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Characterization of Ganciclovir-Resistant Mutants of Human Cytomegalovirus

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CHARACTERIZATION OF GANCICLOVIR-RESISTANT MUTANTS
OF HUMAN CYTOMEGALOVIRUS

by

Nell S. Lurain

A Dissertation submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

April

1990

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LIFE

Nell S. Lurain is the daughter of William P. Snavely and Alice (Pritchett) Snavely. She was born August 1, 1946, in Charlottesville, Virginia.

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Lurain, N.S., K.D. Thompson, and S.K. Farrand. 1986. Rapid detection of cytomegalovirus in clinical specimens by using biotinylated DNA probes and analysis of cross-reactivity with herpes simplex virus. *J. Clin. Microbiol.* 24:724-730.

Lurain, N. and K. Thompson. Development of a probe for rapid detection of cytomegalovirus by DNA-DNA hybridization. Abstr. Ann. Meeting American Society for Microbiology, St. Louis, Missouri, 1984.

Lurain, N., K.D. Thompson and S. Farrand. Detection of cytomegalovirus (CMV) in clinical specimens using a biotinylated probe. Abstr. Ann. Meeting American Society for Microbiology, Washington, D.C., 1986.

Tucker, P., F.-J. Lee, N.S. Lurain, J.P. O'Keefe, and K.D. Thompson. Comparison of DNA probe, monoclonal antibody and culture in the laboratory diagnosis of cytomegalovirus infection. Abstr. American Federation of Clinical Research, Chicago, IL, 1987.

Lurain, N.S., K.D. Thompson, and S.K. Farrand. 1988. Rapid detection of cytomegalovirus in clinical specimens by using biotinylated DNA probes and analysis of cross-reactivity with herpes simplex virus, p. 313-314. In Year Book of Pathology and Clinical Pathology. K.M. Brinkhous (ed.) Year Book Medical Publishers, Inc., Chicago, IL.

Lurain, N.S., W.A. Tatarowicz, G.S. Read, and K.D. Thompson. Characterization of mutants of human cytomegalovirus resistant to ganciclovir. Second International Cytomegalovirus Workshop, San Diego, California, 1989.

Tatarowicz, W.A., N.S. Lurain, and K.D. Thompson. Development of an in situ ELISA for the rapid screening of human cytomegalovirus infected cells for a late nuclear antigen.

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INTRODUCTION

This project is comprised of three main areas of investigation of human cytomegalovirus (HCMV). The first of these areas is the development of DNA probes derived from HCMV strain AD169 for the rapid detection of HCMV in clinical samples. Three probes were selected and tested against a large number of clinical samples. One probe was found to cross-hybridize with herpes simplex virus types 1 and 2 (HSV-1, HSV-2) DNA controls.

This observation led to the investigation of the cross-hybridization, which was localized to specific restriction fragments of HCMV and HSV-1. Since the particular HSV-1 restriction fragment was known to encode the viral DNA polymerase, it appeared likely that the corresponding fragment of HCMV encoded the HCMV DNA polymerase.

The viral DNA polymerases of HCMV and HSV are targets for a number of antiviral agents. Therefore, the third and most extensive area of investigation was the isolation and characterization of drug-resistant mutants of HCMV. Three mutants of HCMV AD169 which were resistant to ganciclovir (GCV) were isolated. Although the viral DNA polymerase is a target for GCV, this drug requires intracellular phosphorylation in order to become therapeutically active.

The three mutants were characterized by high performance liquid chromatography (HPLC) for the study of GCV anabolism in infected cells.

It was found that extracts of cells infected with each of the mutants contained much lower levels of the phosphorylated derivatives of GCV compared to the levels of the same derivatives in wild-type-infected cells. These data suggested that a virus-specific kinase activity responsible for GCV phosphorylation was decreased in mutant-infected cells.

Three experimental approaches were devised to characterize this new HCMV GCV kinase function. The first approach was in vitro translation and expression of enzyme activity. Preston (121) reported that the HSV-1 thymidine kinase (TK) activity could be detected from in vitro translation products of RNA isolated from infected cells. Since the TK of HSV-1 is known to phosphorylate GCV in HSV-1-infected cells, similar in vitro translation experiments were performed using HCMV mRNA.

The second approach was marker transfer (33,114) of GCV resistance from mutant to wild-type virus. A cosmid library was made of one of the mutants. Marker transfer experiments were performed by transfecting human foreskin fibroblasts (HFF) with individually selected cosmids. The HFF were either co-transfected with wild-type genomic DNA or subsequently infected with wild-type virus. The transfected cells were cultured in the presence of GCV for selection of recombinant progeny.

The third approach was to compare infected cell proteins from mutant and wild-type-infected cells by two-dimensional polyacrylamide gel electrophoresis. Comparison of mutant and wild-type proteins by this method revealed a protein which was present in the wild-type HCMV AD169 but absent in uninfected and mutant-infected cells.

LITERATURE REVIEW

Structure and growth characteristics. Human cytomegalovirus (HCMV) is a member of the family Herpesviridae. These viruses generally produce primary infections which become latent and may later reactivate. Other human viruses which are classified in this family include Epstein-Barr virus (EBV), herpes simplex types 1 and 2 (HSV-1, HSV-2) and varicella-zoster (VZV). The growth characteristics of these viruses in cell culture differ with respect to permissive cell type, length of replication cycle and extracellular viral titer (38). HSV types 1 and 2 grow in a variety of mammalian cells with a short replication cycle resulting in release of the virions into the extracellular fluid. By comparison the only permissive cells for HCMV replication are human diploid fibroblasts. Replication occurs very slowly and there is little release of virus into the cell culture fluid. Repeated passage of HCMV isolates in culture may eventually result in measurable viral titer in the extracellular fluid, however, the titer varies with the particular strain of virus. There are a number of well-characterized laboratory strains (36,78,87,126) derived from clinical isolates after multiple cell-culture passage. These strains differ from their initial isolates by producing relatively high extracellular viral titers. The strain used in all of the experiments for this project was AD169 (137).

The complete virion of HCMV is composed of a double-stranded linear

DNA-containing core, an icosahedral capsid and an envelope (158,174). Electron micrographs, however, have demonstrated the presence of many incomplete viral particles, which may contain less than the full complement of viral DNA (103,174). In addition, there are "dense bodies", which appear to be composed of excess viral structural proteins surrounded by an envelope. The dense bodies carry HCMV-specific antigens but have no detectable DNA (137,159). Thus, the ratio of non-infectious particles to plaque-forming units (pfu) resulting from HCMV infection may be as great as 100:1.

The replication steps of HSV and HCMV also have been compared by electron microscopy. Both viruses enter cells by phagocytosis or fusion with the plasma membrane. Both rapidly traverse the cytoplasm to the perinuclear area, but HCMV nucleocapsids appear to acquire a fine fibrillar coat in the cytoplasm and are subsequently disassembled much more slowly than HSV nucleocapsids (141). Adsorption and penetration, therefore, occur equally rapidly in both HSV and HCMV infections, but replication of HCMV DNA proceeds at a much slower rate than that of HSV (38,169,142).

Another difference between HSV and HCMV is that HSV infection rapidly shuts down host macromolecular synthesis while HCMV infection in permissive cells may stimulate the synthesis of cellular macromolecules (37). The physiological state of the permissive cell, however, affects the amount of cellular DNA synthesis as well as the amount of HCMV replication. Subconfluent cells are much more susceptible to HCMV infection than are confluent cells. It was demonstrated by DeMarchi and

co-workers (37), however, that stimulation of cellular DNA synthesis only occurred in abortively infected cells, while DNA synthesis in productively infected cells was eventually inhibited. The rate of inhibition of DNA synthesis is much slower than that which occurs in HSV-infected cells.

Molecular characteristics of HCMV DNA. The size of the infectious HCMV genome is approximately 240 kilobase (kb) pairs, but there is some variation among strains (38,57,90). Replication of full-length genomes requires inoculation of permissive cell monolayers at a multiplicity of infection (MOI) of 0.1 or less. When the MOI is greater than 0.1, there is an increase in production of defective genomes of less than 240 kb in size (161).

The structure of the HCMV genome is similar to that of HSV 1 and 2. There is a long unique sequence (U_L) bounded by inverted repeats (IR_L) and a shorter unique sequence (U_S) also bounded by a different set of inverted repeats (IR_S) (Figure 1). The presence of submolar fragments in restriction endonuclease digests of the viral DNA supports a model in which there are four possible genome arrangements. Inversion of each unique sequence and its repeats relative to the other produces the four structures, which are present in approximately equal amounts in infected cells (67,87,116,165).

Restriction endonuclease cleavage patterns and nucleic acid hybridization techniques have been used to determine strain variation of HCMV (78,147,164). Strains of HCMV from the same species show approximately 80% or greater homology at the DNA level (78,126). A study of several strains of HCMV demonstrated, that in spite of loss or gain of

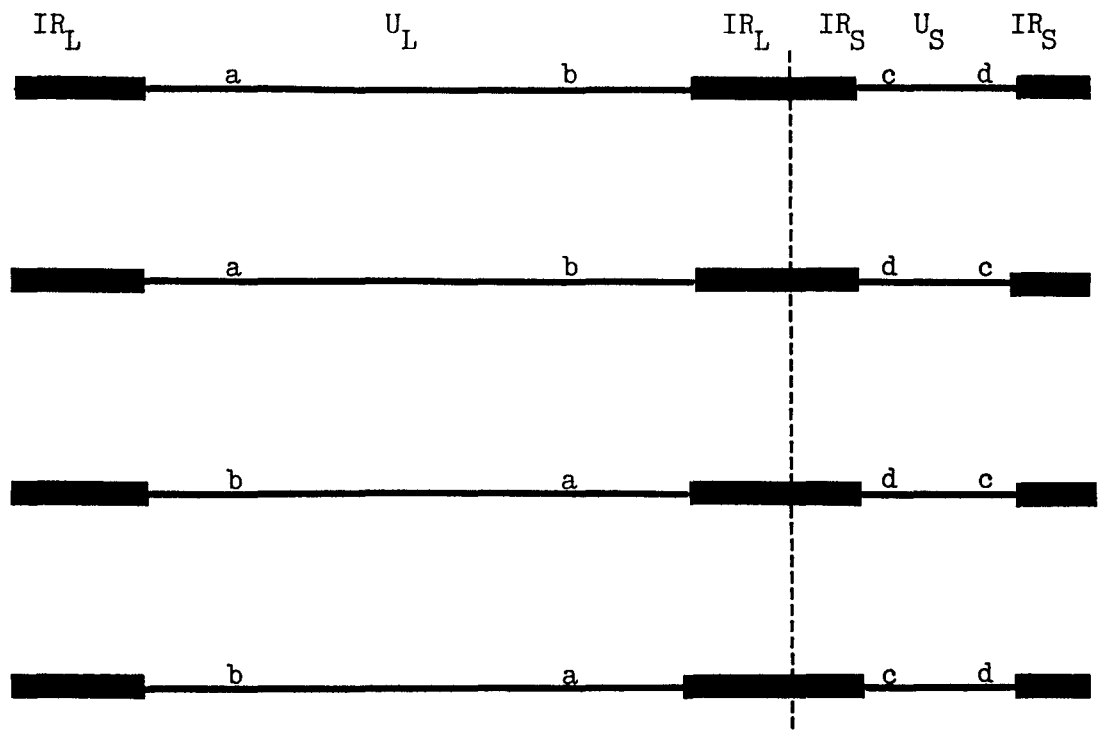
Figure 1. The structure of the genome of HCMV. The four arrangements are generated by the ability of each unique sequence and its associated repeats to invert in relationship to the other.

IR_L = long inverted repeat sequence

IR_S = short inverted repeat sequence

U_L = long unique sequence

U_S = short unique sequence



restriction endonuclease sites, the order of homologous fragments is colinear for all of these strains (26,172).

Digestion of the DNA of HCMV strains with several restriction endonucleases produces strain-specific "fingerprints" (150). Huang et al. (78) studied a group of women and their infants who had evidence of HCMV infection. The patterns of DNA restriction fragments of mother and offspring were identical for at least two restriction enzymes (BamHI and HindIII) and showed only minor changes in band migration with a third enzyme (EcoRI). Unrelated controls carried heterogeneous strains of HCMV with different restriction patterns for all three enzymes. Repeat isolates from some of the same patients over a period of as long as nine years had the same restriction pattern as the initial isolate. In spite of the apparent strain stability, however, there is as yet no classification of HCMV similar to that of HSV, which can be separated into two types by both antigenic and molecular criteria (87,158).

A characteristic that HCMV does have in common with HSV is temporal expression of the viral genome (38,160,168,169). Three groups of virus-specific proteins have been described based on their order of production in the infected cell. Immediate early proteins (IE) require no prior viral protein synthesis for their expression and are produced in lytically-infected cells after treatment with protein synthesis inhibitors or in abortively-infected cells (109,162,163). There is a switch from IE proteins to early (E) proteins within two hours post-infection in lytically-infected cells. Early proteins do not require viral DNA synthesis, and they are produced in the presence of viral DNA replication

inhibitors. Some E proteins are also produced in latently-infected cells (6,81). Late (L) proteins require viral DNA synthesis for expression and are, therefore, not produced in nonpermissive, abortively or latently-infected cells (55,63,102).

Another characteristic of HCMV replication is that the temporal expression of the genome is frequently controlled posttranscriptionally (55,146). HCMV early and late messages are both present early in infection, but the messages are not translated until the appropriate time.

There are therefore, several factors which influence the outcome of HCMV infection; 1) the type and species of cell; 2) the physiological state of the cell (8,35,37); and 3) the temporal expression of the viral genome (38,160,163,169). Both cellular and viral regulatory proteins, involved in the temporal control may be responsible for the prolongation of the HCMV lytic cycle and for the determination of latency or persistence in non-lytic infections (8,169).

Epidemiology. Evidence of HCMV infection is widespread. Detectable antibody titers and active virus excretion can be demonstrated in a high percentage of members of various socially-defined groups. From 20 to 60% of children in day care centers shed virus in their urine (1,84,118). Up to 2% of newborns are infected congenitally (69,154). Between 20 and 80% of the general adult population is seropositive (43,170,173), but seropositivity can be as high as 95% in homosexual men (28). Most HCMV infections are asymptomatic, but serious complications may occur in neonates or in immunocompromised hosts (120,131,155,156).

Congenital infection is defined by isolation of virus from patient

specimens taken during the first week of life (11,156,176). The source of the virus appears to be predominately a reactivation of maternal infection, although occasionally primary maternal disease occurs during pregnancy. Symptoms range from persistent viruria to death. The severity of the symptoms is greatest with primary maternal infection, but factors such as gestational age at the time of infection and the immune response of the fetus may also contribute to the clinical outcome (10,119,120,154,155).

Perinatal infection is also usually maternal in origin, but nosocomial sources may produce disease in some cases (39,69,148). The maternal infection is again generally a reactivation of latent virus and is passed to the infant through the genital tract or in breast milk (75,131). Many of these infants shed virus asymptotically, but in some cases the children have neurologic sequelae even though they are initially asymptomatic. Slow psychomotor development and loss of hearing may become apparent at a later age (131,170).

Current interest in the pathogenicity of HCMV stems from its association with blood transfusion (2,85,125), organ transplantation (16,17,66,105,153), and the acquired immune-deficiency syndrome (AIDS) (43,44,149). Cytomegalovirus infections under these conditions usually are caused by endogenous reactivation of latent virus, however, organ transplant and blood transfusion recipients may also be infected by reactivation of latent virus present in the exogenously-acquired tissue.

HCMV has been recovered from peripheral blood leukocytes (41,175), which are probably the source of post-transfusion HCMV infection.

Multiply-transfused patients show a higher rate of seroconversion, which is further evidence of transmission of HCMV through blood products (2). It would appear, therefore, that donor blood can be a source for HCMV transmission. However, there is evidence that reactivation of latent virus can occur following transfusion. When viral isolates from a group of donors and recipients were compared by DNA restriction endonuclease analysis, the paired isolates appeared to be unrelated. (76). Most patients remain asymptomatic, but neonates and immunosuppressed patients are vulnerable to adverse sequelae (85).

Organ transplant recipients are highly susceptible to HCMV infection (62,123). In many cases the source of the virus is probably a reactivation of latent host infection, but transmission of virus from the donor organ has been frequently documented (16,105,166). Chou (16) analyzed viral DNA from renal donor-recipient paired isolates for restriction enzyme fragment polymorphisms. Seropositive recipients were found to shed virus of donor origin in some cases, but viral strains different from those of the donors were also recovered. The donor strain was detected in isolates from seronegative recipients.

In a series of cardiac transplant patients (123) the donors were demonstrated to be the source of HCMV when the recipients were seronegative. These recipients had primary HCMV infections from latent or infectious virus present in donor tissue or contaminating blood. The recipients who were seropositive for HCMV before transplantation, and who subsequently developed HCMV viremia, most likely had a reactivation of latent host virus.

Finally, HCMV infection is found in close to 100% of patients with AIDS (42,44,52). A significant number of these patients may experience severe HCMV infection in the form of retinitis, colitis or pneumonia. HCMV may cooperatively enhance the pathogenicity of the human immunodeficiency virus (HIV). Nelson et al. (110) reported that HIV and HCMV can co-infect brain cells in patients with AIDS. The mechanism of synergistic interaction, however, has not been determined. In these patients HCMV infection is most frequently a reactivation of latent infection, because the majority of the patients are seropositive for HCMV at the onset of AIDS (45,68).

Latency and oncogenicity. Since reactivation of latent HCMV infection appears to be a significant factor in the pathogenesis of the virus, there have been a number of studies to determine the site of the latent infection. Polymorphonuclear and adherent mononuclear leukocytes seem to be the most likely sources of inactive virus (32,41,83,114,132,139). In situ hybridization experiments have demonstrated the presence of viral DNA and RNA in both of these cell types in the peripheral blood. Human lymphocytes and monocytes can be infected with HCMV in vitro, but this results in abortive infection (132). Factors which may trigger activation of the virus include infection with another agent (43,52), host immunosuppression (85) or host response to allogenic cells (17,114).

There is evidence that HCMV may also be oncogenic (19,108). Three transforming regions of the genome of strains AD169 and Towne have been mapped to the U₁ (XbaI C fragment of AD169). Each of these is designated

as a morphological transforming region (mtr), which is capable of neoplastic transformation of established rodent cell lines. The mtrI is a non-coding region, which can transform NIH 3T3 cells. The sequence can form a stem-loop structure similar to an insertion element (109). Both mtrII and mtrIII transform rodent cell lines (47). There are open reading frames in mtrII in addition to potential stem-loop structures (130). No transforming proteins have been identified. Transcriptional activation or genetic rearrangement by HCMV DNA elements may be responsible for cell transformation.

In a recent study (7) it was reported, that HCMV infection activates the proto-oncogenes c-fos, c-jun and c-myc. Increased production of all three proto-oncogene mRNAs was noted as an immediate early event in HCMV infection. Inactivated virus was still able to cause an increase in the proto-oncogene mRNA levels, therefore, viral protein synthesis was not required. It was postulated that interaction of the virus with the cell surface might be the mechanism of activation, but the role of this activation in HCMV oncogenesis remains speculative.

Susceptibility to Antiviral Agents. Another factor which adds to the seriousness of HCMV infection is the resistance of this virus to many antiviral agents including acyclovir (ACV). One agent which has proven effective in treatment of severe HCMV infection is 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG). DHPG is now more commonly referred to as ganciclovir or GCV.

GCV has been used experimentally to treat severe HCMV infections especially in transplant or AIDS patients (27,24,94). It is currently

approved for treatment of HCMV retinitis. Most clinical and laboratory strains remain sensitive to GCV (106,122), but increased resistance has been reported among both types of isolates (4,24,48). Neutropenia is a side-effect of GCV treatment, which limits the usefulness of this drug for some patients (94).

A second antiviral agent which has been clinically tested for inhibitory activity against HCMV is phosphonoformic acid (PFA or foscarnet) (167). There are also adverse side-effects associated with this agent. Manischewitz et al. (99) demonstrated a synergistic inhibition of HCMV by GCV and PFA for both clinical isolates of HCMV and the laboratory strain AD169. Both drugs inhibit viral DNA polymerase activity, but the target site within the polymerase is probably different for each drug. In addition the toxicities of GCV and PFA differ. The synergy might allow treatment with lower doses of each drug, and thus, minimize the adverse side-effects of the individual agents.

In addition to treatment of HCMV infections antiviral agents have been used in the selection and isolation of resistant mutants. Viral genes related to drug activity can be mapped from these mutants. The HSV-1 DNA polymerase coding sequence was functionally determined in this manner (20,58,71,72,88,127). Agents whose target is the DNA polymerase include phosphonoformic acid (PFA) (112), phosphonoacetic acid (PAA) (40,74) and aphidicolin (Aph) (80,111). Several other agents which target the polymerase also must be activated by cellular or viral kinases. Among these agents are 9- β -D-arabinofuranosyladenine (Ara A) (22), 5'-iodo-2'-fluoroaracytosine (FIAC), 5'-iodo-2'-fluoroarauracil (FIAU) (25,100), ACV

(93,138) and GCV (15,31,50,53,143). Cellular enzymes phosphorylate AraA (30). FIAc, FIAU, ACV and GCV are monophosphorylated in HSV-1-infected cells by the viral TK. These agents are only phosphorylated at low levels by the cellular TK (5,25,30). Di- and tri-phosphorylation is thought to be carried out by cellular enzymes. This means that drug-resistant mutants of the virus may be separated into two classes: 1) thymidine kinase mutants in which the enzyme fails to phosphorylate the drug and 2) polymerase mutants in which the altered enzyme is no longer a target for the drug. Both types of mutants have been described for HSV-1 (23,93,138).

Although cellular thymidine kinase activity is increased in HCMV-infected cells, no viral thymidine kinase activity has been demonstrated (49,53,177). The fact that HCMV is sensitive to GCV, however, suggests that monophosphorylation of this drug in HCMV-infected cells requires a viral function. In addition there is evidence, that the cellular thymidine kinase activity induced by HCMV infection is completely separable from the "GCV kinase" activity (4,5,53). Therefore, there also may be two classes of HCMV GCV-resistant mutants.

There have been only two reports of HCMV drug-resistant mutants. D'Aquila and Summers (34) isolated PAA-resistant mutants and Biron et al.(4) characterized a GCV-resistant mutant. In both cases the mutants were selected by growing the wild-type virus in increasing concentrations of the drug. Unlike HSV there have been no one-step high-level drug-resistant mutants of HCMV reported to date.

Rapid detection of HCMV. The potentially severe consequences of

HCMV infection and the availability of antiviral agents make rapid diagnosis desirable (133). Cell culture is usually very slow, but it is still the standard identification method for comparison with newer more rapid techniques. Two techniques which show the most promise for rapid viral identification are: 1) detection of viral antigens with monoclonal antibodies, and 2) hybridization of viral nucleic acids with labeled probes.

Monoclonal antibodies specific for HCMV are available commercially. Detection of infected cells by immunofluorescence is now widely used in conjunction with cell culture (61,82,96,101,145). The sensitivity of the method is still not high enough to detect all HCMV-positive specimens, but for screening purposes it is very useful. One disadvantage of the immunofluorescence method is that it still requires cell culture for antigen amplification.

The use of DNA probes for detection of HCMV can be applied directly to clinical specimens. Probes labeled with ^{32}P are sensitive (11,18,140,150,151), but the isotope has a short half-life and is hazardous. Nonisotopically-labeled probes have been developed to avoid these problems (9,12,92,95,97,107), but the sensitivity has been lower as compared to that of the radioactive probes.

Amplification of probe target sequences by the polymerase chain reaction (PCR) has improved significantly the sensitivity of hybridization methods (13,73,115). Selection of primers, however, is critical, since variation in clinical isolates may prevent primer binding and subsequent amplification. Hsia et al.(73) used two sets of primers homologous to

sequences at opposite ends of the genome. The PCR amplification produced positive results in all culture-positive specimens. Olive et al. (115) reported that the PCR method of detection in clinical samples was only positive when the same sample was scored as positive with at least one other method (culture, immunofluorescence or direct probe hybridization). The assay has, therefore, been reported to be as sensitive as cell culture and results are available rapidly.

It is apparent that there have been recent advances in detection and treatment of HCMV infections. These advances are in large part the result of a better understanding of the molecular biology of both HSV and HCMV. Detection methods are based on characterization of both virus-specific antigens and selected regions of the DNA genome. The design of antiviral agents requires an understanding of the function of virus-specific proteins and the transcriptional and translational control of their expression. Knowledge of the genome organization and viral products of HSV has provided a basis for many of the experiments for HCMV. HCMV is a much more difficult viral system to work with than HSV, and for this reason the level of understanding of the mechanisms of HCMV pathogenesis still lags behind that of HSV.

MATERIALS AND METHODS

Virus. HCMV strain AD169 (134) was obtained from Dr. Marc Beem at the University of Chicago. Clinical isolates of HCMV, HSV-1 and HSV-2 were collected from the Clinical Microbiology Laboratory at Loyola University Medical Center. Additional clinical isolates of HCMV were collected by Dr. Mary Christensen, Childrens Memorial Hospital, Chicago, IL.

Virus stocks were frozen and maintained at -70° C in medium containing 10% dimethylsulfoxide (DMSO) and 10% fetal calf serum (FCS). Stocks were propagated by inoculation of permissive cell monolayers at an MOI of 0.01-0.001. Virus stocks were harvested 3 days after the appearance of 100% CPE.

Cells. Human foreskin fibroblasts (HFF) at passage 5-7 were purchased from Bartels, Bellevue, WA, and maintained in Eagle's minimum essential medium (MEM) (Hazelton, Denver, PA or Gibco, Grand Island, NY) supplemented with 1% FCS (Gibco), 7.5% NaHCO_3 (to adjust pH to 7.2), L-glutamine (1 mM), HEPES buffer (20 mM) (Flow Laboratories, McClean, VA), gentamicin (50 $\mu\text{g}/\text{ml}$) (M.A. Bioproducts, Walkersville, MD) and amphotericin B (Gibco) (2.5 $\mu\text{g}/\text{ml}$). Confluent monolayers were passaged 1:2 and grown to confluency in Eagle's MEM containing the same supplements except 10% FCS. Earles balanced salt solution (EBSS) (Flow Laboratories) was used for washing cell monolayers.

