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Edited by

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Preface

Human cytomegalovirus is an ubiquitous herpesvirus that rarely causes symptomatic disease in immunocompetent individuals. However, it is a major cause of disease in the immunocompromised, particularly those undergoing solid organ or bone marrow transplants, and infection *in utero* is a major cause of congenital malformation, often resulting in deafness and mental retardation. Clinically, then, cytomeglovirus is a major problem and an effective antiviral therapy is still in its infancy.

As well as being a major medical problem and an important target for antiviral research, cytomegalovirus also represents a viral system that is inherently scientifically interesting. As with all herpesviruses, cytomegalovirus persists in the host for life after primary infection. At least one site of carriage of the virus in the healthy seropositive individual is the peripheral blood monocyte, where the virus remains latent, but terminal differentiation of monocytes in vivo, to tissue macrophages, probably results in sporadic local reactivation in the healthy carrier. The mechanism by which latency and reactivation is controlled in these latently infected cells and just how the virus persists in the face of the host immune response are major areas of interest in cytomegalovirus research.

As with a number of other viruses, once productive infection occurs, cytomegalovirus "hijacks" many normal cellular control mechanisms in order to optimize the cell for viral DNA replication. The mechanisms by which HCMV modifies normal cellular gene expression are also of great current interest. It is clear that the virus has evolved many gene functions that target cellular transcription and DNA replication and these often involve viral proteins that act as transcriptional regulators of cellular genes, *per se*, but also act by interacting directly with cellular proteins by modifying their function.

The early chapters in *Cytomegalovirus Protocols* cover the culture and detection of cytomegalovirus using both immunological and biological techniques. The next four chapters describe techniques that analyze fundamental aspects of the infection cycle—binding and entry of the virus, transcriptional control of viral genes, and regulation of viral and cellular gene expression by direct DNA/protein or protein/protein interactions.

Cytomegalovirus infection is maintained in the infected host despite strong immune surveillance. Chapter 8 details the analysis of the T cell response to cytomegalovirus infection in the human host and the following chapter describes current methods in anticytomegalovirus research.

Much of our understanding of the molecular biology of cytomegalovirus has, to date, depended on the analysis of specific viral genes in isolation. The final chapter of the book describes techniques to generate cytomegalovirus recombinants. Such techniques will allow the generation of specific viral gene deletions and mutations, which are essential tools for investigating the structure and function of viral genes in the context of the whole viral genome.

The aim of the various chapters in *Cytomegalovirus Protocols* is to provide complete protocols in certain techniques that have already and will in the future help us further understand the biology and pathogenesis of this complex and intriguing human herpesvirus.

John Sinclair

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Immunological Methods for the Detection of Human Cytomegalovirus

Andrew D. Yurochko and Eng-Shang Huang

1. Introduction

Human cytomegalovirus (HCMV), a double-stranded DNA virus in the herpesvirus family, is a ubiquitous virus that infects greater than 40–60% of the general population and up to 100% within some subpopulations and/or geographic areas ***(1)***. HCMV has a complex pathobiology because infection of immunocompetent individuals is rarely associated with severe clinical symptoms and in most cases is simply asymptomatic, whereas HCMV infections can cause a wide range of severe diseases, including mononucleosis, mental retardation, deafness, chorioretinitis, and fatal diseases, such as interstitial pneumonia and disseminated virus infections in immunocompromised hosts ***(1)***. As with other herpesviruses, HCMV is thought to establish latent or persistent infections. Reactivation of this infection is frequently encountered during pregnancy and in organ transplant and acquired immune deficiency syndrome (AIDS) patients ***(1)***. In addition, HCMV has been implicated as a co-etiological agent in cervical cancer ***(2)*** and has been found associated with a wide range of other tumors ***(1)***. More recently, HCMV has also been shown to be epidemiologically linked to restenosis ***(3–5)*** and atherosclerosis ***(5,6)***. The severity of these HCMV-associated

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diseases warrants an accurate ability to detect and diagnose persons with HCMV, especially because of the clinical availability of the anti-HCMV agents, ganciclovir and foscarnet, which have been used successfully to treat patients with HCMV viremia. Early and accurate detection is needed especially in cases in which infected individuals are at risk of complications arising from HCMV infection; for example, in patients undergoing organ transplants or angioplasty, expectant mothers, or AIDS patients.

The detection or diagnosis of HCMV infection in the laboratory can be achieved by a number of different approaches, including both classical methods (i.e., virus isolation, some serologic tests, and the search for cytomegalic inclusions in histological sections) and more modern methods (i.e., Western blot analysis, enzyme linked immunosorbent assay [ELISA], nucleic acid hybridization, and the polymerase chain reaction [PCR]). The appropriate method to use depends on the type of technique used to collect the specimens and the type of answer that is desired. Because of limitations, we discuss only the modern immunological techniques for detecting HCMV in the laboratory setting. The focus of this discussion is the common immunological methods for the detection of HCMV, including ELISA, immunohistology (immunofluorescence and immunoperoxidase), and Western blot analysis as it pertains to the use of immunological reagents, antibodies. Since other methods exist ***(7–10)***, we also included a brief synopsis of these additional methods and provided some references for the reader's convenience to allow for a more focused understanding of these additional techniques.

2. Materials

2.1. ELISA

1. As a source of HCMV antigen and control extracts, $2 \times 850\text{-cm}^2$ roller-bottle (Corning, Corning, NY) cultures of human fibroblasts are needed: (1) a roller bottle with 100% of the cells showing cytopathic effects (CPE), and (2) a roller bottle of uninfected cells as a control.
2. 0.15 *M* glycine buffer (pH 9.0), 100 mL is needed.
3. Microtiter plates (Nunc, Corning, or Falcon Elisa Plates [Fisher]).

4. 1–2 L of phosphate-buffered saline (PBS).
5. Blocking solution: 0.1 *M* Tris-HCl (Boehringer, Mannheim, Indianapolis, IN; pH 8.0), 2% protease free bovine serum albumin (BSA; Boehringer Mannheim) and 0.1% thimersol (Sigma, St. Louis, MO; ~50 mL is needed).
6. Human serum sample(s).
7. 100 mL of PBS containing 0.1% Triton X-100 (Sigma).
8. Alkaline phosphatase (AP)-conjugated or horseradish peroxidase (HRP)-conjugated anti-human IgG or IgM secondary antibody (i.e., Sigma, Santa Cruz, Santa Cruz, CA, or Vector Laboratories, Burlingame, CA). *See* **Note 1** for additional comments.
9. The colorimetric substrate (*see* **Note 2**).
   a. For AP-conjugated secondary antibodies, the substrate is *p*-nitrophenyl phosphate (NPP; Sigma) — a solution containing 3 m*M* NPP, 0.05 *M* Na_2CO_3 (Mallinckrodt, Paris, KY), and 0.05 m*M* $MgCl_2$ diluted in dH_2O is needed (10 mL is needed for each 96-well microtiter plate used — this solution should be stored at 4°C).
   b. For HRP-conjugated secondary antibodies, the substrate is 3,3',5,5'-tetramethylbenzidine (TMB) — two solutions should be made:

      Solution A (urea hydrogen peroxide; Sigma): 0.054 g urea hydrogen peroxide, diluted in 100 mL of 0.1 *M* sodium citrate (adjusted to pH 5.0 with H_3PO_4; Fisher)

      Solution B (the colorimetric substrate): 30 mg of TMB, dissolved in 1 mL of DMSO and then diluted to a final concentration of 0.3 mg/mL in a 100-mL solution containing 10% glycerol (10 mL), 30% methanol (30 mL), and dH_2O (59 mL). This solution should be stored in the dark.
10. 0.5 *M* Ethylenedinitrilotetraacetic acid (EDTA).
11. ELISA plate reader.
12. These reagents are generally very stabile and can be stored for months.

2.2. Immunofluorescence and Immunoperoxidase Assays

2.2.1. IFA

1. A freshly prepared paraformaldehyde solution containing 3% paraformaldehyde (3 g), 2% sucrose (2 g) in 100 mL of PBS. The paraformaldehyde solution must be neutralized with 2 N NaOH to a

pH 7.0 and heated to 60°C to allow it to go into solution. Care should be exerci Èd when heating this product because formaldehyde is a carcinogen (this step should be performed in a chemical hood).
2. Wash buffer (PBS).
3. 10% normal goat serum (Accurate Chemical and Scientific Corporation, Westbury, NY; bovine serum albumin [BSA] or the serum from the species of animal in which the primary or secondary antibody was prepared) diluted in PBS.
4. Permeabilization buffer (0.5% Triton X-100 and 300 m*M* sucrose diluted in PBS). Make up 100 mL of this solution and store at 4°C.
5. An appropriate primary antibody targeting the HCMV gene product of interest; some are available commercially (e.g., Vancouver Biotech, British Columbia, Canada; Rumbaugh-Goodwin Institute for Cancer Research, Plantation, FL; Dupont, Boston, MA), but most have been developed in the laboratories of individual researchers (*see* **Note 1**).
6. The appropriate fluorescently-conjugated (usually fluorescein isothiocyanate [FITC]) secondary antibody (e.g., Sigma, Vector Laboratories, etc; *see* **Notes 3** and **4**).
7. 50% glycerol (Fisher) in PBS.
8. A fluorescent microscope (*see* **Note 4**).

2.2.2. IPA

1. PBS.
2. A primary antibody targeting the protein of interest (*see* **Note 1**).
3. The appropriate HRP-labeled secondary antibody (*see* **Note 3**).
4. 3,3'-diaminobenzidine tetrahydrochloride dihydrate (DAB)—a solution of 0.05% DAB (5 mg DAB in 10 mL of PBS) should be prepared fresh (*see* **Note 5**).
5. 30% hydrogen peroxide (Fisher, Pittsburgh, PA).
6. 0.05 *M* EDTA in PBS.
7. A microscope with the required magnification.

2.3. Western Blot Analysis

1. Harvested infected cell lysates in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer *(7)*.
2. Gel electrophoresis and transfer equipment.

3. Nitrocellulose (Immobilon-P; Millipore, Bedford, MA).
4. Ponceau S (0.5 g of Ponceau S is first dissolved in 1 mL of acetic acid and then diluted in 100 mL of dH_2O).
5. Blocking solution (5% skim milk (Carnation nonfat dry milk) and 0.1% Tween-20 diluted in PBS), around 100 mL of blocking solution is needed for each mini-gel performed.
6. Primary anti-HCMV antibodies. There are many commercially available antibodies (Vancouver Biotech., Rumbaugh-Goodwin Institute for Cancer Research, Dupont), as well as a large number of antibodies that have been described in the literature (*see* **Note 1**).
7. 0.5–1 L of washing solution (PBS containing 0.1% Tween-20).
8. HRP-conjugated secondary antibody.
9. The enhanced chemiluminescence kit (ECL) (Amersham Life Sciences or equivalent system) or a colorimetric substrate such as TMB or DAB (*see* **Notes 2** and **5**).

3. Methods

3.1. Detection of Infectious Virus — Virus Isolation

During active infection, HCMV can be found in most tissues and organs, as well as in most bodily fluids (urine, blood, semen, milk, stool, saliva, cervical discharges). However, during in vitro cell culture, HCMV usually replicates only in human fibroblasts (e.g., HEL, WI–38, MRC-5, from American Type Culture Collection). Thus, any attempt to isolate infectious virus from a patient and subsequent culture in vitro, must be performed in the various available human fibroblast lines. Briefly, this simple yet descriptive technique involves the separation or isolation of infectious virus from the various patient samples, infection of human fibroblast cells by these viral isolates, and then the examination 3–4 d postinfection for the typical HCMV induced CPE in these cells (*see* **refs. *8–10*** for more comprehensive details about this technique).

3.2. Serologic and Immunologic Tests for CMV Infections

The diagnosis of HCMV infection can also be rapid and accurately achieved by examining either the patients serum for the pres-

ence of specific antibodies or clinical specimens for the presence of viral antigens. The various tests that can be used to accomplish this task include the complement fixation test, the viral neutralization test, and the ELISA. These techniques have been commonly used for years to measure serum anti-HCMV titers. In addition, several additional procedures can be performed to detect the presence of HCMV and/or specific HCMV antigens in various cell types infectable by HCMV. These procedures include: the IFA, the IPA, and Western blot analysis. This chapter discusses only the more modern immunological tests, the ELISA, IFA and IPA, and Western blot analysis in detail.

3.2.1. Complement Fixation Test

The complement fixation test uses hemolysin-sensitized sheep red blood cells (RBCs) to measure the amount of complement bound by specific antigen–antibody complexes in a defined assay condition ***(8–11)***. The theory behind this technique is simple: if a specific HCMV antigen–antibody reaction occurs, the complement in the serum will bind to the complex and thus not lyse the sensitized RBCs; if no antigen–antibody complex is formed, lysis of the sensitized RBCs will occur. This method was used to measure antibody titers using defined HCMV antigen, or to detect the existence of HCMV antigen using anti-HCMV antibodies, but is now generally outdated because of the use of the newer and more sensitive techniques (ELISAs and Western blot analyses).

3.2.2. ELISA

ELISAs are a very sensitive, straightforward, easy-to-perform assay for detecting either specific antibodies or antigens in human serum. Another advantage of this technique is that no radioactive materials are used and most of the reagents are stable at 4°C for months. This assay is a very valuable tool for the semiquantitative detection of HCMV-specific IgG and IgM titers in the serum of infected patients and for the detection of HCMV antigens in the serum or other body fluids. This assay has been used for the detection of specific antibodies against HCMV in nearly all types of

patients (e.g., AIDS patients, organ transplant patients, expectant mothers, neonates). The theory behind this technique involves the unique and specific association of antigens and antibodies. In the procedure described below, which is an indirect ELISA, microtiter plates are first coated with HCMV antigens and then incubated with the patient's serum. Other procedures are described in detail elsewhere *(7)*. Patients who are seropositive will possess anti-HCMV specific antibodies that will bind to the HCMV antigens coating the wells. Next, the plates are incubated with an enzymatically labeled secondary antibody that is targeted against human immunoglobulins. Finally, a colorimetric substrate is added that changes color as it is hydrolyzed by the enzyme conjugated to the secondary antibody, thus allowing for the quantitation of the levels of anti-HCMV antibodies in the serum. For measuring HCMV-specific IgG or IgM titers in serum samples, the procedure is as follows (*see* **Note 6** for potential problems):

1. The HCMV-specific antigens that are used for coating the wells are harvested from the infected fibroblasts (100% of the cells should have developed CPE) and the control extract is harvested from the uninfected control cultures (*see* **Note 7**). First the roller-bottle cultures are gently washed (2 times) with PBS, once all the PBS has been removed, 10 mL of a 0.15 *M* glycine buffer is added, the cells are carefully removed with a cell scraper and placed in a 15-mL tube. Purified overexpressed HCMV-specific antigens such as the immediate-early gene products, viral membrane glycoproteins, or other viral products that elicit an immune response can also be used (if these products are used, skip to **step 3**).
2. The cells are lysed by sonication and centrifuged at 12,000*g* for 10 min to remove the insoluble cellular debris. The supernatant is the source of the soluble HCMV antigens. These soluble antigens can be stored at –70°C for years.
3. The infected cell extracts, control extracts, and/or purified proteins are then diluted in PBS to a final concentration of approx 100 µg/mL and then added to the microtiter plates at 100 µL per well and incubated overnight at 4°C with constant agitation.
4. The plates are washed three times in PBS after the overnight absorption (*see* **Note 8**).

5. 300 µL of the blocking solution is added to the wells and incubated for 1 h at room temperature with frequent rocking.
6. The blocking solution is then removed and the coated wells are washed three times with PBS. The plates can be stored for weeks at this step by drying the plates under vacuum, sealing them in a plastic bag with silica dry gel, and storing them at 4°C.
7. 100 µL of the diluted patients serum sample (dilute in PBS to 1:10–1:500; *see* **Note 9** for additional comments) is added to the antigen-coated wells and allowed to react at 37°C for 1–2 h or at 4°C overnight. If the nonspecific binding of the patients serum to the control wells is high, the serum should be diluted in the blocking solution instead of PBS.
8. Plates are then washed with PBS (containing 0.1% Triton X-100) three times and air dried for several minutes.
9. 100 µL of an AP- or HRP-conjugated anti-human IgG or IgM secondary antibody diluted in PBS (1/500 to 1/10,000 depending on the affinity of this antibody–this concentration will have to be determined for each lot of secondary antibody used) is added next to each well and incubated at room temperature or 37°C for 1–2 h (if the signal is weak, the length of incubation can be increased to several hours or overnight) with gentle agitation. If the nonspecific binding of this secondary antibody to the control wells is significantly high, the antibody should be diluted in the blocking solution instead of PBS.
10. Wash the wells at least five times with PBS.
11. Add the colorimetric substrate (100 µL of NPP for AP-conjugated or 100 µL of an equal parts mixture of TMB solution A and B for HRP-conjugated secondary antibodies; *see* **Note 2**) to each well and incubate with gentle agitation for 10–30 min at room temperature (longer times can be used if the reaction is weak, e.g., ≥1 h]). As the reaction of the enzyme with substrate proceeds, a yellow color (NPP) or blue color (TMB) begins to appear.
12. Add 50 µL of EDTA (0.5 *M*) to the wells to stop the reaction; then quantitate or read the results on an ELISA reader set to a wavelength of 405 nm for NPP or 655 nm for TMB. If only a positive/negative (yes or no) answer is desired, the results of the colorimetric reaction can be recorded by visual inspection.

3.2.3. IFAs and IPAs

IFAs and IPAs have been successfully used in detecting virus-specific antigens and antibodies in HCMV-infected cells. The prin-

ciples of these techniques are identical except that different labeling materials (fluorescent tag vs enzymatic tag) are used on the primary or secondary antibodies to trace the specific reactions. IFAs and IPAs can be categorized into direct and indirect methods based on whether the primary or the secondary antibody is labeled. FITC is one of the most common fluorescent tags (*see* **Note 4**). Others include tetramethylrhodamine isothiocyanate/Texas red and R-phycoerythrin ***(12–14)***. The common enzymatic conjugates are HRP or AP.

3.2.3.1. IFA

The procedure for an IFA is as follows.

1. Frozen tissue sections of 5-µm thickness, exfoliated cells, or infected cell cultures, on either chamber slides or coverslips cultured by the shell culture method (Viromedical Laboratories, Hopkins, MN; ***15***), are first fixed in freshly prepared paraformaldehyde solution for 10–20 min at room temperature. In all cases, negative control cells or frozen sections should be used and treated identically to the infected cells to determine the level of background staining by the primary or secondary antibodies, or both. Note that for this step and all the subsequent incubation or wash steps involving the various solutions, it is easiest to immerse the slides in a glass staining dish (Fisher, Wheaton), although the various solutions can be carefully placed on the slides covering the entire sample.
2. Wash the cells two times with the PBS wash buffer.
3. Depending on whether the viral protein of interest is an external (membrane) protein or an internal protein, cells might have to be treated with a permeabilization buffer. For the detection of internal viral (or cellular) proteins, the cells need to be treated for 5 min with an ice-cold permeabilization buffer.
4. Wash the slides two times with the wash buffer.
5. Block the cells with a 10% solution of normal goat serum (BSA or serum from the species of animal that the primary or secondary antibody was raised) diluted in PBS for 15 min on ice.
6. Apply the properly diluted primary antibody (dilute the antibody in PBS, usually 1:10–1:1000; the dilution will have to be determined, based on the particular level of background staining for each antibody), which is targeted against the HCMV antigen of interest (*see* **Note 1**), for 1 h at either 37°C or room temperature. The primary and

secondary (*see* **step 8**) antibody incubation steps are most easily performed by placing a sufficient amount of diluted antibody on the slide (cover the entire sample) and then incubating them in a humid chamber (e.g., a 150-mm tissue culture dish in which a moist paper towel is placed on the bottom and several wooden applicator sticks are used to keep the slides off the wet paper towel). If the background staining of this primary antibody is determined to be high, the antibody can be diluted in a PBS/0.5% serum blocking solution (*see* **Note 10**).

7. Wash extensively with PBS (3–5 times, 5 min each).
8. Add the fluorescently labeled secondary antibody to the slides. The antibody should be diluted in PBS; the precise dilution will have to be determined by examining the signal to noise ratio. If the background staining of the secondary antibody is determined to be high, the antibody can be diluted in a PBS/0.5% blocking serum solution (*see* **Note 10**).
9. Slides are then mounted with coverslides with 50% glycerol in PBS and analyzed by fluorescent microscopy.

3.2.3.2. IPA

In the IPA, the antibody is labeled with an enzyme, HRP, instead of a fluorescent tag. These products are available commercially or can be labeled in the laboratory setting *(7)*. The procedure for an IPA is identical to that of the IFA except that the final step involves a colorimetric reaction due to the use of a secondary antibody that has an enzymatic tag instead of a fluorescent tag.

1. The most commonly used reagent for detection of a HRP-conjugated antibodies in slide preparations (i.e., IPA) is DAB. Freshly prepared 0.05% DAB (5 mg DAB in 10 mL of PBS) is mixed together with 10 µL of 30% hydrogen peroxide and applied to the fixed specimens on the slides. Cleavage of the DAB substrate by HRP yields a brownish precipitate that is insoluble in alcohol, xylene, and water (*see* **Note 5** for special considerations when using DAB).
2. The reaction can be stopped after 5 min with 0.05 *M* EDTA in PBS.

3.2.4. Anticomplement Immunofluorescent Test

Besides using the IFA discussed above, an anti-complement immunofluorescent test has been used in the past to detect HCMV in

infected tissues ***(11)***. This test is based on the ability of a specific antigen/antibody reaction to fix a component of the complement cascade. Using FITC-conjugated anti-complement serum, viral antigens can be indirectly demonstrated. This test can detect both the IgM- and IgG-mediated antigen–antibody reactions that fix complement.

3.2.5. Western Blot Analysis

The detection of specific HCMV antigens can also be accomplished by gel electrophoresis and Western blot analysis. This is usually a very specific and sensitive assay that excels in its ability to detect specific proteins of interest. (For further details of this technique, *see* **ref. *7***.) Briefly, this technique involves the resolution of different protein species by molecular weight and then the transfer of these proteins to a solid matrix such as nitrocellulose. Then, in a principle analogous to the indirect ELISA, a primary antibody is incubated first, followed by incubation of a secondary HRP- or AP-conjugated antibody and then developed using a colorimetric reaction in which the specific proteins/antigens of interest are identified by a band on the nitrocellulose matrix. We have only included a brief description of this technique because of its use of immunological reagents and its similarity in principle to the other techniques. To perform a Western blot, use the following procedure:

1. Infected cell lysates or clinically infected tissue for gel electrophoresis and Western blot analysis are harvested in a SDS-PAGE sample buffer, boiled, and then either used immediately or stored at –20°C.
2. Samples are electrophoresed on a 5–15% SDS-PAGE gel, depending on the molecular weight of the protein(s) of interest along with a molecular weight marker. Equal protein amounts are always added to each well. We have not included the detailed information about preparing a gel for electrophoresis or running a protein gel because of the space limitations within this chapter (for more information, *see* **ref. *7***). Instead, we have focused on the actual Western blot protocol.
3. The proteins are transferred to nitrocellulose (Immobilon-P; Millipore). The time used to transfer the proteins varies on the size of the gel, the speed with which it is run, and the molecular weight of the proteins of

interest (smaller proteins transfer faster). For instance, it is possible to transfer nearly all of the proteins in a mini-gel to a nitrocellulose membrane at 14–20 V overnight or at 100 V for 1–1½ h.

4. For accurate measurement of the success of a protein transfer, the reversible protein stain Ponceau S can be used. The nitrocellulose blot(s) is incubated for 5 min at room temperature with enough Ponceau S to barely cover the surface of the nitrocellulose, and then carefully washed in dH_2O for 1–2 min. During this time, the background red color will slowly disappear leaving only the major protein species. At this point, the molecular-weight markers should be marked on the membrane with indelible ink; then, if needed, the membrane can be wrapped in plastic wrap and xeroxed for a permanent record of the protein loading for each lane (*see* **Note 11**).
5. Next, the blots are incubated in a blocking solution for 1 h.
6. The blots are then incubated with an anti-HCMV primary antibody, diluted 1/100–1/5000 in the blocking solution (this dilution will generally have to be determined for each antibody used) for 1 h at room temperature (this time can be increased, depending on the strength of the signal to as long as overnight) with gentle rocking.
7. The blots are washed three times for 10–15 min each in the washing solution with gentle rocking.
8. Incubate the blots for 1 h with the appropriately conjugated secondary antibody (we prefer an HRP-conjugated antibody) with gentle rocking.
9. Wash the blots 3–5 times in the washing solution for 10 min each and then two times in PBS with rocking.
10. The blots are then incubated with the developing agent and developed according to the Amersham Life Sciences ECL protocol (*see* **Note 12** for additional comments about the detection of the various proteins on the nitrocellulose blots).

3.3. Detection of HCMV-Specific DNA and RNA by Nucleic Acid Hybridization

A number of extremely useful nucleic acid hybridization techniques are available for the laboratory investigator. These techniques are very powerful tools in molecular biological research and have been used successfully in the diagnosis and detection of both viral DNA and RNA. These various methods also permit quantita-

tion of HCMV DNA or mRNA levels ***(7,16,17)***. Uses for nucleic acid hybridizations with nitrocellulose membranes as solid supports include Southern blot analysis, dot or slot-blot analysis, and Northern blot analysis. Because of the use of radioactive materials in these procedures, the use of these techniques is not always an option. However, with the recent advances in the development of nonradioactive DNA or RNA probes these techniques may become more popular tools in all areas of clinical virology.

3.4. Detection of HCMV Infection by PCR

The use of PCR offers two main advantages over many other techniques: sensitivity and speed. In this assay, a specific segment of DNA (i.e., cellular or viral) is amplified by a heat stable DNA-dependent DNA polymerase, such as *Taq* or Vent polymerase by using specific oligonucleotide primers that hybridize to the complementary DNA (cDNA) strands flanking the specific DNA fragment of interest, in this case a specific HCMV viral sequence (for a more complete introduction to PCR, *see* **ref.** ***7***). The amplified DNA fragment(s) can be amplified up to one million-fold and can be identified by agarose gel electrophoresis and, if needed by Southern blot analysis as well ***(7)***. PCR has been shown to detect HCMV in the urine of newborns, in the blood of AIDS patients, in organ transplant patients, in Kaposi's sarcoma specimens, in the lungs of patients with interstitial pneumonitis, in the arterial walls of patients suffering from atherosclerosis, and in cervicovaginal cells (*see* **ref.** ***8*** and the references within). The sensitivity of this assay has been estimated to be at the level of 1 viral genome per 40,000 + cells ***(18)***. The superb sensitivity and specificity of PCR assay make it one of the best methods in the clinical laboratory for detecting HCMV in a great variety of clinical samples.

3.5. Direct Microscopic Examination of Clinical Samples

HCMV-infected cells can be found in exfoliated cells from urine, bronchial lavage, saliva, vaginal discharge, milk, and other bodily fluids of CMV-infected individuals. With the various stains avail-

able, cytomegalic intranuclear inclusion-bearing cells are easily distinguished from normal cells under the light microscope (for additional information about this technique, *see* **refs. *8–10***), however, because of the morphological similarities between the herpes viruses, it is necessary to examine specimens in parallel with the other methods described in this chapter.

3.6. Conclusions

Numerous methods and approaches can be used to diagnose and detect HCMV infection; each has its own advantages. The optimal and appropriate method(s) to be used frequently depend(s) on the nature of the clinical sample collected and the clinical manifestation at the time a sample is collected. In general, virus isolation and immunocytochemistry are good choices for the defined and rapid diagnosis of HCMV infection in biopsied or autopsied specimens and exfoliated cells. The ELISA also offers rapid and accurate detection of HCMV infections when anti-HCMV antiserum is available; however, it may not always give accurate results when patients become immunotolerant to HCMV. Thus, it is always a good idea to use a combination of tests to detect HCMV infections. In addition, *in situ* hybridization can also be an effective tool for confirmation of HCMV infection. Nevertheless, it is important to determine the technique or combination of techniques that allows one to address the specific questions that need to be answered. Most of the techniques described above can be used very effectively in both clinical and molecular virology laboratories to detect and characterize the consequences of HCMV infection accurately.

4. Notes

1. It is important to realize that many of the antibodies that can be used in these various techniques are in many cases technique specific. For instance, an antibody that specifically recognizes an HCMV gene product in an ELISA might not work in a Western blot analysis or in the various immunohistochemical procedures (and vise versa), due to specific primary and tertiary structures that individual antibodies

recognize. This is an important point to realize and one that must be taken into account when performing these techniques.

2. A number of different colorimetric substrates can be used for each of the enzymatic tags. With the use of each substrate, along with its corresponding conjugated enzyme, there will be different incubation conditions, different colorimetric reactions resulting in different color changes after hydrolysis of the substrate, and lastly, different wavelength settings on the plate readers, which will have to be used to quantitate the results.
3. In addition to FITC, and the other fluorescent labels, primary or secondary antibodies can be conjugated with AP, HRP, biotin, or any number of other different biochemical reagents allowing for a large amount of flexibility in using and performing these assays. In addition, because of the potential use of multiple enzymatic, fluorescent, or other potential labels, multiple viral (and/or cellular) antigens can be examined at one time. If this is the case, one must clearly determine the appropriate concentrations of each reagent, as well as test must be clearly determined for the presence of background staining before performing the specific reactions with HCMV-infected cells.
4. The type of fluorescent label used dictates the stability of the fluorescent tag and the filters required for accurate visualization of the results.
5. Another commonly used reagent for the development of the colorimetric reaction in which a peroxidase-conjugated antibody is used is DAB. The cleavage of the DAB substrate by HRP yields a brownish precipitate. Because DAB is a potential carcinogen, it should be handled with great care.
6. Unfortunately, false-positive tests and nonspecific IgM activities can arise due to the presence of rheumatoid factors or antinuclear antibodies in the sera of infected cell patients ***(9,19,20)*** or because of the presence of a nonspecific IgM titer against HCMV that can be generated during Epstein-Barr virus and varicella-zoster virus infections ***(9,21,22)***. Therefore, additional tests such as the detection of viral antigen via Western blot analysis ***(7)***, viral DNA via Southern blot analysis ***(7)*** or PCR ***(7)***, or the isolation of virus in tissue culture ***(8–10)*** are also important in the diagnosis of active HCMV infections. Several approaches have been used to eliminate nonspecific IgM activity and to improve the sensitivity of detection of specific anti-HCMV IgM antibodies ***(23–26)***.
7. The cellular antigen control sample from the uninfected cells is used to determine the amount of background or nonspecific binding to essentially give a "zero" control value.

8. During the wash steps, it is easiest to use a plastic squirt bottle filled with PBS to wash the wells. To empty the wells, simply flick the plate while upside down into a sink or onto several paper towels.
9. Several different concentrations of the patients serum should be tested to permit accurate quantitation of the anti-HCMV titers.
10. In the direct or indirect IFAs, background fluorescence is usually seen due to the presence of Fc receptors on the cell surface. Fc receptors by nature are designed to react to the Fc portion of the antibody, thus potentially causing a heightened background fluorescence due to the nonspecific interaction of the cell with FITC- or enzyme-conjugated primary or secondary antibodies, in the case of these tests. To minimize this nonspecific reaction, the conjugated antibody should be diluted with a buffer containing normal goat serum or serum from the same animal species from which the specific antibody is derived.
11. In addition, ponceau S allows one to determine whether there was a complete transfer, if any problems developed during the transfer, and to visually determine whether equal amounts of protein were loaded in each lane. It is also important to point out that this protein stain does not impart specificity, nor is it particularly sensitive, but its strength comes from its reversibility (complete removal during the blocking step, preventing any interference with the antibody incubation steps).
12. A number of different reactions can be used to detect your protein(s) of interest. The ECL protocol is the easiest in our hands and the most sensitive and gives a permanent hard copy of the data. In addition, the substrate TMB can also be used with HRP-conjugated antibodies to produce a purplish precipitate on the membrane. If an AP-conjugated antibody is used, the colorimetric substrate complex of nitro blue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate is needed.

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References

1. Huang, E.-S. and Kowalik, T. F. (1993) The pathogenicity of human cytomegalovirus: an overview, in *Molecular Aspects of Human Cyto-*

megalovirus Diseases (Becker, Y., Darai, G., and Huang, E.-S., eds.), Springer-Verlag, Berlin, pp. 1–45.
2. Shen, C. Y., Ho, M. S., Chang, S. F., Yen, M. S., Ng, H. T., Huang, E.-S., and Wu, C. W. (1993) High rate of concurrent genital infections with human cytomegalovirus and human papillomaviruses in cervical cancer patients. *J. Infect. Disease* **168,** 449–452.
3. Speir, E., Modali, R., Huang, E.-S., Leon, M. B., Shawl, F., Finkel, T., and Epstein, S. E. (1994) Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. *Science* **265,** 391–394.
4. Zhou, Y. F., Leon, M. B., Waclawiw, M. A., Popma, J. J., Yu, Z. X., Finkel, T., and Epstein, S. E. (1996) Association between prior cytomegalovirus infection and the risk of restenosis after coronary atherectomy. *N. Engl. J. Med.* **335,** 624–630.
5. Epstein, S. E., Speir, E., Zhou, Y. F., Guetta, E., Leon, M., and Finkel, T. (1996) The role of infection in restenosis and atherosclerosis: focus on cytomegalovirus. *Lancet* **348,** s13–17.
6. Melnick, J. L., Adam, E., and DeBakey, M. E. (1993) Human cytomegalovirus and atherogenesis, in *Molecular Aspects of Human Cytomegalovirus Diseases* (Becker, Y., Darai, G., and Huang, E.-S., eds.), Springer-Verlag, Berlin, pp. 80–91.
7. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl K. (eds.) (1997) *Current Protocols in Molecular Biology* (Chanda, V. B., series ed.), John Wiley and Sons, Boston.
8. Huang, E.-S. and Kowalik, T. F. (1993) Diagnosis of human cytomegalovirus infection: laboratory approaches, in *Molecular Aspects of Human Cytomegalovirus Diseases* (Becker, Y., Darai, G., and Huang, E.-S., eds.), Springer-Verlag, Berlin, pp. 225–255.
9. Ho, M. (1991) *Human Cytomegalovirus: Biology and Infection*, 2nd ed., Plenum, New York, pp. 101–126.
10. Lennette, E. H. and Schmidt, N. J. (1979) *Diagnostic Procedures for Viral and Rickettsial Infection*, American Public Health Association, New York, pp. 399–439.
11. Huang, E.-S., Kilpatrick, B. A., Huang, Y. T., and Pagano, J. S. (1976) Detection of human cytomegalovirus and analysis of strain variation. *Yale J. Biol. Med.* **49,** 29–43.
12. Hijmans, W., Schuit, H. R. E., and Klein, F. (1969) An immunofluorescence procedure for the detection of intracellular immunoglobulins. *Clin. Exp. Immunol.* **4,** 457–472.

13. Goding, J. M. (1976) Conjugation of antibodies with fluorochromes: modification to the standard methods. *J. Immunol. Methods* **13,** 215–226.
14. Kronick, M. N. (1986) The use of phycobiliproteins as fluorescent labels in immunoassay. *J. Immunol. Methods* **92,** 1–13.
15. Gleaves, C. A., Smith, T. F., Shuster, E. A., and Pearson, G. R. (1984) Rapid detection of CMV in MRC-5 cells inoculated with urine specimens by using low speed centifugation and antibody to an early antigen. *J. Clin. Microbiol.* **19,** 917–919.
16. Huang, E.-S. and Pagano, J. S. (1977) Nucleic acid hybridization technology and detection of proviral genomes. *Methods Virol.* **12,** 1473–1481.
17. Huang, E.-S. and Pagano, J. S. (1977) Comparitive diagnosis of cytomegalovirus: new approaches, in *Human and Related Virus, Part A* (Kurstak, E. and Kurstak, C., eds.), Academic Press, San Diego, pp. 241–285.
18. Cassol, S. A., Poon, M. C., Pal, R., Naylor, M. J., Culver-James, J., Bowen, T. J., Russel, J. A., Krawetz, S. A., Pon, R. T., and Hoar, D. I. (1989) Primer-mediated enzymatic amplification of cytomegalovirus (CMV) DNA. Application to early diagnosis of CMV infection in marrow transplant recipients. *J. Clin. Invest.* **83,** 1109–1115.
19. Reimer, C. B., Black, C. M., Philips, D. J., Logan, L. C., Hunter, E. F., Pender, B. J., and McGrew, B. F. (1975) The specificity of fetal IgM: antibody or antiantibody? *Ann. NY Acad. Sci.* **254,** 77–93.
20. Cremer, N. E., Schmidt, N. J., Jensen, F., Hoffman, M., Osiro, L. S., and Lennette, E. H. (1975) Complement-fixing antibody in human sera reactive with viral and soluble antigens of cytomegalovirus. *J. Clin. Microbiol.* **1,** 262–267.
21. Hanshaw, J. B., Niederman, J. C., and Chessin, L. N. (1972) Cytomegalovirus macroglobulin in cell-associated herpesvirus infection. *J. Infect. Dis.* **125,** 304–306.
22. Horwitz, C. A., Henle, W., Henle, G., Polesky, H., Balfour, H. H., Siem, R. A., Borken, S., and Ward, P. C. J. (1977) Heterophile-negative infectious mononucleosis and mononucleosis-like illnesses: laboratory confirmation of 43 cases. *Am. J. Med.* **63,** 947–957.
23. Joassin, L. and Reginster, M. (1986) Elimination of nonspecific cytomegalovirus immunoglobulin M activities in the enzyme-linked immunosorbent assay by using anti-human immunoglobulin G. *J. Clin. Microbiol.* **23,** 576–581.
24. Stagno, S., Tinker, M. K., Elrod, C., Fuccillo, D. A., Cloud, G., and O'Beirne, A. J. (1985) Immunoglobulin M antibodies detected by

enzyme-linked immunosorbent assay and radioimmunoassay in the diagnosis of cytomegalovirus infections in pregnant women and newborn infants. *J. Clin. Microbiol.* **21,** 930–935.

25. Chou, S. and Scott, K. M. (1988) Latex agglutination and enzyme-linked immunosorbent assays to cytomegalovirus serological screening of transplant donors and recipients. *J. Clin. Microbiol.* **26,** 2116–2119.
26. Chou, S., Kim, D., Scoot, K. M., and Sewell, D. L. (1987) Immunoglobulin M to cytomegalovirus in primary and reactivations in renal transplant recipients. *J. Clin. Microbiol.* **25,** 52–55.

2

Identification of Immunoreactive Viral Proteins

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1. Introduction

Several diagnostic tools are available for the identification of acute and latent viral infections. Although newly developed nucleic acid amplification methods, such as the polymerase chain reaction (PCR), have proved to be very useful diagnostic procedures, conventional methods, such as cell culture and serology, still play an important role in viral diagnostics. Despite the fact that modern serological assays, such as enzyme-linked immunosorbent assay (ELISA), are inexpensive and easy to perform, there is a strong demand to improve the performance of such systems. Most serological tests are based on poorly characterized antigens produced in infected culture cells. It has been shown, however, that only few viral antigens contained in these preparations are essential for serodiagnosis. In addition, numerous viral proteins display homologies with their counterparts from related viruses. Finally, the specificity of serological assays can also be reduced by contaminating proteins from host cells. Selective purification of natural viral antigens using, for example, immunoaffinity chromatography is one possible way to improve the quality of an antibody assay. However, the low concentration of most viral proteins in cell culture-derived antigen preparations reduces the practicability of this approach.

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Recombinant technology and chemical peptide synthesis are alternative strategies to provide large quantities of distinct antigen components. Both methods require the identification of the major reactive viral antigens or antigenic fragments.

Human cytomegalovirus (HCMV) is an ubiquitous member of the herpesvirus family. In most immunocompetent individuals, infection remains asymptomatic. HCMV can cause severe disease in immunocompromised individuals, however, including transplant recipients and acquired immune deficiency syndrome (AIDS) patients ***(1)***. Detection of specific antibodies is a commonly used procedure to identify acute and latent HCMV infection. Previous studies have investigated the immunoreactivity of different viral antigen components ***(2)***. Those studies evaluating recombinant antigens used fragments expressed in fusion with bacterial proteins; in addition, some viral components were omitted simply because of limitations of conventional cloning techniques.

The following discussion describes a strategy we used for the identification of immunodominant antigens of HCMV. DNA fragments coding for the different viral polypeptides were generated by PCR. PCR primers used for amplification contained recognition sequences of restriction endonucleases, which facilitated the subsequent cloning steps. The viral antigens were expressed in fusion with glutathione *S*-transferase (GST) using expression vector pGEX–3X ***(3)***. According to our experience, GST shows only limited reactivity with human sera. In a first series of experiments, bacterial lysates of the recombinant clones expressing the viral antigens were evaluated in immunoblot experiments with a small number of well defined human sera. Using this procedure, we were able to identify the major reactive polypeptides. Selected recombinant antigens were then purified and further analyzed in ELISA experiments.

