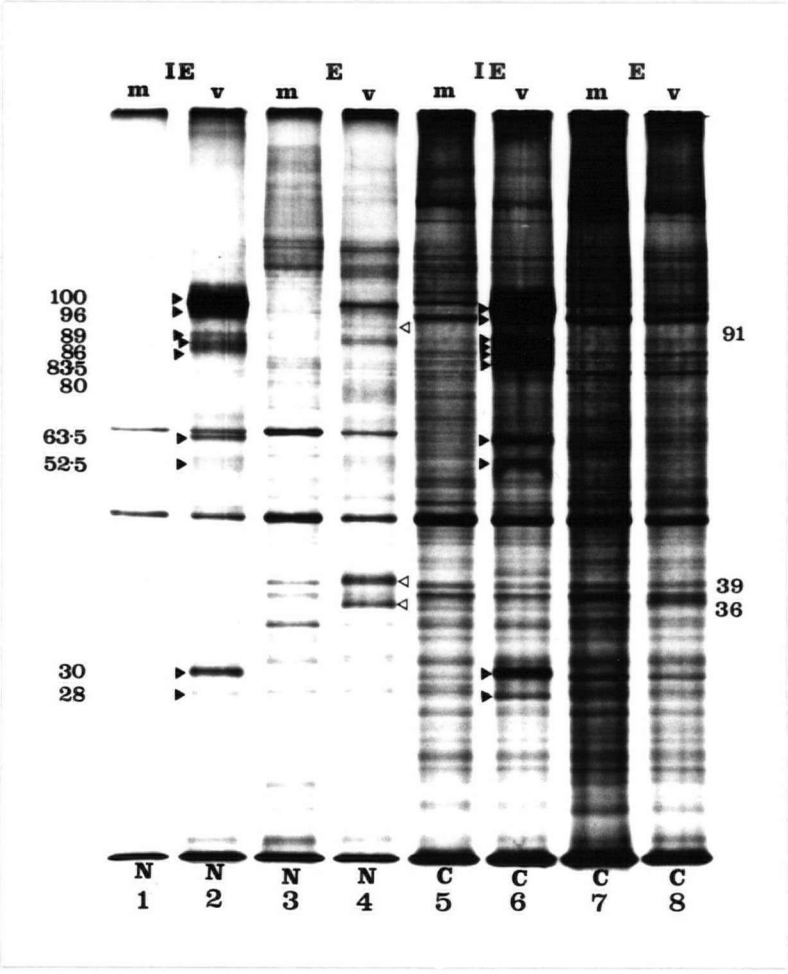


Fig. 7 (B).


IE
E
IE
E
m
v
m
v
m
v
m
v
100
96
89
86
83·5
80
63·5
52·5
30
28
91
39
36
N
1
N
2
N
3
N
4
C
5
C
6
C
7
C
8

detected on Coomassie blue stained gels (not shown). These experiments confirmed the previous classification of the proteins into IE and early classes on the basis of time of synthesis. These designations will be used even for proteins that were not detected in this set of experiments (60K, 54K, 51.5K and 51K).

We have noticed, in this and also later experiments, that the 63.5K protein migrated in an anomalous manner on different gels. This was seen in Fig. 7 A (lane 2) where it had a position above the host nuclear protein and in Fig. 7 B (lane 2), where its position was below this band. In other samples, its mol. wt. has varied from 56K to 63.5K. The 63.5K estimation (obtained from gradient gel estimations, where migration patterns remained constant) will be used to designate this protein even though this will appear anomalous in some cases. It was not possible to determine whether there were different proteins being detected or whether the same protein could migrate differently.

9. Immunoprecipitation. To determine whether the proteins being studied were virus-specific, proteins from infected cells were precipitated with serum derived from MCMV-infected mice. As these proteins are only produced in small amounts during the normal infectious cycle, high titers of antibodies to them do not arise in infected mice. Antibodies obtained from mice following acute MCMV infection (taken 28 days pi.) could only precipitate the 100K IE protein (data not shown). More 'potent' antisera were obtained from mice that were chronically infected with MCMV (taken 6 months pi.). Although it still did not have strong activity, a number of characterized IE and early proteins were precipitated with these antisera.

Fig. 8 A shows the precipitation of proteins derived from cells labeled

to detect IE proteins (lanes 1 and 2) and early proteins (lanes 3 and 4) The proteins were only precipitated with serum from MCMV-infected animals. Due to low amounts of radioactivity, the gel was treated with En^{3}Hance to shorten the exposure time necessary. This accounts for the diffuseness of the bands on the autoradiogram. Two strong bands covering the positions of the 100K and 96K proteins and 89K and 86K proteins, respectively, and faint bands corresponding to the 30K and 28K were detected (lane 2). The unlabeled band (lane 2) does not correspond to an identified viral-induced protein, but has an estimated mol. wt. of 43-44K (equivalent to actin). A diffuse band corresponding to the positions of proteins of 54K, 51.5K and 51K was detected, as were bands corresponding to the early proteins 39K and 36K. In Fig. 8 B, the autoradiogram showed that the 100K, 96K, 89K and 86K could be separately identified after precipitation. The 39K and 36K proteins were not detected, but a different antisera pool had been used in this experiment from that used in Fig. 8 A.

10. Two-dimensional analysis of infected cell proteins. To more clearly resolve the complex mixtures of proteins, two-dimensional separations of infected cell lysates, labeled at IE and early times (details in Fig. 9 legend), were carried out. Autoradiograms of two-dimensional gels of MCMV infected 3T3-L1 and J774A.1 cells, shown in Figs. 9 and 10 respectively, illustrate a number of spots that identified with virus-induced proteins. They were identified by their absence on corresponding gels of lysates prepared from mock-infected 3T3-L1 cells (Fig. 9 C) and J774A.1 cells (not shown) and by having a second dimensional mobility equivalent to that of virus-induced proteins on one dimensional gels.

Fig. 8:

Autoradiographic images of (^{35}S) labeled proteins immunoprecipitated from MCMV-infected cells and separated in 10% polyacrylamide gels. A: Cultures of 3T3-L1 cells were infected with MCMV and treated with cycloheximide 0-3 hr pi. (lanes 1, 2) or 2-5 hr pi. (lanes 3, 4) and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). Cell extracts were precipitated with MCMV antibody positive (+) and negative (-) serum as described in text.

B: Cultures of 3T3-L1 cells were infected with MCMV or mock-infected and labeled 1-4 hr pi. with (^{35}S) methionine (50 μCi/ml).

Lanes: 1, proteins from virus-infected cell extract precipitated with MCMV antibody negative serum (-); 2, proteins from mock-infected cell extract precipitated with MCMV antibody positive serum (+); 3-5, proteins from MCMV-infected cell extracts precipitated with MCMV antibody positive serum (+) (5, 10 and 20 μl respectively). Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. ($x10^{-3}$)

Fig. 8: Proteins immunoprecipitated from MCMV-infected cells with immune mouse serum. Autoradiographic images of labeled proteins immunoprecipitated from MCMV-infected cells and separated in 10% polyacrylamide gels.

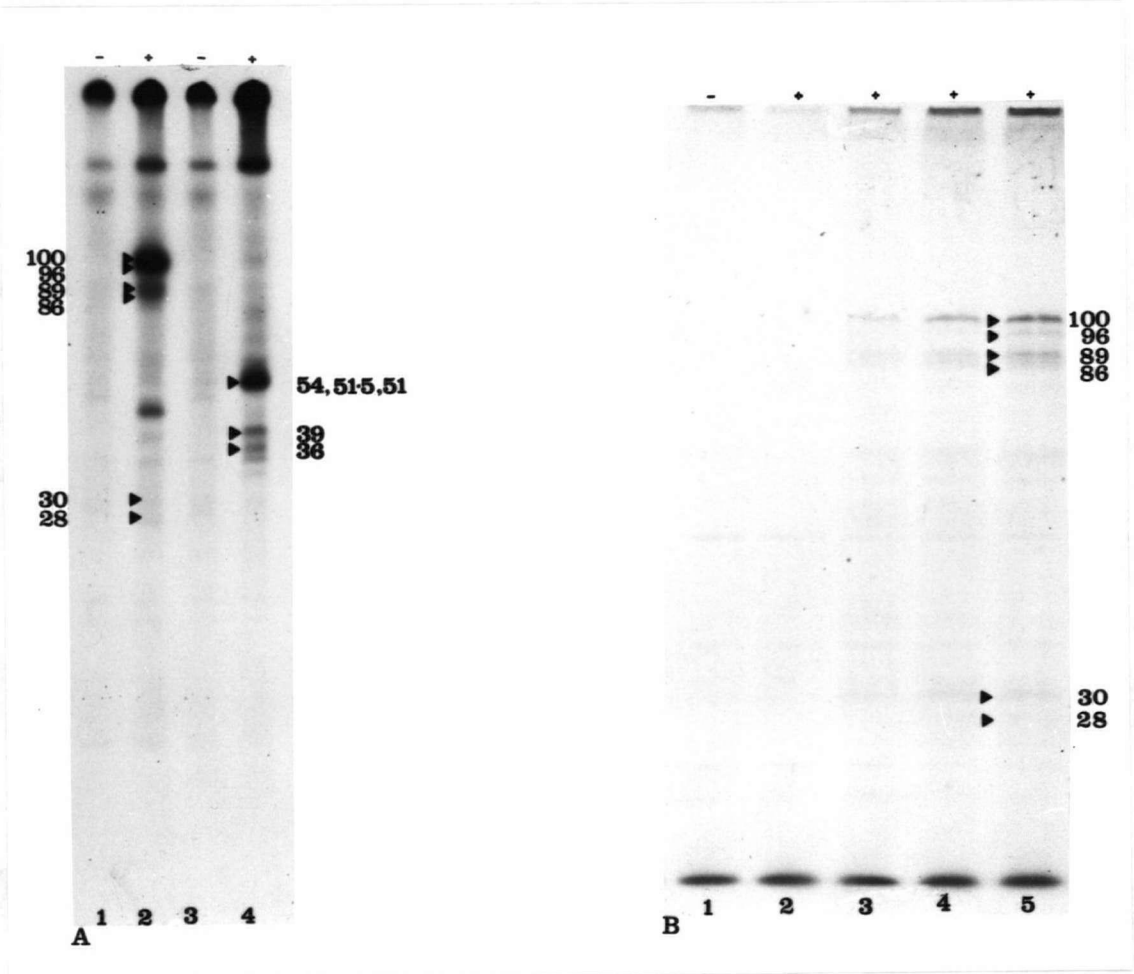


Fig. 9:

Autoradiographic images of (^{35}S) labeled proteins from MCMV-infected 3T3-L1 cells separated in two dimensions by electrophoresis. 3T3-L1 cells were infected with MCMV and incubated with cycloheximide from: A) 0-3 hr and B) 2-5 hr pi., and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). First dimension was run from acidic to basic ends for 3000 V.h.. Arrows indicate virus-induced proteins with their apparent mol. wt. ($x10^{-3}$). Prominent cellular proteins are indicated (h_1, actin). C) 3T3-L1 cells were mock-infected and incubated with cycloheximide from 0-3 hr pi. and then labeled 1 hr with (^{35}S) methionine.

Fig. 9: Two-dimensional separation of proteins in MCMV-infected 3T3-L1 cells at immediate-early and early times and mock-infected 3T3-L1 cells.

Fig. 9 (A) and (B).

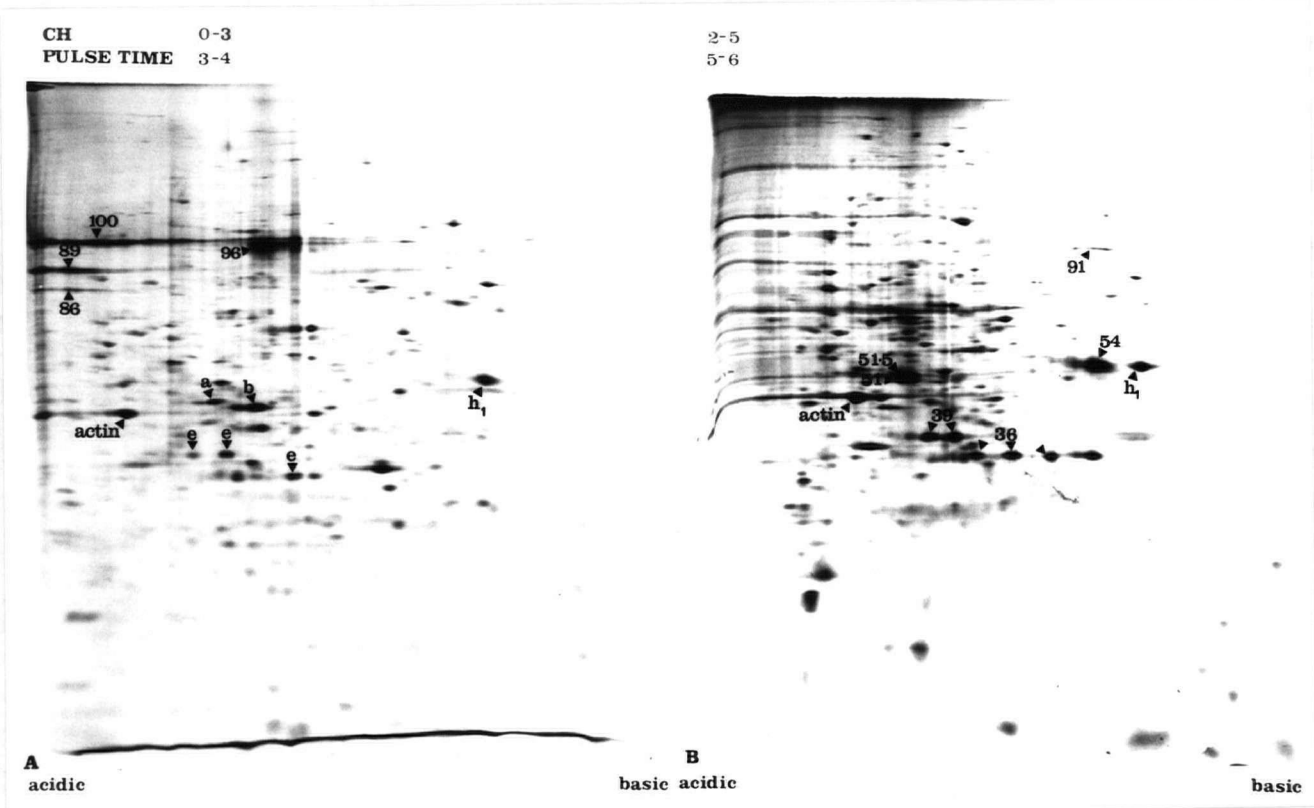


Fig. 9 (C).

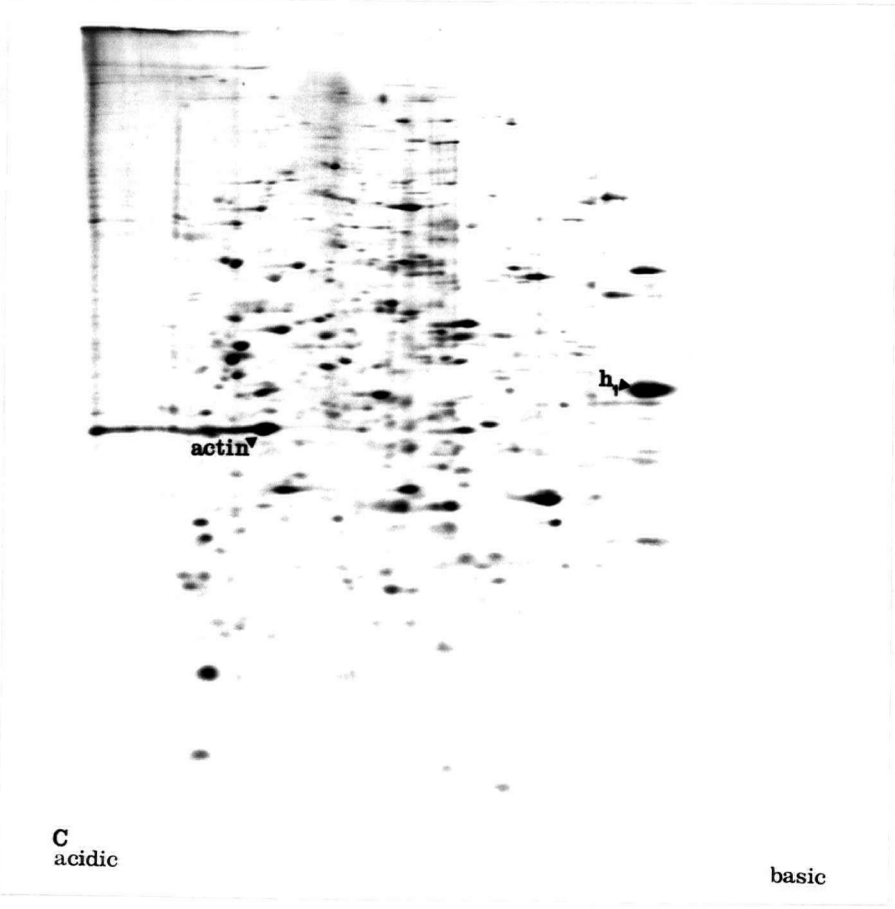
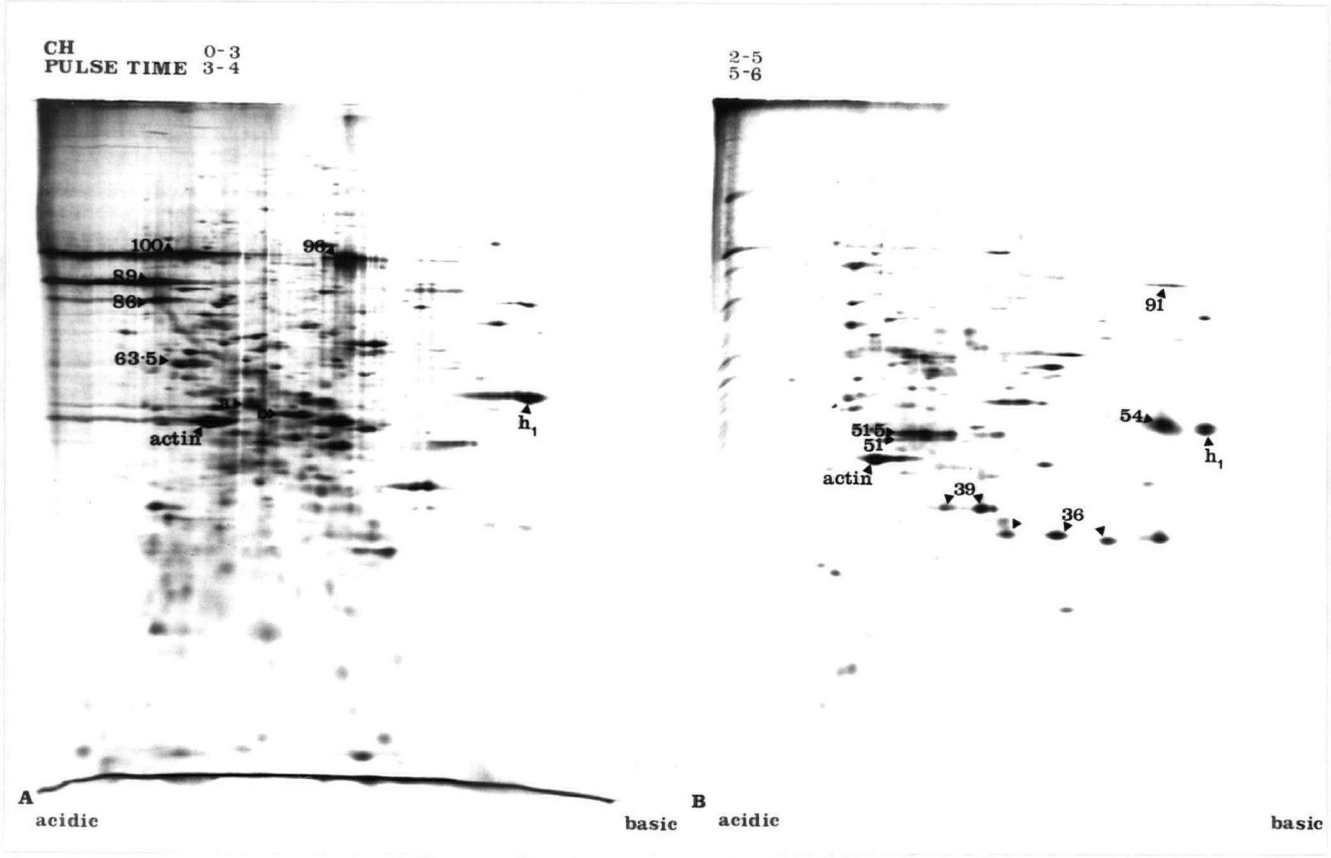


Fig. 10:

Autoradiographic images of (^{35}S) labeled proteins from MCMV-infected J774A.1 cells separated in two dimensions by electrophoresis. J774A.1 cells were infected with MCMV and incubated with cycloheximide from: A) 0-3 hr and B) 2-5 hr pi., then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). C) was prepared as B) but was taken from a different gel. First dimension was run from acidic to basic ends for 3000 V.h.. Arrows indicate virus-induced proteins with their apparent mol. wt. ($x10^{-3}$).

Fig. 10: Two-dimensional separation of proteins in MCMV-infected J774A.1 cells at immediate-early and early times.



C

Some proteins detected by SDS-PAGE are not apparent on these autoradiograms. This was probably due to their insolubility in the urea lysis buffer. The IE proteins of 100K and 89K were poorly soluble in the first dimension lysis buffer as they consistently appeared as streaks on the 2D gels (Figs. 9 A and 10 A). However, the main position of these spots can be seen (Fig. arrowheads) on the acidic side of the gels. The 96K protein can be seen in each case as a diffuse, basic spot. The 86K IE protein had a similar two dimensional separation pattern to the 100K and 89K proteins. The 63.5K IE protein was shown to have an acidic net charge (Fig. 10 A). Although not detectable in Fig. 9 A, this protein has been seen in other gels of infected 3T3-L1 cell proteins. Two spots, marked 'a' and 'b', were seen on gels of infected cell proteins only (Fig. 9 A and 10 A), but because of their closeness in size to actin, they were never detected by SDS-PAGE alone. At present, it is not possible to determine whether they are IE viral-coded proteins or induced cellular proteins, although a band of approximately this size was detected by immunoprecipitation (Fig. 8 A, lane 2). Spots corresponding to the 83.5K and 80K IE proteins could not be identified in either case on these gels.

The 91K early protein was identified as a horizontal streak on the basic side of the gels (Figs. 9 B and 10 B). This probably indicated the presence of several charge isomers that were not being fully resolved. There appeared to be two charge isomers in positions equivalent to the 39K early protein and three corresponding to the 36K early protein (Figs. 9 B and 10 B). Spots marked 'e' on Fig. 9 A refer to early proteins whose synthesis has partially overlapped with the IE time period. The 54K early protein appeared as a diffuse spot on the basic side of the gels in both cases, while there were two sets of twin spots that corresponded to the

51.5K and 51K early proteins (Figs. 9 B and 10 B). This is seen more clearly in Fig. 10 C that was taken from the appropriate section of another gel.

In summary, it can be seen that there was no difference, between the two cell types, in the two-dimensional separation of the major induced proteins.

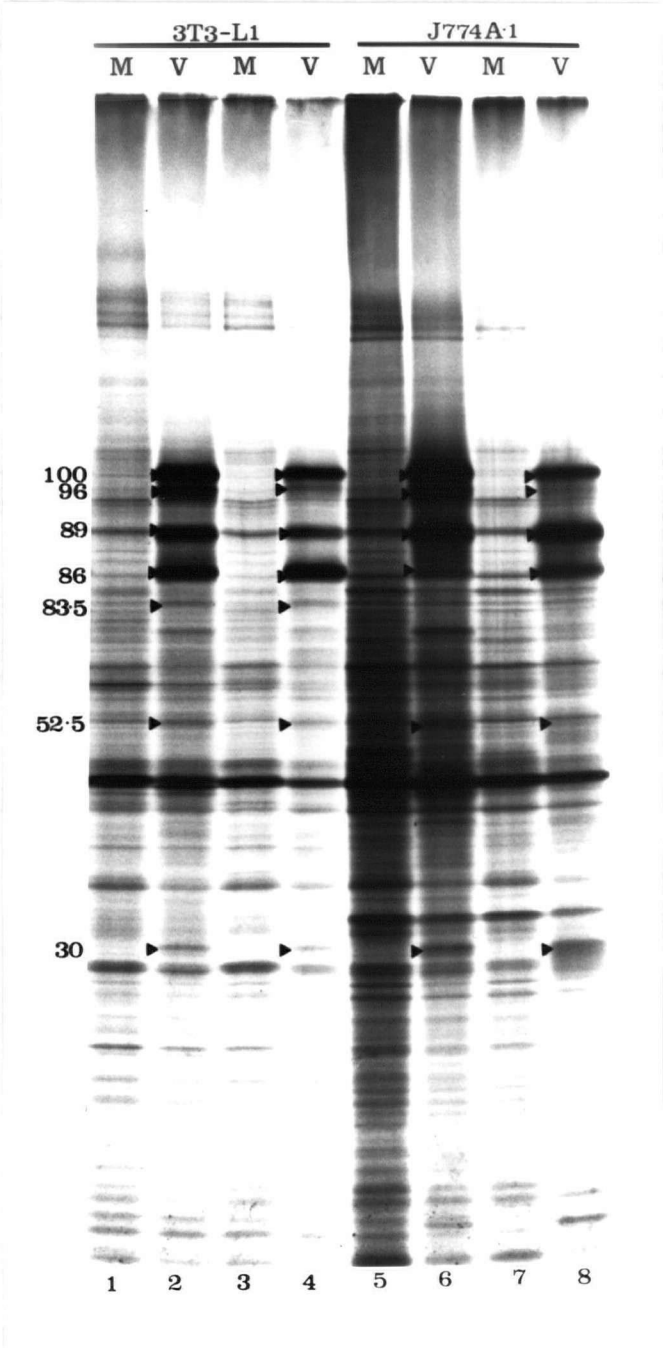
11. Stability and processing of the IE proteins. A series of experiments was performed to study the properties of the identified virus-induced proteins. The first experiment was designed to study the stability and processing of the main IE proteins. Fig. 11 shows a pulse/chase experiment performed with 3T3-L1 and J774A.1 cells that had been treated so as to produce enhanced amounts of labeled IE proteins (details in Fig. legend). The intensities of the bands were measured by scanning densitometry and the areas under each peak were calculated. The intensities of the 96K protein in the 3T3-L1 sample decreased by 69.7% after the chase period and by 77% in samples from J774A.1 cells. The intensities of the 100K and 89K remained within 15% of initial values while the intensities of the 86K proteins increased by 22.8% in 3T3-L1 cells and by 33% in J774A.1 cells. It is not known if the 96K proteins were degraded or processed to the 86K form. As was seen, the 86K protein had an acidic net charge (Fig. 9 A), similar to the 100K and 89K protein, while the 96K protein had a basic net charge. There was no appearance of new bands during the chase period.