Mycoplasma testing. Virus stocks of AD169 and mutant D6/3/1 were tested for mycoplasma contamination by culture on Mycotrim RS and Mycotrim GU triphasic medium (Hana Biologics, Inc. Alameda, CA). In addition HFF monolayers inoculated with each stock were sent to Program Resources, Inc., McClean VA (formerly Flow Laboratories) for more extensive testing. This testing included Hoechst staining of infected cells, monoclonal antibody fluorescent staining specific for M. hyorhynis, and culture of both cell culture fluid and cells.

Antiviral agents. All antiviral agents were reconstituted in distilled water except for aphidicolin, which was reconstituted in 10% DMSO. The stocks were filtered through a 0.2 μm filter for sterility. The following is a list of the agents and manufacturers:

1) Adenine 9- β -D-arabinofuranoside (AraA); thymine-1-D-arabino-furanoside (AraT); aphidicolin (Aph); phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) all from Sigma, St. Louis, MO.

2) 5-iodo-2'-fluoroarauracil (FIAU); 5-iodo-2'-fluoroaracyto-cytosine (FIAC) both a gift from Bristol-Myers, Wallingford, CT.

3) Acyclovir (ACV) from Burroughs Wellcome, Research Triangle Park, NC.

4) Ganciclovir (GCV) a gift from Burroughs-Wellcome, Research Triangle Park, NC and Syntex, Palo Alto, CA.

Plaque assay. The method of Wentworth and French (171) was modified for titering and plaque purifying virus. HFF cells were grown to 80-90% confluency in 24-well tissue culture plates. Ten-fold viral dilutions were made in maintenance medium for inoculation. Growth medium from the

wells was removed and replaced with 0.1 ml inoculum. The virus was allowed to adsorb for 2-4 h at 37° C. The inoculum was removed at the end of the adsorption period and replaced with an overlay of equal parts 0.6% agarose and double strength maintenance medium. Seven days later a second overlay of the same composition was placed on top of the first. Fourteen days were usually required for reading of titers and plaque purification.

Extraction and purification of viral DNA. Virus stocks were propagated in either tissue culture flasks (150 cm²) or roller bottles (850 cm²). Each monolayer was inoculated at subconfluency at an MOI between 0.1 and 0.01. The supernatant was harvested 3 days after 100% CPE was observed.

A modification of the method of La Femina and Hayward (91) was used to extract the DNA. Infected cell culture fluid was centrifuged at 7,000 rpm for 10 min in a Sorvall GSA rotor. The supernatant was centrifuged at 12,000 rpm for 90 min in the same rotor. The pelleted virus was resuspended in a buffer containing 0.1 M NaCl, 0.01 M Tris-HCl pH 8.0, 0.01 M EDTA. Heat-treated RNase A (20 µg/ml) was added to the virus suspension, which was then incubated at 37° for 60 min. Sarkosyl (2%) and SDS (0.5%) were added, and the lysate was incubated at 37° C for 60 min. Predigested Pronase (1 mg/ml) was added and incubation was continued overnight. After extraction with phenol:chloroform:isoamyl alcohol (50:48:2) the aqueous layer was precipitated with two volumes of ice-cold 95% ethanol. The precipitated DNA was centrifuged and DNA pellets were redissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

Cloned restriction fragments. Approximately 1 µg of HCMV strain

AD169 DNA was digested to completion with BamHI. The vector pBR322 was linearized with BamHI. The digests were ethanol precipitated and redissolved in ligase buffer (60 mM Tris-HCl pH 8.0, 33 mM NaCl, 10 mM MgCl₂) containing ATP (0.5 mM) and DTT (5mM). The ligation reaction mixture contained the precipitated digests of insert (HCMV) DNA and vector (pBR322) DNA at a concentration ratio of 10:1 and 1 unit of T₄ ligase.

The streptomycin-resistant E. coli strain SF8 [genotype: hsrR⁻, hsdM⁻, recB, recC, lop-11 (ligase overproducer) supE44), gal-96, leuB6, thi-1, thr; a gift from Dr. Masayasu Nomura, University of Wisconsin] was grown in an overnight preculture in 5 ml of Luria broth (Gibco). A 0.2 ml sample of this preculture was added to 20 ml of Luria broth and grown to a Klett reading of 100. The bacteria were sedimented by centrifugation at 5,000 rpm in a Sorvall SS-34 rotor for 5 min at 2° C. The pelleted cells were resuspended in 10 ml cold 50 mM CaCl₂ and incubated for 60 min at 0° C. The centrifugation and resuspension steps were repeated in a 1 ml volume of CaCl₂. The bacteria were then added in a 2:1 (v/v) ratio to the ligation mixture. The transformation sample was incubated for 2 min at 37° C for 20 min. Dilutions of the cells from the sample were then plated out on Luria agar plates containing ampicillin (100 μg/ml) or tetracycline (10 μg/ml). Colonies which appeared on the plates were numbered and picked to duplicate plates containing either ampicillin or tetracycline. Colonies which were resistant to ampicillin but sensitive to tetracycline were selected for further plasmid analysis. Insert fragments were identified by restriction endonuclease digestion patterns based on the map published by Greenaway et al (67). There are 67

recombinant clones which compose a partial HCMV AD169 genomic library.

EcoRI restriction fragments of HSV-1 strain KOS DNA inserted in the vector pBR328 were kindly provided by Dr. Myron Levine, University of Michigan, Ann Arbor. In particular pSG17 and pSG87 (64) containing the HSV-1 EcoRI M and N fragments respectively were used as radiolabeled probes.

Plasmid DNA Isolation. Large-scale plasmid isolation was performed by the alkaline lysis method described by Maniatis (98). A 5 ml Luria broth preculture containing 100 μ g/ml ampicillin was inoculated with the recombinant bacterial strain and incubated at 37° C with shaking overnight. The bacteria were harvested by centrifugation at 7,000 rpm for 10 min at 4° C in a Sorvall GSA rotor. The pellet was resuspended in 5 ml of a solution containing 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA and transferred to a 50 ml Oak Ridge tube. An additional 5 ml of the same solution containing 10 mg/ml lysozyme was added and the suspension was incubated at room temperature for 5 min. Then 20 ml of a freshly-made solution containing 0.2 N NaOH, 1% SDS was added and incubation at room temperature was continued for 10 min. The tube was filled with an ice-cold solution of 5M potassium acetate (pH 4.8). The tubes were vigorously shaken and placed on ice for 10 min. The tubes were centrifuged at 18,000 rpm for 30 min at 4° C in a Sorvall SS34 rotor. The supernatant was divided into two 30 ml Corex tubes and 0.6 volumes of isopropanol was added. The tubes were incubated at room temperature for 15 min and centrifuged at 10,000 rpm for 30 min also at 20° C in the SS34 rotor. The pellets were resuspended in 7.4 ml TE. To purify the plasmid DNA, 1 g

CsCl and 0.8 ml ethidium bromide (10mg/ml) were added per ml of sample volume. The samples were centrifuged in a Beckman VTi 65 rotor at 60,000 rpm or a Beckman Tl-100 rotor at 86,000 rpm for 16-20 h. Plasmid bands were harvested from the gradients by side puncture. The ethidium bromide was removed by extraction with isopropanol saturated with 10X SSC (1X SSC is 0.15 M NaCl, 0.015 M trisodium citrate). The DNA was precipitated by adding an equal volume of TE to reduce the salt concentration and then adding two volumes of ethanol. The samples were placed at -20° C overnight. DNA was pelleted by centrifugation at 10,000 rpm for 20 min at 4° C. Pellets were resuspended in TE.

For rapid small-scale plasmid isolation a 1.5 ml culture in Luria broth (Gibco) with 100 µg/ml ampicillin was incubated at 37° with shaking overnight. Samples were centrifuged for 1 min in a microcentrifuge. The pellets were resuspended in 100 µl of an ice-cold solution containing 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0, and 4 mg/ml lysozyme. The suspensions were incubated for 5 min at room temperature, and then 200 µl of fresh solution containing 0.2 N NaOH, 1% SDS was added followed by another incubation for 5 min at room temperature. A 150 µl volume of ice-cold 5 M potassium acetate solution pH 4.8 was added, and the samples were placed on ice for 5 min then centrifuged for 5 min at 4° C in a microcentrifuge. The supernatant was removed to a fresh tube and an equal volume of phenol:chloroform:isoamyl alcohol (50:48:2) was added. The samples were centrifuged for 5 min at 4° C. The aqueous layer was removed to a fresh tube. The DNA was precipitated with 2 volumes 95% ethanol at room temperature and pelleted by microcentrifugation. The pellets were

washed with 70% ethanol and re-centrifuged. The pellets were dried and resuspended in 30 μ l TE (10mM Tris-HCl pH 7.5, 1 mM EDTA) containing 20 μ g/ml RNase A.

Agarose gel electrophoresis. Both undigested and restricted DNA samples were routinely analyzed by electrophoresis on horizontal 0.7% agarose gels in buffer containing 89 mM Tris base and boric acid, and 2 mM disodium EDTA (final pH 8.0). After electrophoresis, the gels were stained with 0.5 μ g of ethidium bromide per ml of staining solution.

Removal of insert DNA from recombinant plasmids. Recombinant plasmid DNA was digested with the appropriate restriction endonuclease. Insert DNA was separated from vector DNA by agarose gel electrophoresis, electroeluted from the gel and purified by passage over a DEAE-Sephacel (Pharmacia, Piscataway, NJ) column (98). The DNA was eluted from the column with buffer containing 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.6 M NaCl, and concentrated by ethanol precipitation.

Alternatively restriction fragments were removed from low melting point (LMP) agarose (FMC BioProducts, Rockland, ME) by the method of Gafner et al. (54). Briefly, the agarose containing the fragment was heated at 65° C, extracted twice with phenol equilibrated with 50 mM Tris-HCl pH 8.0 and once with chloroform. The DNA was concentrated by ethanol precipitation.

Processing of clinical specimens for probe detection of HCMV DNA (18,152). Clinical urine specimens were kindly collected by Dr. Mary Christensen at Children's Memorial Hospital, Chicago, IL. The volumes ranged from 1 to 3 ml. The total volume was brought up to 8 ml with EBSS

without indicator. The samples were centrifuged at 2,000 x g for 5 min. In some samples, a portion of this low-speed-centrifugation pellet was examined for cells, crystals, and bacteria. The pellet was then returned to the supernatant, and the total sample was centrifuged at 25,000 x g for 90 min. Alternatively the supernatant from the low-speed centrifugation was transferred to a new tube and centrifuged at 25,000 x g for 90 min.

A 20- μ l volume of a solution containing 0.1 M NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 500 μ g of pronase per ml, and 0.5% SDS was added to the pellets from the high-speed centrifugation, and this mixture was incubated at 37° C for 1 h. The samples were transferred to 1.5 ml microcentrifuge tubes, extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2), extracted once with chloroform-isoamyl alcohol (24:1), and precipitated with ethanol. The precipitates were suspended in 20 μ l of TE. An equal volume (20 μ l) of 0.3 M NaCl/1.0 M NaOH was added; the samples were vortexed briefly and allowed to stand for 15 min at room temperature. Another volume (40 μ l) of 0.8 M Tris-HCl pH 6.8/1M NaCl was added. The entire sample was placed in a well of a Minifold filtration apparatus (Schleicher & Schuell, Keene, NH) for blotting onto a nitrocellulose filter (BA85, Schleicher & Schuell). A slight vacuum was applied after the wells appeared to be empty. The nitrocellulose filter was baked under vacuum at 80° C before further processing.

Samples of HCMV, HSV-1, and HSV-2 were processed in the same manner as were the clinical samples. DNA from each of these viruses was suspended directly in 20 μ l of TE and prepared for dot blotting onto

nitrocellulose filters as described above.

Processing of infected cell monolayers for probe detection of HCMV DNA (151,152). Cell culture fluid was removed and the monolayers were washed with 2 ml EBSS. Monolayers were detached after washing by adding 250 μ l of a filtered 0.25% trypsin solution and incubation at 37⁰ C for 5 min. One milliliter of EBSS without indicator was added to each tube. The samples were transferred to 1.5 ml microfuge tubes and centrifuged at 5,000 x g for 5 min. The supernatant fluid was discarded.

The cell pellets were treated with 200 μ l of lysis solution (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 10 mM EDTA, 500 μ g/ml pronase and 0.5% SDS) and incubated at 37⁰ C for 1 h. The lysate was extracted twice with phenol:chloroform:isoamyl alcohol, once with chloroform:isoamyl alcohol (24:1) and ethanol precipitated. The pellets were redissolved in 20 μ l TE. An equal volume of 1 M NaOH-2 M NaCl was added, and the suspensions were incubated at room temperature for 15 min. The samples were neutralized with 40 μ l of 3 M sodium acetate pH 5.5. Each sample was suctioned onto either a GeneScreen Plus membrane (DuPont NEN, Boston, MA) or a nitrocellulose filter using a Minifold filtration apparatus as described above.

Labeling of DNA probes. Two methods were used to label DNA probes. DNA for biotinylated probes was labeled with biotinylated dUTP by nick translation with reagents purchased in a kit manufactured by BRL. The labeled probes were separated from unincorporated nucleotides by ethanol precipitation. Some radioactive probes were also labeled with ³²P-dCTP by nick translation using the same BRL kit, but unincorporated nucleotides

were removed by Sephadex G-50 chromatography. The Sephadex G-50 (Pharmacia) was equilibrated in buffer containing 50 mM Tris-HCl, 10 mM EDTA. The nick translation mixture was loaded onto the column and the same buffer was used to elute the probe. Separation of probe and unincorporated nucleotide peaks was monitored with a Geiger counter. Approximately 1.5 ml of buffer containing the separated probe was collected directly after the predetermined void volume.

More recently, smaller quantities of DNA (25 ng) were labeled by random priming with a kit purchase from Amersham, Arlington Heights, IL. The unincorporated nucleotides were separated from the labeled probes by spin columns purchased from 5 Prime-->3 Prime, Inc., West Chester, PA.

Southern hybridizations. Agarose gels were prepared for Southern blotting by depurination in 0.4 M HCl, denaturation in 0.5 M NaOH-1.0 M NaCl and neutralization in 0.5 M Tris-HCl-1.5 M NaCl. The gels were blotted onto GeneScreen Plus or Nytran (Schleicher and Schuell) with 10X SSPE (1X SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA). The membranes were prehybridized in 50% formamide, 0.9% SDS, 5X SSPE, 10X Denhardt's (1X Denhardt's is 0.02% Ficoll, polyvinylpyrrolidone and BSA) and 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA for 1-2 h at 42^o C. The prehybridization solution was replaced with hybridization solution containing 6X SSPE, 1% SDS, 50% formamide, and 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA and heat-denatured probe. The membranes were hybridized overnight at 42^o.

GeneScreen Plus membranes were washed according to the manufacturer's protocol: twice with 100 ml 2X SSC at room temperature for 5 min each; twice with 200 ml 2X SSC/1% SDS at 65^o for 30 min each;

twice with 100 ml 0.1X SSC at room temperature for 30 min each. Nytran membranes were washed as follow: once in 1X SSPE for 5 min at room temperature; twice in 1X SSPE/1% SDS at 65° C for 15 min each; twice in 0.1X SSPE at room temperature for 15 min each. Membranes were briefly air-dried and placed in X-ray cassettes with Cronex Lightning Plus intensifying screens (DuPont, Wilmington, DL) and Kodak X-OMAT AR film. The cassettes were held at -70° C for whatever period of time was required for the individual experiment.

Dot-blot hybridization. Dot-blotted nitrocellulose filters were prehybridized in a solution containing 45% formamide, 5X SSC, 0.2% BSA, 0.2% ficoll 400, 25 mM sodium phosphate buffer pH 6.5 and 300 µg/ml heat-denatured calf thymus DNA. The filters were prehybridized in a heat-seal bag at 42° C for 2 h with shaking.

The filters were hybridized in a solution containing the same components as the prehybridization plus 0.1% sodium pyrophosphate, 10% dextran sulfate (Pharmacia), and heat-denatured biotinylated probe. The filters were incubated in heat-seal bags at 42° C for 1-20 h depending on the experiment.

After hybridization, nitrocellulose membranes were washed according to the manufacturer's (BRL) protocol: twice in 2X SSC/0.1% SDS at room temperature for 3 min each; twice in 0.2X SSC/0.1% SDS at 50° C for 15 min each; once in 2X SSC/0.1% SDS at room temperature for 3 min.

Detection of hybridized biotinylated probe. Membranes were rehydrated for 1 min in BRL Buffer I (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.05% Triton X-100). The membranes were blocked for 20-75 min

in freshly prepared Buffer II (Buffer I containing 3% BSA). After blocking, membranes were baked in a vacuum oven at 80° C for 20 min.

Membranes were rehydrated in Buffer II for 10 min and drained. A solution containing 2 µg/ml Streptavidin in Buffer I was constantly pipetted over the membranes for 10 min at room temperature. This was followed by three washings in Buffer I at room temperature for 3 min each. A solution containing 1 µg/ml poly-alkaline phosphatase (poly-AP) in Buffer I was pipetted over the membranes for 10 min at room temperature. The membranes were then washed twice in Buffer I at room temperature for 2 min each and twice in Buffer III (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂) at room temperature for 2 min each. A dye solution [7.5 ml Buffer III, 33 µl nitroblue tetrazolium (NBT), 25 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP)] was added to the blocked membranes in a heat-seal bag. This step was performed in very low light, and the heat-seal bag was placed in the dark for color development. After 2-4 h the reaction was stopped by washing the membranes in a solution containing 5 mM EDTA, 20 mM Tris-HCl pH 7.5 at room temperature for 15 min.

Standard strips spotted with low concentrations of biotinylated DNA were baked for 2 h at 80° C in a vacuum oven. A standard strip was developed along with all test membranes.

Drug resistance measured by per cent survival. Plaque-purified isolates of each mutant were titered in the presence and absence of a single high concentration of each drug. The percent survival was calculated from the ratio of the titer of each mutant in the presence of the drug to the titer of the mutant in the absence of the same drug

multiplied by 100. The percent survival of the wild-type was determined as a control for each experiment.

Drug-resistance measured by plaque reduction. The concentration of each drug which could reduce the number of plaques of a single viral dilution by fifty percent was determined. All monolayers in a 24-well plate were inoculated with a single viral dilution calculated to produce 30-50 plaques per control well. The four control wells were overlaid with medium without drug, and the remaining wells were overlaid in quadruplicate with medium containing a range of drug concentration. The assays were read at 14 days post-infection.

Drug-resistance measured by enzyme-linked immunosorbent assay (ELISA) (3,128). Monolayers in 96-well microtiter plates were inoculated with wild-type or mutant strains of HCMV at a single dilution. Control wells were inoculated with maintenance medium. The inoculum was replaced with medium containing two-fold dilutions of the drug. In one set of wells which had been inoculated with virus, the medium without drug was added to serve as a virus control. The plates were incubated usually for 5-7 days until the virus control demonstrated 50% CPE (2+).

In order to detect the amount of HCMV replication, the cell culture medium was aspirated and replaced with 0.2 ml phosphate buffered saline (PBS contains per liter: 7.65 g NaCl, 1.2688 g disodium phosphate, 0.1 g monosodium phosphate, 0.2133 g monopotassium phosphate) containing 1% BSA. The plates were incubated at room temperature for 30 min. The PBS-BSA was aspirated and replaced with 0.2 ml per well ethanol:acetone 95:5, and the plates were placed at -20° C for 30 min. The ethanol:acetone was

aspirated, and the wells were washed 5 times with PBT buffer (0.05% Tween 20 and 1% BSA in PBS). The washes were performed with an Immuno Wash 8 (Nunc, Kamstrup, Denmark). Monoclonal antibody (Late Nuclear Protein, DuPont NEN) specific for an HCMV late antigen (142) was added to a dilution of 1:5,000 in a solution containing 10% goat serum, 1% FCS, 0.05% Tween 20 in a volume of 0.1 ml per well. The plates were incubated at 37° C for 1 h. The wells were again washed 5 times with PTB buffer, and 0.1 ml of goat anti-mouse Ig conjugated with horse radish peroxidase (Bio-Rad, Richmond, CA) at a dilution of 1:2,000 in a solution containing 10% goat serum and 0.05% Tween 20 was added to each well. The plates were incubated at 37° C for 2 h followed by 5 washes in PTB. A volume of 0.2 ml of substrate solution [2,2'-azinobis(3-ethylbenzthiazoline) sulfonic acid (Sigma) and 0.005% H₂O₂] at a concentration of 1 mg/ml was added to each well. Color was allowed to develop for 15-30 min. The plates were read on a Dynatech MR580 Microelisa Auto Reader at 405 nm.

HPLC analysis of GCV anabolism in virus-infected cells. HFF monolayers in 150 cm² flasks were inoculated with HCMV AD169 or one of the three GCV-resistant mutants (D1/3/4, D10/3/2, D6/3/1) at an MOI of approximately 1.0. One flask was mock-infected for a cell control. At either 72 or 96 h post-infection ³H-GCV (a gift from Syntex, Palo Alto, CA) was added to each flask. The concentration of labeled drug was approximately 1 μM and cold drug was added such that the final GCV concentration was 50 μM and each flask contained approximately 1.67 x 10⁷ cpm.

After 24 h incubation the cells were prepared for perchloric acid

extraction. The radioactive medium was removed from the HFF monolayers and replaced with 1.5 ml 0.25% trypsin per flask to detach the monolayers. An equal amount of growth medium was added to each flask, and the cells were dispersed with a pipette. These cells were placed in a 15 ml conical centrifuge tube. The flasks were washed with an additional 1.5 ml growth medium, which was then added to the 15 ml tubes. The tubes were centrifuged at 2,000 rpm in a Beckman TJ-6 tabletop centrifuge for 10 min. All but 0.5 ml of the supernatant was removed, and the cells were resuspended in the remaining fluid. The cells were transferred to a microfuge tube, washed with 1.5 ml Dulbecco's PBS (DPBS per liter: 0.2 g KCl, 0.2 g KH_2PO_4 , 8 g NaCl, 2.16 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$). The cells were washed in 1.0 ml DPBS two more times. After the final wash the cells were resuspended in 200 μl DPBS, and 65 μl 2 M perchloric acid was added to produce a final concentration of approximately 0.5% perchloric acid. The cell extracts were vortexed and then centrifuged for 20-25 min at 4° C. The supernatant was transferred to a new microfuge tube and either placed on ice for immediate analysis by HPLC or stored at -70° C.

GCV and GCV-monophosphate at a concentration of 100 mM were used as standards for HPLC analysis of products of infected-cell GCV anabolism. These standards were injected in 10 μl volumes for analysis on a Partisil 10 ODS3 250 x 4.6 mm reversed phase HPLC column (Phenomenex, Rancho Palos Verdes, CA). The mobile phase was 0.02 M KH_2PO_4 pH 3.65. The perchloric acid extracts of infected cells labeled with ^3H -GCV were assayed under the same conditions, but fractions were collected in scintillation vials at 0.3 min intervals for 15 min. Scintillation fluid (10 ml Universol, ICN

Biomedicals, Irvine, CA) was added to each vial, and each vial was counted for 2 min on an LKB 1214 Rackbeta liquid scintillation counter.

Messenger RNA isolation from virus-infected cells. Monolayers of HFF in 150 cm² flasks were inoculated at an MOI of 1.0 with wild-type AD169 or mutant strains of virus. Messenger RNA was isolated at 72 h post-infection according to a kit protocol (Fast Track mRNA Isolation Kit, Invitrogen, San Diego, CA). Briefly, the cells were washed with DPBS and lysed in Stock Buffer containing 0.2M NaCl, 0.01 M Tris-HCl pH 7.5, 1.5 M MgCl₂, 2% SDS plus 0.02 volumes RNase Protein Degradar (patented by Invitrogen). The lysates were passed 5-6 times through a 21 gauge needle to shear the cell DNA and then digested for 1-2 h at 45° C to remove protein. The salt concentration of the lysate was adjusted to 0.5 M before incubation with oligo dT for 40-60 min at room temperature. The oligo dT was washed repeatedly with a Binding Buffer [0.1 M EDTA, 0.01 M Tris-HCl (diethylpyrocarbonate-treated)] and then added to a disposable column. Washing was continued until the OD₂₆₀ was less than 0.05. The poly A+ RNA was eluted with kit-supplied Elution Buffer (0.5 M NaCl, 0.01 M Tris-HCl) into four fractions of 0.4 ml each. These fractions were pooled, mixed and realiquoted into four microfuge tubes to ensure that the mRNA concentration was the same in each tube. The RNA was precipitated at -20° C by adding 0.1 volume 2 M sodium acetate and 2 volumes of 100% EtOH. The samples were centrifuged for 30 min at 4° C, dried and redissolved in 50 μl TE. The OD₂₆₀ was taken to determine the concentration of RNA.

In vitro translation of mRNA from virus-infected cells. Messenger

RNA isolated at 72 h post-infection from virus-infected or mock-infected cells was used as a template for in vitro translation. A micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) was used for the translation. The reaction mixture consisted of 35 μ l of the lysate, 1 mM amino acids minus methionine, 40 units of RNasin (Promega), 50 μ Ci 35 S-methionine (DuPont NEN) and 5-10 μ g of mRNA for a final volume of 50 μ l. The translation mixture was incubated at 30° C for 90 min. At the end of the 90 min incubation the translation mix was either analyzed directly or stored at -70° C.

In order to assay for GCV kinase activity associated with the translated proteins, the translation mixture was added to a solution containing 100 mM MES (Sigma) pH 6.1 (or in some experiments 100 mM Tris-HCl pH 7.5), 2 mM magnesium acetate, 10 mM ATP, 1 unit creatine phosphokinase (Sigma), 6 mM creatine phosphate (Sigma), 1% BSA, and 7.5 μ Ci 3 H-GCV. This reaction was incubated at 30° C for 5-11 h. At the end of the incubation period the reaction was extracted with 0.5 M perchloric acid and centrifuged for 30 min at 4° C. The supernatant was transferred to a new tube and recentrifuged for 15 min. The supernatant was again transferred to a new tube and stored at -70° C for HPLC analysis as described above.