2. Materials

2.1. PCR Amplification and Cloning

1. Computer analysis of DNA sequences was performed using the DNA-Star program.

2. PCR-primers were synthesized on an Applied Biosystems 381A machine, using β-cyanoethyl-phosphoramidite chemistry and were subsequently purified with OPC cartridges (Applied Biosystems) using the protocol supplied by the manufacturer.
3. PCR-amplification reaction mix: 100-µL samples containing 10 µL of PCR reaction buffer (Perkin-Elmer Cetus, Norwalk, CT), 200 µ*M* of deoxynucleotides (Sigma, St. Louis, MO), 0.5 µ*M* of purified PCR primers, 50–100 ng of template DNA and 2.5 U of Ampli *Taq* DNA-Polymerase (Perkin-Elmer Cetus).
4. Ultra Pure Agarose (BRL).
5. Biotrap Chamber (Schleicher and Schüll, Dassel, Germany).
6. Elutip D columns (Schleicher and Schüll).

2.2. Immunoblot Evaluation

1. L-Broth (LB): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 mg/L ampicillin.
2. Isopropyl-β-D-thiogalactopyranoside (IPTG, Calbiochem).
3. Electrophoresis sample buffer: 0.125 *M* Tris-HCl, pH 6.8, 17.5% glycerol, 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 0.05% bromphenol blue.
4. Electrophoresis buffer 2X: 12 g/L Tris-HCl, 57.6 g/L glycine, 2 g/L SDS.
5. Electroblotting buffers: cathode buffer: 25 m*M* Tris-HCl, 40 m*M* 6-aminohexane acid, 20% v/v methanol (pH 9.4). Anode buffer 30 m*M* Tris-HCl, 20% v/v methanol (pH 9.4). Concentrated anode buffer: 300 m*M* Tris-HCl, 20% methanol (pH 9.4).
6. PVDF membranes (Immobilon, Millipore).
7. Semidry electroblot chamber (Hoefer).
8. Blocking buffer: 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS).
9. Serum dilution buffer: PBS containing 0.1%BSA.
10. Washing buffer: PBS containing 0.1% Tween-80.
11. Peroxidase-labeled anti-human IgG/IgM raised in rabbits (Dako, Carpenteria, CA), diluted 1:500–1:1000.
12. Diaminobenzidine (Sigma), 10 mg solubilized in 1 mL methanol + 1 mL 0.05 *M* Tris-HCl, pH 7.5.
13. Staining buffer: 0.1 *M* Tris-HCl, pH 7.5.
14. H_2O_2 (30% v/v in H_2O).

2.3. Bacterial Culture and Expression (Preparative Scale)

1. L-Broth (LB): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl (Difco).
2. Na-ampicillin: 25 mg/L in H_2O (Sigma).
3. Isopropyl-β-D-thiogalactoside (IPTG; Calbiochem).
4. Cooling centrifuge with rotor for 6 × 1 L bottles; 3000*g* (Hettich Roto Silenta).
5. PBS: 2.5 m*M* NaH_2PO_4, 7.5 m*M* Na_2HPO_4, 150 m*M* NaCl, pH 7.2.
6. Cooling centrifuge with rotor for 6 × 0.25 l bottles, 10,000*g* (Sorvall RC5).

2.4. Lysis of Bacteria (Preparative Scale)

1. Lysis buffer: Tris-HCl, 20 m*M*, pH 7.5.
2. Nonidet P-40 (NP-40) (Calbiochem).
3. Phenylmethylsulfonylfluoride (PMSF; Merck, Whitehouse Station, NJ) stock: 200 m*M* (saturated) in 2-propanol.
4. Pefabloc (Roth, Darmstadt, Germany): 100 m*M* in H_2O.
5. EDTA: 0.5 *M*, pH 7.5 (titration with solid NaOH).
6. Lysozyme, chicken (Sigma): 100 mg in 10 mL H_2O.
7. Glycerol 87%.
8. 2-Mercaptoethanol.
9. Branson Sonifier II 250/450 (previously B–30), standard horn 3/4 inch.
10. Potter-S (Braun-Melsungen) homogenizer, glass homogenizer vessels (70 mL) with Teflon plungers.
11. Cooling centrifuge with rotor for 8 × 50-mL tubes, 40,000*g* (Sorvall RC5, SS34 rotor).

2.5. Purification of Soluble Fusion Proteins

1. Chromatography column 2.6 × 20-cm, adaptors.
2. Chromatographic system: peristaltic pump, ultraviolet (UV) monitor, recorder, fraction collector, tubes and fittings.
3. Glutathione (GSH)-Sepharose 4B (Pharmacia, Uppsala, Sweden).
4. Filtration system (glass, Millipore Pyrex, Bedford, MA), vacuum pump, membranes 0.2 μm.

5. GSH equilibration buffer: 20 m*M* Tris-HCl, pH 7.5, 1.4 m*M* 2-mercaptoethanol.
6. GSH washing buffer: 20 m*M* Tris-HCl, pH 7.5, 1.4 m*M* 2-mercaptoethanol, 1.0% (v/v) NP-40 (Calbiochem).
7. GSH elution buffer: 20 m*M* Tris-HCl, pH 7.5, 1.4 m*M* 2-mercaptoethanol, 5 m*M* Glutathione red (Sigma).
8. Cleaning solution: 100 m*M* NaOH.

2.6. Purification of Insoluble Fusion Proteins

1. Solubilization buffer A: 20 m*M* Tris-HCl, pH 9.0, 4 *M* urea, 14 m*M* 2-mercaptoethanol.
2. Solubilization buffer B: 20 m*M* Tris-HCl, pH 9.0, 8 *M* urea, 14 m*M* 2-mercaptoethanol.
3. Solubilization buffer C: 20 m*M* Bistris-HCl, pH 6.0, 4 *M* urea, 14 m*M* 2-mercaptoethanol.
4. Solubilization buffer D: 20 m*M* Bistris-HCl, pH 6.0, 8 *M* urea, 14 m*M* 2-mercaptoethanol.
5. Ultrafiltration pressure system, stirring chambers (Amicon, Danvers, MA, 400 mL, 200 mL, 50 mL), UF membranes with 30-kDa exclusion limit (Filtron Omega 30).
6. Refolding buffer: 100 m*M* Tris-HCl, pH 9.0, 10% (v/v) glycerol (87%), 14 m*M* 2-mercaptoethanol.
7. Chromatography column 2.6 × 20 cm, adaptors.
8. Chromatographic system: two pump system or one pump with gradient mixer, UV monitor, conductivity monitor, two-channel recorder, fraction collector, tubes, and fittings.
9. Anion-exchange material: Q-Sepharose Fast Flow (Pharmacia) or Fractogel TMAE-M (Merck).
10. QA buffer: Tris-HCl, 10 m*M*, 8 *M* urea, pH 9.0, 1.4 m*M* 2-mercaptoethanol.
11. QB buffer: QA-buffer with 1 *M* NaCl.
12. Cation-exchange material: SP-Sepharose Fast Flow (Pharmacia) or Fractogel SO3-M (Merck).
13. SA buffer: 10 m*M* Bistris-HCl, 8 *M* urea, pH 6.0, 1.4 m*M* 2-mercaptoethanol.
14. SB buffer: SA buffer with 1 *M* NaCl.
15. Prepacked column: Superdex 200, 2.6 × 60, HiLoad (Pharmacia).
16. S-200 buffer: QA or SA buffer.

17. Cleaning: 100 m*M* NaOH.
18. SDS: 20% w/v in H_2O.

2.7. Analysis

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) equipment (*see* **Subheading 2.2.**).
2. Immunoblot (*see* **Subheading 2.2.**).
3. Bradford reagent *(7)*.

2.8 ELISA Experiments

1. Polystyrene microdilution plates (Nunc, Polysorb).
2. Coating buffer: 0.01 *M* carbonate buffer, pH 9.5.
3. Postcoating buffer: 0.01 *M* carbonate buffer, pH 9.5, containing 60% fetal calf serum (FCS).
4. Serum dilution buffer: PBS containing 20% FCS.
5. Washing buffer: PBS containing 0.05% Tween-20.
6. 1,2-Phenylenediamine (OPD, Sigma) tablets containing 30 mg.
7. Substrate buffer: 0.1 *M* phosphate/citrate buffer pH 5.5, 0.02% H_2O_2.
8. Stop solution: 1 *N* sulfuric acid.
9. Optical densities were determined in an ELISA photometer (Anthos) at 492 nm (reference filter 620 nm).
10. Conjugate dilution buffer: PBS, containing 0.1% BSA.
11. Adhesive foils (Flow Laboratories, McLean, VA).

3. Methods

3.1. Initial Evaluation of DNA Sequences

1. Before amplification, DNA sequences should be analyzed concerning potential sequence homologies. Numerous HCMV proteins show homologies with their counterparts of other herpesviruses, such as human herpesvirus 6 (HHV-6). Thus, those portions of putative antigens that show substantial homology with other proteins should not be considered for cloning.
2. Select DNA fragments of 400–800 bp for amplification. Fragments of this size result in recombinant viral proteins of molecular weight of 15–30 kDa, in most cases efficiently expressed in *Escherichia*

coli. Fragments should not contain large numbers of cysteine residues, as this may reduce the expression rate.
3. Analyze cloned sequences with regard to their content of restriction enzyme recognition sites that are used for the subsequent cloning steps of the amplified DNA.

3.2. Selection of PCR Primers

In addition to the priming sequence, each pair of PCR primers includes a segment that facilitates the subsequent cloning and expression procedures (*see* **Note 1**).

1. Select the original priming sequence of the viral protein with an average length of 18 bp.
2. 5' to the priming sequence, add the recognition sequence GG-ATC-C of restriction enzyme *Bam*HI. Include additional nucleotides between the *Bam*HI-site and the priming sequence to ensure that the ATC of the *Bam*HI and the cloned viral sequence are in the same reading frame. This enables an in frame insertion of the cloned viral DNA into the *Bam*HI site of expression vector pGEX–3X.
3. Select the appropriate priming sequence of the 3'-primer; 3' of this sequence include the recognition sites of *Bgl*II (AGATCA) and *Eco*RI (GAATTC). The ATC stretch of the *Bgl*II site has to be in the same frame as the 5'-*Bam*HI site. Between *Bgl*II and *Eco*RI sites introduce a stop-codon (TAA) within the same frame as the cloned DNA.

This combination of restriction sites not only facilitates the cloning of the ampified DNA in a preferred orientation via *Bam*HI and *Eco*RI sites; it also enables the in-frame assembly of several DNA fragments. As *Bam*HI and *Bgl*II cleavage results in an identical 5'-overhang, it is possible to insert a *Bam*HI-*Eco*RI fragment into the downstream *Bgl*II and *Eco*RI sites. Using this procedure, two or more DNA fragments can be assembled that encode different viral proteins in-frame, in order to synthesize a so-called autologous fusion protein. As both restriction sites, *Bam*HI and *Bgl*II, are lost after ligation, the newly created DNA fragment can be easily subcloned using the remaining *Bam*HI and *Eco*RI sites.

3.3. Amplification by PCR

1. PCR amplification is carried out for 25 cycles in a Perkin-Elmer Cetus DNA Thermal Cycler, using the following conditions: 55°C/1 min, 72°C/1 min, 94°C/1 min. Cosmids and plasmids harboring defined genomic fragments or cDNAs of HCMV strain AD 169 are used as templates.
2. Run a 5-µL aliquot of the reaction mix on a 0.8% agarose minigel.
3. If the mix exhibits a distinct band of the expected size, purify the remaining volume using a preparative agarose gel.
4. Identify the DNA band using a 365-nm UV lamp and cut it out of the gel, reducing the UV exposure time of the DNA as much as possible.
5. Elute the DNA from the gel slice using the Biotrap chamber and further purify it with Elutip D columns according to the manuals supplied by the manufacturer.
6. Digest the purified DNA with restriction endonucleases *Bam*HI and *Eco*RI for 2 h. Reduce volume by ethanol-precipitation (*see* **Note 2**).
7. Dissolve the DNA in 10 µL TBE buffer, and check the purity of an 1-µL aliquot on an agarose-minigel.

3.4. Cloning Procedures

1. Ligate an 50-ng aliquot of amplified DNA with 100 ng of standard vector pUC8, digested with *Bam*HI and *Eco*RI. Use standard techniques described in **ref. *8***.
2. After identification and characterization of the recombinant clones, purified *Bam*HI/*Eco*RI fragments are used without further modification for subcloning in PGEX–3X. Clone and identify recombinants by using standard techniques (*see* **Note 3**).

3.5. Immunoblot Evaluation (See Note 4)

1. Grow the individual clones expressing the different viral antigens at 37°C in 50 mL of LB medium to an optical density of 0.5–0.7 (600 nm).
2. Induce expression by adding IPTG up to a final concentration of 1 m*M*, and incubate for 2 h.
3. Harvest bacteria from 1.5-mL aliquots of cell suspension by centrifugation in Eppendorf tubes.
4. Remove the supernatant and lyse the cells by adding 200 µL of electrophoresis buffer, boil for 5 min, and centrifuge for 5 min in a microfuge.

5. Aliquot samples and store at –20°C until further use.
6. Before immunoblot experiments, all bacterial lysates should be separated at different dilutions on a 15% SDS-PAGE and stained with Coomassie brilliant-blue. Immunoblots are performed at those dilutions in which the protein band representing the recombinant viral protein is faintly visible.
7. Run 5 µL each of the diluted lysate samples on a 15% SDS-PAGE. Each run should include a lysate of a control clone expressing only GST, in order to identify antibody reactivity with the fusion partner of recombinant viral proteins.
8. Transfer the separated proteins to a PVDF membrane under semidry conditions.
9. Block the membranes with blocking buffer for 1 h.
10. Perform serum incubation at room temperature with human sera diluted 1:100.
11. Wash the membranes four times and incubate with peroxidase labeled anti-human IgG/IgM conjugates diluted 1:1000 for 2 h.
12. Wash four times and develop the membranes for 5 min. Stop reaction by soaking the membranes in ethanol.

3.6. Bacterial Culture and Expression

The upstream procedures are standardized for all clones. A batch of 3 L in six shaking flasks is sufficient. Depending on the expression rate and stability, this can result in 10–100 mg purified fusion protein.

1. Pick an isolated colony from a recently prepared agar plate (< 10 d), and inoculate it into a 500-mL flask containing 150 mL LB medium, supplemented with 300 µL freshly prepared ampicillin solution.
2. Incubate for 12–16 h (overnight) at 37°C with moderate shaking.
3. Transfer 20 mL each of the overnight culture into 6 prewarmed (37°C) 2000-mL flasks containing each 500 mL LB, supplemented with 1 mL freshly prepared ampicillin solution. Six parallel flasks have to be inoculated.
4. Incubate at 37°C while shaking.
5. Follow the growth by measuring the absorbance at 600 nm in a photometer.
6. Induce the expression at A(600) = 0.7, by adding 0.5 mL IPTG solution into each flask. Continue incubation.

7. Stop the incubation 4 h after induction, and harvest the bacterial cells by centrifugation. Use a precooled (4°C) rotor. Conditons: 6 × 1-L bottles, 3000 g, 30 min. Avoid foaming.
8. Resuspend the sediments in ice-cooled PBS in a final volume of 200 mL.
9. Centrifuge the suspension at 5000 rpm/20 min in a precooled GSA-Rotor (1 × 250 mL).
10. Remove the supernatant thoroughly, keep the sediment in the centrifugation vial and freeze the material at –20°C. The sediment can be stored frozen for several weeks.
11. The expression rate should be analyzed by SDS-PAGE (i.e., compare aliquots taken both before and at the end of induction.

3.7. Lysis of Bacteria

Lysis is a key step for the downstream procedures. The method is standardized. However, some modifcations are advantageous for those proteins, which undergo degradation (*see* **Note 7**). The efficiency of the lysis is of crucial importance for insoluble proteins. Residual unlysed bacteria will co-purify during washing steps and thereby contaminate the purified antigen.

The following lysis protocol is a combination of freezing-thawing, lysozyme activity, and mechanical disruption by sonification and potter homogenization. All steps, except the lysozyme incubation, should be performed on ice. The relatively high EDTA concentration destabilizes membranes and inhibits metal proteases, whereas PMSF and pefabloc inhibit serine proteases.

1. Thaw and resuspend the bacterial sediment in 60 mL of lysis buffer using a plastic transfer pipet (Pasteur pipet). Use for the final homogenization the 70-mL glass homogenizer and a Teflon plunger. Three plunges at 1000 rpm are sufficient.
2. Transfer the suspension to a 200-mL flask. Use additional 20 mL of lysis buffer for rinsing the potter, and add this to the suspension. Put the suspension on a magnetic stirrer and mix at room temperature.
3. While stirring, add the following components: 100 µL NP-40, 50 µL PMSF solution, 200 µL pefabloc solution, 10 mL EDTA solution, and 10 mL freshly prepared lysozyme solution.
4. Stir for 60 min at room temperature.

5. Add 14 mL of glycerol (87%) and 140 µL of 2-mercaptoethanol while stirring. Adjust the volume to 140 mL, using lysis buffer.
6. Sonify the suspension in a 250-mL plastic beaker under the following conditions (referring to the use of the Branson sonifier II or B–30): ice bath, 20 kHz, pulse 40%, power 6–7, 3/4-inch titan horn, 5 min.
7. Homogenize the suspension in two portions in the 70-mL glass homogenizer with a Teflon plunger. Three times plunging at 1000 rpm is sufficient.
8. Centrifuge in 4 × 50-mL tubes using a SS-34 precooled (4°C) rotor at 20,000 rpm for 30 min.
9. Separate supernatants and sediments and store at –20°C or, if solubility of the fusion protein is known, directly continue purification.
10. Analyze the distribution of the fusion protein between the sediment and the supernatant, as well as its stability (fragmentation during lysis?) by SDS-PAGE.

3.8. Purification of Soluble Fusion Proteins

The purification is based on the GSH affinity of the GST moiety. The purification is a standard protocol ***(3)***. Typically, the eluted GST proteins have high purity and no further steps are necessary (*see* **Notes 6** and **7**).

1. Use a column with the size 2.6 × 20 cm. A volume of 50 mL GSH-Sepharose 4B is desired (*see* **Note 5**).
2. Equilibrate the column with equilibration buffer (5 vol).
3. Adjust UV (280-nm) monitor and record the baseline.
4. Deliver the sample (130–140 mL) onto the column. Flow rate should not exceed 5 mL/min. The 280-nm absorbance has to be recorded at full scale with the sample.
5. After application of the sample, wash with 100 mL washing buffer (*see* **Note 8**), and subsequently with equilibration buffer.
6. When the recorded UV absorbance has reached the baseline, elute the GST-protein with elution buffer containing 5 m*M* freshly dissolved GSH.
7. When the absorbance once again reaches the baseline level, perform a cleaning in-place step with 0.1 *M* NaOH.

3.9. Purification of Insoluble Fusion Proteins

Insoluble fusion proteins display better stability and often higher yields. However, purification is more difficult. No general recom-

mendations for the purification can be given. In our experience, most of the insoluble GST fusion proteins are expressed as so-called inclusion bodies. A possible strategy is to leave the inclusion bodies intact and purify them as far as possible by differential washing and centrifugation steps. The washing buffers vary in pH, detergent concentration, and content of chaotropic reagents. A number of protocols for this have been published ***(4,5)***. We recommend the following strategies for insoluble GST proteins (*see* **Notes 3** and **4**).

3.9.1. Refolding to a Soluble GSH-Binding Protein

1. Thaw and resuspend the sediment after lysis in 50 mL solubilization buffer A. For further homogenization, use the 70-mL glass homogenizer and a Teflon plunger. Three plunges at 1000 rpm are sufficient (*see* **Notes 10** and **11**).
2. Sonify the suspension in a 100-mL plastic beaker under the following conditions (referring to the use of the Branson sonifier II or B–30): ice-bath, 20-kHz, pulse 40%, power 6, 3/4-inch titan horn, 5 min.
3. Centrifuge in 2 × 50-mL tubes using a SS-34 precooled (10°C) rotor at 20,000 rpm for 20 min. Separate supernatant and sediment. If there is a significant residual pellet, repeat **steps 1–3** using 30 mL buffer B (*see* **Note 9**).
4. Withdraw the final sediment. Analyze the distribution with SDS-PAGE. Select one supernatant or combine both. Adjust to 8 *M* with urea.
5. Dilute the protein solution slowly under stirring with equal volume of refolding buffer.
6. Concentrate the solution by ultrafiltration to 50 mL (ice bath).
7. Dilute the protein slowly to 100 mL while stirring within the UF chamber with refolding buffer.
8. Repeat **steps 6** and **7** twice until the solution has an urea concentration of approx 0.5 *M* and a volume of 100 mL.
9. Collect the samples from the ultrafiltration chamber. Dilute once again to 200 mL with refolding buffer.
10. Perform the GSH-affinity chromatography according to **Subheading 3.8.**, with the following modifications: use pH 9.0 in all buffers instead of pH 7.5.
11. The recorded absorption peak of the GSH elution immediately indicates whether the strategy was successful (*see* **Notes**), regarding precipitation of proteins.

3.9.2. Standard Chromatographies in 8 M *Urea*

1. Calculate the theoretical pI value of the recombinant fusion protein by computer. Consider all expressed aminoacids. If the pI value is <7.0, use solubilization buffers A and B. For higher pI, use buffers C and D.
2. Solubilize the proteins as described in **Subheading 3.9.1.**, **steps 1–4**.
3. Use a column with the size 2.6 × 20 cm. A volume of 50 mL is desired.
4. Use Q-Sepharose and Q buffers for pI <7. Use SP-Sepharose and S buffers for pI >7. Alternatively, Fractogel materials can be used (*see* **Note 12**).
5. Equilibrate the column with equilibration buffer A (5 vol QA or SA).
6. Adjust UV (280-nm) monitor and conductivity monitor and record the baselines.
7. Apply the sample followed by 100 mL buffer A. Flow should not exceed 5 mL/min.
8. Elute bound proteins with a linear gradient of B in A (dC/dV = 1.5 m*M*/mL).
9. Clean column with NaOH.
10. Collect fractions with 10 mL.
11. Analyze fractions by SDS-PAGE with respect to content and purity of recombinant protein.
12. Combine desired fractions. If purity is acceptable, continue with **step 21**.
13. The facultative final step is gel chromatography on prepacked 2.6 × 60 Superdex 200 column. Maximum sample volume is 5 mL. Therefore, the pooled fractions of the first chromatography have to be concentrated by ultrafiltration.
14. Depending on the amount of protein, S-200 chromatography has to be repeated 1–2 times.
15. Equilibrate the column with S-200 buffer. Maximum flow is 3 mL/min.
16. Apply the sample and record UV 280 nm.
17. Clean with NaOH.
18. Collect 5-mL fractions.
19. Analyse fractions by SDS-PAGE.
20. Combine desired fractions.
21. Reduce the volume and remove urea by ultrafiltration.
22. Use the procedure described in **Subheading 3.9.1.**, **steps 5–8**. The final concentration should result in 1–5 mg/mL protein.
23. If protein precipitates during filtration, add SDS to the final solution, and solubilize with sonification.

3.10. Analysis

1. Determine the protein concentration according to Bradford *(6)*
2. Analyze the purity with SDS-PAGE and immunoblot using antigen-specific, GST-specific, and *E. coli*-specific antisera, if available.

3.11. Evaluation of Purified Viral Proteins in ELISA Experiments

1. Coat polystyrene microdilution plates with 100 µL coating buffer containing 100 ng of purified antigen. Incubate in a moist chamber for 12–18 h.
2. Add 100 µL of postcoating buffer and incubate for additional 2 h.
3. Remove the fluid and tap the inverted plate thoroughly onto a paper.
4. Dilute human sera 1:50 and add 100 µL to the wells of the microdilution plates. Two wells should be incubated in each run with 100 µL serum dilution buffer only, in order to recognize nonspecific binding of the conjugate. Seal the plate with an adhesive foil and incubate for 2 h at 40°C in a waterbath. Each ELISA evaluation of a recombinant viral protein should be performed in comparison with an ELISA plate coated with purified GST, to identify reactivity caused by the fusion partner of the viral antigens.
5. After intensive washing, each well is filled with 100 µL of anti-human IgG or IgM specific conjugate, diluted 1:1000. Seal the plate and incubate for 1h at 40°C in a waterbath.
6. At 5 min before the end of the conjugate incubation, dissolve 1 OPD-tablet in 12 mL of substrate buffer.
7. Wash the plate and add 100 µL of the chromogen/substrate solution. Incubate for 15 min at room temperature protected from light.
8. Stop the color development by adding 100 µL of stop solution and read the results in an ELISA reader at 495 nm. Samples with OD values of >0.5 and a GST-specific reactivity of <0.25 are considered positive.

4. Notes

1. The combination of restriction enzymes (*Bam*HI, *Bgl*II, *Eco*RI), which were introduced into the PCR primers, were used to clone more than 50 different DNA-fragments coding for viral antigens. Because of the low incidence of these sites in viral sequences, it was

possible to avoid interference with restriction sites within the cloned viral sequence.

2. To ensure complete digestion of amplified DNA with restriction enzymes *Bam*HI and *Eco*RI and high efficiency of the subsequent cloning step, the DNA should be pure. We therefore combined purification by electrophoresis and affinity chromatography.
3. Each cloned fragment should be carefully characterized after initial cloning in pUC8, to avoid confusion. This can be done most easily by looking for characteristic restriction sites. We sequenced only the clones encoding those viral proteins, which have proved to be essential antigens.
4. Immunoblot evaluation is a useful tool for the initial identification of those antigens that can be used for serodiagnosis. These experiments can be performed only with a small number of well characterized samples. This method is of limited value, however, to evaluate the specificity of a recombinant protein. This information can only be obtained in more standardized ELISA experiments with a large number of samples.
5. To reduce blocking of the GSH column caused by particles or precipitated proteins, a special 10-µm filter (the net of an column adaptor) should be used at the inlet of the pump.
6. The purity after the GSH-affinity step is typically sufficient for ELISA experiments. Very rarely, an additional purification step is necessary.
7. More rarely, rapid and complete degradation to GST (M_r = 26–28 kDa) may occur. In such cases, we were successful to overcome this by using the following strategy: Change the bacterial host. Use BL21 instead of JM109. BL21 lacks two periplasmatic proteases. Increase the concentration of pefabloc during lysis (four- to fivefold). Perform the lysis completely on ice. Reduce the lysozyme incubation to 5 min (on ice). Perform GSH chromatography immediately after the centrifugation of the lysate.
8. The washing buffer containing 1% NP-40 reduces hydrophobic binding of an *E. coli* protein (65–70 kD), the main contaminant after GSH-chromatography. Instead of NP-40, Triton X-100 may be used.
9. Most insoluble proteins will be dissolved in 4 *M* urea. Sometimes only partial solubilization occurs. The residual amount should appear in the 8 *M* urea fraction.
10. The refolding out of 4–8 *M* urea is a critical step. At best we obtained 50% of total protein which has the right conformation to bind to GSH. Typical yields are 10–30%. Recycling is possible, that is, the

nonbound fraction can be adjusted to 8 *M* urea, and further refolding and chromatography can improve the final yield. Once bound to GSH-sepharose, the eluted protein is of high purity, even better than that of soluble proteins.

11. Most initially insoluble GST fusion proteins can be refolded to soluble proteins. A common problem encountered while dealing with a very insoluble protein is its precipitation after removal of urea. If this happens nearly completely, the protein can be recovered by centifugation, resuspended in buffer without urea, and solubilized with SDS (0.1–0.5%) by sonification. Remarkably, most antigens keep their reactivity under such conditions.
12. Sometimes there seems reduced capacity of the Sepharose ion-exchange material under 8 *M* urea conditions. This could be related to high-molecular-weight aggregates, which are excluded from the Sepharose beads. If a significant amount of the GST-protein partitions in the nonbinding fraction, change to Fractogel. This material has better properties for binding, but less performance compared with Sepharose.
13. It has been reported that the solubility and stability of GST proteins can be improved by lowering the temperature of the culture to 20°C ***(6)***. In our hands, this was not successful.
14. The major advantages of the GST system are the GSH affinity purification and the low prevalence of anti-GST antibodies in human sera. Some disadvantages of GST are related to its biochemical properties. GST contains four cysteines, which can favor the synthesis of insoluble inclusion bodies when the cloned sequence has additonal cysteines. The pI value of GST is rather high (6.35); if basic sequences are fused, the pI is often shifted to 7–7.5. This reduces the solubility in vivo and during lysis.

References

1. Britt, W. J. and Alford, C. A. (1996) Cytomegalovirus, in *Virology* (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Lippincott-Raven, Philadelphia, pp. 2493–2524.
2. Landini, M. P. and Mach, M. (1995) Searching for antibodies specific for human cytomegalovirus: is it diagnostically useful? When and how. *Scand. J. Infect. Dis. Suppl.* **99,** 18–23.
3. Smith, D. B. and Johnson, K. S (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusion with glutathione *S*-transferase. *Gene* **67**, 31–40.

4. Frangioni, J. V. and Neel, B. G. (1993) Solubilization and purification of enzymatically active glutathione *S*-transferase (pGEX) fusion proteins. *Anal. Biochem.* **210,** 179–187.
5. Marston, F. A. (1986) The purification of eukaryotic polypeptides synthesized in Escherichia coli. *Biochem. J.* **240,** 1–12.
6. Ghosh, S., Basu, S., Strum, J. C., and Bell, R. M. (1995) Identification of conditions that facilitate the expression of GST fusions as soluble, full-length proteins. *Anal. Biochem.* **225,** 376–378.
7. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254.
8. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning — A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

3

Analysis of Human Cytomegalovirus Using the Polymerase Chain Reaction

Marc Mendelson

1. Introduction

As with numerous other branches of science, the study of human cytomegalovirus (HCMV) infection has been revolutionized by the polymerase chain reaction (PCR) method first devised by Mullis and Faloona ***(1)***. PCR allows the in vitro amplification of HCMV DNA sequences by the simultaneous primer extension of complementary DNA strands. Similarly, reverse transcription-PCR (RT-PCR) allows the study of targeted gene expression, by reverse transcription of RNA to complementary DNA (cDNA), followed by amplification of target DNA using predetermined primers. The PCR method is used in the clinical diagnosis of HCMV infection, particularly in the setting of transplantation medicine and in those patients infected with the human immunodeficiency virus (HIV). In addition, the advent of PCR and RT-PCR has transformed our understanding of the pathogenesis of HCMV infection, central to which is the definition of the sites of latency, the degree and type of gene expression within the latently infected cell, and the factors influencing both the maintenance of latency and reactivation of the virus during immunosuppression.

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Before the advent of the PCR method, studies of the sites of HCMV latency were hampered by the use of methods too insensitive to detect the viral genome reliably at the low copy number at which it is thought to exist (*see* **Note 1**). Early *in situ* hybridization studies ***(2,3)*** suggested low level infection by HCMV of both monocytes and lymphocytes within the healthy seropositive host. However, it required PCR to confirm peripheral blood monocytes ***(4,5)*** as the site of latent infection within peripheral blood. Furthermore, PCR has demonstrated that bone marrow progenitors are also a site of latency within healthy seropositives ***(5,6)***. In terms of gene expression within latently infected cells, RT-PCR has defined CMV latency-associated transcripts in bone marrow progenitors ***(7)***, and lytic reactivation of HCMV from latently infected peripheral blood monocytes has been demonstrated ***(8)***.

By its very nature, the PCR method, which is capable of generating 10^{12} molecules in a typical 100-µL amplification reaction ***(1)***, is exquisitely sensitive to contamination by previously amplified product. Methods designed to prevent such "carryover" PCR contamination (*see* **Note 2**) should be rigorously applied to all procedures outlined in this chapter. In addition, incorporation of an equal number of negative controls within each PCR is vital to the interpretation of results.

The choice of primers used to analyze HCMV DNA differs between different laboratories and includes regions within the major immediate early (IE) genes, the major immediate early promoter (MIEP) and the glycoprotein B gene sequence. We have found the use of primers directed toward a conserved region within exon 4 of the immediate early gene IE-1 ***(4)*** as the most sensitive PCR for detection of the latent HCMV genome within cells of the human host. In addition, comparison of different DNA extraction techniques produced both "in house" and commercially has shown that a modified sodium perchlorate method ***(9)*** is optimal for use with our PCR method.

This chapter details our method for extraction of cellular DNA using the modified sodium perchlorate technique, coupled with the

protocol for amplification of HCMV IE-1 exon 4 DNA, first described by Taylor-Wiedeman, et al. ***(4)***. The same PCR protocol can be used for amplification of HCMV plasmid DNA, using a reduced cycle number, compared with that required for study of latent infection. A β-globin gene PCR is also described, used as a control for the integrity of DNA and the presence of inhibitors to the PCR reaction within the PCR mix.

A description of the method for amplification of HCMV DNA is followed by a discussion of the method used in our laboratory for studying HCMV lytic gene expression by RT-PCR. As with PCR, all possible steps should be taken to avoid contamination of preamplification materials by the same methods described in **Note 2**.

2. Materials

2.1. Extraction and Purification of Cellular DNA Using the Modified Sodium Perchlorate Method

1. Starting cell population. In this example, 10^6 purified peripheral blood monocytes are used to illustrate the method.
2. Solution 1: 100 m*M* NaCl, 50 m*M* EDTA, pH 8.0.
3. Solution 2: 25% w/v sodium dodecyl sulfate (SDS).
4. Solution 3: 5 *M* sodium perchlorate.
5. Phenol–chloroform–isoamyl alcohol (25:24:1).
6. Chloroform–isoamyl alcohol (24:1).
7. Isopropanol, 70% ethanol solution, sterile water (Sigma, St. Louis, MO).
8. Dialysis tubing and clips.

2.2. Preparation of PCR Oligonucleotides

1. Oligonucleotide primers for all PCRs were synthesized on an Applied Biosystems (Warrington, UK) model 381A DNA synthesizer.
2. Ammonia solution.
3. Potassium acetate (5 *M* potassium acetate, 3 *M* glacial acetic acid).
4. Ethanol, nuclease-free sterile water.

2.3. PCR Amplification of HCMV exon 4 IE-1 DNA.

1. Genomic DNA (*see* **Subheading 2.1.**) or plasmid containing the coding sequence of interest.
2. Oligonucleotide primers. For the study of lytic infection or amplification of HCMV IE-1 DNA from plasmid, external oligonucleotides sense (S1), and antisense (AS1) primers are used in a single PCR. For study of latency where HCMV copy number is low, a nested internal PCR is used requiring internal sense (S2) and antisense (AS2) primers:
   - S1 5'-GGTCACTAGTGACGCTTGTATGATGACCA TGTACGG-3'
   - AS1 5'-GATAGTCGCGGGTACAGGGGACTCT-3'
   - S2 5'-AAGTGAGTTCTGTCGGGTGCT-3'
   - AS2 5'-GTGACACCAGAGAATCAGAGGA-3'
3. 10X PCR buffer: 0.1 *M* Tris-HCl, pH 8.5, 0.5 *M* KCl, 0.02 *M* $MgCl_2$, 0.1% gelatin, 2.5% (v/v) glycerol (*see* **Note 4**).
4. 1 μ*M* each oligonucleotide primer.
5. 2 m*M* dATP, dTTP, dCTP, and dGTP mix.
6. Tween-20.
7. Light mineral oil.
8. *Taq* Polymerase (*see* **Note 5**) from Promega (Madison, WI).
9. Perkin-Elmer Cetus Thermocycler or equivalent.

2.4. PCR Amplification of β-Globin Gene and Histidyl tRNA Synthetase Gene DNA

1. Sense (S) primer and antisense (AS) primer sequences for β-globin gene PCR:
   - S: 5'-TGTCCACTCCTGATGCTGTT-3'
   - AS: 5'-GGATTCTAAACTGTACCCTG-3'
2. Sense (S) primer and antisense (AS) primer sequences for histidyl tRNA synthetase gene PCR:
   - S: 5'-TCATCAGGACCCAGCTGTGC-3'
   - AS: 5'-CTTCAGGGAGAGCGCGTGCG-3'
3. 10X PCR buffer for β-globin and histidyl tRNA synthetase gene PCR: 0.1 *M* Tris-HCl, pH 8.5, 0.5 *M* KCl, 0.01 *M* $MgCl_2$, 0.1% gelatin, 2.5% (v/v) glycerol.

4. 1 m*M* each oligonucleotide primer.
5. 2 m*M* dATP, dTTP, dCTP, and dGTP mix.
6. Tween-20.
7. Light mineral oil.
8. *Taq* Polymerase from Promega.
9. Perkin-Elmer Cetus Thermocycler or equivalent.

2.5. Radiolabeling of HCMV Exon 4 IE-1 Probe

1. Solution X: 10 m*M* spermidine, 0.2 *M* Tris-HCl, pH 9.5, 1 m*M* EDTA.
2. 1 µg/µL Probe (5'-GCCGCATTGAGGAGATCTGCATGAAG GTCT-3').
3. 10X Blunt-ended buffer: 0.1 *M* $MgCl_2$, 0.5 *M* Tris-HCl, pH 9.5, 50 m*M* DTT, 50% (v/v) glycerol.
4. γ-^{32}P-dATP (70 mCi/mL).
5. T4 polynucleotide kinase.
6. Sterile water (Sigma), Tris-EDTA, pH 8.0 (TE).
7. DE-52 0.2 *M* NaCl slurry, 0.2 *M* NaCl/TE, 0.5 *M* NaCl/TE.
8. 2-mL syringe, autoclaved pollyallomer wool.

2.6. RNA Extraction and Purification

Extraction of cellular total RNA is performed using the RNAgents isolation system (Promega), a method based on that of Chomczynski and Sacchi ***(10)***.

1. RNAgents isolation system (Promega).
2. RQ1 buffer: 40 m*M* Tris-HCl, pH 8.3, 10 m*M* NaCl, 6 m*M* $MgCl_2$, 10 m*M* $CaCl_2$.
3. rRNasin (Promega).
4. RQ1™ DNase (Promega).
5. Stop buffer: 50 m*M* EDTA, 1.5 *M* Na acetate, 1% sodium dodecyl sulfate (SDS).
6. RNase-free water (Promega).
7. Acidified phenol; chloroform; isoamyl alcohol.
8. Isopropanol.

2.7. Reverse Transcription

1. For first-strand cDNA synthesis, we use Superscript II reverse transcriptase (Gibco-BRL); however, other standard RT protocols can be employed.
2. RNase-free tRNA.
3. Oligo $(dT)_{12–18}$ primer (Pharmacia).
4. RNase-free water.
5. 5X First-strand buffer (Gibco-BRL): 250 m*M* Tris-HCl, pH 8.3, 375 m*M* KCl, 15 m*M* $MgCl_2$, 0.1 *M* DTT.
6. 0.1 *M* DTT.
7. 1 m*M* dATP, dTTP, dCTP, and dGTP.
8. Superscript II reverse transcriptase.
9. Thermal heating block.

2.8. Nested PCR Amplification of Intron-Spanning Immediate-Early 1 DNA

1. cDNA resulting from reverse transcription of cellular tRNA.
2. Oligonucleotide primers for nested intron-spanning immediate-early 1 (IE-1) amplification include external sense (S1) and antisense (AS1) primers and the nested sense (S2) and antisense (AS2) primers. The probe oligonucleotide sequence (P) is also shown.

   S1: 5'-GGTGCATTGGAACGCGGATT-3'
   AS1: 5'-ATTCTATGCCGCACCATGTCCA-3'
   S2: 5'-ATGGAGTCCTCTGCCAAGAG-3'
   AS2: 5'-CATAGTCTGCAGGGACGTCGT-3'
   P: 5'-GACCCTGATAATCCTGACGA-3'
3. 10X PCR buffer: 0.1 *M* Tris-HCl, pH 8.5, 0.5 *M* KCl, 0.01 *M* $MgCl_2$, 0.1% gelatin, 2.5% (v/v) glycerol. For the nested PCR, 0.015 *M* $MgCl_2$ is used.
4. 1 m*M* each oligonucleotide primer.
5. 2 m*M* dATP, dTTP, dCTP, and dGTP mix.
6. Tween-20.
7. Light mineral oil.
8. *Taq* Polymerase (Promega).
9. Perkin-Elmer Cetus Thermocycler or equivalent.