12. Distribution of IE and early proteins within infected cells. The distribution of viral-induced proteins within infected cells, particularly their distribution at the time of initiation of viral DNA synthesis, might

Fig. 11:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV infected 3T3-L1 and J774A.1 cells separated in a 10-30% polyacrylamide gel. Replicate cultures of 3T3-L1 and J774A.1 cells were infected with MCMV (V) or mock-infected (M), incubated with cycloheximide 0-3 hr pi. and then labeled 1 hr with (^{35}S) methionine (50 µCi/ml). At 4 hr pi, one culture was harvested. A duplicate was washed and incubated for a further 5 hr. Lanes: 1 : proteins in mock-infected, pulsed 3T3-L1 cells; 2 : proteins in virus-infected, pulsed 3T3-L1 cells; 3 : proteins in mock-infected, chased 3T3-L1 cells; 4 : proteins in virus-infected, chased 3T3-L1 cells; 5 : proteins in mock-infected, pulsed J774A.1 cells; 6 : proteins in virus-infected, pulsed J774A.1 cells; 7 : proteins in mock-infected, chased J774A.1 cells; 8 : proteins in virus-infected, chased J774A.1 cells. Closed arrowheads indicate positions of virus-induced proteins with mol. wt. ($x10^{-3}$) on the left side

Fig. 11: Stability and processing of MCMV proteins in 3T3-L1 and J774A.1 cells at immediate-early times.



give an indication of their function in the replication of the virus. In addition, a defect in cellular transport of a protein, in nonpermissive cells, could indicate a reason for the failure of the virus to replicate (Preston, 1979b).

It had been noticed that there was an overall stimulation of synthesis of cellular nuclear proteins in infected samples (seen in Fig. 12, lane 5 and 7 compared to 1 and 3). This feature will be noticed on a number of other gels, particularly at IE times.

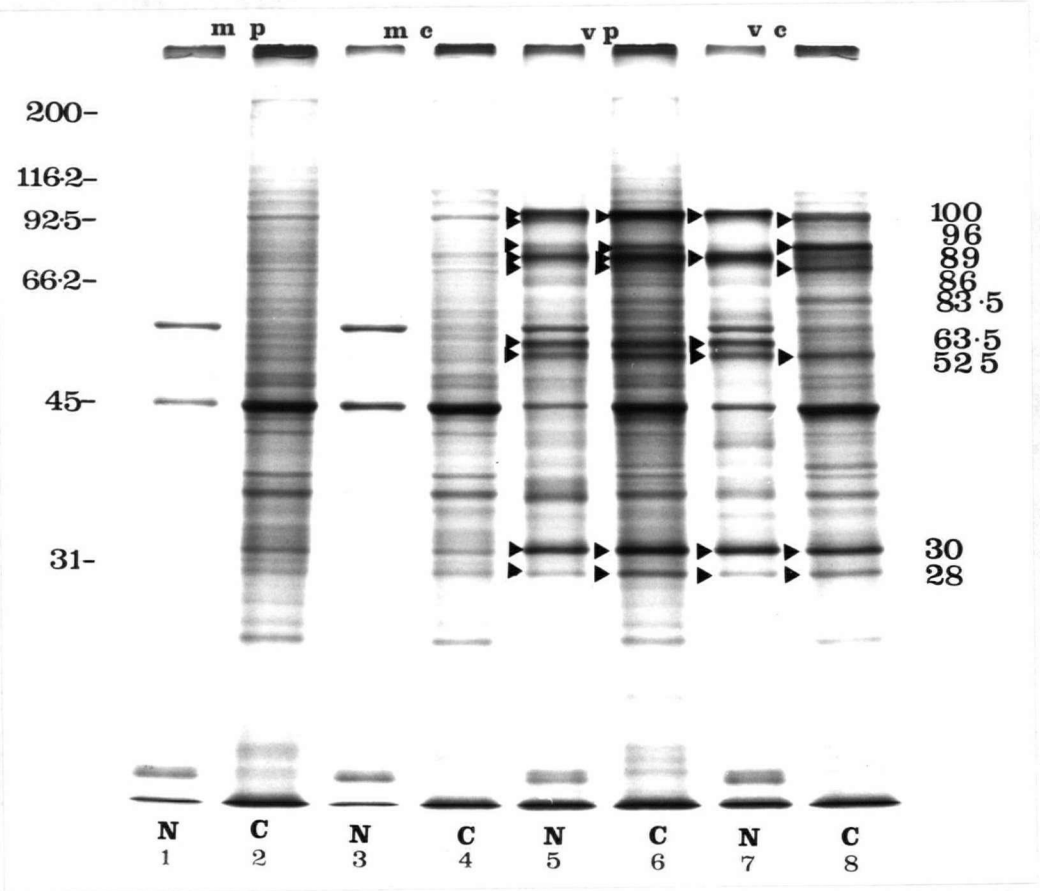
The fractionation technique was designed to handle a number of samples quickly, as proteins can leak from the nuclei if separation takes too long. In addition, the technique was relatively gentle so as not to cause damage to nuclei. Fig. 12 shows an alteration in the distribution of some of the IE viral proteins in 3T3-L1 cells, at the end of the chase period.

At the end of the labeling period, all the IE proteins appeared evenly distributed between nuclei and cytoplasm (Fig. 12, lanes 5 and 6), with the exception of the 96K protein. Although the 96K protein was not clearly separated from the 100K protein in this gel, it appeared only to be associated with the nuclear fraction (lane 5). At the end of the 5 hr chase period, the 100K protein was present in the nuclear fraction (lane 7) and a form with a slightly increased electrophoretic mobility was in the cytoplasmic fraction (lane 8). The 96K protein band had diminished over the chase period, similar to what was shown in Fig. 11. Because of the observed turnover of the 96K protein that was seen in Fig. 11, it could be suggested that the highest mol. wt. virus-induced band in the cytoplasm (lane 8) represented a form of the 100K protein, rather than the 96K protein. At the end of the chase period, the 89K and 83.5 K IE proteins partitioned almost exclusively with the cytoplasmic fraction, while the 86K IE protein was

Fig. 12:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV infected 3T3-L1 cells separated in a 10% polyacrylamide gel. Replicate cultures of 3T3-L1 cells were infected with MCMV (v) or mock-infected (m) and incubated with cycloheximide 0-3 hr pi., then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). At 4 hr pi., one culture was harvested (p) and separated into nuclear and cytoplasmic fractions. A duplicate was washed and further incubated for 5 hr. (c). Lanes: 1 and 2: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, pulsed cells; 3 and 4: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, chased cells; 5 and 6: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected pulsed cells; 7 and 8: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected chased cells. Closed arrowheads indicate positions of virus-induced proteins with mol. wt. ($x10^{-3}$) on the right side.

Fig. 12: Location of IE proteins of MCMV from 3T3-L1 cells after pulse and chase periods. Autoradiographic image of labeled proteins from mock and MCMV infected 3T3-L1 cells separated in a 10% polyacrylamide gel.



mainly nuclear. In addition, the 63.5K protein became exclusively nuclear, while the 52.5K, 30K and 28K proteins remained equally distributed between the nuclear and cytoplasmic fractions. It can be seen that no new band appeared, equivalent to the 91K protein, during the chase period. This indicated that the 91K protein was not a processed form of one of the IE proteins.

The results for the equivalent experiments covering the main time period for early protein synthesis are shown in Fig. 13. The early proteins (91K, 39K and 36K) were detected solely within the nuclear fraction at the end of the pulse labeling period (lane 3). This association remained stable during the chase period (lane 7). The minor early proteins (60K, 54K, 51.5K and 51K) were not detected in these experiments.

These same experiments were performed using MCMV-infected J774A.1 cells. The partitioning of some of the IE viral proteins after the chase period was not as distinct as that observed in infected 3T3-L1 cells (Fig. 14 A). The 100K protein was present in the nucleus and cytoplasm after the chase period. In these experiments, the 96K IE protein was more clearly separated from the 100K protein. Although the intensity of the band had decreased, the gel showed that the 96K protein had remained associated with the nuclei after the chase period. The 89K, 86K, 83.5K and 63.5K proteins did not partition, after the chase period, in the distinct manner described for 3T3-L1 cells. In lane 7 and 8 (Fig. 14), these bands appeared to remain evenly distributed between the nuclear and cytoplasmic fractions. The significance of this is uncertain. The 52.5K, 30K and 28K maintained an equal distribution between nucleus and cytoplasm. In the equivalent gel for early proteins (Fig. 14 B), the 91K, 39K and 36K proteins were present solely in the nuclear fractions during both periods studied.

Fig. 13:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV-infected 3T3-L1 cells separated in a 10% polyacrylamide gel. Replicate cultures of 3T3-L1 cells were infected with MCMV (v) or mock-infected (m) and incubated with cycloheximide from 2-5 hr pi., then labeled 1 hr with (^{35}S) methionine (50 µCi/ml). At 6 hr pi., one culture was harvested (p) and separated into nuclear and cytoplasmic fractions. A duplicate was washed and further incubated for 5 hr (c). Lanes: 1 and 2: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, pulsed cells; 3 and 4: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected, pulsed cells; 5 and 6: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, chased cells; 7 and 8: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected, chased cells. Virus-induced IE proteins are indicated by closed arrowheads and early proteins by open arrowheads, with apparent mol. wt. ($x10^{-3}$).

Fig. 13: Location of early proteins of MCMV from 3T3-L1 cells after pulse and chase periods. Autoradiographic image of labeled proteins from mock and MCMV-infected 3T3-L1 cells separated in a 10% polyacrylamide gel.

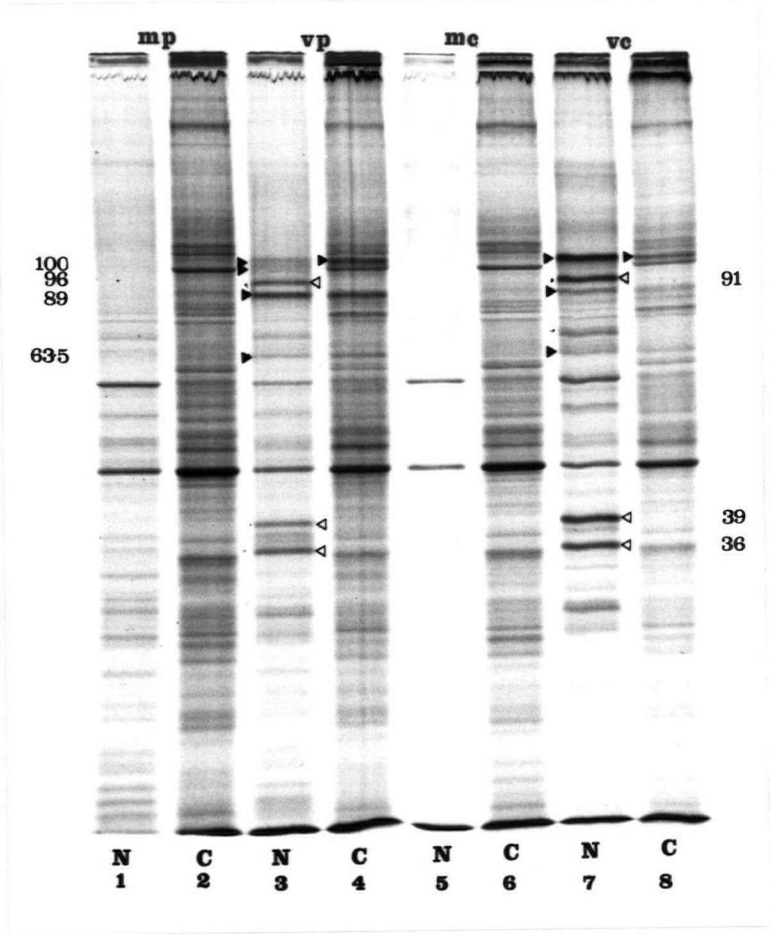


Fig. 14:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV-infected J774A.1 cells separated in a 10% polyacrylamide gel. Replicate cultures of J774A.1 cells were infected (V) or mock-infected (M) and incubated with cycloheximide from 0-3 hr pi. (A) or 2-5 hr pi. (B) then labeled 1 hr with (^{35}S) methionine (50 µCi/ml). At 4 hr pi. (A) or 6 hr pi. (B), one culture was harvested (p) and separated into nuclear and cytoplasmic fractions. A duplicate was washed and further incubated for 5 hr (c). Lanes: 1 and 2: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, pulsed cells; 3 and 4: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected, chased cells; 5 and 6: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected, pulsed cells; 7 and 8: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected, chased cells.

Virus-induced proteins indicated by closed arrowheads with apparent mol. wt. ($x10^{-3}$) on right side.

Fig. 14: Location of IE and early proteins of MCMV from J774A.1 cells after pulse and chase periods. Autoradiographic image of labeled proteins from mock and MCMV infected J774A.1 cells separated in a 10% polyacrylamide gel.

Fig. 14 (A).

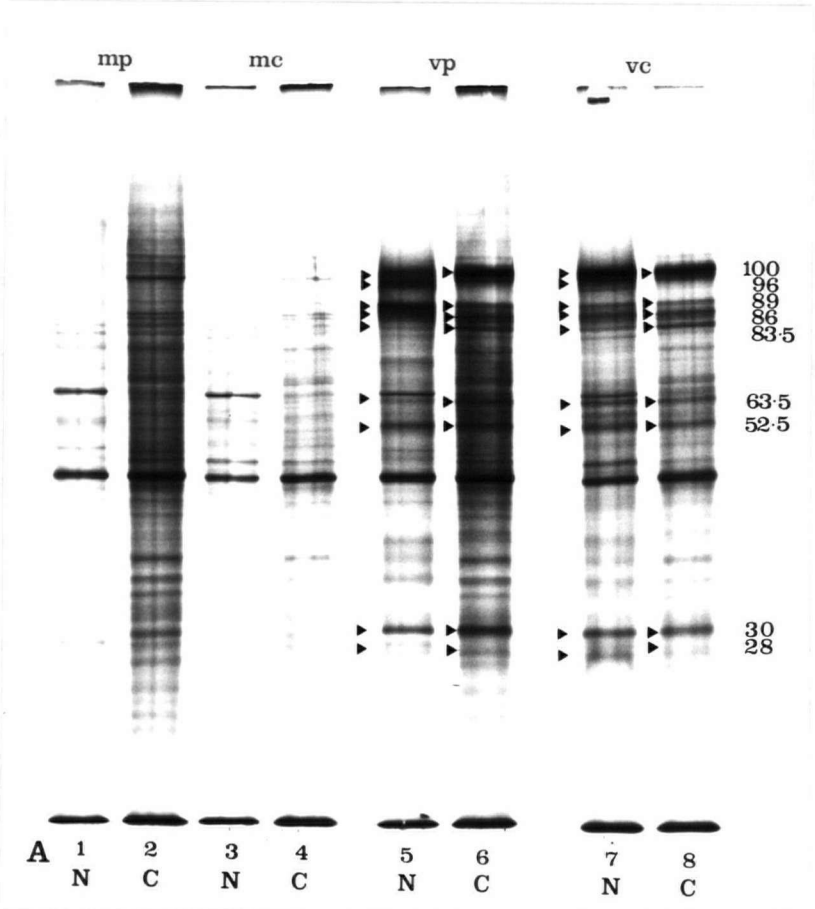
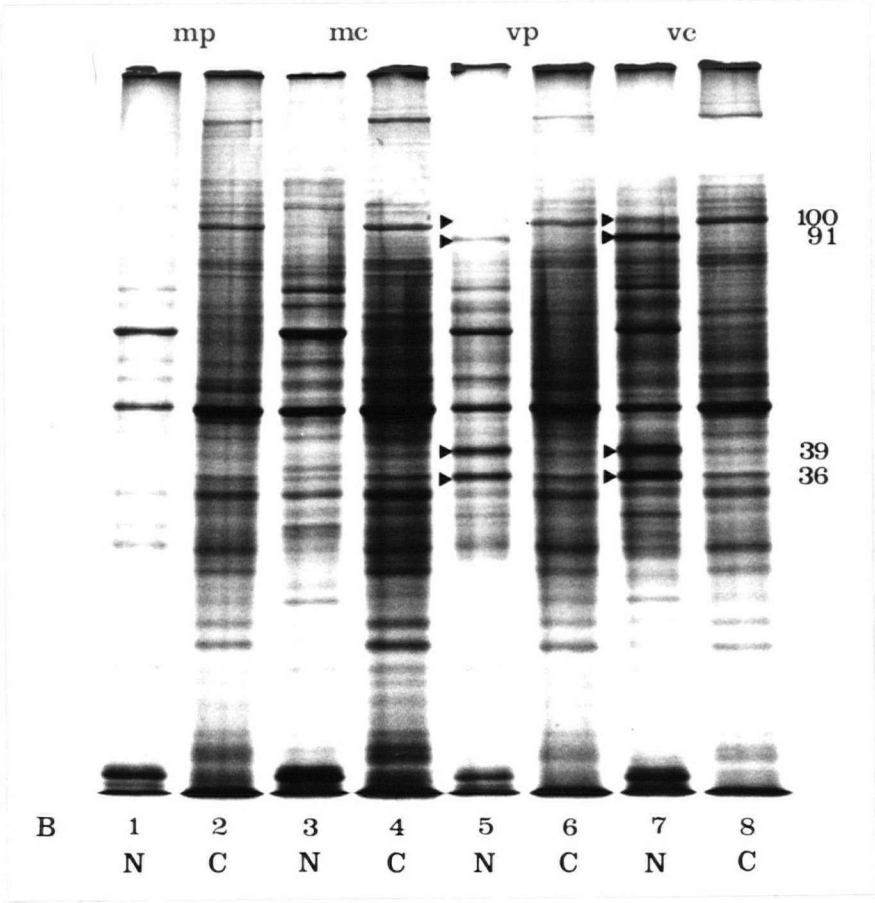


Fig. 14 (B).



13. IE and early protein distribution under unenhanced conditions. To determine if cycloheximide-enhancement had an effect on the synthesis or localization of any of the virus-induced proteins, cultures of mock and MCMV-infected 3T3-L1 cells and J774A.1 cells were labeled, without cycloheximide pretreatment, from 1-4 hr pi. This time period covered the main period of synthesis of the IE and early proteins.

Fig 15 shows that 8/10 of the IE and 4/6 of the early proteins could be identified in nuclear fractions of unenhanced infected 3T3-L1 and J774A.1 cells, with the same estimated mol. wts. as determined previously. The 83.5K and 80K IE and the 54K, 51.5K and 51K early proteins were not detected using these labeling conditions. The main feature to note from this experiment was the distribution of the induced proteins under unenhanced conditions. In previous experiments (shown in Fig. 7 A and 12 A), where enhancement of the viral proteins had occurred due to cycloheximide pretreatment, a higher or equal proportion of the 100K, 89K, 83.5K, 52.5K, 30K and 28K proteins partitioned with the cytoplasmic fractions. Using unenhanced conditions, the virus-induced proteins adopted a predominantly nuclear association and only a small proportion of the 100K, 89K and 30K IE proteins were detected in cytoplasmic fractions of infected cells. The same numbers of virus-induced proteins, with the same distribution, were observed in 3T3-L1 cells as in J774A.1 cells in these experiments.

To study further the distribution of viral-induced proteins, nuclei from NP40-solubilized cells were treated to remove the cytoskeletal framework material that adheres to the nuclear membrane (Penman, 1966). Treatment with a Tween40/DOC detergent mixture removes the cytoplasmic 'tags' without disrupting the nuclei.

Fig. 16 A and B shows the distribution of proteins in 3T3-L1 and

Fig. 15:

Autoradiographic images of (^{35}S) labeled proteins from MCMV-infected (v) and mock-infected (m) 3T3-L1 and J774A.1 cells separated into nuclear (A) and cytoplasmic (B) fractions and separated in 10% polyacrylamide gels. Cultures of 3T3-L1 and J774A-1 cells were infected with MCMV or mock-infected and labeled with (^{35}S) methionine (25 μCi/ml) from 1-4 hr pi. Closed arrowheads indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$) on left side.

Fig. 15: Location of IE and early proteins of MCMV from 3T3-L1 and J774A.1 cells produced under unenhanced conditions. Autoradiographic image of labeled proteins from mock and MCMV infected 3T3-L1 and J774A.1 cells separated in 10% polyacrylamide gels.

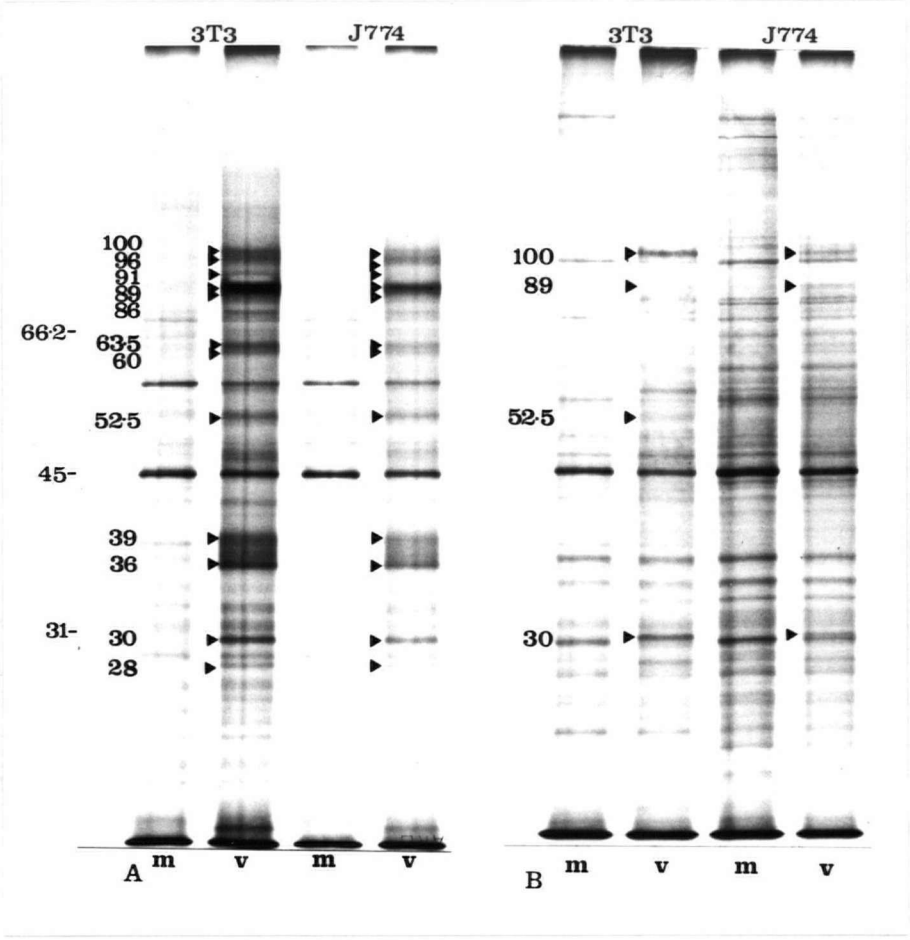


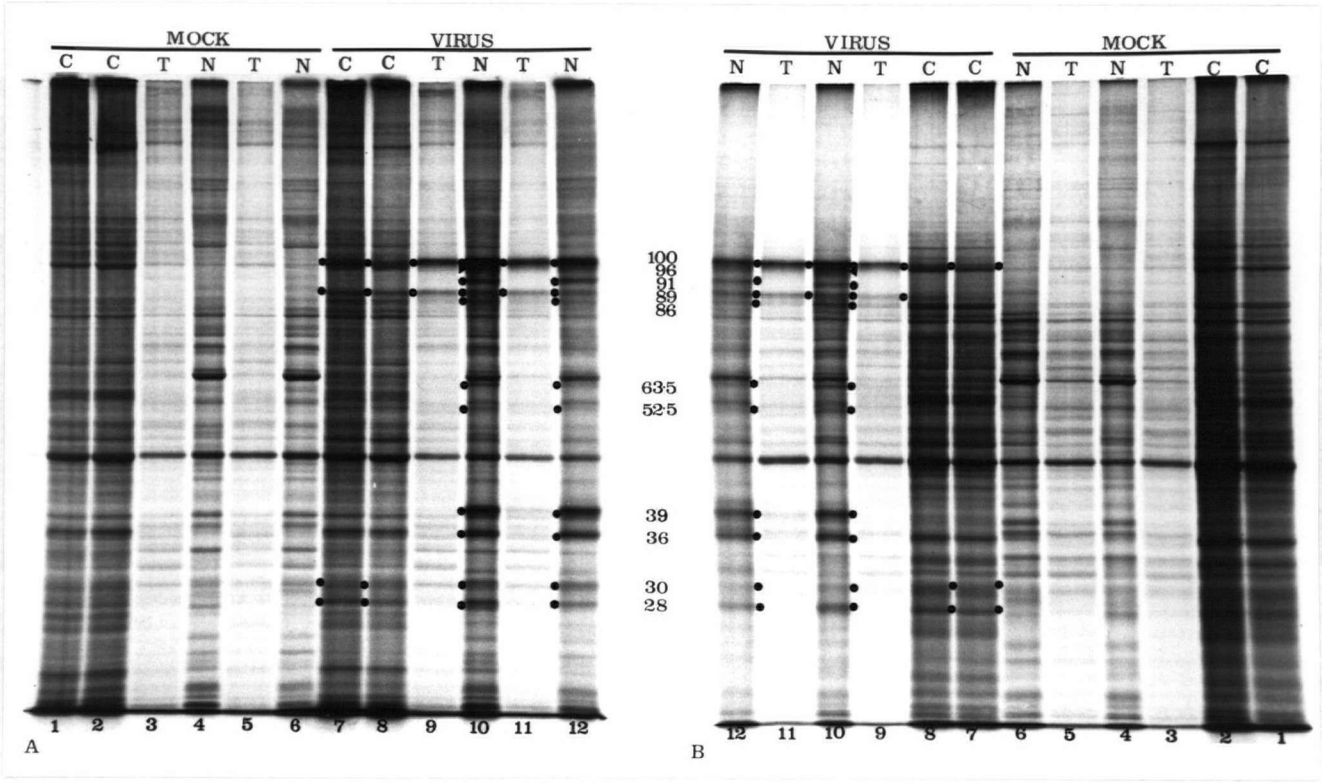
Fig. 16:

Autoradiographic images of (^{35}S) labeled proteins from mock and MCMV-infected 3T3-L1 (A) and J774A.1 (B) cells separated into nuclear (N), cytoskeletal 'tag' (T) and cytoplasmic (C) fractions and separated in 10% polyacrylamide gels. Duplicate sets of cultures were MCMV or mock-infected and labeled 1-4 hr pi. with (^{35}S) methionine (25 μCi/ml). One set was harvested at 4 hr pi. and fractionated, while the other set was rinsed and incubated for a further 5 hr before fractionation.