Cosmid cloning of mutant D6/3/1. Virion DNA isolated from mutant D6/3/1 was partially digested with BamHI by serial dilution of the enzyme. The two concentrations of enzyme which produced fragments between 30 and 50 kb in size were selected for the actual cloning steps. One microgram of D6/3/1 DNA was partially digested with either 4.2 or 2.1 units of

enzyme. The products were checked for size on 0.3% agarose gels. Cosmid pWE15 (Stratagene) was linearized with BamHI and dephosphorylated with calf intestinal alkaline phosphatase (Boehringer-Mannheim, Indianapolis, IN). The cosmid and partially digested DNA fragments were ligated overnight at 4° C. The components of the ligation were: 1.5 μ l partially digested HCMV DNA (1 μ g/ μ l); 1 μ l linearized pWE (1 μ g/ μ l); 2 μ l 10X ligation buffer (0.5 M Tris-HCl pH 7.5, 50 mM MgCl₂, 50 mM DTT); 2 μ l ATP 10 M ; 13.5 μ L H₂O. The ligation products were packaged into phage with a packaging kit (Gigapack Gold, Stratagene). Briefly 4 μ l of ligated DNA was added to a freeze/thaw extract (kit supplied). Fifteen microliters of a sonic extract (also kit supplied) was then added. This reaction mixture was incubated at room temperature for 2 h. The packaged phage were then treated with 20 μ l chloroform.

A culture of E. coli strain NM554 [genotype F⁻, recA, araD139, (ara leu)7696 lacY74, galU⁻, galK⁻, hsr⁻, strA, mcrA(-), mcrB(-)] (supplied by Stratagene) was grown overnight in Luria broth containing 0.2% maltose and 10 mM MgSO₄ at 30° C. The bacteria were pelleted for 10 min at 4,000 rpm in a Sorvall SS34 rotor at 4° C. The bacteria were resuspended in 10 mM MgSO₄ to an OD₆₀₀ of 0.5. Equal volumes of NM554 cells and packaged phage solution were incubated at room temperature for 45 min. In order to allow expression of beta lactamase, Luria broth (200 μ l) was added to each tube and the tubes were incubated at 37° C for 60 min. During the incubation the tubes were gently shaken every 15 min. The suspension was pelleted for 1 min at 14,000 rpm in a Beckman microcentrifuge. The supernatant was removed, and the bacteria were resuspended in 50 μ l of fresh Luria broth.

The cultures were then plated on Luria agar plates containing 100 μg ampicillin per ml and incubated at 37° C overnight. Ampicillin-resistant colonies were picked to new ampicillin plates. Each recombinant was analyzed for cosmid content by the alkaline lysis procedure (98). Cosmids containing overlapping regions of the mutant D6/3/1 genome were isolated by the large-scale alkaline lysis method (98) followed by CsCl purification.

Transfection of HFF with cosmid DNA. Slightly subconfluent monolayers of HFF in 6-well tissue culture plates were washed with serum-free Opti-MEM (Gibco). Cosmid DNA was diluted in Opti-MEM (6.7 $\mu\text{g}/0.5$ ml). Lipofectin reagent (BRL) (14) was diluted separately in Opti-MEM (17 $\mu\text{g}/0.5$ ml). Equal portions of cosmid and lipofectin solutions were added to a new tube and 1 ml of the combined reagents was added dropwise to the HFF monolayers. The cells were incubated with the transfection medium for 24 h. The transfection medium was then removed, and the cells were infected with HCMV AD169 at an MOI of 1.0. The virus was adsorbed for 2 h, at which time the inoculum was removed and replaced with 3 ml of maintenance medium. After 24 h incubation the medium was removed and replaced with maintenance medium containing 100 μM GCV. Incubation was continued for 3-7 days. Infected monolayers were then scraped from the plates and passed to uninfected monolayers for further selection in the presence of 100 μM GCV.

Alternatively the monolayers were infected with AD169 2 h before transfection with cosmid DNA. At the end of the transfection period maintenance medium containing 100 μM GCV was added directly. In some

experiments monolayers were co-transfected with cosmid and AD169 DNA instead of infection with intact virus. Selection with GCV was delayed for 24-48 h post-transfection.

Control monolayers were transfected with cosmid vector pWE15 DNA. The plasmid carries the gene for neomycin resistance for selection. The neomycin analogue G418 (400 $\mu\text{g}/\text{ml}$) was added at 48-72 h post-transfection. In addition there were control monolayers which were mock-transfected to determine cell viability after Lipofectin exposure.

Two-dimensional gel electrophoresis. Translation products from in vitro translation of infected cell mRNA were analyzed by two-dimensional polyacrylamide gel electrophoresis (113). The first dimension was an isoelectrofocusing (IEF) gel. Tube gels consisting of 8.8 M urea, 4% acrylamide, 0.5% bisacrylamide, 2% NP-40, 4% ampholine pH 5-7 (Pharmacia LKB), 1% ampholine pH 3-10 (LKB), 0.01% ammonium persulfate and 0.067% TEMED (Bio-Rad) were cast in a 20-gel apparatus (Electro-Nucleonics, Siversprings, MD) and allowed to polymerize for 2 h. The gels were pre-run for 1 h at 200 V. The anode buffer was 10 mM phosphoric acid and the cathode buffer was 20 mM NaOH. A 20 μl volume of the translation reaction mixture was added to 50 μl of sample buffer containing 50 mM CHES, 2% SDS, 1% DTT, 10% glycerol. The samples were run for 16-17 h at 800 V. The gels were then extruded into equilibration buffer containing 0.125 M Tris-HCl pH 6.8, 2% SDS, 8.6 mM dithiothreitol, 10% glycerol and a trace of bromphenol blue.

The first dimension gels were placed at the top of SDS polyacrylamide slab gels (10% acrylamide, 0.9% bisacrylamide, 0.3% SDS,

0.05% ammonium persulfate and 10 μ l TEMED per 30 ml volume). The second dimension gels (SDS-PAGE) were electrophoresed for 2.5-3 h at 30 mA per gel in a buffer containing 25 mM Tris-HCl pH 8.3, 192 mM glycine. The gels were fixed in 30% methanol, 10% acetic acid for 1 h and then enhanced in 5 volumes of Enlightning (DuPont NEN) for 30 min. The gels were dried with heat and vacuum and placed in X-ray cassettes with Kodak XO-MAT AR film for 48-90 h. The films were developed for 4 min in GBX Developer (Kodak). Development was stopped by rinsing the films in tap water. The films were fixed for 2 min in GBX Fixer (Kodak).

Proteins extracted from infected cells were also analyzed by 2-D gel electrophoresis. HFF monolayers in 150 cm² flasks were infected with stocks of mutant or wild-type strains at an MOI of 1.0-2.0. Monolayers were incubated for 48 h in normal maintenance medium. The medium was then replaced with methionine-free medium (Gibco) containing 1% FCS, gentamicin (50 μ g/ml), amphotericin B (2.5 μ g/ml) and ³⁵S-methionine (25 μ Ci/ml, specific activity 1,000 Ci/mmol) plus 10% normal maintenance medium.

After 24 h the cells were harvested as follows: the monolayers were washed with 1X DPBS; the cells were scraped into 4 ml PBS, dispersed by pipetting and centrifuged at 1,000 x g for 10 min. Cells were resuspended in 1X DPBS and centrifuged again. This step was repeated two more times. After the final pelleting, cells were resuspended in 2 ml DPBS and transferred to 4 microfuge tubes. The cells were pelleted in a microcentrifuge for 10 min. The supernate was removed, and the cells were solubilized in 20-30 μ l of urea solubilizer [9 M urea, 4% NP-40, 2% ampholyte (9-11 range), 2% mercaptoethanol] for 2 h with frequent

vortexing. Solubilized proteins were stored at -70° C until analyzed by 2-dimensional gel electrophoresis. The 2-dimensional gel analysis was performed by Lori Berg in the laboratory of Dr. Chung Lee at Northwestern University Medical Center, Chicago, IL.

RESULTS

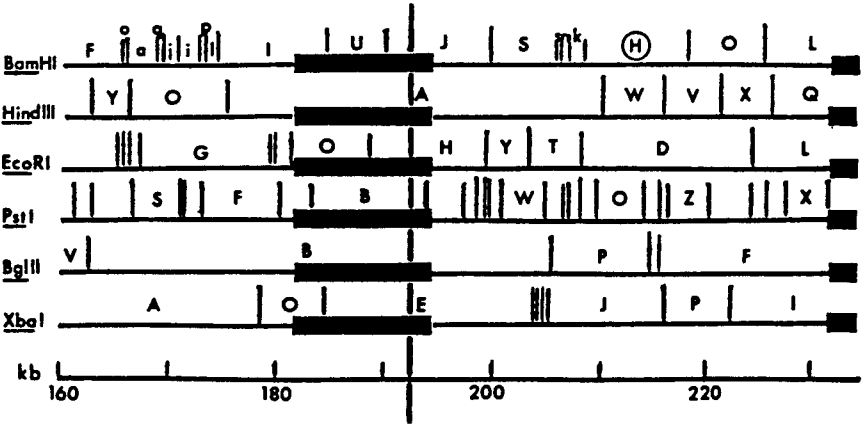
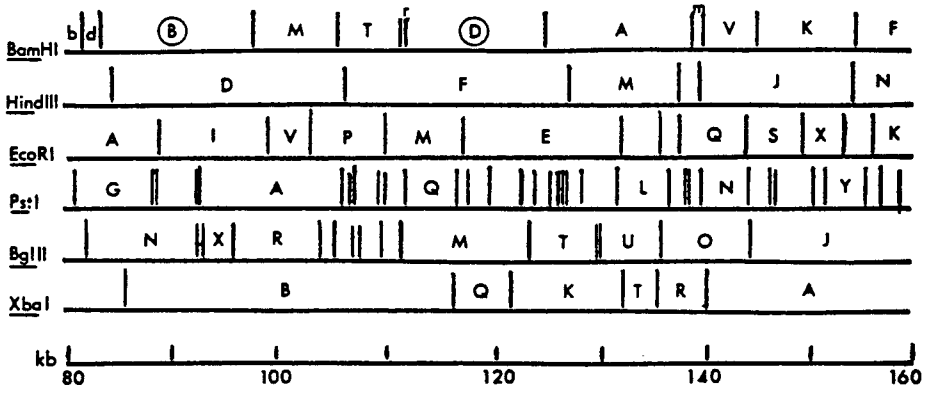
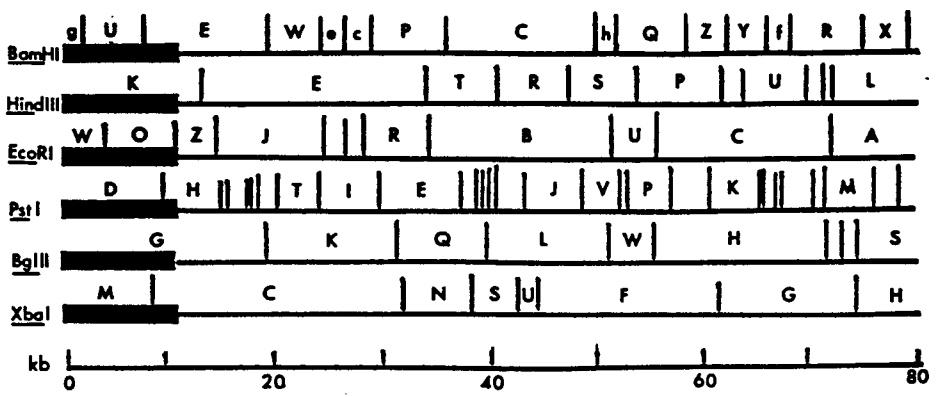
Selection of HCMV probes. In order to develop DNA probes for the rapid detection of HCMV in clinical specimens, regions of the HCMV AD169 genome were selected. The feasibility of this method was based on the fact that there is at least 80% homology among the genomes of HCMV strains which have been tested (26). The greatest variability has been reported around the junction of the unique and repeat segments (90,147). The unique segments are colinear even though restriction endonuclease sites may be altered (26). Large restriction fragments from unique regions of the genome of HCMV AD169, therefore, would contain sufficient homology with comparable regions of the genomes of clinical strains of HCMV to allow hybridization under moderately stringent conditions.

The HCMV AD169 BamHI B fragment inserted in pBR322 was selected initially as a probe because of its size (15 kb) and position in the genome (U_L sequence; Figure 2). The intact recombinant plasmid carrying this fragment was used as the probe in all experiments.

The BamHI D (13 kb, U_L) and H (10 kb, U_S) fragments cloned in pBR322 were also selected as probes because of size and position in the genome. The vector sequences were removed from the fragments before they were used as probes.

In order to determine the minimum amount of homologous HCMV AD169 DNA which each probe could detect, each of the three probes was hybridized

Figure 2. Restriction enzyme map of the HCMV AD169 genome. From Greenaway et al. (67).



to 100 ng to 10 pg quantities of HCMV AD169 DNA. All of the probes detected a minimum of 30 pg of homologous HCMV DNA (Figure 3).

Detection of HCMV DNA in clinical specimens. A total of 61 urine specimens submitted for culture to the Virology Laboratory at Children's Memorial Hospital were processed for direct detection of HCMV with the biotinylated BamHI B fragment as a probe. Samples of 1 to 3 ml from each specimen were prepared for dot-blotting on nitrocellulose filters as described in Materials and Methods. Similarly processed samples containing DNA extracted from laboratory strains of HCMV, HSV-1 and HSV-2 were included as controls.

The comparison of culture and hybridization results for the B fragment are shown in Table 1. Additional information concerning the length of time required to produce visible CPE and the amount of CPE present in the culture-positive specimens was collected after the hybridization reactions had been recorded. There were 15 specimens which were scored positive by both methods, whereas 14 specimens were scored positive by culture but negative with the probe. Four specimens were weakly positive with the probe but remained culture negative. The sensitivity of the probe detection method was 52%, and the specificity was 87.5%. There was 70.5% agreement between the results of hybridization and culture methods. The amount of CPE in cell culture, however, did not correlate with the probe results. In several samples the reported CPE was 1+ to 2+, and the probe did not detect the presence of virus. Conversely, there were specimens which produced a strong color reaction with the probe but which yielded only a few lesions in the cell culture tubes.

Figure 3. Hybridization of selected probes with dot-blotted HCMV AD169 DNA. Dilutions of CsCl-purified total genomic HCMV AD169 DNA were hybridized with each single-probe BamHI fragment (B,D, and H), as well as with the combined D and H probes. Amounts of HCMV DNA are shown at the top of each column. Control solutions containing no DNA are in column C. Hybridization was performed for 18 h, and color development for detection of hybridized probe was allowed for the maximal 4 h recommended by the manufacturer (BRL).

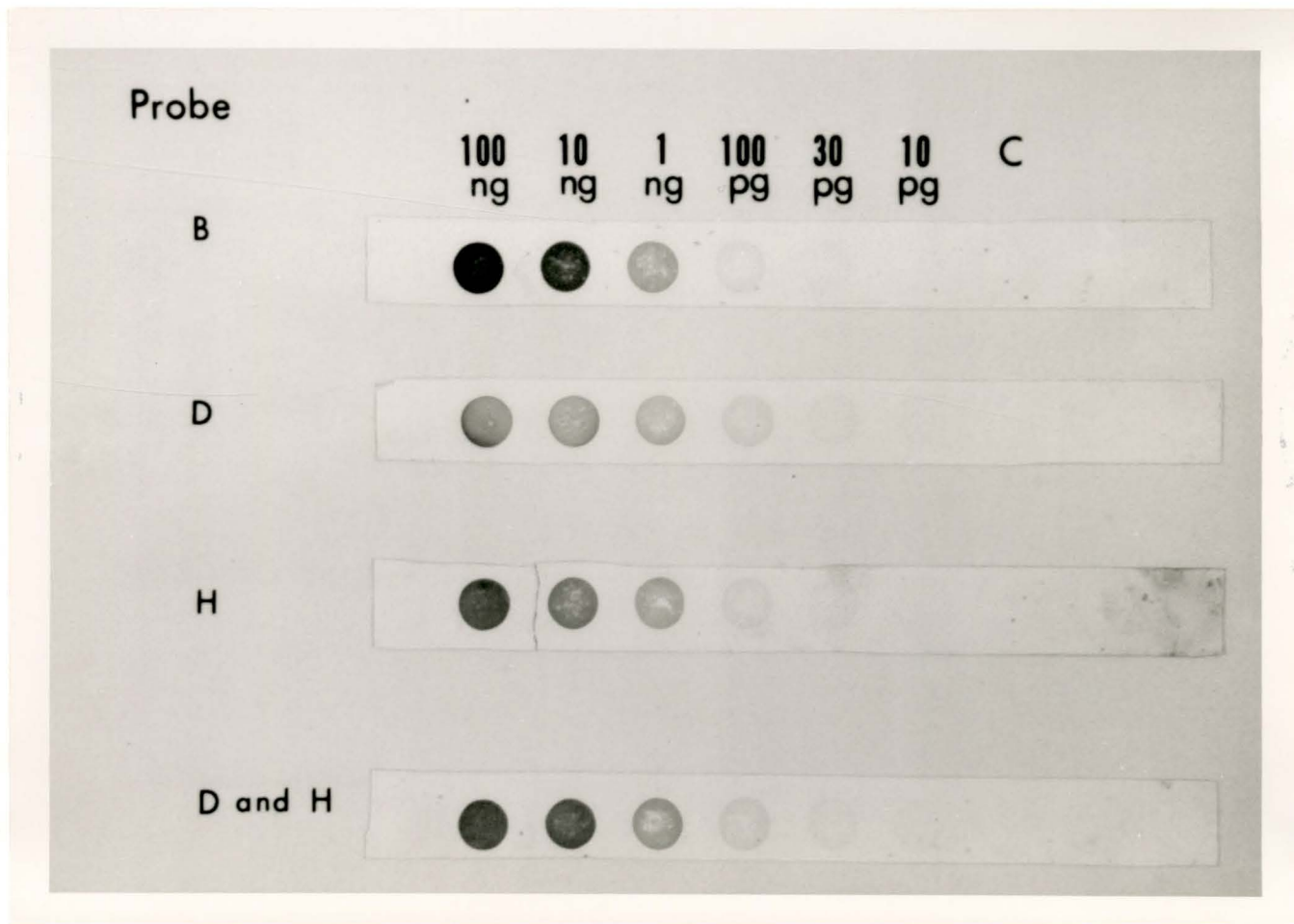


TABLE 1. Comparison of results for detection of HCMV in clinical specimens by cell culture and DNA probe hybridization.

DNA probe results	Cell culture		Sensitivity (%)	Specificity (%)	Agreement (%)
	Positive	Negative			
<u>BamHI B</u>					
Positive	15	4	52	87.5	70.5
Negative	14	28			
<u>BamHI D+H</u>					
Positive	17	5	81	68.8	75.7
Negative	4	11			
<u>BamHI H</u>					
Positive	11	5	78.6	76.2	77.1
Negative	3	16			

Detection of HCMV with combined BamHI D and H fragment probes.

During the course of these experiments it was reported that a portion of the HCMV EcoRI restriction fragment I contains sequences which weakly hybridize with mammalian cellular DNA (135). The region of the EcoRI fragment which is homologous with the cellular DNA is also contained within the BamHI B fragment. In order to avoid the possibility of cross-hybridization, the HCMV BamHI D and H restriction fragments (Figure 2) were used instead of the B fragment in subsequent experiments. Each of these fragments is in a region of the genome which has no reported homology with mammalian DNA. Two changes were made in the detection procedure in these experiments, both aimed at increasing the sensitivity of the detection method. The first change was the use of two fragments (BamHI D and H) as combined probes (151). The second change was the elimination of the initial low-speed centrifugation of the urine samples to prevent the loss of any cell-associated virus present in the specimens.

A total of 37 specimens submitted for culture were processed for direct detection with the combined biotinylated D and H probes (Table 1). There were 17 specimens which were positive by both methods, 4 specimens which were probe negative and culture positive, and 5 specimens which were weakly positive with the probe but which remained culture negative. The sensitivity of the combined probes was 81.0%, and the specificity was 68.8%. The agreement between probe and culture results was 75.7%. The amount of CPE again did not correlate with the color intensity of the probe hybridization. Of the five probe-positive, culture-negative specimens, one was from a patient who had had previous positive cultures.

Probe hybridization with HSV virion DNA. There was significant hybridization of the new probes with HSV controls prepared along with the clinical samples (Figure 4). To determine how the observed hybridization with HSV was related to the use of the new probes, duplicate filters were prepared with dot blots of HSV-1, HSV-2, and HCMV virion DNA prepared in the same manner as the clinical specimens. Each filter was hybridized with one of the fragments. Comparison of these two filters shows that the D fragment hybridized to each of the HSV samples, whereas the H fragment produced a positive signal only with HCMV DNA (Figure 5).

Analysis of cross-reactivity with HSV-1. Southern blots of EcoRI digests of HSV-1 (KOS) DNA were probed with the ³²P-labeled HCMV BamHI D fragment. Hybridization was observed only with the 4.1 kb HSV-1 EcoRI M fragment (Figure 6A). No hybridization was detected between the HSV-1 EcoRI M fragment and HCMV BamHI fragments other than D (Figure 6B).

Southern blots of restriction digests of the HCMV BamHI D fragment were then probed with the ³²P-labeled EcoRI M fragment of HSV-1. The probe hybridized with the smaller HCMV EcoRI fragment (M), the largest PstI fragment (Q), the largest XbaI fragments (B,Q), and the largest BglII fragment (M) (Figure 6C).

Conversely, Southern blots of the HSV-1 M fragment digested with KpnI and BamHI were probed with the radiolabeled HCMV D fragment. Hybridization occurred only with the smaller HSV-1 BamHI fragment and the larger HSV-1 KpnI fragment (Figure 6D). The region of homology is shown in Figure 7. Although the EcoRI M fragment of HSV-1 also contains part of the coding sequences for the major DNA binding protein (104), the

Figure 4. Dot blot of processed patient urine samples and HSV and HCMV controls hybridized with the combined biotinylated HCMV BamHI D and H probes. Rows 1 to 3 columns A through E, contain patient samples. Row 4 contains the control virion samples: A, background; B, HSV-1; C, HSV-2; D, HCMV AD169; and E, clinical isolate of HCMV. Note the probe reactivity with the HSV controls.

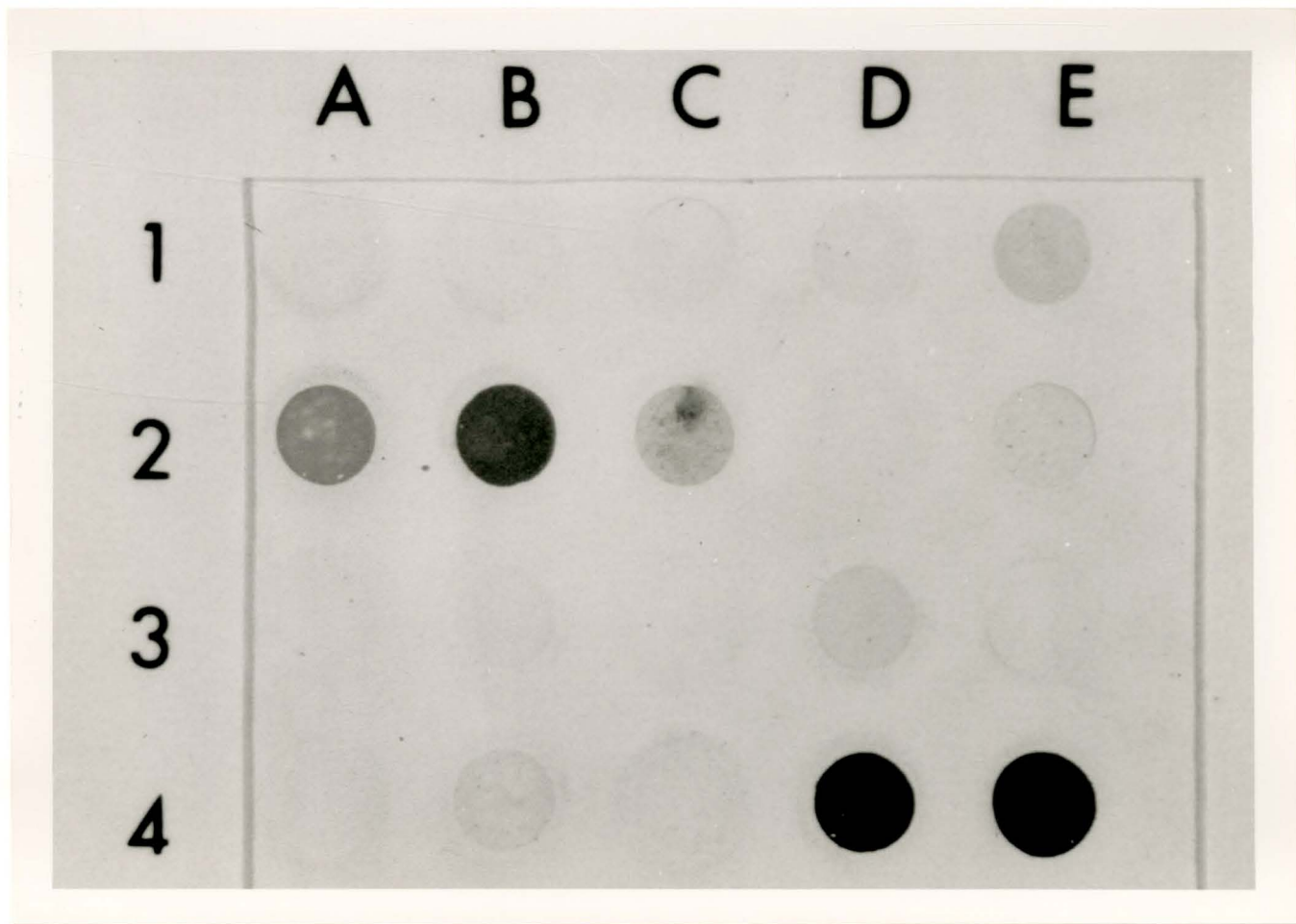


Figure 5. Duplicate dot blots of HSV and HCMV controls hybridized with single HCMV BamHI D or H fragment. Dot blots I (left) were hybridized with the H probe; dot blots II (right) were hybridized with the D probe. Row 1: HSV-1 virion DNA from cell culture supernatant: 1-ml (A) and 0.1 ml (B) samples. Row 2: HSV-1 virion DNA from cell culture supernatant: 1-ml (A) and 0.1 ml (B) samples. Row 3: HCMV AD169 virion DNA from cell culture supernatant: 1-ml (A) and 0.1 ml (B) samples. Row 4: HCMV AD169 CsCl-purified DNA: 20-ng (A) and 200 pg (B) samples. Note the reactivity of the D probe with HSV samples and corresponding lack of reactivity of the same samples with the H probe.

