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*J. Virol.* 2004, 78(21):11988. DOI: 10.1128/JVI.78.21.11988-12011.2004.

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Global Analysis of Host Cell Gene Expression Late during Cytomegalovirus Infection Reveals Extensive Dysregulation of Cell Cycle Gene Expression and Induction of Pseudomitosis Independent of US28 Function†

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Received 26 March 2004/Accepted 24 May 2004

Replication of human cytomegalovirus (CMV) depends on host cell gene products working in conjunction with viral functions and leads to a dramatic dysregulation of cell cycle gene expression. Comprehensive transcriptional profiling was used to identify pathways most dramatically modulated by CMV at late times during infection and to determine the extent to which expression of the viral chemokine receptor US28 contributed to modulating cellular gene expression. Cells infected with the AD169 strain of virus or a fully replication competent US28-deficient derivative (RV101) were profiled throughout the late phase of infection (50, 72, and 98 h postinfection). Although sensitive statistical analysis showed striking global changes in transcript levels in infected cells compared to uninfected cells, the expression of US28 did not contribute to these alterations. CMV infection resulted in lower levels of transcripts encoding cytoskeletal, extracellular matrix, and adhesion proteins, together with small GTPases and apoptosis regulators, and in higher levels of transcripts encoding cell cycle, DNA replication, energy production, and inflammation-related gene products. Surprisingly, a large number of cellular transcripts encoding mitosis-related proteins were upmodulated at late times in infection, and these were associated with the formation of abnormal mitotic spindles and the appearance of pseudomitotic cells. These data extend our understanding of how broadly CMV alters the regulation of host cell cycle gene products and highlight the establishment of a mitosis-like environment in the absence of cellular DNA replication as important for viral replication and maturation.

Human cytomegalovirus (CMV) infection has a dramatic impact on the cell that starts immediately after infection (4) and continues through late times (34, 67). The replication cycle follows a temporal cascade of events that depends upon both viral and host cell functions. Viral DNA replication begins between 14 and 24 h postinfection (hpi), and release of progeny starts between 36 and 48 hpi, reaching maximal levels between 72 and 96 hpi (67). This process causes profound changes in host cell shape, metabolism, and gene transcription, components of which are suspected to be critical for efficient replication. Previous studies in primary fibroblasts have revealed the global impact of viral infection on signaling and transcriptional changes that start as early as 15 min and last as long as 48 hpi (4, 16, 50, 51, 85, 112, 113). These studies have largely focused on the immediate impact of the virus on cells and have revealed a dramatic upmodulation of cellular inflammatory and immune gene expression due to virus binding and penetration. Based on this work, selected cellular signaling events (51, 103) and cellular proteins (13, 89, 114) have been implicated as important regulators of infection. There has been a less concerted effort to understand the global impact of CMV infection at late times during infection (16), despite the fact that maximal modulation would be expected at late times. Also, remarkably little information has been presented on the contribution of virus-encoded signaling proteins that are expressed at late times, such as the CMV chemokine receptor US28.

CMV encodes at least four apparent seven transmembrane-spanning proteins (21, 42), pUL33, pUL78, pUS27, and pUS28, one of which (pUS28) is a G-protein-coupled receptor that binds a wide range of CC chemokines (9, 38, 56, 72, 100), as well as fractalkine/CX3CL1 (55). pUS28-mediated signaling is both chemokine ligand dependent (38) and constitutive (19, 101) and stimulates mitogen-activated protein kinase extracellular-signal-regulated kinase 2, focal adhesion kinase 1, and Src (9, 19, 91, 101). pUS28-mediated signaling occurs in CMV-infected cells (9, 98), as well as in US28-transfected cells. US28 transcripts are readily detected by 24 hpi and continue to rise through late times when pUS28 scavenges chemokines from the infected cell culture fluid (12, 98). Although many of the US28 mutant viruses exhibit modest replication defects, US28 itself is completely dispensable for replication in cultured fibroblasts (12, 98), as shown through studies on one viral mutant in particular, RV101 (12). The phenotypic consequences of signaling through this receptor during infection in fibroblasts have not been investigated.

CMV infection of resting primary fibroblasts dysregulates expression of several genes encoding cell cycle regulators (34), such as the G2/M cyclin B (26); cell cycle checkpoint proteins, such as p53 and pRb (49); and DNA replication effectors,
including components and regulators of the prereplication complex (10, 107). Through the induction of these changes, CMV has long appeared to stimulate the generation of an intracellular environment similar to S phase based initially on cellular activation (4) while simultaneously inhibiting cellular DNA synthesis in a manner analogous to a G1/S block (34, 67). The execution of these strategies presumably allows the virus to maximize the availability of cellular functions required for successful replication of the viral genome, to eliminate the need to use the cellular genome, and to avoid the onset of apoptosis.

We used cDNA microarrays to evaluate the impact of CMV infection, as well as the contribution of US28 expression, on cellular gene transcription in fibroblasts at late times postinfection. We used a replication-competent US28 mutant virus, RV101 (12), to avoid contributions from adventitious mutations that are a common occurrence in CMV mutants (67). The levels of a large number of transcripts encoding functions involved in a wide range of cellular pathways were altered by infection, but none of these changes could be ascribed to US28 expression. The most remarkable findings revealed a substantial increase in the expression levels of mitochondrial genes, a considerable alteration in expression levels of multiple GTPase family members, and an intense dysregulation of cell cycle pathways that normally control mitosis. These latter changes were linked to the appearance of pathological mitosis in culture and were likely part of a virus-induced strategy to enhance viral genome replication and virion maturation.

(These results were presented at the 9th International Cytomegalovirus Workshop and the First International Betaherpesvirus Workshop in Maastricht, The Netherlands, on 20 to 25 May 2003.)