Lanes: 1: mock-infected cytoplasmic proteins (4 hr); 2: mock-infected cytoplasmic proteins (9 hr); 3: mock-infected 'tags' proteins (4 hr); 4: mock-infected nuclear proteins (4 hr); 5: mock-infected 'tags' proteins (9 hr); 6: mock-infected nuclear proteins (9 hr); 7: virus-infected cytoplasmic proteins (4 hr); 8: virus-infected cytoplasmic proteins (9 hr); 9: virus-infected 'tags' proteins (4 hr); 10: virus-infected nuclear proteins (4 hr); 11: virus-infected 'tags' proteins (9 hr); 12: virus-infected nuclear proteins (9 hr).

Spots indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$) in center. The 96K IE protein is designated by a closed arrowhead.

Fig. 16: Location of IE and early proteins of MCMV from 3T3-L1 and J774A.1 cells produced under unenhanced conditions. Autoradiographic image of labeled proteins from mock and MCMV infected 3T3-L1 and J774A.1 cells fractionated into nuclear, cytoplasmic 'tags' and cytoplasmic fractions and separated in a 10% polyacrylamide gel.



J774A.1 cells at 4 hr and 9 hr pi. The experimental details are described in Fig. 16 legend. These autoradiograms showed that virus-induced proteins from both types of cells partitioned into the three fractions in the same manner. Of particular interest was the behaviour of the major IE proteins. The 100K and 89K proteins were shown to be present in each fraction in both pulse and chase samples. The distribution did not appear to change over the 5 hr chase period. This experiment does not give any indication as to the site of action of these proteins. The efficiency of the extraction procedure was checked by phase contrast microscopy. Following Tween 40/DOC treatment, the nuclei appeared 'clean', with only a small percentage having any adhering material visible. This means that the location of the 100K and 89K proteins, attached to the cytoskeletal framework and also in the nuclei would be related to their function within the infected cell. The 96K protein was present only in the nuclear fraction after the pulse labeling and this band had become undetectable at the end of the chase period. The 63.5K IE and 91K, 39K and 36K early proteins had a stable association with the nuclei. The 52.5K, 30K and 28K proteins could be detected mainly in the nuclear fractions, but small amounts also appeared in the cytoplasmic fractions.

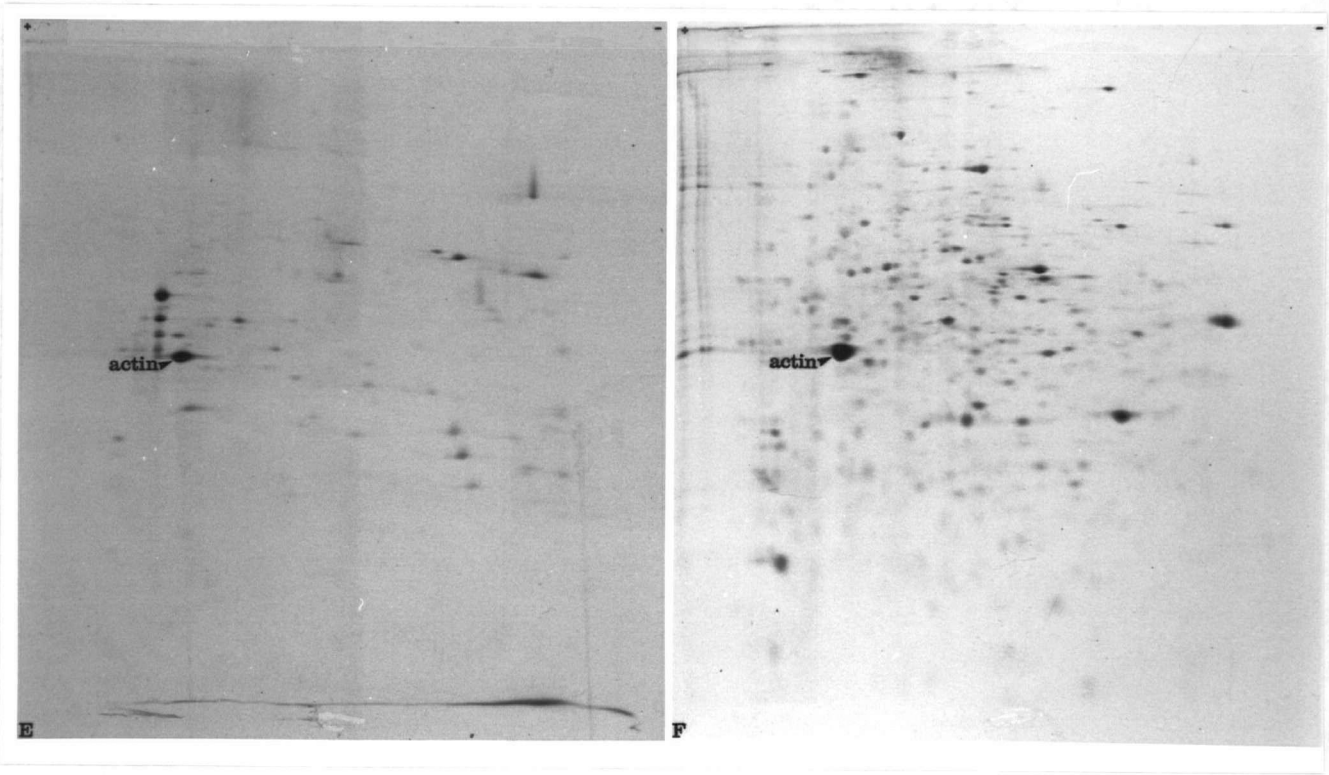
14. Two-dimensional analysis of fractionated samples. Two-dimensional analysis of fractionated samples identified some properties of the virus-induced proteins not shown by the other experiments. In the 2D gels of proteins from the nuclear and cytoplasmic fractions of MCMV-infected 3T3-L1 cells (Fig. 17 A and B), labeled to detect IE proteins, only the major IE virus-induced proteins were detected. In Fig. 17 A, the positions of the IE proteins of 100K, 89K and 86K are now shown clearly as spots on

Fig. 17:

Autoradiographic images of (^{35}S) labeled proteins of lysates of nuclear A), C) and E) and cytoplasmic B), D), and F) fractions of MCMV (A-D) and mock-infected (E, F) 3T3-L1 cells separated in two dimensions by electrophoresis. Replicate cultures were infected with MCMV and incubated with cycloheximide 0-3 hr pi. (A and B) or 2-5 hr pi. (C and D) and labeled with (^{35}S) methionine (50 µCi/ml) for 1 hr. Mock-infected cultures were incubated with cycloheximide 0-3 hr pi. and labeled with (^{35}S) methionine for 1 hr. Cells were fractionated into nuclear and cytoplasmic fractions, dissolved in urea lysis buffer and separated in the first dimension (left to right) (acid (+) to base (-)) for 3000 V.h.. Closed arrowheads indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$).

Fig. 17: Two-dimensional separation of proteins from nucleus and cytoplasm of mock and MCMV-infected 3T3-L1 cells at immediate-early and early times.

Fig. 17 (cont.)



the acidic side of the gel, as compared to previous examples (Figs. 9 and 10) where extensive streaking of these proteins in the first dimension was observed. The poor solubility of the 100K and 89K proteins is still seen as demonstrated by some streaking in the first dimension. There was no evidence of the 96K IE protein being present in the cytoplasmic fraction (Fig 17 B).

Figs. 17 C and D show the autoradiograms for cells labeled to detect early viral-induced proteins. A horizontal streak on the basic side of the gel appears to correspond to the 91K protein (this was resolved more clearly in Fig. 19 B into three or four charge isomers). Two spots, that appeared to correspond to the 39K protein, and three spots, that appeared to correspond to the 36K early protein, were only detected in the nuclear fractions. Without any further evidence, it is being assumed that each set of spots represent charge isomers of the same protein. Also shown are two spots corresponding to the 30K IE protein. Fig. 19 B has two spots each, that appear to correspond to the 30K and 28K IE proteins. The minor early proteins (54K, 51.5K and 51K), that could not be detected by one-dimensional electrophoresis on gels of uniform acrylamide composition (Fig. 7), were shown to be exclusively cytoplasmic (Fig. 17 D). The profiles of nuclear and cytoplasmic fractions from mock-infected 3T3-L1 cells are shown in Fig. 17 E and F. These represent fractions from cells labeled at IE times, but the same proteins were shown in mock-infected cells labeled at early times (data not shown).

Figs. 18 A and B shows that the same major IE and early proteins were detected in nuclear fractions of MCMV-infected J774A.1 as in 3T3-L1 cells. These proteins all had the same two-dimensional separation as those synthesized in permissive 3T3-L1 cells.

Fig. 19 shows the two-dimensional profile of proteins from nuclear

Fig. 18:

Autoradiographic images of (^{35}S) labeled proteins of lysates of nuclear fractions of MCMV-infected J774A.1 cells separated in two dimensions by electrophoresis. Cultures were infected with MCMV and incubated with cycloheximide from 0-3 hr pi. (A) or 2-5 hr pi. (B) and labeled with (^{35}S) methionine (50 μCi/ml) for 1 hr. Cells were fractionated into nuclear and cytoplasmic fractions by treatment with NP40 buffer, and nuclear fractions were dissolved in urea lysis buffer and separated in the first dimension (left to right) (acid (+) to base (-)) for 3000 V.h.. Closed arrowheads indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$).

Fig. 18: Two-dimensional separation of proteins from nuclear fractions of MCMV-infected J774A.1 cells at immediate-early and early times.

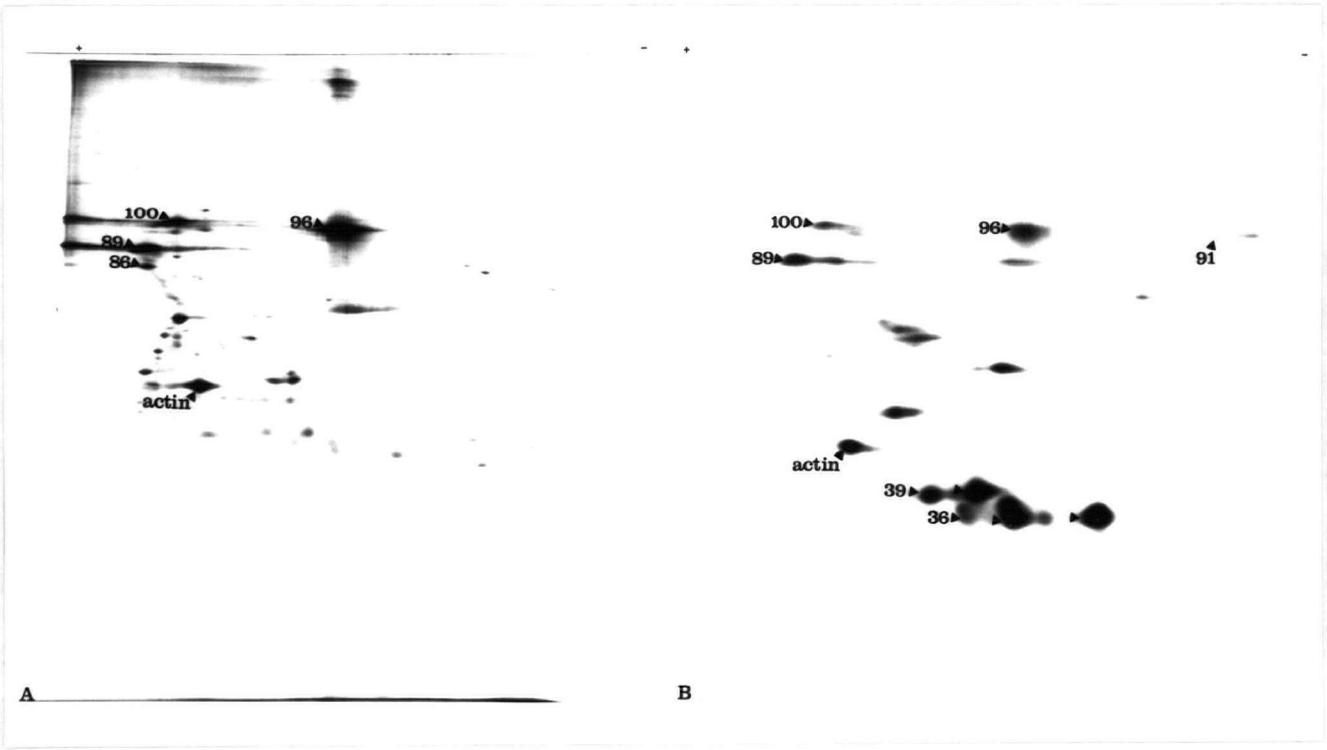
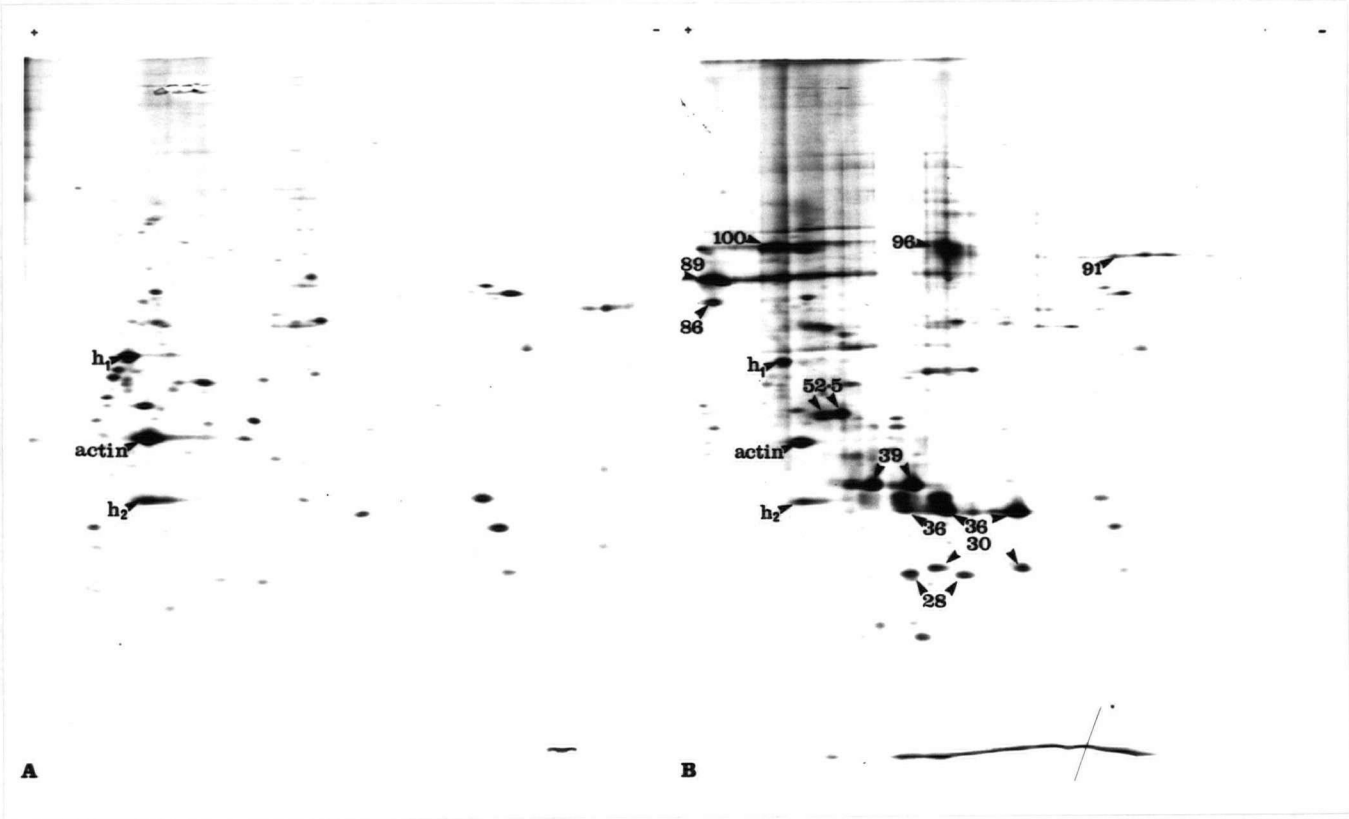


Fig. 19:

Autoradiographic images of (^{35}S) labeled proteins of lysates of nuclear fractions of mock (A) and MCMV (B)-infected 3T3-L1 cells separated in two dimensions by electrophoresis. Cultures of 3T3-L1 cells were infected with MCMV or mock-infected and labeled with (^{35}S) methionine (25 μCi/ml) from 1-4 hr pi. The first dimension was run from left to right (acid (+) to base (-)) for 3000 V.h.. Closed arrowheads denote virus-induced proteins with apparent mol. wt. ($x10^{-3}$). h_1, h_2 and actin designate major cellular proteins.

Fig. 19: Two-dimensional separation of proteins from nuclear fractions of mock and MCMV-infected 3T3-L1 cells labeled from 1-4 hr. pi.



fractions of mock (A) and MCMV-infected (B) 3T3-L1 cells, that were labeled from 1-4 hr pi. without cycloheximide treatment. This gel showed that cycloheximide pretreatment did not affect the migration properties of the proteins synthesized. In addition to the features referred to, two spots that appeared to correspond to the 52.5K protein were identified.

15. Identification of infected cell phosphoproteins. The major IE proteins of several strains of human and simian cytomegalovirus have been shown to be phosphoproteins (Gibson, 1981), as have the main IE proteins of herpes simplex virus (Pereira et al., 1977).

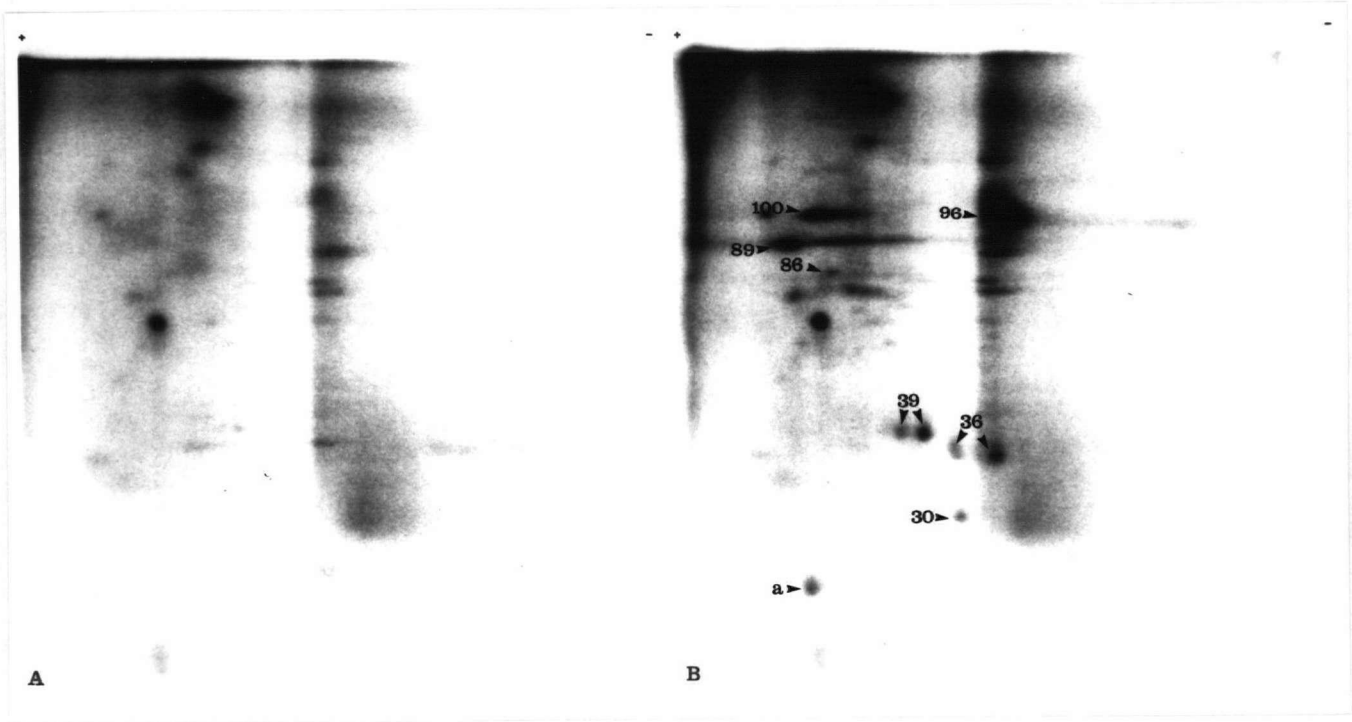
The 100K, 96K, 89K (and 86K) IE proteins and the 39 and 36K early proteins were biosynthetically labeled when MCMV-infected 3T3-L1 cells were incubated with (^{32}P) orthophosphate (Fig. 20 B). One of the isomers of the 30K protein was also phosphorylated. The same proteins were also phosphorylated when synthesized in J774A.1 cells, except for the 30K isomer (Fig. 20 C). This may only represent a difference in sensitivity between the two cell types in detecting minor labeled proteins. In addition, a spot (marked 'a' on the gels) of around 15K mol. wt. was observed only in infected extracts of both cell types. This did not coincide with any of the identified viral proteins. The indicated position of the 86K phosphoprotein in Fig. 20 B and C was different from its position on gels of (^{35}S) methionine labeled proteins (e.g. Fig. 19 B). It can be suggested that this may represent a minor isomer of the 86K protein, which is not easily detectable on the gels of total proteins, or this may represent a cellular protein that becomes phosphorylated as a consequence of virus infection.

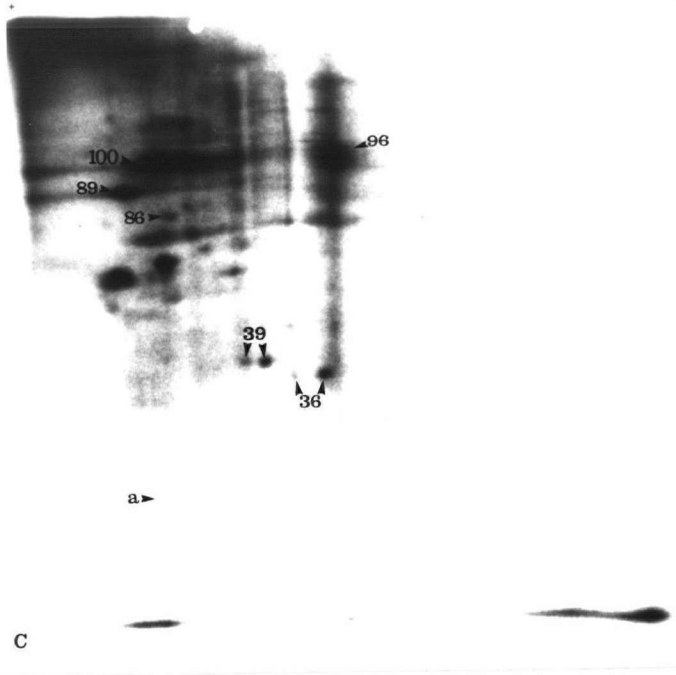
16. MCMV-induced IE and early proteins in human fibroblasts. It has

Fig. 20:

Autoradiographic image of (^{32}P) labeled proteins of nuclear fractions of mock-infected (A) and MCMV-infected 3T3-L1(B) and MCMV-infected J774A.1 (C) cells treated under the following conditions and separated in two dimensions by electrophoresis. Cultures were infected with MCMV and incubated with cycloheximide 2-5 hr pi. and then labeled with (^{32}P) orthophosphate (200 μCi/ml) for 2 hr in the presence of 10 μg/ml actinomycin D. Cells were separated into nuclear and cytoplasmic fractions. The first dimension was run from left to right (acid to base) for 3000 V.h. Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. ($\times 10^{-3}$).

Fig. 20: Two-dimensional separation of phosphate-labeled proteins from nuclear fractions of mock and MCMV-infected 3T3-L1 and MCMV-infected J774A.1 cells labeled at early times after infection.





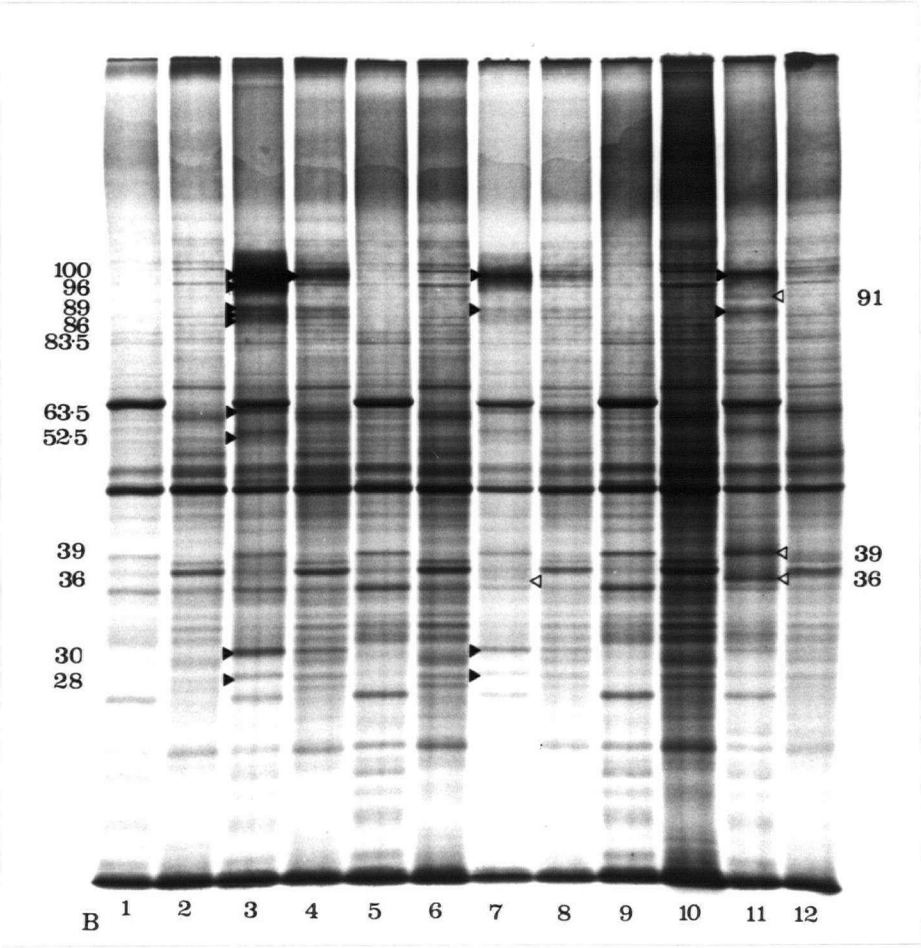
been shown that a small amount of MCMV replication occurred following infection of J774A.1 cells, while human fibroblasts were totally nonpermissive to MCMV. As the immediate-early and early proteins appeared to be synthesized in J774A.1 cells in the same pattern as those seen in 3T3-L1 cells, it was necessary to answer the question as to whether MCMV gene expression in J774A.1 cells was different from that seen in a fully nonpermissive cell system. The IE and early proteins synthesized in infected human foreskin fibroblasts (HFF) cells were compared with those from 3T3-L1 and J774A.1 cells.