I

II

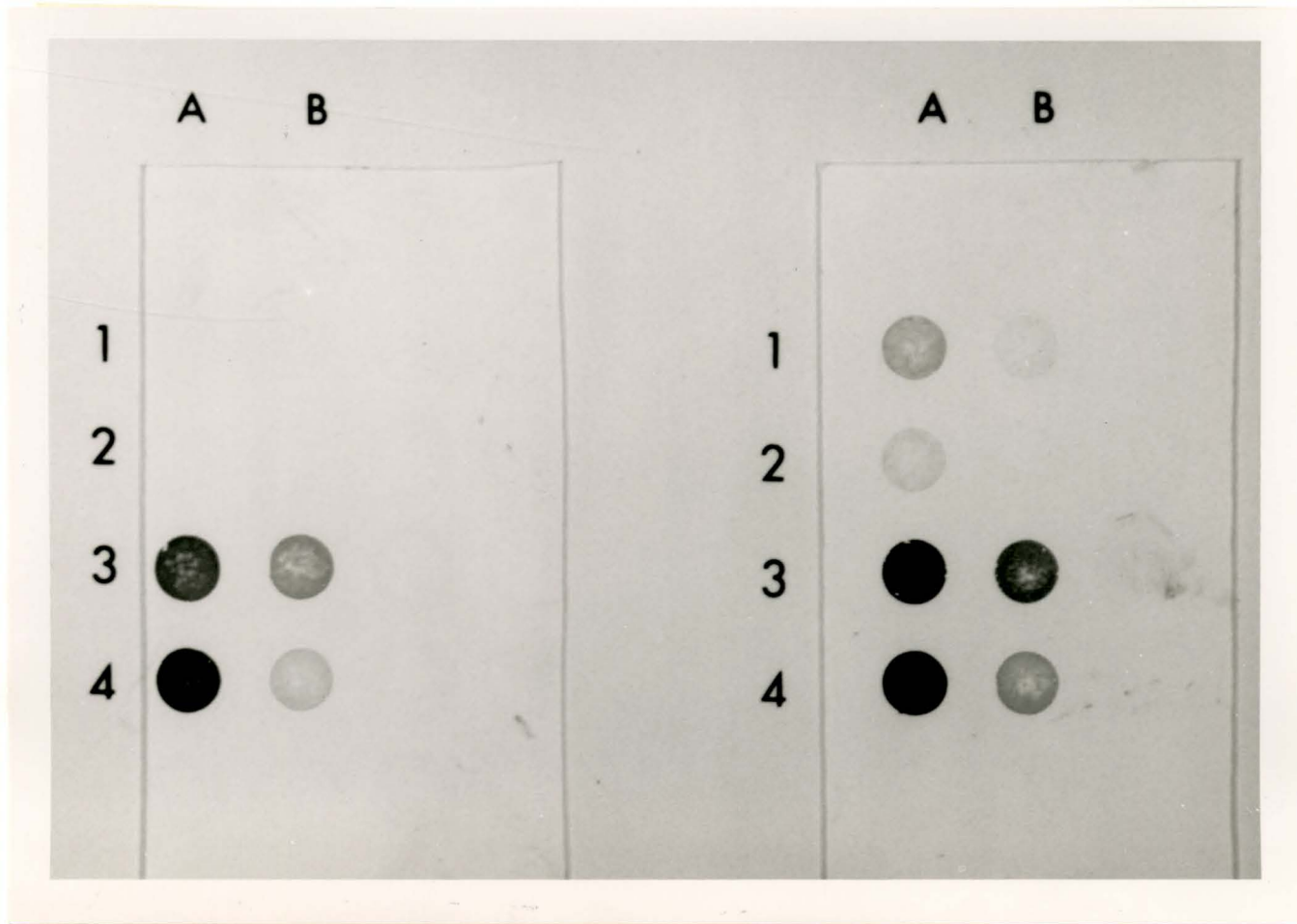


Figure 6. Southern hybridization analysis showing regions of homology between restriction fragments of HCMV and HSV-1. Left-hand lanes in each panel (A through D) show DNA restriction enzyme digests electrophoresed in 0.7% agarose gels and stained with ethidium bromide, and right-hand lanes show the corresponding autoradiograms of the Southern blots made from the same gels and hybridized with ³²P-labeled probes. Autoradiograms were exposed for 20 h (A and C) or 48 h (B and D). (A) Lane 1, EcoRI digest of HSV-1 (KOS) genomic DNA; lane 2, hybridization with HCMV AD169 BamHI D fragment. (B) Lane 3, BamHI digest of HCMV AD169 genomic DNA; lane 4, hybridization with pSG17 (HSV-1 EcoRI M insert). (C) Lanes 5 to 8, HCMV AD169 D fragment digested with (left to right): EcoRI, PstI, XbaI, and BglIII, respectively; lanes 9 to 12, hybridization with HSV-1 EcoRI M fragment. Faint bands on gel and autoradiogram represent partial digestion products. (D) Lanes 13 and 14, HSV-1 M fragment digested with BamHI and KpnI, respectively; lanes 15 and 16, hybridization with HCMV AD169 BamHI D fragment. Numbers to the left of panels C and D are molecular size markers.

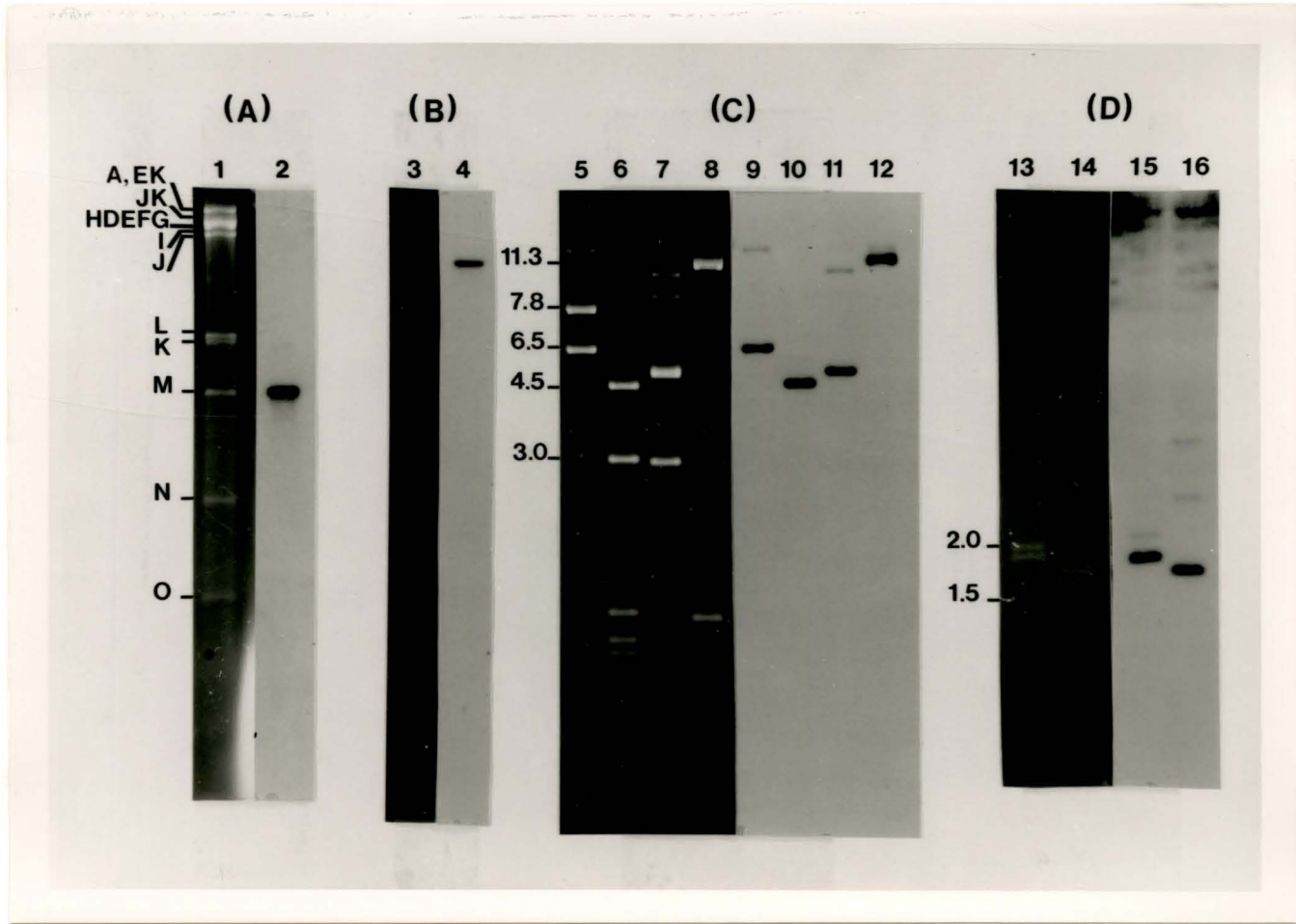
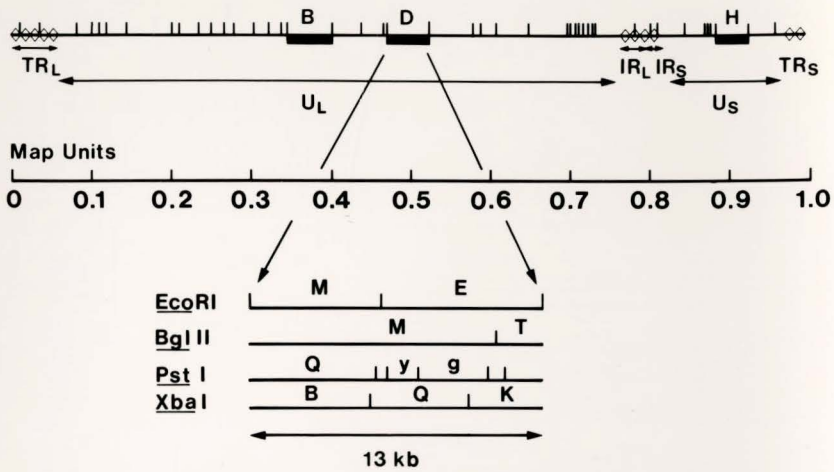
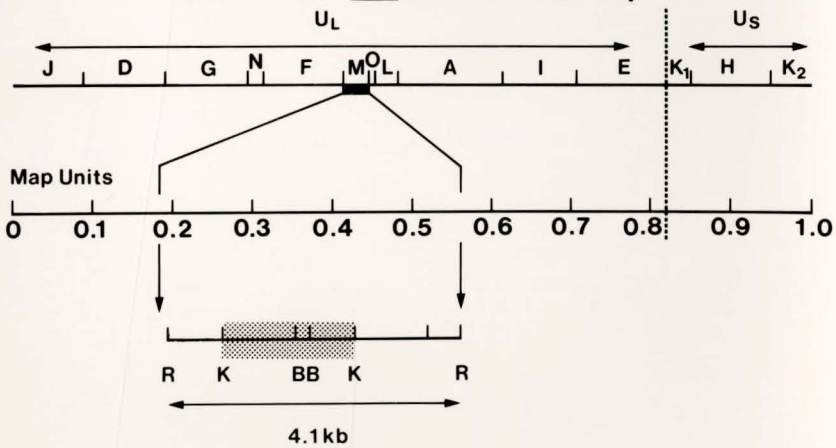


Figure 7. Restriction endonuclease maps of HCMV AD169 (67) and HSV-1 KOS (59). (A) BamHI map of HCMV AD169 showing location of selected probe fragments B, D, and H. An expanded map of the D fragment showing EcoRI, BglIII, PstI, XbaI sites is shown below. (B) EcoRI restriction map of Hsv-1 (KOS) showing location of the M fragment. An expanded map of this fragment is also shown. Enzyme sites are EcoRI (R), KpnI (K), and BamHI (B). The shaded area of the expanded HSV-1 M fragment represents the region of homology with HCMV. U_L, Long unique sequence; U_S, short unique sequence; TR_L and TR_S, terminal repeats; IR_L and IR_S, inverted repeats.

HCMV AD169 BamHI Restriction Map



HSV-1 KOS EcoRI Restriction Map



region of homology between HSV-1 and HCMV includes only sequences from the DNA polymerase open reading frame (104).

Detection of HCMV with the H fragment probe. Since the H fragment did not cross-react with HSV DNA, it was used alone to see whether a single fragment would have the same sensitivity as the combined probes. A total of 35 urine samples were prepared for detection of HCMV in the same manner as the previous specimens, and the samples were hybridized with the biotinylated H fragment as the probe. The results are given in Table 1. Of 14 culture-positive specimens, 11 were probe positive (sensitivity, 78.6%), whereas 5 specimens were probe positive but culture negative. One of the latter samples was from a patient whose previous specimens had been culture positive. Sixteen specimens were negative by both methods. The specificity for this probe was, therefore, 76.2%. Probe and cell culture agreement was 77.1%.

Although sensitivity and specificity were similar for the combine (D and H) and single (H) probes, there was a difference in color intensity of the positive samples. None of the samples tested with the H fragment alone showed a color reaction greater than plus/minus. With the combined probes, on the other hand, there were several samples which showed 2+ or greater color reactivity (Figure 4).

Screening of HCMV recombinant library for sequences homologous to HSV-1 TK. The homology discovered between the HSV-1 and putative HCMV DNA polymerase sequences prompted a similar search for homology between the HSV-1 TK and HCMV. In order to screen dot blots of DNA isolated from HCMV recombinant clones for homology to the HSV-1 TK gene, it was

necessary to remove the HSV-1 EcoRI N fragment from the vector DNA (pBR328) of pSG87 (64). The vector sequences would otherwise hybridize to the pBR322 vector sequences of the HCMV recombinants. Small-scale DNA preparations from the 67 recombinant clones of the HCMV AD169 library were dot-blotted onto nitrocellulose and hybridized with the HSV-1 EcoRI N fragment labeled with ^{32}P . After 22 h of hybridization under moderately stringent condition, two isolates, numbers 279 and 306, produced a stronger signal with the probe than any of the other isolates (Figure 8). All of the recombinant samples produced a weak signal, which was probably the result of hybridization between small residual amounts of vector DNA in the probe preparation and the vector sequences of the HCMV recombinant plasmids.

DNA isolated from the 67 HCMV recombinants was digested with BamHI, subjected to agarose gel electrophoresis, and blotted onto GeneScreen Plus membranes. The membranes were hybridized with the intact HSV-1 pSG87 plasmid (64) labeled with ^{32}P . This meant that both vector (pBR328) and insert (EcoRI N fragment) were present in the probe. Figure 9 shows the results for a representative group of the recombinants including 279 and 306. The common band in all of the digests represents probe hybridization with the pBR322 vector of the recombinants. The other faint bands of hybridization do not correspond to any insert bands on the original gels (Figure 9C and 9D). There are also faint bands in the gel lanes which occur at the same position as the non-vector bands on the autoradiograms, but these probably represent undigested or partially digested plasmid DNA which still contains vector sequences. The lanes which contain DNA

Figure 8. Dot-blot of HCMV strain AD169 BamHI library for detection of homology with HSV TK.

Rows 1-6: Plasmid pBR322 with HCMV BamHI restriction fragment inserts. No DNA in positions 5L and 6L. Positions 6H,I,J, and K, HindIII fragments A,B,C,D of VZV DNA inserted into the vector pBR322 (negative controls).

Row 7: Position 7F, HSV EcoRI N fragment without vector. Position 7H, pBR322 DNA. Position 7J, 100 ng HCMV AD169 DNA. The blotted membrane was hybridized with HSV EcoRI N fragment without vector sequences. This fragment contains the coding region for the TK gene. Autoradiogram was exposed for 20 h.

Note: positions 3L and 4G are clone numbers 279 and 306 respectively.

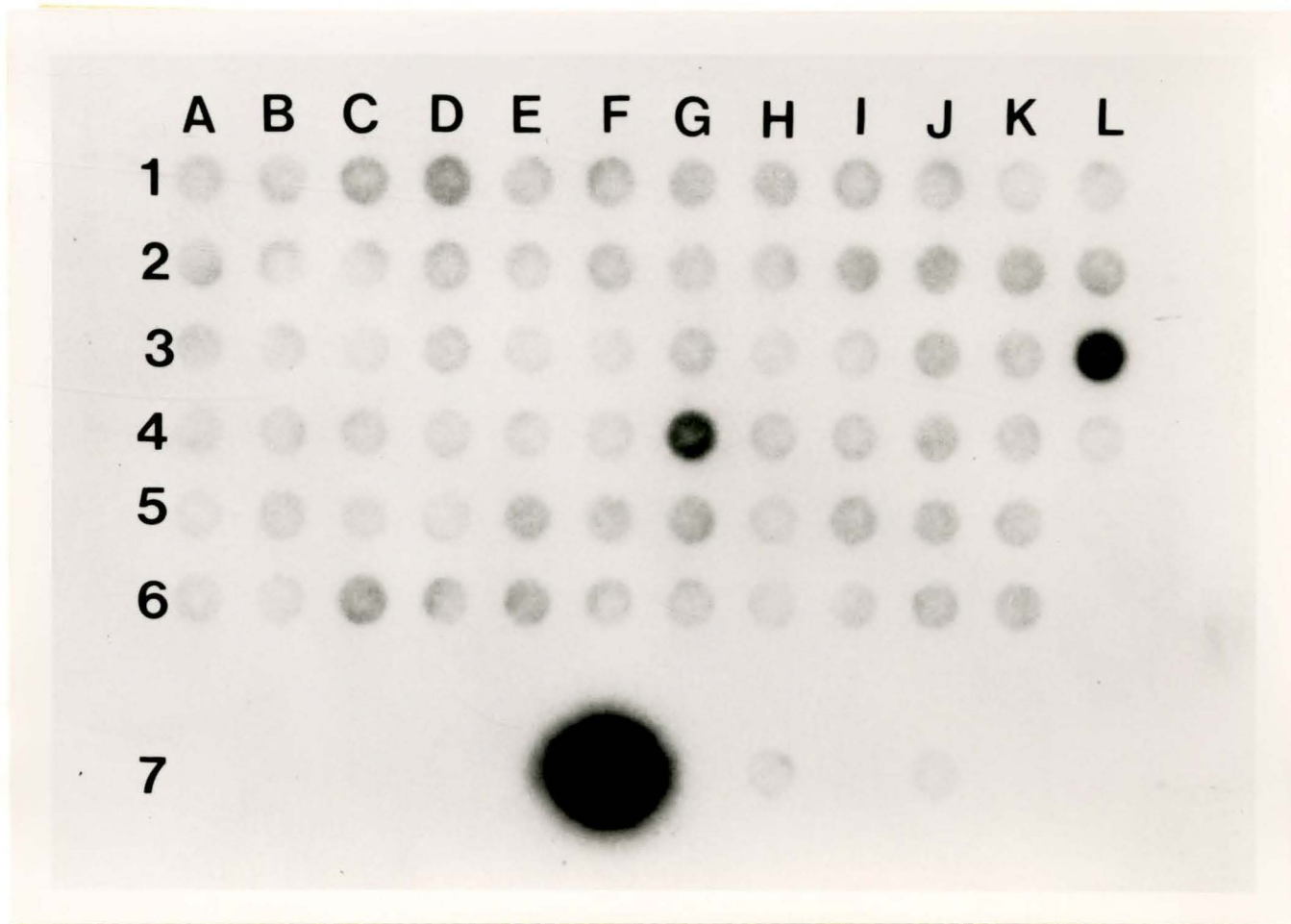


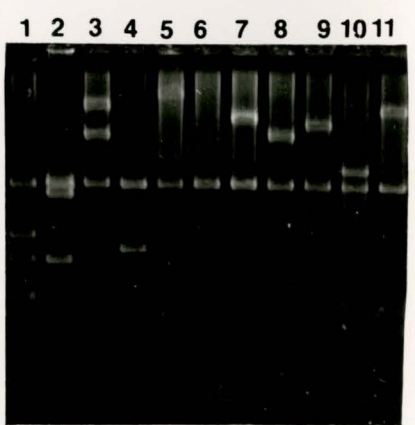
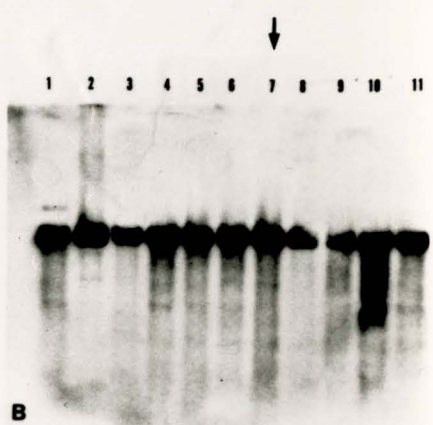
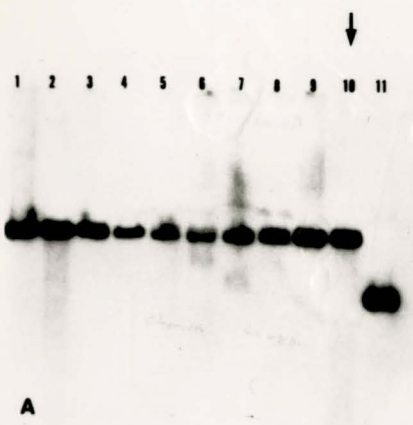
Figure 9. Southern blots of restriction endonuclease digests of HCMV DNA cloned fragments to detect homology with HSV TK.

A and C: Southern blot with corresponding agarose gel. Lanes 1-10: BamHI restrictions of HCMV fragments inserted in the BamHI site of pBR322. Lane 11: pUC18 plasmid DNA.

B and D: Southern blot with corresponding agarose gel. All lanes contain BamHI restrictions of HCMV fragments inserted in the BamHI site of pBR322.

Blots were hybridized with pSG87 (64) which has HSV EcoRI N fragment insert (HSV TK) in the vector pBR328. Autoradiograms were exposed for 20 h.

Note lanes A-10 and B-7 (arrows) contain clone numbers 279 and 306 respectively.



C

D

isolated from recombinants 279 and 306 (arrows) are A10 and B7 respectively. Unlike the dot-blot preparations there are no insert bands hybridizing with the intact HSV-1 pSG87 plasmid probe.

BamHI restriction digests of recombinants 8, 215, 279, 306, and 452 were performed to try to resolve the conflicting results between the dot-blot and Southern blots of the HCMV recombinants. In addition total genomic HCMV DNA was digested with BamHI, HindIII, and XbaI. Southern blots of all of these digests were probed with the HSV-1 EcoRI N fragment. There was no hybridization with any restriction fragments under moderately stringent conditions. The only visible hybridization was to the N fragment control (data not shown).

Since the Southern blots of these selected recombinants failed to show hybridization with the HSV-1 probe, dot-blot of newly isolated DNA from the same recombinants was hybridized with the same probe (HSV-1 EcoRI N fragment without vector). There was no consistent increase in hybridization with recombinants 279 and 306 as had been previously observed on the original dot-blot (Figure 10D, compare with Figure 8). In fact the pBR322 vector control (Figure 10D column F1,2) had as strong a signal as some of the recombinants.

In order to address the possibility that there was an error in applying the HCMV recombinant DNA preparations in the original dot-blot (Figure 8), the entire set of HCMV recombinant plasmids was freshly isolated and dot-blotted onto nitrocellulose membranes. These dot-blot were again probed with the HSV-1 EcoRI N fragment. Figures 10A, 10B and 10C show no marked increase in intensity of probe hybridization for any

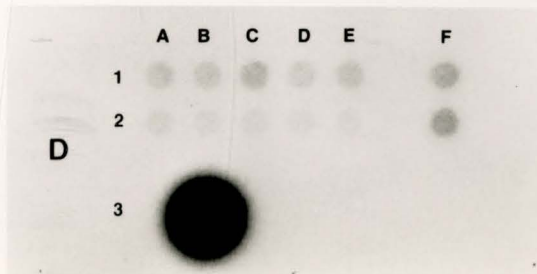
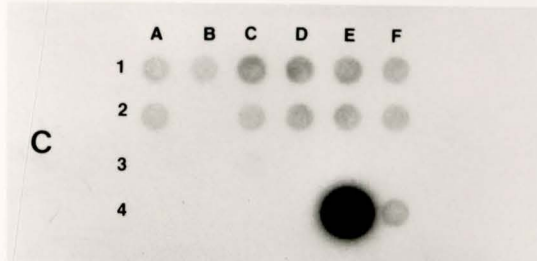
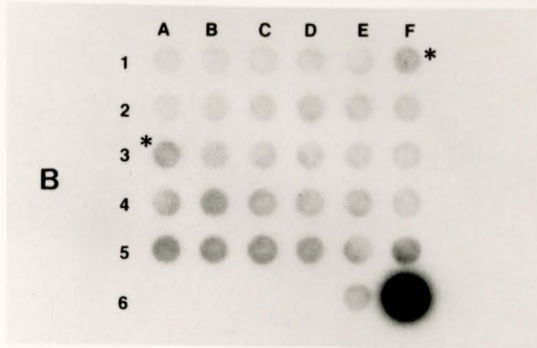
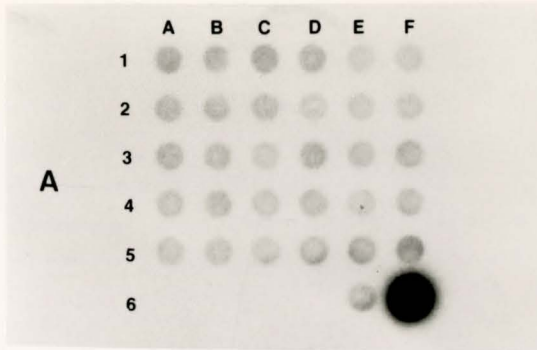
Figure 10. Repeat dot-blot of HCMV strain AD169 BamHI library to detect homology with HSV TK (see Figure 8). The probe for all dot-blot is the HSV-1 EcoRI N fragment with vector removed. Exposure was for 24 h.

Panel A: Rows 1-5, plasmid DNA from HCMV library samples. Row 6, E, pBR322 DNA; F, HSV-1 pSG87 DNA (positive probe control).

Panel B: Rows 1-5, BamHI HCMV library samples. 1F, clone 279; 3A, clone 306 (asterisks). Row 6, same controls as in panel A.

Panel C: Row 1, plasmid DNA samples from HCMV library. Row 2, A, last sample from HCMV library; C-F, HindIII fragments A,B,C,D of VZV (negative controls). Row 3, C 100 ng HCMV DNA. Row 4, E, HSV-1 pSG87 DNA ; F, pBR322.