3. Methods

3.1. Extraction and Purification of Cellular DNA Using the Modified Sodium Perchlorate Method

1. In a 2-mL centrifuge tube, 10^6 monocytes are resuspended in 600 µL of solution 1; add 50 µL of solution 2; then add 150 µL of solution 3 to the tube (*see* **Note 3**); the mix is pipetted up and down vigorously until the consistency changes from viscous to fluid.
2. Phenol–chloroform–isoamyl alcohol extract once, centrifuging at 10,000*g* for 10 min at room temperature.
3. Extract the aqueous phase with chloroform's isoamyl alcohol and precipitate with an equal volume of isopropanol for 30 min on ice.
4. Wash the cell pellet with 70% ethanol and resuspend in 200 µL of sterile water.
5. Dialyze the sample against 1000-fold volume of sterile water at 4°C.
6. Estimate the concentration of 1 µL of the DNA by spectrophotometry and the DNA is phenol/chloroform and adjusted to a concentration of 1 µg/µL in sterile water.

3.2. Preparation of PCR Oligonucleotides

1. Empty the contents of the oligonucleotide column into a 2-mL centrifuge tube.
2. Add 1 mL ammonia solution (BDH) and incubate for 1 h at RT.
3. Centrifuge at 9000 rpm in a benchtop microcentrifuge for 3 min and remove the aqueous layer into a fresh tube.
4. Incubate for 18 h at 55°C.
5. Precipitate oligonucleotides on dry ice for 10 min with 1/10 volume potassium acetate and 3 vol absolute ethanol.
6. Precipitate the pellet at 10,000*g* for 10 min and wash in 80% ethanol.
7. Resuspend the oligonucleotide pellet in 100 µL of nuclease-free water and determine stock concentration by spectrophotometry at 260 nm. Store stock solution at –70°C, making aliquots stored for immediate use at –20°C.

3.3. PCR Amplification of HCMV Exon 4 IE-1 DNA

For the purpose of this description, we will use as an example the nested PCR used in our laboratory for study of low copy number latent HCMV infection.

1. Into a 0.5-mL PCR amplification tube (Perkin-Elmer), pipet 1× PCR buffer on ice, adding 1 μ*M* of each primer, 200 μ*M* of each dNTP, 0.5% Tween-20, 1 μg of genomic DNA (*see* **Note 6**) and 1.25 U of *Taq* polymerase, made up to a volume of 50 μL with nuclease-free water. Keep the reaction mix on ice. For each PCR amplification, an identical control is set up, substituting sterile water for the 1 μg of genomic DNA.
2. Overlay with 50 μL of mineral oil maintaining the PCR mix on ice to prevent mis-priming events.
3. Heat the thermocycler block to 94°C, and transfer the PCR tube from the ice into the block, thus providing a modified hot start reaction (*see* **Note 7**). Denature the reaction at 94°C for 4 min 30 s.
4. Optimized cycling parameters for the primary external PCR and the nested internal PCR were 35 and 30 cycles, respectively, of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 90 s.
5. PCR mix for the nested PCR is made up within the pre-PCR suite laminar flow hood and is identical to the external PCR mix excluding the genomic DNA, which is substituted by 2 μL of primary product added in a hood outside the pre-PCR suite.
6. PCR products are analyzed by standard horizontal agarose gel electrophoresis, using a 1% agarose gel run against a 1-kilobase (kb) DNA marker (Gibco-BRL). The target amplicon for the primary PCR is 373 bp and the nested PCR is 293 bp.
7. The PCR products are denatured with 0.4 *M* NaOH and Southern blotted according to standard technique ***(11)***.

3.4. PCR Amplification of β-Globin Gene DNA

The β-globin gene PCR is used as a control PCR for the exon 4 IE-1 amplification. It controls for DNA integrity and inhibitors of *Taq* polymerase within the PCR mix. Amplification of β-globin gene DNA is undertaken on the same starting concentration of DNA used for the IE-1 exon 4 PCR.

1. Set up the PCR reaction with 1× PCR buffer on ice, adding 1 μ*M* of each primer, 200 μ*M* of each dNTP, 0.5% Tween-20, 1 μg of genomic DNA and 1.25 U of *Taq* polymerase, made up to a volume of 50 μL with nuclease-free water.

2. Overlay with 50 µL of mineral oil.
3. Heat the thermocycler block to 94°C and transfer the PCR tube to the heated block.
4. Optimized cycling parameters are 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 90 s.
5. The target amplicon is 246 bp, analyzed by horizontal electrophoresis on a 1% agarose gel.

3.5. Radiolabeling of HCMV Exon 4 IE-1 Probe

1. Set up a mix containing 4 µL of solution X, 3 µL of probe, 5 µL 10X blunt end buffer, 3 µL γ-^{32}P-dATP, 1 µL of T4 polynucleotide kinase, and 34 µL of sterile water.
2. Incubate the reaction mix at 37°C for 60 min.
3. Assemble the column over which the radiolabeled probe will be passed to remove unincorporated γ-^{32}P-dATP. Pack autoclaved pollyallomer wool into the 2-mL syringe up to approx 0.75 ml. Pass 1 mL of DE–52 0.2 *M* NaCl slurry over the column, and repeat until the packed resin constitutes approx 0.5 mL within the syringe. Wash the column with 4 mL of TE before loading the probe.
4. Add 50 µL TE to the incubated reaction mix, and pass the total volume over the resin column. Wash the column with 4 mL 0.2 *M* NaCl/TE and elute the cleaned probe with 1 mL 0.5 *M* NaCl/TE, storing at –70°C.
5. Hybridization of probe onto the product and autoradiography are performed as pre standard technique ***(11)***.

3.6. RNA Extraction and Purification

1. Total RNA is extracted from cells using the RNAgents isolation system (Promega) according to the manufacturer's instructions (*see* **Note 8**).
2. Following the final wash step with ice-cold 75% ethanol and pelleting of RNA at 10,000*g* for 5 min, the remaining genomic DNA is removed using RQ1™ DNase:
3. Cell pellet is resuspended in 500 µL of buffer RQ1.
4. Add 2 U of rRNasin.
5. Add 15 U RQ1™ DNase and mix.
6. Incubate for 15 min at 37°C.

7. Stop the reaction by adding ½ vol stop solution.
8. Precipitate overnight at –20°C with equal volume isopropanol after extracting the DNase-free RNA with an equal volume of acidified phenol–chloroform–isoamyl alcohol supplied with the RNagents kit.
9. Resuspend the RNA pellet in an appropriate volume of RNase-free water, depending on the anticipated RNA concentration. Store at –70°C.

3.7. Reverse Transcription

Controls for the reverse transcription reaction are provided by setting up an equal number of tubes substituting RNase-free water for Oligo $(dT)_{12–18}$ primer, and in separate reactions, substituting RNase-free water for Superscript II reverse transcriptase.

1. On ice, add 1–5 µg of cellular total RNA to 1 µL of Oligo $(dT)_{12–18}$ primer and make up the reaction volume to 12 µL with RNase-free water.
2. Heat to 70°C for 10 min followed by quick chill on ice for 30 s.
3. Pulse centrifuge the mix and add 4 µL of 5X first-strand buffer.
4. Add 2 µL DTT and 1 µL of each dNTP.
5. Incubate for 10 min at 25°C, followed by 2 min at 42°C.
6. Add 1 µL of Superscript II and reverse transcribe at 42°C for 50 min.
7. Inactivate the Superscript II by heating to 70°C for 15 min.
8. Store cDNA at –20°C.

3.8. Nested PCR Amplification of Intron-Spanning Immediate-Early 1 DNA

1. Set up the PCR reaction with 1X PCR buffer on ice, adding 1 µ*M* of each primer, 200 µ*M* of each dNTP, 0.5% Tween-20, 1 µg of cDNA (*see* **Note 9**) and 1.25 U of *Taq* polymerase made up to a volume of 40 µL with nuclease-free water. The PCR mix for the nested PCR is identical to the above, except 2 µL of primary product acts as template and the total reaction volume is 20 µL.
2. Overlay with 50 µL of mineral oil maintaining the PCR mix on ice.
3. Heat the thermocycler block to 94°C and transfer the PCR tube from ice into the block. Denature the reaction at 94°C for 4 min 30 s.
4. Optimized cycling parameters for both the primary external PCR and the nested internal PCR were 50 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 90 s.

5. PCR mix for the nested PCR is made up within the pre-PCR suite laminar flow hood. A similar modified hot start procedure is employed; 2 μL of primary product is added to the reaction mix in a hood outside the pre-PCR suite.
6. PCR products are analyzed by standard horizontal agarose gel electrophoresis using a 2% agarose gel run against a 1-kb DNA marker (Gibco-BRL). The target amplicon for the primary PCR is 353 bp and the nested PCR is 117 bp.
7. Southern blotting, radiolabeling of probe, hybridization, and autoradiography are carried out as described in **Subheadings 3.3.** and **3.4**.

3.9. PCR Amplification of Histidyl tRNA Synthetase Gene DNA

The histidyl tRNA synthetase gene is used as a control PCR for the nested intron-spanning IE-1 amplification. It controls for cDNA integrity and inhibitors of *Taq* polymerase within the PCR mix. Amplification of histidyl tRNA synthetase gene DNA is undertaken on the same starting concentration of cDNA as used for the nested intron-spanning IE-1 PCR.

1. The materials used are detailed in **Subheading 2.4.** and the amplification protocol is the same as that for the β-globin gene PCR detailed in **Subheading 3.4.**, except for the difference of 50 cycles being used, with an annealing temperature of 55°C. The target amplicon for histidyl tRNA synthetase gene amplification is 128 bp, analyzed by horizontal electrophoresis on a 2% agarose gel.

4. Notes

1. We have found that the minimum concentration of DNA from peripheral blood monocytes or $CD34^+$ bone marrow progenitors required to detect HCMV DNA using our PCR method is approx 1 μg. This equates to a starting number of approx 1×10^6 cells, estimating the copy number of HCMV in latent infection to be approx 1 copy per 10^6 cells.
2. Prevention of carryover PCR contamination requires the physical separation of work involving steps in the methods before PCR amplification (pre-PCR), and work undertaken in amplification of DNA sequences and their analysis. This is particularly critical in the

analysis of HCMV latency, where HCMV copy number is low. In our laboratory studies of latency, all pre-PCR work is carried out in a laminar flow hood, located in a dedicated separate laboratory suite into which no infected cell material or amplified product is allowed. All equipment therein is dedicated to pre-PCR work, and all pipet tips are sterile ART™ (aerosol resistant tips, Northern Biologicals Ltd) or equivalent, used on positive displacement pipets. In addition, all reagents used in the PCR are purchased as liquids or are reconstituted in laboratories that do not handle HCMV. Stock solutions of components of the PCR reaction mix should be aliquoted immediately into volumes ready for PCR and stored at –20°C within the pre-PCR laboratory. After preparation of the PCR mix, we routinely irradiate the laminar flow hood for >5 min, which reduces carryover contamination ***(12)***. To prevent handling contamination, gloves should be changed on entering the pre-PCR suite and regularly if manipulation of samples occurs outside the laminar flow hood ***(13)***. For nested PCR, reaction mix is prepared within the pre-PCR suite, whilst the primary amplicon is added in a laminar flow hood in a separate laboratory.

3. The volumes of solutions 1–3 can be scaled up or down, but keep the ratios constant.
4. The constituents of the buffer mix have been optimized for the primer sets used within the reaction. We found that co-solvents other than Tween-20 and glycerol failed to improve DNA amplification. PCR amplification of HCMV exon 4 IE-1 DNA without the use of Tween-20 and glycerol increased nonspecific priming during amplification and decreased sensitivity. If different primers are used from those outlined, the PCR reaction should be re-optimized, particularly with respect to magnesium concentration.
5. We have found that Promega *Taq* polymerase gives optimal results with the HCMV exon 4 IE-1 PCR, when tested against other available *Taq* polymerases.
6. If using the HCMV IE-1 exon 4 PCR to identify an insert within plasmid DNA, the PCR reaction need only be spiked with 10–20 ng of plasmid DNA, and *Taq* polymerase can be added to the reaction mix after initial denaturation in the thermocycler.
7. The optimum temperature range for *Taq* polymerase is 70–75°C ***(14)***; however, *Taq* is able to function at lower temperatures. In an attempt to prevent mis-priming events before reaching the initial denaturation step, one can employ a “hot start” procedure. The addition of

one component of the PCR mix after attainment of the denaturation temperature or having a physical interface between components of the mix such as achieved using AmpliWax PCR gems (Perkin-Elmer) constitutes a true hot start. However, concerns about contamination of samples if the PCR tube is opened once in the thermocycler preclude the addition of a component of the mix at denaturation temperature. We have compared using AmpliWax PCR gems with a modified hot start, providing a rapid ramping time by transferring the PCR mix from ice directly to the 94°C denaturation temperature. We found no difference in specificity or sensitivity between the two methods.

8. The critical factor in extraction of cellular RNA is avoiding introduction of RNases from the environment. To this end, fresh gloves should be worn at all times when handling materials involved in the method. Bench-top microcentrifuges should be run at 4°C unless specified in the manufacturer's instructions; likewise, the reactions are undertaken on ice to avoid any contaminating RNase from working.
9. We routinely use 1 μg of cDNA for detection of immediate early transcripts by nested intron-spanning IE-1 amplification.

References

1. Mullis, K. B. and Faloona, F. A. (1987) Specific synthesis of DNA in vitro via a polymerase-catalysed chain reaction. *Methods Enzymol.* **155**, 335–350.
2. Schrier, R. D., Nelson, J. A., and Oldstone, M. B. A. (1985) Detection of human cytomegalovirus in peripheral blood lymphocytes in a natural infection. *Science* **230**, 1048–1051.
3. Stanier, P., Taylor, D. L., Kitchen, A. D., Wales, N., Tryhorn, Y., and Tyms, A. S. (1989) Persistence of cytomegalovirus in mononuclear cells in peripheral blood from blood donors. *Br. Med. J.* **299**, 897–898.
4. Taylor-Wiedeman, J. A., Sissons, J. G. P., Borysiewicz, L. K., and Sinclair, J. H. (1991) Monocytes are a major site of persistence of human cytomegalovirus in peripheral blood mononuclear cells. *J. Gen. Virol.* **72,** 2059–2064.
5. Mendelson, M., Monard, S., Sissons, J. G. P., and Sinclair, J. H. (1996) Detection of endogenous human cytomegalovirus in $CD34^+$ bone marrow progenitors. *J. Gen. Virol.* **77**, 3099–3102.
6. Von Laer, D., Meyer-Konig, U., Serr, A., Finke, J., Kanz, L., Fauser, A. A., Neumann-Haefelin, D., Brugger, W., and Huffert, F. (1995)

Detection of cytomegalovirus in $CD34^+$ cells from blood and bone marrow. *Blood* **86**, 4086–4090.

7. Kondo, K., Xu, J., and Mocarski, E. S. (1996) Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. USA* **93**, 11,137–11,142.
8. Soderberg-Naucler, C., Fish, K., and Nelson, J. A. (1997) Reactivation of latent human cytomegalovirus by allogeneic stimulation of blood cells from healthy donors. *Cell* **91,** 119–126.
9. Weetman, A. P., Zhang, L., Webb, S., and Shine, B. (1990) Analysis of HLA-DQB and HLA-DPB alleles in Graves' disease by oligonucleotide probing of enzymatically amplified DNA. *Clin. Endocrinol.* **33**, 65–71.
10. Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156–1159.
11. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Approach*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
12. Sarkar, G. and Sommer, S. S. (1990) Shedding light on PCR contamination. *Nature* **343**, 27.
13. Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* **339**, 237–238.
14. Saiki, R. K. (1989) The design and optimisation of the PCR. In *PCR Technology-Principles and Applications for DNA Amplification* (Ehrlich, H. A., ed.), Stockton Press, New York, pp. 7–16.

4

Analysis of Cytomegalovirus Ligands, Receptors, and the Entry Pathway

Teresa Compton

1. Introduction

The process of virus entry accomplishes the delivery of the viral genetic information into the cell so that replication can take place. Entry of enveloped viruses into mammalian cells requires that the virus attach to the host cell surface through an interaction between an envelope component and a cellular molecule that serves as a receptor. After attachment, the virus must cross the plasma membrane during a phase of the life cycle termed *penetration*. For enveloped viruses, a fusion reaction occurs between the viral envelope in either the endosomal membrane or the plasma membrane. In simple viral systems that have one or two envelope glycoproteins, generally, a single, specific interaction between a viral protein and a cellular constituent is necessary and sufficient to result in infection. The herpesviruses, including human cytomegalovirus (HCMV), are structurally much more complex and often exhibit broad, diverse cellular tropism. Thus, all evidence to date points to the involvement of multiple cellular and viral proteins with overlapping or compensatory mechanisms possibly responsible for entry into specialized cell types.

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1.1. HCMV Envelope Proteins

Analysis of the nucleotide sequence of HCMV showed a total of 54 open reading frames (ORFs) that have characteristics of glycoprotein genes or of exons of glycoprotein genes ***(1)***. Some have homology to immunoglobulin superfamily proteins, others are multiple membrane spanning, and a few have recognized homology to glycoproteins of other herpesviruses. Three major glycoprotein complexes present in the envelope of HCMV have been biochemically characterized. It is probable that other glycoproteins are present in the virion envelope, but they remain uncharacterized and undetected. The gC-I complex, the most abundant component of the envelope, contains the HCMV glycoprotein B (gB) homologue glycoprotein ***(2–4)***. The UL55 ORF of the HCMV genome encodes gB, a 907-amino acid (aa) protein ***(1,5)***. In its native state, gB appears to be a homo-oligomer composed of disulfide-linked gB (gp93/gp55) monomeric subunits ***(3,6,7)***. HCMV gB is also one of the major targets of the immune response and elicits the production of neutralizing monoclonal antibodies (MoAbs) ***(5,8–10)***. A second envelope glycoprotein complex, designated gC-III ***(2)*** or gp86 ***(11)***, contains the gH and gL homologues, as well as a third component that is not genetically defined ***(12–15)***. A third major envelope glycoprotein complex, gC-II, is composed of very large disulfide-linked oligomers that reduce completely to 47–52 kDa ***(2,16)***. One component of gC-II is thought to be UL100, or the gM homologue ***(17)***. All three presently characterized HCMV glycoprotein spikes appear to have some role in HCMV entry into cells. Recently, it was discovered that low passage clinical isolates of HCMV contain an additional 13 kb of genetic information, potentially encoding as many as 12 additional glycoproteins ***(18)***. Although probably not part of the basic replication machinery, these glycoproteins may have specialized functions for entry into defined cell types.

1.2. The HCMV Entry Pathway and the Involvement of Cellular Molecules

HCMV infection is initiated by attachment to heparin sulfate proteoglycans (HSPGs) ***(19,20)***. Both gB and the gC-II complex

have heparin-binding ability ***(20–22)***. Attachment to HSPGs is dissociable but rapidly converts to a stable binding state presumably mediated by a second receptor ***(20)***. Both stable binding and HSPG binding can be measured at 4°C and are therefore thought to be attachment receptors. One candidate for the stable attachment was cellular annexin II ***(23,24)***. Although annexin II does form an association with the HCMV gB protein, annexin II is not required for entry and initiation of infection ***(25,26)***. Similarly, CD13 has been proposed as an attachment receptor; however, the requirement for CD13 can only be demonstrated in certain mouse cell lines ***(27)***. After HCMV is bound to the surface of cells, the pathway of entry for the virus is direct fusion between the viral envelope and the plasma membrane ***(28)***. At least one other cellular molecule is needed for fusion, as the virus can bind but not enter, some cell lines ***(23)***. In addition, antibodies blocking a 92.5 cellular protein recognized by gH prevent fusion ***(29,30)*** but the identity of this cellular protein is unknown.

2. Materials

2.1. Purification of Labeled Virions

1. Cell lines: Human fibroblasts (HF).
2. Cell culture medium: Dulbecco's minimal essential media (DMEM) (Biowhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, UT), 1.0% penicillin-streptomycin-fungizone (PSF; Biowhittaker), 0.3% L-glutamine (Biowhittaker) and 100 µg/mL geneticin (Gibco-BRL, Gaithersburg, MD).
3. The AD169 strain was used for all experiments.
4. Radiolabel. EXPRE^{35}S^{35}S Protein Labeling Mix (DuPont NEN, Boston, MA), containing both ^{35}S-methionine and ^{35}S-cysteine is used to label viral and recombinant proteins metabolically.
5. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl.
6. Sorbitol solutions: 20%, 30%, 40%, 50%, and 60% sorbitol solutions (w/v) were prepared in TBS.

2.2. Purification of Viral Ligands: Recombinant, Soluble gB in Baculovirus

1. Insect cells: Adherent cultures of *Trichoplusia ni* (TN-5) insect cells ***(11)*** were cultured in ExCell 401 media (JRH Biosciences, Lenexa, KS) supplemented with 5% FBS and 1% penicillin-streptomycin-fungizone (PSF; BioWhitaker).
2. Heparin agarose beads.
3. Phosphate-buffered saline (PBS) (10 m*M* $NaPO_4$, 140 m*M* NaCl, pH 7.4).
4. 0.65 *M* NaCl.
5. Nickel-nitrilotriacetic acid (NTA) agarose beads (Qiagen).
6. Binding buffer (100 m*M* $NaPO_4$, pH 7.8, 10% glycerol).
7. Wash buffer (15 m*M* imidazole in 50 m*M* $NaPO_4$, pH 6.0, 10% glycerol).
8. Elution buffer (0.5 *M* imidazole in 0.5 *M* $NaPO_4$, pH 7.8, 300 m*M* NaCl, 10% glycerol).

2.3. Purification of the gH-Complex from Cytomegalovirus Infected Cells

1. CNBr-activated Sepharose 4B (Pharmacia Biotech).
2. HCMV infected fibroblasts.
3. RIPA (1% NP-40, 0.5% DOC, 0.1% SDS, 5% glycerol in 150 m*M* NaCl, 50 m*M* Tris, pH 7.5, 1 m*M* EDTA).
4. Protease inhibitor cocktail (PIC): 2 µg/mL each of antipain, aprotinin, chymostatin, leupeptin, and pepstatin.
5. Iodoacetamide.
6. 100 m*M* glycine, pH 2.5.
7. 1 *M* Tris, pH 8.0.

2.4. Cellular Binding Assay

1. PBS-GC (10 m*M* Na_2HPO_4, 1.5 m*M* KH_2PO_4, 140 m*M* NaCl, 3 m*M* KCL, 0.5 m*M* $MgCl_2$.6H2O, 1 m*M* $CaCl_2$, 0.1% glucose, 1% inactivated FCS, pH 6.5).
2. 5 mg/mL ovalbumin.
3. Purified radiolabeled virions or ligands (described in **Subheadings 3.1.–3.3.**).

4. Lysis buffer (1% Triton X-100, 1% sodium dodecyl sulfate [SDS] in PBS).
5. Heparinase I or heparinase III (Sigma).
6. Soluble heparin (10-mg/mL stocks in PBS).

2.5. Virus Entry Assays: Detection of the Major Immediate-Early Protein (IE) by Immunofluorescence (IF)

1. Human fibroblasts grown on sterile glass coverslips.
2. Citrate inactivation buffer (40 m*M* citric acid/10 m*M* KCl/135 m*M* NaCl, pH 3.0).
3. 3% paraformaldehyde in PBS.
4. 0.1% Triton X-100 in PBS prepared from a 10% stock.
5. Gelatin.
6. Anti-species second antibodies conjugated to a fluorochrome. For example, rhodamine-conjugated goat anti-rabbit.
7. Hoechst dye (1 mg/mL water solution).

2.6. Detection of IE by Quantitative Western Blot

1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents.
2. Nitrocellulose membrane.
3. Horseradish peroxidase (HRP)-conjugated anti-species antibodies.
4. Chemiluminesence detection reagents (LumiGLO HRP Substrate Kit; Kirkegaard and Perry).

2.7. Virus Entry Assay: Uptake of pp65

1. Materials are the same as described in **Subheading 2.5.**

3. Methods

3.1. Purification of Labeled Virions

1. Infect human fibroblasts cells (HF) when they are approx 80% confluent with HCMV (MOI-1-5).
2. For ^{35}S-methionine labeled virions, at about 5 d postinfection, incubate the infected cells in methionine-free medium for 1 h, followed

by the addition of ^{35}S-methionine (25 µCi/mL) in deficient medium supplemented with 10% normal methionine.
3. Continue to incubate for 3–5 d.
4. Collect the supernatant and remove cells by centrifugation.
5. Concentrate virions by centrifugation at 20,000*g* through a 20% sorbitol cushion.
6. Resuspend virion pellet in TBS plus 100 µg/mL bacitracin and apply to 20–70% sorbitol step gradients.
7. Band virus by centrifugation at 100,000*g* for 1 h. Collect the 50–60% interface for complete enveloped virions.

3.2. Purification of viral ligands: recombinant, soluble gB in baculovirus. (protocol for a polyhistidine tagged, truncated gB, see Note 1)

1. Collect supernatant containing gB-S (either insect cell supernatant infected with a recombinant baculovirus encoding gB-S or stably transfected mammalian cells; ^{35}S-methionine labeled or not).
2. Passage the gB-S supernatant over a 5-mL heparin-agarose column by gravity flow.
3. Wash the column with at least 10 vol of PBS.
4. Elute gB-S (and other heparin-binding proteins) with 0.65 *M* NaCl.
5. Adjust the salt to 0.1 *M* and apply to a 1-mL nickel agarose column.
6. Wash with 10 vol of 100 m*M* $NaPO_4$/10% glycerol, pH 7.8, and 3 vol of the same buffer, pH 3.0.
7. Wash with 3 vol of the phosphate buffer containing 10 m*M* imidazole.
8. Elute gB-S with 500 m*M* $NaPO_4$/300 m*M*, NaCl/0.5 *M* imidazole/10% glycerol.
9. Dialyze gB-S against PBS, aliquot, and freeze at –80°C.

3.3. Purification of the gH Complex from Cytomegalovirus-Infected Cells

1. The anti gH monoclonal antibody, 14–4b, which recognizes a conformational epitope of the mature gH-containing complex [31] was used to generate an affinity column by covalent linkage to preactivated CNBr sepharose (Pharmacia), according to the manufacturer's instructions.
2. Collect HF cells that were infected with HCMV (MOI = 5) for 5–7 d.

3. Lyse the cells by the addition of 10 mL RIPA supplemented with PIC [15] and 10 m*M* iodoacetamide (*see* **Note 2**). Lyse on ice for 30 min and remove insoluble material by centrifugation.
4. Apply the lysate to the 14-4b column that was equilibrated with RIPA/PIC/10 m*M* iodoacetamide. Circulate the lysate over the column with a peristaltic pump.
5. Wash with 20 column volumes of 20 mL RIPA containing 0.1× detergents/PIC/iodoacetamide
6. Elute the gH complex with 3 mL 100 m*M* glycine, pH 2.5, neutralized immediately by the addition of 1.0 *M* Tris, pH 8.0.

3.4. Cellular Binding Assays

1. Plate HF cells in 24- or 96-well plates and incubate overnight. Cells can be treated with 3 U/mL heparinases to measure contribution of HSPGs or various antibodies to candidate receptors (*see* **Note 3**).
2. Wash the cells with PBS-GC plus 5 mg/mL ovalbumin for 30 min on ice.
3. Add labeled virions or purified ligand to cells in PBS-GC for 90 min on ice.
4. Remove unbound ligand and save for counting, wash two times with PBS-GC, and combine with unbound material.
5. Lyse the cell monolayer with 1% Triton X-100, 1% SDS in PBS, and count as the bound fraction.

3.5. Virus Entry Assays: Detection of IE by Immunofluorescence (IF)

1. Plate HF cells on sterile glass coverslips placed in the bottom of 24- or 12-well dishes.
2. Chill the cells at 4°C for 15 min before the addition of virus in the presence or absence of competing ligand, heparin, or receptor antibodies. Incubate for 90 min.
3. Shift the cultures to 37°C for 30 min, to allow for penetration.
4. Wash the cultures with a low pH citrate inactivation buffer for 1 min, followed by two washes with medium. This step inactivates any exterior but nonpenetrated virus.
5. Incubate at 37°C for 15–24 h.
6. Fix the cells with 3% paraformaldehyde in PBS for 20 min at room temperature.

7. Wash one time with PBS and permeabilize the membrane by the addition of 0.1% Triton X-100 in PBS for 10 min. Wash the cells twice with PBS plus 0.2% gelatin.
8. Incubate the cells with anti-IE antibody for 1 h (*see* **Note 4**).
9. Wash the cells three times with PBS plus 0.2% gelatin.
10. Incubate with the appropriate second antibody conjugated to a fluorochrome and 10 µg/mL Hoechst dye (to visualize the nucleus of all cells).
11. Score the number of IE-positive cells as a percentage of the total.

3.6. Detection of IE by Quantitative Western Blot

1. Perform **steps 1–5** as described in **Subheading 3.5**.
2. Harvest the cells by centrifugation and resuspend in 1× Laemmli sample buffer.
3. Resolve on 7.5% SDS-PAGE, and transfer to nitrocellulose.
4. Perform immune blot with anti-IE antibody, followed by HRP-second antibody (*see* **Note 4**).
5. Detect with chemiluminesence and image with a Bio-Rad Molecular Imager. Quantify with Molecular Analyst Software. Compare relative with mock-infected cells.

3.7. Virus Entry Assay: Uptake of pp65 (see Note 5)

1. Plate HF cells on sterile glass coverslips placed in the bottom of 24- or 12-well dishes.
2. Chill the cells at 4°C for 15 min before the addition of virus in the presence of absence of competing ligand, heparin, or receptor antibodies. Incubate for 90 min.
3. Shift the cultures to 37°C for 30 min, to allow for penetration.
4. Wash the cultures with a low pH citrate buffer (40 m*M* citric acid, 10 m*M* KCl, 135 m*M* NaCl, pH 3.0) for 1 min, followed by two washes with medium. This step inactivates any exterior but nonpenetrated virus.
5. Incubate at 37°C for 2–6 h.
6. Perform immunofluoresence protocol as above (**Subheading 2.3.1.1.**, **steps 6–11**), but use anti-pp65 antibody.

4. Notes

1. The protocol for purification of soluble gB (**Subheading 3.2.**) is for a recombinant protein containing a 6 histidine tag. We have sucessfully used other affinity tags such as streptavidin affinity ***(15)***.
2. Iodoacetamide is added during glycoprotein complex isolation to stabilize the disulfide bonding of the gC-III complex (**Subheading 3.3.**).
3. A variety of compounds can be added to the binding assays (**Subheading 3.4.**) as competitive inhibitors. For example, soluble heparin is frequently used as a negative control for HCMV binding. Antibodies can also be added to candidate receptor proteins.
4. Expression of the major immediate-early protein, a nonstructural protein synthesized soon after infection ***(32)*** can be used as a marker for entry and initiation of infection. Two formats that can be used to measure and detect IE expression are indirect immunofluorescence (IF) (**Subheadings 3.5.** and **3.6.**) and quantitative immune blots. Use of the immune blot protocol requires appropriate analytical software.
5. To confirm that inhibitory effects observed with IE gene expression are due to specific blocks in entry, rather than to a negative modulation of the transcription environment, follow uptake and nuclear localization of the major tegument protein, pp65 (**Subheading 3.7.**).

5. Challenges and Future Directions

HCMV is an opportunistic pathogen that causes diverse disease manifestations in immune deficient individuals. Intrinsic to the broad range of clinical syndromes associated with HCMV infection is the ability of the virus to infect cells of distinct and divergent developmental lineages, including epithelial, endothelial, neuronal, and blood cells. From the perspective of virus entry, this either means that the virus uses very broadly distributed cell surface molecules to facilitate the entry pathway or has distinct or overalapping or compensatory mechanisms available. It is likely that certain steps in the entry pathway may be conserved. For example, the initial attachment to the ubiquitously distributed HSPG molecules may facilitate stable attachment in most cell types. In addition, the fusion machinery responsible for the final aspect of penetration is prob-

ably conserved. However, considering the coding capacity of HCMV and the number of potential glycoproteins, specialized ligand–receptor interactions may mediate entry into distinct cell types. Similarly, functional compensation mechanisms may exist making negative phenotypes for null mutations difficult to interpret.

To establish definitively that a cellular molecule serves as an entry facilitator, one needs to express that molecule in a cell that is resistant to HCMV entry and confer the ability to enter when the molecule is present. Similarly, if a receptor is unknown, an entry-deficient cell can be used for expression cloning, as was recently done for the herpes simplex virus (HSV) entry mediator protein ***(33)***. The irony of HCMV is that while the virus has exquisite in vitro tropism for productive replication and infection, HCMV entry is quite promiscuous. The virus has the ability to bind, enter, and initiate infection in most laboratory cell lines ***(23)***; thus, we have lacked an important technical tool to identify cellular receptors. We recently found murine L cells to be highly resistant to HCMV entry, and this cell line may prove very useful in the future for HCMV receptor biology. Another approach that may prove useful is ligand affinity purification. As individual HCMV envelope glycoproteins become characterized with respect to their ligand properties, they may lend themselves to purification tools and the receptor identified by protein sequence analysis. Finally, entry analysis in biologically targeted cell types such as endothelial cells and monocytes/macrophages may yield important insights into the conservation and/or divergence of the HCMV entry mechanism as well as lend clues to the identity of cellular receptors.

References

1. Chee, M. S., et al. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154,** 125–169.
2. Gretch, D. R., Kari, B., Rasmussen, L., Gehrz, R. C., and Stinski, M. F. (1988) Identification and characterization of three distinct families of glycoprotein complexes in the envelopes of human cytomegalovirus. *J. Virol.* **62,** 875–881.

3. Gretch, D. R., Gehrz, R. C., and Stinski, M. F. (1988) Characterization of a human cytomegalovirus glycoprotein complex (gcI). *J. Gen. Virol.* **69,** 1205–1215.
4. Kari, B. and Gehrz, R. (1988) Isolation and characterization of a human cytomegalovirus glycoprotein containing a high content of O-linked oligosaccharides. *Arch. Virol.* **98,** 171–188.
5. Cranage, M. P., et al. (1986) Identification of the human cytomegalovirus glycoprotein B gene and induction of neutralizing antibodies via its expression in recombinant vaccinia virus. *EMBO J.* **5,** 3057–3063.
6. Britt, W. J. and Vugler, L. G. (1992) Oligomerization of the human cytomegalovirus major envelope glycoprotein complex gB (gp55–116). *J. Virol.* **66,** 6747–6754.
7. Kari, B., Goertz, R., and Gehrz, R. (1990) Characterization of cytomegalovirus glycoproteins in a family of complexes designated gC-II with murine monoclonal antibodies. *Arch. Virol.* **112,** 55–65.
8. Britt, W. J. (1984) Neutralizing antibodies detect a disulfide-linked glycoprotein complex within the envelope of human cytomegalovirus. *Virology* **135,** 369–378.
9. Pereira, L., Hoffman, M., Tatsuno, M., and Dondero, D. (1984) Polymorphism of human cytomegalovirus glycoproteins characterized by monoclonal antibodies. *Virology* **139,** 73–86.
10. Rasmussen, L., Mullenax, J., Nelson, R., and Merigan, T. (1985) Viral polypeptides detected by a complement dependent neutralizing murine monoclonal antibody to human cytomegalovirus. *J. Virol.* **55,** 274–280.
11. Rasmussen, L., Nelson, R., Kelsall, D., and Merigan, T. (1984) Murine monoclonal antibody to a single protein neutralizes the infectivity of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **81,** 876–880.
12. Cranage, M. P., Smith, G. L., Bell, S. E., Hart, H., Brown, C., Bankier, A. T., Tomlinson, P., Barrell, B. G., and Minson, T. C. (1988) Identification and expression of a human cytomegalovirus glycoprotein with homology to the Epstein-Barr virus BXLF2 product, varicella-zoster virus gpIII, and herpes simplex virus type 1 glycoprotein H. *J. Virol.* **62,** 1416–1422.
13. Kaye, J. F., Gompels, U. A., and Minson, A. C. (1992) Glycoprotein H of human cytomegalvirus (HCMV) forms a stable complex with the HCMV UL115 gene product. *J. Gen. Virol.* **73,** 2693–2698.

14. Spaete, R. R., Perot, K., Scott, P. I., Nelson, J. A., Stinski, M. F., and Pachl, C. (1993) Coexpression of truncated human cytomegalovirus GH with the UL115 gene product or the truncated human fibroblast growth factor receptor results in transport of gH to the cell surface. *Virology* **193,** 853–861.
15. Huber, M. T. and Compton, T. (1997) Characterization of a novel third member of the human cytomegalovirus glycoprotein H-glycoprotein L complex. *J. Virol.* **71,** 5391–5398.
16. Kari, B., Liu, Y. N., Goertz, R., Lussenhop, N., Stinski, M. F., and Gehrz, R. (1990) Structure and composition of a family of human cytomegalovirus glycoprotein complexes designated gC-I (gB). *J. Gen. Virol.* **71,** 2673–2680.
17. Kari, B., Li, W., Cooper, J., Goertz, R., and Radeke, B. (1994) The unman cytomegalovirus UL100 gene encodes the GC-II glycoproteins recognized by Group 2 monoclonal antibodies. *J. Gen. Virol.* **75,** 3081–3086.
18. Cha, T.-A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996) Human cytomegalovirus clinical isolates carry at least 19 gene not found in laboratory strains. *J. Virol.* **70,** 78–83.
19. Neyts, J., Snoeck, R., Schols, D., Balzarini, J., Esko, J. D., Van, S. A., and De, C. E. (1992) Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology* **189,** 48–58.
20. Compton, T., Nowlin, D. M., and Cooper, N. R. (1993) Initiation of human cytomegalovirus infection requires initial interaction with cell surface heparan sulfate. *Virology* **193,** 834–841.
21. Boyle, K. A. and Compton, T. (1997) Receptor binding properties of a soluble form of the glycoprotein B of human cytomegalovirus.
22. Kari, B. and Gehrz, R. (1992) A human cytomegalovirus glycoprotein complex designated gC-II is a major heparin-binding component of the envelope. *J. Virol.* **66,** 1761–1764.
23. Nowlin, D. M., Cooper, N. R., and Compton, T. (1991) Expression of a human cytomegalovirus receptor correlates with infectibility of cells. *J. Virol.* **65,** 3114–3121.
24. Wright, J. F., Kurosky, A., and Wasi, S. (1994) An endothelial cell-surface form of annexin II binds human cytomegalovirus. *Biochem. Biophys. Res. Commun.* **198,** 983–989.
25. Pietropaolo, R. and Compton, T. (1997) Cellular annexin II is not required for entry of human cytomegalovirus into fibroblast cells.

26. Pietropaolo, R. and Compton, T. (1997) Direct interaction between human cytomegalovirus glycoprotein B and cellular annexin II. *J. Virol.* in press.
27. Soderberg, C., Giugni, T. D., Zaia, J. A., Larsson, S., Wahlberg, J. M., and Moller, E. (1993) CD13 (human aminopeptidase-N) mediates human cytomegalovirus infection. *J. Virol.* **67,** 6576–6585.
28. Compton, T., Nepomuceno, R. R., and Nowlin, D. M. (1992) Human cytomegalovirus penetrates host cells by pH-independent fusion at the cell surface. *Virology* **191,** 387–395.
29. Keay, S. and Baldwin, B. (1992) The human fibroblast receptor for gp86 of human cytomegalovirus is a phosphorylated glycoprotein. *J. Virol.* **66,** 4834–4838.
30. Keay, S. and Baldwin, B. (1991) Anti-idiotype antibodies that mimic gp86 of human cytomegalovirus inhibit viral fusion but not attachment. *J. Virol.* **65,** 5124–5128.
31. Bogner, E., Reschke, M., Reis, B., Reis, E., Britt, W., and Radsak, K. (1992) Recognition of compartmentalized intracellular analogs of glycoprotein H of human cytomegalovirus. *Arch. Virol.* **126,** 67–80.
32. Stinski, M. F. (1990) Cytomegalovirus and its replication, in *Virology* (Fields, B. N., Knipe, D. M., et. al., eds.), Raven Press, New York, pp. 1959–1980.
33. Montgomery, R. I., Warner, M. S., Lum, B. J., and Spear, P. G. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87,** 427–436.

5

Analysis of Cytomegalovirus Gene Expression by Transfection

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1. Introduction

Human cytomegalovirus (HCMV), a human herpesvirus, is the leading viral cause of birth defects ***(1,2)***. In acquired immune deficiency syndrome (AIDS) patients, HCMV can cause severely debilitating colitis and retinitis; it is now an increasingly common cause of life-threatening pneumonitis in transplant patients ***(3–5)***. HCMV has a severely restricted host range in culture. It grows efficiently only in primary human diploid fibroblasts (HFF) and a few selected human cell lines ***(6–9)***. Monocytes serve as a major reservoir of latent HCMV in humans and HCMV can be grown in vitro in primary monocyte-derived macrophages (M/M) ***(10–14)***.