Infection of HFF cells with MCMV produced, within 4 hrs, the characteristic early cytopathic effect that was seen in infected 3T3-L1 and J774A.1 cells. This indicated that some viral gene expression occurred in these cells. The previous types of experiments were repeated with MCMV-infected HFF cells. After the reversal of a cycloheximide block, the major IE proteins (100K, 96K and 89K) were synthesized in elevated amounts (Fig. 21, lane 3). The other previously identified MCMV IE proteins of 86K, 83.5K, 63.5, 52.5K, 30K and 28K were also detected in nuclear fractions of these cells. The switch to early protein synthesis occurred in these cells. The 91K and 36K could be detected by 3 hr pi. (Fig. 21, lanes 7), but the initial appearance of the 39K early protein was not detected, as human fibroblasts have a cellular protein of the same mol. wt.. Higher levels of the 91K, 39K and 36K proteins were detected in lane 11, which detected proteins labeled over the period equivalent to 3-4 hr pi.. The synthesis of these early proteins appeared to be slightly delayed in these samples compared with MCMV-infected 3T3-L1 cells. However, the significance of this is uncertain as it seemed to have a variable pattern, perhaps related to the physiological state of the cells.

Fig. 21:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV-infected human fibroblast (HFF) cells separated in a 10% polyacrylamide gel. Cultures of HFF cells were infected with MCMV or mock-infected and incubated with cycloheximide 0-3 hr pi. (lanes 1-4), 2-5 hr pi. (lanes 5-8) or 3-6 hr pi. (lanes 9-12) and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). After labeling, cells were separated into nuclear and cytoplasmic fractions. Lanes: 1, 5 and 9: proteins in mock-infected nuclear fractions; 2, 6 and 10: proteins in mock-infected cytoplasmic fractions; 3, 7 and 11: proteins in virus-infected nuclear fractions; 4, 8 and 12: proteins in virus-infected cytoplasmic fractions.
Closed arrowheads indicate positions of virus-induced IE proteins and open arrowheads indicate positions of virus-induced early proteins, with apparent mol. wts. ($x10^{-3}$).

Fig. 21: Proteins from mock and MCMV-infected human fibroblast (HFF) cells labeled at IE and early times after infection.



The same number of nuclear IE and early proteins, with the same mol. wts., were synthesized in MCMV-infected HFF cells as were synthesized in 3T3-L1 cells under the same conditions.. In addition, the one and two-dimensional separations of the proteins, that had been synthesized without cycloheximide treatment (Figs. 22 and 23), showed the same number of bands (spots) as equivalent samples from 3T3-L1 cells (Figs. 15 and 19). One feature seen in Figs. 21 and 22 (compared to Figs. 12 and 15) was the stronger partitioning of the 100K protein with the nucleus. The second experiment showed that the abnormal partitioning was not caused by the cycloheximide treatment of HFF cells.

In a similar experiment to that shown in Fig. 16 for 3T3-L1 and J774A.1 cells, MCMV-infected HFF cells were fractionated into nuclear, cytoskeletal 'tags' and cytoplasmic fractions (Fig. 24). The numbers of proteins detected were the same as observed with 3T3-L1 and J774A.1 cells (Fig. 16), but there was again a difference in the partitioning of the 100K protein and also 89K protein. The 100K IE protein remained mainly with the nucleus (lanes 10 and 12), with only a small proportion being associated with the cytoskeletal framework (T)(lanes 9 and 11) and none detected in the cytoplasm (lanes 7 and 8). This partitioning remained the same over the 5 hr chase period. It could be seen that the 89K protein also partitioned mainly with the nucleus, but the proportion in the cytoplasm increased over the 5 hr chase period (Fig. 24, lane 8). This was in contrast to what was seen with 3T3-L1 and J774A.1 cells (Fig 16 A and B) where a significant proportion of the 100K and 89K proteins partitioned with the cytoplasm and the cytoskeletal framework.

These results give an indirect indication of a cytoplasmic site of action for the major IE proteins or of a repressor function when localized in the nucleus.

Fig. 22:

Autoradiographic images of (^{35}S) labeled proteins from MCMV-infected and mock-infected HFF cells separated into nuclear and cytoplasmic fractions and separated in 10% polyacrylamide gels. Cultures of HFF cells were mock or MCMV-infected and labeled with (^{35}S) methionine (25 μCi/ml) from 1-4 hr pi.

Lanes: 1, proteins from nuclear fraction of mock-infected cells; 2, proteins from nuclear fraction of virus-infected cells; 3, proteins from cytoplasmic fraction of mock-infected cells; 4, proteins from cytoplasmic fraction of virus-infected cells.

Closed arrowheads indicate positions of virus-induced IE proteins and open arrowheads indicate positions of virus-induced early proteins, with apparent mol. wt. ($x10^{-3}$).

Fig. 22: Location of IE and early proteins of MCMV from HFF cells produced under unenhanced conditions. Autoradiographic image of labeled proteins from mock and MCMV infected HFF cells separated in a 10% polyacrylamide gel.

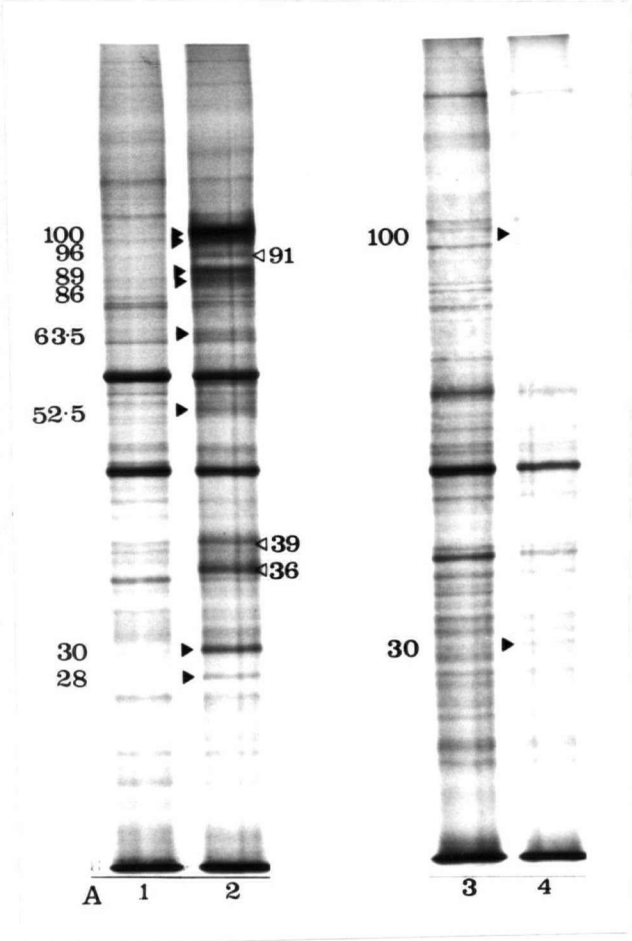


Fig. 23:

Autoradiographic images of (^{35}S) labeled proteins in lysates of nuclear fractions of mock (A) and MCMV (B)-infected HFF cells separated in two dimensions by electrophoresis. Cultures of HFF cells were MCMV or mock-infected and labeled with (^{35}S) methionine (25 µCi/ml) from 1-4 hr pi. The first dimension was run from left to right (acid (+) to base (-)) for 3000 V.h.. Closed arrowheads denote virus-induced proteins with apparent mol. wt. ($x10^{-3}$).

Fig. 23: Two-dimensional separation of proteins from nuclear fractions of mock and MCMV-infected HFF cells labeled from 1-4 hr. pi.

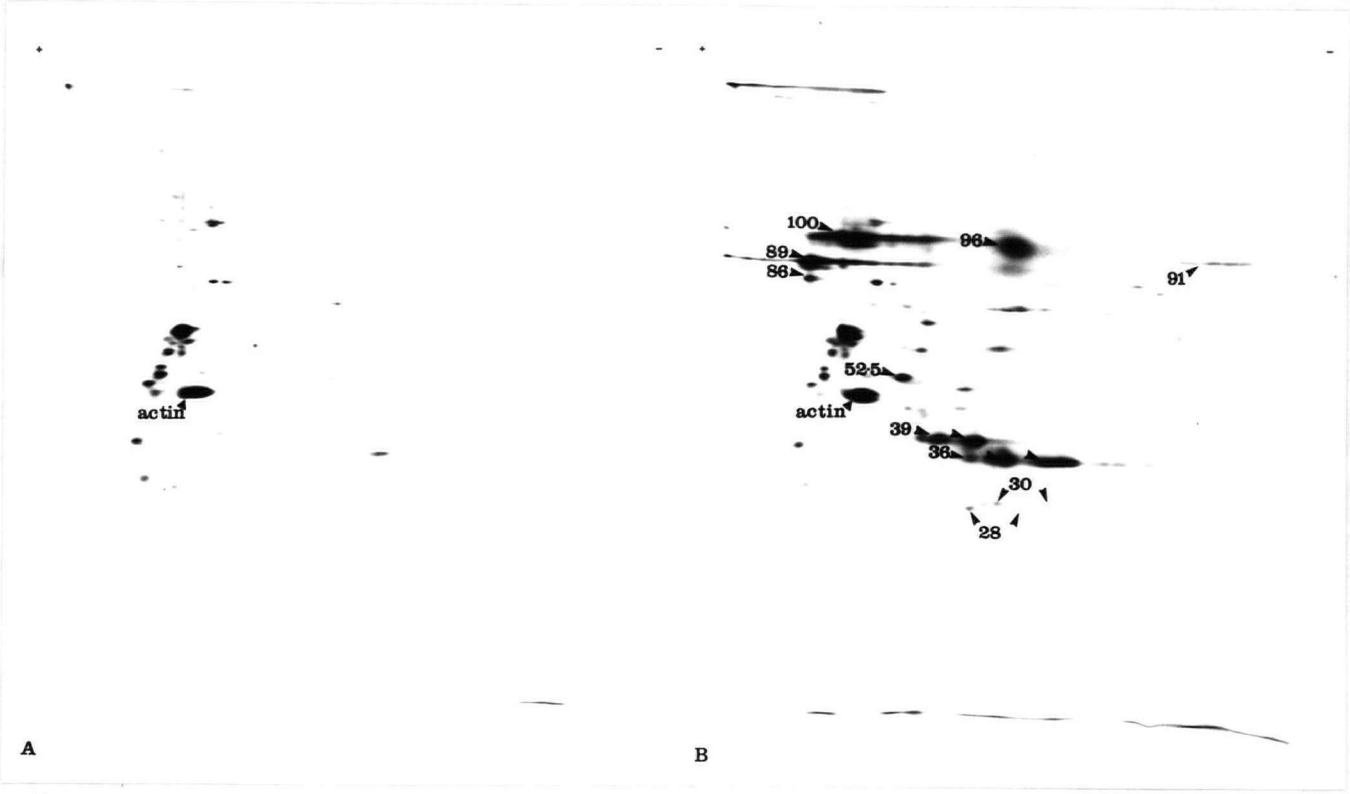


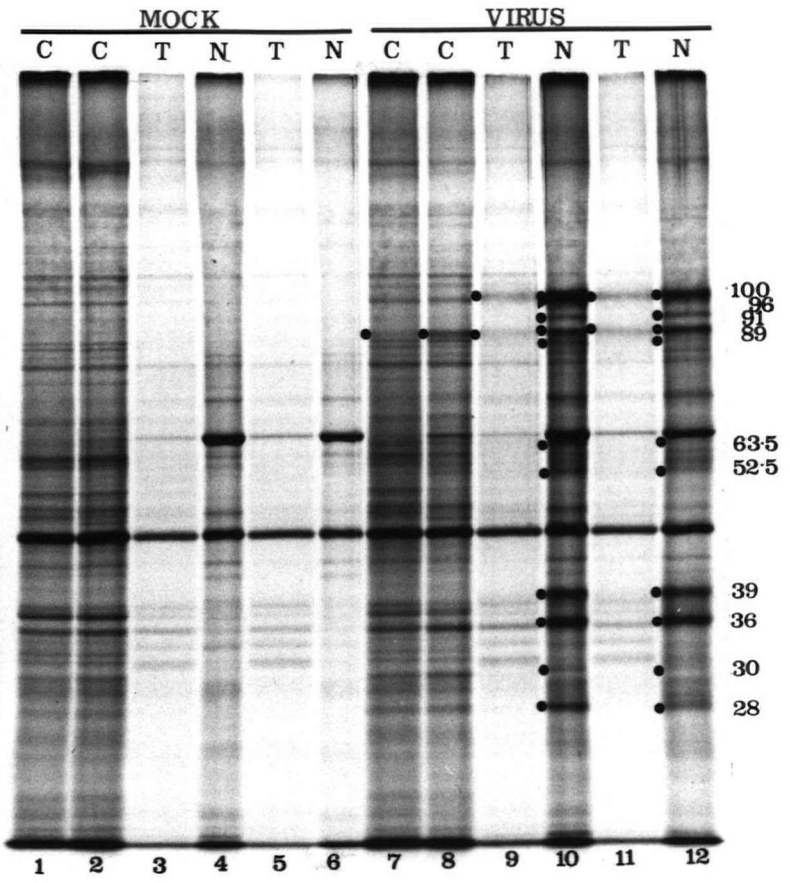
Fig. 24:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV-infected HFF cells separated into nuclear (N), cytoskeletal 'tag' (T) and cytoplasmic (C) fractions and separated in 10% polyacrylamide gels. Duplicate sets of cultures were MCMV or mock-infected and labeled 1-4 hr pi. with (^{35}S) methionine (25 µCi/ml). One set was harvested at 4 hr pi. and fractionated, while the other set was rinsed and incubated a further 5 hr before fractionation.

Lanes: 1, mock-infected cytoplasmic proteins (4 hr); 2, mock-infected cytoplasmic proteins (9 hr); 3, mock-infected 'tags' proteins (4 hr); 4, mock-infected nuclear proteins (4 hr); 5, mock-infected 'tags' proteins (9 hr); 6, mock-infected nuclear proteins (9 hr); 7, virus-infected cytoplasmic proteins (4 hr); 8, virus-infected cytoplasmic proteins (9 hr); 9, virus-infected 'tags' proteins (4 hr); 10, virus-infected nuclear proteins (4 hr); 11, virus-infected 'tags' proteins (9 hr); 12, virus-infected nuclear proteins (9 hr);

Spots indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$) on right side. The 96K IE protein is designated by a closed arrowhead.

Fig. 24: Location of IE and early proteins of MCMV from HFF cells produced under unenhanced conditions. Autoradiographic image of labeled proteins from mock and MCMV infected HFF cells fractionated into nuclear, cytoplasmic 'tags' and cytoplasmic fractions and separated in a 10% polyacrylamide gel.



17. Identification of virus-induced DNA-binding proteins.

a) Extraction procedure. The extraction procedure used to isolate proteins had been reported to be superior to others (Purifoy and Powell, 1976) for producing the best yields of soluble proteins that are essentially DNA-free. The efficiency of recovery of high-salt extracted total proteins following dialysis to 50 mM NaCl was on average 26% (data not shown).

b) DNA-cellulose chromatography: whole cell extracts. Proteins were prepared from mock and MCMV-infected cells that had been treated with cycloheximide from 0-3 hr pi. and then labeled for 6 hr to include the synthesis of all IE and early proteins. Samples were prepared and DNA affinity chromatography performed as described in Materials and Methods. The elution procedure was the same as that used by Purifoy and Powell (1976).

Table III shows the recovery of radioactivity during DNA-cellulose chromatography of protein extracts from 3T3-L1 cells. Similar results were obtained for J774A.1 cells. Approximately 20% of input c.p.m. bound to denatured DNA-cellulose while 6% bound to native DNA-cellulose. These results were approximately the same for both mock and virus-infected samples. The bound proteins were efficiently retained by the DNA-cellulose, as the columns were washed each time with 30 bed volumes of low salt column buffer prior to elution with column buffer of increasing NaCl molarity. Approximately 50% of the bound radioactivity from the denatured DNA columns and 60% from the native DNA columns was eluted by the 0.3 M NaCl buffer.

The gels shown (Figs. 25, 26 and 27) have complex patterns due to continued host protein synthesis throughout the six hour labeling period. This has hampered clear interpretation of the results as there are several

Table III:

Recovery of radioactivity following DNA-cellulose chromatography.

Preparation.	MOCK-INFECTED		VIRUS-INFECTED	
	% of total cpm recovered	% of bound cpm	% of total cpm recovered	% of bound cpm
Denatured DNA column				
Column load	100	-	100	-
0.05 M NaCl	81.8	0	78.4	0
0.1 M NaCl	3.9	21.5	4.9	24.7
0.3 M NaCl	9.1	50.7	10.2	50.9
0.5 M NaCl	4.2	23.9	3.4	17.4
1.0 M NaCl	1.0	5.2	1.1	5.8
Total bound	18.2	-	21.6	-
Native DNA column				
Column load	100	-	100	-
0.05 M NaCl	94.2	0	94.3	0
0.1 M NaCl	2.1	27.5	1.3	23.8
0.3 M NaCl	3.1	60.0	3.7	66.5
0.5 M NaCl	0.2	5.6	0.5	7.2
1.0 M NaCl	0.4	6.8	0.2	2.4
Total bound	5.8	-	5.7	-

bands in the lanes containing samples from mock-infected cells that have the same electrophoretic mobility as several infected-cell-specific DNA-binding proteins. As we did not have MCMV antiserum that could precipitate out proteins from dilute solutions, identification of virus specific DNA-binding proteins was based on the presence in viral-infected extracts of a band of greater intensity than an equivalent band in mock-infected extracts, and which had the same mol. wt. as the viral proteins already characterized.

Fig. 25 shows the viral-induced proteins extracted from mock and MCMV-infected 3T3-L1 cells with affinity for native DNA-cellulose. Seven of eight IE proteins previously detected on gradient gels were shown to have affinity for native DNA-cellulose and 3/6 early proteins also had DNA-binding properties. It should be noted that there were at least 3 low mol. wt. protein bands with affinity for DNA that were present in viral-infected cell extracts only (Fig. 25, lane 8 marked with open arrowhead and dots). These had not been identified previously as viral proteins and were not further investigated. The heterogeneous nature of some of these proteins (100K, 89K, 63.5K, 52.5K and 30K) can be seen in that they are eluted at concentrations ranging from 0.05 M NaCl (unbound) to 0.3 M NaCl. The same viral-induced proteins with affinity for native DNA-cellulose were detected from infected J774A.1 cells as from 3T3-L1 cells (not shown).

The viral-induced proteins extracted from mock and MCMV-infected 3T3-L1 and J774A.1 cells and chromatographed on denatured DNA-cellulose columns are shown in Figs. 26 and 27 respectively. The number of proteins that had affinity for denatured DNA-cellulose was less than for native DNA, but as was shown, the total amount of bound protein was about 3 fold more (Fig. 25 was exposed for 37 days compared to 15 days for Figs. 26 and 27). The major

Fig. 25:

DNA-binding proteins from mock and MCMV-infected 3T3-L1 cells. Autoradiographic image of (^{35}S) labeled proteins from high salt extracts of mock and MCMV-infected 3T3-L1 cells, which were chromatographed on a native DNA-cellulose column and separated in a 10-30% polyacrylamide gel. Mock and MCMV-infected cells were treated with cycloheximide 0-3 hr pi., labeled 6 hr with (^{35}S) methionine (25 µCi/ml), extracted with 1.7 M NaCl and the soluble fraction, after dialysis with 0.05 M NaCl, was applied to a column of double stranded DNA-cellulose. Lanes: 1,2: Input mock (1) and virus-infected (2) proteins applied to column. 3,4: Unbound fractions of mock (3) and virus (4) proteins eluted from column. 5,6: Mock (5) and virus (6) proteins eluted with 0.1 M NaCl. 7,8: Mock (7) and virus (8) proteins eluted with 0.3 M NaCl. 9,10: Mock (9) and virus (10) proteins, eluted with 0.5 M NaCl. 11,12: Mock (11) and virus (12) proteins eluted with 1.0 M NaCl.

Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. ($\times 10^{-3}$) in left margin.

Lane 2 has approximately four times the amount of protein as lane 1.

Fig. 25: Proteins from mock and MCMV-infected 3T3-L1 cells with affinity for native DNA-cellulose. Autoradiographic image of labeled proteins from mock and MCMV-infected 3T3-L1 cells, which were chromatographed on a native DNA-cellulose column and separated in a 10-30% polyacrylamide gel.

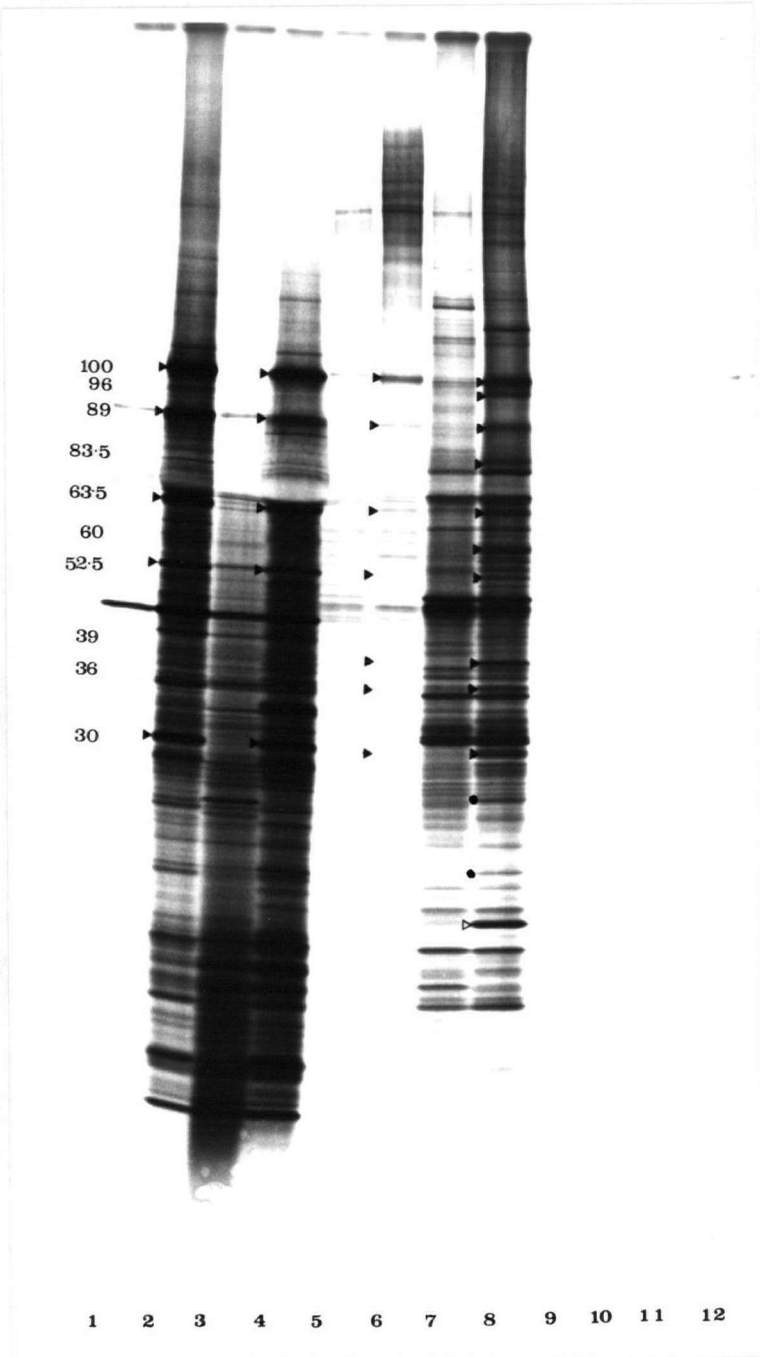


Fig. 26:

DNA-binding proteins from mock and MCMV-infected 3T3-L1 cells. Autoradiographic image of (^{35}S) labeled proteins from high salt extracts of mock and MCMV-infected 3T3-L1 cells, which were chromatographed on a denatured DNA-cellulose column and separated in a 10-30% polyacrylamide gel. Mock and MCMV-infected cells were treated with cycloheximide 0-3 hr pi., labeled 6 hr with (^{35}S) methionine (25 µCi/ml), extracted with 1.7 M NaCl and the soluble fraction, after dialysis with 0.05 M NaCl, was applied to a column of single stranded DNA-cellulose. Lanes: 1,2: Input mock (1) and virus infected (2) proteins applied to column. 3, 4: Unbound fractions of mock (3) and virus (4) proteins. 5, 6: Mock (5) and virus (6) proteins, eluted with 0.1 M NaCl. 7,8: Mock (7) and virus (8) proteins, eluted with 0.3 M NaCl. 9,10: Mock (9) and virus (10) proteins, eluted with 0.5 M NaCl. 11,12: Mock (11) and virus (12) proteins eluted with 1.0 M NaCl.

Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. (x 10^{-3}) in left margin. Right margin shows positions and mol. wt. (x 10^{-3}) of marker proteins.

Lanes 3 and 4 were accidentally overloaded, they have 6-8 fold more protein than lanes 1 and 2.

Fig. 26: Proteins from mock and MCMV-infected 3T3-L1 cells with affinity for denatured DNA-cellulose. Autoradiographic image of labeled proteins from mock and MCMV-infected 3T3-L1 cells, which were chromatographed on a denatured DNA-cellulose column and separated in a 10-30% polyacrylamide gel.