Panel D: Repeat isolates of plasmid DNA from clones which originally hybridized with probe (Figure 8) and random negative clones from library. Rows 1 and 2: Upper and lower dots in each column are two different DNA preparations of each recombinant clone. Columns A-E recombinant plasmids in order: 8, 215, 279, 306, and 452. Column F: pBR322 vector control. Row 3: B, plasmid pSG87 positive probe control.



of the samples including 279 and 306, which are both labeled with asterisks on dot blot B (1F and 3A respectively) The variability in hybridization signal among the recombinants was interpreted to be non-specific binding of the probe to the DNA samples.

From these experiments it was concluded that although there was homology between the DNA polymerase coding sequences of HSV-1 and a specific region of HCMV DNA, there was no evidence that there was homology between a fragment encoding the HSV-1 TK and HCMV DNA.

HCMV AD169 stock for selection of drug-resistant mutants. The BamHI D fragment of HCMV DNA had been found to hybridize with a subfragment of the HSV-1 EcoRI M fragment (Figures 6 and 7). This evidence plus sequence data from two published reports (70,89) indicates, that the HCMV DNA polymerase probably is encoded by a 5.28 kb subfragment of BamHI D. Further evidence that this region encodes the polymerase would be provided by mapping mutations which produce resistance to drugs which target the DNA polymerase. In order to select for HCMV isolates which have such mutations, a sample of HCMV strain AD169 was cultured for large-scale production of a "wild-type" stock.

A three-times plaque-purified frozen sample of HCMV AD169 (24/6A/2D, harvested 4/30/82) was used to inoculate tube monolayers of HFF at an MOI of 0.01. The supernatant fluid was harvested three days after the cultures reached 100% CPE. Virus stock from these tubes was frozen in 10% DMSO and 10% FCS at -70° C for future use. All experiments to isolate drug-resistant mutants started with this "wild-type" stock.

Selection for drug-resistance by plaque assay. Mutants of HSV-1

resistant to antiviral agents have been selected after a single exposure to each agent (23,40). This selection method is most likely to produce high-level resistance resulting from single mutations. In order to try to isolate similar mutants of HCMV the wild-type AD169 stock was titered in the presence and absence of high concentrations of PAA, Aph and GCV.

The concentration of drug originally used for selection of PAA mutants, 700 μM (100 $\mu\text{g/ml}$), was chosen based on a reported method of selection for HSV-1 mutants (40). Only a few small plaques were obtained in all of the wells at this concentration. Attempts to pass these plaques were not successful. The concentration of drug was lowered to 175-350 μM (25-50 $\mu\text{g/ml}$). PAA even at 175 μM (25 $\mu\text{g/ml}$) produces a more than 20-fold reduction in titer (Table 2), but a few of the plaques which did appear in the drug wells were passed to tube monolayers. Two of these tubes yielded viable virus, which was retitered. The percent survival of each of these plaque isolates (P25A and P50A) was no higher than the wild-type.

The second drug which was used to try to select for resistant mutants was Aph. The drug concentrations chosen to titer the stock strain were based on similar experiments with HSV-2 (111) and preliminary testing of HCMV (65). Concentrations from 1.5 to 15 μM produced reductions in titer ranging from 5 to 100-fold (Table 2). Plaques were selected from wells containing 6 or 15 μM and retitered for resistance. Again no increased resistance was noted even with three-times plaque-selected isolates (see isolates Aph 5B/5A/5D and Aph 5B/5F/5D in Table 2).

The third drug used for selection of resistance was GCV. There was at least a 100-fold reduction in titer of the wild-type in the presence

TABLE 2. Selection for drug-resistant mutants from direct plaque isolates.

Isolate Number	Drug Selection	Drug Concentration	% Survival**
AD169	PAA	700 μ M	<<1%
AD169	PAA	350 μ M	<1%
AD169	PAA	175 μ M	4.4%
AD169	PAA	87.5 μ M	30%
P25A	PAA	140 μ M	2.5%
P50A	PAA	140 μ M	2.3%
AD169	Aph	15 μ M	<1%
AD169	Aph	6 μ M	5.5%
AD169	Aph	1.5 μ M	22.5%
Aph 5C	Aph	15 μ M	<1%
Aph 5D	Aph	15 μ M	<1%
Aph 5B/5A/5D	Aph	15 μ M	<1%*
Aph 5B/5F/5D	Aph	15 μ M	<1%*
AD169	GCV	100 μ M	<1%
AD169	GCV	50 μ M	<1%
AD169	GCV	25 μ M	<1%
AD169	GCV	12.5 μ M	14%

TABLE 2. (Continued)

D25B	GCV	12.5 μ M	10%
D12B	GCV	12.5 μ M	22%
D12A/50A	GCV	12.5 μ M	7.6%
D25B/12A	GCV	12.5 μ M	21.4%
D12B/25A/12B	GCV	12.5 μ M	17.6%*
D12B/25A/12C	GCV	100 μ M	<1%*
D12B/25A/12D	GCV	100 μ M	<1%*

*Plaque-selected three times.

** $\frac{\text{Titer with drug}}{\text{Titer without drug}} \times 100$

of 25 μM GCV (Table 2). Individual plaques which did appear at that concentration were passed to tube monolayers and cultured in the absence of drug. Viral progeny from these tubes were retitered in the presence and absence of GCV. Percent survival of plaque-selected isolates was essentially the same as the wild-type AD169 at both low (12.5 μM) and high (100 μM) concentrations of drug. For example in Table 2 isolate D25B was an initial plaque isolate with a 10% survival in 12.5 μM GCV. Isolates D12B/25A/12C and D12B/25A/12C were plaque-selected three times, but the percent survival at 100 μM GCV was still less than 1%.

Whenever one of these three drugs (GCV, PAA, Aph) was present in the culture medium at low concentration, the plaques were smaller than those produced in the absence of drug. With GCV and PAA the cells within the plaques were large and usually syncytial. Cells remained attached to the monolayer surface, and initial foci of infection enlarged only slightly during the course of the assay (14-18 days). At the higher drug concentrations no plaques were formed and only rare single enlarged cells were observed.

Selection for resistant mutants in increasing concentrations of antiviral agents. A second method for selection of resistant mutants was to expose the wild-type stock to increasing concentrations of antiviral agents. Individual tube monolayers were inoculated with wild-type HCMV AD169 virus stock. The virus was propagated initially in the presence of low concentrations of each of the three antiviral agents PAA, Aph or GCV. Each tube monolayer was passed to a new monolayer, whenever there was close to 100% CPE, or the monolayers were too old to support further viral

replication. The time between passages was usually at least two weeks but often 4-6 weeks. The progeny of each of the original tubes were always kept separate, so that any potential mutants would represent individual mutational events.

The initial concentration of PAA was $140 \mu\text{M}$ ($20 \mu\text{g/ml}$). The monolayers were incubated at this concentration up to 4 weeks. If the CPE was less than 100% after this time, the monolayers were passed to new monolayers, and the same concentration of drug was added to the culture medium. If 100% CPE was observed before 4 weeks of culture, the monolayers were passed to new monolayers, and the concentration of PAA was increased by a $10 \mu\text{M}$ increment. Eventually after 8-10 passages, there was close to 100% CPE in three tubes which had been exposed to $700 \mu\text{M}$ ($100 \mu\text{g/ml}$) PAA. When the extracellular virus was retitered at $700 \mu\text{M}$ PAA, however, the percent survival was less than 10%. Although these isolates may be slightly more resistant to PAA than the wild-type, this does not represent high-level resistance. The CPE may have been the result of passage of virus monolayers at a high MOI, which increases the apparent level of resistance to antiviral agents (see effect of MOI on GCV mutants measured by ELISA below).

Aphidicolin above $7.5 \mu\text{M}$ is slightly toxic for uninfected HFF. Five tubes were inoculated with HCMV AD169, and Aph at a concentration of $1.5 \mu\text{M}$ was added to the cell culture medium. The same protocol was followed for passage of monolayers as described above for PAA selection, except that the levels of increasing drug concentration were 6, 7.5 and $15 \mu\text{M}$. The number of passages at each drug concentration was usually 3 to 4, and

the total number of passages was 8 to 10. One of the original 5 tube lines initiated for selection of resistance to Aph retained evidence of viral infection at a 15 μM concentration, but there was never 100% CPE, and only monolayers could be passed for infectivity.

The only drug selection which yielded high-level resistant virus was GCV. For GCV selection the frequency of passage of monolayers again depended on the amount of CPE up to 4 weeks of culture. The initial selection was at a drug concentration of 6.25 μM . The levels for increasing the concentration of drug were 10, 12.5, 25, 50 and 100 μM . Virus from three of the original 10 tube lines produced 100% CPE at 25 μM GCV. These virus lines were spontaneously able to produce 100% CPE at 50 and 100 μM . High-level resistance, therefore, was achieved after exposure to 25 μM GCV.

Each of these three GCV-resistant strains of HCMV AD169 was plaque-purified three times. One plaque-purified sample from each strain was selected for further characterization. These isolates are designated D6/3/1, D1/3/4 and D10/3/2. All three GCV-resistant mutants produce true plaques which enlarge during the period of culture in the presence of the drug. The plaques are slightly smaller and slightly slower to appear when the drug is present, but both D6/3/1 and D1/3/4 have extracellular titers equivalent to the wild-type in the absence of GCV. D10/3/2 appears to replicate to lower extracellular titer than the wild-type in the absence of drug, and the plaque morphology differs from both the wild-type and the other mutants in appearing more syncytial. The relative reduction in titer of D10/3/2 in the presence of GCV, however, is no greater than that

of the other two mutants (see below).

Resistance of wild-type and GCV-resistant mutants to other antiviral agents measured by plaque dilution. Table 3 summarizes the cross-resistance of the three GCV-resistant mutants to other antiviral agents measured by percent survival. All three mutants were equally sensitive to relatively high concentrations of PAA, Aph and ACV. All three mutants were slightly more sensitive to AraA compared to the wild-type, but this hypersensitivity was less than four-fold. GCV is the only antiviral agent to which the mutants are much more resistant than the wild-type.

One problem in determining percent survival is that the wild-type does not form plaques in the presence of GCV at higher dilutions of the stock. Instead only isolated enlarged cells appear in the monolayer. The titer of AD169 in the presence of GCV, therefore has been recorded as the highest dilution in which only rare single infected cells are present. These individual cells are probably abortively infected, since adjacent cells never appear to be infected. Counting these enlarged cells as plaques means that the calculated titer based on number of plaque forming units (pfu) in the presence of GCV is probably too high. Thus, an accurate measurement of percent survival for the wild-type is difficult to determine by this method.

Resistance of mutants and wild-type AD169 measured by plaque reduction. A second method which was used to determine the relative resistance of both mutant and wild-type HCMV strains to antiviral agents was plaque reduction. To perform the assay a dilution of each virus strain containing 30 to 50 pfu was inoculated on monolayers in 24-well

TABLE 3. Percent survival of AD169 and mutants.

	Ganciclovir (100 μM)	AraA (150 μM)	PAA (700 μM)	Aph (15 μM)	ACV (300 μM)
AD169	<1*	33	<1	<1	6
D6/3/1	85	9	<1	<1	12
D1/3/4	77	10	<1	<1	14
D10/3/2	72	4	<1	<1	14

*Values are $\frac{\text{Titer with drug}}{\text{Titer without drug}} \times 100$.

Titers were determined by plaque dilution assay.

plates. The overlay medium contained serial two-fold dilutions of the drug. The ID_{50} was determined by the micromolar concentration of each antiviral agent which reduced the number of plaques by 50% relative to controls without the antiviral agent. The results of these assays are summarized in Table 4. All of the mutants were equally sensitive or resistant to GCV, PAA, Aph, and ACV. The mutants appeared again to be slightly hypersensitive to AraA compared to the wild-type. The mutant D6/3/1 was no more resistant to PFA than the wild-type but was slightly hypersensitive to AraT, FIAC and FIAU.

Since this assay also involves counting plaques, the same apparent wild-type abortive infection that is observed in plaque dilution assays is a problem. Even at the lowest drug concentration of GCV ($6.25 \mu M$), the size of the wild-type plaques was significantly reduced compared to those produced by the mutants.

Resistance of mutants and wild-type AD169 measured by ELISA. The problems encountered with counting wild-type plaques in the presence of GCV were addressed by using a third method to measure antiviral drug resistance. Both the percent survival and plaque reduction assays may have overestimated the titer of the wild-type in the presence of GCV.

In order to perform the ELISA, monolayers in 96-well microtiter plates are infected with the HCMV strain at an MOI of 0.01. Replication is allowed to proceed until the virus control wells show approximately 50% CPE (2+). This usually takes 4 to 5 days post-infection. The monolayers are then fixed to the bottom of the microtiter plate wells. Primary murine monoclonal antibody to an HCMV late antigen (see ELISA

TABLE 4. Plaque reduction assays.

	GCV	AraA	PAA	Aph	ACV	PFA	FIAC	FIAU	AraT
	—	—	—	—	—	—	—	—	—
AD169	5*	150	180	6	150	200	4	10	1,000
D6/3/1	150	50	180	6	150	200	1.5	5	290
D1/3/4	>100	50	180	6	150	-	-	-	-
D10/3/2	>100	50	-	6	75	-	-	-	-

*Values are the micromolar concentrations of each antiviral agent which reduce the number of plaques by 50% relative to controls without the antiviral agent.

section of Materials and Methods) is added to the fixed infected monolayer followed after a period of incubation with a secondary antibody, which is tagged with horse radish peroxidase. A substrate for horse radish peroxidase is added after another period of incubation and a colored product results from enzyme activity on the substrate. The amount of color is measured spectrophotometrically and is related to the amount of viral antigen produced. Inhibition of viral replication by GCV is measured by a reduction in optical density in virus-infected wells containing GCV in comparison to virus-infected control wells. The ELISA endpoint is the drug concentration producing a 50% reduction in the optical density (OD_{50}) of colored product. The main purpose of this assay was to confirm the previously observed sensitivity of the wild-type to GCV, which was difficult to quantitate by the other two methods described above. Only one of the mutants, D6/3/1 was tested by this method. The endpoints of the wild-type and D6/3/1 for GCV were very similar to those obtained by the plaque reduction assay. The mutant was also hypersensitive to AraA (Table 5).

The advantage of the ELISA is that production of the viral late antigen is detected colorimetrically. This measurement is proportional to the actual amount of virus replication within each cell, because the production of late antigen requires viral DNA replication. Plaque assays measure the ability of an infectious particle to produce additional infectious progeny, which in turn infect adjacent cells. In comparison with plaque assays the ELISA should more accurately reflect the amount of virus produced by the wild-type within the large single infected cells

TABLE 5. Resistance to antiviral agents determined by ELISA.

	<u>GCV</u>	<u>AraA</u>	<u>PAA</u>	<u>Aph</u>
AD169	3*	125	45	2
D6/3/1	125	50	45	2

*Values are the micromolar concentrations which cause 50% reduction of optical density of colored substrate.

which are observed in the presence of GCV.

It should be noted, however, that the actual value of the 50% endpoint in the ELISA is dependent on the MOI. In Figure 11 the effect of increasing MOI on the OD_{50} is demonstrated for both mutant and wild-type. The OD_{50} of the wild-type is approximately $1 \mu\text{M}$ at an MOI of 0.01 and $20 \mu\text{M}$ at an MOI of 0.1. At the lower MOI of the mutant D6/3/1 the OD_{50} is approximately $80 \mu\text{M}$ while the OD_{50} at the higher MOI is approximately $190 \mu\text{M}$. Even at the highest MOI, however, the level of resistance of the mutant is close to 10-fold higher than that of the wild-type.

Analysis of restriction enzyme digests of mutant and wild-type genomic DNA. The assays for cross-resistance of the GCV-resistant mutants to other antiviral agents did not provide any evidence for a mutation in the DNA polymerase gene. In order to investigate the possibility of a polymerase mutation further, virion DNA was isolated from the wild-type and the three mutants D6/3/1, D1/3/4 and D10/3/2. The samples of DNA were digested with EcoRI, PstI, HindIII or BglII. Southern blots of the digests were hybridized with a plasmid containing the EcoRI M restriction fragment inserted into the vector pUC18. The EcoRI M fragment hybridizes with the HSV-1 DNA sequences which encode the HSV-1 DNA polymerase (89,97). No differences in the positions of the bands hybridizing with the probe were noted for any of the enzyme digests (Figure 12). The probe hybridized with fragments of the predicted size (Figure 2) in all digests.

On some gels on which the restriction enzyme patterns of all three mutants were compared to that of the wild-type, an extra restriction

Figure 11. Effect of MOI on OD_{50} for GCV. Upper graph, wild-type AD169.
Lower graph, mutant D6/3/1.

Squares, MOI=0.1; triangles, MOI=0.01.

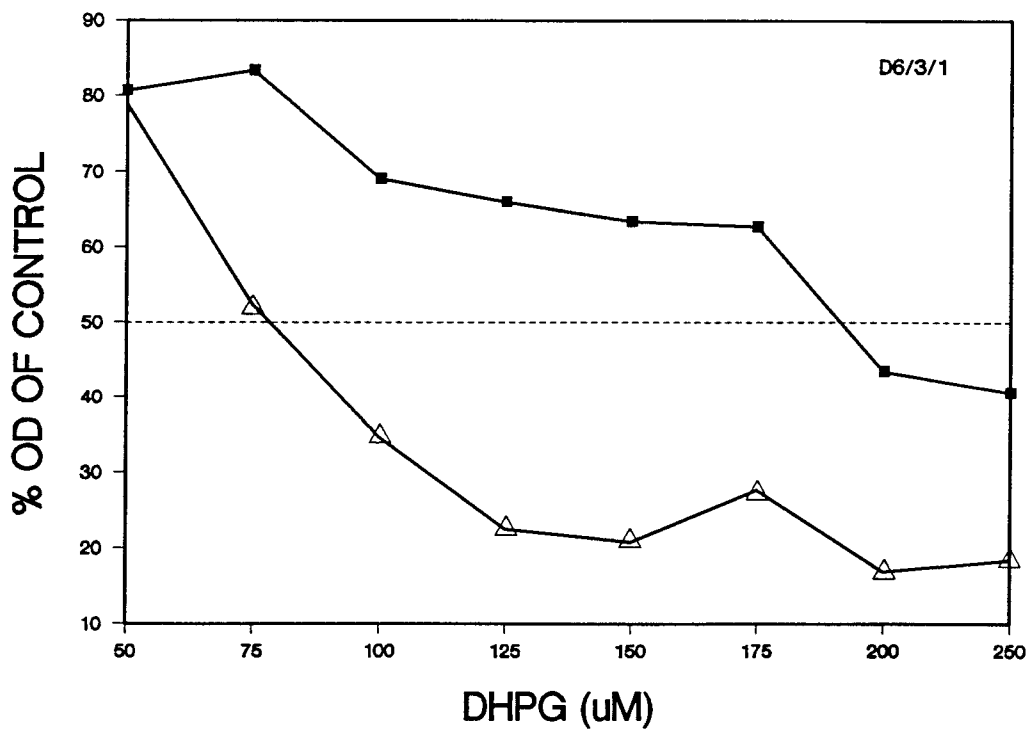
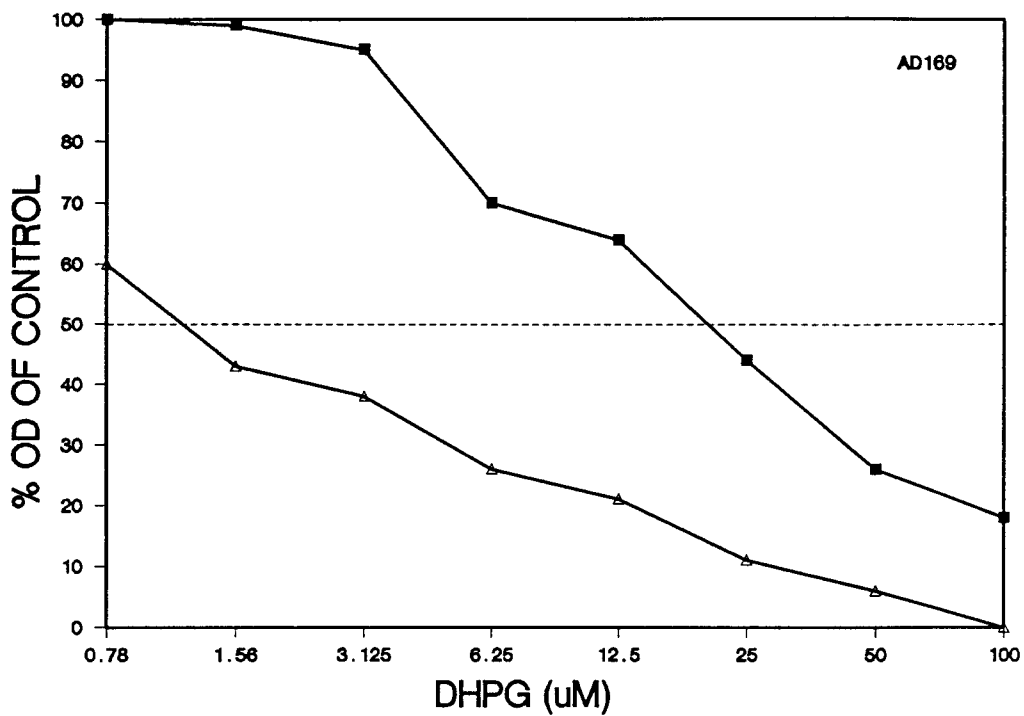


Figure 12. Southern blot with corresponding agarose gel of restriction endonuclease digests of DNA from mutant D10/3/2, D1/3/4, and D6/3/1 and wild-type AD169 to detect restriction site polymorphism in the DNA polymerase gene.

Lanes A, F, K, and P: AD169 DNA.

Lanes B, G, L, and Q: D10/3/2 DNA.

Lanes C, H, M, and R: D1/3/4 DNA.

Lanes D, I, N, and S: D6/3/1 DNA.

Lanes E, J, O, and T: EcoRI M fragment of AD169 inserted in pUC18.

Restriction enzymes used for digestion were as follows: Lanes A-E EcoRI; lanes F-I PstI; lane J PstI and EcoI; lanes K-N HindIII; lane O HindIII and EcoRI; lanes P-S BglII; lane T BglII and EcoRI.

Probe for Southern blot was HCMV AD169 EcoRI M fragment inserted in pUC18. Exposure time of autoradiogram was 24 h.

fragment of about 2 kb in size was noted in the BglIII digests of the mutants (see lower left gel in Figure 13). The intensity of the fragment was less than that of the majority of bands in the digest suggesting that it might represent a new terminal fragment which is present in less than 1 M concentration. In order to test this hypothesis Southern blots of BglIII digests (Figure 13) were hybridized with plasmids containing BamHI restriction fragment J (see Figure 2). This fragment contains terminal (isomeric inversion see Figure 1) and junction sequences surrounding the short unique region of the genome. The probe hybridized with this new fragment (Figure 13 autoradiogram) as well as to the large fragments at the top of the gel. These large bands are the junction fragments, which span the repeat regions of both the U_s and U_l segments of the genome. These bands would be expected to hybridize with the probe, because they contain the common repeat sequences.

There are two other slightly larger fragments which hybridized with the probe. These bands may contain some of the repeat sequences in common with the probe and may represent additional variation in restriction bands in the repeat region. This part of the HCMV genome has been shown to be the most variable among HCMV strains (90,126).

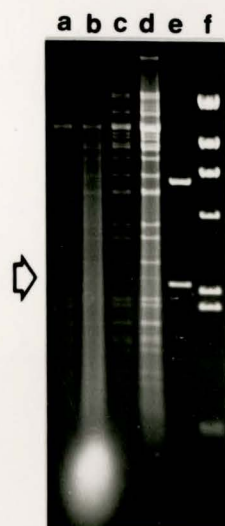
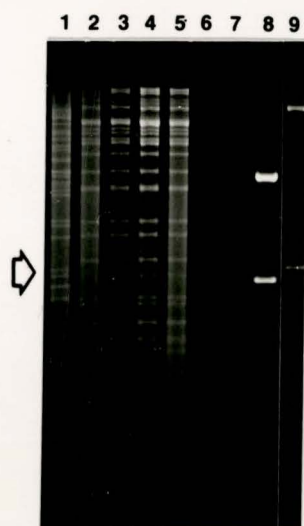
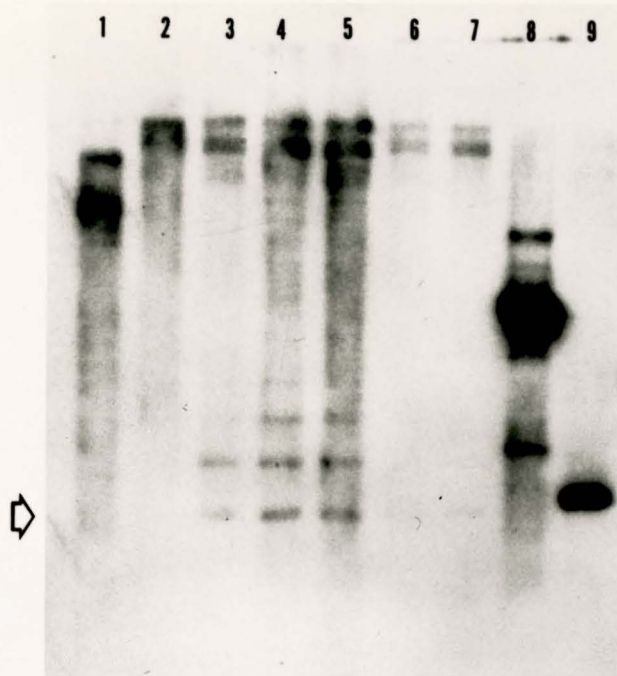
Further investigation of the extra BglIII restriction fragment in the mutants, demonstrated, however, that the fragment is associated with the source of the viral DNA. The sample of AD169 DNA which had been analyzed with the mutant DNAs in Figure 13 (lane 2), was purified from intracellular DNA. When a sample of extracellular wild-type virion DNA was digested with BglIII, the extra restriction band was present in the

Figure 13. Analysis of BglIII restriction site polymorphism of mutant and wild-type AD169 DNA.