Analysis of HCMV gene function, gene regulation, and promoter sequences or enhancer elements, as well as isolation of HCMV viral mutants, have relied heavily on the use of transfection assays. Because of the cell culture growth properties of HCMV and of its host cells, these studies have been most often performed in permissive HFF cells or in nonpermissive, transformed human cells such as HeLa cells ***(15–17)***. HFF cells can be derived directly from human tissues such as foreskins or can be purchased from any of

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several vendors (Biowhittaker, Walkersville, MD; Viromed, MN). As primary diploid cells, HFF cells senesce within several passages and, for best results, should be used at an early passage number (*see* below). Although HeLa cells are nonpermissive for virus growth, they are nonetheless useful because of their human origin, the ease of their growth and handling and their high efficiency of gene expression after DNA transfection. Moreover, the patterns of transactivation by HCMV gene products in HeLa and HFF cells are often similar although the magnitude of gene expression is not ***(17)***. Nonetheless, caution is warranted in the interpretation of results obtained in nonpermissive cells, particularly with respect to events required for HCMV growth.

Although biologically significant for the study of HCMV gene expression during latency and reactivation, primary human M/M are not often used for transfection assays, as they are difficult to culture and transfect efficiently ***(18)***. M/M are commonly generated by differentiation of primary human monocytes obtained directly from healthy donors or via the American Red Cross (Baltimore, MD). Monocytes can be differentiated for these studies by allogeneic stimulation or treatment with phytohemagglutinin (PHA, Sigma-Aldrich, St. Louis, MO) or concanavalin A (Con A, Sigma-Aldrich) ***(13,14)***. Unlike HFF cells and HeLa cells, however, differentiated M/M can not be passaged in culture and must be repeatedly obtained from healthy donors and differentiated. The need to obtain specimens from different donors introduces an uncontrollable variable in the experimental materials. To overcome this difficulty, a number of laboratories have used established cell lines (e.g., THP-1, U937, or HL60 cells) of monocytic lineages for transient transfection studies.

HCMV gene expression after transfection can be studied either within the context of the whole viral genome or in the context of plasmids carrying particular genes of interest. To generate mutant progeny, infectious HCMV DNA (isolated from gradient purified virions) or partially overlapping cosmids reconstituting the HCMV genome are necessary (***19–21***) (*see* **Note 1**). Conversely, expression plasmids containing individual HCMV genes or clusters of HCMV genes may be used ***(15–17)***. Plasmid DNA or cosmid DNAs

to be used for transfections can be purified either by binding to Qiagen DNA columns (Qiagen, Valencia, CA, tip 500, #10063) or by double banding in cesium chloride (CsCl) gradients.

We have tested four commonly used methods of transfection to introduce HCMV genes or viral DNA into human cells: (1) calcium phosphate precipitation ***(15–17,24)***, (2) DEAE dextran ***(15)***, (3) electroporation ***(19,21)***, and (4) lipofection ***(22)***. Because of its efficiency, reproducibility, and simplicity, this chapter deals exclusively with lipofection of HFF or HeLa cells. Nonetheless, for specific purposes (*see* **Note 2**), we use the other protocols as published previously ***(15–17,24)***. To determine gene expression, dependent on the transfected indicator gene, we commonly use one or more enzymatic assays. The chloramphenicol acetyltransferase assay (CAT) ***(15–17)*** and β-galactosidase assay using chlorophenol red-β-D-galactoside (CPRG, Boehringer Mannheim, Indianapolis, IN) as a substrate are published ***(15,23)***. To determine the location of HCMV proteins, we have used indirect immunofluorescence (IFA) after transfection ***(24)***. To determine promoter usage and RNA abundance from the transfected DNA, we use primer extension analysis ***(15)***. This chapter details our unpublished modification of the luciferase assay, which is an exquisitely sensitive enzymatic assay.

2. Materials

2.1. Lipofection

1. LipofectAMINE Reagent (Gibco-BRL, Gaithersburg, MD, 18324-012).
2. 6-mL sterile tubes (12 × 75, Falcon, Becton Dickinson, Lincoln Park, NJ, 2063).
3. 6 deep-well cell culture plates (Falcon, 3046).
4. Sterile eppendorf tubes (1.5-mL, e.g., Sarstedt, Newton, NC, 72.688.999).
5. Opti-MEM I reduced serum media (Gibco-BRL, 31985-070).
6. Dulbecco's modified Eagle's medium (DMEM; Biowhittaker, 12-604F).
7. Fetal calf serum (FCS; HyClone, Logan, UT, SH30070).
8. Penicillin and streptomycin (P/S; Biowhittaker, 17-602E).

9. 1X Phosphate-buffered saline (1× PBS, Biowhittaker, 17-516F).
10. CO_2 Water jacketed incubator (e.g., Forma Scientific, Marietta, OH, model 3354).

2.2. Quantitation of Protein

1. 96-well Flexible microtiter assay plates (Falcon, 3912).
2. Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA, 500-0006).
3. Protein Standard (Bio-Rad, 500-0005).
4. Multichannel pipetter (Rainin, Woburn, MA, P220-M8).
5. Molecular Devices (Sunnyvale, CA) Plate Reader with a filter in the 570- to 600-nm range.

2.3. Luciferase Assays

1. Luciferase assay reagent (Promega, Madison, WI, E1483).
2. Cell culture lysis reagent (Promega, E1531).
3. Bovine serum albumin (Sigma, A–3425).
4. Luciferase (Roche Diagnosis, Indianapolis, IN, 411523).
5. Glass disposable scintillation vials (7 mL, Fisher, Scientific, Pittsburgh, PA, 03-337-26).
6. Beckman β-Scintillation Counter (such as LS6500) with Single Photon Monitor (Beckman Coulter, Fullerton, CA, Part 501006).

3. Methods

3.1. Lipofection

This protocol has been modified from the vendor's procedure for the optimal detection of HCMV immediate-early (IE) protein transactivation of viral promoters driving a luciferase indicator gene in HFF or HeLa cells. It can be readily modified, however, for the detection of other indicator enzymes or gene products.

1. Split HFF cells (1:2) or HeLa cells (1:3) about 18–24 h before transfection into 6-well plates with 2 mL of culture medium (DMEM/10% FCS/1% P/S). Incubate the cells overnight at 37°C in a 5% CO_2 incubator.
2. The next morning, examine cells for viability and cell density. If cells are 50–80% confluent, proceed with the transfection (*see* **Note 3**).

3. For each well, add 1 µg of DNA to 100 µL of Opti-MEM without serum or antibiotics in a sterile 6-mL tube (*see* **Note 4**). Separately add 6 µL of LipofectAMINE (2 mg/mL) to 100 µL of Opti-MEM. Mix DNA-Opti-MEM with the LipofectAMINE-Opti-MEM. Let the DNA-lipid complex form for 30–45 min at room temperature. Add 800 µL of Opti-MEM to DNA-LipofectAMINE mixture, and mix gently.
4. Meantime, wash cells twice with Opti-MEM without serum or antibiotics. Aspirate and add 1 mL of the LipofectAMINE-DNA mixture to the cells in each well. Incubate lipofected cells for 4 h at 37°C (*see* **Note 5**).
5. After incubation, add 1 mL of DMEM/20% FCS/2% P/S to each well. Incubate lipofected cells for an additional 20 h at 37°C (*see* **Note 6**).
6. Wash cells once with 1 mL of DMEM/10% FCS/1% P/S. Aspirate and add 2 mL of DMEM/10% FCS/1% P/S to each well. Incubate at 37°C.
7. Harvest HeLa cells 24 h later or HFF cells 72 h later (*see* **Note 7**). To harvest, wash cells twice with 1× PBS, aspirate, and add 50 µL of lysis buffer per well. Incubate at room temperature for 15 min. Scrape cells into lysis buffer and transfer lysate into sterile Eppendorf tubes. Store at –70°C until ready for assay (*see* **Note 8**).

3.2. Quantitation of Protein

We adapted the Bio-Rad Protein Determination assay to a microtiter plate format. This assay is sensitive and convenient for the simultaneous detection of minute amounts of protein from multiple transfection assays.

1. Prepare 1:5 dilution of Bio-Rad Protein Assay Reagent using ultrapure water. Aliquot 150 µL of diluted reagent per well in a 96-well microtiter plate into sufficient wells for standards, blanks, and unknown samples.
2. Make twofold serial dilutions of the protein standard (*see* **Note 9**). Add 10 µL of each standard (at least in duplicate) into each well with diluted reagent. Add 10 µL of water to each of two wells for the blank control.
3. Add 1–10 µL of each unknown sample (or 1:5 or 1:10 dilutions of unknown samples, as necessary) in duplicate into each of the remaining wells. At least one of the samples or its dilution must be within the linear range of the standard control.

4. Mix carefully (avoiding bubble formation) by pipetting repeatedly, using a multichannel pipetter. Let the reactions stand 5–10 min before reading.
5. Read microtiter plate assay using a plate reader with a filter within the 570- to 600-nm range. Determine protein concentration by linear regression of the standard curve and interpolation.

3.3. Luciferase Assays

The luciferase assay is a modification of the vendor's protocol, optimized for detection of rapidly decaying photons, using a single photon monitor in a β-scintillation counter (*see* **Note 10**).

1. Thaw the samples to be assayed and the luciferase assay reagent (protect from light) until they reach room temperature. Lower temperatures decrease luciferase activity.
2. Add 100-µL aliquots of the luciferase assay reagent into scintillation vials.
3. To initiate assay, use the single photon monitor program.
4. Prepare one blank sample by adding 20 µL of lysis buffer into the luciferase assay substrate in vial, mix by pipetting, and cap vial. Read sample immediately. Repeat at least 2 more times.
5. After blank samples have been read, assay 20 µL of samples as above. Repeat twice.
6. As a positive control for these reactions, use firefly luciferase. Resuspend the firefly luciferase in 0.5 *M* Tris-acetate buffer, pH 7.5. Aliquot and store at –70°C.
7. Thaw an aliquot of the enzyme, and make serial twofold dilutions in lysis buffer supplemented with BSA (1 mg/mL). Assay as above by adding 20 µL of diluted enzyme to 100 µL of luciferase reagent (*see* **Note 11**).

3.4. Internal Controls

Internal control plasmids encoding another indicator gene may be included and assayed separately. However, it is often the case that promoters driving the expression of the indicator gene in the internal control plasmids are regulated either positively or negatively by HCMV transactivators, limiting their use as an independent standard in these studies. It is therefore prudent to repeat these

experiments at least three times, to verify the experimental findings. Statistical analyzes are valuable in determining significant differences between groups.

4. Notes

1. The production of infectious progeny from HCMV DNA is exceedingly low compared with many other herpesviruses and is greatly increased by the cotransfection of an HCMV ppUL82 (pp71) expression vector ***(21)***.
2. Although lipofection introduces DNA efficiently into cells, this protocol alters the morphology of HFF cells dramatically. For assays that require intact cell morphology, such as IFA ***(17,24)***, we use calcium phosphate as the transfection method of choice. For production of infectious virus from DNA, electroporation is the method of choice ***(19,21)***.
3. At transfection, cells should be 50–80% confluent. We have found that HFF cells are sensitive to the toxicity of LipofectAMINE and, when less than 50% confluent, mostly die after lipofection. By contrast, if the HFF cells are more than 80% confluent, the DNA expression is quite low. Because HFF cells senesce rapidly with multiple passages in culture, we use HFF cells prior to passage 15 (about 45 cell doublings) for transfection experiments. Older HFF cells do not express transfected DNA efficiently.
4. We have tested various amounts of DNA transfected into HeLa cells, using the described protocol and found that 0.5–1 µg of DNA reach plateau levels of gene expression (**Fig. 1**). For experiments in which gene expression is to be inhibited (e.g., with dominant negative mutants), the transfected DNA amount should be in linear portion of curve (i.e., <0.5 mg in our assays). Otherwise, inhibitory effects may not be discerned. We have tested various ratios of lipid to milligrams of DNA and found the optimal ratio to be 12µg of LipofectAMINE per µg of DNA.
5. We have optimized the exposure time of HeLa cells to the DNA-LipofectAMINE mixture and found 4 h to be optimal (**Table 1**).
6. We tested different wash times at 20 and 24 h after DNA addition without significantly different results at either time.
7. The optimal harvest time for different assays should be determined experimentally (**Table 1**). For example, we found that transactivation

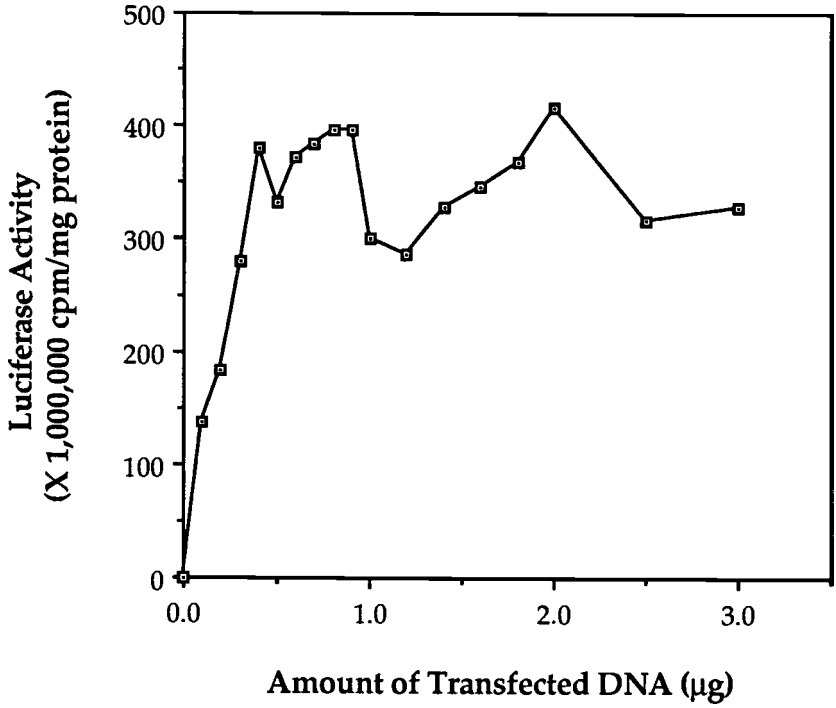

Fig. 1. Dose-responsive luciferase production after lipofection of HeLa cells. HeLa cells were lipofected as detailed above with different amounts of a plasmid carrying the firefly luciferase gene under the control of the Rous sarcoma virus LTR promoter *(25)*. Cell extracts were harvested at 48 h post-transfection and stored at –70°C. They were assayed as above and standardized to the protein concentration of each sample. The results shown are the average of four independent experiments.

of viral promoters in HFF cells was more efficient at 96 h post-transfection, whereas HCMV protein detection by IFA was improved at 40–48 h and transactivation of cellular promoters in HeLa cells was most efficient at 48 h post-transfection.

8. Avoid multiple freeze–thaw cycles. Luciferase activity is retained for at least two freeze–thaw cycles; nevertheless, repeated freeze–thaw cycles will result progressively in denaturation of proteins and their loss of solubility.
9. We have found that the linear range of this microtiter protein determination assay is within 1.4–0.02 mg/mL.

Table 1
Optimization of HeLa Cell Lipofection Using the HCMV Major IE Promoter-*lacZ* Expression Vector

	Average CPRG Conversion (OD_{570})[a] Harvest Time (h)		
Exposure Time (h)[b]	48	72	96
2 h	0.41	0.39	0.21
4 h	1.55	1.39	0.59
6 h	0.70	0.68	0.53
Blank[c]	0.07	0.07	0.07

[a]The CPRG assay was performed as described previously ***(23)***. Values for experimental groups shown are the averages of four samples and for blank, the averages of duplicate samples.

[b]Time of exposure to the DNA-LipofectAMINE complex.

[c]Water was added to the assays to obtain the blank value.

10. If a single photon monitor is unavailable, the coincidence circuit of the β-scintillation counter should be turned off. The photons produced by the luciferase reaction decay rapidly. It is therefore extremely important that the samples are read as fast as possible and with constant time between the addition of sample to the substrate.
11. The linear range for detection of luciferase activity by this assay is between 0.156 and 2 pg of luciferase. Thus, the luciferase assay is exquisitely sensitive and quantitative.

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References

1. Stagno, S., Pass, R. F., and Alford, C. A. (1981) Perinatal infections and maldevelopment. *Birth Defects* **17**, 31–50.

2. Britt, W. J. and Alford, C. A. (1996) Cytomegalovirus, in *Fields' Virology*, 3rd ed. (Fields, B. N., Knipe, D. M., and Howley, P. M., eds.), Lippincott-Raven Press, Philadelphia, pp. 2493–2523.
3. Ho, M. (1995) Cytomegalovirus, in *Principles and Practice of Infectious Diseases* (Mandell, G. L., Bennett, J. E., and Dolin, R., eds.), Churchill Livingstone, New York, Edinburgh, London, Melbourne, Tokyo, pp. 1351–1364.
4. Rubin, R. H. (1990) Impact of cytomegalovirus infection on organ transplant recipients. *Rev. Infect. Dis.* **12S7**, 754–766.
5. Winston, D. J., Ho, W. G., and Champlin, R. E. (1990) Cytomegalovirus infections after allogeneic bone marrow transplantation. *Rev. Infect. Dis.* **12S7**, S776–S792.
6. Gönczöl, E., Andrews, P. W., and Plotkin, S. A. (1984) Cytomegalovirus replicates in differentiated but not in undifferentiated human embryonal carcinoma cells. *Science* **224**, 159–161.
7. Smith, J. D. (1986) Human cytomegalovirus: demonstration of permissive epithelial cells and nonpermissive fibroblastic cells in a survey of human cell lines. *J. Virol.* **60**, 583–588.
8. Compton, T. (1993) An immortalized human fibroblast cell line is permissive for human cytomegalovirus infection. *J. Virol.* **67**, 3644–3648.
9. Sinzger, C. and Jahn, G. (1996) Human cytomegalovirus cell tropism and pathogenesis. *Intervirology* **39**, 302–319.
10. Taylor-Wiedeman, J., Sissons, P., and Sinclair, J. (1994) Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *J. Virol.* **68**, 1597–1604.
11. Kondo, K., Xu, J., and Mocarski, E. S. (1996) Human cytomegalovirus latent gene expression in granulocyte-macrophage progenitors in culture and in seropositive individuals. *Proc. Natl. Acad. Sci. USA* **93**, 11,137–11,142.
12. Sinclair, J. and Sissons, P. (1996) Latent and persistent infections of monocytes and macrophages. *Intervirology* **39**, 293–301.
13. Soderberg-Naucler, C., fish, K. N., and Nelson, J. A. (1997) Reactivation of latent human cytomegalovirus by allogenic stimulation of blood cells from healthy donors. *Cell* **91,** 119–126.
14. Lathey, J. L. and Spector, S. A. (1991) Unrestricted replication of human cytomegalovirus in hydrocortisone-treated macrophages. *J. Virol.* **65**, 6371–6375.

15. Colberg-Poley, A. M., Santomenna, L. D., Harlow, P. P., Benfield, P. A., and Tenney, D. J. (1992) The HCMV US3 and UL36–38 immediate early proteins regulate gene expression. *J. Virol.* **66**, 95–105.
16. Colberg-Poley, A. M., Huang, L., Soltero, V. F., Iskenderian, A., Schumacher, R. -F., and Anders, D. G. (1998) The acidic domain of pμL37x1 and gp μL37 plays a keyrole in transactivation of HCMV DNA replication gene promoters. *Virology* **246,** 400–408.
17. Zhang, H., Al-Barazi, H. O., and Colberg-Poley, A. M. (1996) The acidic domain of the human cytomegalovirus UL37 immediate early protein is dispensable for its transactivating activity and localization but is not for its synergism. *Virology* **223**, 292–302.
18. Aldovini, A. and Feinberg, M. B. (1990) Transfection of molecularly cloned HIV genomes, in *Techniques in HIV Research* (Aldovini, A. and Walker, B. D.), Stockton Press, New York, pp. 147–175.
19. Jones, T. R., Muzithras, V. P., and Gluzman, Y. (1991) Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. *J. Virol.* **65**, 5860–5872.
20. Kemble, G., Duke, G., Winter, R., and Spaete, R. (1996) Defined large-scale alteration of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. *J. Virol.* **70**, 2044–2048.
21. Baldick, C. J., Jr., Marchini, A., Patterson, C. E., and Shenk, T. (1997) Human cytomegalovirus tegument protein pp71 (ppUL82) enhances the infectivity of viral DNA and accelerates the infectious cycle. *J. Virol.* **71**, 4400–4408.
22. Iskenderian, A. C., Huang, L., Reilly, A., Stenberg, R. M., and Anders, D. G. (1996) Four of eleven loci required for transient complementation of human cytomegalovirus DNA replication cooperate to activate expression of replication genes. *J. Virol.* **70**, 383–392.
23. Eustice, D. C., Feldman, P. A., Colberg-Poley, A. M., Buckery, R. M., Neubauer, R. H. (1991) A sensitive method for detection of β-galactosidase in transfected mammalian cells. *BioTechniques* **11**, 739–743.
24. Al-Barazi, H. O. and Colberg-Poley, A. M. (1996) The human cytomegalovirus UL37 immediate-early regulatory protein is an integral membrane *N*-glycoprotein which traffics through the endoplasmic reticulum and Golgi apparatus. *J. Virol.* **70**, 7198–7208.
25. DeWet, J. R., Wood, K. V., DeLuca, M., Helinski, D. R., and Subramani, S. (1987) Firefly luciferase gene: structure and expression in mammalian cells. *Mol. Cell. Biol.* **7**, 725–737.

6

Analysis of Protein–Protein Interactions During Cytomegalovirus Infection

Richard C. Caswell

1. Introduction

A key area in the study of infection by cytomegalovirus (CMV), or that of any other virus, is to gain an understanding of the manner in which viral proteins interact with those of the host cell. The most widely used method to identify interactions between viral and cellular proteins in the infected cell is that of co-immunoprecipitation; lysates from infected cells are treated with antibody which recognises, say, a viral protein of interest, and the resulting immune complexes are then screened for the presence of a cellular protein of interest: the presence of the second protein in the immune complexes is indicative of an interaction between the two proteins in vivo. However, such interaction need not necessarily be direct, as immunoprecipitation of a viral protein that could interact with a single component of a multiprotein complex might be expected to co-precipitate all the proteins in that complex. Therefore assays might be said to demonstrate protein–protein associations in the cell, rather than direct interactions. The resolving power of co-immunoprecipitations is also limited in other respects. First, the success of the assay relies heavily on the quality of the immunological reagents used; it is also possible that antibody binding will actually disrupt the

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interaction to be studied. Second, interactions between regulatory proteins (whether regulation of transcription, translation, cell cycle, or other processes) are often of a dynamic or transient nature; for instance, a cellular protein which is complexed with other cellular proteins might only be able to interact with a viral protein once the cellular protein has been released from that complex, or alternatively, interaction might be regulated by protein modifications such as phosphorylation, which might in turn be regulated by the phase of the cell cycle. Such dynamic or transient interactions might be extremely difficult, if not impossible to detect by co-immunoprecipitation assays.

For these reasons, numerous methods have been developed for the analysis of protein–protein interactions in vitro. Of these, probably the most widely used involves expressing one of the proteins of interest as a fusion protein, though not necessarily in bacteria, in which the protein is "tagged" with a moiety facilitating rapid purification. The fusion protein can then be purified and immobilized on an appropriate matrix, and tested for its ability to bind from solution a second protein of interest, which has been expressed and radiolabeled in vitro. Any interaction between the two proteins results in retention of the labeled protein on the matrix; labeled proteins can then be eluted and are detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. This type of method offers a number of advantages for the study of protein interactions, in that assays are quick and relatively simple to perform, and it becomes possible to study interactions between two proteins in the absence of other factors which might otherwise compete for binding. Furthermore, by expressing fragments of the proteins of interest, it is possible to identify the regions responsible for interaction independent of the presence or biological activity of the whole protein. Such functional dissection might be difficult to perform using in vivo methods such as co-immunoprecipitation, particularly when the proteins to be studied are important for viability or replication; moreover, immunological reagents might not be available for defined subregions of the protein and, even if this were the case, as the region to be studied grows smaller, it is more likely

that antibody binding would disrupt interactions with other proteins. However, as with all in vitro methods, care must be taken to ensure that observations made using such systems are of biological significance; the identification of a novel protein–protein interaction is of limited value in the absence of appropriate functional data. A further consideration to be made when using this type of assay is the possible role that post-translational modifications of the proteins of interest might play in regulating interactions, and whether or not the expression system used supports such modifications.

A number of vector systems for the cloning and expression of fusion proteins in bacteria, and more recently, in various eukaryotic cell types are now available, and the properties of some of these are summarized in **Table 1**. Despite the apparent diversity, all these systems are designed to permit the expression (often to very high levels) and rapid purification of the fusion protein of interest. This chapter will concentrate specifically on the use of one of these systems, that for bacterial expression and purification of glutathione-*S*-transferase (GST) fusions ***(1)***, one that has been widely used by many laboratories in the study of protein–protein interactions in CMV infection ***(2–11)***. In vitro expression and radiolabeling of the protein to be tested for binding to the immobilised fusion protein can be performed using cDNA clones in any vector containing a bacteriophage RNA polymerase promoter (T3, T7, or SP6) flanking the cloned gene at the 5' end; commonly used vectors such as pBlueScript or pGEM and their derivatives are suitable. In addition, many mammalian expression vectors, such as pSG5 or pcDNA3, contain a T7 promoter between the eukaryotic promoter and the cloned gene, allowing the same plasmid construct to be used for both in vivo and in vitro expression of the protein. The most convenient method for in vitro expression is the coupled transcription/translation system, which as the name implies, allows both the transcription and translation reactions to be carried out in a single tube, using plasmid DNA containing the cDNA of interest as substrate.

In the following procedure, the specific examples given have been used to test interactions between the CMV immediate-early (IE) proteins, IE1 and IE2, and the cell cycle regulatory proteins p21 and p27.

Table 1
Some Systems Available for Expression and Rapid Purification of Fusion Proteins[a]

Fusion moiety	Affinity matrix	Host system	Vectors available
Glutathione-*S*-transferase (GST)	Glutathione Sepharose/agarose	*E. coli*	pGEX series (Pharmacia, AMRAD)
—	—	Yeast	pYEX (AMRAD); pESP (Stratagene)
Oligohistidine	Nickel-chelating resins	*E. coli*	pRSET, pTrcHis series (Invitrogen); various pET vectors (Novagen)
—	—	Insect (baculovirus)	pBlueBacHis (Invitrogen); pBacPAK-His (Clontech)
—	—	*Pichia pastoris*	pPICZ, pGAPZ vectors (Invitrogen)
—	—	Mammalian	pcDNA3.1/His, pSecTag, pEBVHis (Invitrogen)
Maltose-binding protein	Amylose resin	*E. coli*	pMAL series (NEB)
Calmodulin-binding peptide	Calmodulin resin	*E. coli*	pCAL series (Stratagene)
Protein A	IgG resins	*E. coli*	pRIT2T (Pharmacia)

[a]Full details of the various systems listed are available from the suppliers shown.

However, in principle, the techniques described can be used for the study of any protein–protein interaction.

2. Materials

2.1. Choice of Bacterial and In Vitro Expression Vectors

Obviously the nature of the plasmids used in experiments will be determined by the identity of the proteins of interest. Assuming that the two proteins to be tested for binding have been cloned previously, the requirements are for one cDNA to be present in a suitable vector with a bacteriophage RNA polymerase promoter 5' to the cloned gene and for the other to have been cloned into a suitable pGEX vector. In the case of the former, most cDNA clones will contain a region of 5' untranslated sequence, followed by the wild-type initiator methionine codon and the rest of the open reading frame (ORF); in such cases, the reading frame of the cloning sites used is not important (*see* **Note 1**). However, if a suitable GST fusion clone is not available for one of the proteins, this must be constructed by cloning the cDNA into one of the pGEX vectors as a translational fusion. In this case, it is vital that the cDNA is cloned in the same reading frame as the GST ORF of the vector. To facilitate this, a family of pGEX vectors have been developed containing polylinkers in all three reading frames, although it may be necessary to introduce a suitable restriction site at the 5' end of the gene of interest by PCR amplification using a mutagenic primer.

2.2. Expression and Purification of GST Fusion Proteins

1. Luria-Bertani (LB) broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl per liter.
2. Ampicillin (sodium salt), 100-mg/mL solution, filter-sterilized.
3. Isopropyl-β-D-thiogalactoside (IPTG), 0.1 *M* solution, filter-sterilized.
4. Phosphate-buffered saline (PBS) buffer: 150 m*M* NaCl, 16 m*M* Na_2HPO_4, 4 m*M* NaH_2PO_4, pH 7.3.
5. Phenylmethylsulfonyl fluoride (PMSF), 125 m*M* in acetone.

6. Soniprep 150 sonicator (MSE) or similar.
7. Triton X-100, 10% (v/v) solution in PBS.
8. Glutathione Sepharose 4B beads (Pharmacia, Uppsala, Sweden).
9. Glycerol, 20% (v/v) solution in PBS.
10. Equipment and reagents for SDS-PAGE ***(12)***, and Coomassie staining of gels.

2.3. Generation of Radiolabeled Probe Proteins

1. Plasmid DNA (*see* **Note 2**) containing the cDNA of interest in a suitable vector for in vitro expression (e.g., pBS-IE2, pSV_2-IE1, pGelsolin) ***(11)***.
2. Reagents for coupled transcription/translation in vitro (e.g., Promega TNT® kit) (*see* **Note 3**).
3. RNase inhibitor (e.g., RNasin; Promega, Madison, WI), if not included in the coupled transcription/translation kit (*see* **Note 3**).
4. L-[^{35}S] Methionine, translation grade, 1000 Ci/mmol, 10 mCi/mL (*see* **Note 4**).
5. Glycerol, 60% v/v solution in water.
6. Equipment and reagents for SDS-PAGE.
7. Fixing solution: 50% (v/v) methanol, 10% (v/v) acetic acid.
8. Fluorographic reagent for gels, e.g., Amplify® (Amersham, Arlington Heights, IL).
9. Gel dryer, autoradiography cassette, and X-ray film.

2.4. Protein–Protein Interaction Assay

1. GST fusion protein bound to Sepharose beads (*see* **Subheading 2.2.**).
2. Bovine serum albumin (BSA): 2-mg/mL solution.
3. ^{35}S-labeled protein (*see* **Subheading 2.3.**).
4. Nonidet P-40 nonionic detergent (*see* **Note 5**).
5. EBC buffer: 50 m*M* Tris-HCl, 140 m*M* NaCl, 10 m*M* NaF, 200 µ*M* Na_3VO_4, pH 8.0.
6. NET buffer: 20 m*M* Tris-HCl, 100 m*M* NaCl, 1 m*M* EDTA, pH 8.0.

3. Methods

3.1. Expression and Purification of GST Fusion Proteins

1. Inoculate 10 mL of LB broth, containing ampicillin at a final concentration of 100 µg/mL, with bacteria harbouring the appropriate recombinant pGEX plasmid, and grow overnight.

2. Use the starter culture to inoculate 400 mL fresh LB/ampicillin, and continue to grow cells at 30–37°C (*see* **Note 6**) until mid-log phase (i.e., to an absorbance at 600 nm of 0.5–0.6).
3. Add IPTG to a final concentration of 0.1 m*M*, and continue to grow for an additional 2–3 h (*see* **Note 6**).
4. Harvest cells by centrifugation at 2500*g* (4500 rpm in a Sorvall RC-5B, using GS3 or GSA rotor) for 20 min.
5. Resuspend cells in cold (4°C) PBS buffer; the exact volume used depends on how efficiently cells grow after IPTG induction, as expression of some proteins may be detrimental to cell growth, but is typically 2–8 mL.
6. Add PMSF to a final concentration of 0.5 m*M*. Cells are then disrupted by sonication, using a probe approx 5 mm in diameter at the tip, set at 15-μm amplitude. If possible, the sample should be sonicated in a small metal vessel, set in ice, to conduct away the heat generated. Sonication should be carried out in bursts of 15–30 s, allowing the sample to cool between bursts; a total of 60- to 120-s sonication is usually sufficient for adequate cell lysis (*see* **Note 7**).
7. Add 1/10 volume of 10% (v/v) Triton X-100 solution to the lysate, and mix gently by inversion. A final concentration of 1% (v/v) Triton X-100 will reduce nonspecific binding to the Sepharose beads but will not interfere with specific binding by GST fusion proteins. Centrifuge at 12,500*g* (12,000 rpm in a Sorvall SS34 rotor) for 15 min at 4°C. Efficient cell lysis will produce a straw-colored supernatant; if the supernatant remains pale or colorless, cells should be resuspended and subjected to a further round of sonication.
8. During centrifugation of the lysate, transfer 400 μL of a 50% (v/v) slurry of glutathione Sepharose 4B beads (*see* **Note 8**) into a universal tube, and wash with 3 × 10 mL cold PBS; between each wash, beads are harvested by centrifugation at 1000 rpm for 3 min in a benchtop centrifuge, and the supernatant removed by careful aspiration. After the final wash, remove the supernatant (it is not necessary to remove the last traces), and store on ice. If multiple GST fusions are to be purified, the bead pellet should be resuspended in PBS to the original volume, to yield a 50% slurry, and 400-μL aliquots of this slurry used for each sample.
9. Decant the cleared cell lysate (from stage 7) from the pellet of cell debris. Retain a small aliquot of the lysate for analysis by SDS-PAGE; then add the bulk of the lysate to the washed bead

slurry and incubate at 4°C for 30–60 min with constant gentle agitation (e.g., on a tube rotator).

10. Pellet the beads by centrifugation as before, and remove the supernatant. Depending on the level of expression of the particular protein, significant amounts of GST fusion may remain in solution after binding to the glutathione Sepharose beads, as judged by SDS-PAGE analysis. If this is the case, the amount of beads used to purify the GST fusion may be increased, to maximise recovery of the protein from the lysate.
11. Wash the beads with 4 × 10 mL cold PBS, as above. After the final wash, resuspend the beads in approx 1 mL PBS and transfer to a 2-mL microcentrifuge tube; rinse the universal tube used for washing the beads with a further 1 mL PBS, and add this to the bead slurry. Pellet the beads by centrifugation at 5000–6500 rpm for 30 s in a microcentrifuge, remove the supernatant, then add an equal volume of 20% (v/v) glycerol solution in PBS to the volume of packed beads and resuspend gently by inversion. Remove a small aliquot (10–20 µL) for analysis by SDS-PAGE; the beads should then be stored at –70°C pending use in protein–protein interaction assays.
12. Analyze samples by SDS-PAGE; the sample prepared from the beads should show a protein of approximately the size expected for a fusion of GST (27 kDa) to the protein of interest (**Fig. 1**). The concentration of GST fusion bound to the beads may vary quite widely according to how well the fusion is expressed in bacteria but will typically be with in the range of 0.1–5 µg/µL of bead slurry.

3.2. Generation of Radiolabeled "Probe" Proteins

1. Coupled in vitro transcription and translation reactions should be carried out in accordance with protocols supplied with the particular kit or system used. Generally, plasmid DNA is incubated at 30°C for 60–120 min in a reaction mixture containing the appropriate RNA polymerase (T7 RNA polymerase, in the case of the examples shown here) and nucleotide triphosphates, which provide the necessary components for the transcription reaction, in addition to reticulocyte lysate and all amino acids (including the radiolabeled amino acid), which provide the necessary components for translation of the RNA generated in the transcription reaction. An RNase inhibitor is also included,

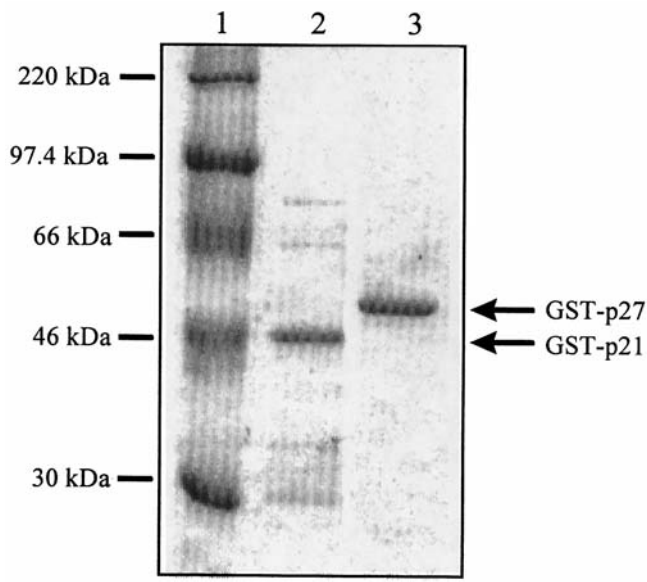

Fig. 1. Coomassie-stained SDS-PAGE gel showing GST-p21 and GST-p27 fusions after purification on glutathione Sepharose beads. Lane 1, Rainbow molecular-weight markers (Amersham); lane 2, GST-p21; lane 3, GST-p27. The pGEX vectors for the expression of GST fusions to p21 and p27 were a kind gift of T. Hunter, Salk Institute, La Jolla, CA, USA).

to prevent degradation of the RNA by possible nuclease contamination. Reactions are typically carried out in a final volume of 50 µL.

2. Following incubation of the coupled transcription/translation reaction, add 60% (v/v) glycerol solution to a final concentration of 10% glycerol (i.e., 10 µL per 50 µL reaction).
3. Remove a 3 µL aliquot for analysis by SDS-PAGE, and store the bulk of the reaction at –70°C.
4. Run one-half of the analytical sample on SDS-PAGE; after running, transfer the gel to fixing solution (15 min for 0.75-mm-thick gels, 30 min for 1–1.5-mm thick gels), rinse briefly in water; then transfer to the fluorographic reagent for 30 min.
5. The gel should then be dried onto a suitable support (Whatman 3MM paper is routinely used in this laboratory), then exposed to X-ray film overnight at –70°C. Bands of radiolabeled protein are usually clearly visible after an overnight exposure of gels loaded with 1.5 µL of reaction mixture.

3.3. Protein–Protein Interaction Assay

1. A few minutes before use, prepare sufficient EBC/N and NET/N buffer for use in binding assays from EBC and NET buffer stocks, by the addition of Nonidet P-40 to a final concentration of 0.5% (v/v). Nonidet P-40 is a nonionic detergent, which is included to reduce nonspecific protein binding; this should be thoroughly dispersed by stirring or gentle inversion, and buffers containing Nonidet P-40 should be freshly prepared for each experiment. Each reaction requires 0.18 mL EBC/N and 3 mL NET/N.
2. Each binding reaction uses 500 ng of GST fusion protein, bound to Sepharose beads, as the immobilized target. Transfer an appropriate volume of the beads, containing this amount of bound fusion protein, to a microcentrifuge tube; then adjust the volume of all samples to 10 µL by the addition of PBS-washed glutathione Sepharose 50% bead slurry (as prepared in **Subheading 3.1.8.**), which contains no bound protein. This controls for any nonspecific binding to the Sepharose matrix, and also makes the bead pellet easier to handle during subsequent washing steps. In addition to the specific GST fusion to be tested, reactions should also be carried out using beads bound with a negative control GST protein. This could be GST itself, prepared from bacteria transformed with the empty pGEX vector; however, in the examples shown here, GST-IE1 is used as a negative control, as this has been shown previously not to interact with any of the probe proteins used ***(11)***.
3. Add 10 µL of 2 mg/mL BSA, mix gently, and incubate at room temperature for 5 min.
4. Add 0.18 mL EBC/N buffer to each tube, then add an appropriate volume of the radiolabeled probe protein (*see* **Subheading 3.2.**). Again, the precise volume used will depend on the efficiency of expression and incorporation of the label in vitro, but is typically 0.5–5 µL. A negative control radiolabeled protein (gelsolin, in the examples shown here) should also be tested for binding to each GST fusion, and the amount of each probe protein used should be such that each would give a band of approximately equal intensity after autoradiography. This can usually be judged reasonably accurately by inspection of the autoradiograph of the analytical SDS-PA gel (*see* **Subheading 3.2.**) and will facilitate comparison of the degree of binding to GST fusions by the different probe proteins.

5. Incubate binding reactions for 1 h at room temperature, with constant gentle agitation (e.g., on a rocker or tube rotator).
6. Pellet the beads by centrifugation at 5000–6500 rpm for 30 s in a microcentrifuge, and remove the supernatant by careful aspiration. The supernatant will contain unbound radiolabeled protein, and unincorporated L-[^{35}S] methionine; it should be handled and disposed of accordingly.
7. Wash beads with 3 × 1 mL NET/N buffer, harvesting beads and removing supernatant at each step as above. After the final wash, remove as much supernatant as possible. If binding is to be compared across a number of reactions, equal volumes of beads from each reaction should be analyzed by SDS-PAGE. This can be achieved either by addition of SDS-PAGE sample buffer to the wet beads, to give an equal sample volume in each case, or as is routinely carried out in this laboratory, by drying the bead pellet in a vacuum centrifuge (Savant Speedvac, Illkirch, France, or similar) for 10–20 min at a medium heat setting, then resuspending the pellet in a known volume, typically 40 µL, of 1× SDS-PAGE sample buffer (*see* **Note 9**).
8. Bound proteins are eluted from the beads by boiling in SDS-PAGE buffer for 10 min. After boiling, samples are chilled on ice and then centrifuged at high speed in a microcentrifuge for 1 min. Analyze one-half of each sample by SDS-PAGE; then treat the gel for autoradiography as in **Subheading 3.2**. The gel should also be loaded with a reference sample of each radiolabeled probe protein used in the experiment. Typically, if one-half of the sample from each binding reaction is analyzed, reference lanes are loaded with 10% of the amount of probe protein used in each binding reaction; therefore, if the reference lane and the sample lane produce bands of equal intensity after autoradiography, this indicates retention of 20% of the labeled probe protein on the beads during the binding reaction (**Fig. 2**). In practice, 5% specific binding of the probe protein can readily be detected, while strong protein–protein interactions may result in the binding of greater than 25% of probe to the beads (*see* **Note 10**).