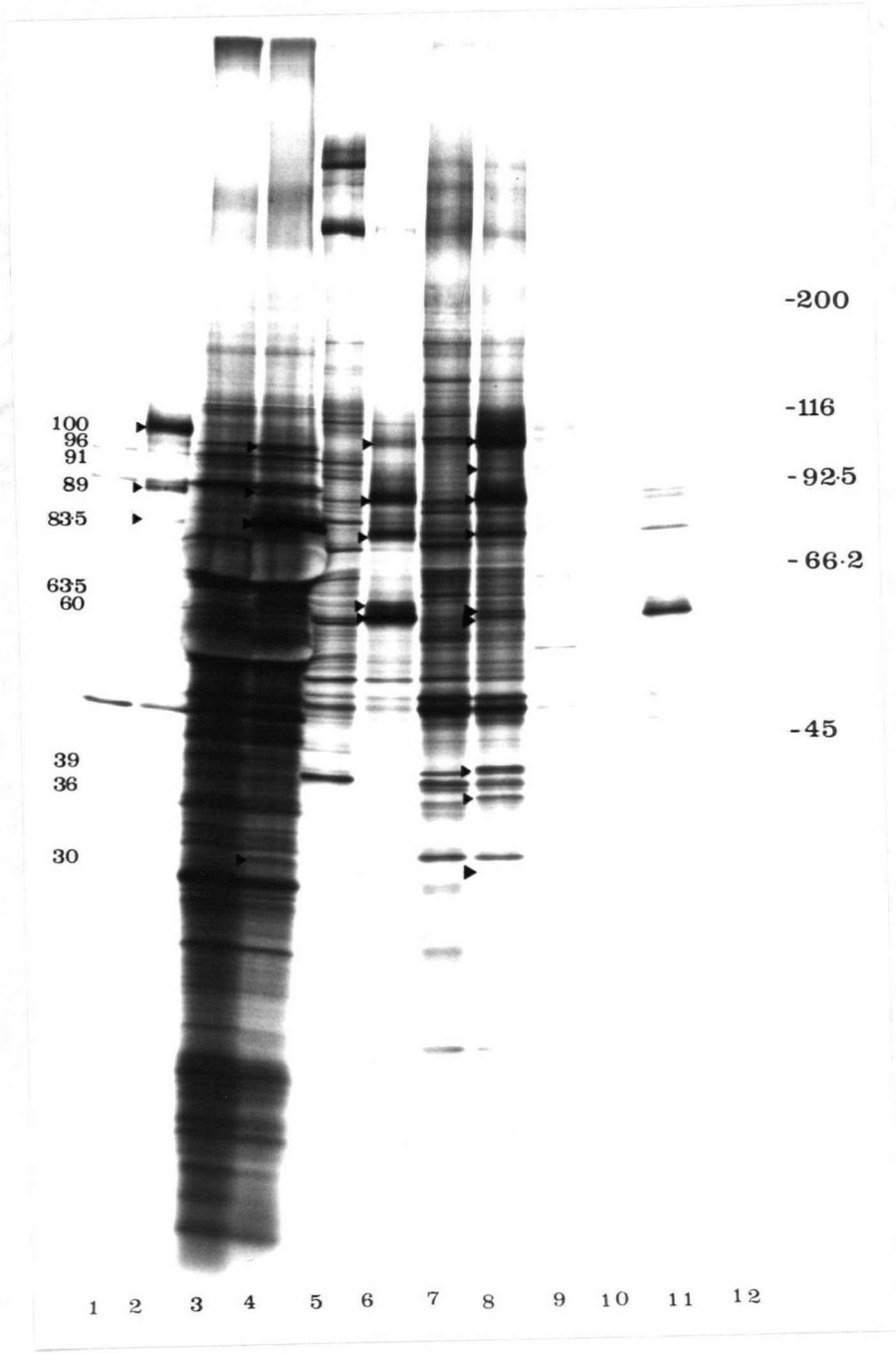
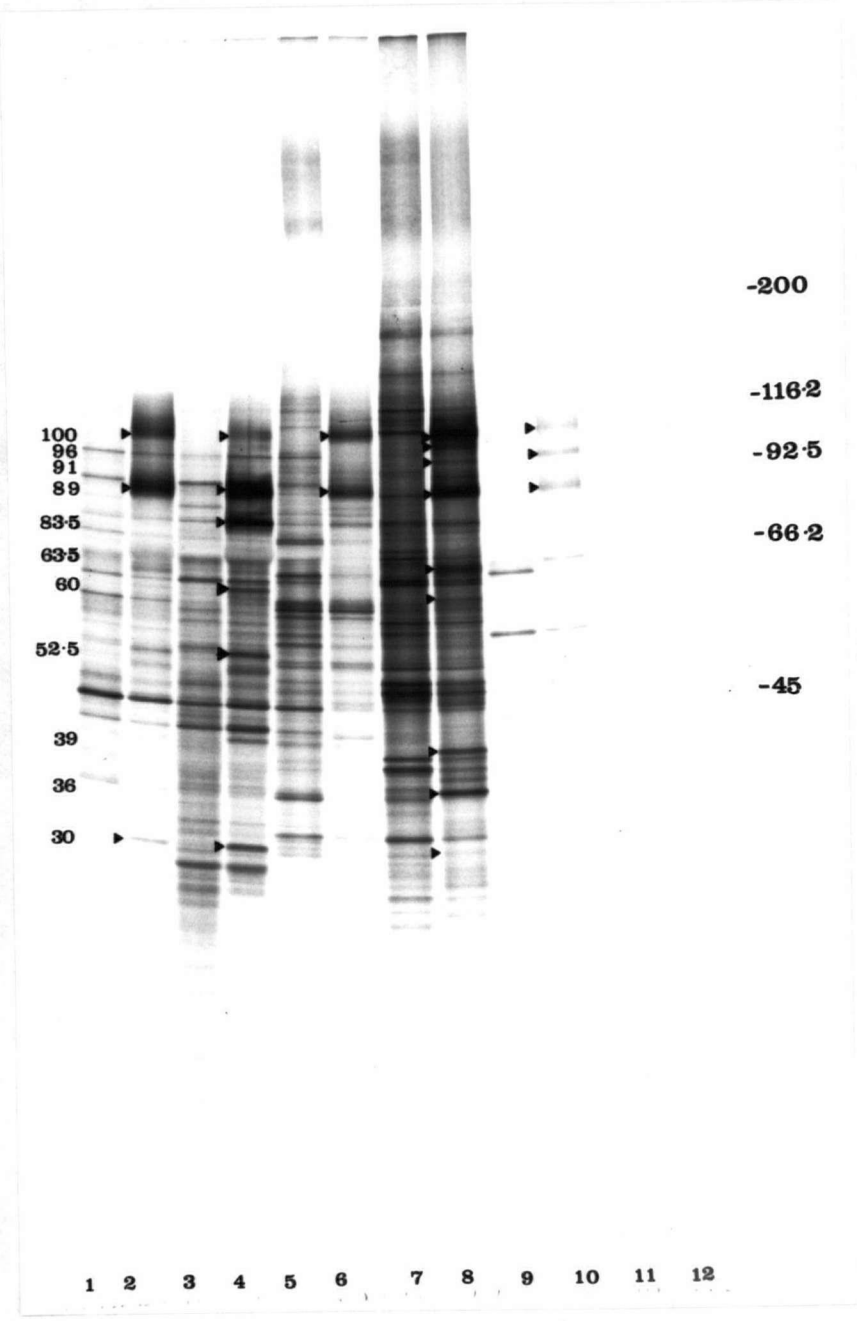


Fig. 27:

DNA-binding proteins from mock and MCMV-infected J774A.1 cells. Autoradiographic image of labeled proteins from high salt extracts of mock and MCMV-infected J774A.1 cells, which were chromatographed on a denatured DNA-cellulose column and separated in a 10-30% polyacrylamide gel. Mock and MCMV-infected cells were treated with cycloheximide 0-3 hr pi., labeled 6 hr with (^{35}S) methionine (25 µCi/ml), extracted with 1.7 M NaCl and the soluble fraction, after dialysis with 0.05 M NaCl, was applied to a column of single-stranded DNA-cellulose. Lanes: 1,2: Input mock (1) and virus infected (2) proteins applied to column. 3,4: Unbound fractions of mock (3) and virus (4) proteins. 5,6: Mock (5) and virus (6) proteins eluted with 0.1 M NaCl. 7,8: Mock (7) and virus (8) proteins eluted with 0.3 M NaCl. 9,10: Mock (9) and virus (10) proteins eluted with 0.5 M NaCl. 11,12: Mock (11) and virus (12) proteins eluted with 1.0 M NaCl.

Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. ($x10^{-3}$) in left margin. Right margin shows positions and mol. wt. ($x10^{-3}$) of marker proteins.

Fig. 27: Proteins from mock and MCMV-infected J774A.1 cells with affinity for denatured DNA-cellulose. Autoradiographic image of labeled proteins from mock and MCMV-infected J774A.1 cells, which were chromatographed on a denatured DNA-cellulose column and separated in a 10-30% polyacrylamide gel.



IE proteins (100K and 89K) had affinity for both native and denatured DNA-cellulose but a greater affinity for denatured DNA can be observed (Figs. 26 and 27, lanes 8). Differences were seen when affinity for denatured DNA-cellulose was compared. In particular, the 96K protein had affinity for denatured DNA when synthesized in J774A.1 cells (Fig. 27, lane 8) but not when produced in 3T3-L1 cells (Fig. 26, lane 4). This protein had been shown to have affinity for native DNA in both cases. The 83.5K IE protein did not appear to have affinity for denatured DNA when synthesized in J774A.1 cells (Fig. 27, lane 4). The 52.5K IE protein was observed to have affinity for native DNA (Fig. 25), however this protein could not be detected in Fig. 26 and only in the unbound fraction in Fig. 27 (lane 4).

The 91K early protein was only observed to have affinity for denatured DNA-cellulose. The major early proteins (39K and 36K) had affinity for both native and denatured DNA-cellulose.

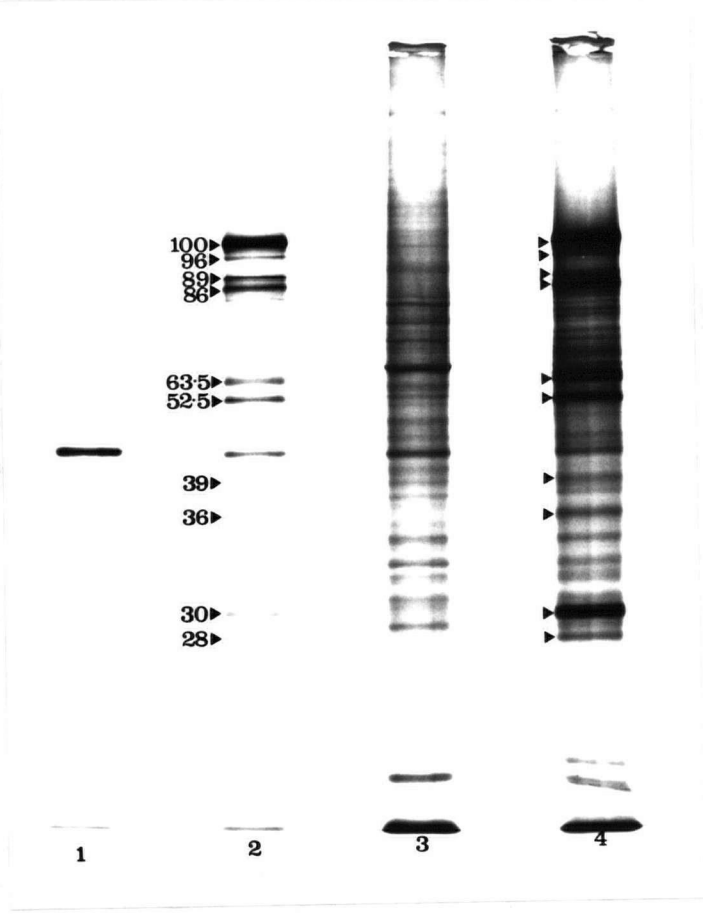
c) DNA-cellulose chromatography: nuclear extracts. To further analyze the differences observed with the 96K IE protein, protein extracts from nuclear fractions of infected cells were prepared. The original high salt procedure used did not solubilize the nuclear viral proteins efficiently. Fig. 28 shows that a proportion of the characterized viral-induced proteins remained with the insoluble nuclear fraction after high salt treatment (lane 2). This could indicate that a proportion of the viral proteins were attached to the nuclear matrix. Studies have shown that some herpes simplex virus proteins were resistant to high salt extraction when attached to the nuclear matrix (Bibor-Hardy et al., 1982; Bibor-Hardy et al., 1985). In addition, another portion of the viral proteins precipitated, following dialysis of the extraction solution to 0.05 M NaCl (Fig. 28, lane 4). As an

Fig. 28:

Autoradiographic image of insoluble proteins from extracts of nuclear fractions of MCMV and mock-infected 3T3-L1 cells separated by electrophoresis. Cells were MCMV-infected and mock-infected, treated with cycloheximide 0-3 hr pi. and then labeled 6 hr with (^{35}S) methionine (10 µCi/ml). Nuclei were released by treatment of infected cells with 1% NP40. Extracts were prepared from nuclei by extraction in buffer containing 1.7 M NaCl. The insoluble material (fraction A) was sedimented by centrifugation (30,000g/30 min) and the supernatant dialysed against buffer containing 50 mM NaCl. The precipitate (fraction B) was removed by centrifugation (100,000g/1 hr). Fractions A) and B) were dissolved in SDS-sample buffer and separated by electrophoresis in a 10% polyacrylamide gel.

Lanes: 1, 2; proteins from fraction A) from mock-infected and virus-infected nuclear fractions. 3, 4; proteins from fraction B) from mock-infected and virus-infected nuclear fractions. Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. (x 10^{-3}) in left margin.

Fig. 28: Insoluble proteins from extracts of nuclear fractions of MCMV and mock-infected 3T3-L1 cells.



alternative, extraction with high salt and spermine (described by Blair and Honess, 1983) was used as it had been reported to solubilize efficiently the early proteins of Herpesvirus saimiri from nuclear fractions of infected cells.

Protein extracts were prepared from nuclear fractions of mock and MCMV infected cells and used as starting material for denatured DNA-cellulose chromatography. The peak fractions eluted from the columns were precipitated and run on 7.5% polyacrylamide gels. This concentration of acrylamide more clearly separated the 100K and 96K proteins. This extraction method produced some differences in the DNA-binding proteins detected, presumably as a result of differences in the population of proteins that were solubilized. In particular, it can be seen that the major early proteins (39K and 36K) did not adhere to DNA-cellulose in either case (Fig. 29 A and B, lanes 4). However, the 100K and 89K proteins from both cell types had DNA-affinity. In addition, this experiment confirmed the previous observation that the 96K protein only had DNA-binding property when synthesized in J774A.1 cells, but not when synthesized in 3T3-L1 cells (Fig. 29 B, lanes 6 and 8).

d) DNA-binding proteins detected by filter affinity. An alternative method was used to analyze proteins with DNA-binding properties. The difference in this method is that proteins can be analyzed that are insoluble under the high salt extraction/dialysis conditions used in previous experiments. However this method analyzes proteins that have been denatured and which have only partially renatured after blotting. DNA-binding properties of some proteins may not be detected if the protein structure necessary for this property remained denatured.

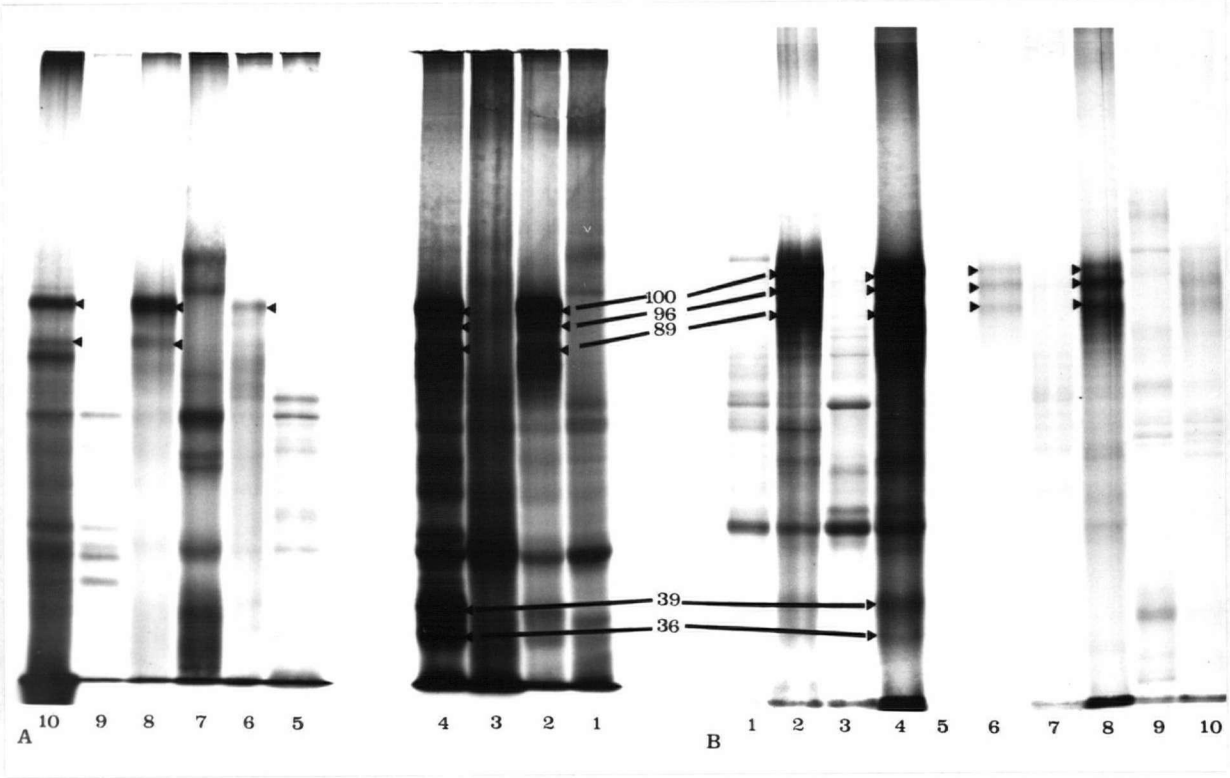
Fig. 29:

Autoradiographic images of DNA-binding proteins from nuclear fractions of mock and MCMV-infected 3T3-L1 (A) and J774A.1 (B) cells separated in 7.5% polyacrylamide gels. Mock and MCMV-infected cells were treated with cycloheximide 0-3 hr pi., labeled 6 hr with (^{35}S) methionine (20 µCi/ml) and released nuclei were extracted with 2.0 M NaCl and 5 mM spermine. The soluble fractions, after dialysis with 0.05 M NaCl and 5 mM spermine, were applied to columns of single stranded DNA-cellulose. Lanes: 1,2: Input mock (1) and virus infected (2) proteins applied to column. 3,4: Unbound fractions of mock (3) and virus (4) proteins. 5,6: Mock (5) and virus (6) proteins eluted with 0.1 M NaCl. 7,8: Mock (7) and virus (8) proteins eluted with 0.3 M NaCl. 9,10: Mock (9) and virus (10) proteins eluted with 0.5 M NaCl. 11,12:

Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. (x 10^{-3}).

Fig. 29: Proteins from nuclear fractions of mock and MCMV-infected 3T3-L1 and J774A.1 cells with affinity for denatured DNA-cellulose.

Autoradiographic image of labeled proteins from nuclear fractions of mock and MCMV-infected 3T3-L1 and J774A.1 cells, which were chromatographed on a denatured DNA-cellulose column and separated in 7.5% polyacrylamide gels.



The proteins were probed with labeled MCMV-DNA, as opposed to calf thymus DNA, which had been used for making the DNA-cellulose used in the columns. This method seems to have limited sensitivity. Fig. 30 (lane 2) shows the proteins with DNA-binding properties from the nuclear fractions of infected cells that were synthesized under IE conditions (described in Fig 4). The 100K protein has DNA-binding properties by this method. The equivalent cytoplasmic sample is shown in lane 6. The 100K protein was detected here along with the 89K and 80K IE proteins. The 39K and 36K proteins could not be assessed by this method as they comigrated with and were masked by strongly binding nuclear cellular proteins which will include histones and non-histone chromosomal proteins. The mol. wts. of the virus-induced DNA-binding proteins were determined by comparison with a gel, run in parallel, of samples of (^{35}S) methionine labeled proteins from cells infected under the same conditions as described in Fig. 30 legend (not shown).

Of interest was the fact that the major DNA-binding protein present in purified virions (Fig. 30 - VIR) also had a mol. wt. of approximately 100K. At present, it is not known if it is the same protein as the 100K IE protein.

A summary of the preceding experiments is listed in Table IV. This lists the complete number of MCMV-induced proteins, with their kinetic class, cellular localization, phosphorylation and DNA-binding properties.

18. Attempts to detect viral-induced IE glycoproteins. It had been reported that a cytotoxic T cell response developed in MCMV-infected mice that was reactive in vitro against MCMV-infected cells expressing only IE proteins (Reddehasse and Kozinowski, 1984). This led to the speculation

Fig. 30:

Autoradiographic image of proteins with affinity for denatured MCMV-DNA. MCMV or mock-infected 3T3-L1 cells were treated with cycloheximide 0-3 hr pi. and then incubated 3 hr in medium with 10 µg/ml actinomycin D (1,2,5,6)(IE) or infected and maintained in the presence of PAA (100 µg/ml) until 5 hr pi. (3,4,7,8)(Early). Cells were harvested, separated into nuclear (NUC) and cytoplasmic (CYT) fractions, separated in a 10% polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The filters were incubated with 10^6 c.p.m. of denatured MCMV-DNA labeled with (α^{-32}P) dATP by nick translation.

Lanes: 1, proteins from nuclear fractions of mock-infected cells (IE). 2, proteins from nuclear fractions of virus-infected cells (IE). 3, proteins from nuclear fractions of mock-infected cells (Early). 4, proteins from nuclear fractions of virus-infected cells (Early). 5, proteins from cytoplasmic fractions of mock-infected cells (IE). 6, proteins from cytoplasmic fractions of virus-infected cells (IE). 7, proteins from cytoplasmic fractions of mock-infected cells (Early). 8, proteins from cytoplasmic fractions of virus-infected cells (Early).

VIR. DNA-binding to separated proteins from gradient purified MCMV virions.

Closed arrowheads indicate virus-induced proteins with corresponding mol. wt. (x 10^{-3}) on left side.

Fig. 30: Autoradiographic image of proteins from mock and MCMV-infected 3T3-L1 cells, electroblotted onto nitrocellulose and probed for affinity to labeled denatured MCMV-DNA.

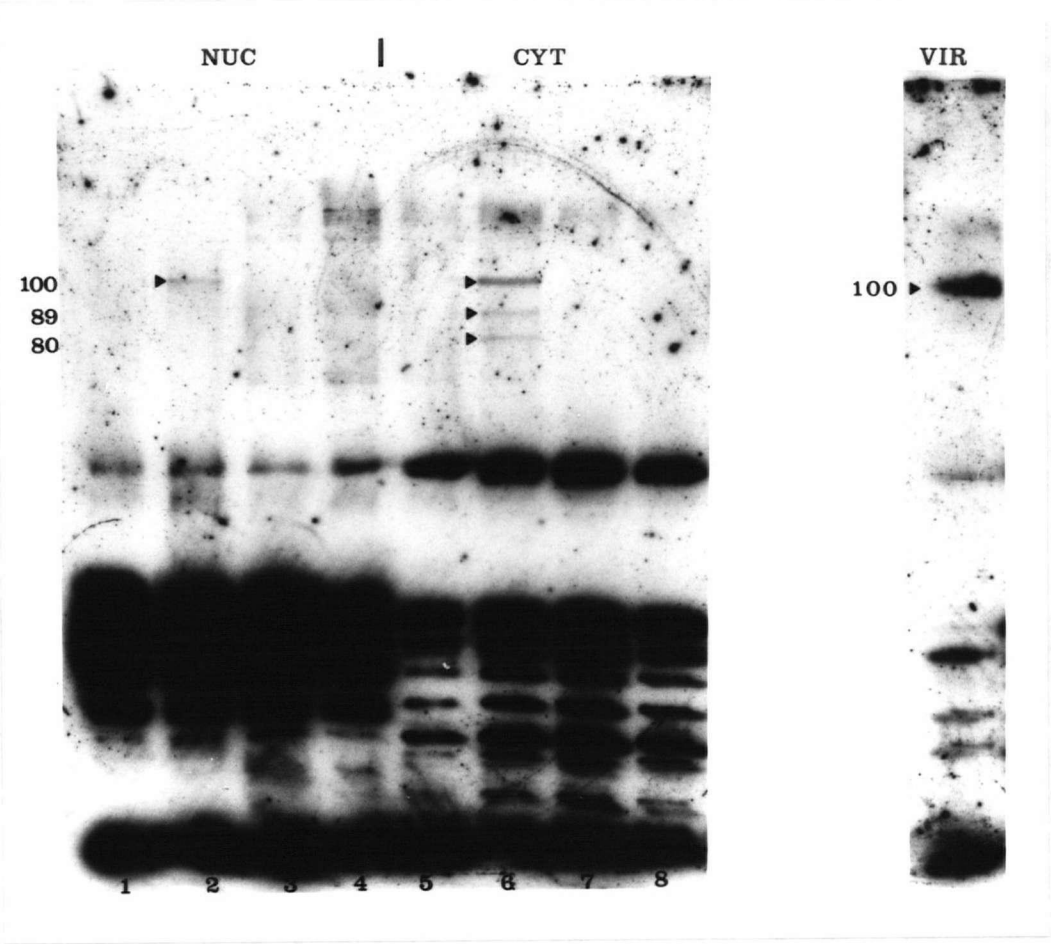


Table IV:

Properties of MCMV-induced Immediate Early and Early Proteins

MOLECULAR WEIGHT ($X\ 10^{-3}$)	KINETIC CLASS	DNA AFFINITY	PHOSPHORYLATED	CELL LOCATION
100	IE	ds & ss	+	Nucleus & cytoplasm.
96	IE	ds & ss[1]	+	Nucleus.
91	E	ss	-	Nucleus.
89	IE	ds & ss	+	Nucleus & cytoplasm.
86	IE	-	+(?)	Mainly nucleus.
83.5	IE	ds & ss[2]	-	Nucleus & cytoplasm.
80	IE	+[3]	-	Cytoplasm.
63.5	IE	ds & ss	-	Mainly nucleus.
60	E	ds & ss	-	N.D.
54	E	-	-	Cytoplasm.
52.5	IE	ds	-	Nucleus & cytoplasm.
51.5	E	-	-	Cytoplasm.
51	E	-	-	Cytoplasm.
39	E	ds & ss	+	Nucleus.
36	E	ds & ss	+	Nucleus.
30	IE	ds & ss	+ (?)	Nucleus & cytoplasm.
28	IE	N.D.	-	Nucleus & cytoplasm.

IE : immediate early; N.D. : not determined

E : early

ds : double stranded calf thymus DNA-cellulose

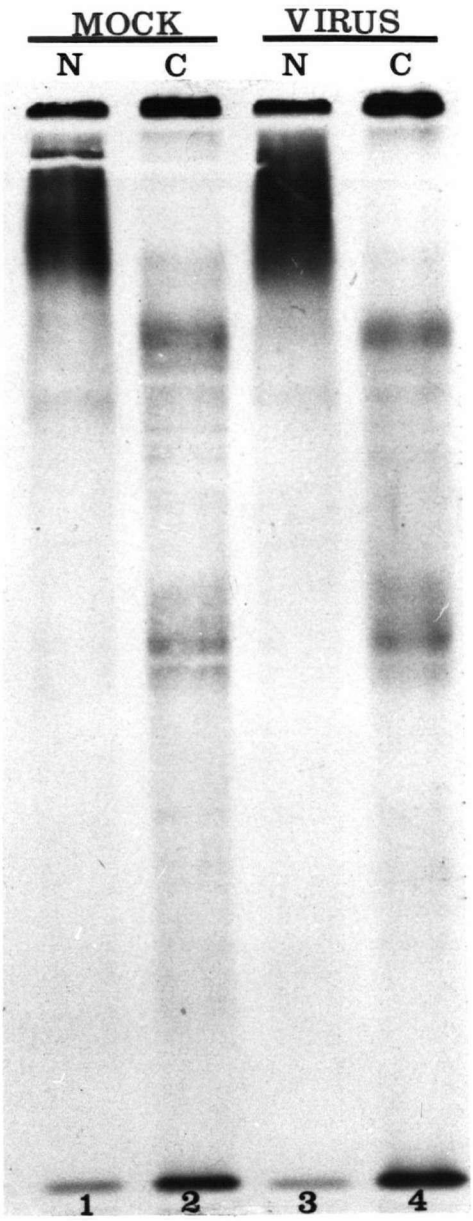
ss : single stranded calf thymus DNA-cellulose

1. 96 K IE protein - no ss affinity from 3T3-L1 cells.
2. 83.5K IE protein - no ss affinity from J774A.1 cells.
3. Measured by DNA-affinity to electroblotted proteins on NC membrane.