Southern blot (upper figure): lane 1, intracellular AD169 DNA digested with BamHI; lane 2, intracellular AD169 DNA digested with BglIII; lane 3, extracellular D6/3/1 DNA digested with BglIII; lane 4, extracellular D10/3/2 DNA digested with BglIII; lane 5 extracellular D1/3/4 DNA digested with BglIII; lanes 6 and 7, extracellular AD169 DNA digested with BglIII; lane 8 pSG87 (HSV-1 EcoRI N fragment, vector pBR328) digested with BglIII; lane 9 plasmid containing HCMV EcoRI D fragment in vector pUC18 digested with EcoRI. Probe was HCMV AD169 recombinant clone number 279 which has BamHI J fragment inserted into pBR322. Exposure time for autoradiogram was 24 h.

Agarose gel (lower left): corresponding gel for Southern blot above.

Agarose gel (lower right): Lane 1, extracellular AD169 DNA ; lane 2, intracellular AD169 DNA; lane 3, extracellular D6/3/1 DNA; lane 4 extracellular D1/3/4 DNA; lane 5 HSV-1 pSG87 (EcoRI N fragment, TK); lane 6, lambda DNA. Lanes 1-4 were digested with BglIII, lane 5 with EcoRI and lane 6 with HindIII.



wild-type digests as well as the mutants (Figure 13 lower right gel). There is extracellular AD169 DNA digested with BglIII in lanes 6 and 7 of the lower left gel in figure 13. The concentration of DNA is too low to visualize the extra BglIII band, but on close examination of the upper autoradiogram, faint bands co-migrating with the hybridization bands in lanes 3 through 5 can be seen. Therefore, this band is found in extracellular DNA in both mutant and wild-type strains and does not represent an altered restriction site that could be associated with GCV resistance.

HPLC analysis of drug anabolism of the GCV-resistant mutants.

Neither cross-resistance to other antiviral agents nor restriction fragment polymorphisms provided any suggestion as to the site of the mutation responsible for GCV resistance. Biron et al. (4) used ion exchange HPLC to demonstrate that their GCV-resistant mutant of HCMV AD169 was unable to phosphorylate the drug.

In order to analyze the GCV anabolism by D6/3/1, D1/3/4 and D10/3/2, a reversed phase HPLC system was developed. Unlabeled GCV and GCV-monophosphate (a gift from Syntex) were used as standards to determine retention times and drug stability under the conditions required for sample preparation. No standards for the di- and tri-phosphates of GCV were available, but it was assumed that these compounds would be more polar than the monophosphate, and therefore, would have shorter retention times.

On the Partisil ODS-3 column with a mobile phase of 0.02 M KH_2PO_4 , GCV-monophosphate had a retention time between 4.5 and 5.0 min depending

on the flow rate, which was either 1.5 or 2.0 ml/min. The retention time for GCV under the same conditions was between 10 and 11 min. Two different perchloric acid extraction methods were tested. When both standards were exposed to 0.5 M perchloric acid for several hours at room temperature, the unmodified drug was unstable, but the monophosphate was unaffected. When perchloric acid extracts were neutralized with KOH, only the monophosphate was unstable. In order to avoid degradation of either GCV or GCV-monophosphate, infected cell monolayers which had been labeled with ^3H -GCV (Syntex), were washed and resuspended in a small volume of DPBS and extracted in a 200 μl volume at a final concentration of 0.5 M perchloric acid. The samples were then analyzed immediately by HPLC. If necessary, the samples were stored briefly at -70°C .

The analysis of labeled cell extracts was repeated three times, and the results were the same for each virus strain. Uninoculated control cells had low counts in fractions 34-37, which represent unphosphorylated drug (Figure 14). The early fractions collected up to five minutes (Fractions 8-18) have very few counts above background, therefore, there is very little phosphorylation of GCV by uninfected cells. The same analysis of wild-type AD169 infected cells showed very high peaks for both unphosphorylated GCV (fractions 34-37) and its phosphorylated derivatives (fractions 8-18) (Figure 15). By contrast all three mutants have much lower peaks corresponding to the retention times of the phosphorylated derivatives (fractions 8-18 in Figures 16 through 18). From these results it can be concluded that AD169 phosphorylates GCV as predicted, while the mutants do not phosphorylate GCV to any great extent. Since mono-

Figure 14. HPLC analysis of GCV anabolism in uninfected HFF cells. Fractions were collected at 0.3 min intervals over a Partisil ODS3 reversed phase column. The flow rate was 1.5 ml/min. The mobile phase was 0.02 M KH_2PO_4 . Fractions 8-18 correspond to the retention times of phosphorylated derivatives. Unphosphorylated drug fractions are 34-37.

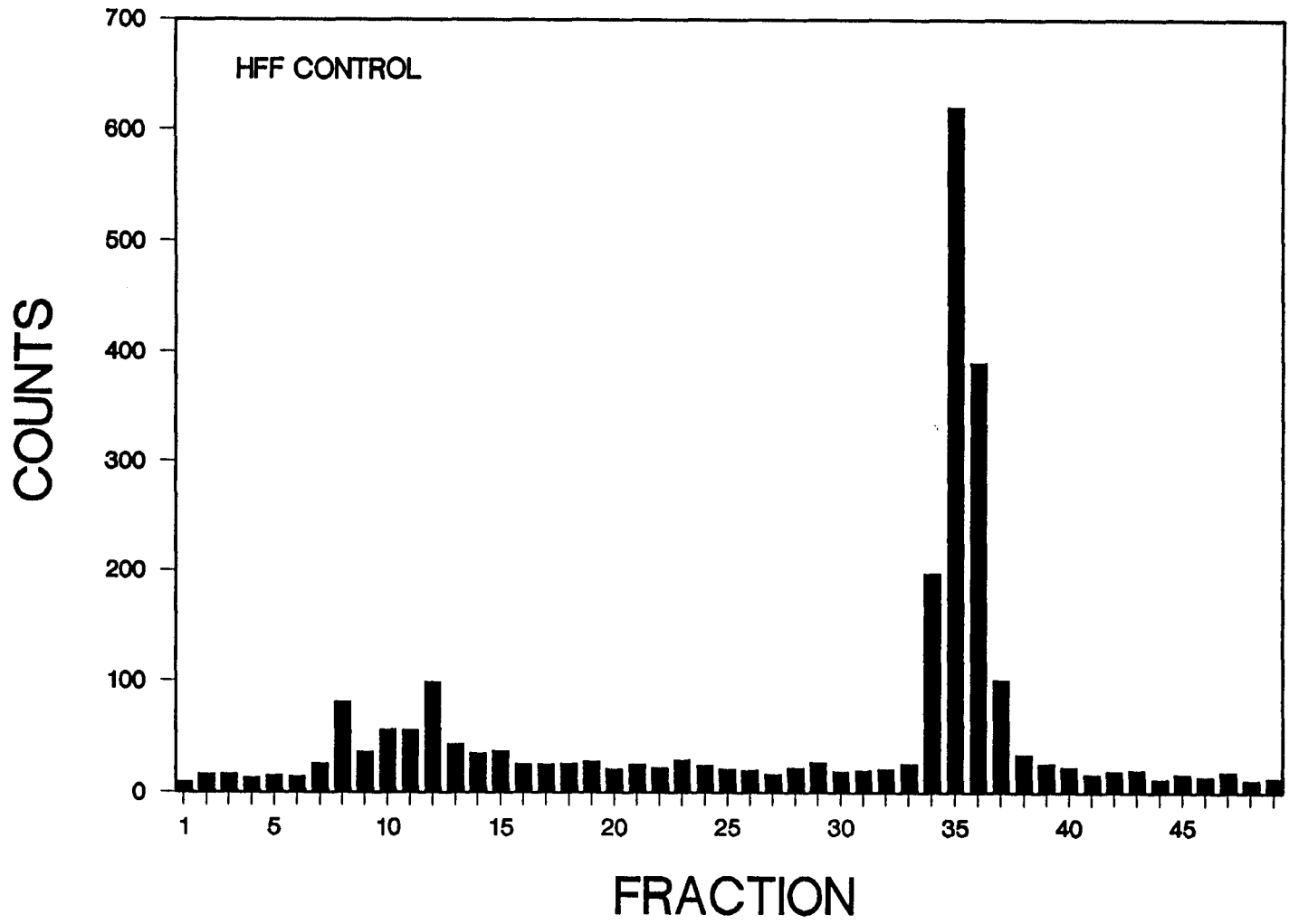


Figure 15. HPLC analysis of GCV anabolism in AD169-infected cells. HPLC conditions were the same as in Figure 14. Fractions 8-18 correspond to retention times of phosphorylated derivatives of GCV. Unphosphorylated drug fractions are 34-37.

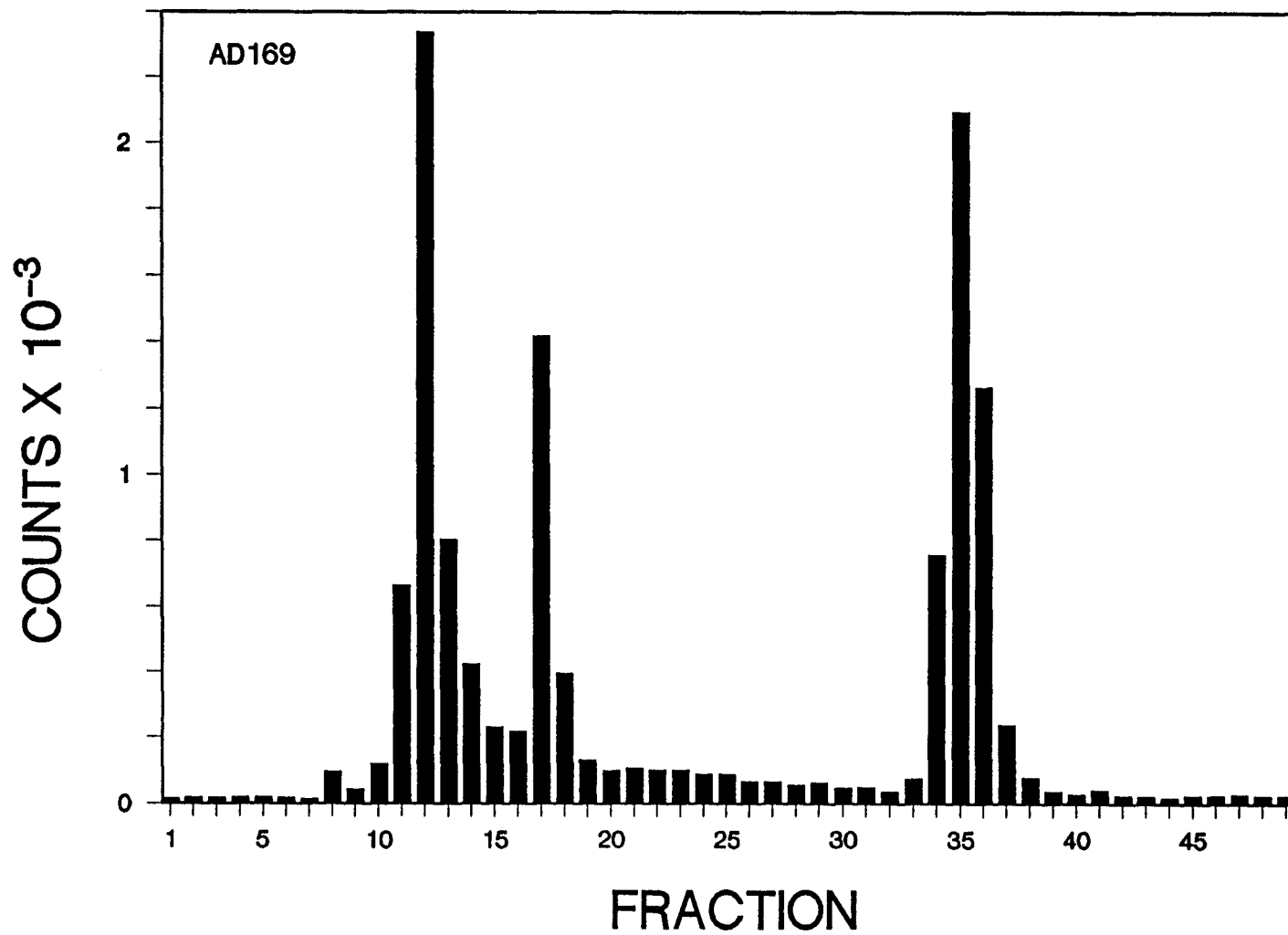


Figure 16. HPLC analysis of GCV anabolism in mutant D6/3/1-infected cells. HPLC conditions were the same as in Figure 14. Fractions 8-18 correspond to the retention times of phosphorylated derivatives of GCV. Unphosphorylated drug fractions are 33-38.

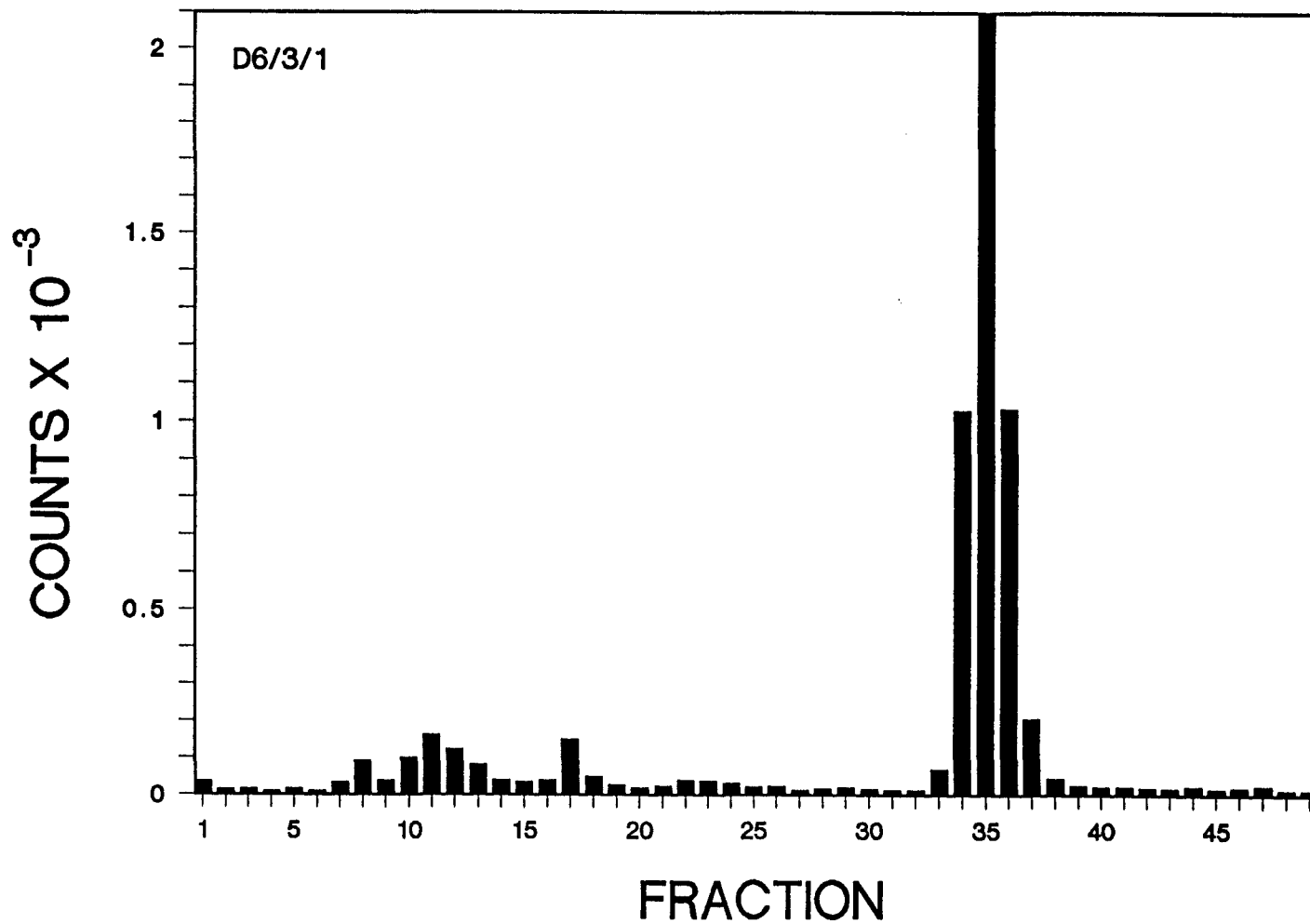


Figure 17. HPLC analysis of GCV anabolism in mutant D1/3/4-infected cells. HPLC conditions were the same as in Figure 14. Fractions 8-18 correspond to the retention times of phosphorylated derivatives of GCV. Unphosphorylated drug fractions are 33-38.

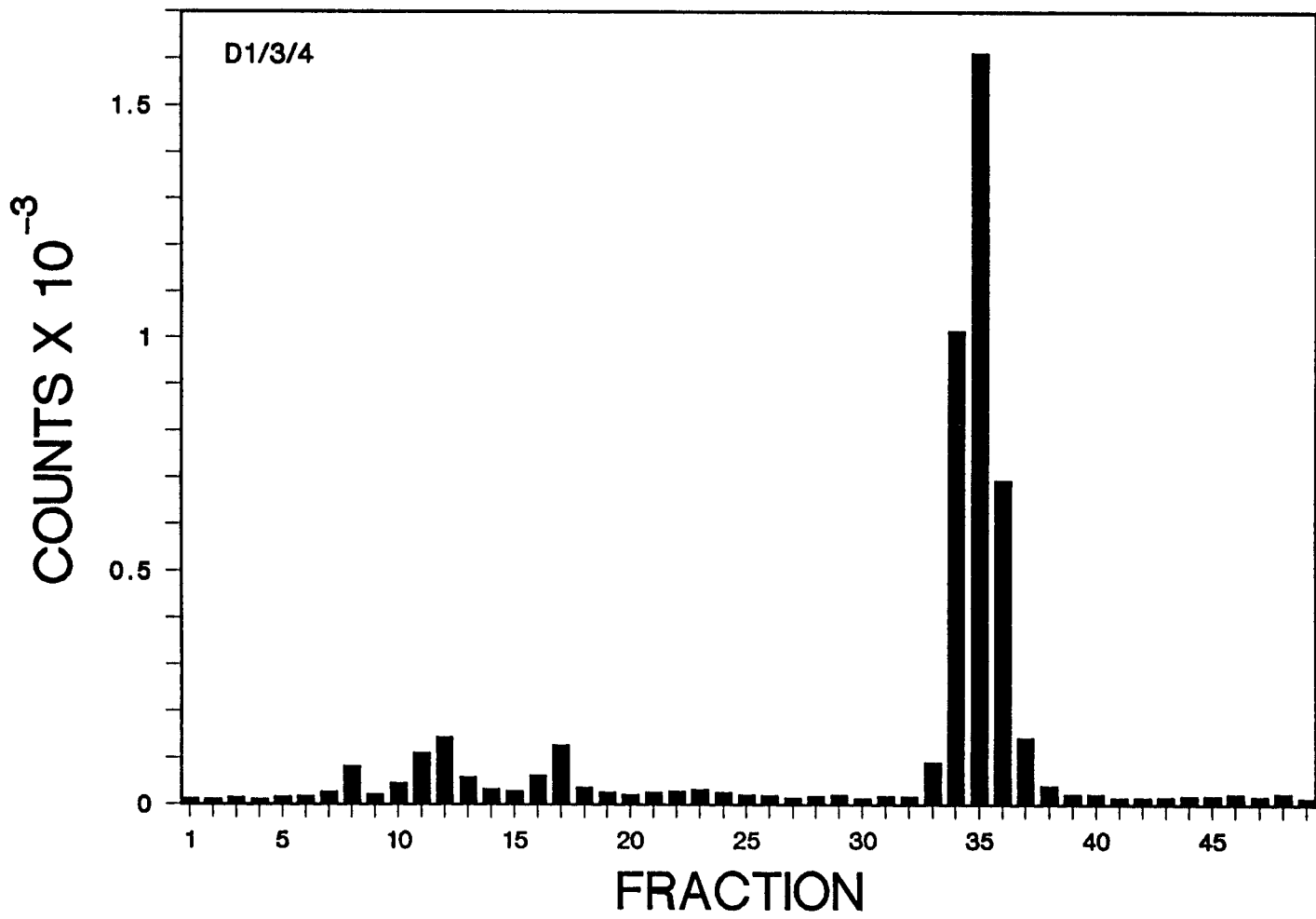
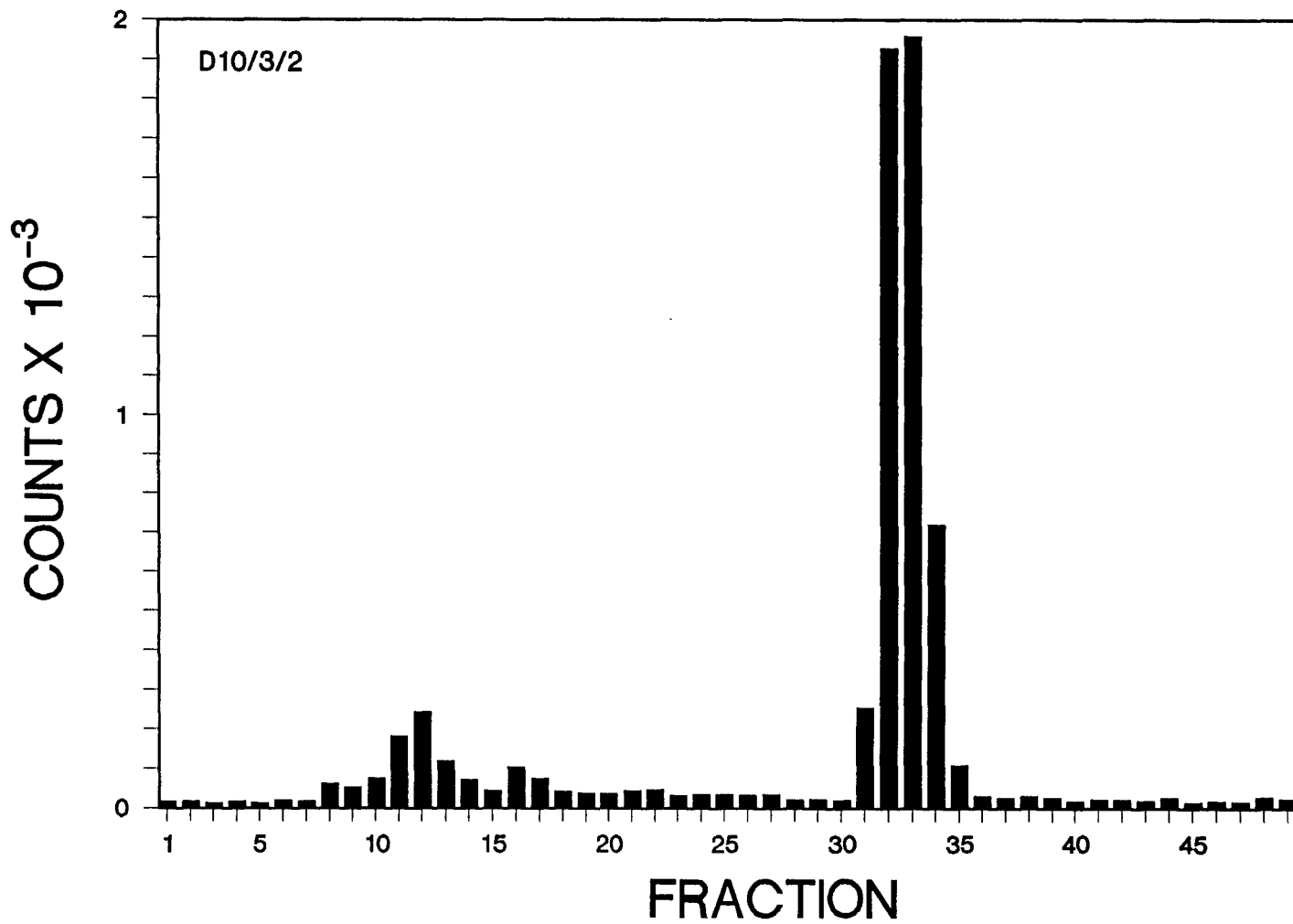


Figure 18. HPLC analysis of GCV anabolism in mutant D10/3/2-infected cells. HPLC conditions were the same as in Figure 14. Fractions 8-17 correspond to retentions times of phosphorylated derivatives of GCV. Unphosphorylated drug fractions are 31-35.



phosphorylation is necessary to activate the drug intracellularly, the increased resistance of the mutants to GCV can be attributed to the altered anabolism.

Analysis of GCV anabolism in co-infected cells. The interaction of wild-type and mutant D6/3/1 in co-infected cells was investigated next. HFF were co-infected with AD169 and D6/3/1 at an MOI of 1.0 for each virus. Control flasks were also infected with either AD169 or the mutant alone at an MOI of 2.0. The cultures were pulsed with ^3H -GCV at 72 h post-infection, and the cells were harvested 24 h later. The infected cells were extracted with perchloric acid and the extracts were analyzed by reversed phase HPLC. In Figure 19 the wild-type and mutant controls, each at an MOI of 2.0, are compared. The profiles are consistent with the previous HPLC analysis where drug anabolism by the mutant occurs at a much lower level than that of the wild-type. In the upper graph in Figure 20 the wild-type AD169 control at an MOI of 2.0 is compared to co-infected cells for which the total MOI was 2.0, but the MOI of each individual virus was 1.0. Drug anabolism in co-infected cells in this experiment was approximately 10-fold lower than that of the wild-type. The experiment was repeated except that the AD169 control was infected at an MOI of 1.0 instead of 2.0. The HPLC profiles were similar to those in the previous experiment, but as might be predicted from the Poisson distribution, the number of counts in the GCV and GCV-derivative peaks of the wild-type AD169 control were lower. At an MOI of 2.0 the percent of uninfected cells would be predicted to be 13.5%, while at an MOI of 1.0 the percentage of uninfected cells would be expected to increase to 36.8%.

Figure 19. HPLC analysis of AD169 and mutant D6/3/1 controls for co-infection experiment. Cell monolayers for each control were infected at an MOI of 2. HPLC conditions were the same as for the anabolic studies in Figures 14-18 (phosphorylated derivatives of GCV in fractions 7-17, unphosphorylated GCV in fractions 32-37).

Cross-hatched bars, AD169 control.

Solid bars, D6/3/1 control.