4. Notes

1. For cDNAs lacking an initiator codon, one can be introduced by PCR, using a mutagenic primer, at the same time as a 5' restriction site, by using sites that contain an ATG triplet (e.g., *Nco*I or *Nde*I).

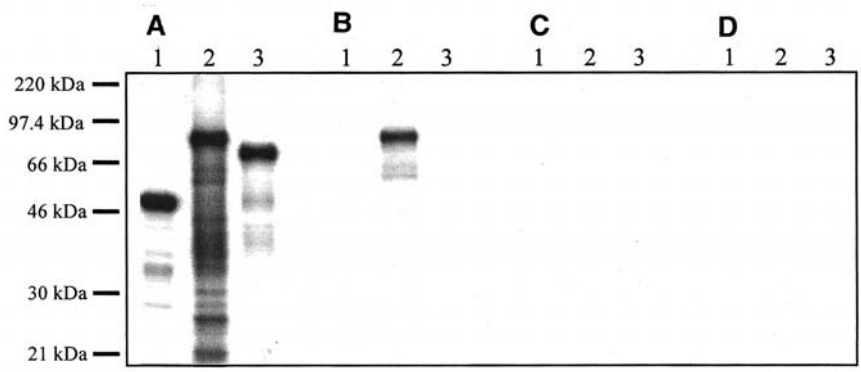

Fig. 2. Autoradiograph of SDS-PAGE gels showing that IE2 binds to GST-p21 but not GST-p27, and that IE1 binds to neither p21 nor p27. (A) The three radiolabeled probe proteins used. Each lane was loaded with 10% of the amount of protein used in each binding assay, as reference: lane 1, gelsolin (negative control); lane 2, IE2; lane 3, IE1. These proteins were used in binding assays with GST-p21 (B), GST-p27 (C), and GST-IE1 (D). In each case, one-half of the sample from the binding assay was analyzed by SDS-PAGE. Lane numbering in B–D corresponds to that in lane A. The position of molecular-weight markers is shown to the left.

In such cases, it is vital to ensure that the primer used contains the ATG triplet in the same reading frame as that of the following cDNA.

2. Although the efficiency of coupled transcription/translation reactions is usually higher using supercoiled plasmid DNA as template, linear DNA can also be used. This also provides a convenient way of producing nested C-terminal truncations of the protein, by digesting the plasmid with a restriction enzyme that cuts partway through the cDNA; processing RNA polymerase molecules simply drop off the template DNA at the point of digestion, and the resulting RNA is then translated to give a truncated protein. For instance, pBS-IE2 can be digested with either *Xho*I or *Stu*I, which after transcription/translation give truncated proteins containing amino acids 1–290 or 1–542, respectively. An enzyme that has more than one site in the plasmid may also be used; the only requirement is that there is no site between the phage RNA polymerase promoter and the start of the cDNA ORF.
3. Some commercial kits provide all reagents required needed for the coupled transcription/translation reaction, with the exception of the

radiolabeled amino acid to incorporated into the protein. Because these kits include a phage RNA polymerase, one should ensure that the kit purchased contains the appropriate enzyme for use with the cDNA clone; pBS-IE2, pSV_2-IE1, and pGelsolin all use T7 RNA polymerase. These kits will also include an RNase inhibitor, whereas with other systems this must be obtained separately. Whereas coupled transcription/translation provide the most convenient way of generating labeled protein from a cDNA clone, it is not actually necessary to carry out the coupled reactions in a single step and, if required, transcription and translation reactions can be carried out separately. The plasmid DNA template is first used in an in vitro transcription reaction, containing the appropriate RNA polymerase and nucleotide triphosphates, and subsequently the RNA generated is translated using nuclease-treated rabbit reticulocyte lysate.

4. Labelling of proteins in this laboratory is routinely carried out using L-[^{35}S] methionine, which is used in conjunction with an amino acid mixture lacking methionine. Kits such as the Promega TNT® Coupled Reticulocyte Lysate system are also supplied with an amino acid mixture lacking leucine, permitting incorporation of L-[^{14}C] leucine into the protein, which may be necessary for proteins that have little or no methionine content. However, it should be noted that at the time of writing, a recent development of this kit, the Promega TNT® T7 Quick system, is available only for use with L-[^{35}S] methionine as the radiolabeled amino acid.
5. Note that Nonidet P-40 (octylphenoxy polyethoxy ethanol) is not identical to NP-40 (nonylphenoxy polyethoxy ethanol) but is chemically indistinguishable from Igepal CA-630.
6. The exact conditions used for induction of fusion proteins may differ. Growth and induction at lower temperatures (e.g., 25–30°C), may improve the solubility of some fusion proteins, which might otherwise tend to form insoluble inclusion bodies in the host cells. If the fusion expressed is found to be detrimental to cell growth after induction, cells may be grown to a higher density before induction, then induced for a shorter period. Many fusion proteins are also subject to degradation by host cell proteases; this will be apparent when purified fusion proteins are analyzed by SDS-PAGE. Stability of the fusion protein may be improved by induction at lower temperatures; in our laboratory, we find that in some cases a higher proportion of nondegraded fusion protein is obtained if cells are grown to mid-log phase at 30°C, then allowed to cool to room temperature and induced

with IPTG overnight at room temperature, shaking the culture on a rotating platform. In addition, protease-negative strains of *E. coli* may be beneficial and have been shown to improve the stability of some fusion proteins dramatically ***(13)***. Determining which strain is best suited for the particular fusion protein of interest is a matter of trial and error, however, as strains that improve the expression of one fusion may not necessarily improve the stability of another.

7. Cell lysis can be enhanced if necessary by the addition of lysozyme to a final concentration of 0.5–1 mg/mL, after resuspension of cells in PBS/PMSF, followed by incubation on ice for 10 min, before sonication.
8. Glutathione Sepharose is supplied preswollen, as a 50% (v/v) slurry. If required, glutathione agarose can be used instead, although this is usually obtained as a powder and therefore requires swelling before use. However, Sepharose beads tend to form a more compact pellet and are therefore somewhat easier to handle during washing steps. This is especially important in the actual protein–protein interaction assay, as only small volumes of beads are used in the binding reactions.
9. The general method described above can be modified to estimate the strength of protein–protein interactions by varying the salt concentration. This can be done in either of two ways: (1) multiple reactions can be performed in which binding reactions are carried out in EBC/N buffer containing different salt concentrations (e.g., 0.1–0.5 *M* NaCl), followed by washing with NET/N as described; and (2) reactions can be performed using EBC/N at a single salt concentration, and after the final NET/N wash, proteins can be eluted stepwise by the addition of small volumes (e.g., 20 µL) of NET/N of progressively higher salt concentrations. At each elution step, beads would be allowed to equilibrate with the buffer for, say, 10 min, before centrifugation and removal of the supernatant for analysis by SDS-PAGE, followed by the application of the next aliquot of high-salt NET/N elution buffer. An alternative to this method would be to scale up the entire binding reaction by, say, fivefold and then, after washing, to split the beads into five equal aliquots, and elute each bead sample with NET/N of a different salt concentration.
10. Some proteins may give high levels of nonspecific binding. This may be reduced by increasing the amount of detergent in the EBC/N binding buffer, either by raising the Nonidet P-40 concentration, or by the additional presence of another detergent such as Sarkosyl,

typically at a concentration within the range 0.1–0.5% (v/v) final. However, the optimum conditions for specific binding should be determined empirically and may require different combinations of detergents for different protein–protein interactions.

References

1. Smith, D. B. and Johnson, K. S. (1988) Single step purification of polypeptides expressed in *E. coli* as fusions with glutathione-*S*-transferase. *Gene* **67**, 31–40.
2. Caswell, R. C., Bryant, L., and Sinclair, J. (1996) Human cytomegalovirus immediate-early 2 (IE2) protein can transactivate the human *hsp70* promoter by alleviation of Dr1-mediated repression. *J. Virol.* **70**, 4028–4037.
3. Hayhurst, G. P., Bryant, L. A., Caswell, R. C., Walker, S. M., and Sinclair, J. H. (1995) CCAAT box-dependent activation of the TATA-less human DNA polymerase α promoter by the human cytomegalovirus 72-kilodalton major immediate-early protein. *J. Virol.* **69**, 182–188.
4. Lang, D., Gebert, S., Arlt, H., and Stamminger, T. (1995) Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. *J. Virol.* **69**, 6030–6037.
5. Lukac, D. M., Manuppello, J. R., and Alwine, J. C. (1994) Transcriptional activation by the human cytomegalovirus immediate-early proteins: requirements for simple promoter structures and interactions with multiple components of the transcription complex. *J. Virol.* **68**, 5184–5193.
6. Margolis, M. J., Pajovic, S., Wong, E. L., Wade, M., Jupp, R., Nelson, J. A., and Azizkhan, J. C. (1995) Interaction of the 72-kilodalton human cytomegalovirus IE1 gene product with E2F1 coincides with E2F-dependent activation of dihydrofolate reductase transcription. *J. Virol.* **69**, 7759–7767.
7. Poma, E. E., Kowalik, T. F., Zhu, L., Sinclair, J. H., and Huang, E.-S. (1996) The human cytomegalovirus IE1–72 protein interacts with the cellular p107 protein and relieves p107-mediated transcriptional repression of an E2F-responsive promoter. *J. Virol.* **70**, 7867–7877.
8. Schwartz, R., Helmich, B., and Spector, D. H. (1996) CREB and CREB-binding proteins play an important role in the IE2 86-kilodalton

protein-mediated transactivation of the human cytomegalovirus 2.2-kilobase RNA promoter. *J. Virol.* **70**, 6955–6966.

9. Scully, A. L., Sommer, M. H., Schwartz, R., and Spector, D. H. (1995) The human cytomegalovirus IE2 86-kilodalton protein interacts with an early gene promoter via site-specific DNA binding and protein–protein interactions. *J. Virol.* **69**, 6533–6540.
10. Yoo, Y. D., Chiou, C.-J., Choi, K. S., Yi, Y., Michelson, S., Kim, S., Hayward, G. S., and Kim, S.-J. (1996) The IE2 regulatory protein of human cytomegalovirus induces expression of the human transforming growth factor β1 gene though an Egr-1 binding site. *J. Virol.* **70**, 7062–7070.
11. Caswell, R. C., Hagemeier, C., Chiou, C.-J., Hayward, G., Kouzarides, T., and Sinclair, J. (1993) The human cytomegalovirus 86K immediate early (IE) 2 protein requires the basic region of the TATA-box binding protein (TBP) for binding and interacts with TBP and transcription factor TFIIB via regions of IE2 required for transcriptional regulation. *J. Gen. Virol.* **74**, 2691–2698.
12. Laemmli, U. K. (1970) Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* **227**, 680.
13. Nakano, H., Yamazaki, T., Ikeda, M., Masai, H., Miyatake, S., and Saito, T. (1993) Purification of glutathione *S*-transferase fusion proteins as a non-degraded form by using a protease-negative *E. coli* strain, AD202. *Nucleic Acids Res.* **22**, 543–544.

7

Analysis of Protein–DNA Interactions During Cytomegalovirus Infection

Stefan Flöß and Thomas Stamminger

1. Introduction

One characteristic of human cytomegalovirus (HCMV) is the high complexity of its genome: the double-stranded DNA of approx 240 kbp contains the coding capacity for more than 200 different proteins ***(1,2)***. The genes encoding those proteins are expressed coordinately during the replication of this virus ***(3)***. Although post-transcriptional regulation has been described for HCMV, transcriptional regulation by promoter activation is believed to play a key role in mediating the cascade fashion of gene expression observed during the replicative cycle ***(4,5)***. Both viral and cellular proteins contribute to promoter activation. For instance, during the immediate-early (IE-) phase of gene expression, a strong enhancer-promoter drives transcription from the major IE gene region ***(6,7)***. This enhancer-promoter can both be regulated by cellular proteins such as transcription factors of the ATF/CREB and NF-κ-B family and viral proteins such as the tegument constituents pp71 and ppUL69 or the immediate early protein IE2-p86 ***(8–12)***.

In addition to interactions of proteins with other polypeptides, the identification of direct contacts with specific DNA-sequences

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appears to be of paramount importance in order to understand the complex process of promoter activation. The basic methods for detecting protein–DNA interactions are the gel retardation assay and various protection methods such as DNase I protection analysis ***(13,14)***. The main advantage of gel retardation analysis is its high sensitivity, whereas DNase I protection analysis is able to localize the site of contact more accurately. Once the site of interaction has been identified, several additional methodologies (e.g., hydroxyl radical interference, methylation, and ethylation interference) are available for fine mapping of protein contacts on DNA. One problem often encountered when performing studies on protein–DNA interactions is the fact that transcription factors are minor constituents of the cells total protein mass. Although a protein may have clear DNA-binding activity, one might not be able to detect this with protein extracts from cells, as the respective factor is either present in low amounts or is rapidly degraded in whole cell or nuclear extracts. By contrast, the presence of multiple proteins within a complex mixture of polypeptides may mask a DNA-binding site due to competition of other factors for the respective *cis*-acting sequence. Although it can be argued that this may reflect the in vivo situation, a potential interaction site may be missed that could turn out to be of importance for promoter regulation.

Therefore, in order to identify and map a contact site of a protein with DNA accurately we prefer prokaryotically expressed transcription factors as a source of protein for DNA interaction studies. This circumvents many problems encountered with cell or nuclear extracts such as the limiting amount of active protein. In addition, it permits the rapid identification of functional protein domains by using deletion mutants of the investigated factor. For prokaryotic expression, we have made extensive use of His-tagged polypeptides that permit one-step purification under denaturing conditions by metal chelate affinity chromatography ***(15)***. Although the purified protein has to be renatured in order to regain function, this method proved useful even for factors that could not be expressed and purified using alternative systems such as fusions with glutathione-S-transferase. Moreover, the respective factor is only minimally

modified either at the N- or the C-terminus by the addition of a few amino acids encoding the His-tag.

Using this approach, we were able to identify a DNA-binding activity of the IE2-p86 protein of HCMV, which is the major transactivator of viral early gene expression ***(16)***. This chapter describes the procedures for prokaryotic expression and purification of IE2-p86 and the subsequent use of this protein in gel retardation and DNase I protection analysis. However, the described procedure has also been used for expression of various ATF/CREB factors ***(17)*** and is easily adaptable to further transcription factors and viral proteins for which the cDNA is available. In addition, the gel retardation and DNase I protection assays are generally applicable and can also be performed using nuclear extracts ***(18)***, although the results critically depend on the quality of the extract used and may not be as unequivocal as with purified proteins.

2. Materials

2.1. Construction of a Prokaryotic Expression Vector for IE2-p86

For successful expression of the IE2-p86 protein as a His-tagged polypeptide, the respective cDNA has to be cloned in-frame with the coding sequence for six consecutive histidines into a prokaryotic expression vector. This can be achieved by insertion of a synthetic oligonucleotide encoding the six histidines into a preexisting prokaryotic expression vector, at either the amino- or the C-terminus of the respective protein ***(16)***. Alternatively, various His-tag expression vectors with multiple cloning sites in all three reading frames are available from several companies (e.g., from Qiagen, Santa Clarita, CA, from Novagen, Madison, WI).

1. Plasmid DNA containing the coding sequence of interest. For subcloning experiments we used a pBS plasmid containing the full length IE2-p86 cDNA which had been constructed by PCR ***(19)***.
2. Restriction enzyme: *Sal*I. Enzyme buffer supplied by the manufacturer.

3. 1X TA buffer: 40 m*M* Tris-acetate, 2 m*M* EDTA, pH 8.0. For staining of nucleic acids ethidium bromide is added at a concentration of 0.5 µg/mL.
4. Agarose of low melting temperature (e.g., from Bio-Rad Laboratories, München, Germany).
5. Elutip-d chromatography columns (Schleicher and Schüll, Dassel, Germany).
6. Low salt buffer: 0.2 *M* NaCl, 20 m*M* Tris-HCl, pH 7.3, 1.0 m*M* EDTA. High salt buffer: 1.0 *M* NaCl, 20 m*M* Tris-HCl, pH 7.3, 1.0 m*M* EDTA.
7. Phenol/chloroform ***(20)***, chloroform/isoamylalcohol (24:1), ethanol, 70% ethanol solution, sterile H_2O.
8. Prokaryotic His-tag expression vector pQE10 (from Qiagen, Santa Clarita, CA).
9. Shrimp alkaline phosphatase (from USB, Cleveland, OH).
10. 4 *M* lithium chloride solution.
11. T4 DNA ligase, 5X ligase buffer supplied by manufacturer (e.g., Gibco-BRL, Gaithersburg, MD).
12. Competent *Escherichia coli* DH5α.
13. Sequencing primer for pQE10 vector: 5'GGCGTATCACGAGGCC CTTTCG3'.

2.2. Expression and Purification of His-Tag Fusion Proteins

1. Bacterial strain M15[rep4] (from Qiagen) (*see* **Note 1**).
2. Luria broth: 10 g tryptone, 5 g yeast extract, and 10 g NaCl/L H_2O.
3. Kanamycin, ampicillin.
4. Isopropyl-β-D-thiogalactoside (IPTG).
5. 1 *M* Imidazole.
6. Ni^{2+}-NTA resin (from Qiagen).
7. Lysis buffer: 6 *M* guanidine hydrochloride, 0.01 *M* Tris-HCl, pH adjusted to 8.0 with NaOH (*see* **Note 2**).
8. Wash buffer: 8 *M* urea, 0.01 *M* Tris-HCl, 20 m*M* imidazole, pH adjusted to 8.0 with NaOH (*see* **Note 2**).
9. Elution buffer: 8 *M* urea, 0.01 *M* Tris-HCl, 200 m*M* imidazole, pH adjusted to 8.0 with NaOH (*see* **Note 3**).
10. Disposable 1-mL polypropylene columns for chromatography (e.g., from Qiagen).

11. SDS-PAGE equipment ***(20)***.
12. Microdialyzer system 500 (e.g., from Pierce, Rockford, IL).
13. Buffer D: 20 m*M* HEPES, pH 8.0, 20 m*M* KCl, 1 m*M* $MgCl_2$, 0.5 m*M* DTT, 0.5 m*M* PMSF, 0.1 m*M* EDTA, pH 8.0, 20% glycerol. Add DTT and PMSF immediately before use. PMSF is highly toxic and should be prepared as a 100 m*M* stock solution in ethanol, which is stable for 1–2 wk at –20°C.
14. Bio-Rad protein assay (Bio-Rad).

2.3. Gel Retardation Analysis

2.3.1. Preparation of the DNA Probe

1. Synthetic oligonucleotides corresponding in sequence to a putative binding site of a protein. For gel retardation analysis of IE2-p86, we used oligonucleotides representing the *cis*-repressive sequence (CRS) of the IE1/2 enhancer-promoter of HCMV ***(21)***. CRS, CGTTTAGTGAACCGTCAGAT//CTAGATCTGACGG-TTCAC TAAACGAGCT.
2. T4 polynucleotide kinase (e.g., from Gibco-BRL). Reaction buffer supplied by the manufacturer.
3. γ-^{32}P-ATP (e.g., from Amersham).
4. Gel loading buffer (6X): 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in H_2O.
5. 10X TBE buffer, stock solution: 108 g Tris base, 55 g boric acid, 4 mL 0.5 *M* EDTA, pH 8.0 , add H_2O to a final volume of 1 L.
6. 12% Nondenaturating polyacrylamide gel: prepare the gel solution by mixing 5 mL 10X TBE, 15 mL of a 40% acrylamide/bisacrylamide (19:1) solution and 30 mL H_2O. Start the polymerization reaction by adding 300 µL of a 10% ammonium persulfate solution and 50 µL TEMED. Pour the gel and allow to polymerize for 1 h.
7. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
8. Equipment for autoradiography ***(20)***.

2.3.2. DNA Binding Reaction and Gel Electrophoresis

1. Binding buffer (10X): 250 m*M* Tris-HCl, pH 8.0, 62.5 m*M* $MgCl_2$, 5 m*M* EDTA, pH 8.0, 5 m*M* DTT.
2. Nonspecific competitor DNA: 1 µg/µL calf thymus DNA (Merck, Darmstadt, Germany) (for alternative competitors, *see* **Note 6**).

3. 80% Glycerol in H_2O.
4. 4% Nondenaturating polyacrylamide gel: Prepare the gel solution by mixing 4.8 mL 2% bisacrylamide solution, 16 mL 30% acrylamide solution, 6 mL 10× TBE, 240 µL 1 *M* $MgCl_2$, 60 µL 1 *M* DTT and 92.86 mL H_2O. Start the polymerization reaction by adding 720 µL of a 10% ammonium persulfate solution and 120 µL TEMED. Pour the gel and allow to polymerize for 1 h. We use glass plates that are 28 × 18 cm (w × l) in size, 1.5-mm spacer and a comb with 1.5 × 2 cm (w × l) slots.

2.4. DNase I Protection Analysis

2.4.1. Preparation of Probe for DNase I Protection Analysis

1. Plasmid DNA containing a fragment with a putative binding site of the protein to be analyzed. The fragment should be flanked by unique restriction enzyme sites in order to release the probe from the plasmid backbone.
2. Appropriate restriction enzymes. Enzyme buffers supplied by the manufacturer.
3. Shrimp alkaline phosphatase (from USB).
4. TE buffer: 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
5. Phenol/chloroform, chloroform/isoamyl alcohol, 4 *M* lithium chloride solution, ethanol, 70% ethanol solution.
6. T4 polynucleotide kinase and Klenow enzyme (e.g., from Gibco-BRL). Reaction buffers supplied by the manufacturer.
7. γ-^{32}P-ATP and α-^{32}P-dXTP (X indicates a nucleotide complementary to one found in the 5' overhang produced by the restriction enzyme) (e.g., from Amersham).
8. Gel loading buffer (6×): 0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol in water.
9. 10X TBE buffer, stock solution: 108 g Tris base, 55 g boric acid, 4 mL 0.5 *M* EDTA, pH 8.0, dissolve in 1 L H_2O.
10. 6% Nondenaturing polyacrylamide gel: prepare the gel solution by mixing 7.5 mL of a 40% 19:1 acrylamide/bisacrylamide solution with 5 mL 10X TBE buffer and 37.5 mL H_2O. Start the polymerization reaction by the addition of 300 µL of a 10% ammonium persulfate solution and 50 µL TEMED. Pour the acrylamide gel mix between the plates and insert the appropriate comb. Allow to polymerize for at least 30 min.
11. Equipment for autoradiography ***(20)***.

2.4.2. DNase I Footprinting Reaction

1. Nonspecific competitor-DNA: e.g., 1 µg/µL calf thymus DNA (e.g., from Merck), 1 µg/µL poly(dA-dT)-poly(dA-dT), 1 µg/µL poly-(dG-dC)-poly(dG-dC) (e.g., from Pharmacia Biotech Europe, Freiburg, Germany) (*see* **Note 6**).
2. MSK buffer: 20 m*M* KCl, 4 m*M* $MgCl_2$, 4 m*M* spermidine.
3. DNase I (e.g., grade 2, from Roche Diagnostics, Indianapolis, IN). Dissolve in 500 m*M* Tris-HCl, pH 7.5, 100 m*M* $MgCl_2$ at a concentration of 1 mg/mL. Store in aliquots at –80°C.
4. Stop buffer: 20 m*M* EDTA, pH 8.0, 0.2 *M* NaCl, 50 µg/mL yeast tRNA, 0.5 mg/mL proteinase K (add proteinase K immediately before use).
5. Phenol/chloroform/isoamyl alcohol (25:24:1), ethanol.
6. Sequencing gel-loading buffer: 98% deionized formamide, 10 m*M* EDTA, pH 8.0, 0.025% xylene cyanol, 0.025% bromphenol blue.
7. 6% Sequencing gel: Dissolve 21 g urea in a solution containing 5 mL 10X TBE buffer, 7.5 mL 40% acrylamide/bisacrylamide (19:1), and adjust the volume with H_2O to 50 mL. Add 300 µL of a 10% ammonium persulfate solution and 50 µL TEMED. Pour between glas plates and insert the appropriate comb.
8. Equipment for autoradiography ***(20)***.

3. Methods

3.1. Construction of a Prokaryotic Expression Vector for IE2-p86

1. Digest 20 µg of pBS plasmid containing IE2-p86 cDNA with 20 U of *Sal*I at 37°C for 3 h.
2. Carry out electrophoresis of the digested plasmid on a 1% agarose gel (1 g agarose/100 mL 1X TA buffer containing 0.5 µg/mL ethidium bromide) at 120 V for 1 h.
3. Visualize the band of interest using a long-wavelength ultraviolet (UV) lamp.
4. Using a scalpel, cut out a gel slab in front of the band of interest. Fill in a 1% low- melting-temperature agarose (in 1X TA-buffer).
5. When the agarose of low melting temperature has solidified, continue electrophoresis until all DNA to be recovered has entered the agarose.

6. Cut out a slice of agarose of low melting temperature containing the band of interest and transfer to a disposable 10 mL plastic tube.
7. Add about 10 vol of low salt buffer and melt the agarose by incubation for 10 min at 65°C. Then, cool the sample to 37°C.
8. Purify the DNA by Elutip-d column chromatography. For this, prime the column with 2 mL of high salt buffer and wash with 5 mL of low salt buffer that has been prewarmed to 37°C. Apply the sample at a low flow rate (2 drops/s). Wash with 3 mL of low salt buffer and elute the DNA with 0.4 mL of high salt buffer.
9. Add an equal volume of phenol/chloroform to the DNA solution, mix by vortexing, and spin at 13,000 rpm in a microcentrifuge for 5 min. Transfer the aqueous layer to a new microcentrifuge tube. Repeat the extraction using an equal volume of chloroform/isoamylalcohol.
10. Add 1 mL of ethanol to the eluted DNA. Incubate the mixture for 15 min at –80°C, and then recover the DNA by centrifugation at 13,000 rpm for 15 min, using a microcentrifuge. Carefully remove the ethanol and add 1 mL of 70% ethanol at room temperature. Recentrifuge for 5 min at 13,000 rpm. Dry under vacuum for 10 min, and resuspend the pelleted DNA in 20 µL of sterile H_2O.
11. Digest 4 µg of pQE10 expression plasmid with 10 U of *Sal*I in a volume of 50 µL at 37°C for at least 3 h. Include 10 U of shrimp alkaline phosphatase in the reaction mix in order to dephosphorylate the DNA.
12. Add 150 µL of TE buffer. Extract the DNA with an equal volume of phenol/chloroform and chloroform/isoamylalcohol as described in **step 9**.
13. Precipitate the vector DNA by the addition of 1 vol 4 *M* lithium chloride solution and 2.5 vol of ice-cold ethanol. Incubate at –80°C for 15 min, and then centrifuge at 12,000 rpm for 15 min. Remove the ethanol, wash the pellet in 70% ethanol solution, recentrifuge for 5 min, and dry under vacuum for 10 min. Resuspend the vector DNA in 20 µL of sterile H_2O.
14. Set up ligation reaction containing 2 µL of 5X ligase buffer, 1 µL of dephosphorylated vector DNA, 2 µL of gel-purified insert, 1 U of T4 DNA ligase, and 4 µL of sterile H_2O. Incubate at 14°C overnight.
15. Transform competent *E. coli* DH5α cells with 1 µL of the ligation mixture. Identify clones with the correct insertion by restriction analysis, and confirm the in-frame fusion of the IE2-p86 cDNA to the N-terminal His-tag by nucleotide sequence analysis.

3.2. Expression and Purification of His-Tagged Fusion Proteins

1. Transform competent *E. coli* M15 cells containing the pREP4 plasmid with the pQE10IE2 expression plasmid (*see* **Note 1**). Plate transformants on LB-agar plates containing 25 µg/mL kanamycin and 100 µg/mL ampicillin. Incubate overnight at 37°C.
2. Inoculate 100 mL of Luria broth containing 25 µg/mL kanamycin and 100 µg/mL ampicillin with the recombinant bacteria. Grow the culture overnight at 37°C.
3. Dilute the overnight culture 1 in 10 with prewarmed Luria broth containing the appropriate antibiotics. Grow the cells at 37°C for a further 2 h.
4. Add IPTG to a final concentration of 0.5 m*M*. Incubate for an additional 2–3 h at 37°C.
5. Harvest cells by centrifugation (5000 rpm in a Sorvall GS-3 rotor for 10 min).
6. Resuspend cells in 10 mL of lysis buffer containing 6 *M* guanidine hydrochloride.
7. Stir the cell suspension for 1 h at room temperature using a magnetic stirrer.
8. Remove insoluble material by centrifugation of the extract at 10,000 rpm in a Sorvall SS34 rotor for 10 min. Transfer the supernatant to a new tube and add 1 m*M* imidazole (*see* **Note 2**).
9. Prepare a 1 mL Ni^{2+}-NTA column: mount the column and rinse it once with 5 mL H_2O. Mix about 2 mL of Ni^{2+}-NTA resin with an equal volume of H_2O. Pour the slurry into the column and leave to settle. Rinse the column with 10 mL of H_2O.
10. Equilibrate the Ni^{2+}-NTA column with 5 mL of lysis buffer. Stop the flow of the column, and apply the clarified protein solution at a slow flow rate (1–2 drops/s).
11. Wash the column with 2 mL of lysis buffer; then wash with 10 mL of wash buffer in order to reduce the amount of contaminating proteins that bind with low affinity to the Ni^{2+}-NTA resin (*see* **Note 2**).
12. Elute the recombinant His-tag protein with elution buffer. Collect fractions of 500 µL and determine the OD_{280}. Assay the peak fractions by SDS-PAGE.
13. Renature the recombinant protein by dialysis against buffer D for 24 h at 4°C using a microdialyzer system (*see* **Note 3**).

14. Clarify the extract by centrifugation at 13,000 rpm for 10 min at 4°C, using a microcentrifuge. Store in aliquots at –80°C.
15. Analyze 1 aliquot of the dialyzed protein by SDS-PAGE. Determine the protein concentration using the Bio-Rad protein assay.

3.3. Gel Retardation Analysis

The gel retardation analysis is a very sensitive assay to detect protein/DNA interactions. This method is based on the effect that a protein–DNA complex migrates slower during native polyacrylamide gel electrophoresis than DNA alone. The oligonucleotide or DNA fragment used as probe is radioactively end-labeled and can be visualized by autoradiography. Owing to the high sensitivity of this assay, proteins that bind to DNA in a nonspecific fashion can also result in a retardation of the probe. Therefore, the binding specificity of a protein has to be demonstrated by competition experiments using various nonlabeled oligonucleotides and DNA fragments as specific or nonspecific competitors.

3.3.1. Preparation of the DNA Probe

The probes for bandshifting that contain the putative protein binding site should be as small as possible (ideally <100 bp), because this minimizes the binding of proteins that interact nonspecifically with DNA. This is most easily achieved by using synthetic oligonucleotides (e.g., CRS oligonucleotide, containing a high affinity binding site for IE2-p86), which can be labeled to a high specific activity using T4 polynucleotide kinase (*see* **Note 5**).

1. Mix 200 ng of each oligonucleotide with 2.5 µL 10X kinase buffer, 50 µCi γ-^{32}P- ATP, 25 U T4 polynucleotide kinase and add H_2O to a final volume of 25 µL. Incubate for 60 min at 37°C.
2. For annealing of the oligonucleotides, incubate for 4 min at 94°C, 10 min at 65°C, 10 min at 37°C, 10 min at room temperature, and 5 min on ice. Add 6 µL gel loading buffer.
3. Prepare a 12% nondenaturing polyacrylamide gel for purification of the probe. Load the sample and perform gel electrophoresis with 1X TBE as running buffer. Run at 180 V until the bromophenol blue marker has reached two-thirds of the gel.

4. Autoradiograph the gel for 1 min in order to locate the required band. Cut out the band with the minimum amount of polyacrylamide using a scalpel.
5. Transfer the gel slice to a 1.5-mL microcentrifuge tube. Puncture a hole in the bottom of the tube, and place inside another one. Spin in a microcentrifuge until all the polyacrylamide is in the lower tube. Add 200–500 µL TE buffer (depending on the amount of polyacrylamide) and incubate for 12 h at 4°C in a shaking incubator.
6. Pellet the gel fragments for 5 min, using a microcentrifuge. Transfer the supernatant solution containing the radioactive probe to a new tube, carefully avoiding any polyacrylamide pieces.
7. Remove an aliquot of the probe for Cerenkov counting. Adjust the volume to give 10,000 cpm/µL.

3.3.2. DNA Binding Reaction and Gel Electrophoresis

1. Set up the reaction by mixing 1.5 µL 10X binding buffer, 1.5 µL glycerol, 1µL nonspecific competitor DNA, 15,000–20,000 cpm DNA probe, recombinant IE2-p86 and H_2O to a final volume of 15 µL (increasing the volume ≤40 µL is possible). It is useful to titrate the recombinant polypeptide by adding increasing amounts of the protein in order to determine the optimal binding conditions. As a control, prepare a reaction without protein.
2. The specificity of the interaction can be determined by competition experiments: Prepare additional reactions with a constant amount of IE2-p86 and perform titrations with various nonspecific competitor DNAs (e.g., heterologous oligonucleotides) using amounts between 100 ng and 2µg. The formation of specific protein–DNA complexes should not be affected, even in the presence of high amounts of nonspecific competitor. Perform also titrations with a specific competitor (e.g., CRS oligonucleotide, without radioactive label), using amounts of 1–200 ng. The intensity of bands that represent specific protein–DNA complexes should be reduced with low amounts of the specific competitor.
4. Incubate the samples for 20 min at room temperature (*see* **Note 7**).
5. Load the samples directly onto a 4% nondenaturing polyacrylamide gel that has been pre-run for 1 h at 100 V, using 0.5X TBE as running buffer. Load 5 µL gel loading buffer into an additional gel slot as marker (*see* **Note 8**).

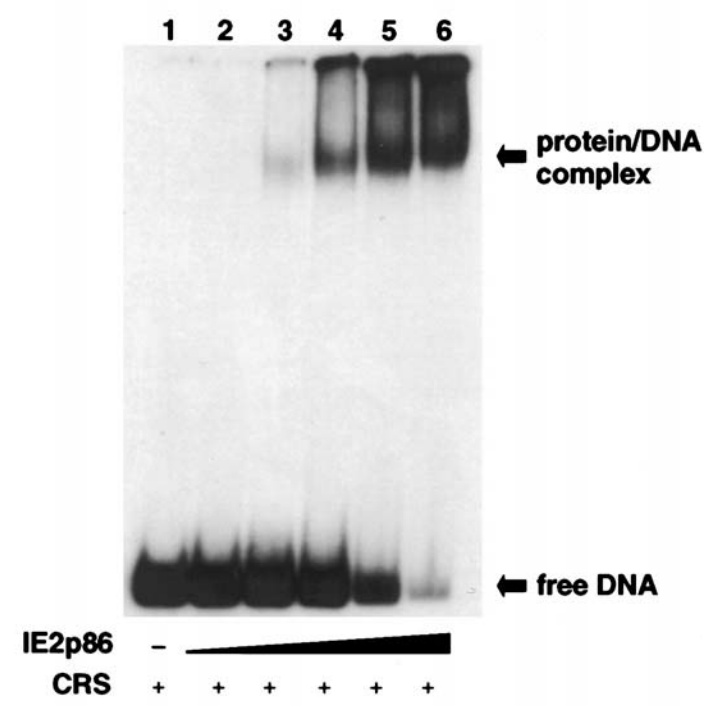

Fig. 1. Gel retardation analysis of prokaryotically expressed IE2-p86, using the CRS-element as probe. The labeled CRS oligonucleotide was either incubated without IE2-p86 (lane 1) or with increasing amounts of the recombinant protein (lanes 2–6). After incubation for 20 min, the reactions were separated via nondenaturing PAGE. Arrows at the right of the autoradiograph indicate the positions of free DNA and protein/DNA complexes.

4. Run the gel at 10.5 V per cm gel length until the bromophenol blue marker has reached about three-fourths of the gel (*see* **Note 9**).
5. Dry the gel on Whatman 3MM paper under vacuum for 1 h at 80°C.
6. Autoradiograph the gel for 10–20 h with intensifying screens at –80°C (**Fig. 1**).

3.4. DNase I Protection Analysis

The method (also termed DNase I footprinting) is essentially a comparison of the DNase I cleavage pattern of a known DNA fragment in the absence or presence of a protein or proteins that bind to

specific DNA sequences. This method is able to localize the site of interaction on a DNA fragment containing a binding site for the protein under investigation (e.g., IE2-p86). In order to visualize the cleavage pattern by autoradiography, the DNA fragment should be labeled radioactively, generally using ^{32}P.

3.4.1. Preparation of Probe for DNase I Protection Analysis

Probes for DNase I protection analysis are linear, double-stranded (ds) DNA molecules that contain a radioactive label at one specific end of the DNA. The ideal size of this fragment is 100–350 bp in order to allow good resolution on sequencing gels. Specific end-labeling of a fragment is achieved by (1) cleavage of a plasmid containing the respective fragment with a restriction enzyme, (2) labeling with either polynucleotide kinase or Klenow enzyme, (3) cleavage with a second enzyme in order to release the labeled fragment from the plasmid, and (4) purification of the probe by polyacrylamide gel electrophoresis.

1. Linearize 20 µg of plasmid DNA containing the respective fragment to be labeled in a volume of 50 µL by using the appropriate restriction enzyme, which should leave a 5' overhang. If labeling by polynucleotide kinase is intended, include 10 U of shrimp alkaline phosphatase in the reaction mix.
2. Add 150 µL of TE buffer. Extract with phenol/choroform, chloroform/isoamylalcohol and precipitate the DNA as described in **Subheading 3.1.**, **steps 12** and **13**.
3. For 5' labeling of probes, incubate the linearized and dephosphorylated DNA for 1 h at 37°C with 10 U of polynucleotide kinase in the presence of 40 µCi of γ-^{32}P-ATP using the reaction buffer supplied by the manufacturer.
4. Alternatively, for 3' labeling of probes, set up the reaction in 1X Klenow buffer with 50 µCi α-^{32}P-dXTP (where X is a nucleotide complementary to one found in the 5' overhang produced by the restriction enzyme), 1 µL dNTP mix (containing the other three dNTPs) and 5 U of Klenow enzyme. Incubate at room temperature for 30 min.
5. Proceed as described in **step 2**.
6. Perform a second restriction enzyme digestion to release the labeled fragment from the plasmid vector.

7. Add gel loading buffer, and load mixtures from the second cleavage reaction directly onto a 6% nondenaturing polyacrylamide gel containing 1X TBE buffer (when pouring the gel, use comb with wide slots). Run at 180 V until the bromphenol blue marker has reached about two-thirds of the gel.
8. Proceed as described in **Subheading 3.3.1.**, **steps 4–7**.

3.4.2. DNase I Footprinting Reaction

DNase I cleavage has to be optimized in order to create conditions that result on average in one cleavage reaction per DNA molecule. For this, the appropriate reaction conditions have to be determined empirically by titrating the amount of DNase I and analysing the cleavage pattern, which should show a rather even distribution of all fragment lengths. This has to be done for each combination of probe and protein extract, as DNase I cleavage may be inhibited in the presence of additional polypeptides.

1. Set up the binding reaction: mix 1 µL of nonspecific competitor DNA, 5 µL protein, and 3 µL of MSK-buffer for each reaction. For control lanes, use 5 µL of dialysis buffer without protein. Incubate for 10 min on ice (*see* **Note 10**).
2. Add 2 µL (= 20,000 cpm) probe to the reaction mixture and leave for a further 10 min on ice (*see* **Note 7**).
3. Prepare dilutions of DNase I using ice-cold water: for the control reactions (no protein added) prepare dilutions of 2.5–40 µg/mL, for the test reactions (recombinant protein added) use 10–200 µg/mL DNase I (*see* **Note 11**).
4. Add 2 µL of appropriately diluted DNase I to the reactions. Mix by pipetting, and incubate for exactly 90 s at 20°C.
5. Stop the reaction by the addition of 100 µL stop buffer. Mix vigorously.
6. Incubate for 15 min at 37°C and then, heat for 2 min to 95°C.
7. Add 200 µL phenol/chloroform/isoamylalcohol, vortex, and centrifuge for 5 min at 13,000 rpm using a microcentrifuge.
8. Transfer the aqueous phase to a new 1.5-mL microcentrifuge tube. Carefully avoid to take material from the interphase.
9. Precipitate the DNA by the addition of 200 µL ice-cold ethanol, incubation at –80°C for 10 min and centrifugation at 13,000 rpm for 15 min.