Fig. 31:

Autoradiographic image of (^{3}H) glucosamine labeled glycoproteins of nuclear and cytoplasmic fractions from mock infected and MCMV-infected 3T3-L1 cells separated by electrophoresis in a 10% polyacrylamide gel. Cultures of 3T3-L1 cells were mock and MCMV-infected, treated with cycloheximide from 0-3 hr pi. and then labeled with (^{3}H)glucosamine (20 μCi/ml) for 5 hr in presence of 10 μg/ml actinomycin D. The gel was treated with En^{3}Hance prior to drying. Lanes: 1, proteins in nuclear fraction of mock-infected cells. 2, proteins in cytoplasmic fraction of mock-infected cells. 3, proteins in nuclear fraction of virus-infected cells. 4, proteins in cytoplasmic fraction of virus-infected cells.

Fig. 31: Identification of glycosylated proteins in nuclear and cytoplasmic fractions of mock and MCMV-infected 3T3-L1 cells, labeled at IE times.



that one of the IE proteins may also be located in the cell membrane. As most membrane proteins are glycosylated, attempts were made to detect IE proteins labeled with (^{3}H) glucosamine.

Fig. 31 shows that no viral-induced IE glycoproteins of any mol. wt. were detected using the labeling conditions described (see Fig. legend). This was confirmed by Keil et al. (1985), but contrary to the findings of V. Misra (personal communications).

19. MCMV-infection of P388D.1 macrophage and NS-1 myeloma cells. MCMV infections of two other murine lymphoid cell lines were studied. P388D.1 cells are a line of interleukin-1 secreting macrophage-like cells and NS-1 cells are a line of B lymphocyte myeloma cells.

The cells were infected under standard conditions using cycloheximide pretreatment. Fig. 32 shows a comparison of proteins synthesized in P338D.1 (lanes 1-4) and J774A.1 (lanes 5-8) cells using labeling conditions specific for IE proteins. In this gel, 8 of the MCMV IE proteins can be detected in nuclear fractions of infected J774A.1 cells, but no viral-induced proteins were identified in infected P388D.1 cells.

Some MCMV-induced IE and early proteins were synthesized in NS-1 cells (Fig. 33). The high degree of host protein synthesis, even of proteins that partitioned with the nuclear fractions of cells, may have hindered detection of minor bands. However, the major IE proteins of 100K, 96K and 89K were detected. In addition, the switch to early protein synthesis occurred in these cells as the 91K, 39K and 36K early nuclear proteins were detected.

Fig. 32:

Autoradiographic image of (^{35}S) methionine labeled proteins of nuclear and cytoplasmic fractions from mock infected and MCMV-infected P338D.1 (lanes 1-4) and J774A.1 cells (lanes 5-8) separated by electrophoresis in a 10% polyacrylamide gel. Cells were mock and MCMV-infected, treated with cycloheximide 0-3 hr pi. and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). The gel was treated with En^{3}Hance prior to drying. Lanes; 1, proteins in nuclear fractions of mock-infected P388D.1 cells. 2, proteins in cytoplasmic fractions of mock-infected P388D.1 cells. 3, proteins in nuclear fractions of virus-infected P388D.1 cells. 4, proteins in cytoplasmic fractions of virus-infected P388D.1 cells. 5, proteins in nuclear fractions of mock-infected J774A.1 cells. 6, proteins in cytoplasmic fractions of mock-infected J774A.1 cells. 7, proteins in nuclear fractions of virus-infected J774A.1 cells. 8, proteins in cytoplasmic fractions of virus-infected J774A.1 cells.

Closed arrowheads indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$) in right column.

Fig. 32: Proteins in nuclear and cytoplasmic fractions of mock and MCMV-infected P338D.1 and J774A.1 cells, labeled at IE times.

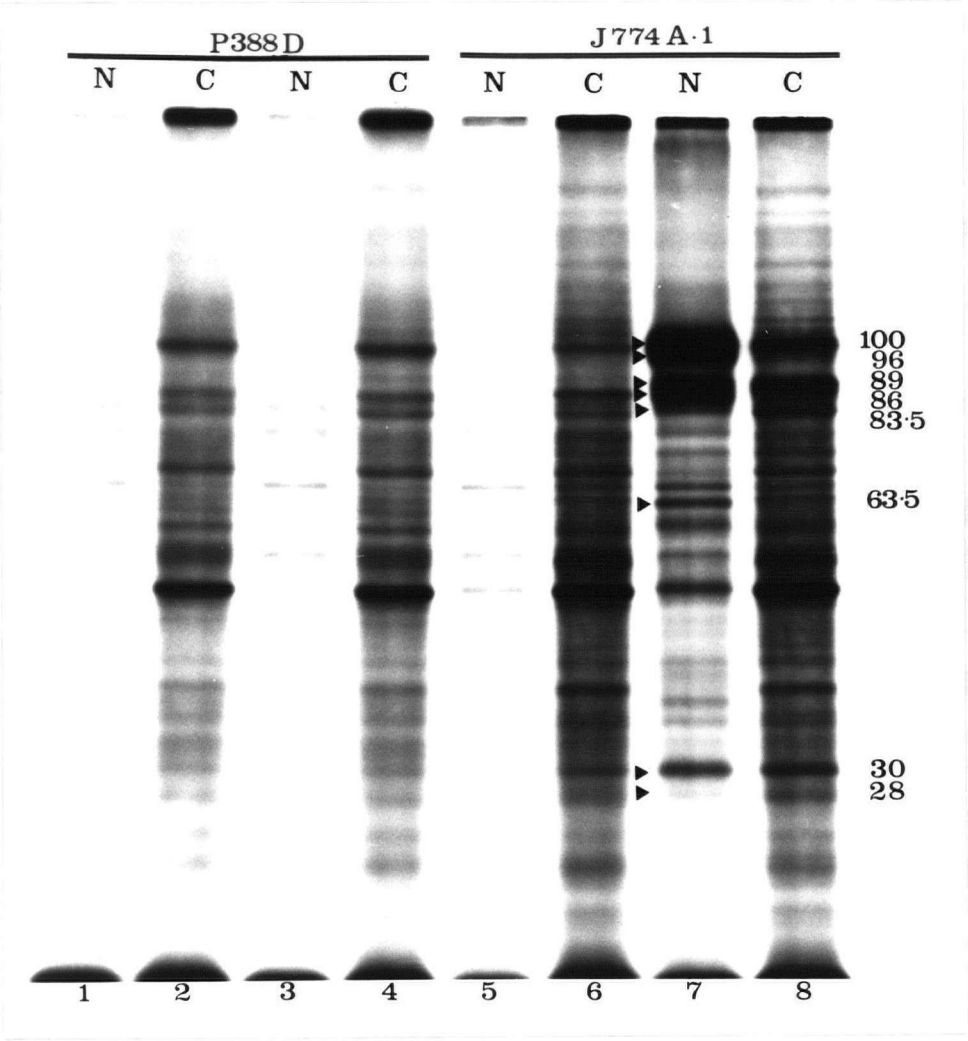
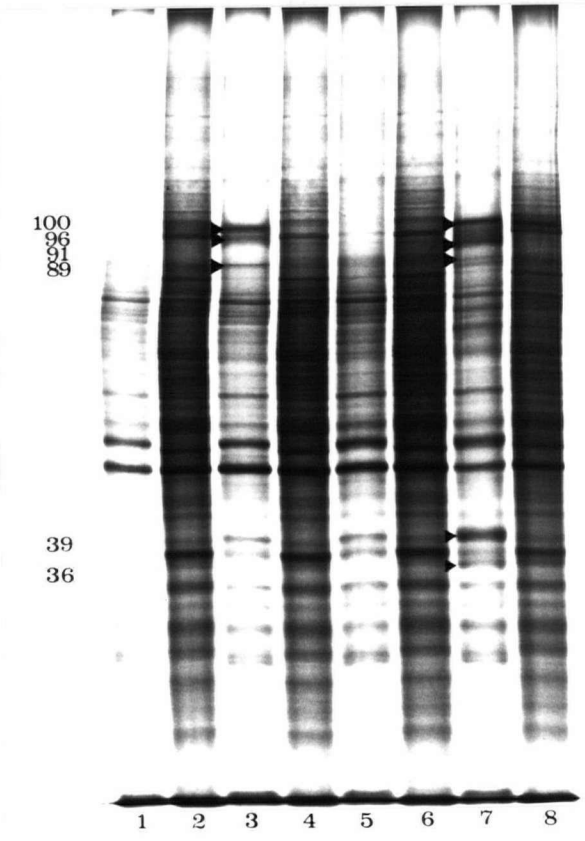


Fig. 33:

Autoradiographic image of (^{35}S) methionine labeled proteins of nuclear and cytoplasmic fractions from mock infected and MCMV-infected NS-1 cells separated by electrophoresis in a 10% polyacrylamide gel. Cells were mock and MCMV-infected, treated with cycloheximide 0-3 hr pi. (lanes 1-4) or 2-5 hr pi. (lanes 5-8) and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). Lanes: 1,5; proteins in nuclear fractions of mock-infected NS-1 cells. 2,6; proteins in cytoplasmic fractions of mock-infected NS-1 cells. 3,7; proteins in nuclear fractions of virus-infected NS-1 cells. 4,8; proteins in cytoplasmic fractions of virus-infected NS-1 cells.

Closed arrowheads indicate virus-induced proteins with apparent mol. wt. ($x10^{-3}$) in left column.

Fig. 33: Proteins in nuclear and cytoplasmic fractions of mock and MCMV-infected NS-1 cells, labeled at IE and early times.



DISCUSSION.

The detailed analysis of proteins induced by MCMV during the early phase of infection (prior to DNA synthesis) has been described. These proteins were characterized according to their temporal order of synthesis, synthesis in the presence of transcriptional and viral DNA synthesis inhibitors, two-dimensional separation, localization in cells under enhanced and unenhanced synthesis conditions, phosphorylation and DNA-binding properties. In addition, the synthesis of these proteins in two nonpermissive cell types was analyzed and the features of these nonpermissive infections were also investigated.

A number of significant findings were made during this study. Firstly, a total of 10 immediate-early (IE) and 7 early MCMV-induced proteins were identified in permissively infected cells, during the period studied, with estimated mol. wts. ranging from 28K - 100K. This was a greater number than had previously been detected (Chantler and Hudson, 1978; Chantler, 1978; Moon *et al.*, 1979). These results do not imply the synthesis of 17 separate gene products. Thirteen of 17 of the proteins were shown to partition exclusively or partly with the nuclei during the infectious cycle and 12 of them had affinity for DNA.

Secondly, analysis of nonpermissive MCMV infections indicated that they have different characteristics to those described for HCMV (Jeang *et al.*, 1982; DeMarchi, 1983b). Sixteen of 17 proteins found in permissively infected 3T3-L1 cells were also found in nonpermissively infected J774A.1 cells (the equivalent experiment to identify the 80K cytoplasmic IE protein was not performed). There was no evidence of a restriction of early gene expression occurring at any stage prior to viral DNA synthesis. In addition,

all the nucleus-associated proteins found in permissively infected cells were identified following MCMV infection of J774A.1 cells and human fibroblast cells.

Thirdly, two properties of major IE proteins were observed to be different in the nonpermissively infected cells. In one case, the 96K IE protein produced in J774A.1 cells was seen to have affinity for denatured DNA-cellulose, while the same protein produced in 3T3-L1 cells did not have this property. In the other case, a higher proportion of the 100K IE protein in HFF cells remained nucleus-associated compared to that in 3T3-L1 cells.

The major limitation of these experiments has been the almost exclusive study of virus-induced proteins in total cell or fractionated cell lysates and the absence of immunoprecipitation studies with defined antiserum. Although 9-11 of the 17 proteins could be precipitated with serum from MCMV infected mice, it was not possible to prove that they were virus coded. However, this work has identified and provided a detailed description of some of the properties of the proteins that are present in infected cells during the early stage of infection.

A comparison of these results with those published for the same strain of MCMV showed the following. In this work, we have found that three major IE proteins (100K, 96K and 89K) were produced in greatest abundance after the reversal of a cycloheximide block. Present with these, in less abundance, were two other IE proteins (86K and 83.5K). Chantler and Hudson (1978) identified three 'IE' proteins of 86.5K, 74K and 69K mol. wt. and four major proteins in a later study (Chantler, 1978). Moon et al. (1979) detected four abundant MCMV IE proteins and one minor species, whose mol. wts. more closely matched those of the proteins identified in this thesis.

During the preparation of this thesis, a report describing the characterization of the major IE proteins of MCMV was published (Keil et al., 1985). The two works will be compared in detail as, although there were a number of similarities with the proteins we have identified, there were also a number of differences. They identified three major IE proteins of 89K, 84K and 76K estimated mol. wts. These proteins had some of the properties of the 100K, 96K and 89K proteins that had been identified in this thesis, in that they were highly enhanced by cycloheximide pretreatment of infected cells. For the purposes of comparison, it will be assumed that these two groups of proteins are the same. One might conclude that the differences in mol. wt. estimations occurred because of the use of different PAGE procedures. However, it can be suggested that there is a genetic basis for the differences. In their results and those reported here, the same early protein of 91K estimated mol. wt. has been identified which can be used as a marker for comparison. In the first case, it was larger than the major IE proteins they had characterized (Keil et al., 1985), while the same protein identified in this thesis had a faster electrophoretic mobility than two of the major IE proteins (Fig. 7). It can be suggested that the IE proteins of the MCMV strain used in this thesis were actually larger than those described by Keil et al. (1985).

A surprising difference between the two studies was the number and abundance of the other IE proteins. We have found a larger number of abundant IE proteins than reported by Keil et al. (1985). The minor IE proteins they identified (31K and 15K), were present in very small amounts in cell lysates and two others (69K and 51K) were only found after in vitro translation of whole cell RNA. The IE proteins of 30K and 52.5K identified in this thesis would be equivalent to the 31K and 51K proteins. These

proteins were readily detectable in lysates of infected cells. They had no equivalent to the 86K and 83.5K proteins we have identified. These proteins were characterized in several different experiments. The distinctly different localization of the 86K and 83.5K proteins from the 100K and 89K proteins, under enhancing conditions, would seem to indicate that they were not non-specific proteolytic cleavage products of the major IE proteins. In addition, they were regularly detected in gels of proteins from three different infected cell types.

Differences were also seen between this work and that reported by Keil et al. (1985) in the early proteins identified. A unifying feature was the presence of a protein of around 91K. However, we have no obvious counterparts to the 46K and 65K early proteins they have identified, and they have no equivalents to the early proteins of 39K and 36K described in this thesis. Proteins with similar mol. wts. and time of synthesis (Moon et al., 1979) and migration patterns (Chantler, 1978) as the 39K and 36K proteins have been identified in these other reports. The 46K early protein was reported to be exclusively cytoplasmic (U.H. Koszinowski, personal communications), while the 39K and 36K proteins were exclusively nuclear. As these proteins are likely to be the components of the MCMV DNA polymerase and thus a part of the viral DNA-replication complex, a nuclear association would be expected. In addition, a group of cytoplasmic proteins of 54K, 51.5K and 51K mol. wt. was identified in this thesis.

It was noticeable that, with the exception of the 96K protein, there was an absence of detectable post-translational processing of these proteins. Although in some cases there was a change in the distribution of the proteins within the infected cells (86K and 63.5K proteins), there was no evidence of post synthetic modification of any of the bands on the gels.

In each case, the bands remained the same mol. wts. during the chase periods. This does not take into account processing that has taken place during the labeling period. This was shown to occur with the proteins that appear to be equivalent to the 100K and 89K proteins (89K and 76K, Keil et al., 1985).

The apparent differences in partitioning of the IE proteins, depending on whether they had been synthesized under enhanced or unenhanced conditions can be explained in the following manner. Under unenhanced (normal) conditions, functional amounts of each protein are synthesized and transported to the nucleus. With enhancement, 'redundant' amounts of each protein are produced and only a portion of these can be accomodated by the nucleus. The amounts of the remaining proteins in the cytoplasmic fraction would depend on the degree of enhancement. This may give the appearance of a higher proportion being in the cytoplasm. This should be interpreted as an experimental artifact as no functional significance can be attached to the greater cytoplasmic localization. This feature was seen with the 96K protein. Under unenhanced conditions or with moderate degrees of enhancement (Fig. 15 and Figs. 17 A and B), this protein was exclusively nuclear, but with greater amounts of enhancement (Fig. 7 A , lanes 6), a portion was found in the cytoplasm. Similarly, with prolonged enhancement, the 100K and 89K proteins were present in much larger amounts in the cytoplasm, while with no enhancement (normal amounts of proteins synthesized), a higher proportion of each protein was found in the nucleus.

Recent detailed analyses of the transcription of MCMV and HCMV have shown them to have the same patterns of gene expression (Marks et al., 1983; Keil et al., 1984; McDonough and Spector, 1983; Stinski et al., 1983). It is on this basis that one would expect the patterns of early protein

synthesis to be the same. However, it has been difficult to directly correlate the finding of 10 immediate-early and 7 early proteins of MCMV with similar results obtained for HCMV, particularly as the experiments were performed under different conditions.

The major IE protein of HCMV (Towne) migrated as a heterogeneous band on gels, having a mol. wt. of around 72K (Stinski et al., 1983). This protein might be analogous to the 100K protein identified for MCMV. The major MCMV IE proteins have different features to those of HCMV. Two distinct bands, of 100K and 89K, were identified in MCMV-infected cells rather than a single band. These two proteins had features in common as identified by cell fractionation, two-dimensional gel separation and degree of enhanced synthesis following cycloheximide pretreatment of infected cells. In addition, apparently equivalent proteins (described by Keil et al. 1985) were reported to be precipitated by the same monoclonal antibody, thus indicating antigenic relatedness, and also, in vitro translation data has shown that the lower mol. wt. protein was derived from the higher mol. wt. protein by proteolytic cleavage (Keil et al., 1985).

The two-dimensional separation of the main MCMV IE proteins was similar to those of two strains of HCMV and two strains of simian CMV. The major IE proteins of the human and simian strains had acidic net charges (Gibson, 1983). The MCMV 100K IE protein migrated to a position just on the acidic side of actin, similar to the HCMV IE protein, while the MCMV 89K IE protein had a consistently more negative charge, similar to the major IE protein of simian CMVs.

A prominent feature of MCMV IE proteins was the presence of the 96K IE protein intermediate between the two major IE bands. This protein was a consistent feature at IE times in MCMV-infected cells, whether permissively

or nonpermissively infected. It had distinctly different properties from the 100K and 89K proteins. Upon two-dimensional separation, it migrated as a diffuse spot with a net basic charge. In addition, it was shown to be nuclear except when produced under conditions that greatly enhanced the amount of protein synthesized (Fig. 7). However, recently published data indicated that an equivalent protein was precipitated by a monoclonal antibody that recognized the other two equivalent major IE proteins (Keil et al., 1985). It was reported that proteases present in normal mouse serum could process the major IE protein (in their case 89K) to the intermediate form (84K). These two proteins appear to be equivalent to the 100K and 96K proteins we have referred to. The fact that the 96K protein may be related to the 100K and 89K proteins seems surprising considering the differences in charge. Distinctly different fragments of the polypeptide would have to be cleaved in order to cause a large increase in positive charge in one case (100K to 96K) and a small increase in negative charge in the other (100K to 89K). Experiments will be needed to determine if all these IE proteins are functional.

Various types of experiments have been done to study HCMV IE proteins but emphasis has always been on a single major protein or related group of proteins (Gibson, 1981; Stinski et al., 1983). Although this was similar to the results for MCMV published by Keil et al. (1985), this approach did not fit the results obtained in this thesis. The observation of ten MCMV IE proteins could lead to several conclusions when comparison is made with HCMV. 1) MCMV IE gene expression was actually different from that of HCMV; 2) some of the observed MCMV IE bands represented early proteins; or 3) some of the observed bands represented induced cellular proteins.

To take the first point, it can be suggested that the number of HCMV IE

proteins reported has been underestimated. If one compares the results from work done with HCMV (Towne) by in vivo infection of cells and in vitro translation of RNA (Stinski, 1978 ; Stinski et al., 1983), a larger number of HCMV IE proteins can be identified. Most of the proteins produced by in vitro translation of hybrid-selected IE RNA, (Wathen and Stinski, 1981; Stinski et al., 1983), except for the major IE proteins, could be identified with those that had been been characterized as early proteins in the previous in vivo study (Stinski, 1978). Thus, most of the early proteins listed do in fact appear to be IE proteins. In addition, the proteins produced by in vitro translation of early mRNA represented a different population from those previously characterized as early proteins (Wathen and Stinski, 1981). From these results one can then conclude that 9 HCMV IE proteins are synthesized. In addition, the six IE proteins detected by Gibson (1981) partly confirmed these reassessed results of Stinski. Thus it can be suggested that the total number of HCMV and MCMV IE proteins are similar. There does not appear to be a reason for the differences between studies, with both MCMV and HCMV, in the numbers and abundance of IE proteins.

Taking the second point, the proof that some of the IE MCMV proteins identified were not early proteins will only be answered definitely once in vitro translation experiments can be performed using hybrid-selected IE mRNA. However, the repeatability of detection of these proteins in the first hour of protein synthesis in infected cells, and also their synthesis following the use of a combination of cycloheximide and actinomycin D, would seem to indicate otherwise.

Taking the third point, as there was coordinated synthesis of the designated IE proteins that followed a similar pattern, with most of their

syntheses declining by 2 hr pi., and likewise only a transient period of synthesis of the designated early proteins, it would seem to argue against these being cellular proteins that were induced following MCMV infection. Separation of these proteins in two dimensions showed each induced protein to be a new species. In addition, immunoprecipitation studies showed that most of the proteins designated as virus-induced were only precipitated from MCMV-infected cell extracts with serum from MCMV-infected mice. Heat shock proteins have been shown to be induced in cells following infection by a number of different viruses, including herpes simplex virus ts mutant (Notarianni and Preston, 1983). Attempts were made to induce heat shock proteins by incubation of cultures of 3T3-L1 cells at elevated temperatures, followed by labeling with (^{35}S) methionine. However, cellular protein synthesis was almost totally inhibited. Thus it was not possible to determine whether any of the proteins characterized might be induced cellular stress proteins.

In comparison to the identification of 7 early proteins detected for MCMV, 10 early protein bands were detected after <u>in vitro</u> translation of HCMV mRNA (Wathen and Stinski, 1981) and 16 early proteins were identified by immunoprecipitation (Blanton and Tevethia, 1981), although only 6-8 were present in abundance.

For understanding the role of IE proteins in controlling viral gene expression, most of the information has come from experiments with HSV, in particular with HSV mutants of the protein ICP 4 (Preston, 1979a,b). It is not clear though how much relevance these have for understanding CMV gene regulation. There are not many similarities in the physical properties of the IE proteins of HSV compared to MCMV (and other strains of CMV). ICP 4, 0 and 27 were each shown to have five charge isomers (of neutral to basic

net charge), also ICP 4 was processed to two higher mol. wt. nuclear forms (which also each had several charge isomers)(Ackermann et al., 1984). The major IE proteins of MCMV and HCMV were only detected in one form by two-dimensional electrophoresis and were processed in a different manner, namely from higher to lower mol. wt. forms.

This study has not been able to indicate a function for any of the major MCMV IE proteins. The fact that they were phosphorylated, were similar in charge and also partitioned to some extent like those of HCMV (Gibson, 1981) would suggest that they might have the same function within the infected cell. However, the MCMV major IE proteins of 100K and 89K remained partly nucleus-associated following treatment of nuclei to remove the adhering cytoskeletal 'tags'. Treatment of CMV (Colburn)-infected cells by this method could completely remove the 94K IE protein from the nuclei (Jeang and Gibson, 1980). The significance of this difference is not known. The results obtained with human fibroblasts showed that the 100K protein had a stronger affinity for the nucleus in these cells, compared to 3T3-L1 cells, with much less of the protein being present in the cytoplasmic fraction. This could suggest that this protein had either a cytoplasmic site of action, or else a repressor function in the nucleus which is reversible in permissively infected cells. However, recent results have shown that ICP 4 of HSV can transactivate the early HSV genes (O'Hare and Hayward, 1985; Persson et al., 1985). Although this protein and the major CMV IE proteins have different physical properties, based on the similarities in patterns of gene expression, a similar function might be anticipated for the major CMV IE proteins. If this proves to be correct, a nuclear site of action would be expected.

DNA-binding properties were observed for 7 IE proteins by affinity to

DNA-cellulose and for another one (80K IE protein) by DNA-affinity to electroblotted proteins. Four of 6 early proteins also had affinity for DNA-cellulose. None of these proteins appeared to have a tight affinity for DNA, as shown by the fact that most of each of the proteins was eluted with 0.3 M NaCl containing buffer. Difficulties in interpreting these results arose because of the heterogeneity in binding of most proteins. A proportion of several of the IE proteins (100K, 89K, 83.5K, 63.5K and 30K) was present in the unbound as well as the bound fractions from DNA-cellulose columns. The amounts of IE proteins produced in the cells had been elevated by cycloheximide treatment prior to labeling, and only a proportion of these might be 'active' binding forms. This problem of heterogeneity was indicated following attempts to extract virus-induced proteins from nuclear fractions of cells. These proteins appeared to be poorly soluble (Fig. 28) or else a proportion of the proteins were attached to the nuclear matrix, rendering them resistant to extraction procedures. A similar characteristic had been observed with HSV-infected cells (Wilcox et al., 1980). Several HSV proteins were insoluble under standard conditions, while some others (eg. ICP 4a, 4c, 27 and 29) were shown to be soluble when extracted from cytoplasmic fractions but insoluble when extracted from nuclear fractions. These differences, which we also seem to have encountered with nucleus-associated MCMV proteins, compared to proteins extracted from whole cells, may represent a further modification of the proteins located in the nuclei (or they may become aggregated with other proteins or nucleic acid, rendering them less soluble). Thus one must consider that the DNA-binding properties observed may only represent analysis of a subset of each type of protein, which may or may not be the active form. This was noticeable on analysis of proteins of nuclear fractions extracted by high salt/spermine

treatment. It appeared that a subset of the major early proteins (39K and 36K) was extracted that did not have DNA-binding properties, where previously DNA-affinity had been observed for these proteins.

Differences in the behaviour of the major IE proteins of MCMV (100K) and CMV (Colburn) (94K) were observed in that DNA-binding properties were seen in the former but not the latter case (Gibson et al., 1981). In addition, the MCMV 100K protein was shown to bind DNA in the filter affinity assay, which would only detect direct protein-DNA interactions. This was different from IE 175 (ICP 4) of HSV-1 which required an intermediate cellular protein to mediate its binding to DNA; the purified form was shown not to bind to DNA-cellulose (Freeman and Powell, 1982).