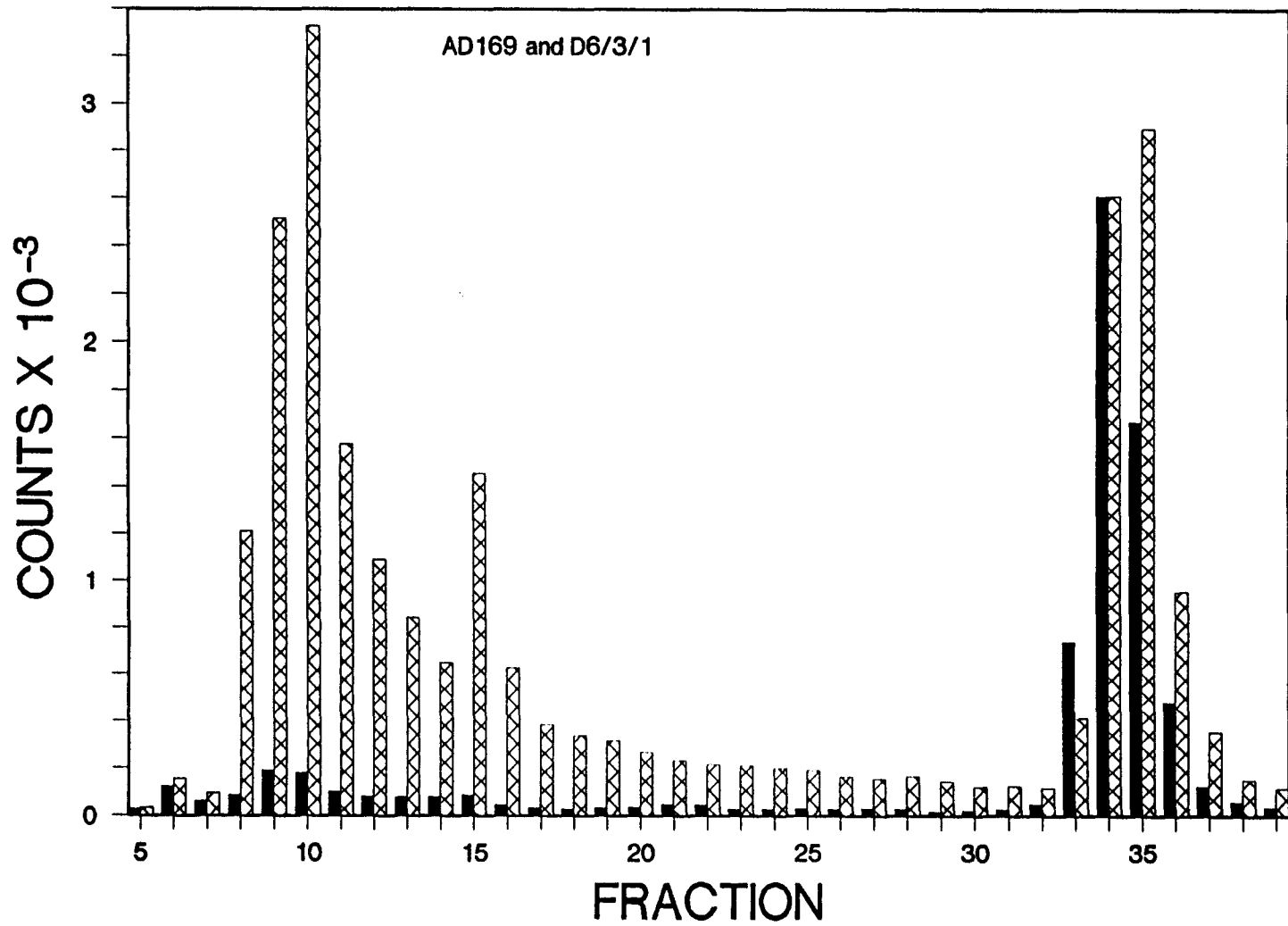
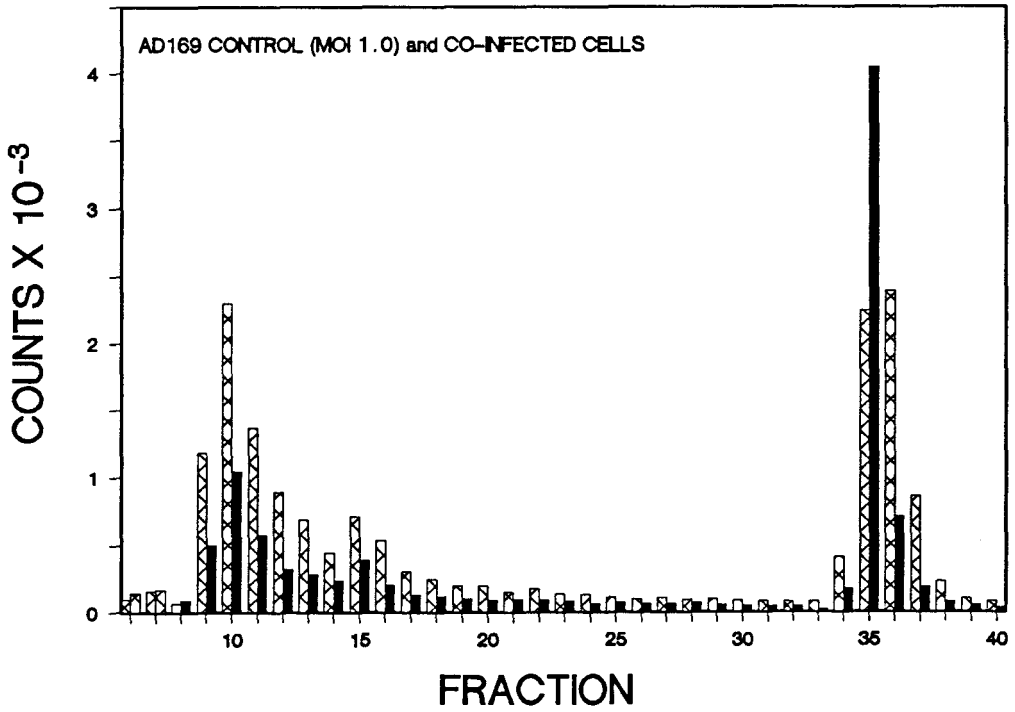
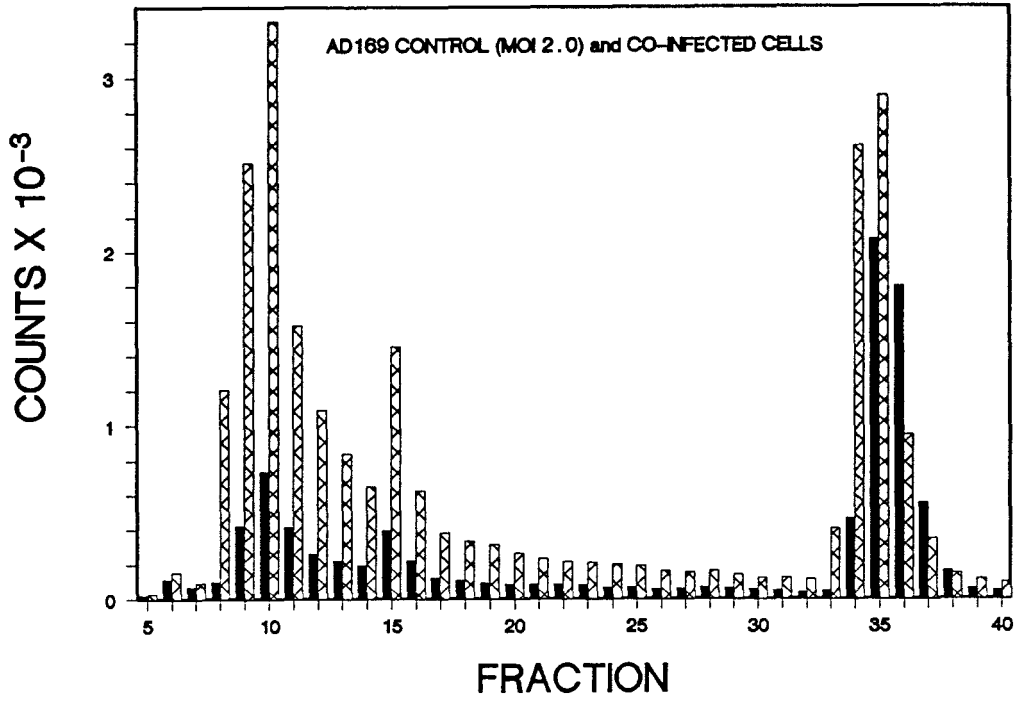


Figure 20. HPLC analysis of GCV anabolism in co-infected cells. **Upper graph:** Cell monolayer was co-infected at an MOI of 1.0 for each virus (total MOI=2). HPLC conditions same as for anabolic studies in Figures 14-18 except the flow rate was 2.0 ml/min. Phosphorylated derivatives of GCV in fractions 7-17, unphosphorylated drug in fractions 32-37. Control monolayer infected with AD169 at an MOI of 2.0. **Lower graph:** same as above except AD169 control monolayer infected at an MOI of 1.0.

Cross-hatched bars, AD169 control.

Solid bars, co-infected cells.



As the number of uninfected cells increases, the amount of phosphorylation, and therefore the number of counts associated with phosphorylated derivatives, decreases. The GCV derivative fractions, however, still had more than twice the counts as the corresponding fractions in the co-infected cell samples (Figure 20 lower graph).

The wild-type "kinase" would be predicted to be dominant over the mutant, if there is no interaction between the enzymes when they are present in the same cell. These experiments demonstrate that the mutant activity does have an effect on the wild-type, because the total level of GCV phosphorylation in co-infected cells is greatly reduced compared to the wild-type control.

In vitro translation of mRNA from virus-infected cells. From the previous HPLC analysis it is apparent that a viral mutation produces an alteration in the kinase activity which is responsible for the phosphorylation of GCV in virus-infected cells. This suggests that either the virus encodes the enzyme for this function or that there is a viral product which modifies a cellular kinase. Work by Preston (124) with the HSV TK demonstrated that it is possible to obtain a functional protein by in vitro translation of RNA isolated from HSV-1-infected cells. A similar set of experiments was designed to try to express the GCV kinase function in vitro.

Tritiated-GCV or ^{14}C -labeled-GCV was added to translation products from mRNA isolated from cells 72 h post-infection with either wild-type AD169 or mutant D6/3/1. In the initial experiments the pH of the reaction was 7.5, and the period of incubation for expression of enzyme activity

was 5 h. The reason for choosing this pH was that Biron and co-workers (5) had used pH 7.5 in attempts to express kinase activity from infected cell extracts. The products of the in vitro translation reaction were extracted with perchloric acid and the extracts were analyzed by HPLC. The conditions for the HPLC analysis were the same as those for the studies of GCV anabolism (Figures 14-18). There was no peak corresponding to phosphorylated derivatives of GCV from wild-type infected cells (Figure 21, upper graph). The pH of the reaction was changed to 6.1 for the second set of experiments, and the incubation was increased to 11 h, but the results were the same (Figure 21, lower graph). This lower pH was used in experiments by Preston (124) for expression of HSV-1 TK activity. Since GCV is a substrate for the HSV-1 TK, the optimal reaction conditions of the "GCV kinase" might be similar.

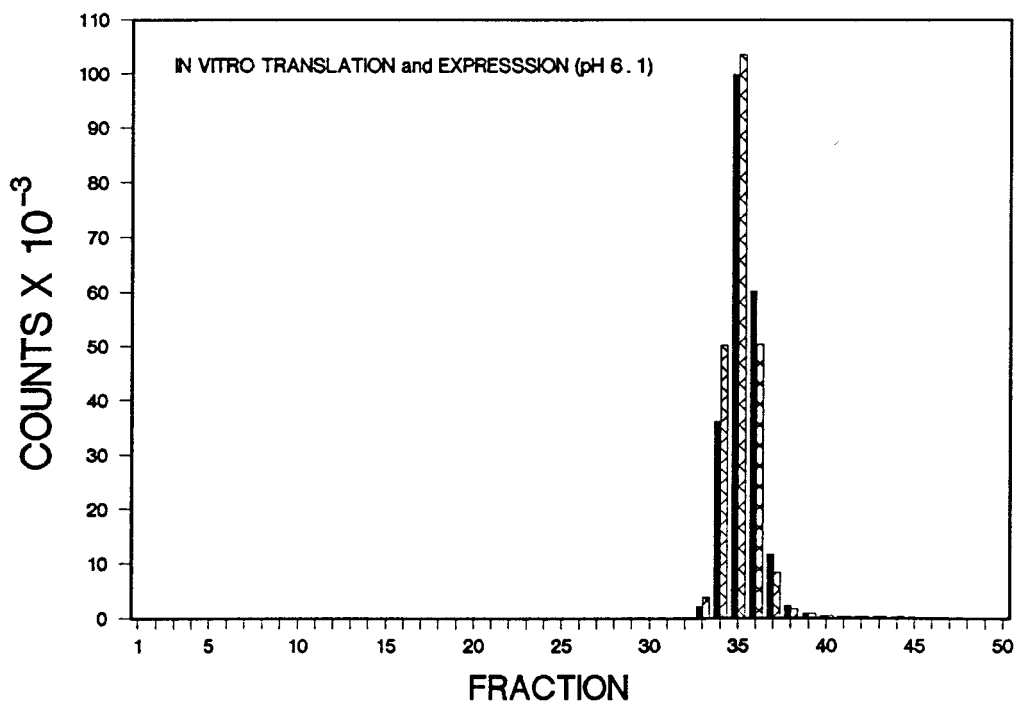
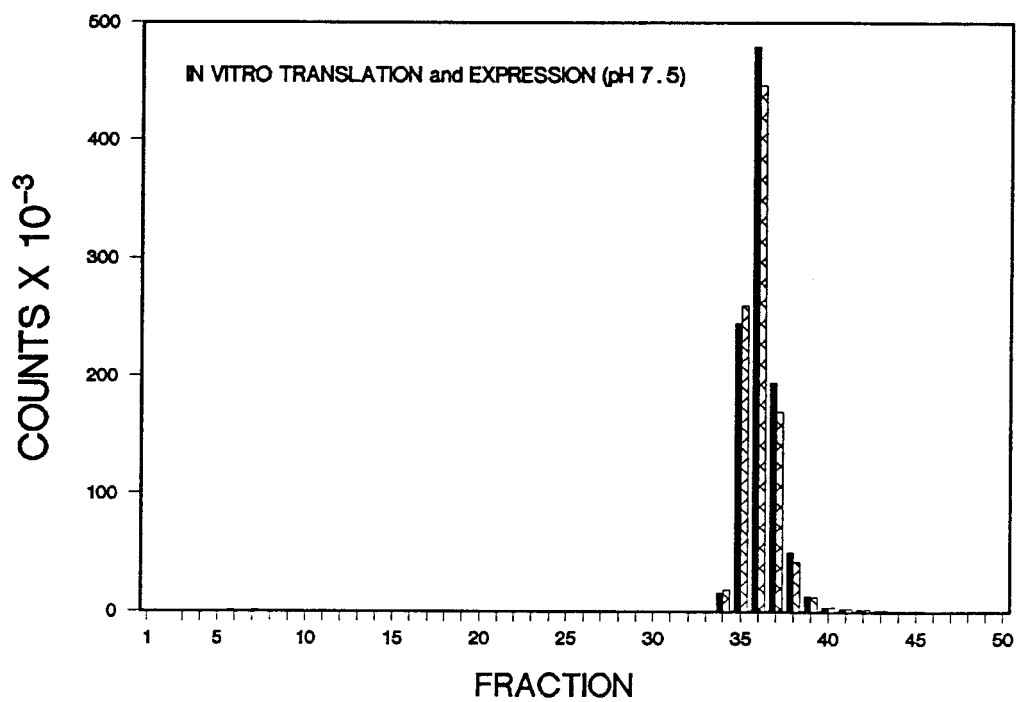
The in vitro translation reactions produced proteins, because the same mRNA preparations were used in the analysis of infected-cell proteins by 2-dimensional gel electrophoresis as described below, and consistent discrete spots were present on all fluorograms of these gels. The lack of detectable kinase activity is assumed to be the result of either low specific activity label or suboptimal conditions for enzyme activity or both.

Cosmid cloning of D6/3/1 DNA. Marker transfer would be a definitive method for mapping the enzyme function lacking in the mutants (117). DNA was isolated from extracellular virions of D6/3/1 and partial BamHI restriction fragments were cloned in the cosmid vector pWE15 (Stratagene). Over 500 ampicillin-resistant colonies were obtained and 120 of these were

Figure 21. HPLC analysis of GCV anabolism following incubation with products of in vitro translation of mRNA.

Upper graph: Analysis of GCV anabolism following incubation with in vitro translation products of mRNA isolated from uninfected (cross-hatched bars) and AD169-infected (solid bars) cells. HPLC conditions were the same as for co-infection studies in Figure 20. The pH of the expression reaction was 7.5 and the period of expression was 5 h.

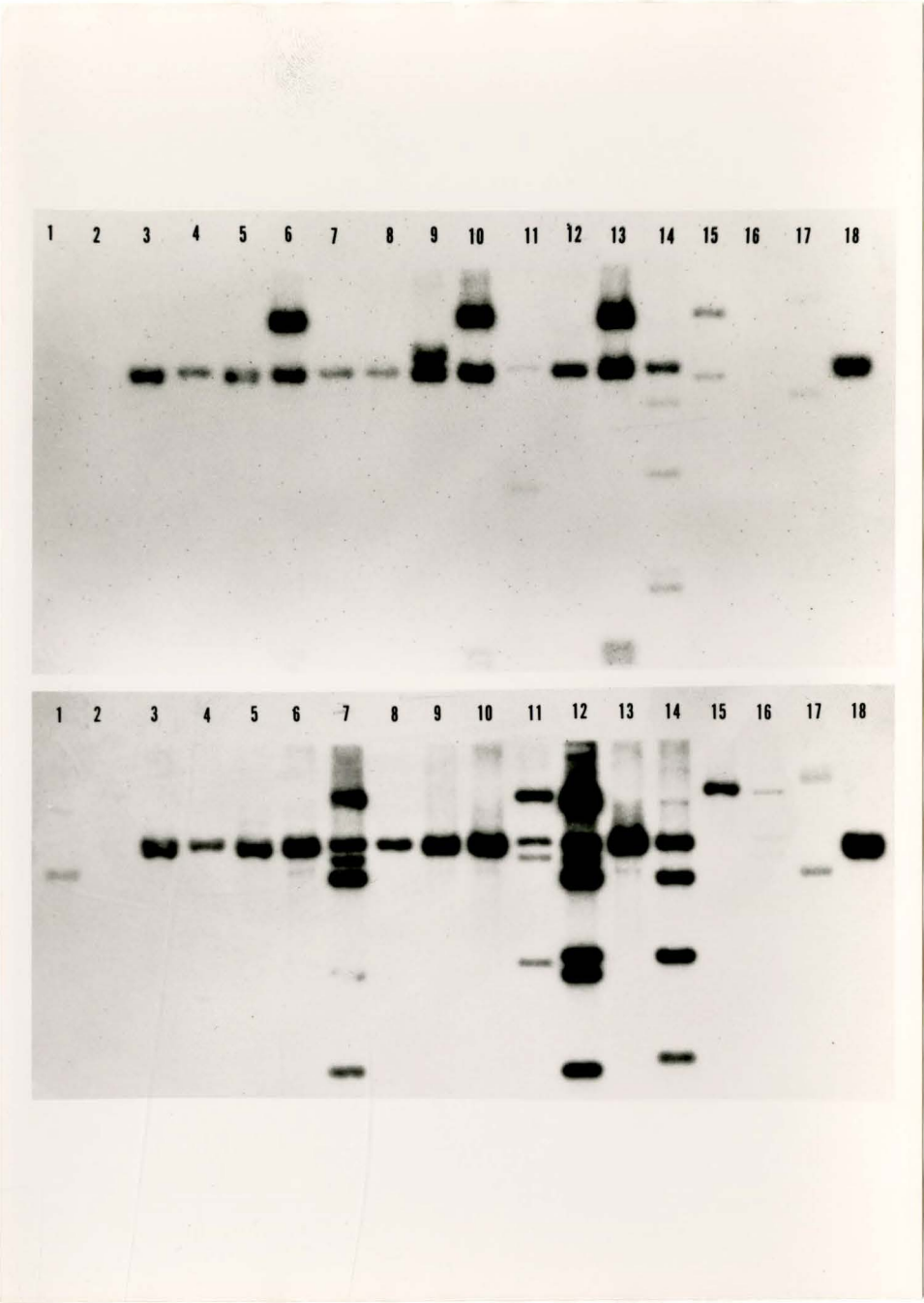
Lower Graph: Analysis of GCV anabolism following incubation with in vitro translation products of mRNA isolated from mutant D6/3/1 (cross-hatched bars) and AD169-infected (solid bars) cells. HPLC conditions were the same as for the co-infection studies in Figure 20. The pH of the expression reaction was 6.1, and the time of expression was 11 h.



subjected to plasmid DNA analysis. The DNA from each isolate was digested with BamHI to determine the size of the fragments present in the cosmid. In addition the DNA was blotted and probed with AD169 cosmids obtained from Bernhard Fleckenstein (51) or previously cloned fragments of AD169 DNA (97). Cosmid inserts were identified by fragment size and hybridization pattern. Figure 22 shows a group of mutant cosmids, which were hybridized with two different known AD169 cosmids to identify the region of the genome represented in the mutant. The same blot was first hybridized with cosmid 1075 which spans the HindIII R to L fragments. The membrane was stripped and then rehybridized with cosmid 1106, which spans the HindIII L through D fragments. The mutant cosmids were derived from BamHI partial digests of D6/3/1 DNA, while the wild-type cosmid probes are composed of HindIII partial digestion products. Note all cosmids have different hybridization patterns with the two probes except those in lanes 14 and 15. The cosmid in lane 14 was found on further study to have non-contiguous fragments, and the cosmid in lane 15 appeared to have rearranged vector sequences in addition to HCMV DNA inserts. Note there is no band in lane 15 which migrates with the common vector band (upper autoradiogram) in all of the other digests. The hybridization in the lambda lanes (1 and 17) results from the common cos sequences present in the pHc79 cosmid vector of the probe.

Eight cosmids were selected for more extensive analysis to see if the entire genome was represented. These eight cosmids were chosen, because they all had different restriction patterns and different patterns of hybridization with the HCMV AD169 cosmid probes. It is difficult to

Figure 22. Southern blot of restriction endonuclease digestions of cosmid clones of HCMV mutant D6/3/1 DNA. The same blot was hybridized first with AD169 cosmid 1075 (ref. 51) (upper autoradiogram). The probe was then stripped, and the blot was hybridized with AD169 cosmid 1106 (ref. 51) (lower autoradiogram). Lanes 1 and 17 of both autoradiograms, lambda DNA digested with HindIII. Hybridization of lambda fragments with the probe results from the lambda cos site, which is part of the cosmid probe construct. The vector portion of the AD169 cosmid probes is pHC79. The vector for the D6/3/1 cosmids is pWE15 (Stratagene). Lane 18 of both autoradiograms, pWE15 DNA, restricted with BamHI (single site). Lanes 3-16 of both autoradiograms, cosmid D6/3/1 DNA restricted with BamHI.



analyze cosmids which span any of the repeat regions, since the wild-type cosmid probes will hybridize with any fragments containing common repeat sequences regardless of the attached unique sequences (see Figure 2). Since there are four isomeric forms of the virus, smaller probes are required to determine which conformation is present in a given cosmid. Smaller probes were not available for this region without extensive subcloning. For this reason extra cosmids with different restriction patterns, which hybridized with repeat regions of the genome were selected to try to ensure, that all repeat and junction regions were included. From the somewhat superficial analysis of these eight cosmids, it would appear that most, if not all, of the genome is represented.

The DNA from each of these cosmids was used for a series of transfection experiments. Each experiment was set up as follows: 1) In the first experiment cosmid DNA was co-transfected with AD169 purified virion DNA. In addition some monolayers were transfected with cosmid DNA and then infected with wild-type virus. After 48 h 20 μM GCV was added to the cell culture medium. Monolayers were passed to new monolayers in tubes of flasks and selected with 100 μM GCV. 2) In the second experiment all monolayers were transfected with cosmid DNA and infected with wild-type virus after 24 h. GCV (100 μM) was added after another 24 h. Infected monolayers were passed to new monolayers after 5 to 7 days and again incubated with 100 μM GCV. 3) In the third experiment some of the monolayers were infected first with AD169 and transfected after 2 h. GCV was added after 22 h. The remaining monolayers were transfected first with cosmid DNA and infected after 22 h with AD169. After a 2-hour

adsorption period, GCV (100 μm) was added to the cell culture medium. Monolayers were passed to tubes and flasks at 5 to 7 day intervals. The cell culture medium always contained 100 μM GCV.

The reason for combining infection with transfection is that it is difficult to transfect the intact AD169 genome because of its large size, and it is also difficult to obtain large quantities of purified virion DNA. The disadvantage of supplying the wild-type DNA in the form of infectious virus is that there is a high background of wild-type CPE, which must be titered on new monolayers to test for recombinant virus. The order of transfection and infection was varied to see if either method might be more efficient for producing resistant virus.

In all three experiments tubes and flasks were held 4 to 6 weeks and repeatedly checked for plaque development. No resistant virus plaques were detected in any of the three experiments, although single enlarged cells often persisted after passage to successive new monolayers.

Two-dimensional gel electrophoresis. Since it was possible to obtain in vitro translated products from mRNA isolated from virus-infected cells, these products were analyzed by 2-dimensional gel electrophoresis. The proteins were labeled with ^{35}S -methionine and subjected to isoelectrofocusing in the first dimension and SDS-PAGE in the second. It is possible that there is a point mutation in D6/3/1, which is responsible for the altered kinase activity. Isoelectrofocusing would detect a change in isoelectric point resulting from a single amino acid change or possibly a small deletion. Point mutations which result in premature stop codons or frameshifts would be expected to produce lower molecular weight

proteins. A second possibility would be that the proteins might be unstable and would not be detected on the gels. Deletions, if large enough, would change the molecular weight of the protein.

Comparison of 2-dimensional gels of mutant and wild-type in vitro translation products did not reveal any consistent protein change between the two strains. The majority of proteins were consistently present on all fluorograms. There were occasional quantitative or qualitative changes which were not reproducible. Technical variations in 2-dimensional gel electrophoresis may contribute to the lack of reproducibility.

Comparison by 2-dimensional gel analysis of proteins extracted from mutant and wild-type-infected cells, however, revealed a protein which was present in wild-type-infected cells but missing in the mutant-infected cells. The arrows in Figure 23 indicate the position of the protein. The estimated molecular weight of this protein is 43,000.