10. Air-dry the samples for 30 min, and resuspend in 4 µL sequencing gel-loading buffer.
11. Heat the samples at 90°C for 5 min, and load immediately onto a 6% sequencing gel. In parallel, load a Sanger sequencing reaction of a known nucleotide sequence to provide precise size markers (*see* **Note 12**).
12. Run the gel until the bromophenol blue marker has reached the bottom of the gel. After electrophoresis, dry the gel and autoradiograph at –80°C, using intensifying screens (**Fig. 2**).

4. Notes

1. The plasmid pREP4 is a multicopy plasmid that carries the *neo* gene for selection with kanamycin and the *lac*I gene encoding the *lac* repressor. Overexpression of the lac repressor is required in order to ensure repression of protein expression without induction when using the pQE10 vector. This is critical in cases in which the expressed protein has toxic effects on cell growth. As an alternative, *E. coli* strains containing the *lac*I^q gene (e.g., JM109, TG1) can also be used, however, regulation may not be as tight as with *E. coli* strains carrying the pREP4 plasmid.
2. Great care should be taken to ensure the proper pH of the lysis and wash buffers, as the His-tagged protein cannot bind to the Ni^{2+}-NTA under conditions of low pH (<pH 8.0). Because of the dissociation of urea, the pH of the solutions should be checked immediately before use.
3. Imidazole can bind to the Ni^{2+}-NTA and displace the tagged protein. High concentrations of imidazole (100–200 m*M*) are used for elution of the recombinant protein. However, it is recommended to add imidazole at low concentrations (1–50 m*M*) to the protein extract and the wash buffer in order to remove contaminating *E. coli* proteins that bind with low affinity to the Ni^{2+}-NTA resin. Optimal concentrations of imidazole for washing and elution, which will vary for each protein, can be determined by eluting with an imidazol gradient.
4. For many proteins, renaturation of at least a fraction of the polypeptide can simply be achieved by careful dialysis against buffer D. Use dilute solutions of the purified protein to avoid the formation of insoluble aggregates. For some proteins, a stepwise dialysis against buffer D containing decreasing concentrations of urea may result in an improved refolding. Moreover, the addition of salt (≤1 *M* NaCl), glycerol (≤30%) and various nonionic detergents at low concentra-

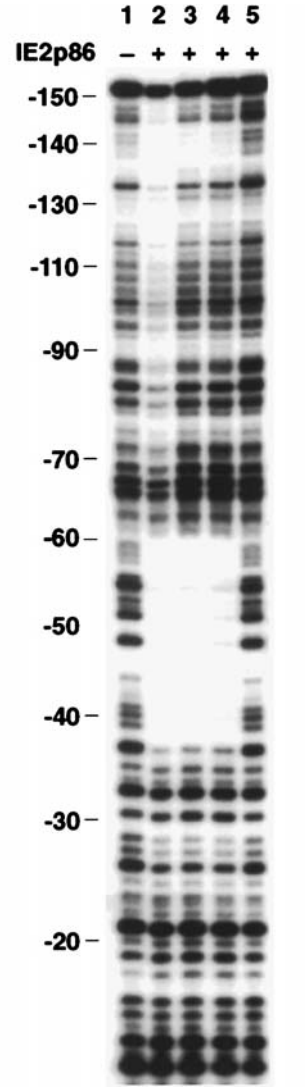

Fig. 2. DNase I protection analysis of the UL84 promoter region with the recombinant IE2-p86 of HCMV. A DNA fragment comprising the UL84 promoter of HCMV was used as probe. Lane 1, no added protein; lanes 2–5, recombinant IE2-p86 was added; lane 4, specific competition was performed by the addition of 100 ng of unlabeled CRS oligonucleotide; lane 5, nonspecific competition was performed by the addition of 100 ng of a heterologous oligonucleotide. Numbers on the left refer to nucleotide positions of the UL84 promoter.

tions (e.g., 0.1% Triton X-100, 0.1% Tween-20) may help keep the protein in solution.

5. Alternatively, labeling of oligonucleotides with protruding 5' ends can be achieved by a filling-in reaction with Klenow polymerase. Restriction enzyme fragments labeled as described in **Subheding 3.4.1.** are suitable both for gel retardation analysis and DNase I footprinting, however, should be no longer than 100 bp when nuclear extracts are used. For purified proteins with a known DNA-binding activity, longer fragments are possible.
6. Various DNAs can be used as nonspecific competitors. They are added in high excess in order to avoid an interaction of the radioactive probe with proteins that bind DNA without sequence specificity. Most widely used nonspecific competitors are the synthetic homopolymers poly(dI-dC)-poly(dI-dC), poly(dA-dT)- poly(dA-dT) and poly(dG-dC)-poly(dG-dC). Note that some protein–DNA interactions are sensitive to high concentrations of certain homopolymers. We have observed that IE2-p86 of HCMV binds with high affinity to poly(dI-dC)-poly(dI-dC); the addition of this nonspecific competitor to the DNA binding reaction will therefore result in a disruption of the specific protein–DNA complex ***(16)***. This has also been observed for other transcription factors such as the TATA-binding protein TBP. Alternatively, calf thymus DNA or poly(dA-dT)-poly(dA-dT) can be used in the case of IE2-p86. When a new protein–DNA interaction is investigated, it is worth trying various nonspecific competitors in order to exclude a negative effect.
7. The temperature used for incubation of DNA-binding reactions can be varied between 4°C and 37°C. Most factors bind well to DNA at 4°C. Incubation at 4°C has the advantage that DNases and proteases are inhibited; this is most useful when working with crude cellular or nuclear extracts. Some factors, however, such as the TATA binding protein TBP, require energy in order to interact with DNA: DNA-binding of TBP is only observed after incubation at 30°C for 20 min. If the optimal incubation temperature is unknown for the factor under investigation, several conditions should be tested.
8. Since we observed that the addition of gel loading buffer to the gel retardation reaction can have a destabilizing effect on some protein–DNA interactions, we omit this: the reactions are loaded directly onto the polyacrylamide gel. As a marker, gel loading buffer is added in an empty slot of the gel.

9. Performing the gel run at 4°C in a cold room can increase the stability of some protein/DNA complexes during electrophoresis.
10. As described in **Subheading 3.3.2.**, for the gel retardation reaction competition with specific and nonspecific oligonucleotides can also be performed in the DNase I footprinting reaction. For this, 1–200 ng of oligonucleotide should be included in the reaction mix.
11. We have observed that DNase I cleavage is inhibited considerably when using extracts with high protein concentrations such as nuclear extracts. The amount of DNase I has to be adjusted accordingly.
12. Alternatively, a Maxam and Gilbert sequencing reaction of the footprinted DNA fragment can be loaded as a size marker. However, we have observed that a Sanger reaction of a known DNA sequence using ^{35}S-d-ATP as the labeled nucleotide permits an accurate measurement of protected regions and avoids the use of hazardous chemicals required for the Maxam and Gilbert reactions.

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References

1. Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A., Kouzarides, T., Martignetti, J. A., et al. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154,** 125–169.
2. Cha, T. A., Tom, E., Kemble, G. W., Duke, G. M., Mocarski, E. S., and Spaete, R. R. (1996) Human cytomegalovirus clinical isolates carry at least 19 genes not found in laboratory strains. *J. Virol.* **70,** 78–83.
3. Wathen, M. W. and Stinski, M. F. (1982) Temporal patterns of human cytomegalovirus transcription: mapping the viral RNAs synthesized at immediate early, early, and late times after infection. *J. Virol.* **41,** 462–477.
4. Goins, W. F. and Stinski, M. F. (1986) Expression of a human cytomegalovirus late gene is posttranscriptionally regulated by a 3'-end-

processing event occurring exclusively late after infection. *Mol. Cell. Biol.* **6,** 4202–4213.

5. Stamminger, T., Puchtler, E., and Fleckenstein, B. (1991) Discordant expression of the immediate-early 1 and 2 gene regions of human cytomegalovirus at early times after infection involves posttranscriptional processing events. *J. Virol.* **65,** 2273–2282.
6. Thomsen, D. R., Stenberg, R. M., Goins, W. F., and Stinski, M. F. (1984) Promoter-regulatory region of the major immediate early gene of human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **81,** 659–663.
7. Boshart, M., Weber, F., Jahn, G., Dorsch-Hasler, K., Fleckenstein, B., and Schaffner, W. (1985) A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus. *Cell* **41,** 521–530.
8. Stamminger, T., Fickenscher, H., and Fleckenstein, B. (1990) Cell type-specific induction of the major immediate early enhancer of human cytomegalovirus by cyclic AMP. *J. Gen. Virol.* **71,** 105–113.
9. Winkler, M. and Stamminger, T. (1996) A specific subform of the human cytomegalovirus transactivator protein pUL69 is contained within the tegument of virus particles. *J. Virol.* **70,** 8984–8987.
10. Liu, B. and Stinski, M. F. (1992) Human cytomegalovirus contains a tegument protein that enhances transcription from promoters with upstream ATF and AP-1 cis-acting elements. *J. Virol.* **66,** 4434–4444.
11. Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W., and Mocarski, E. S. (1989) NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *EMBO J.* **8,** 4251–4258.
12. Pizzorno, M. C. and Hayward, G. S. (1990) The IE2 gene products of human cytomegalovirus specifically down-regulate expression from the major immediate-early promoter through a target sequence located near the cap site. *J. Virol.* **64,** 6154–6165.
13. Fried, M. and Crothers, D. M. (1981) Equilibria and kinetics of lac repressor-operator interactions by polyacrylamide gel electrophoresis. *Nucleic Acids Res.* **9,** 6505–6525.
14. Galas, D. J. and Schmitz, A. (1978) DNAse footprinting: a simple method for the detection of protein-DNA binding specificity. *Nucleic Acids Res.* **5,** 3157–3170.
15. Crowe, J., Dobeli, H., Gentz, R., Hochuli, E., Stuber, D., and Henco, K. (1994) 6xHis-Ni-NTA chromatography as a superior technique in recombinant protein expression/purification. *Methods Mol. Biol.* **31,** 371–387.

16. Lang, D. and Stamminger, T. (1993) The 86-kilodalton IE-2 protein of human cytomegalovirus is a sequence-specific DNA-binding protein that interacts directly with the negative autoregulatory response element located near the cap site of the IE1/2 enhancer-promoter. *J. Virol.* **67,** 323–331.
17. Lang, D., Gebert, S., Arlt, H., and Stamminger, T. (1995) Functional interaction between the human cytomegalovirus 86-kilodalton IE2 protein and the cellular transcription factor CREB. *J. Virol.* **69,** 6030–6037.
18. Lang, D., Fickenscher, H., and Stamminger, T. (1992) Analysis of proteins binding to the proximal promoter region of the human cytomegalovirus IE-1/2 enhancer/promoter reveals both consensus and aberrant recognition sequences for transcription factors Sp1 and CREB. *Nucleic Acids Res.* **20,** 3287–3295.
19. Plachter, B., Britt, W. J., Vornhagen, R., Stamminger, T., and Jahn, G. (1993) Analysis of proteins encoded by IE-regions 1 and 2 of human cytomegalovirus using monoclonal antibodies generated against recombinant antigens. *Virology* **193,** 642–652.
20. Sambrook J., Fritsch E. F., and Maniatis T. (1989) *Molecular Cloning—A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
21. Cherrington, J. M., Khoury, E. L., and Mocarski, E. S. (1991) Human cytomegalovirus ie2 negatively regulates alpha gene expression via a short target sequence near the transcription start site. *J. Virol.* **65,** 887–896.

8

Analysis of Cytotoxic T-Cell Responses to Human Cytomegalovirus Infection

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1. Introduction

Inferential evidence from studies of immunosuppressed humans indicates the control of disease due to human cytomegalovirus (HCMV) in the persistently infected host (virus carrier) depends on the cellular immune response: cytotoxic T lymphocytes (CTL) appear to be a particularly important component of this response ***(1,2)***.

A number of groups have published the antigen specificity of HCMV specific $CD8^+$ CTL generated in response to HCMV infection in humans ***(3–8)***. More recently, the frequency of CTL to individual HCMV antigens has been investigated using limiting dilution techniques ***(7,8)*** three independant reports have shown that CTL specific to the major tegument protein (pp65) comprises most HCMV-specific CTL. In addition, the fine peptide specificity of CTL that recognize pp65 for a number of HLA class I molecules (A2, B7, B8, B35) has been elucidated ***(8)***.

The most often used technique for generating HCMV specific CTL is to stimulate peripheral blood mononuclear cells (PBMCs) from HCMV-seropositive subjects with autologous primary fibroblasts that have been infected with HCMV. However workers have also used autologous fibroblasts incubated with CNBr-digested pro-

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tein ***(6)***, protein that has undegone alkaline hydrolysis ***(9)*** and autologous PBMC pulsed with synthetic peptides ***(8)***. This chapter describes in detail how to establish primary fibroblast lines and stimulate HCMV specific CTL using HCMV infected fibroblasts and peptide pulsed PBMC. In addition protocols are included for chromium^{51} release cytotoxicity assays and cloning/maintenance of antigen-specific T cells. It is beyond the scope of this chapter, however, to include protocols and analysis techinques for the quantitation of HCMV-specific CTL, using limiting dilution analysis.

A number of new techniques have very recently been published for the analysis of HCMV specific T cells, these include tetramer staining, epitope mapping by flowcytometry and molecular clonotype analysis ***(10–12)***. These techniques are not discussed further in this chapter, however the references contain technical details. HCMV pp65 specific HLA A*02012 and B*07027 tetramers have recently been constructed by Paul Moss (P.Moss@bham.ac.uk,personal communication).

2. Materials

2.1 Establishment of Primary Fibroblast Line

1. Sterile coverslips.
2. 6-well tissue culture plate (e.g., Falcon).
3. Curved sterile scalpel.
4. Sterile forceps.
5. Eagle's minimal essential medium (EMEM) (e.g., Gibco-BRL): supplemented with 10% fetal calf serum (FCS), 2 m*M* L-glutamine, 10^5 IU of penicillin per liter and 100 mg streptomycin per liter (EMEM-10).
6. Trypsin EDTA (e.g., Sigma): 9 mL 10X trypsin EDTA in 81 mL PBS.
7. Phosphate-buffered saline (PBS) (e.g., Oxoid).

2.2. Establishment of Transformed B-Cell Lines

1. B95.8 supernatant (*see* **Note 1**).
2. RPMI 1640 supplemented with 10% FCS, 2 m*M* L-glutamine, 10^5 IU of penicillin per liter and 100 mg streptomycin per liter, (RPMI-10).

3. Cyclosporin A: 1 mg/mL in RPMI-10.
4. Sterile 24-well tissue culture plate (e.g., Falcon).
5. Fresh PBMC (as prepared in **Subheading 3.3.**).

2.3. Generation of HCMV-Specific CTL

2.3.1. Preparation of PBMC

1. Heparin: 100 U/mL in PBS.
2. Ficoll-Hypaque (e.g., Lymphoprep, Nyegaard).
3. Anti-human monoclonal antibodies to CD4, CD16, and CD56 (e.g., Becton Dickinson, Franklin Lakes, NJ).

2.3.2. Antigen Stimulation

2.3.2.1. Virus Co-culture

1. Autologous primary fibroblasts (as prepared in **Subheading 3.1.**).
2. HCMV stock.
3. Interleukin-2 (IL-2).

2.3.2.2. Peptide Stimulation

1. Peptide in RPMI 1640 (*see* **Note 2**).
2. Fresh autologous PBMC (as prepared in **Subheading 3.3.**).

2.4. Cytotoxicity Assays

1. Sodium Chromate^{51} 5 mCi/mL (e.g., Amersham).
2. RPMI 1640: supplemented with 10% FCS, 2 m*M* L-glutamine, 10^5 IU of penicillin per liter and 100 mg streptomycin per liter (RPMI-10).
3. Target cells (e.g., HCMV-infected fibroblasts or peptide pulsed B cells).
4. Sterile 96 well U bottom tissue culture plate with lid (e.g., Corning, Corning, New York).
5. 1% Nonidet P-40 (NP-40) (e.g., BDH).

2.5. Maintenance of CTL Lines

1. Allogenic fresh PBMC.
2. Phytoheamaggluttin 1 mg/mL (e.g., Sigma, St. Louis, MO).

3. IL-2.
4. RPMI 1640: supplemented with 10% FCS, 10% human AB serum, 2 m*M* L-glutamine, 10^5 IU of penicillin per liter and 100 mg streptomycin per liter (RPMI-HU AB).

2.6. T-Cell Cloning

1. As for **Subheading 2.5.**

3. Methods

3.1. Establishment of Primary Fibroblast Cultures

Primary fibroblasts cultures are relatively easy to establish from donors of interest. The cell lines take 6–8 wk to establish from the initial biopsy material and can be readily frozen down in liquid nitrogen for long term storage. Primary fibroblasts are fully permissive to HCMV infection and provide an excellent way of presenting HCMV antigens in the context of the autologous MHC class I molecules to cytotoxic T-cell precursors in order to generate antigen specific CTL cultures. Skin biopsies are performed by qualified medical personnel. Depending on your individual circumstances, this procedure may also require ethical committee approval.

1. Obtain skin biopsy material.
2. Under sterile tissue culture condition cut the skin biopsy into strips (*see* **Note 3**).
3. Place a single strip into each well of a 6-well plate.
4. Place a coverslip over the strip.
5. Add a small drop of EMEM-10 to the edge of the coverslip; this should be sucked under by capillary action.
6. Add 2 mL of EMEM-10 to each well.
7. Incubate at 37°C 5% CO_2 (*see* **Note 4**).
8. When fibroblasts are confluent under coverslip, lift the coverslip and remove growth medium.
9. Wash with PBS and remove.
10. Add 0.5 mL of trypsin/EDTA.
11. Incubate at 37°C, check every minute to see whether fibroblasts have detached from the plastic.

12. Harvest trypsin fibroblast mixture pooling all wells into a centrifuge tube, top up with EMEM-10 to neutralize trypsin.
13. Centrifuge cells (2000 rpm, 5 min) and resuspend in 10 mL EMEM-10.
14. Seed cells into a 25-cm^2 tissue culture flat and incubate at 37°C 5% CO_2.
15. Expand primary fibroblasts when tissue culture flats become confluent, the cells should not be split higher than 1 in 2.
16. Primary fibroblasts can be cryopreserved and stored in liquid nitrogen.

3.2. Establishing Transformed B-Cell Lines

1. Take 1×10^7 fresh PBMC and pellet in a centrifuge tube.
2. Resuspend the cell pellet in 2 mL of B95.8 supernatant (*see* **Note 1**).
3. Incubate at 37°C 5% CO_2 for 3 h.
4. Add 2 mL of RPMI 1640 and 8 µL of Cyclosporin A (1 mg/mL).
5. Put 2 mL of sterile PBS in every outside well of a 24-well tissue culture plate.
6. Put 2 mL of cell suspension into each of two inner wells.
7. Incubate at 37°C 5% CO_2.
8. Check every few days that the outer wells are still filled with PBS.
9. Every week remove one-half the media from the wells, and replace with fresh RPMI-10 supplemented with 2 µg/mL cyclosporin A.
10. When B-cell colonies are visible to the naked eye split the wells 1 in 2, when 4 wells are confluent transfer the cells into a 25-cm^2 tissue culture flat, top up to 12 mL with RPMI 1640.
11. The tissue culture flats should be incubated upright with the cap lose and split 1/2 every 3–4 d.

3.3. Generation of HCMV-Specific CTL

Expanded populations of HCMV-specific CTL are generated from the $CD8^+$ T-cell fraction of PBMC isolated from HCMV seropositive donors. Polyclonal HCMV specific CTL can be stimulated using autologous HCMV-infected fibroblasts as antigen presenting cells. If a particular HCMV peptide antigen is known, peptide-specific CTL lines can be generated using irradiated autologous PBMC which have been pulsed with the peptide. Donor PBMC are used as a source of $CD8^+$ T cells but also to provide a feeder

layer for the growing T cell cultures. In our experience, high-quality CTL lines can be produced if natural killer cells ($CD16^+$ and $CD56^+$) and $CD4^+$ T cells are removed from PBMC prior to antigen stimulation. There are numerous techniques for subfractionating PBMC populations which would be suitable, however, we use flow cytometry and negative cell sorting, PBMC are stained with florescent monoclonal antibodies to CD4, CD16, and CD56; cells that do not stain are collected. This gives a highly enriched $CD8^+$ cell population (>75%) the rest of the population being B cells.

3.3.1. Preparation of PBMC

1. Dilute fresh heparinized venous blood 50:50 v/v with PBS.
2. Layer 25 mL of the diluted blood onto 12.5 mL of Ficoll-Hypaque.
3. Centrifuge at 2000 rpm for 15 min, with the centrifuge brake switched off.
4. Harvest the PBMC band.
5. Wash twice with PBS.
6. Count PBMC.
7. Stain required amount of PBMC with anti-human monoclonal antibodies to CD4, CD16, and CD56 (using amount of antibody according to manufacturers recommendations), the negative staining cells can be selected for using a cell sorter; this gives a highly enriched $CD8^+$ T-cell population.
8. Add 50 µL of $5 \times 10^5 - 1 \times 10^6$ cells/mL of sorted cells to each of the central 60 wells of a 96-well U-bottom tissue culture plate, the outer wells should have 300 µL of sterile PBS added to them.

3.3.2. Antigen Stimulation

3.3.2.1. Virus Co-Culture

1. Wash a confluent (75-cm^2) monolayer of the appropriate primary human fibroblasts with PBS.
2. Remove all PBS.
3. Add sufficient HCMV virus stock to the monolayer to achieve an multiplicity of infection (MOI) of 10.
4. Every 10 min rock the tissue culture flask to distribute the virus.

5. After one hour add fresh EMEM-10 to the tissue culture flask and incubate at 37°C 5% CO_2.
6. Remove growth media and wash with PBS, trypsinize cells, and resuspend in fresh EMEM-10.
7. Count fibroblasts and adjust to a cell concentration of 1.5×10^5/mL in RPMI 1640 10% human AB serum 10% FCS.
8. Prepare a suspension of PBMC at 2×10^6 cell/mL in RPMI 1640 10% human AB serum 10% FCS 10 IU/mL IL-2.
9. Irradiate PBMC suspension (2400 rad).
10. Mix equal volumes of infected fibroblasts and irradiated PBMC.
11. Add 50 µL of this mixture to each T-cell culture well (as prepared in **Subheading 3.3.1.**).
12. The cultures are incubated at 37°C 5% CO_2 and refed with 50 µL of RPMI 1640 10% human AB 10% FCS supplemented with 5 IU/mL IL-2 on days 5 and 10.

3.3.2.2. Peptide Stimulation

1. Aliquot enough PBMC suspension to stimulate the T-cell cultures (50,000 PBMC per well of a 96-well plate) and centrifuge cells.
2. Remove tissue culture media and resuspend the cell pellet in 50 µL of peptide at 40 µg/mL.
3. Incubate at 37°C 5% CO_2 for 1 h.
4. Irradiate cells (2400 rad).
5. Make cells up to 1×10^6 cells/mL with RPMI 1640 10% human AB serum, 10% FCS 10 IU/mL IL-2.
6. Add 50 mL of this mixture to each T-cell culture well.
7. The cultures are incubated at 37°C 5% CO_2 and refed with 50 µL of RPMI 1640 10% human AB 10% FCS supplemented with 5 IU/mL IL-2 on days 5 and 10.

3.4. Cytotoxicity Assays

3.4.1. HCMV Infected Fibroblasts as Targets

1. Infect confluent 75-cm^2 tissue culture flats of autologous and mismatched fibroblasts with HCMV as in **Subheading 3.3.2.1.**, **steps 1–5**); also, mock-infect a monolayer of autologous fibroblasts with media to provide an uninfected control.

2. The infected fibroblasts are incubated for 48 h before use as target cells.
3. After 48-h incubation, wash infected fibroblasts and trypsinise cells.
4. Wash trypsinized cells in EMEM-10 once and count.
5. Wash cell in PBS once.
6. Remove all PBS from fibroblast cell pellet with a pipet.
7. Resuspend cell pellet in Sodium Chromate (Cr^{51}) using 10 µL of sodium chromate per 1×10^6 fibroblasts.
8. Incubate cells at 37°C 5% CO_2 for 45 min.
9. Wash cells three times in RPMI-10 (*see* **Note 5**).
10. Resuspend cells in 5 mL RPMI-10 and count.
11. Adjust cell suspension to 1.7×10^4 cells/mL.

3.4.2. Peptide Pulsed B-Cells as Targets

1. Pellet sufficient autologous and mismatched B cells for the assays (*see* **Subheading 3.4.3.**).
2. Wash once in PBS.
3. Remove all PBS from cell pellets with a pipet.
4. Resuspend cell pellet in sodium chromate (Cr^{51}) using 10 µL of sodium chromate per 1×10^6 B-cells.
5. Incubate cells at 37°C 5% CO_2 for 45 min.
6. Add 50 µL of 40 µg/mL solution of target peptide to autologous and mismatched B cells.
7. Incubate cells at 37°C 5% CO_2 for 1 h.
8. Wash cells three times in RPMI 1640 10% FCS (*see* **Note 5**).
9. Resuspend cells in 5 mL RPMI 1640 10% FCS and count.
10. Adjust cell suspension to 3.2×10^4 cells/mL (4000 cells/well).

3.4.3. Cytotoxicity Assays and Analysis

In order to determine the percentage of specific lysis of a given effector CTL population, a gamma counter is used to measure the spontaneous, maximum, and test chromium release in counts per minute (cpm) for a given target cell. These values are determined from:

Labeled targets no effector cells: Spontaneous
Labeled targets with detergent: Maximum
Labeled targets with effector cells: Test

Percentage specific lysis is calculated for each target using the formula:

$$\frac{(\text{cpm test release} - \text{cpm spontaneous release})}{(\text{cpm maximum release} - \text{cpm spontaneous release})} \times 100$$

Cytotoxicity assays can be performed over a wide range of effector to target (E:T) ratios, we use 2000 fibroblast or 4000 B-cell targets per test in our assays and typically E:T values of 40:1, 20:1, and 10:1. It is usual to perform cytotoxicity assays in triplicate and derive the mean cpm for the spontaneous, maximum and test releases and use these to calculate the percentage specific lysis.

1. Pool T-cells from individual 60 well cultures and count.
2. Dilute T-cell cultures in RPMI-10 to give the desired range of E:T ratios per 50 µL (e.g., using 4000 target cells per well to get an E:T ratio of 40:1 would require 1.2×10^5 T cells in 50 µL, for a triplicate 3.6×10^5 cells in 150 µL).
3. Using a 96-well U-bottom tissue culture plate aliquot out in triplicate the range of E:T ratios (50 µL per well).
4. Each set of E:T ratios will also require a triplicate of spontaneous release wells comprising 50 µL of media.
5. Each set of E:T ratios will also require a triplicate of maximum release wells comprising 50 µL of 1% NP-40.
6. Add 120 µL of target cell suspension (*see* **Subheading 3.4.1.** or **3.4.2.**) to EVERY well, add target cells to NP-40 wells last.
7. Incubate the CTL assay at 37°C 5% CO_2 for 8 h (fibroblasts targets) or 4 h (B-cell targets).
8. After incubation, remove 70 µL of supernatant from each well, taking care not to remove any of the cell pellet.
9. Count each 70-µL aliquot in a gamma counter.

3.5. Maintenance of CTL Lines

HCMV-specific polyclonal T-cell lines as generated in **Subheading 3.3.** can be maintained for relatively long periods using a combination of allogenic and lectin stimulation with a higher dose of IL-2.

1. Plate out T-cell line at 1000–10,000 cells per 50 µL per well in the central 60 wells of a 96-well plate. The outer wells should have 300 µL of sterile PBS added to them.
2. Prepare fresh PBMC (*see* **Subheading 3.3.1.**) from any donor other than the donor the CTL line was originally derived from (MHC mismatch).
3. Irradiate PBMC (2400 rad).
4. Dilute PBMC in RPMI 1640 10% human AB 10% FCS supplemented with 50 IU/mL IL-2 and 2 µg/mL of phytohemagglutinin (PHA) to give 1×10^6 cells/mL.
5. Feed each well of the T-cell line with 50 µL of this mixture.
6. Incubate at 37°C 5% CO_2.
7. Refeed the cultures 3–4 d later with RPMI 1640 10% human AB serum 10% FCS supplemented with 50 IU/mL IL-2.
8. 3–4 d later repeat from **step 2**.
9. The cultures can be split 1 in 2 about every week.

3.6. T-Cell Cloning

1. Harvest T-cell line and count cells.
2. Dilute T cells to give 240 cells in 6 mL of RPMI 1640 10% human AB 10% FCS.
3. Plate out 50 µL per well in the central 60 wells of a 96-well plate. The outer wells should have 300 µL of sterile PBS added to them.
4. Add 3 mL of RPMI 1640 10% human AB 10% FCS to the remainder of the T cells.
5. Using a second 96-well plate, add 50 µL of the T cells per well in the central 60 wells.
6. Add 3 mL of RPMI 1640 10% human AB 10% FCS to the remainder of the T cells.
7. Using a second 96-well plate, add 50 µL of the T cells per well in the central 60 wells.
8. Prepare fresh PBMC (*see* **Subheading 3.3.1.**) from any donor other than the donor from which the CTL line was originally derived.
9. Irradiate PBMC (2400 rad).
10. Dilute PBMC in RPMI 1640 10% human AB 10% FCS supplemented with 50 IU/mL IL-2 and 2 µg/mL of PHA to give 1×10^6 cells/mL.
11. Feed each well with 50 µL of this mixture.

12. Incubate at 37°C 5% CO_2.
13. Refeed the cultures 3–4 d later with RPMI 1640 10% human AB 10% FCS supplemented with 50 IU/mL IL-2.
14. 3–4 d later repeat from **step 8**.
15. The T-cell clones are ready for testing for antigen specificity after 2–3 wk growth; once antigen-specific clones are identified, they can be expanded and maintained as detailed in **Subheading 3.5.**

4. Notes

1. B95.8 cells ATCC number CRL–1612. These cells produce Epstein-Barr virus. They should be grown in RPMI 1640 supplemented with 10% FCS, 2 m*M* L-glutamine, 10^5 IU of penicillin per liter and 100 mg streptomycin per liter in upright 25-cm^2 flask. When the growth media is quite acidic (yellow), the cells can be split 1 in 2. The second split can be centrifuged to remove cells and aliquoted into 2-mL amounts and frozen at –70°C for use as stock B95.8 supernatant.
2. Peptides are dissolved in unsupplemented RPMI 1640, when dissolved they are best stored in small aliquots at –70°C. Peptides containing a large number of hydrophobic residues may not be soluble in RPMI 1640.
3. Fibroblasts grow out of skin sections cut with a very clean edge. This can be achieved by using a curved scalpel, the point is placed at one edge of the biopsy material and used as a pivot to cut the material in a single smooth action.
4. The fibroblast cultures need to be examined using a tissue culture microscope every few days. The first cells to grow out of the biopsy will be epithelial cells (cubic like morphology) and these will grow out from clean cut edges of the biopsy material. Some weeks later, fibroblasts will grow out from the biopsy under the epithelial layer, the epithelial cells are not long lived and often die or detach to reveal the underlying fibroblast culture. Old growth media should be removed every week and replaced with fresh.
5. In our experience, it is best to have the RPMI-10 quite acidic, this can be achieved by adding concentrated hydrochloric acid dropwise until the media turns a strong orange/yellow color. Maintaining acid media decreases the spontaneous $chromium^{51}$ release.

References

1. Quinnam, G. V., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Moreschi, G., Santos, G. W., Saral, R., and Burns, W. H. (1982) Cytotoxic T cells in cytomegalovirus infection: HLA restricted T lymphocyte and non T lymphocyte cytotoxic responses correlate with recovery from cytomegalovirus in bone marrow transplant recipients. *N. Engl. J. Med.* **307,** 7–13.
2. Quinnam, G. V., Burns, W. H., Kirmani, N., Rook, A. H., Manischewitz, J. F., Jackson, L., Santos, G. W., and Saral, R. (1984) HLA restricted cytotoxic T lymphoctes are an early immune response and important defense mechanism in cytomegalovirus infections. *Rev. Infec. Dis.* **6,** 156–163.
3. Borysiewicz, L. K., Hickling, J., Graham, S., Sinclair, J., Cranage, M., Smith, G., and Sissons, J. G. P. (1988) Human Cytomegalovirus specific cytotoxic T cells — relative frequency of stage specific CTL recognising the 72KDa immediate early protein and glycoprotein B expressed by recombinant vaccinia viruses. *J. Exp. Med.* **168,** 919–931.
4. Alp, N. J., Allport, T. D., Van-Zanten, J., Rodgers, B., Sissons, J. G. P., and Borysiewicz, L. K. (1991) Fine specificity of cellular immune responses in humans to human cytomegalovirus immediate-early 1 protein. *J. Virol.* **65(9),** 4812–4820.
5. Riddell, S. R., Rabin, M., Gaballe, A. P., Britt, W. J., and Greenberg, P. D. (1991) Class I MHC restricted cytotoxic T lymphocyte recognition of cells infected with human cytomegalovirus does not require endogenous viral gene expression. *J. Immunol.* **146,** 2795–2804.
6. McLaughlin-Taylor, E., Pande, H., Forman, S., Tanamachi, B., Li, C., Zaia, J., Greenberg, P., and Riddell, S. (1994) Identification of the major late human cytomegalovirus matrix protein pp65 as a target antigen for $CD8^+$ virus-specific cytotoxic T lymphocytes. *J. Med. Virol.* **43,** 103–110.
7. Boppana, S. B. and Britt, W. J. (1996) Recognition of human cytomegalovirus gene products by HCMV-specific cytotoxic T cells. *Virology* **222(1),** 293–296.
8. Wills, M. R., Carmichael, A. J., Mynard, K., Jin, X., Weekes, M. P., Plachter, B. and Sissons, J. G. P. (1996) The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity and T-cell receptor usage of pp65 specific CTL. *J. Virol.* **70(11),** 7569–7579.

9. Gavin, M. A., Gilbert, M. J., Riddell, S. R., Greenberg, P. D., and Bevan, M. J. (1993) Alkali hydrolysis of recombinant proteins allows for the rapid identification of class I MHC-restricted CTL epitopes. *J. Immunol.* **151(8),** 3971–3980.
10. Altman, J. D., Moss, P. A., Goulder, H. P. J., Barouch, D. H., McHeyzer Williams, M. G., Bell, J. I.,NcNucgaekm A. J., and Davis, M. M. (1996) Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274,** 94–96.
11. Kern, F., Surel, I. P., Brock, C., Freistedt, B., Radtke, H., Scheffold, A., Blasczyk, R., Reine, P., Schneider-Mergener, J., Radbruch, A., Walden, P., and Volk, H. (1998) T-cell epitope maplping by flow cytoetry, *Nature Medicine.* **4,** 975–978.
12. Wills, M. R., Carmichael, A. J., Weekes, M. P., Mynard, K., Okecha, G., Hicks, R., and Sissons, J. G. (1999) Human virus-specific CD8+ CTL clones revert from CD45ROhigh to CD45RAhigh in vivo: CD45RAhighCD8+ T cells comprise both naive and memory cells. *J. Immunol.* **162,** 7080–7087.

9

Methods in Anti-HCMV Research

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1. Introduction

The study of strategies for the treatment of human cytomegalovirus (HCMV) infection starts with the discovery of compounds that are potent and selective inhibitors of HCMV replication. Selectivity means that the window between the concentrations that block viral replication on the one hand and those that affect host cell functions on the other is sufficiently wide. Because HCMV induces an obvious cytopathic effect (CPE) in human fibroblast cultures, potential antiviral activity can easily be scored microscopically. For this type of assay, a relatively low input of virus should be used, so that the CPE does not appear within the first 2–3 d after infection. If too high a multiplicity of infection (MOI) is used, the input virus will result in a substantial amount of CPE shortly (i.e., within 24 h) after infection. As the CPE induced by this input virus will not be blocked by compounds that inhibibit new progeny virus formation, the use of a high MOI will mask a potential inhibitory effect of a given compound on HCMV replication. Scoring of CPE can be done either by counting the HCMV-induced plaques (although this is a rather labor-intensive method for large-scale screening purposes) or simply by scoring the CPE on a scale of 1–5 (or 10). Each assay should

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always be validated by using a reference compound with proven anti-HCMV activity (e.g., ganciclovir, cidofovir, foscarnet).

Unlike many other viruses (i.e., herpes simplex virus [HSV], human immunodeficiency virus [HIV], influenza), HCMV does not result in (rapid) death of the infected cells but rather stimulates the host cell metabolism. For this reason, assays based on cell viability (MTT or MTS assays) cannot be used for (a [semi-]automated) quantification of the effect of compounds on HCMV replication. Based on our observation that cytoplasmic esterase activity is markedly increased in fibroblast cultures infected with HCMV, we developed a rapid, sensitive fluorometric assay (in a 96-well format) that allows a (semi)-automated in vitro evaluation of anti-HCMV activity ***(1)***.

It should be kept in mind that compounds that prevent the initial interactions of HCMV with the host cell (i.e., the different steps in the binding and fusion processes) will only be picked up provided that the compound and virus are added together to the cell cultures. The reference compound to be used in such assays is dextran sulfate ***(2)***. In the event that the compound under study is not expected to interact with this initial stage of infection, it should be added after the initial virus adsorption period. In this case, ganciclovir or cidofovir can be used as reference compounds.

Once a selective inhibitor of HCMV replication has been identified, experiments should be set up to unravel at what stage of the viral life cycle the compound acts. A simple "time-of-addition" assay, in which the compound under study is added at well-defined times before, during, or after infection of the cells often yields important information concerning the mechanism of action of the drug. In parallel in infected, but untreated, cultures this type of study can be further documented by determining the appearance of different markers of the viral life cycle (i.e., immediate-early, early, or late antigens and the time of onset of viral DNA synthesis).

Next, the effect of the compound on different events in the viral cycle should be studied. If a molecule is thought (as derived from the time-of-addition experiments) to interfere with the intial interaction of the virus with the host cell, its effect on the binding of

(radiolabeled) virus particles to the host cell should be studied. A molecule exhibiting anti-HCMV activity may also act immediately after viral attachment takes place, i.e., at the level of the virus–cell fusion process. This can be studied by allowing the virus to bind at 4°C to the cells (a temperature at which binding but no fusion occurs), after which the temperature of the culture (either in the absence or presence of compound) is switched to 37°C. Shortly thereafter (at i.e., 10–16 h), cells can be monitored for the expression of immediate-early viral antigens.

If a compound does not act on the initial steps in the viral life cycle, its effect on the expression of immediate-early, early, or late viral antigens should be studied. This can be done by means of immunofluorescence, flow cytometry ***(3)*** or Western blotting. The effect on viral DNA synthesis can be conveniently followed by means of a slot-blot DNA–DNA hybridization assay, using a digoxigenin-labeled probe.

Nucleoside or nucleotide analog inhibitors of the replication of HCMV (and other herpesviruses) may be specifically phosphorylated in the virus-infected cells ***(4,5)***. The specific phosphorylation of ganciclovir to its 5'-monophosphate in HCMV-infected cells is mediated by a protein kinase encoded by the HCMV UL97 ***(6,7)***. Further phosphorylation of nucleoside analogs to the triphosphate metabolite is accomplished by cellular kinases ***(8)***. The metabolism of nucleoside-type inhibitors of viral replication can be easily monitored by incubating (HCMV-infected) cell cultures for a given period of time with the radiolabeled compounds after which extracts are analyzed by means of anion-exchange high-performance liquid chromatography (HPLC). The phosphorylated metabolites of the antivirally active nucleoside analogs may specifically inhibit the HCMV DNA polymerase. For example, this is the basis for the selective anti-HCMV activity of ganciclovir, cidofovir, and lobucavir ***(8)***.

The following description is not exhaustive, but the techniques covered are sufficient for general anti-HCMV studies and mechanism of action studies for some, but not all, types of compounds. Specific assays may be used or need to be developed, to study, for example, drug effects on uncoating, transcription, promotor function, DNA processing and viral assembly.