An unexplained phenomenon was the differential affinity for denatured DNA of the 96K IE protein, depending on whether it was synthesized in 3T3-L1 or J774A.1 cells. This observation was repeatable under the conditions used and also shown on the gels of DNA-binding proteins from nuclear fractions extracted by high salt spermine treatment. No alterations in the physical properties of this protein were observed in other experiments to explain this difference in DNA-binding properties. The protein had the same two dimensional separation, the same partitioning characteristics and was phosphorylated when synthesized in either type of cells. The significance of this is uncertain, as it may or may not be related to the nonpermissive nature of MCMV infections in J774A.1 cells. At present, it is not known if the 96K protein is a functional derivative of the 100K protein. It can be suggested that binding of this protein to single-stranded (replicating) viral DNA in vivo may have the effect of inhibiting DNA replication. In 3T3-L1 cells, this protein may initially bind to viral DNA but at a later time be released (due to some modification). In J774A.1 (and other

nonpermissive) cells, this protein may remain bound to viral DNA thus inhibiting DNA replication. Further investigation of this observation will require the purification of this protein from both cell types.

A noticeable feature for MCMV was the absence of a high mol. wt. (early) major DNA-binding protein equivalent to the HSV and pseudorabies proteins, of 130K (ICP 8) and 136K respectively, that have multifunctional roles in regulating viral DNA replication and various stages of gene expression (Powell et al., 1981; Ben-Porat et al., 1983; Godowski and Knipe, 1983). In addition, preliminary characterization of a 140K early DNA-binding protein of HCMV has indicated that it may be equivalent to the major DNA-binding protein of HSV (Anders and Gibson, 1984). The closest equivalent protein for MCMV was the 91K early protein. This protein was not studied in detail as it was not produced in abundance. It was identified in four different cell types and shown to have a nuclear association. It was observed to have affinity for denatured DNA-cellulose but not for native DNA-cellulose. This was similar to the behaviour of HSV (ICP 8) which has a much weaker affinity for native DNA compared to denatured DNA (Powell et al., 1981).

The nature of nonpermissive infections by MCMV would seem to follow a different pattern to those studied previously with HCMV.

While some of the MCMV-infected J774A.1 cells were permissive for virus replication, the overall population appeared to behave in the same manner as the nonpermissive HFF cells. These infections had some of the same characteristics as other nonpermissive CMV infections in that there was absence of detectable viral DNA and structural protein synthesis and no viral replication occurred (or a small amount in J774A.1 cells). However, examination of the products of early gene expression did not provide any

evidence that restriction of MCMV early gene expression was occurring, thus providing a reason for the inhibition of viral replication. None of the early gene products appeared to be absent. The results from other nonpermissive CMV systems had all shown evidence of a certain degree of restriction of early gene expression, though this had varied depending on the cell system used. However, there was also other evidence, from the results of the nonpermissive MCMV cell system described by Muller et al. (1978), that MCMV behaves in a different manner to HCMV. It had been shown that there was restriction in the amount of MCMV transcription occurring at early times in infected G_0 phase 3T3 cells. The unique feature of this system was that there was no synthesis of the main MCMV IE protein, equivalent to the 100K IE protein. However, the main early proteins (equivalent to the 39K and 36K proteins) were still synthesized in G_0 phase cells and viral DNA polymerase activity was detected in vitro, although its activity was repressed in the cells (Muller and Hudson, 1978).

Different characteristics were seen in cells nonpermissively infected with HCMV. There was evidence of restricted early protein expression in HCMV-infected guinea pig (GP) cells (Stinski, 1978) and an absence of HCMV DNA polymerase activity (Hirai et al., 1976). HCMV and CMV (Colburn) infection of BALB/c-3T3 and Rat-1 cells resulted in the synthesis of only the major IE protein in each cell type. The proteins necessary for activation of early gene expression were not expressed or were not functional (Jeang et al., 1982; LaFemina and Hayward, 1983). A degree of restriction of early gene transcription and absence of viral DNA polymerase activity was seen in HCMV-infected rabbit kidney (RK) cells (DeMarchi, 1983b, c).

The appropriateness of any of these model systems for describing the

events occurring *in vivo* is still uncertain. Rice *et al.* (1984) described nonpermissive types of infections by HCMV of human peripheral blood mononuclear cells. Although it was shown that IE antigens were produced but not late antigens, the stage of the block to replication was not further described. It was not determined if there was synthesis of any of the early proteins. This was the first *in vitro* system that could closely model the events occurring following nonpermissive HCMV infections *in vivo*. However, it is still not known whether HCMV gene expression *in vivo* is blocked at the IE stage, whether there is a restriction at the early stage of gene expression or whether the block comes at the stage of viral DNA synthesis as modelled by the MCMV systems we have described in this thesis.

From this work (and other published reports), several suggestions can be made on the potential mechanisms of nonpermissive interactions of CMVs with cells.

Firstly, evidence *does* seem to suggest that some of the mechanisms involved in regulating MCMV and HCMV replication are different. A fundamental difference between the two viruses in the control of DNA replication has been identified. MCMV is very sensitive to the antiviral agent acyclovir (ACV) while HCMV is much less so (Sandford *et al.*, 1985). The reason for MCMV sensitivity to ACV is not known, but it is believed to be due to the extreme sensitivity of the MCMV DNA polymerase to ACV that has been phosphorylated by cellular enzymes. Mutations in one gene (believed to be the MCMV-DNA polymerase) rendered the virus resistant to ACV (Sandford *et al.*, 1985). The lack of sensitivity of HCMV to ACV could indicate that the MCMV and HCMV DNA polymerases have fundamentally different properties.

Another difference appeared to be the amount of restricted gene expression following nonpermissive infections by MCMV and HCMV. This is

best illustrated if one compares the amount of gene expression that occurred following nonpermissive infection by MCMV of human fibroblasts (described in this thesis) and nonpermissive infection by HCMV of mouse fibroblasts (LaFemina and Hayward, 1983). In the former case, there was no evidence for restricted early gene expression, while in the latter case, viral gene expression was limited to the synthesis of the major IE protein. This could indicate that HCMV nonpermissive infections occur for different reasons to those with MCMV. A possible mechanism for restricted MCMV replication will be suggested and is based on the assumption that the viral DNA polymerase is present in nonpermissively infected cells.

MCMV has been shown to be more dependent on cell cycle factors for its replication than HCMV. Viral DNA replication may require the participation of cellular factors. Recently, two cellular factors have been isolated from uninfected human cells that were required for adenovirus DNA replication to occur in vitro (Nagata et al., 1983; Friefeld et al., 1984). Nuclear factor 1, a 47K protein, was required for the initiation and partial elongation of replicating adenovirus DNA in vitro. Nuclear factor II was required for the formation of full length adenovirus genomes. These proteins interacted with three viral proteins and viral DNA. Nuclear factor I was shown to be a sequence specific DNA-binding protein that recognized a particular sequence of adenovirus DNA within the origin of replication site. In addition, similar specific DNA sequences for nuclear factor I were identified in the human cellular genome (Gronostajski et al., 1984). It can be suggested that MCMV DNA replication may also depend on the presence of this or a similar protein. In nonpermissive infections of cells of the same species, replication may be limited by the amount of nuclear factor I or a similar protein, required for initiating DNA replication. In nonpermissive

infections of cells of a different species, replication may be inhibited because of the inability of the particular nuclear factor of that species to recognize the origin of replication of a different species of CMV. Origins of replication have been identified in the genomes of HSV (Stow et al., 1982) and EBV (Yates et al., 1985), so one may assume that CMVs also have similar sequences. The origins of replication of each species of CMV may vary such that they are only recognized by nuclear factor(s) specific for each species of host. This might occur if each species of CMV had acquired its origin of replication sequences during some stage of its evolution, by incorporation of corresponding cellular replication sequences.

HCMV does not appear to be so dependent on the cell cycle for its replication (DeMarchi and Kaplan, 1976) and also the virus can stimulate host cell DNA synthesis (DeMarchi and Kaplan, 1977b: DeMarchi, 1983b). One can suggest that in nonpermissive HCMV infections, different host cell factors (or viral proteins) inhibit a stage of early gene expression, perhaps by binding to the promoter section of certain genes. This would account for the observed restricted early gene expression. Some evidence for this came from the studies of StJeor and Rapp (1973), where pretreatment of nonpermissive human kidney cells with IUdR made them partially permissive to HCMV replication. It was suggested that the IUdR treatment caused the inhibition of synthesis of a cellular protein(s) that regulated gene expression.

In summary, a general description of the number and properties of the murine cytomegalovirus proteins that were detected during the early phase of the replication cycle in permissively infected cells has been provided, as well as a description of the synthesis of these proteins in nonpermissively infected cells. This work has provided sufficient information to permit

detailed investigation of individual proteins, in subsequent studies of their role in virus replication. Any further studies will require the preparation of monospecific antibodies in order to be able to analyse the role of each protein within the complex of proteins induced by this virus. Studies are indicated to investigate the interaction of the major IE proteins (particularly the 96K protein) with virus DNA within the infected cell. Also, it should be determined if the 91K protein performs functions similar to the major DNA-binding protein of HSV. In addition, identification of the proteins of the viral DNA-polymerase will provide a basis for comparing this enzyme in permissive and nonpermissive cell types.

SECTION 2.

COMPARISON OF THE PROTEINS OF MURINE CYTOMEGALOVIRUS STRAINS SMITH AND K181.

INTRODUCTION.

Experiments with murine cytomegalovirus (MCMV) have mostly been performed with two particular laboratory strains. These are the Smith strain and the K181 strain. The latter was a derivative of the Smith strain, that was selected following prolonged passage *in vivo*. The K181 strain has been shown to have a greater virulence (Osborn, personal communication) and also replicated to significantly higher titers in the submaxillary glands of young mice (Misra and Hudson, 1980).

Initial studies of the restriction endonuclease digestion profiles of the DNA of the two strains had shown that there were minor differences between the genomes (Misra and Hudson, 1980; Loh and Hudson, unpublished data.), as seen by an alteration in the migration of several of the bands.

The aim of the work in this section was to compare the proteins produced by both strains of virus, at various stages of the replication cycle, to determine if these differences in nucleotide sequences are reflected in changes in any of the gene products.

MATERIALS AND METHODS.

Virus and cells. The Smith and K181 strains of MCMV were used in these studies. These have been described in sections 1 and 3.

Virus was grown and proteins labeled in 3T3-L1 mouse fibroblast cells.

Infection and labeling of cells and SDS-PAGE. To detect immediate early proteins, cells were infected (m.o.i. 25) and maintained in the presence of cycloheximide (50 µg/ml) from 0-3 hr pi. The cells were rinsed three times and labeled 1 hr with 50 µCi/ml (^{35}S) methionine. To detect early proteins also, cells were infected (m.o.i. 25) and treated with cycloheximide from 2-5 hr pi. and labeled 1 hr as described. To detect late proteins, cells were infected (m.o.i. 10) and labeled from 28-32 hr pi..

In each experiment, infected cells were separated into nuclear and cytoplasmic fractions by the method described in section 1 (Materials and Methods).

The samples were separated by electrophoresis in 10% polyacrylamide gels according to the procedures described in section 1 (Materials and Methods).

Purification of virions and nuclocapsids. Virions were purified from the tissue culture media of MCMV-infected 3T3-L1 cells. The purification procedure of Chantler and Hudson (1978) was used, with some modifications.

3T3-L1 cells were grown in roller bottle cultures (850 cm^2- Falcon Labware, Becton-Dickinson, Oxnard, Calif.) and infected with MCMV at an m.o.i. of 0.1. For each preparation, the media from two roller culture bottles were used. The cells were incubated with methionine-free MEM

supplemented with 2% FBS and 20 μCi/ml (^{35}S) methionine when they showed 90-100% cytopathic effect (days 2-3 pi.). The cells were labeled for 2 days.

Cellular debris was removed from the media by low speed centrifugation (5,000 r.p.m. for 10 min at 4°C in an SS34 Sorvall rotor). The virions were pelleted by centrifugation at 16,000 r.p.m. for 90 min using an SS34 Sorvall rotor. The virions were suspended in 3 ml of phosphate buffered-saline (PBS), with the use of a ground glass homogenizer to produce a homogeneous preparation. This was layered onto a preformed 20-50% potassium tartrate gradient (dissolved in 0.15 M NaCl/0.015M Na Citrate) and centrifuged at 25,000 r.p.m. for 2 hr using a Beckman SW 27 rotor. The virus banded as a wide white band. This was harvested by side puncture of the centrifuge tube. The virus was suspended in 15 ml PBS, pelleted (20,000 r.p.m. for 1 hr in a Beckman SW 27 rotor), resuspended in 3 ml of PBS and centrifuged through a second 20-50% potassium tartrate gradient. The virions were harvested from the gradient and pelleted as before. A portion was removed for electron microscopical examination and the rest was dissolved in SDS sample buffer.

Nucleocapsids were extracted from virions from the cytoplasm of infected cells. The cells were scraped from the roller bottles into PBS and harvested by centrifugation (3,000 r.p.m. for 10 min, in an SS34 Sorvall rotor). The pellet was resuspended in 1% NP40 in PBS (5 ml/10^8 cells) and incubated on ice, with occasional vortexing, for 10 min. The nuclei and cellular debris were removed (9,000 r.p.m. for 15 min) and the supernatant spun through a 30% glycerol cushion (23,000 r.p.m. for 1 hr in a Beckman SW 27 rotor). This pellet was resuspended in 3 ml of 1% NP40 in PBS by homogenization and then banded by centrifugation through a 20-50% potassium tartrate gradient, as described for the virions.

RESULTS AND DISCUSSION.

1. Comparison of immediate-early and early proteins. The IE and early proteins induced by MCMV (Smith) and MCMV (K181) are illustrated in Figs. 34 A and 34 B respectively. Eleven of 17 proteins that had previously been characterized (section 1) were identified in this experiment. The main feature of this experiment was that the same number of major nuclear IE and early proteins, with the same mol. wts., were synthesized in cells infected with the different strains of MCMV. In particular, it should be noted that the mol. wts. of the major IE proteins of the two strains were the same. This was different from what had been found with some HCMV strains, where the mol. wts of the major IE proteins were shown to differ (Gibson, 1981; Cameron and Preston, 1981). The 80K IE and 60K, 54K, 51.5K and 51K early proteins were not identified on these gels because the labeling and electrophoretic conditions used were not suitable for the detection of these bands (discussed in section 1).

2. Comparison of late infected cell proteins. Fig. 35 shows the proteins synthesized in MCMV (Smith) (lanes 3 and 4) and MCMV (K181) (lanes 7 and 8) infected cells from 28-32 hr pi. The viral-induced proteins were designated according to their synthesis in infected cells with no equivalent band in mock-infected samples, or a band of lesser intensity. Twelve virus-induced proteins were identified according to these criteria. The same bands could be identified in both Smith and K181 infected cells.

3. Comparison of virion proteins. A comparison of the proteins of the purified virions of the two strains of MCMV was made. The proteins present

Fig. 34:

Autoradiographic images of (^{35}S) labeled proteins from mock and MCMV (Smith) and MCMV (K181) infected 3T3-L1 cells separated in 10% polyacrylamide gels. 3T3-L1 cells were infected with MCMV (Smith or K181)(v) (m.o.i. 25) or mock-infected (m) and incubated with cycloheximide from 0-3 hr pi. (lanes 1-4) or from 2-5 hr pi. (lanes 5-8) and then labeled 1 hr with (^{35}S) methionine (50 μCi/ml). Lanes: 1 and 2: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected cells; 3 and 4: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected cells; 5 and 6: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected cells; 7 and 8: proteins in nuclear (N) and cytoplasmic (C) fractions of virus-infected cells.

Arrowheads indicate positions of virus-induced proteins with mol. wts. ($x10^{-3}$) by the side.

Fig. 34: IE and early proteins of MCMV strain Smith and MCMV strain K181. Autoradiographic images of labeled proteins from mock and MCMV (Smith) and MCMV (K181) infected 3T3-L1 cells, at IE and early times pi., separated in 10% polyacrylamide gels.

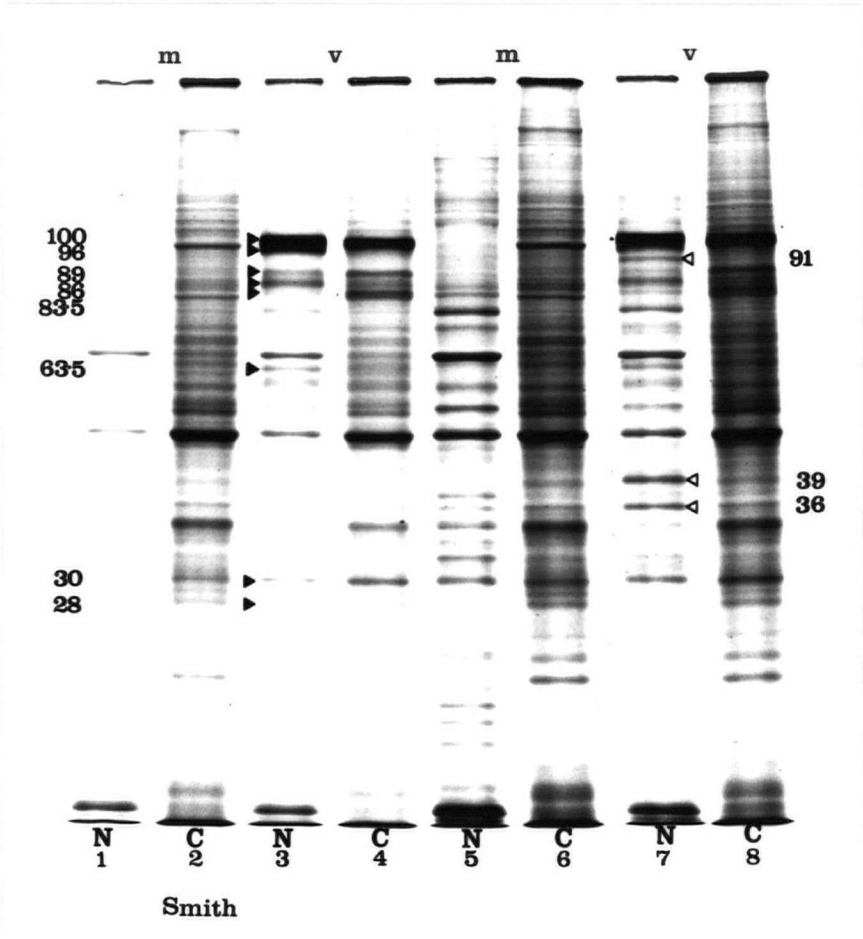


Fig. 34 (cont.)

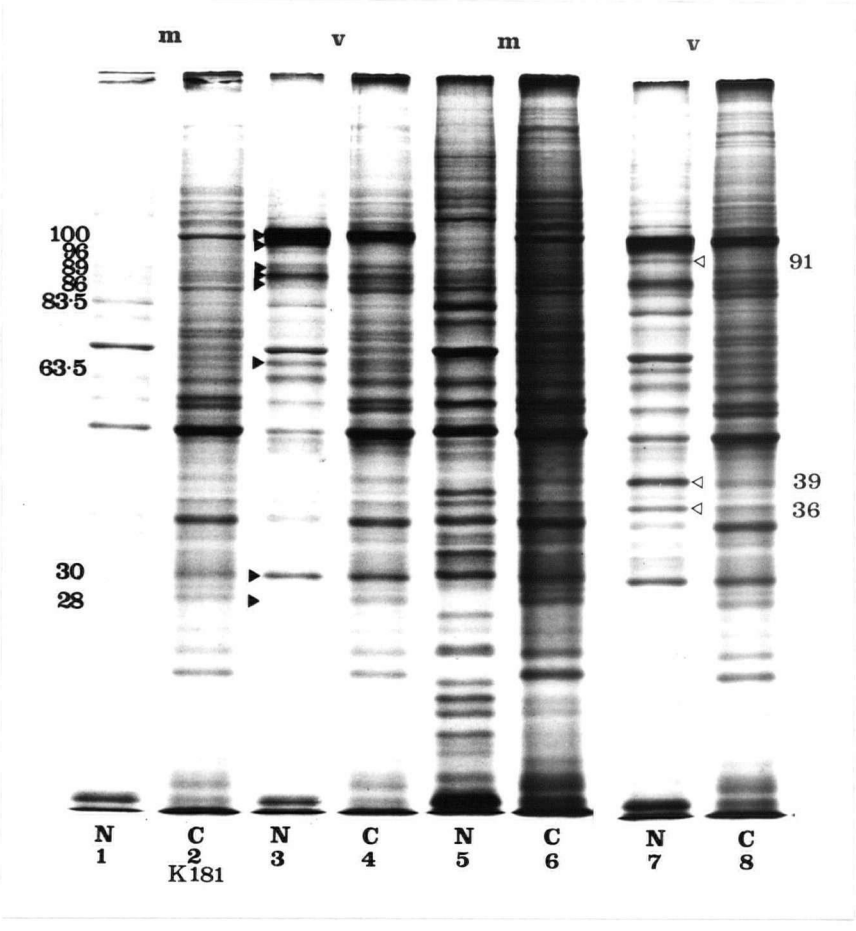
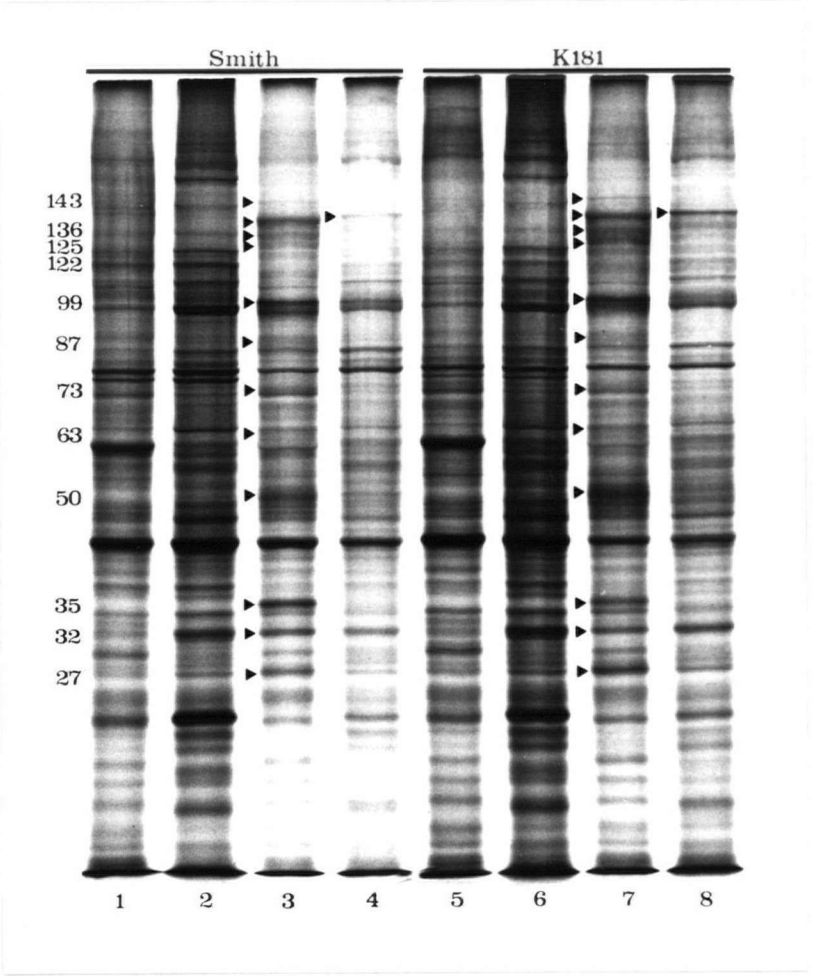


Fig 35:

Autoradiographic image of (^{35}S) labeled proteins from mock and MCMV (Smith) and MCMV (K181) infected 3T3-L1 cells separated in a 10% polyacrylamide gel. 3T3-L1 cells were infected with MCMV (Smith or K181)(v) (m.o.i. 10) or mock-infected (m) and labeled from 28-32 hr pi. with (^{35}S) methionine (25 μCi/ml). Lanes: 1 and 2: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected cells; 3 and 4: proteins in nuclear (N) and cytoplasmic (C) fractions of MCMV (Smith) infected cells; 5 and 6: proteins in nuclear (N) and cytoplasmic (C) fractions of mock-infected cells; 7 and 8: proteins in nuclear (N) and cytoplasmic (C) fractions of MCMV (K181) infected cells.

Closed arrowheads indicate positions of virus-induced proteins with mol. wts. ($x10^{-3}$) on the left side.

Fig. 35: Late proteins of MCMV strain Smith and MCMV strain K181. Autoradiographic image of labeled proteins from mock and MCMV (Smith) and MCMV (K181) infected 3T3-L1 cells labeled from 28-32 hr. pi and separated in a 10% polyacrylamide gel.



in the nucleocapsids were also analysed in parallel. The main aim of the experiment was to compare the proteins present in the virion envelope of the two strains. The absence of an envelope glycoprotein had been found in an attenuated strain of pseudorabies virus (Ben-Porat et al., 1984). The envelope will be absent from the nucleocapsids extracted from the cytoplasm of NP40-treated cells. An electron micrograph of the nucleocapsids, purified from MCMV (Smith) infected cells, is shown in Fig. 36. Samples from MCMV (K181) infected cells were the same. All particles seen were lacking the characteristic herpesvirus envelope.

The proteins present in the virions and nucleocapsids of the two strains of MCMV are shown in Fig. 37. The same proteins with the same mol. wts. were detected in Smith and K181 virions. In this experiment, 23 virion proteins were identified. Due to the lower yield of K181 virions, some of the minor proteins identified in the Smith virions (Fig. 37, lane 1) could not be seen clearly in the gel of the K181 virion proteins (Fig. 37, lane 3). These had all been identified in other gels (data not shown). The molecular weights obtained in this experiment were, with expected experimental variation, similar to those previously reported for the Smith strain of MCMV (Chantler and Hudson, 1978). In both strains, a protein of 205K was present in the Coomassie brilliant blue stained gels that did not show on the autoradiograms (not shown). A high mol. wt. protein has previously been characterized as a virion component (Chantler and Hudson, 1978; Moon et al., 1976b).

It can be seen that the nucleocapsid preparations (lanes 2 and 4) were contaminated with host cell proteins. However, there were six bands present in the virion preparations of each strain that were clearly absent or greatly reduced in the nucleocapsid preparations. These can be considered

Fig. 36:

Electron micrograph of nucleocapsid particles purified from the cytoplasm of MCMV (Smith)-infected cells by the method described. Magnification approximately 71,000x. Bar represents 100 nm.