Both the wild-type AD169 and mutant D6/3/1 stocks were extensively tested for mycoplasma contamination to eliminate the possibility, that the protein could be of mycoplasmal origin. The tests included Hoechst staining, monoclonal antibody specific for M. hyorrhinis, and culture of both cells and cell culture fluid. All test results were negative.

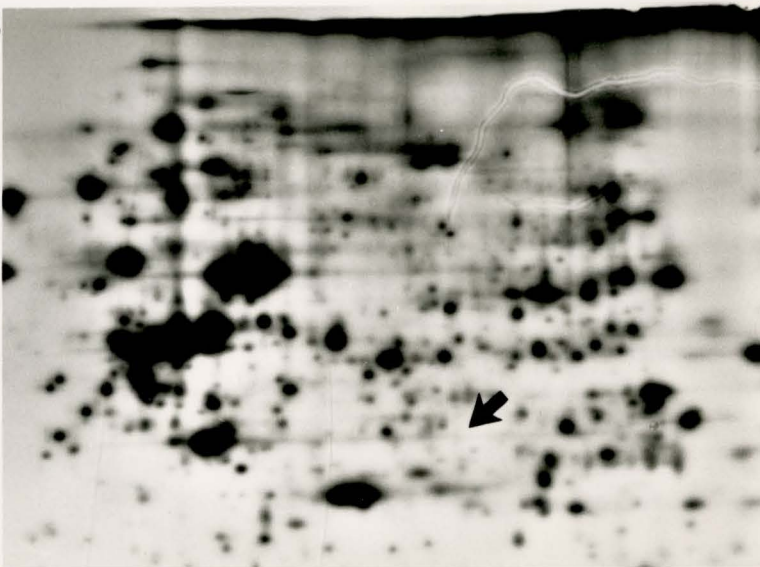
The experiment was repeated two more times, and the same protein difference appeared on the fluorograms. The protein was not present in either uninfected HFF cells or in mutant D1/3/4-infected cells (data not shown).

Figure 23. Fluorograms of 2-dimensional gels of proteins extracted from AD169-infected cells (A) and D6/3/1-infected cells (B). Proteins were labeled 48 h post-infection with ^{35}S -methionine and harvested for gel analysis 72 h post-infection. Arrows indicate position of 43,000 MW protein present in AD169, missing in mutant D6/3/1.

A



B



DISCUSSION

There were several significant findings from the experiments performed for this project. First of all DNA probes were found to be capable of direct detection of HCMV in clinical specimens. Coincidentally one of the probes cross-hybridized with the region of the HSV-1 genome which encodes the HSV-1 DNA polymerase. Since the HCMV DNA polymerase had not yet been mapped, this observation was a significant piece of evidence for the location of the polymerase on the HCMV genome.

Drug-resistant mutants have been very useful for analyzing the DNA polymerase locus of HSV. However, there have been very few HCMV mutants of any kind reported to date (4,33,34). Three mutants of HCMV (D6/3/1, D1/3/4 and D10/3/2), which are resistant to GCV were isolated. These mutants were found by HPLC to be deficient in intracellular anabolism of GCV. Since GCV must be activated by phosphorylation, the phenotype of the mutants suggested that there is a viral function required for this activation. No HCMV virus encoded TK has yet been found, although GCV is a substrate for the HSV-1 TK.

Detection of a protein in wild-type infected cells, which is absent in D6/3/1- and D1/3/4-infected cells is probably the most significant finding of this investigation. No kinase activity has yet been demonstrated for this protein, but the MW (43,000) and the temporal appearance (early post-infection) are similar to those of the HSV-1 TK.

Development of DNA probes. The biotinylated probes developed for this project were each able to detect HCMV in clinical specimens, provided that enough viral DNA was available to be detected by probe hybridization. Control experiments (Figure 3) indicated that the limit of detection was around 30 pg for totally homologous DNA. The studies using the HCMV BamHI B probe detected cell-free virus, since the low-speed centrifugation step of the protocol would have removed any virus associated with intact host cells. The sensitivity of the test with this probe was low for two possible reasons. (1) The nonspecific color background of the membrane was high. (2) There may have been a significant proportion of intracellular virus which was removed by the low-speed centrifugation. Color background is a technical problem and was corrected in later experiments by maintaining a constant flow of the streptavidin solution over the nitrocellulose membranes. The low-speed centrifugation step was eliminated to preserve intact cells. This meant, however, that mammalian cellular DNA could be present in the dot blot preparations.

During the time that the B fragment probe was being tested, there was a report (135) that DNA sequences within this fragment could hybridize weakly with cellular DNA sequences. In other studies (18,151) two probes from different regions of the genome were used. In order to avoid any possibility of cellular DNA hybridization and to investigate the possibility that a combination of two probes would raise the sensitivity of the procedure, both BamHI D and H fragments were chosen for further development as probes (Figure 2). With these new probes the sensitivity of the test procedure increased from 52 to 81%, but the specificity

declined from 87.5 to 68.8%. The decreased specificity could be the result of nonspecific binding of the biotinylated probe to substances in the urine samples. Microscopic analysis of materials pelleted by low-speed centrifugation showed that some urine specimens contained a significant number of cells, crystals, or bacteria.

There was unexpected cross-reactivity between the HCMV D fragment and HSV DNA controls, which will be discussed below. For this reason the H fragment was tested as a single probe. The sensitivity and specificity results were similar to those of the combined D and H probes, but it was noted that the positive specimens produced only a plus/minus color reaction. This suggests that a single probe might prove to be less sensitive than the combined probes. One reason for the decreased sensitivity would be that there is only one region of each genome which can hybridize with the probe, therefore the total probe signal per genome would be reduced. In addition there is the possibility of variation in DNA sequence among clinical strains of HCMV, which might decrease the homology between the probe and the corresponding region of the clinical strain to be detected.

Homology between HSV-1 and HCMV. The cross-reactivity between the HCMV BamHI D fragment and the HSV controls was investigated further. The HSV EcoRI M fragment specifically hybridized to this fragment. The total genomic homology between HSV-1 and HCMV was originally reported to be only 5% (79). HSV-1 and Epstein-Barr virus show homology which is confined to specific sequences coding for proteins having related functions (29,60,121). Similar limited homology has now been shown to exist between

HSV-1 and HCMV (89,97).

The HSV-1 M fragment codes for part of the viral DNA polymerase (21,59). Cross hybridization with the HCMV BamHI D fragment was observed within a region containing approximately 2.8 kb of the coding sequences of the HSV DNA polymerase (Figure 7). It has been confirmed by others that the specific hybridization between HCMV and HSV-1 observed in this study represents homology of that portion of the two polymerases which is highly conserved functionally among herpesviruses (58,89).

Similar hybridization experiments were also performed to determine if there is homology between the HSV-1 TK and any portion of the HCMV genome. No separate viral TK activity has been reported to be associated with HCMV infection (49,177), and there have been no reports of experiments performed to determine possible homology between the HSV-1 TK gene and HCMV DNA. The fact that ACV is not phosphorylated in HCMV-infected cells has been interpreted as further evidence that HCMV does not encode a TK (5). HCMV is sensitive to GCV, however, and GCV is phosphorylated by the HSV-1 TK.

When the first dot-blot which was made from the HCMV BamHI library was probed with the HSV-1 TK sequences, there were two samples which hybridized more strongly with the probe (Figure 8). Attempts to reproduce these results by probing restriction endonuclease digests of the BamHI library (Figure 9) or repeat dot-blot (Figure 10) were unsuccessful. The two recombinants which hybridized with the HSV-1 TK region on the original dot-blot contained the BamHI G and J fragments. These fragments contain sequences from the short repeat, which has a high G+C content (129). The

high G+C content could cause non-specific probe hybridization, which might explain why the original results were not reproducible. It should also be noted that the 67 BamHI recombinant clones tested for HSV-1 TK homology may not constitute a complete HCMV library, since not all HCMV fragment inserts have been identified. The conclusion from these experiments and those of others (49,177) is, that there is not a gene encoded by HCMV, which is homologous at the DNA level to the HSV-1 TK.

GCV-resistant mutants. The fact remains, however, that GCV is an effective therapeutic agent for treatment of HCMV infections and GCV requires intracellular phosphorylation for its antiviral activity. Resistance to GCV theoretically could be caused by mutations in two different genes of HCMV, the polymerase or the "GCV kinase". Biron and co-workers(4) demonstrated that a GCV-resistant mutant of HCMV AD169 was deficient in kinase activity. The three GCV-resistant mutants D6/3/1, D1/3/4 and D10/3/2, which were isolated during this project, were similarly characterized to determine what function was altered.

Multiple restriction digests of all three mutants were probed with the wild-type EcoRI M fragment, which encodes the polymerase gene (Figure 12). The purpose of this experiment was to determine if a restriction site might fortuitously have been gained or lost in the polymerase gene at the site of the mutation. No differences in the hybridization patterns could be detected.

In BglIII digests of the mutant DNAs, however, there was an extra band which was present in less than one molar concentration. Quarter or half molar concentrations are typical of terminal and junction fragments

of HCMV (90). For this reason the BglIII digests were probed with a plasmid containing the BamHI J fragment of AD169. There was hybridization with the extra BglIII fragment as well as two other faint fragments (Figure 13 lower gel). These other fragments may represent terminal variability, which has been described for this region (172). On further investigation, however, it was demonstrated that although the extra fragment was absent in digests of AD169 DNA extracted from infected cells, it was present in extracellular virion DNA of AD169 (Figure 13). HCMV DNA is replicated intracellularly as concatamers, therefore, the new terminal fragments may be generated when the DNA is packaged into virions.

Restriction enzyme analysis did not yield any evidence of altered restriction sites either in the region of the HSV polymerase gene homology or in digests of the total genome. This does not, however, rule out a mutation in the polymerase gene, since there are many sites where a base change would not affect a restriction enzyme recognition sequence. Similarly changes in any other region of the genome would not necessarily produce detectable restriction polymorphisms, especially since only five restriction endonucleases were tested. Another approach to determining whether the GCV resistance of the mutants was a result of changes in polymerase or kinase function was to develop a drug-resistance profile for each mutant. HSV-1 drug-resistant mutants were found to cluster in two genes, the TK and the polymerase depending on the target of the individual drug. There was often cross-resistance among drugs affecting the same gene. For example PAA-resistant mutants were also resistant to PFA and AraA (20,21,22). Cross-resistance of the GCV-resistant HCMV mutants was

tested first by plaque dilution to determine percent survival (Table 3). Relatively high concentrations of each antiviral agent were chosen. The mutants were similar to the wild-type in their sensitivity to PAA and Aph. Both mutants and wild-type were relatively resistant to ACV. High-level GCV-resistance of the mutants was confirmed. The mutants, however, appeared to be slightly hypersensitive to AraA. This was somewhat surprising, since AraA is thought to be phosphorylated by cellular kinases (93).

There are two problems with this method of quantitation. First of all only one concentration of drug is tested. It is conceivable, that lower concentrations might indicate more subtle differences in sensitivity or resistance between mutants and wild-type. The second problem is that the wild-type does not form plaques in the presence of GCV. At higher dilutions of the stock (above 1×10^{-3} , MOI=0.01) only enlarged single cells appear. These cells probably represent abortive infection, therefore, the amount of infectious virus produced, if any, is much lower than that of plaques produced in the absence of drug. The determination of the titer in the presence of GCV, which is measured by the highest dilution with only single cells, is, therefore, overestimated.

Plaque reduction assays for quantitation of drug resistance are performed at a single dilution of virus. This dilution is usually selected such that 30-50 pfu are inoculated onto a monolayer in a 24-well plate. Inoculation of this amount of virus represents a relatively low MOI. A range of drug concentrations can, therefore, be tested for inhibition of plaque formation at a single MOI. This avoids one of the

problems with the plaque dilution method. The results of the plaque reduction assays confirmed the pattern of sensitivity of mutants and wild-type determined by plaque dilution. No differences were detected among the mutants, and the resistance to GCV and hypersensitivity to AraA relative to the wild-type were reproducible. Mutant D6/3/1 was further tested for susceptibility to AraT, FIAC and FIAU by plaque reduction assay. This mutant was found to be slightly hypersensitive to all three drugs. This is somewhat unexpected, because although AraA is probably phosphorylated by the cellular TK (93), AraT, FIAC and FIAU at least in HSV-infected cells are phosphorylated by the viral TK.

The plaque reduction assays still have a problem similar to one associated with plaque dilution, namely the fact that the wild-type virus does not form plaques in the presence of GCV. In order to better quantitate the sensitivity of the wild-type virus to this agent an ELISA was developed.

The ELISA detects a late HCMV antigen which requires viral DNA replication for its production (142). Since GCV inhibits DNA replication, synthesis of this antigen is especially sensitive to the effects of the drug. The measurement of the reduction in optical density with increasing concentrations of GCV is proportional to the amount of virus produced in each infected monolayer. This is a much better method for determining actual virus replication beyond abortive infection. The results of the ELISA confirmed the level of sensitivity of the wild-type to GCV previously determined by plaque dilution and plaque reduction. The high-level resistance to GCV and slight hypersensitivity to AraA of D6/3/1 were

also confirmed.

There was no evidence for cross-resistance of the mutants to any of the drugs dependent only on polymerase activity (PAA, PFA, or Aph), but there still could be a mutation in the polymerase gene which did not affect the interaction of the enzyme with these three antiviral agents.

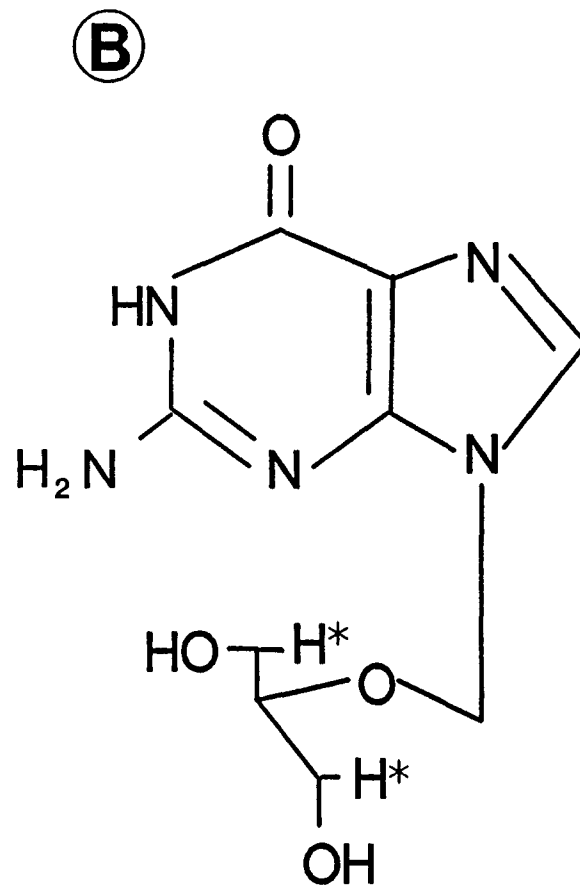
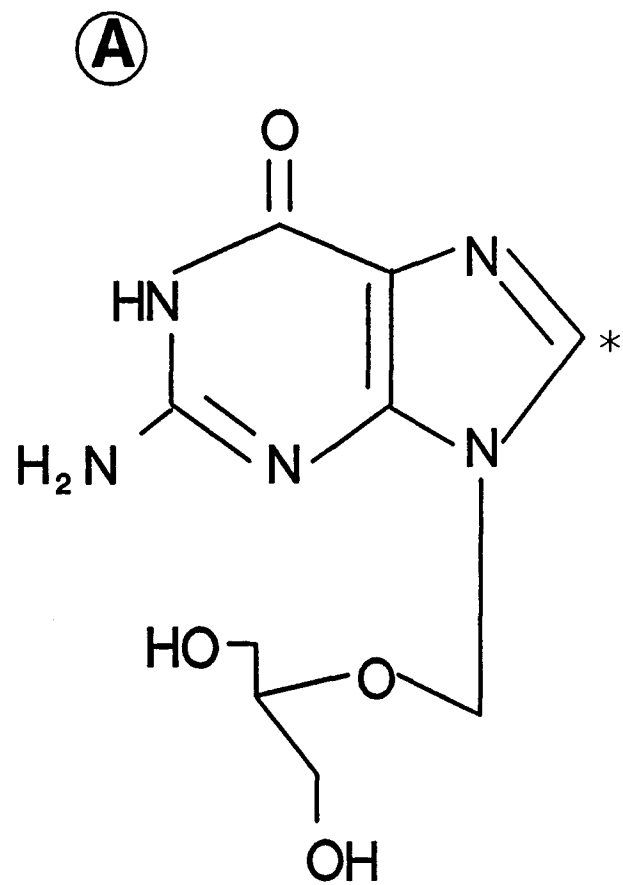
In order to determine whether these mutants were phenotypically similar to the one described by Biron et al.(4), HPLC analysis of GCV anabolism within cells infected with either mutants or wild-type AD169 was performed. A reversed phase system was developed to distinguish GCV from its phosphorylated derivatives. This was different from the ion exchange HPLC method of Biron and co-workers (4), but the results were highly reproducible. All three mutants were remarkably deficient in the ability to phosphorylate GCV. The lack of activation of GCV within mutant-infected cells could account for the increased resistance, but there could be another mutation contributing to the high-level resistance, which is phenotypically masked by the lack of kinase activity.

GCV anabolism was also studied in co-infected cells. It might be expected that the wild-type enzyme would be dominant over the mutant, therefore, in co-infected cells the wild-type enzyme would be functional and GCV would be phosphorylated. The amount of phosphorylation of GCV in co-infected cells was approximately 10% of that of wild-type-infected cells at the same MOI. These results suggest that the mutant enzyme competes for the GCV substrate, or that the enzyme is a multimer. In the latter case mutant subunits mixed with wild-type could cause loss of function for the complete multimer. The mechanism of the interaction of

wild-type and mutant enzyme activities, however, cannot be determined from these experiments.

An experiment which would be helpful in studying enzyme activity in co-infected cells, would be to vary the relative amounts of each virus for co-infection at the same total MOI. Such an experiment was attempted, but the only radiolabeled-GCV which was available was the ^{14}C -GCV received from Burroughs-Wellcome. The specific activity of this labeled drug was 500 mCi/mmol) while the specific activity of the ^3H -GCV from Syntex was three times higher. The number of counts of the GCV taken up by the infected cells from the medium was not high enough to produce significantly increased peaks of phosphorylated derivatives from even wild-type-infected cells. One other problem with this label is that the ^{14}C is in the guanine portion of the molecule (Figure 24A). The bond between guanine and the acyclic ring is somewhat labile, therefore when it is broken, labeled guanine remains, which can be phosphorylated by cellular kinases. These kinases are present in both mutant and wild-type-infected cells, and the reversed phase HPLC system cannot distinguish the phosphorylation products of GCV and guanine. There were similar peaks of less than 100 cpm in control and co-infected cells when the co-infection experiment was performed with ^{14}C -GCV. From Figure 24B it is apparent that the lability of the bond between guanine and the acyclic ring is not a problem with the ^3H -GCV, because the label is in the acyclic ring. If the bond is broken the remaining guanine is not labeled. The labeled acyclic ring apparently does not co-migrate with any GCV anabolic products, because mutant and wild-type HPLC profiles are dramatically different. Unfortunately the

Figure 24. The structure of GCV from different sources with the position of the label indicated (asterisk). (A) ^{14}C -GCV (Burroughs-Wellcome); label on C8. (B) ^3H -GCV (Syntex); label on two hydrogens of acyclic ring.



only source of radiolabeled GCV is as a gift of the pharmaceutical companies which developed the drug, and the higher-specific activity tritiated compound is no longer available.

Strategies for mapping the GCV kinase activity. The HPLC analysis of the mutants, however, did at least show that a kinase activity, whether virally encoded or virally modified, was greatly reduced. Three different approaches were taken to try to map the mutation: 1) in vitro translation and expression of kinase activity; 2) transfer of resistance from a mutant cosmid library to wild-type AD169; 3) 2-dimensional gel electrophoresis analysis of mutant and wild-type infected cell proteins.

The experimental design for the in vitro translation approach to mapping the kinase function was based on the HSV-1 TK experiments performed by Preston (124) in which in vitro translated infected cell RNA produced an active TK product. In addition some experiments were based on methods used by Biron and co-workers (5) to try to express HCMV "GCV kinase" activity from cytosolic extracts of wild-type infected cells.

If wild-type-infected cell mRNA could be translated and expressed in vitro, individual cosmid clones of the HCMV genome could be used to arrest the translation and expression of the enzyme activity. In this manner the translation product could be mapped to a specific cosmid. More specific mapping within the cosmid region could be achieved by the same type of experiment using subclones of the cosmid to arrest translation.

The optimal conditions for the activity of the HCMV enzyme are entirely unknown, therefore, in order to try to develop an in vitro expression system similar to that of the HSV-1 TK, two different sets of

experimental conditions were used (Figure 21). For the first set of experiments the pH was 7.5 and the period of time allowed for expression was 5 hours. The pH was later changed to 6.1 and the expression time was either 5 hours or 11 hours. There were never any measurable peaks of phosphorylated GCV anabolic products after HPLC analysis of the expression reactions. The most likely explanations for the negative results are 1) the optimal reaction conditions for the kinase activity differ significantly from those of the HSV-1 TK, and therefore, were not met by the *in vitro* expression system; 2) the mRNA which was isolated at 72 h post-infection did not represent the peak of temporal transcription of kinase message, therefore there was insufficient message present for translation of the kinase product; 3) the specific activity of the labeled GCV substrate was not high enough to detect the phosphorylated products. Any combination of these explanations could also be responsible for the negative findings. In support of the last possibility, it was shown that the expression of the HSV-1 TK required labeled substrate of very high specific activity (124). The highest specific activity of labeled GCV that was available was approximately 30-fold lower than that of the HSV-1 TK substrate.

Another approach to mapping the kinase function was transfer of the resistance from mutant to wild-type virus by specific cosmid-cloned regions of the mutant genome. A cosmid library of the mutant D6/3/1 was made. The wild-type genome was represented by either purified extracellular virion DNA or infectious virus. The large size of the HCMV DNA genome makes it difficult to transfect intact. An additional problem

with transfection of the DNA is that HFF take up exogenous DNA much less readily than do many cell lines, and unfortunately there is no cell line which is permissive for HCMV replication. Three different methods of transfecting HFF with a marker plasmid (cosmid pWE15 which encodes neomycin resistance) were tested: 1) calcium phosphate precipitation; 2) electroporation; and 3) liposome-mediated transfer with Lipofectin (BRL). Transfection was measured by resistance of cells to the neomycin analogue G418. The only method which was successful was the third method.

In order to try to transfer resistance from mutant to wild-type virus different combinations of transfection order and form of the wild-type genome were used. In some experiments both mutant cosmid and wild-type naked DNA were co-transfected. In other experiments the wild-type genome was in the form of infectious virus. HFF were either infected first and then transfected with individual mutant cosmids (46) or the order of transfection and infection was reversed (157). No recombinant virus was isolated from any of these experiments. The technical difficulty of transfection of DNA into fibroblasts may have been the major reason for the lack of detectable recombination. It is also possible that the high-level resistance of the mutant to GCV is the result of more than one mutation. If this is the case, marker transfer of the GCV resistance with a single cosmid may not be possible.

The third approach which was taken to map the kinase activity was analysis of viral proteins by 2-dimensional gel electrophoresis. The initial strategy was to compare in vitro translation products from mRNA isolated from mutant D6/3/1 and wild-type AD169. If differences in

protein products could be detected, it might be possible to arrest translation of particular proteins with cosmid DNA. The disappearance of specific proteins from the 2-dimensional gels could serve to map those proteins to a 30 to 40 kb region of the genome.

No reproducible differences in in vitro translated protein products of the mutant and wild-type were observed. It is possible that the mRNA isolated at 72 h post-infection, which was used for the translation reaction, did not contain sufficient message to produce a detectable protein. The reason that the mRNA was isolated at 72 h post-infection was that the HPLC analysis of GCV anabolism indicated that the enzyme activity was present between 72 and 96 h post-infection. It could be that the protein is stable, but the message is not, therefore mRNA isolated at 24-48 h post-infection might represent the peak of kinase mRNA production.

Proteins extracted from cells infected with virus were also analyzed by 2-dimensional gel electrophoresis. A reproducible protein difference between the two extracts was found by this analysis. A protein is present in wild-type-infected cells and missing in mutant D6/3/1-infected cells (Figure 23). It is also missing in D1/3/4-infected cells and uninfected cell controls (data not shown). Interestingly the approximate molecular weight of the protein appears to be 43,000, which is very close in size to that of the monomers of HSV-1 TK (136). It is possible that the protein may be an induced or modified cellular protein, and that similarity to the size of the HSV-1 TK is purely coincidental. The fact that the protein is not present in either uninfected or mutant-infected cells, however, strongly suggests that it is associated with GCV kinase

activity. Further evidence of the specific function of the protein will be required to confirm this association.

Since the most promising experimental results were obtained with the 2-dimensional gel analysis of infected cell proteins, a logical next step would be to extract the protein from the gel and to try to determine a partial amino acid sequence. The amino acid sequence could then be used to map the coding region on the HCMV genome. If the coding sequence of the protein is found to be part of the viral genome, there are several possible approaches to determine its function and its role, if any, in GCV anabolism.

The wild-type gene could be used as a probe to identify the corresponding mutant DNA sequences. The availability of both mutant and wild-type genes would permit DNA sequencing, which could determine if there is indeed a difference in this region of the genomes of the two virus strains. Finding a change at the DNA level, however, still does not link this region with GCV kinase function.

The gene from the mutant, however, could be used for marker transfer studies to see if recombinant virus resistant to GCV can be detected. This may not work, however, if more than one mutation is required for high-level GCV resistance.

A more direct approach to determine function would be to clone the gene or cDNA in an expression vector. Selection of the best vector and cell system would require some preliminary testing. It might then be possible to detect kinase activity in cell lines which are much easier to work with than HFF.

HCMV is the only member of the human herpesviruses for which no TK gene has been found. The GCV kinase activity defined by these mutants is the closest HCMV viral function to that of the HSV-1 TK, which has been characterized to date. Since GCV is the most effective antiviral agent for HCMV available at the present time, it will be important to characterize the kinase activity which is responsible for HCMV resistance.

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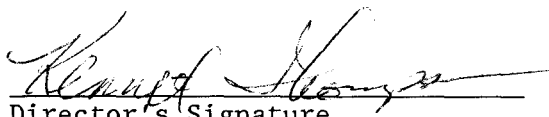
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The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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