2. Materials

2.1. Cell Culture Assays

2.1.1. Virus Titration

1. Human embryonic lung (HEL) fibroblasts (ATCC CCL 137) used at low passage, grown in 96-well microtiter plates.
2. Stocks of HCMV reference strains: Davis (ATCC VR–807) and AD-169 (ATCC VR–538).
3. MEM 10%: Minimum essential medium (MEM, Life Technologies) supplemented with 10% inactivated fetal calf serum (FCS), 1% L-glutamine, and 0.3% sodium bicarbonate, are used to culture HEL cells.
4. MEM 2%: MEM supplemented with 2% FCS, 1% L-glutamine, and 0.3% sodium bicarbonate are used for dilution of virus and compounds.
5. 70% Ethanol.
6. 2.5% Giemsa solution in distilled water.

2.1.2. Plaque and CPE Reduction and Cell Growth Assay

1. HEL cells grown to confluency in 96-well microtiter plates.
2. Stocks of a well defined titer of AD-169 and Davis.
3. Serial dilutions of the antiviral compounds in MEM 2% with inclusion of the reference compounds ganciclovir and cidofovir.
4. 70% ethanol.
5. 2.5% Giemsa solution in distilled water.

2.1.3. Virus Yield

1. HEL cells grown in 24- or 6-well microplates.
2. Stocks of a well defined titer of AD-169 and Davis.
3. Serial dilutions of the antiviral compounds in MEM 2% with inclusion of the reference compounds ganciclovir and cidofovir.

2.2. Fluorometric Anti-HCMV Assay

1. Fluorescein diacetate (FDA) (Janssen Chimica, Beerse, Belgium): make a 20 mg/mL stock in dimethyl sulfoxide (DMSO).

2. A Fluoroscan able to measure fluorescence in a 96 well plate format (e.g., Titertek Fluoroscan II, Flow Laboratories).

2.3. Virus Binding Assay

1. [*methyl*-^{3}H]dThd (specific radioactivity: 30–60 Ci/mmol).
2. Angle TI rotor (Beun de Ronde, Amsterdam, The Netherlands).
3. Sucrose (p.a.).
4. GF/C filters (25 mm, Whatman, Clifton, NJ, 1822025).

2.4. Detection of Viral Antigens

2.4.1. Immunofluorescence

1. Two-, four-, or eight-well chamberslides (Nunc, Naperville, IL).
2. Acetone (–20°C).
3. Monoclonal antibodies.
   a. E13 (Biosoft, Cambridge, UK), diluted 1/50.
   b. 2A2 (Biosoft), diluted 1/40.
   c. CCH2 (Dako, Copenhagen, Denmark), diluted 1/20.
4. Rabbit anti-mouse-IgG-F(ab')$_2$-FITC (Dako).

2.4.2. Flow Cytometry

1. HEL cells grown in 24-well microtiter plates.
2. Stocks of a well defined titer of AD-169 and Davis.
3. MEM 10% and MEM 2%.
4. Trypsin (Life Technologies, Bethesda, MD).
5. Serial dilutions of the antiviral compounds in MEM 2% with inclusion of the reference compounds ganciclovir and cidofovir.
6. Fixation solution: acetone/phosphate-buffered saline (PBS) (v/v: 66:34) kept at –20°C.
7. 1% gelatin (Vel, Haasrode, Belgium) in PBS.
8. Monoclonal antibodies E13, 2A2, CCH2, or others.
9. Rabbit anti-mouse-IgG-F(ab')2-FITC (Dako).
10. 0.5% formaldehyde solution (Merck) in PBS/gelatin.
11. Fluorescence-activated cell sorter (FACS) (e.g., Facstar, Becton Dickinson, San José, CA).

2.5. DNA–DNA Hybridization Assay

2.5.1. DNA Extraction

1. Qiamp blood kit 50 or 250 (Qiagen 29104).

2.5.2. Probe Generation by Means of PCR

1. dNTP set (dATP, dCTP, dGTP, and dCTP each at 100 m*M*) (Gibco-BRL 10297–018).
2. dNTP mix for use in digoxigenin (DIG) labeling:

100 m*M* dATP	5 µL
100 m*M* dGTP	5 µL
100 m*M* dCTP	5 µL
100 m*M* dTTP	3.25 µL
MilliQ wate	81.75 µL
	100 µL

Primers:

IEA-1: 5' GTCCTCTGCCAAGAGAAAGATGGAC 3'
IEA-2: 5' ACATCTTTCTCGGGGTTCTCGTTGC 3'
IEA-3: 5' GCCACGGTACGTGTCGGGGTTTGTG 3'
IEA-4: 5' TTGCAATCCTCGGTCACTTGTTCAA 3'

For PCR–1 (*see* **Subheading 3.6.4.**), use 5 µL instead of 3.25 µL dTTP. Divide dNTP stock in 5× 20-µL aliquots and freeze at –20°C.

1. *Taq* polymerase (Sphaero Q, Leiden, The Netherlands) or *Taq* polymerases from other manufacturers.
2. DIG-11-dUTP (1 m*M*, Boehringer Mannheim 1093 088).
3. 10X PCR buffer, including $MgCl_2$ (Gibco-BRL).
4. DNA gel extraction kit (e.g., Qiaquick gel extraction kit [Qiagen 28704]).

2.5.3. DNA–DNA Hybridization Assay

2.5.3.1. Reagents

1. Hybond-N membrane (20 cm × 3 m) (Amersham RPN-203N).
2. DIG-easy Hyb (Boehringer Mannheim 1603558).
3. Blocking reagent (Gibco 1096176).

4. Anti-DIG conjugate-AP (Boehringer Mannheim 1093274).
5. CSPD-Tropix 25 m*M* (Clontech, Palo Alto, CA, 8055-2).
6. Maleic acid (p.a.) (Serva 28337).
7. Diethanolamine (≥98.5%) (Sigma M-1028).

2.5.3.2. Solutions

1. SSC buffer (20X): 3 *M* sodium chloride, 300 m*M* sodium citrate.
2. Denaturation solution: 1.5 *M* NaCl, 0.5 *M* NaOH.
3. Neutralization solution: 0.5 *M* Tris-HCl, 3 *M* NaCl, pH 7.5.
4. Washbuffer 1: 2X SSC, 0.1% SDS.
5. Washbuffer 2: 0.1X SSC, 0.1% SDS.
6. Maleic acid buffer: 100 m*M* maleic acid, 150 m*M* NaCl, pH 7.5.
7. Blocking buffer: 1% blocking reagent in 0.1% Tween-20 in PBS.
8. Washbuffer 3: 0.3% Tween-20 in PBS.
9. Diethanolamine solution: 0.1 *M* diethanolamine in water (warm stock solution diethanolamine to 37°C before use), shield bottle containing solution from light by using aluminum foil.
10. Assay buffer: 0.1 *M* diethanolamine solution, 1 m*M* $MgCl_2$.

2.5.4. Equipment

1. Slot-blot device (e.g., Hoefer, San Francisco, CA, PR600).
2. Ultraviolet (UV) light.
3. Hypercassette™ (Amersham RPN 2642).
4. Curix Blue HC-S plus medical X-ray film (Agfa-Gevaert).
5. Dark room and equipment for film development.

2.6. HPLC Analysis

1. $(NH_4)H_2PO_4$ (Acros organics, 19370-5000).
2. Partissphere SAX radial compression column (Pharmacia 4621–05051).
3. Natural nucleosides and nucleotides (all available from Sigma).
4. Acrodisc 0.45 μ*M* (Gelman Sciences, PN 4473).
5. HPLC apparatus: available from different manufacturers.
6. Liquid scintillation counter.

2.7. DNA Polymerase Assay

1. [2,8-^{3}H]dATP (specific activity 20–40 Ci/mmol).
2. Activated calf thymus DNA (Pharmacia).
3. Trichloroacetic acid (TCA) p.a. (Merck 1.00807.1000).
4. GF/C filters (Whatman).

3. Methods

3.1. Cell Culture Assays

3.1.1. Virus Titration

A titration of the viral stocks must be carried out before performing a plaque or CPE reduction assay.

1. Prepare 5- to 10-fold dilutions of the virus stock in MEM 2%. Dilutions within the range of 10^{-1}–10^{-5} are appropriate for most virus stocks. Use a fresh pipet or tip for each dilution to prevent carryover of virus (which might otherwise result in an anomalously high virus titer).
2. Use HEL cells grown to confluency in 96-well microtiter plates. Remove the medium from the plates and apply 100 µL of diluted virus per well; use six wells per dilution.
3. Incubate the cell cultures at 37°C in a 5% CO_2 atmosphere for 2 h.
4. Remove the inoculum and replace with MEM 2%.
5. Incubate the cells at 37°C in a CO_2 atmosphere for 7 d.
6. Fix the microplates with ethanol and stain them with 2.5% Giemsa solution for 2 h, rinse with distilled water, air-dry, and monitor viral plaque formation under the microscope.
7. Calculate the titer of the virus stock in PFU/mL.

3.1.2. CPE and Plaque Reduction Assay

1. Dilute the virus stock in MEM 2% ultimately obtaining 20 PFU/0.1 mL (plaque reduction assay) or 100 PFU/0.1 mL (CPE reduction assay); 10 mL of virus preparation is needed per 96-well microtiter plate.
2. Remove the medium (by means of aspiration) from the HEL cell monolayers (grown to confluency in 96-well microtiter plates) and inoculate them with 0.1 mL of the diluted virus per well. It is advisable to include only one viral strain per microplate.

3. Incubate the infected cells at 37°C in a 5% CO_2 atmosphere for 2 h.
4. Remove the virus inoculum by means of aspiration.
5. Add serial dilutions of the test compounds in duplicate.
6. Incubate the infected cells at 37°C in a 5% CO_2 atmosphere for 7–8 d.
7. Fix the cells with 70% ethanol, remove ethanol, and stain with 2.5% Giemsa solution for 2 h. Rinse with distilled water and let air dry.
8. Virus plaque formation or viral CPE is evaluated microscopically. Evaluate CPE microscopically using a scale of 0–5 (or 0–10), with 0 indicating an absence of CPE and 5 (or 10) 100% CPE (comparable to the untreated controls), or count the number of plaques in treated and untreated cultures.
9. Express the minimal antiviral inhibitory concentration as the EC_{50}, or the concentration required to inhibit virus-induced CPE or plaque formation by 50%. IC_{50} values are estimated from (semilogarithmic) graphic plots of the number of plaques (percentage of control) or percentage of CPE as a function of the concentration of the test compound. It may be necessary to calculate EC_{90} values as well.

3.1.3. Virus Yield Assay

1. Remove the medium from confluent HEL cells grown in 24- or 6-well microplates and infect them with the AD-169 strain or Davis strain at an MOI of approx 0.05 PFU/cell.
2. Incubate the infected cells at 37°C in a 5% CO_2 atmosphere for 2 h.
3. Remove the virus inoculum by means of aspiration.
4. Add serial dilutions of the test compounds in MEM 2% in duplicate.
5. Incubate the cells for the desired period (generally, virus titers start to rise from day 4 postinfection) of time at 37°C in 5% CO_2 atmosphere.
6. Harvest the supernatants and clarify by centrifugation at 1500*g* for 10 min. Store at –80°C.
7. Determine virus titers by a plaque assay in HEL cells as described in **Subheading 3.1.1.**
8. Antiviral activity is expressed as the IC_{90} or IC_{99}, or concentration required to reduce viral production by 90% or 99%, respectively.

3.1.4. Cell Growth Assays

To be able to determine whether a compound that inhibits the replication of HCMV does so selectively, its effect on cell growth

Table 1
Activity of Different Compounds Against CMV (Strain AD-169) as Evaluated Microscopically or Fluorometrically

	EC_{50} (μg/mL)			
	FDA[a] (5 μg/mL)	Micr.[b]	FDA[c] (1 μg/mL)	Micr.[d]
Ganciclovir	0.3	0.9	0.8	0.2
Foscarnet	6.5	10	7.0	8.5
Cidofovir	0.012	0.010	0.012	0.015

[a,c]EC_{50} values obtained by the fluorometric assay after incubation of the cell cultures with FDA at 5 μg/mL (a) or 1 μg/mL (c) at 37°C for 30 min [for FDA 5 μg/mL (a), virus control was 133 ± 25 absolute fluorescent units (AFU) and cell control 20.8 ± 3.7 AFU; for FDA 1 μg/mL (c), the virus control was 38 ± 5.7 AFU and cell control 7.3 ± 0.9 AFU].

[b,d]EC_{50} values obtained after microscopic evaluation (inhibition of viral CPE) of the cell cultures mentioned under a and c, respectively. Data taken from **ref. *1***.

should be determined. Alternatively, the effect of compounds on the viability of confluent HEL cell cultures can be assessed. However, measuring the effect on cell growth appears to be a more sensitive way of assessing interactions of molecules with the host cell machinery than testing possible cytotoxic effects on quiescent cells.

1. HEL cells are seeded at 4000 cells/100 μL in 96-well trays in MEM 10%. After the cells have been allowed to attach to the plates for 12 h at 37°C, different dilutions of the test compounds (MEM 10%) are added.
2. Cells are further incubated for 3–4 d at 37°C.
3. Cultures are washed with 100 μL PBS and trypsinized.
4. Triplicates of each condition are counted by means of a Coulter Counter.
5. The concentration of compound responsible for 50% inhibition of cell growth (CC_{50}) is calculated.

3.2. Fluorometric Anti-HCMV Assay (Table 1, Fig.1)

1. Prepare HCMV-infected and uninfected HEL cell cultures in 96-well plates (as described in **Subheading 3.1.2.**).

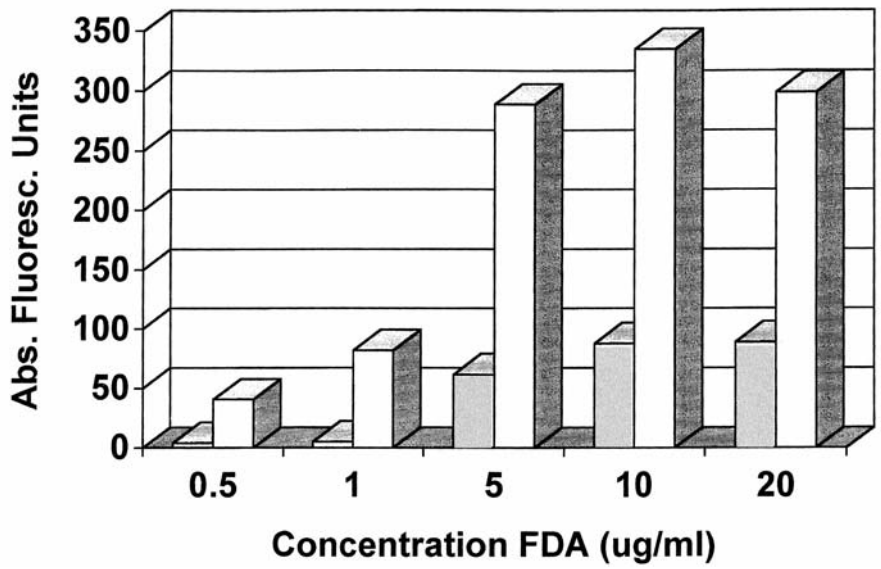

Fig. 1. Effect of HCMV infection of HEL cells (open bars) on FDA-associated fluorescence as compared with uninfected HEL cells (gray bars) when incubated with different concentrations of FDA for 60 min at 37°C.

2. Treat infected and uninfected cultures with serial dilutions of the compound to be tested (*see* **Subheading 3.1.2.**).
3. Remove supernatants in all wells by means of aspiration at 7 d postinfection when CPE in infected untreated cultures has progressed to 100%.
4. Wash each well two times with 100 µL of PBS.
5. Add to each well 100 µL of 5 µg/mL of FDA.
6. Incubate cultures (usually) for 30 min at 37°C.
7. Remove the FDA solution by aspiration and wash each well with 200 µL of PBS.
8. Measure the fluorescence intensity (as absolute fluorescence units [AFU]) in each well (at 0.1 s per well) by means of a Fluoroscan equipped with a 485-nm excitation filter and a 538-nm emission filter
9. The percentage reduction in HCMV-induced CPE by a given compound must be calculated as follows:

$$100 - [\text{AFU(conc} \times) - \text{AFU(cc)} / \text{AFU(vc)} - \text{AFU(cc)} \times 100] = y$$

3.3. Virus Binding Assay

3.3.1. Preparation of Radiolabelled HCMV Particles

1. Infect confluent cultures of HEL cells with the desired strain of HCMV at a multiplicity of infection (MOI) of 1.
2. After 4–5 d, remove supernatant and add 1 μCi of [*methyl*-^{3}H]dThd per mL of MEM 2%. Use a volume of medium that is just sufficient to cover the whole cell culture.
3. Three days later, collect the supernatant and remove cell debris by two subsequent centrifugation steps in a Minifuge T at 1000*g* for 10 min at 4°C.
4. Pellet virus for 2 h by ultracentrifugation at 45,000*g* in a Angle TI rotor at 4°C.
5. Resuspend the (small) viral pellet in 100 μL of culture medium.
6. Layer the resuspended radiolabeled virus pellet on top of a 4-mL sucrose gradient (20–60%) that has been prepared by means of a gradient maker.
7. Ultracentrifugate (swinging-out rotor) the sucrose gradient at 45,000*g* for 1 h at 4°C.
8. Collect 0.25-mL fractions, and determine the virus band by counting radioactivity in a 5-μL volume of each fraction.
9. Collect the fractions containing the virus (radioactivity) and add 20 mL of PBS.
10. Pellet the virus by ultracentrifugation for 2 h at 4°C.
11. Resuspend pellet in 100 μL of PBS and store at –80°C until use.

3.3.2. Binding of radiolabelled HCMV to HEL cells (Fig. 2)

1. Prepare confluent cultures of HEL cells in 48-well plates.
2. Add the compound to be tested in a volume of 140 μL to each well in MEM 2%.
3. Add 10 μL of radiolabeled virus suspension (which should have a titer of $\geq 10^8$ PFU/mL and a specific radioactivity of 5×10^5–10^6 cpm/mL).
4. Include a "blanco condition," i.e., one or two cultures to which virus is added for only 5 s after which the inoculum is removed and the cultures washed six times with 250 μL PBS.
5. Incubate test cultures for 30 min and 60 min at 37°C.

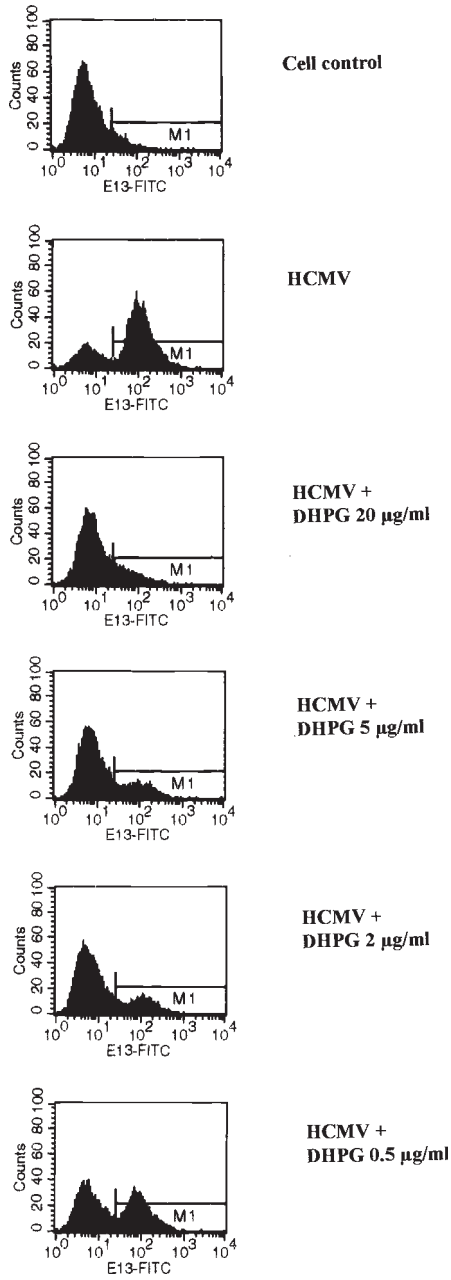

Fig. 2. Flow cytometric analysis of expression of immediate early antigens in HCMV-infected HEL cell cultures at 5 d postinfection after treatment with ganciclovir at different concentrations. The reduction in the number of virus infected cells reflects the inhibitory effect of ganciclovir on new progeny virus formation.

6. Remove the radiolabeled inoculum and wash each well six times with 250 µL PBS.
7. Trypsinize the cells.
8. Harvest the trypsinized cells and spot on GF/C filters mounted on a manifold hooked up to a vacuum pump.
9. Wash each filter three times with 10 mL of 5% TCA.
10. Dry each filter with 70% EtOH and quantify radioactivity by means of liquid scintilation counting.

3.4. Virus Fusion Assay

1. Confluent cultures of HEL cells are prepared in 2- or 4-well chamberslides.
2. Cells are incubated for 30 min at 4°C.
3. Culture medium is removed by aspiration, and cells are covered with a concentrated stock of HCMV (i.e., 10^6 PFU/mL, precooled to 4°C) and further incubated for 1 h at 4°C.
4. Cultures are washed three times with 500 µL of cold culture medium.
5. Test compounds are added to the cells in cold culture medium at the desired concentration.
6. The cultures are rapidly switched to an incubator at 37°C and incubated for 12–18 h.
7. Expression of immediate early antigens is determined by means of immunofluorescence (*see* **Subheading 3.5.1.**).

3.5. Detection of Viral Antigens in HCMV-Infected HEL Cell Cultures

3.5.1. Immunofluorescence

1. Confluent cultures of HEL cells are prepared in chamberslides.
2. Cultures are infected with the desired strain of HCMV and at the MOI needed, either in the absence or presence of the test compound.
3. Cultures are further incubated for the required length of time (e.g., 12–24 h for immediate early antigen expression or 4–5 d for detection of late antigen expression).
4. Culture medium is removed and slides are fixed in ice-cold acetone for 20 min.
5. Slides are air-dried.

6. Slides are incubated in a humidified atmosphere for 1 h with the appropiate dilution of (monoclonal) antibody.
7. After rinsing with PBS, slides are incubated for 30 min with RaM-IgG-F(ab')$_2$-FITC-mouse IgG.
8. Slides are rinsed three times in PBS and once briefly with distilled water.
9. Slides are examined under a fluorescence microscope, and the number of antigen-positive cells is counted in 20 random microscopic fields.

3.5.2. Flow Cytometry (Fig. 3)

1. Remove the medium from confluent HEL cells grown in 24- or 6-well microplates, and infect them with the AD-169 strain or Davis strain at a MOI of approx 0.05 PFU/cell, or as needed.
2. Incubate the infected cells at 37°C in a 5% CO_2 atmosphere for 2 h.
3. Remove the virus inoculum.
4. Add serial dilutions of the test compounds in MEM 2% in duplicate.
5. Incubate the cells for the desired period of time at 37°C in a CO_2 atmosphere.
6. Remove the medium from the cell cultures and trypsinize the cells.
7. Harvest the cells with MEM 10% and spin at 1500*g* for 10 min.
8. Resuspend the cells in 1 mL PBS/acetone. Incubate 20 min at 4°C.
9. Centrifuge at 1500*g* for 10 min.
10. Remove supernatant and resuspend the cells in 1 mL of PBS/gelatin.
11. Add 100 µL of the appropriate dilution of the MAb. Incubate for 1 h in a shaking waterbath at 37°C.
12. Centrifuge at 1500*g* for 10 min.
13. Rinse the cells with 1 mL PBS/gelatin.
14. Centrifuge at 1500*g* for 10 min.
15. Resuspend the cells in 1 mL PBS/gelatin, and add 100 µL of RaM-IgG-F(ab')$_2$-FITC diluted 1/40.
16. Incubate for 30 min in a shaking water bath at 37°C.
17. Centrifuge at 1500*g* for 10 min.
18. Resuspend the cells in 200 µL of 0.5% formaldehyde in PBS.
19. Analyze the samples with a Facstar.

3.5.3. Western Blotting

Western blotting on extracts of HCMV-infected cells is performed using standard methods ***(9)***.

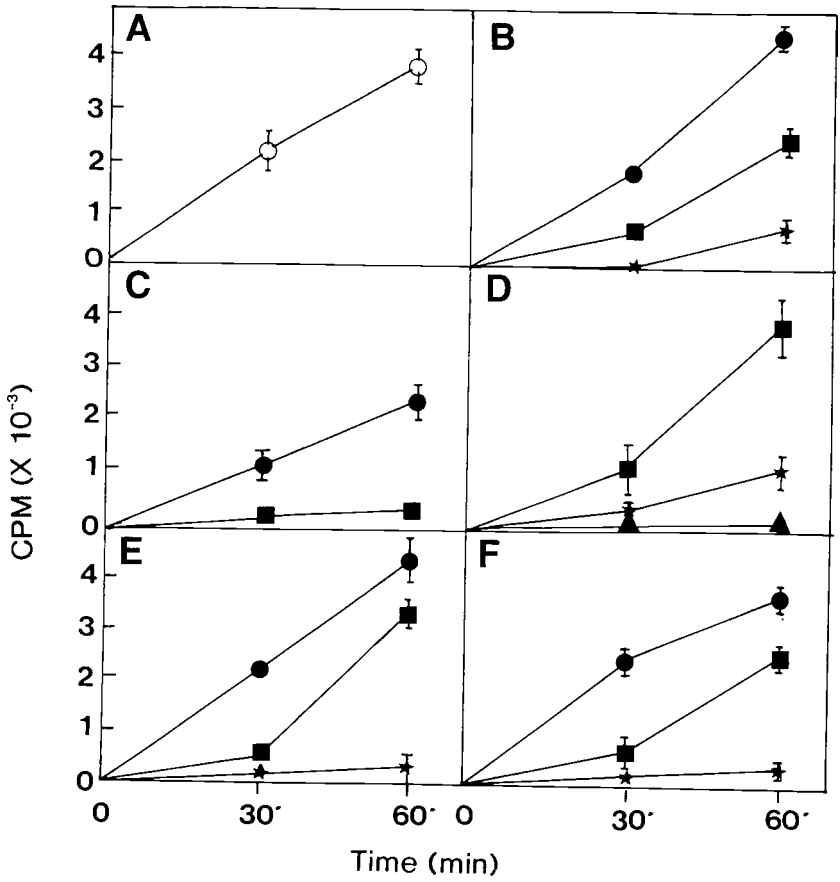

Fig. 3. Inhibitory effect of different sulfated polymers on the binding of radiolabeled HCMV particles to confluent HEL cell monolayers. Virus control (A), dextran sulfate (mol wt 5000) (B), PAVAS (C), a-cyclodextrin hexasulfate (D), pentosan polysulfate (E), heparin at various concentrations (40 μg/mL [s], 4 μg/mL [v], 0.4 μg/mL [n], and 0.04 μg/mL [l] or 0 μg/mL [m]).

3.6. DNA–DNA Hybridization Slot Blot Assay or Detection of HCMV DNA

3.6.1. Preparation of HCMV-Infected Cell Cultures

1. Infect confluent cultures of HEL cells grown in 25-cm^2 culture flasks with the desired strain of HCMV at the required MOI.

2. Remove the viral inoculum after a 2-h adsorption period.
3. Add the compound under study in MEM 2% (include control cultures with reference anti-HCMV compounds such as ganciclovir or cidofovir).
4. Incubate the cultures for 5–8 d until CPE is readily visible (depending on the input virus).
5. Remove the supernatant by aspiration.
6. Wash the culture with 10 mL of PBS at room temperature.
7. Trypsinize the cells.
8. Recover trypsinized cells in 5 mL of MEM containing 10% FCS.
9. Pellet the cells by centrifugation at approx 250 g.
10. Wash the pellet in 5 mL PBS.
11. Resuspend pellet in 200 µL of cold PBS (at this stage, the samples can be stored at 4°C; do not freeze, as this will interfere with the subsequent DNA extraction).

3.6.2. Extraction of DNA

1. The most convenient way to obtain DNA is to use commercial kits. We routinely use the DNA extraction kit from Qiagen (Qiamp Blood Kit 29104).
2. Determine the amount of DNA by measuring the OD at 260 nm. (Because of the low yield of DNA from a 25-cm^2 HEL cell culture, it is suggested to use all recovered DNA for OD measurements, after which the DNA is recovered and the cuvet rinsed extensively.)

3.6.3. Blotting of DNA

1. Calculate the volume of the DNA preparation needed to obtain 10 (or 20) µg of DNA.
2. Boil this amount of DNA for 5 min.
3. Put DNA on ice for 5 min.
4. Add to every sample the same volume of 20X SSC.
5. Blot the DNA with a vacuum slot blot device.
6. Before use: wet an appropiate piece of Hybond-N membrane in distilled water, followed by 10X SSC.
7. After blotting, leave the membrane for 5 min in denaturation solution.
8. Drip off denaturation solution and incubate the membrane for 1 min with neutralization solution.

9. UV-crosslink the membrane for 25 min (at this stage the membrane can be stored dry at 4°C).

3.6.4. Preparation of a Digoxigenin-Labeled HCMV Probe (Method Adopted from ref. 10).

PCR 1:

As template, total DNA extracted from HCMV-infected HEL cell cultures showing full CPE is used :

10X PCR buffer	5 µL
dNTP mix	2 µL
Milli Q water	39.5 µL
Primer IEA-1	1 µL
Primer IEA-2	1 µL
Template (0.1 µg/µL)	1 µL
Amplitaq DNA polymerase	0.5 µL
	50 µL

PCR 2:

PCR 1 yields a 351-bp DNA fragment. Use the purified fragment from PCR 1 as template in the PCR 2 reaction. For probe purification, *see* below.

PCR protocol:	
10× PCR buffer	5 µL
dNTP mix	2 µL
Milli Q water	36 µL
Primer IEA-3	1 µL
Primer IEA-4	1 µL
Template (0.05 µg from PCR1)	1 µL
DIG-dUTP (1 m*M*)	3.5 µL
Amplitaq DNA polymerase	0.5 µL
	50 µL

Run the following PCR program for 28 cycles:

1. 5 min at 94°C

2. 30 cycles of 60 s 95°C
   60 s 57°C
   90 s 72°C
3. 7 min at 72°C

Purification of the DIG-labeled probe

1. Run 50 µL of the PCR reaction in a 1% agarose gel in 1X TBE containing EtBr.
2. Inspect the gel under UV light and carefully excise the desired PCR fragment.
3. Elute the amplified DNA by using a commercial kit. We routinely use the Qiaquick Gel extraction kit (Qiagen 28704).
4. Store the probe in aliquots at –20°C.

3.6.5. Hybridization

1. Add to the membrane containing the blotted, UV-linked DNA, 20 mL/100 cm^2 of DIG-easy hyb solution. Incubate for 30 min at 42°C (in a sealed plastic bag or a closed plastic box of the appropriate size); rock gently.
2. Boil the required amount of DIG-labeled probe for 1 min (usually 1 µL of probe to which 10 µL of water is added), and chill the mixture immediately after boiling on ice.
3. After 5 min on ice, add the denatured probe to a prewarmed (42°C) amount of DIG-easy hyb; mix well (use 3.5 mL of DIG-easy hyb per 100 cm^2). This makes the hybridization solution.
4. Decant the prehybridization solution and add the hybridization solution to the membrane. Incubate overnight at 42°C; rock gently.
5. Decant the hybridization solution.
6. Wash the membrane twice for 5 min with wash buffer 1 at RT.
7. Wash the membrane twice for 15 min with wash buffer 2 at 65°C.

3.6.6. Chemiluminescence Detection

1. Incubate the membrane twice for 15 min in blocking buffer.
2. Incubate the membrane for 60 min with anti-DIG-conjugated AP (diluted 5000-fold in blocking buffer).
3. Preincubate membrane for 5 min with assay buffer.
4. Prepare 0.25 m*M* CSPD in assay buffer (prepare a volume just sufficient to cover the entire membrane).

5. Decant the preincubated assay buffer and add the CSPD solution for exactly 5 min.
6. Decant off the CSPD solution, drip off excess fluid.
7. Expose membrane to a Curix blue HC-S Medical X ray film in a film cassette, and expose for 2–12 h depending on the intensity of the signal.
8. Develop the film.
9. Scan the film and calculate the OD × surface of each signal by means of an image analyzer.

3.7. Metabolism of Nucleoside Analogs with Anti-HCMV Activity in HEL Cells

1. Confluent monolayers of HEL cells in 25-cm^2 culture flasks are infected with the desired strain of HCMV so that CPE reaches about 70% by 5 d postinfection.
2. Remove medium and incubate the cultures with 3 mL of culture medium containing (a predetermined amount) of radiolabeled nucleoside.
3. Incubate cultures for 12–24 h at 37°C.
4. Trypsinize the cell cultures, harvest by centrifugation, and wash two times with 5 mL of PBS.
5. Extract the cell pellets with 70% ice-cold methanol and leave them on ice for 10 min.
6. Spin in a microfuge and filter the supernatant through an Acrodisc 0.45-μ*M* filter.
7. The formation of the metabolites is analyzed by means of HPLC on an anion-exchange Partisphere radial compression column. A linear gradient of 7 m*M* $(NH_4)H_2PO_4$ (pH 3.8) to 250 m*M* $(NH_4)H_2PO_4$ + 500 m*M* KCl (pH 4.5) is used to separate the different metabolites. Extracts should be "spiked" with natural nucleosides and nucleotides and chemically or enzymatically prepared mono-, di-, and triphosphate metabolites of the compounds under study.
8. Two-mL fractions are collected and radioactivity determined by means of liquid scintillation counting.

3.8. DNA Polymerase Assay

3.8.1. Isolation of HCMV DNA Polymerase

Different methods have been published to purify the HCMV DNA polymerase from HCMV-infected cell cultures or from

recombinant sources. Therefore, we will not expand on the purification procedure of this enzyme, but rather refer to methods published by others ***(11–13)***. For initial studies on inhibition of HCMV DNA polymerase, the enzyme can be partially purified from HCMV-infected HEL cultures using "DEAE-column chromatography," as initially described by Huang ***(11)***.

A particular feature of herpesvirus DNA polymerases is that their activity is markedly stimulated at high salt concentrations (100 m*M* $[NH_4]_2SO_4$ is well suited for this purpose), whereas the activity of the cellular DNA polymerases is diminished under these conditions. This characteristic allows us to obtain initial information on the kinetics of inhibition of partially purified HCMV DNA polymerase by a certain compound, in a relatively fast way.

3.8.2. DNA Polymerase Assay

1. HCMV DNA polymerase activity is determined in 20 m*M* Tris-HCl (containing 100 m*M* $[NH_4]_2SO_4$), 100 µ*M* each of dCTP, dGTP and dTTP and 2 µ*M* of (2,8–^{3}H)dATP (specific radioactivity: 24 Ci/mmol), 100 µ*M* dithiotreitol, 3 m*M* $MgCl_2$, 500 µg/mL bovine serum albumin (BSA) and 100 µg/mL of activated calf thymus DNA and the appropiate amount of enzyme.
2. The reaction is incubated for 30 min at 37°C, at which time 1 mL of 10% TCA is added.
3. TCA-insoluble material is precipitated on GF/C filters, filters are washed three times with 10 mL of 10% TCA and analyzed for radioactivity by liquid scintillation.

4. Notes

1. Use *Mycoplasma*-free HEL cells of good quality (i.e., cells should be of a rather young passage and grow rapidly to confluency after plating). The use of older cells will markedly affect the replication of the virus and the reproducibility of the assays.
2. For determination of a potential effect of a compound on HCMV replication, use not more than 100 PFU/100 µL of HCMV (in a 96-well plate format) and a 2-h incubation of the viral inoculum with the cells. If not, the input virus may be too high, which will mask

potential inhibitory effects. When titrating a new virus stock it is convenient to run in parallel at each virus dilution, a dilution series of a reference anti-HCMV compound (e.g., ganciclovir or cidofovir). The virus dilution at which ganciclovir yields an EC_{50} value of about 0.5–1 μg/mL and cidofovir an EC_{50} value of about 0.1 μg/mL is suited for this purpose.

3. It is important to carefully remove all serum-containing medium in the fluorometric anti-HCMV assay before adding FDA, because serum contains esterases that will result in a high background signal.
4. When preparing radiolabeled HCMV particles, one should make sure that the radiolabel is associated with the viral DNA. Therefore, the radiolabeled pellet should be titrated on HEL cells for determination of virus titer, and the specific radioactivity should be determined by quantifying the amount of incorporated label in viral DNA. A fraction of the purified virus should then be spotted on GF/C filters, followed by extensive washing of the filters with 10% TCA, so that all radiolabeled dThd that is not incorporated into viral DNA is being removed.
5. For determination of viral antigens in HCMV-infected HEL cells by means of flow cytometry, cells should not cluster after trypsinization and in the subsequent staining and fixation steps. This can be prevented by incorporation of 1% gelatin in the buffers.
6. For the preparation of the digoxigenin-labeled probe, it is important to carefully purify the probe to remove any contaminating free DIG-11-dUTP. The precise amount of probe (dilution of the labeled stock probe) required in the hybridization assay should be determined for each newly generated probe. Probes can easily be stored for over a year at –20°C without decrease in activity. It is therefore advisable to prepare at once sufficiently large quantities of probe.
7. For the study of the intracellular phosphorylation of nucleoside analogs, a reference molecule such as ganciclovir should be included for which it is known that the phosphorylation is markedly increased in cells infected with HCMV. Several companies prepare radiolabeled nucleoside analogs at the request of the customer (e.g., Moravek, Brea, CA).

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References

1. Neyts, J., Snoeck, R., Schols, D., Himpens, B., and De Clercq, E. (1991) Sensitive, reproducible and convenient fluorometric assay for the in vitro evaluation of anti-cytomegalovirus agents. *J. Virol. Methods* **35,** 27–38.
2. Neyts, J., Snoeck, R., Schols, D., Balzarini, J., Esko, J. D., Van Schepdael, A., and De Clercq, E. (1992) Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology* **189,** 48–58.
3. Schols, D., Snoeck, R., Neyts, J., and De Clercq, E. (1989) Detection of immediate early, early and late antigens of human cytomegalovirus by flow cytometry. *J. Virol. Methods* **26,** 247–254.
4. Biron, K. K., Stanat, S. C., Sorrell, J. B., Fyfe, J. A., Keller, P. M., Lambe, C. U., and Nelson, D. J. (1985) Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human diploid fibroblasts infected with human cytomegalovirus. *Proc. Natl. Acad. Sci. USA* **82,** 2473–2477.
5. Neyts, J., Balzarini, J., Andrei, G., Chaoyong, Z., Snoeck, R., Zimmermann, A., Mertens, T., Karlsson, A., and De Clercq, E. (1998) Intracellular metabolism of the N7-substituted acyclic nucleoside analog 2-amino-7-(1,3-dihydroxy-2-propoxymethyl)purine, a potent inhibitor of herpesvirus replication. *Mol. Pharmacol.* **53,** 157–165.
6. Littler, E., Stuart, A. D., and Chee, M. S. (1992) Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the natural nucleoside analog ganciclovir. *Nature* **358,** 160–162.
7. Sullivan, V., Talarico, C. L., Stanat, S. C., Davis, M., Coen, D. M., and Biron, K. K. (1992) A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature* **358,** 162–164.
8. De Clercq, E. (1995) Trends in the development of new antiviral agents for the chemotherapy of infections caused by herpesviruses and retroviruses. *Rev. Med. Virol.* **5,** 149–164.
9. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) *Molecular Cloning — A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

10. Emanuel, J. R. (1991) Simple and efficient system for synthesis of non-radioactive nucleic acid hybridization probes using PCR. *Nucleic Acids Res.* **19,** 2790.
11. Huang, E. S. (1975) Human cytomegalovirus III: virus-induced DNA polymerase. *J. Virol.* **16,** 298–310.
12. Ertl, P. F. and Powell, K. L. (1992) Physical and functional interaction of human cytomegalovirus DNA polymerase and its accessory protein (ICP36) expressed in insect cells. *J. Virol.* **66,** 4126–4133.
13. Cihlar, T., Fuller, M. D., and Cherrington, J. M. (1997) Expression of the catalytic subunit (UL54) and the accessory protein (UL44) of human cytomegalovirus DNA polymerase in a coupled in vitro transcription/translation system. *Protein Expr. Purif.* **11,** 209–218.

10

Construction of Recombinant Human Cytomegalovirus

Richard F. Greaves

1. Introduction

The use of reverse genetics to generate recombinant viruses allows the researcher to investigate the exact functional significance of particular viral genes during the virus life cycle, by means of their deletion or modification in the viral genome. These studies can extend to the introduction of viral or foreign genetic material into ectopic sites in the viral genome, to investigate viral *cis*-control sequences or to phenotypically modify or tag the recombinant virus. Genetically modified viruses may also have applications in biotechnology and gene therapy. Such studies are well advanced for herpes simplex virus (HSV), a relative of human cytomegalovirus (HCMV), but have been relatively limited for HCMV itself.