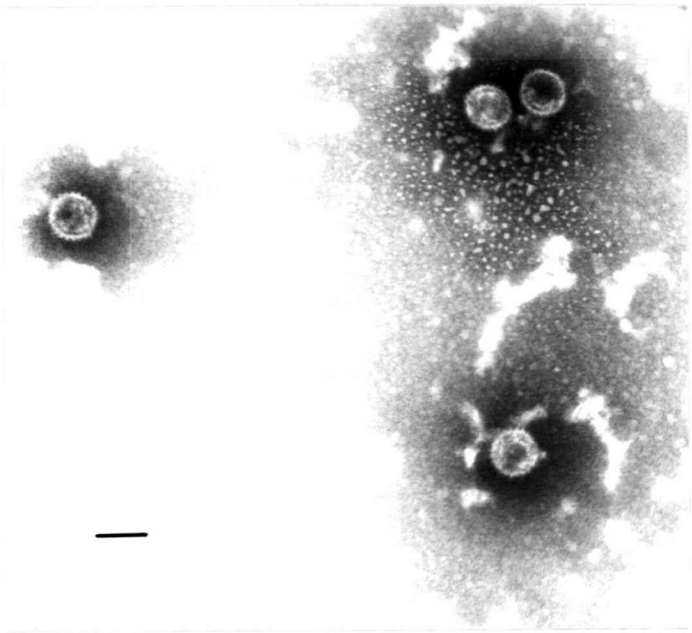


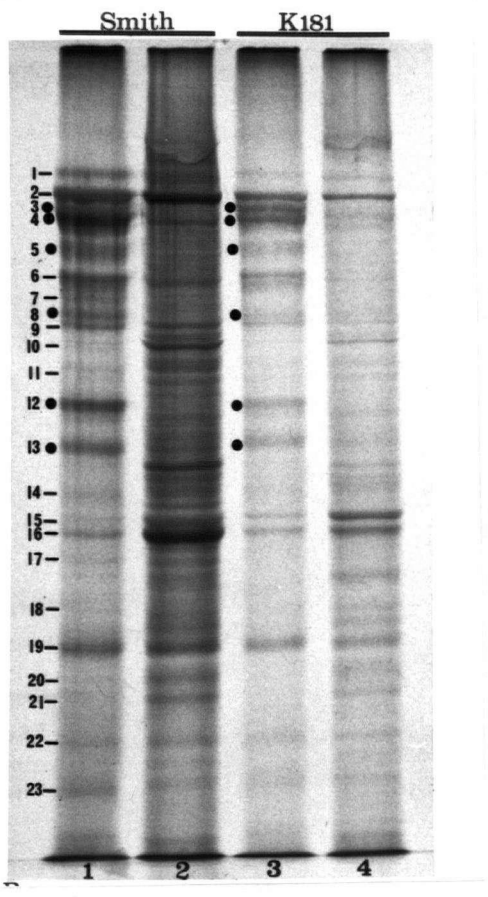
Fig. 37:

A. Autoradiographic image of (^{35}S) methionine labeled proteins of purified virions and nucleocapsids of MCMV (Smith) and MCMV (K181). Virions and nucleocapsids were purified from the tissue culture media and cytoplasm of infected 3T3-L1 cells (described in Materials and Methods). Lanes: 1, proteins in virions of MCMV (Smith). 2, proteins in nucleocapsids of MCMV (Smith). 3, proteins in virions of MCMV) (K181). 4, proteins in nucleocapsids of MCMV (K181). Numbers represent designation of virion proteins. Suggested virion envelope proteins indicated by spots.

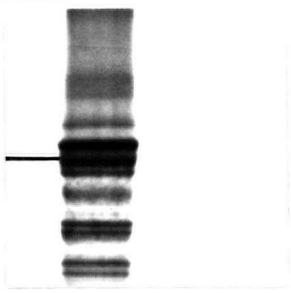
B. Autoradiographic image of (^{35}S) methionine labeled proteins of purified MCMV (Smith) virions (different preparation to that shown in A.). Virion protein 3 (125K) indicated.

Fig. 37: Proteins of purified virions and nucleocapsids of MCMV (Smith) and MCMV (K181).

A.



B. Protein 3 (125K)



as putative envelope proteins, although it must be considered that they may include released tegument proteins. These were the proteins numbered 3, 4, 5, 8, 12 and 13 which had mol. wts. of 125K, 122K, 107K, 87K, 63K and 50K respectively. The inset (Fig. 37 B) shows the 125K band of the Smith virions that was not clearly seen in the virions preparation shown in lane 1 (Fig. 37). Proteins of 106K, 87K, 62K and 51K had previously been identified as glycoproteins by Moon et al. (1976b). There were no glycosylated proteins in that report that might be equivalent to the major proteins (125K, 122K) shown in this study, that appear to be part of the envelope or tegument.

The experiments described in this section show that the two strains of MCMV share the same major IE and early proteins, the same major late proteins and the same major virion proteins (on the basis of mol. wt.). Thus there was no clear difference found between the proteins produced by the Smith and K181 strains of MCMV, at any stage of infection.

The minor differences in the restriction endonuclease profiles of the genomes may result from basepair substitutions. There is no indication of deletions in the K181 genome (Loh and Hudson, unpublished observations). The substitutions result in an alteration of some restriction enzyme recognition sites and may also result in amino acid substitutions in protein(s). This could alter the properties of a particular protein, but this change might not be detectable by SDS-PAGE. Further analyses by two-dimensional gel electrophoresis are indicated.

Although this approach to understanding the nature of virulence of K181 was an initial effort, it may prove to be somewhat simplistic. The mechanisms will probably be much more complex. Previous studies with herpesviruses (Lomniczi et al., 1984), reoviruses (Weiner et al., 1977) and myxoviruses (Rott, 1979) have shown virulence to be a polygenically controlled phenomenon.

SECTION 3.

INVESTIGATION OF MURINE CYTOMEGALOVIRUS LATENCY.

INTRODUCTION.

Murine cytomegalovirus has proven to be a valuable model for HCMV infections *in vivo*. However, in contrast to herpes simplex virus and Epstein Barr virus, the site(s) of latency of MCMV (and HCMV) are not known. The mechanisms involved in causing reactivation are also not known. Various *in vitro* studies have been made of populations of cells from latently infected mice to define some of the characteristics of latency. Most of the investigations have been done with mononuclear lymphoid cells from the spleen or peritoneal cavity (Olding *et al.*, 1975; Brautigam *et al.*, 1979; Jordan and Mar, 1982; Jordan *et al.*, 1982). It appeared that virus could be reactivated from splenic B lymphocytes and also peritoneal macrophage cells. However, evidence is available that MCMV can also remain latent at another site, as *in vivo* reactivation, caused by treatment with immunosuppressive agents, showed that MCMV could be reactivated from splenectomized mice. In addition, virus could be reactivated *in vivo* from mice infected subcutaneously (sc), but not by *in vitro* cocultivation of spleen cells (Jordan *et al.*, 1982). Other tissues that have been implicated as sites of latency were the salivary and prostate glands (Cheung and Lang, 1977), kidneys, ovaries and testes (Brautigam and Oldstone, 1980) and liver (Shanley *et al.*, 1979).

Studies that showed that MCMV replication was cell cycle regulated provided a potential mechanism for latency and reactivation *in vivo* (Hudson

et al., 1979). It can be suggested that if MCMV can not replicate in nondividing cells, latency may arise when the virus infected these types of cells in vivo. In addition, reactivation may occur after latently infected cells receive some 'stimulus' to divide.

The main aim of this investigation was to establish a population of latently infected mice and to study reactivation in vitro through the culture of cells from various tissues of these animals, with the intention of developing a system that could be used to test the effect of various biologically active compounds on promoting reactivation. In addition, replication of MCMV in some primary cell cultures was studied.

MATERIALS AND METHODS.

Virus. The K181 strain of MCMV was used throughout this study. The K181 strain of MCMV, a derivative of the Smith strain, was originally obtained from Dr. June Osborn, University of Wisconsin. All animal infections were performed with K181 strain MCMV that had been passaged in salivary glands of mice. A stock of virus was prepared by infection of 12 SWR/J mice with 10^5 PFU of K181 by intraperitoneal (ip) injection. Fourteen days after infection, the animals were sacrificed, the submaxillary glands were removed and 10% (w/v) suspensions of these organs were made by homogenization. The cellular debris was removed by centrifugation (5000 r.p.m. for 15 min, G.S.A. Sorvall rotor). The virus containing supernatant was aliquoted into 1 ml amounts and frozen (-70°C). The titer of the stock virus was estimated by plaque assay (performed as described in section 1).

Cells. Tertiary mouse embryo cells were used in all experiments for assaying and growing virus. The cells were derived from embryos, from 12-17 day pregnant mice, that had been dissociated by successive trypsin treatments. They were used in assays after two subcultures. These cells were grown in Dulbecco's modified Eagles Medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS) and maintained in DMEM with 5% FBS.

Mice. Male and female SWR/J mice, purchased originally from Jackson Laboratories (Bar Harbor, Me), were bred in the U.B.C. Animal Care Unit.

Inoculation regimes. Several inoculation regimes were tested for use in establishing latent infections. All infections were made by ip

injection. Animals were infected at: 1. 24 hr after birth with 100 PFU; 2. at 14 days after birth with 100 PFU; 3: at 4-6 weeks after birth with 10^4 PFU.

In accordance with criteria established by Jordan et al. (1977), animals were not used in reactivation experiments until at least 4 months after infection.

Assays for free virus. To determine that latent, rather than chronic, infections had been established in the animals, tissues from a sample of each group of animals were tested for free virus, prior to performing reactivation experiments. Submaxillary glands, kidneys and spleens were excised from the animals and 10% (w/v) homogenates made by grinding the tissues with sterile sand in DMEM with 5% FBS. The debris was removed by centrifugation (2000g for 15 min) and 0.5 ml of a 1:10 dilution of each extract was added to 3° mouse embryo cells grown in 24 well plates (Falcon, B-D). The virus was adsorbed for 1 hr, then the cells were rinsed and 1.0 ml of maintenance media added to each well. Cells were observed for cytopathic effects (c.p.e.) for up to 14 days. Media were changed every 4 days.

In vitro cultivation of organs.

a) Salivary glands. The method used was a modification of that described by Owens et al. (1974). Each tissue sample was processed separately. The submaxillary glands were removed from mice by sterile procedures, placed in dishes of warm Hanks Balanced salt solution (HBSS) and trimmed of fat. The tissues were cut into small pieces and incubated 18 hr at 37°C in growth medium (DMEM with 10% FBS) containing 100 U/ml of collagenase (Sigma).

After the incubation period, the pieces dissociated easily by gentle pipetting. The cell material was transferred to centrifuge tubes and stood vertically for 5-10 min. Cell clusters sedimented more rapidly under gravity than isolated cells and debris. The supernatants were removed and disgarded and the sedimented cells resuspended in DMEM. This procedure was repeated three times in total. The sedimented cells were resuspended in growth media (DMEM with 10% FBS, 10% horse serum (GIBCO) and 10 µg/ml insulin (Sigma)) and transferred to 25 cm^2 culture flasks. The epithelial-like cells grew in tightly adherent clusters. Contaminating fibroblasts were removed by selective trypsinization.

Treatment of cultures with 0.05% trypsin, 0.002% EDTA in calcium-magnesium free saline (STV) for 1-2 min would remove most of the fibroblasts. The epithelial cells were very adherent and required 10-15 min treatment with STV before releasing from the flasks.

b) Spleens. Standard dissociation techniques were used to isolate spleen cells (Hudson et al., 1978). In brief, the spleen was teased apart with forceps into Hanks balanced salt solution (HBSS) and the clumps broken up by gentle repetitive pipetting through a hypodermic syringe. The cells were collected by centrifugation at 1000g for 5 min. Red blood cells were lysed by resuspension and treatment with 0.16M NH_4Cl for 4 min. Cells were washed twice with media (RPMI 1640 with 10% FBS) and viability estimated by counting trypan blue excluding cells in a hemocytometer.

Cocultivation assays were performed by mixing spleen cells and mouse embryo cells in RPMI 1640 with 10% FBS and allowing them to settle and adhere to 60 mm petri dishes. Cultures were observed regularly for typical MCMV cytopathic effect. Half the media of each culture was changed every 3-4 days and stored at - 70°C for subsequent plaque assay.

c) Kidneys. Kidneys were dissociated by treatment with 0.25% trypsin in HBSS. The kidneys were cut into small fragments and suspended in 50 ml trypsin solution. This was stirred for 30 min, then the fragments were allowed to settle for 10 min and the released cells were harvested from the supernatant. This was repeated 2-3 times until the fragments had been completely dissociated. The released cells were pooled, washed once and resuspended in growth media (DMEM with 10% FBS) and plated in petri dishes.

d) Liver. Isolated hepatocytes were prepared from mice livers by the technique of Arnheiter (1980). Adult mice were anesthetized with sodium pentobarbital solution (100 mg/kg) containing 1000 U/kg heparin, the abdomen was opened, intestines displaced and the portal vein exposed. The portal vein was canulated with a 21 gauge needle and the inferior vena cava was cut. The liver was perfused in situ via the portal vein, with 12.5 ml of perfusion buffer, held at 37°C (8.4 NaCl, 1.2g $Na_2HPO_4.2H_2O$ and 0.13g KH_2PO_4 per liter of water, pH 7.5) and then with 50 ml of aerated perfusion buffer containing 0.05% collagenase. The liver was then excised, transferred to a petri dish containing DMEM with 10^{-7}M insulin and teased apart by gently forcing through a cell sieve. The cell suspension was washed four times in DMEM with 10^{-7}M insulin by centrifugation (30g for 2 min) and resuspension. Cell viability was determined by trypan blue exclusion. Cells were diluted in DMEM with 15% FBS and 10^{-7}M insulin and plated at 3 - 5 x 10^5 cells/ml in 35 or 60 mm petri dishes. After 2-4 hr, the cultures were rinsed, to remove nonviable cells, and refed with growth medium.

e) Muscle. Muscle fibroblast cultures were initiated by explant culture of fragments of muscle. A section of muscle was excised from a hind leg and cut with a pair of scalpels into 1-2 mm^3 fragments. The fragments were

placed individually onto petri dishes, and then a small amount of growth media was added. Adhesion of the fragments was improved if the surfaces of the petri dishes were scored before the fragments were added. Additional medium was added after 24 hr when adhesion had occurred. Cells could usually be observed growing out from the fragments after 4-7 days.

In vitro replication of MCMV. To determine if MCMV replicated in certain cell types in vitro, cultures were prepared from tissues of uninfected mice by the methods described.

Cultures were infected at various multiplicities of infection (m.o.i.) with tissue culture passaged MCMV (K181). The titers of virus containing supernatants from the cultures were measured by plaque assay, by the method described in section 1.

RESULTS AND DISCUSSION.

1. Establishment of latent MCMV infections in mice. Great difficulty was encountered in establishing latent MCMV infections in SWR/J strain mice. Three different infection regimes were tested before successful establishment of latency could be demonstrated in the majority of animals. This was assessed as the absence of infectious virus in homogenates of salivary glands, kidneys and spleens of mice infected at least 4 months previously.

Infection of mice at 24 hr after birth with 100 PFU of virus (as used by Olding et al., 1975) resulted in a 96% mortality (80/83) within two weeks of infection.

Infection of mice at 14 days after birth with 100 PFU of virus also resulted in a high mortality rate (37%).

Infection of mice at 4-6 weeks after birth with 10^4 PFU of virus did not produce any observable deaths due to MCMV infection.

The other problem encountered was the tendency of the virus to establish prolonged infections in certain tissues of the animals. This was particularly seen in animals inoculated by method 2, where nearly all the animals developed long standing chronic infections and virus was even detected 9 months after infection in the kidneys of 2/3 animals and 11 months after infection in the salivary glands of 1/2 animals. A summary of the outcome of MCMV infection in the groups of mice, at various times after infection, is shown in Table V.

With the use of inoculation regime 3, 88% of animals were virus free after five months. These results appear to illustrate the importance of a functional immune system for limiting MCMV infection of tissues,

Table V:

Outcome of infection of mice of different ages with MCMV.

Inoculation regime.	% Survivors.	Date post infection/months.	Presence and location of virus.
1.	4	4.5	3/3[a] kidney 1/3 s.g.
2.	63	4.5	2/2 kidney 2/2 s.g. 2/2 spleen
2.		5.5	2/2 kidney 1/2 s.g.
2.		5.0	1/1 s.g.
2.		4.0	2/2 kidney 1/2 s.g.
2.		7.5	2/2 kidney
2.		7.0	1/1 neg.
2.		9.0	2/3 kidney 1/3 s.g. 1/3 neg.
2.		11.0	1/2 s.g. 1/2 neg.
3.	100	5.0	15/17 neg. 2/17 kidney
3.		5.5	6/7 neg. 1/7 s.g.

Notes:
a - no. of mice with detectable free virus / no. of mice tested
s.g. - salivary glands. neg. - no virus detected.
inoculation regime 1. Mice inoculated at 24 hr with 100 PFU virus.
inoculation regime 2. Mice inoculated at 14 days with 100 PFU virus.
inoculation regime 3. Mice inoculated at 4-6 weeks with 10^4 PFU virus.
Virus used was salivary gland passaged MCMV (K181).

particularly when the virulent strain of MCMV is used. It appeared that once infection had become established in the salivary glands and kidneys, the virus was not readily accessible to immune clearance. These results have similarities to some published after the termination of this study (Jordan et al., 1982). They showed that 46% of C3H and 62% of DBA mice infected (ip) before 1 week of age with 10^3 PFU of attenuated MCMV were virus positive in the salivary glands 4 months after infection. The high incidence of chronic infection observed in this study may be a characteristic of the SWR/J resistance to MCMV infection (Hudson, 1984).

2. Reactivation of MCMV by cell cultivation methods. As difficulties were encountered in repeatably obtaining viable murine hepatocytes for culture, by in situ perfusion, this organ was not included in the group studied for the detection of reactivated virus.

A summary of the results of a number of reactivation experiments is listed in Table VI. It can be seen that in the first group of experiments (1-5), reactivation was detected in spleens from mice that had free virus in other tissues. A portion of the spleens used in each of these experiments was also tested for the presence of free virus and shown to be negative.

The results in expts. 2 and 3 indicated that virus could be reactivated from spleen cells of SWR/J mice. This was first identified in the cultures after 8 and 10 days, respectively, of cocultivation. This period was slightly shorter than the cocultivation periods of 2-3 weeks reported by Olding et al. (1975) and Jordan et al. (1982), but longer than when free virus had been present in the tissues. However, the presence of infectious virus in other tissues invalidated any conclusions that could be drawn from the reactivation that occurred in these spleen cells.

Table VI:

Reactivation of MCMV from latently infected tissues using cell cultivation methods.

Expt.no.	Inoc reg.	ORGAN TYPE. spleen.	kidney.	s.g.	muscle.
1.	1.	-	F.V.[b]	-	N.D.[c]
2.	1.	+ 8d[a]	F.V.	-	N.D.
3.	1.	+ 10d[a]	F.V.	F.V.	N.D.
4.	2.	- [d]	F.V.	-	N.D.
5.	2.	- [d]	F.V.	-	N.D.
6.	2.	$3/6.75x10^{7}$[e]	-	+	-
7.	3.	$2/1.2x10^{8}$[e]	-	-	-
8.	3.	+	-	-	-
9.	3.	+	-	-	-
10.	3.	+	-	-	-
11.	3.	$2/6.5x10^{7}$[e]	-	+	-
12.	3.	-	-	-	-
13.	3.	-	-	-	-
14.	3.	$2/4x10^{7}$[e]	-	-	-

Notes:
a. no. of days for virus to be detected.
b. F.V. = free virus. c. N.D. = not done.
d. spleen cells fractionated before cocultivation.
e. no. of reactivation foci / total no. of spleen cells assayed.
-. no virus detected. +. virus detected.

In expts. 4 and 5, the spleen cells were separated into plastic adherent and nonadherent populations prior to cocultivation. Virus was not detected in these cases. One would have expected to be able to detect virus in one of the populations, however, Jordan and Mar (1982) had shown that the frequency of reactivation was noticeably less when using spleen cells that had been manipulated.

In expts, 6, 7, 11 and 14, the frequency of MCMV reactivation was determined by cocultivation of all the cells present in each spleen. The spleen cells and mouse embryo cells were mixed in a ratio of 10:1 (5×10^6 spleen cells : 5×10^5 mouse embryo cells) in RPMI 1640 with 10% FBS and added to 60 mm petri dishes. In a preliminary experiment, it had been shown that reactivation had not occurred when the cells were cultured, in the same proportions, in Dulbecco's modified Eagle's medium. It appeared necessary to adjust the culture conditions to ensure optimum survival of the spleen cells, rather than of the fibroblasts. The dishes were examined every second day for the presence of MCMV cytopathology. The time after infection that this was observed was noted. In expt. 6, viral cytopathic effect was observed in three separate dishes after 8 days, 13 days and 18 days respectively. The spread of the cytopathic effect was initially slow and by frequent examination of the dishes, the appearance of an initial 'reactivation foci' could be observed.

A cytopathic effect in the other spleen cultures was first observed between 10-15 days, with the exception of one, where it was noticed after 6 days (expt. 7). This does raise the question as to whether chronic infections can also occur in the spleen under certain circumstances.

A most noticeable feature was the very low frequency of separate reactivation events occurring in each animal. From our figures, it appeared

that 1 spleen cell in 2-6 x 10^7 was carrying MCMV in a latent form from which it could be reactivated. This was considerabaly less than the 1 lymphocyte in 1.2 x 10^5 estimated by Jordan and Mar (1982). It was not possible to determine whether these differences represent a genetic difference in immune response to the virus by the different strains of mice or were due to the fact that, in the latter case, the mice were infected with 10 times more virus. In comparison, 3-4 MCMV genome equivalents per 100 spleen cells were detected by DNA reassociation hybridization (Olding et al., 1976). The very low frequency of reactivation indicated that this system was not suitable for studying the biochemical modulation of reactivation.

In two experiments, MCMV was apparently reactivated from cultured submaxillary gland cells. In expts. 6 and 11, virus was detected in the supernatants taken 9 and 15 days, respectively, after culture. However, it can not be ruled out that low levels of MCMV had persisted in the tissue and had replicated to detectable levels in the fibroblasts that were contaminating the cultures. In no case was a cytopathic effect observed in the epithelial cells of these cultures.

Virus was not detected from cultures of kidney cells or muscle fibroblasts. The viability of the cultures varied from animal to animal and it was difficult to keep them in a viable state past 3 weeks. The kidney cells could not be passaged without loss of a large percentage of the cells.

3. Replication of MCMV in primary cultures.

a). murine hepatocytes. As stated earlier, extreme difficulty was encountered in repeatably producing viable cultures of murine hepatocytes by in situ perfusion. Considerable variability in the number of viable cells and the efficiency with which they adhered to the petri dishes was observed

between animals. Several factors are known to control the survival of these cells during the dissociation stage. The most critical steps involve procedures to minimize shearing, as the cell membranes are known to be reversibly damaged following the two perfusion steps. A study of variable factors in the isolation procedure for producing viable murine hepatocytes was published after the termination of this work (Klaunig et al., 1981). This listed features that varied from the method used (Arnheiter, 1980), including the concentration of collagenase, method of perfusion and rates of centrifugation of the separated cells. All these factors can contribute to reduced yields of viable cells. Table VIIa shows the mean titers from two separate experiments for the replication of MCMV in murine hepatocytes. In these cultures, the cell viability and attachment had been high. The cells showed a progressive cytopathic effect, characteristic of MCMV infection, that eventually involved all the cells. This indicated that the MCMV was being replicated. The results indicated a typical one step infectious cycle, though it appeared more protracted than the MCMV replication cycle in fibroblast cells.

An interesting feature was the fact that MCMV appeared to replicate in cells that do not undergo replicative DNA synthesis in vitro. Arnheiter (1980) had shown that only 0.2-0.5% of unstimulated hepatocyte cells underwent DNA synthesis.

In some of the other cultures established, no virus replication occurred. These cells degenerated more quickly than the productively infected cultures.

b). murine kidney cells. The results for the replication of MCMV in cultured secondary mouse kidney cells are shown in Table VIIb. The results are difficult to interpret as the cultures were contaminated with a small

TABLE VII.

Replication of MCMV in primary cell cultures.

a) Replication of MCMV in murine hepatocytes.

Days post infection.	Titer: PFU/ 10^6 cells.
1.	360.
2.	9.2×10^4
3.	5.2×10^5
4.	8.6×10^5

Cells were infected at a m.o.i. of 1

b) Replication of MCMV in mouse kidney cells.

Days post infection.	Titer: PFU/ 10^6 cells.
1.	6.8×10^2
3.	5.1×10^3
4.	1.1×10^4
5.	1.4×10^4
6.	3.0×10^4

Cells were infected at a m.o.i. of 1

c). Replication of MCMV in IUdR treated and untreated mouse kidney cells.

Days post infection.	Titer: PFU / 10^6 cells.	
	IUdR treated.	Untreated.
1.	4.8×10^2	1.6×10^3
3.	1.4×10^4	1.8×10^4
4.	1.0×10^4	2.6×10^4
5.	6.2×10^3	3.4×10^4
7.	7.1×10^4	4.1×10^4

Cells were infected at a m.o.i. of 2

percentage of fibroblasts. However, a cytopathic effect was observed in a small percentage of epithelial-like cells.

Pretreatment of murine kidney cells with 5-Iodo 2'-deoxyuridine (IUdR) (100 μg/ml) for three days, in a similar manner to that described by St. Jeor and Rapp (1973) and Plummer and Goodheart (1974), did not have the effect of making them more permissive to virus replication (Table. VIIc). In these experiments, it appeared that IUdR pretreatment was having an inhibitory effect.

However, in a similar experiment, cells were pretreated with IUdR for 18 hr before infection with MCMV at a m.o.i. of 10-20. The aim of this experiment had been to detect immediate-early and early viral proteins by labeling infected cells with (^{35}S) methionine. These could not be detected by polyacrylamide gel electrophoresis (data not shown). However, the treated cells all showed a viral cytopathic effect at 24 hr pi. (both epithelial and fibroblast cells), while about 30% of the untreated cells showed a cytopathic effect. This seemed to indicate that IUdR pretreatment was increasing the susceptibility of murine kidney epithelial cells, in some fashion, to MCMV infection.

c). murine salivary gland cells. Infection of cultures from submaxillary glands with MCMV did not result in productive infection of the epithelial cells. MCMV did not produce a cytopathic effect in these cells, even with a high m.o.i..

These results showed that in vitro cultivation of cells from tissues of SWR/J mice, latently infected with MCMV, was not a suitable system for studying the modulation of reactivation. The very low frequency of reactivation of MCMV from spleen cells, the apparent absence of reactivation

from the other tissues cultured, along with the variable ability to culture cells from tissues of adult animals, entailed the abandonment of this system and studies were performed instead with continuous cell lines (described in section 1).

The most interesting feature from this study was that fact that MCMV could replicate in hepatocyte cultures. As these cells do not undergo cellular DNA synthesis in vitro, it illustrated that MCMV replication may not be cell cycle regulated in all cell types. In addition, the fact that MCMV replicated in epithelial types of cells was surprising. With the improved murine hepatocyte isolation technique referred to (Klaunig et al., 1981), it would be valuable to continue with the preliminary studies reported here.

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