Genetic modification of HCMV was first achieved by the insertion of coding sequences for β-galactosidase ***(1)***, an enzyme detectable by conversion of a chromogenic substrate. The marker enzymes β-galactosidase and β-glucuronidase have since been used to generate viruses that lack many open reading frames (ORFs) not essential for HCMV replication in cell culture ***(2–5)***. Standard methods require recombination between transfected plasmid and transfected viral DNA and, although selection both for and against the markers

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is possible ***(1,2,6)***, it is achieved manually and is both technically difficult and time-consuming. The potential for automation does however exist, through the use of fluorogenic substrates and infected cell sorting. More recently, several methods have been developed that simplify the isolation of recombinant HCMV. These include the generation of recombinant viruses by reconstruction from overlapping co-transfected cosmids ***(7)***, the use of neomycin phosphotransferase as a drug-selectable marker ***(8,9)***, and the use of the drug-selectable marker gene *gpt* ***(10–12)***. The use of drug-selectable markers simplifies the delivery of the viral genome for recombination, as the lower recombination frequencies obtained after infection become exploitable. The particular advantage of *gpt* is that drug selection both for and against the marker gene is possible, permitting straightforward isolation of mutants carrying *gpt* by drug selection, and the rapid selection of phenotypic rescues or second-generation mutants ***(10)***.

The most recent advances in recombinant cytomegalovirus technology include the use of green fluorescent protien as a marker gene ***(13)***, and the cloning of infectious cytomegalovirus genomes as bacterial artificial chromosomes ***(14)***. The latter approach permits the use of bacterial genetics to achieve directed and ramdom mutagenesis of the viral genome, prior to the recovery of recombinant virus upon the transfection of permissive cells. These techniques promise rapidity and high throughput, but will not be detailed further this chapter.

This chapter describes the use of drug selection to introduce *gpt* into, and remove *gpt* from, the viral genome. Although it follows the simple example of removing and restoring a viral ORF, it is possible to use these techniques to make other modifications to the virus. Such changes might include large- or small-scale alterations to flanking viral sequences ***(10,12)***, or the introduction of other foreign genes.

Techniques are described for the retroviral transfer of HCMV genes into fibroblasts, and for the immortalization or growth extension of these cells by the retroviral delivery of the E6 and E7 genes of human papillomavirus 16 (HPV 16). The generation of long-lived

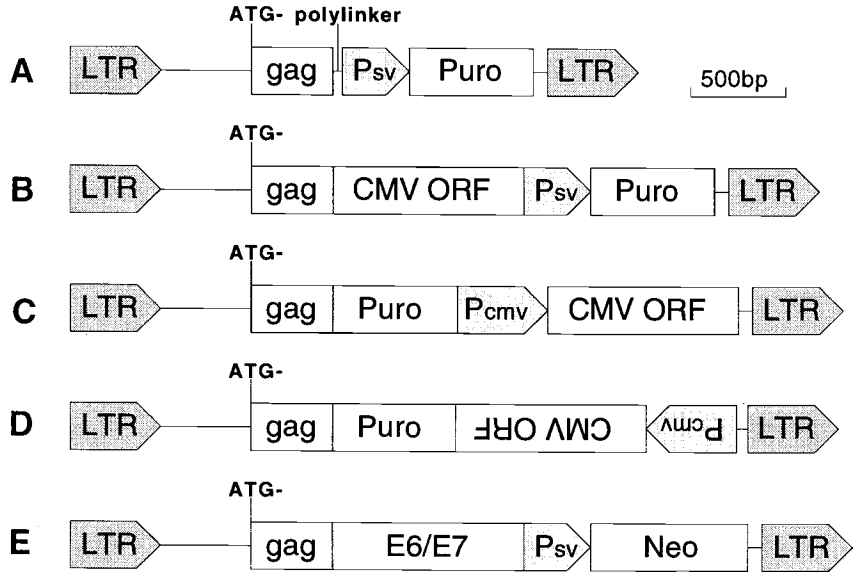

Fig. 1. (A) Proviral portion of retroviral expression vector pBABE Puro (13). (B–D) Expression vectors for HCMV ORFs. (E) Immortalizing vector pLXSN16E6E7. LTR, Retroviral long terminal repeats; gag, Initiator-less retroviral gag coding sequence; Psv, Simian virus 40 early promoter; Puro, Puromycin acetyltransferase coding sequence; CMV ORF, HCMV open reading frame; Pcmv, HCMV promoter; Neo, Neomycin phosphotransferase coding sequence

fibroblast lines allows the complementation in *trans* of viral mutants with disruptions in genes that are otherwise essential for growth in cell culture ***(12)***.

2. Materials

2.1 Design of Plasmid Vectors for the Generation of Complementing Cell Lines and of gpt*-Containing HCMV*

Retroviral vectors that express HCMV gene products are generated by inserting open reading frames from the HCMV genome into pBABEPuro ***(15)*** or similar vectors (**Fig. 1A**,**B**). Fragments may be isolated from plasmid or cosmid libraries ***(16–18)*** or by polymerase

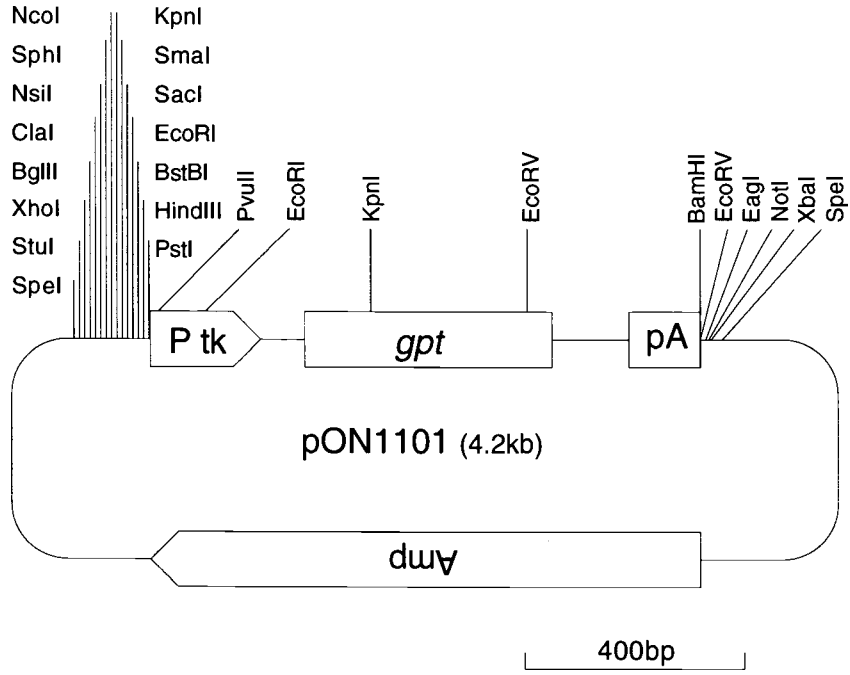

Fig. 2. Vector pON1101 (10), containing a eukaryotic gpt expression cassette. P tk, Herpes simplex virus type 1 thymidine kinase promoter; gpt, E. coli guanosine phosphoribosyl transferase coding sequence; pA, Simian virus 40 polyadenylation signal.

chain reaction (PCR) amplification from the viral genome, using published sequence information ***(19)***. Ensure that ATG codons are absent from upstream noncoding sequences, and that polyadenylation signals from HCMV are not introduced into the retroviral vector. If control by the cognate HCMV promoter is desirable, the vector may be structured differently (**Fig. 1C**). Toxicity of viral gene products may force the redesign of vectors. For delayed-early genes, inducible on HCMV infection, the best solution may be to include the cognate promoter, and clone the HCMV fragment antisense with respect to the vector LTR (**Fig. 1D**). Alternatively, the use of self-contained inducible retroviral vectors may be considered ***(20)***. Vector pLXSN16E6E7 ***(21)*** is required to immortalize HF cells (**Fig. 1E**).

To insert gpt into the viral genome, a gpt expression cassette from plasmid pON1101 (**Fig. 2**) is introduced into a plasmid containing an HCMV DNA fragment. Viral sequences flanking gpt will direct

homologous recombination into the HCMV genome. Flanking viral sequences should ideally be 1 kbp or longer, though I have obtained recombinants using shorter (500-bp) overlaps. HCMV DNA fragments are routinely sub-cloned from plasmids or cosmids, while ensuring that the intact viral fragment can readily be excised from the backbone of the resultant plasmid. The gpt expression cassette is usually inserted in the same orientation as prevailing viral transcription, replacing a complete ORF. Consensus promoter sequences may also be removed, though polyadenylation signals are usually left intact, and flanking ORFs should not be disrupted. Restriction enzyme sites may exist which match these criteria, or may be generated by directed mutagenesis or during PCR cloning.

Ideally, there should be no overlap of HCMV sequences between vectors used to establish cell lines and replacement vectors which direct HCMV mutagenesis. This reduces the likelihood of phenotypic rescue occurring as a result of recombination between virus and cell line, but is not always possible.

2.2. Selection for gpt *in* E. Coli

1. LB Agar plates + 10 µg/mL tetracycline (LB-Tet).
2. *E. coli* strain WB-1 ***(10)***.
3. 10 X M9 salts, autoclaved (6 g/L K_2HPO_4, 3 g/L KH_2PO_4, NaCl 0.5 g/L, NH_4Cl 1 g/L).
4. Agar (Difco, Detroit, MI).
5. Vitamin-free casamino acids (Difco).
6. 20% (w/v) glucose in ddH_2O, filter sterile, store at –20°C.
7. 1% (w/v) thiamin hydrochloride in ddH_2O, filter sterile, store at –20°C.
8. 100 m*M* $CaCl_2$ in ddH_2O, filter sterile.
9. 1 *M* $MgSO_4$ in ddH_2O, filter sterile.
10. 5 mg/mL xanthine in 0.1 *M* NaOH, store at –20°C
11. 0.1 *M* HCl.
12. 100 mg/mL ampicillin, filter sterile; store at –20°C.
13. Plasmid pON1101 ***(10)***.
14. Plasmid containing HCMV genomic fragment (*see* **Subheading 2.1.**).
15. LB agar plates + 100 µg/mL ampicillin (LB-Amp).

2.3. Generation of Human Fibroblast Lines Expressing HCMV Proteins

1. Low passage HF culture: human foreskin fibroblasts (HFF), or human fetal lung fibroblasts (HFL).
2. Amphotropic retroviral packaging cell line, suitable for transient packaging of transfected vectors. ΦNX-A cells (Garry Nolan, Stanford) are available from ATCC.
3. Dulbecco's Modified Eagle Medium (DMEM, Gibco-BRL) plus antibiotics.
4. Fetal calf serum (FCS; Gibco-BRL).
5. Nuserum (Collaborative Biomedical, Bedford, MA; Becton Dickinson, Franklin, NJ).
6. Phosphate-buffered saline (PBS) + 0.2 mg/mL EDTA, autoclaved (PBS-EDTA).
7. 6- and 10-cm tissue culture dishes, 25-cm^2 flasks, 6- and 24-well tissue culture dishes (Nunc or similar).
8. Tissue culture incubator, 5% CO_2, humidified, 37°C.
9. Double distilled or de-ionized water, autoclaved (ddH_2O).
10. Retroviral expression vector (*see* **Subheading 2.1.**). Transfection quality plasmid DNA, CsCl gradient purified.
11. Chloroquine (Sigma) 50 m*M* in ddH_2O, sterile filtered, store at –20°C.
12. 2X HBS: 50 m*M* HEPES, 10 m*M* KCl, 12 m*M* dextrose, 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, pH to 7.05 with NaOH, made in ddH_2O, sterile filtered, store at 4°C.
13. 2 *M* $CaCl_2$, in ddH_2O, sterile filtered, store at 4°C.
14. 5 mL syringe + sterile 0.45-μm syringe filter.
15. Polybrene (Sigma) 5 mg/mL in ddH_2O, sterile filtered, store at –20°C.
16. Puromycin (Sigma) 1 mg/mL in ddH_2O, sterile filtered, store at –20°C.
17. G418 (Gibco-BRL) 100 mg/mL (active conc.) in ddH_2O, sterile filtered, store at –20°C.
18. Cloning cylinders (Sigma or similar).

2.4 Recombination and Enrichment of gpt-Containing HCMV

1. High-titer wild-type HCMV stock.
2. HF cells (or complementing fibroblast cells).

3. Plasmid DNA containing *gpt* cassette and flanking HCMV sequences, transfection quality (*see* **Subheading 2.1.**).
4. DMEM (Gibco-BRL) plus antibiotics.
5. Nuserum (Collaborative Biomedical).
6. 2X BBS (50 m*M* *N*,*N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES, Sigma), 280 m*M* NaCl, 1.5 m*M* Na_2HPO_4, pH to 6.95 with NaOH), made in ddH_2O, sterile filtered, store at 4°C.
7. 2.5 *M* $CaCl_2$ in ddH_2O, filter sterile.
8. 6-cm tissue culture dishes, 75-cm^2 flasks, 175-cm^2 flasks.
9. Tissue culture incubator, 3% CO_2, 37°C, humidified.
10. Sterile syringes and 0.45-µm syringe filters.
11. Mycophenolic acid (Gibco-BRL) 10 mg/mL in 0.1 *M* NaOH, store at –20°C
12. Xanthine (Sigma) 5 mg/mL in 0.1 *M* NaOH, store at –20°C.
13. 0.1 *M* HCl.
14. 9% (w/v) nonfat milk in ddH_2O, autoclaved twice on successive days for 15 min.

2.5. Preparation of HCMV DNA from Infected Cell Cytoplasm

1. HCMV-infected cells.
2. TE-NP-40-DOC: 10 m*M* Tris.Cl pH8.0, 1 m*M* EDTA, 1% (v/v) Nonidet-P40, 0.5% (w/v) sodium deoxycholate, store at –20°C.
3. 10 mg/mL proteinase K in ddH_2O, store at –20°C.
4. 10 mg/mL RNAse A in ddH_2O, store at –20°C.
5. 20% Sodium dodecyl sulfate (SDS) in ddH_2O.
6. Water-saturated phenol, neutralized.
7. Chloroform.
8. 3 *M* Sodium acetate, pH to 5.5 with acetic acid, autoclaved.
9. Isopropanol.
10. 70% (v/v) ethanol in ddH_2O.
11. TE, autoclaved (10 m*M* Tris-HCl, pH8.0, 1 m*M* EDTA).

2.6. Plaque Purification of gpt+ Virus Under Agarose

Materials as in **Subheading 2.4.**, with the following additions:

1. 1.25% Agarose in ddH_2O, autoclaved.

2. 2X DMEM, made from 10× DMEM (Gibco-BRL), sterile ddH_2O, and sodium bicarbonate solution (Gibco-BRL).
3. 10-cm tissue culture dishes.
4. Thermometer.
5. Sterile pasteur pipets.

2.7. Selection Against gpt

Materials as in **Subheadings 2.4.–2.6.**, with the following additions:

1. High-titer *gpt*$^+$ HCMV stock
2. Plasmid DNA containing HCMV sequences spanning *gpt* insertion in virus.
3. *hprt*$^-$ fibroblasts (e.g., ILN7 cells) ***(10)***.
4. 6-Thioguanine (Sigma), 5 mg/mL in 0.1 *M* NaOH.

3. Methods

3.1. Selection for gpt in E. coli

See **Note 1**.

1. *E. coli gpt* mutant strain WB-1 is propagated on LB Agar plates + 10 µg/mL tetracycline.
2. Make Min-X-Amp plates: Autoclave 1.5 g Agar + 1 g casamino acids in 84 mL ddH_2O, cool to 55°C. Add 10 mL 10× M9 salts, 1 mL 20% glucose, 0.1 mL 100 m*M* $CaCl_2$, 1 mL 1 *M* $MgSO_4$, 50 µL 1% thiamine.HCl, 0.1 mL 100 mg/mL ampicillin, 2 mL 5 mg/mL xanthine in 0.1 *M* NaOH, 2 mL 0.1 *M* HCl. Mix and pour 20 mL per Petri dish.
3. Digest plasmids, purify fragments, and ligate *gpt* cassette into the plasmid containing HCMV DNA.
4. Generate competent WB-1 cells by a standard protocol.
5. Transform competent WB-1 with ligation reaction from **step 3**.
6. Divide transformed cells. Plate one-half on LB-Amp plates, and one-half on Min-X-Amp plates. Culture at 37°C.
7. *gpt*$^+$ colonies may take 2–4 d to appear on Min-X-Amp plates. If none arises, take Amp^R colonies from LB-Amp plates, and duplicate plate onto LB-Amp and Min-X-Amp plates. Isolate plasmids from colonies viable on Min-X-Amp and analyze.
8. Prepare Min-X-Amp media, as in **step 1**, but omitting Agar.

9. Culture positive clones to saturation in 500 mL liquid Min-X-Amp media (24 h, 37°C, shaking), and isolate transfection quality plasmid DNA by a standard protocol.

3.2. Generation of Complementing Human Fibroblast Lines Expressing HCMV Proteins

Expression of HCMV proteins in human fibroblast lines is achieved by using the amphotropic ΦNX-A packaging cell line developed by Philip Acacoso and Garry P. Nolan at Stanford University. ΦNX-A cells transiently package transfected Moloney virus-based retroviral vectors, rapidly, and free of helper virus. Primary fibroblasts transduced with retroviral expression vectors will express HCMV gene products and may be used as a bulk (i.e., polyclonal) population, although their life span is limited. The use of clonally purified lines derived from primary cells is generally not feasible, owing to life span constraints. Treatment of fibroblasts with a retroviral vector expressing HPV16 E6 and E7 gene products ***(21)*** results in "immortalized" (or at the very least growth-extended) fibroblasts, which are highly permissive for HCMV ***(22)*** and can be used either as a bulk population or as purified clones. To generate immortalized cell lines, the procedure described below is followed twice, once to introduce the HCMV gene product, and once to introduce the immortalizing pLXSN16E6E7 vector. Packaging of oncogenic papillomavirus sequences into amphotropic vectors is necessarily hazardous, as the vectors are designed to infect human cells. Strict local safety procedures should be established for this work. Delivery of HCMV genes by amphotropic vectors should likewise be treated with appropriate caution.

1. Culture ΦNX-A cells in DMEM + 10% FCS, in 10-cm dishes. Split at 50–70% confluence, using 2 mL PBS-EDTA to detach cells.
2. *Day 1*: Detach ΦNX-A cells from a 70% confluent 10-cm dish, add 8 mL DMEM + 10% FCS, pellet cells (2000 rpm, 5 min), re-suspend in 15 mL DMEM +10% FCS and plate 5 mL in three 6-cm dishes.
3. *Day 2*: Add 2.5 µL chloroquine solution to each 6cm dish 5 min before transfection, return ΦNX-A cells to incubator.
4. In a 15-mL tube, combine 438 µL ddH_2O, 61 µL 2 *M* $CaCl_2$, and 8 µg retroviral vector plasmid DNA. Mix. Add 500 µL 2X HBS, and

bubble air for 10 s to mix. A cloudy precipitate should form. Include a positive control of empty retroviral vector (e.g., pBABEPuro), and a negative control of, e.g., pGEM (*see* **Note 2**).

5. Add transfection solutions dropwise to 6-cm dishes of ΦNX-A cells, rock to mix, and return to incubator.
6. *Day 3*: Replace medium on transfected ΦNX-A cells with 5 mL DMEM, 10% FCS, return cells to incubator.
7. Trypsinize low-passage fibroblasts from a confluent 25-cm^2 flask into six wells of a 6-well dish, place cells in incubator.
8. *Day 4*: Remove medium from ΦNX-A cells, pass through a 0.45-μm filter (*see* **Note 3**), and mix 3 mL with 3 μL polybrene solution. Immediately aspirate medium from one well of 6-well dish of fibroblasts, add packaged supernatant, and return cells to incubator.
9. *Day 5*: Trypsinize transduced fibroblasts, and separate cells thoroughly. Plate in DMEM + 10% Nuserum + 10% FCS, in two 6-well dishes (4 mL/well), starting with 50% of the cells in the first well, then 25% etc., making 11 twofold dilutions. Treat positive and negative control cultures similarly.
10. *Day 8*: Apply drug selection (1 μg/mL puromycin or 400 μg/mL G418).
11. Change media (DMEM + 10% Nuserum + 10% FCS) twice a week, removing dead cells. Maintain drug selection, and guard against dessication. Puromycin resistance is obvious after 4 d, G418 1 wk. E6/E7 transduced cells have an obviously enhanced growth rate.
12. *Approximately 2–3 wk*: Survival is usually highest at intermediate dilutions — high levels of cell death in the undiluted cultures inhibit colony formation. Trypsinize and pool confluent wells for polyclonal populations. Isolate large, well-separated colonies by trypsinization, using cloning cylinders (*see* **Note 4**). Grow clones on in 24-well dishes. Maintain drug selection.
13. Confirm synthesis of HCMV gene products in cell lines by western blot and immunocytochemical staining, using specific antibodies (*see* **Note 5**).

3.3. Recombination and Enrichment of gpt-Containing HCMV

Before commencing this work, local safety procedures for the generation and handling of wild-type and recombinant HCMV strains should be established. Recombinations should be performed

in duplicate, to allow the parallel isolation of independent isolates. A negative control recombination using wild-type virus and e.g., pGEM should be carried through the whole procedure, to give an indication of the effectiveness of selective protocols.

1. *Day 1*: Plate 5×10^5 HF cells in 6-cm dishes, one for each recombination reaction.
2. Digest 8 µg of *gpt*-containing plasmid (or pGEM) with restriction enzyme(s) to remove the plasmid backbone.
3. Phenol/chloroform extract, then chloroform extract DNA, add salt and ethanol precipitate in 1.5-mL tubes. Store at –20°C.
4. *Day 2*: 2–4 h before transfection, change media on 6-cm dishes for 5 mL DMEM + 10% Nuserum. Return to 3% CO_2 incubator.
5. Pellet digested DNA, wash in 70% ethanol, air dry in sterile conditions, and dissolve in 250 µL ddH_2O.
6. Add 250 µL 2X BBS to digested DNA and mix, add 25 µL 2.5 *M* $CaCl_2$ and mix gently. Leave for 20 min at room temperature (*see* **Note 6**).
7. Add mixture dropwise to HFs in 6-cm dishes and mix. Incubate overnight, 3% CO_2, 37°C.
8. *Day 3 (am)*: Observe fine precipitate on cells, change media for 6 mL DMEM + 10% Nuserum.
9. *Day 3 (pm)*: Infect cells with approx 10 PFU/cell wild-type HCMV, diluted in 2 mL DMEM (serum-free). Absorb with rocking for 1 h; then change media for 6 mL DMEM + 10% Nuserum.
10. *Day 4*: Inspect cells for cytopathic effect (c.p.e.); 100% of cells should be infected (*see* **Note 7**).
11. *Day 7*: Divide HFs (or complementing fibroblasts), one confluent 175-cm^2 flask into five 75-cm^2 flasks.
12. *Day 8*: Remove culture medium from infected 6-cm dishes, and pass through 0.45-µm syringe filter (*see* **Note 8**). Freeze an aliquot at –70°C, mixed 1:1 with autoclaved 9% nonfat milk (*see* **Note 9**), and retain remainder.
13. Inoculate fresh 75-cm^2 flasks with various volumes (e.g., 500, 100, 20 µL) of freshly filtered culture medium, diluted in 5 mL serum-free DMEM. Rock to absorb for 1 h.
14. Make culture medium for *gpt* selection: 90 mL DMEM + 10 mL Nuserum + 1 mL 5 mg/mL xanthine in 0.1 *M* NaOH + 1 mL 0.1 *M* HCl + 100 µL 10 mg/mL mycophenolic acid in 0.1 *M* NaOH. Add 20 mL to each 75-cm^2 flask, and return to incubator (*see* **Note 10**).

15. *Day 9*: Assess 24-h rounding c.p.e. Large inocula may give obvious c.p.e., lower dilutions less so. Continue culturing flasks which show little or no c.p.e. at this stage. Do not replace medium.
16. Note when 100% c.p.e. is reached (approx 1½–3 wk)
17. 4 d after 100% c.p.e., plate out fresh 75-cm^2 flasks of HFs or complementing fibroblasts.
18. 5 d after 100% c.p.e., filter culture medium from infected 75-cm^2 flasks through 0.45-µm syringe filters, freeze a sample at –70°C, mixed 1:1 with autoclaved 9% nonfat milk, and retain remainder to inoculate fresh cells. Use infected cells for DNA preparation (*see* **Subheading 3.4.**).
19. Repeat **steps 13–18** two more times (*see* **Note 13**).

3.4. Isolation of HCMV DNA from Infected Cell Cytoplasm

See **Note 12**.

1. Wash infected cells in an infected 75-cm^2 flask (or one well of a 6-well dish) with 5 mL (1 mL) PBS, drain.
2. Lyse cells *in situ* with 500 µL (100 µL) TE-NP-40-DOC. Scrape lysed cells into 1.5-mL microcentrifuge tube.
3. Incubate 5 min on ice, spin 5 min at 2000 rpm.
4. Retain supernatant, discard nuclear pellet. Add 10 µL (2 µL) 10 mg/mL Proteinase K, and 10 µL (2 µL) 10 mg/mL RNAse A. Incubate at 60°C, 15 min.
5. Add 25 µL (5 µL) 20% SDS. Incubate at 60°C, 90 min.
6. Cool on ice, extract digested extract with an equal volume of phenol, then 1:1 phenol/chloroform, then chloroform.
7. Add 1/10 vol 3 *M* sodium acetate, pH 5.5, to aqueous phase and mix, add 1 vol isopropanol and mix. Incubate overnight, 4°C.
8. Spin 15,000 rpm, 10 min, wash pellet in 70% ethanol, air dry and dissolve in 50 µL (10 µL) TE.

3.5. Analysis of Viral DNA

Detailed analysis of viral DNA is carried out by Southern blot, although PCR and DNA dot-blotting may also be useful. DNAs from stocks enriched for *gpt* are probed for *gpt* and for viral sequences near to the expected *gpt* insertion but absent from the original *gpt*-containing plasmid; 2–4 µL of DNA is used as prepared

in **Subheading 3.4.**, and restriction enzymes should be selected to emphasise any changes to the expected restriction map of the recombinant viral genome. Note that the *gpt* cassette (**Fig. 2**) introduces *Eco*RI, *Kpn*I, and *Eco*RV sites. By carefully choosing the restriction enzyme and viral probe, the analysis should measure the proportion of viral genomes in wild type and recombinant configurations. The recombinant proportion frequently will reach 10–30% after three rounds of *gpt* enrichment. At this stage, recombinants may be plaque purified. Analysis of DNA from plaque-purified clones should be used to demonstrate the presence of *gpt*, the absence of replaced viral sequences, and the disruption of neighboring loci.

3.6. Plaque Purification of gpt+ *Virus Under Agarose (See Note13)*

1. Set up 10-cm dishes of HF cells or complementing fibroblasts, seven dishes from a confluent 175-cm^2 flask.
2. Next day, melt 40 mL sterile 1.25% agarose in a microwave oven and place in a 55°C water bath. Warm Nuserum, 2X DMEM, xanthine, mycophenolic acid and HCl to 37°C.
3. After 1 d, infect cells with varying inocula either from a gpt-enriched virus population or from a previously plaque-purified virus stock. Inocula of 10, 1, 0.1, and 0.01 µL should be sufficient, diluted in 2 mL serum-free DMEM. Absorb for 1 h with rocking.
4. Prepare agarose overlay: Add 50 mL 2X DMEM, 10 mL Nuserum, 1 mL xanthine, 0.1 mL mycophenolic acid, 1.1 mL 0.1 M HCl, and mix. Sterilize a thermometer by soaking in ethanol, air dry in hood, and use to measure temperature of liquid overlay solution.
5. As soon as overlay reaches 37°C, remove inocula from 10-cm dishes, replace with 15-mL overlay agarose, and mix by swirling onto cells. Use plastic pipets and work rapidly. Leave overlays still until set (10 min), and transfer to incubator.
6. Next day, seal dishes with parafilm to prevent evaporation.
7. Observe the formation of plaques by microscopic examination from 7 d onward. Mark well-separated plaques on the dishes by drawing circles around them.
8. 13 d after infection, set up several 6-well dishes, each well containing 2×10^5 HFs (or complementing cells).

9. 14 d after infection replace medium on 6-well dishes with 1 mL/well DMEM (serum-free).
10. Using a sterile pasteur pipet, remove the plug of agarose above a marked plaque, and transfer to HFs in a six well dish. Scrape underlying infected cells with Pasteur's pipet and transfer. Transfer each plaque thus to a fresh well. Absorb with rocking, 1 h.
11. Replace medium with 4 mL *gpt* selective medium (*see* **Subheading 3.3.**, **step 13**).
12. Note when 100% c.p.e. is reached in each well (1–3 wk).
13. 5 d after 100% c.p.e., freeze an aliquot of culture medium at –70°C, mixed 1:1 with 9% autoclaved milk.
14. Isolate and analyze viral DNA from infected cells (*see* **Subheadings 3.4.** and **3.5.**).
15. Repeat plaque purification of positive clones twice.

3.7. Selection Against gpt

See **Note 13**.

1. Recombination is carried out as in **Subheading 3.3.**, **steps 1–9**, except a *gpt*$^+$ virus and a plasmid containing wild-type viral sequences are used. Include recombination with, e.g., pGEM as a negative control.
2. *Day 7*: Split a 175-cm^2 flask of *hprt*- fibroblasts into five 75-cm^2 flasks.
3. *Day 8*: Remove culture medium from infected 6-cm dishes, and pass through 0.45-µm syringe filter. Freeze an aliquot at –70°C, mixed 1:1 with autoclaved 9% nonfat milk, retain remainder.
4. Inoculate *hprt*$^-$ cells in 75-cm^2 flasks with various volumes (e.g., 500, 150, 50 µL) of freshly filtered culture medium, diluted in 5 mL serum-free DMEM. Rock to absorb for 1 h.
5. Make culture medium for *gpt* backselection: 80 mL DMEM, 10 mL Nuserum, 10 mL FCS, 100 µL 6-thioguanine (6-TG) solution.
6. Remove inoculum and add 20 mL medium to each flask.
7. *Day 9*: Assess c.p.e at 24 h. Ideally, 10–50% cells should be infected.
8. Almost all infected cells will die within 2–3 d, and the monolayer should recover. Change media frequently to remove dead cells, maintaining 6-TG selection.
9. Wait two weeks for *gpt*$^-$ plaques to form. Rescue flasks should give more plaques than the pGEM control, although there is usually a background of spontaneous *gpt*$^-$ virus.

10. Wait for 100% c.p.e. If plaques are sparse, trypsinize and re-plate infected cells to spread the infection.
11. When 100% c.p.e. is reached, wash cells in drug-free culture medium, and then culture in 20 mL drug-free medium.
12. 5 d after 100% c.p.e., harvest culture medium for virus, and cells for DNA.
13. Prepare and analyze DNA as in **Subheadings 3.4.** and **3.5**. Plaque purify viruses as in **Subheading 3.6.**, but omit mycophenolic acid, xanthine, and HCl additions to agarose overlay and liquid media.

4. Notes

1. *E. coli* are metabolically affected by the expression of *gpt*, and selection may occur against high-level expression. The use of positive selection for *gpt* function in *E. coli* (**Subheading 3.1.**) ensures that an active *gpt* gene is transferred to virus. Different plasmid backgrounds in *gpt* constructs confer different growth characteristics on host *E. coli* cells. Cells containing some *gpt* plasmids may grow well on LB-Amp plates and badly on Min-X-Amp plates, and *vice-versa*. These differences may be due to the use by bacteria of cryptic promoters in HCMV-derived sequences. I have not yet made a *gpt*-containing plasmid that cannot be selected on Min-X-Amp plates, although colony formation may take 3 d or more. Plasmid pON1101 is a good positive control for these procedures.
2. Calcium phosphate transfection using HBS buffer (**Subheading 3.2.4.**) is very effective, but may require optimization. The pH value of 2X HBS is particularly important, and the optimal air bubbling time (**Subheading 3.2.4.**) may vary from batch to batch of 2X HBS.
3. Filtration of packaged retrovirus (**Subheading 3.2.8.**) ensures that packaging cells do not contaminate fibroblast cultures.
4. E6 E7 containing fibroblast clones (**Subheading 3.2.**, **step 12**) show extremely variable morphology and longevity. Colonies that grow rapidly at early stages and that closely resemble primary cells are usually the best choice for cloning. There may be a high level of rounded and detached cells in bulk immortalized populations and in many immortalized clones. Although HCMV c.p.e. becomes harder to assess in such cell populations, they often remain fully permissive for HCMV infection.
5. Levels of HCMV proteins in transduced cells (**Subheading 3.2.**, **step 13**) are variable, and not all cells in a polyclonal population

may express antigen. The proportion of expressing cells may change over time. Clones generally express more evenly and stably.

6. Calcium phosphate transfection using BES-buffered saline (BBS) buffer (**Subheading 3.3.6.**) gives good results for recombination. pH of 2X BBS is critical, CO_2 concentration should be 3%. High [CO_2] may cause a crystalline precipitate which kills cells. If cell death is a persistent problem, check CO_2 levels, or experiment with shorter periods of precipitate absorption. Although the authors of this transfection protocol ***(23)*** found low transfection efficiencies for linear DNA, I routinely obtain recombinants this way.
7. HCMV infected fibroblasts are discernible as rounded and more refractile at 24 h postinfection. Transfected fibroblasts become relatively refractory to HCMV infection. Thus, high multiplicities must be used to achieve 100% infection. If infection is not 100% (**Subheading 3.3.**, **step 10**), repeat with a higher inoculum.
8. Filtration of HCMV-containing culture supernatants (**Subheading 3.4.**, **step 12**) ensures that virions and not infected cells are transferred.
9. Addition of milk protein to HCMV stocks (**Subheading 3.3.**, **step 12**) stabilizes virions to freeze–thaw cycles, removing the need for multiple aliquots. Double autoclaving ensures sterility.
10. *gpt* selection (**Subheading 3.3.**, **step 14**) works because mycophenolic acid blocks *de novo* purine synthesis, and the *E. coli* gpt enzyme salvages xanthine, which cannot be salvaged by the mammalian equivalent hprt. FCS contains guanine, which is a substrate for hprt and can interfere with selection. Although dialyzed serum would be ideal, Nuserum (a partly defined serum substitute) has low guanine levels and works well. The recommended concentrations of mycophenolic acid (10 μg/mL) and xanthine (50 μg/mL) do not kill HFs but are inhibitory to growth. Wild type virus spreads slowly under these conditions, but gpt^+ virus should spread much more rapidly. If necessary, selection can be tightened by raising mycophenolic acid levels and dropping xanthine levels.
11. Viral titers in culture medium after early rounds of selection for *gpt* (**Subheading 3.3.**, **step 19**) may be quite low but should recover as gpt^+ virus becomes prevalent.
12. DNA isolated from cytoplasmic extracts (**Subheading 3.4.**) will contain prominent mitochondrial DNA bands.
13. Viruses should be plaque purified (**Subheading 3.6.**) to homogeneity by three plaque picks. If an enriched virus cannot be purified to homogeneity in this way, complementation may be required.

14. The guanine analog 6-thioguanine (6-TG) is normally toxic to human cells, and selection (**Subheading 3.7.**) works only in resistant hprt$^-$ cells, derived from Lesch-Nyhan syndrome patients. Selection kills cells infected by *gpt*$^+$ virus, and so depends on single *gpt*$^-$ virus particles infecting cells. Infecting 10–50% of cells should give a large number of single-hit infections and leave enough uninfected cells for the fibroblast monolayer to recover. Although 6-TG selection is far tighter than forward selection for *gpt*, there is a much higher background of undesired mutant viruses, probably attributable to spontaneous changes in the *gpt* gene. In practice, back-selection is more rapid, but is not as reliable as forward selection. Repetition may be necessary.

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References

1. Spaete, R. R. and Mocarski, E. S. (1987) Insertion and deletion mutagenesis of the human cytomegalovirus genome. *Proc. Natl. Acad. Sci. USA* **84,** 7213–7217.
2. Jones, T. R., Muzithras, V. P., and Gluzman, Y. (1991) Replacement mutagenesis of the human cytomegalovirus genome: US10 and US11 gene products are nonessential. *J. Virol.* **65,** 5860–5872.
3. Jones, T. R. and Muzithras, V. P. (1992) A cluster of dispensable genes within the human cytomegalovirus genome short component: IRS1, US1 through US5, and the US6 family. *J. Virol.* **66,** 2541–2546.
4. Browne, H., Churcher, M., and Minson, T. (1992) Construction and characterization of a human cytomegalovirus mutant with the UL18 (class I homolog) gene deleted. *J. Virol.* **66,** 6784–6787.
5. Kaye, J., Browne, H., Stoffel, M., and Minson, T. (1992) The UL16 gene of human cytomegalovirus encodes a glycoprotein that is dispensable for growth in vitro. *J. Virol.* **66,** 6606–6615.

6. Kohler, C. P., Kerry, J. A., Carter, M., Muzithras, V. P., Jones, T. R., and Stenberg, R. M. (1994) Use of recombinant virus to assess human cytomegalovirus early and late promoters in the context of the viral genome. *J. Virol.* **68,** 6589–6597.
7. Kemble, G., Duke, G., Winter, R., and Spaete, R. (1996) Defined large-scale alterations of the human cytomegalovirus genome constructed by cotransfection of overlapping cosmids. *J. Virol.* **70,** 2044–2048.
8. Wolff, D., Jahn, G., and Plachter, B. (1993) Generation and effective enrichment of selectable human cytomegalovirus mutants using site-directed insertion of the neo gene. *Gene* **130,** 167–173.
9. Schmolke, S., Kern, H. F., Drescher, P., Jahn, G., and Plachter, B. (1995) The dominant phosphoprotein pp65 (UL83) of human cytomegalovirus is dispensable for growth in cell culture. *J. Virol.* **69,** 5959–5968.
10. Greaves, R. F., Brown, J. M., Vieira, J., and Mocarski, E. S. (1995) Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the *Escherichia coli* guanosine phosphoribosyl transferase (*gpt*) gene. *J. Gen. Virol.* **76,** 2151–2160.
11. Prichard, M. N., Duke, G. M., and Mocarski, E. S. (1996) Human cytomegalovirus uracil DNA glycosylase is required for the normal temporal regulation of both DNA synthesis and viral replication. *J. Virol.* **70,** 3018–3025.
12. Greaves, R. F. and Mocarski, E. S. (1998) Defective growth correlates with reduced accumulation of a viral DNA replication protein after low-multiplicity infection by a human cytomegalovirus *ie1* mutant. *J. Virol.* **72,** 366–379.
13. Vieira, J., Schall, T. J., Corey, L., and Geballe, A. P. (1998) Functional analysis of the human cytomegalovirus US28 gene by insertion mutagenesis with the green fluorescent protein gene. *J. Virol.* **72,** 8158–8165.
14. Messerle, M., Crnkovic, I., Hammerschmidt, W., Ziegler, H., and Kosinowski, U. H. (1997) Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. *Proc. Natl. Acad. Sci. USA* **94,** 14759–14763.
15. Morgenstern, J. P. and Land, H. (1990) Advanced mammalian gene transfer: high titre retroviral vectors with multiple drug selection markers and a complementary helper-free packaging cell line. *Nucleic Acids Res.* **18,** 3587–3596.
16. Thomsen, D. R. and Stinski, M. F. (1981) Cloning of the human cytomegalovirus genome as endonuclease XbaI fragments. *Gene* **16,** 207–216.

17. Fleckenstein, B., Muller, I., and Collins, J. (1982) Cloning of the complete human cytomegalovirus genome in cosmids. *Gene* **18,** 39–46.
18. Oram, J. D., Downing, R. G., Akrigg, A., Dollery, A. A., Duggleby, C. J., Wilkinson, G. W., and Greenaway, P. J. (1982) Use of recombinant plasmids to investigate the structure of the human cytomegalovirus genome. *J. Gen. Virol.* **59,** 111–129.
19. Chee, M. S., Bankier, A. T., Beck, S., Bohni, R., Brown, C. M., Cerny, R., Horsnell, T., Hutchison, C. A. I., Kouzarides, T., Martignetti, J. A., Preddie, E., Satchwell, S. C., Tomlinson, P., Weston, K. M., and Barrell, B. G. (1990) Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Curr. Top. Microbiol. Immunol.* **154,** 125–170.
20. Paulus, W., Baur, I., Boyce, F. M., Breakefield, X. O., and Reeves, S. A. (1996) Self-contained, tetracycline-regulated retroviral vector system for gene delivery to mammalian cells. *J. Virol.* **70,** 62–67.
21. Halbert, C. L., Demers, G. W., and Galloway, D. A. (1991) The E7 gene of human papillomavirus type 16 is sufficient for immortalization of human epithelial cells. *J. Virol.* **65,** 473–478.
22. Compton, T. (1993) An immortalized human fibroblast cell line is permissive for human cytomegalovirus infection. *J. Virol.* **67,** 3644–3648.
23. Chen, C. A. and Okayama, H. (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7,** 2745–2752.

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