MATERIALS AND METHODS

Cells, viruses, and microarrays. Primary human foreskin fibroblasts (HFs) were cultured as described previously (23) and used between passage 15 and 17 postisolation. The human CMV strain AD169 obtained from the American Type Culture Collection (ATCC) has been denoted AD169varATCC (AD) to distinguish it from other variants of this strain in current use (41, 87). The US27/US28 deletion mutant virus RV101 (RV) was made by using AD169varATCC by T. R. Jones (Wyeth-Ayerst Research, Pearl River, N.Y.) and exhibits growth characteristics indistinguishable from parental virus (12) that we independently confirmed for the present study (data not shown). Microarrays were purchased from the Stanford Functional Genomics Facility (http://www.microarray.org/sfgp/pp/home.jsp). About 85% of the spots printed on the polylysine-coated glass microscope slides were from the I.M.A.G.E. consortium clones from the Research Genetics Sequence Verified clone set, 10% were from the Cancer Genome Anatomy Project clone set (http://www.ncbi.nlm.nih.gov/ncicgap), and 5% were from a set of Methanococcus jannaschii controls. The batch of arrays (SHDB) used in the present study contained 41,792 total spots (39,781 unique and 2,011 repeated spots) representing 29,593 unique genes. After purchase, the microarrays were postprocessed as described previously (http://derisilab.ucsf.edu/).

Cell infections. CMV was propagated at a multiplicity of infection (MOI) of 0.01 in confluent HFs and purified as described previously (23). For microarray analysis, HF monolayers plated at a density of 3 \times 10^5 cells/cm^2 in 850-cm^2 roller bottles were infected at confluence (day 5 postseeding) with purified virions (23) at an MOI of 10. After virus adsorption for 1 h at 37°C in 5% CO2, the inoculum was removed, and the cells were washed twice with culture medium prior to the addition of fresh medium. At 50, 72, and 96 hpi, infected monolayers were harvested, snap-frozen, and stored at −80°C until the time course was completed. Uninfected confluent HF cultures were used as a reference. For all other analyses described here, HF monolayers were plated at a density of 5 \times 10^5 cells/cm^2 on coverslips in 24-well plates 3 days before infection with purified AD virions at an MOI of 10. After virus adsorption for 1 h, cells were washed twice prior to the addition of fresh medium. For drug treatment, medium plus 300 μg of phosphonomiformic acid (PFA; Sigma, St. Louis, Mo.), 1mM or medium plus 1 mM or 10 mM hydroxyurea (HU; Sigma) was added to the washed cells. Virus titers in the supernatant of wells treated with each of the drugs were determined by plaque assay and were consistent with previous reports (6, 9).

RNA isolation and microarray hybridization. mRNA populations extracted by using the FastTrack 2.0 mRNA Isolation Kit (Invitrogen, Carlsbad, Calif.). Only RNA preparations reaching optical density at 260/280 nm (OD260/280) ratio values of ≥ 2.0 were used. Labeled cDNA populations were synthesized from 2 μg of mRNA per sample by reverse transcription and simultaneous incorporation of Cy3-dUTP (reference sample, green) or Cy5-dUTP (infected samples, red) (Amersham Biosciences, Piscataway, N.J.) with an oligo-DT primer and the SuperScript II Reverse Transcriptase (Invitrogen). Reference and sample cDNA populations were mixed, and unincorporated nucleotides were removed by using a CyScribe GEX purification kit (Amersham Biosciences). Then, 5 μg of human Cot-1 DNA (Intrivogen), 20 μg of poly(A) RNA (Sigma), 20 μg of yeast rRNA (Invitrogen), 3.4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), and 0.3% sodium dodecyl sulfate were added to the probe prior to denaturation by heating at 100°C for 2 min and application on the arrays. Hybridization of two arrays per virus per time point (total of 12 arrays) were performed at 65°C for 16 to 18 h in a custom slide chamber with humidity maintained by a small reservoir of 3× SSC. The arrays were then washed as described previously (29) and scanned with a GenePix 4000A Scanner (Axon Instruments, Foster City, Calif.).

Microarray data analysis. Array images were analyzed with the Gene Pix Pro 4.0 software and unreliable spots identified by visual inspection were marked with a “flag.” After submission to the Stanford Microarray Database (SMD; http://genome-www.stanford.edu/microarray/), infected/uninfected ratio values were log transformed, normalized by using the default normalization protocol of the SMD, and retrieved by spot number so that no average of the ratio values (from duplicate spots was performed). To eliminate spots of poor quality from subsequent analyses, spot data were filtered by applying three criteria: no flag, brightness (channel 1 [Cy3]-net mean and channel 2 [Cy5]-normalized net mean intensities) of ≥ 150 fluorescence units, and even distribution of color brightness across the whole spot area (regression correlation values of ≥ 0.6). Statistical analysis was performed by using the one- or two-class (unpaired) response type of Significance Analysis of Microarrays (SAM), version 1.20 (http://www-stat.stanford.edu/~tibs/SAM) (96), to three sets of four arrays each (at 50, 72, or 98 hpi) or one set of 10 arrays (50, 72, and 98 [50-72-98] hpi). The number of permutations was set to 24 (4-array sets) or 72 (3-array set), and the random number seed was set to 94329928 (one class) or to 91797601 (two classes, unpaired). The lists of SAM significant spots generated by the one-class analysis of the 50-, 72-, and 98-hpi array sets were intersected with the list generated from the 50-72-98-hpi array set by using the I.M.A.G.E. number as spot identifier. The identity of SAM significant spots was obtained through the SOURCE database (http://source.stanford.edu; time of the analysis, April 2003) (25), and gene grouping in functional categories was performed according to information retrieved from Online Mendelian Inheritance in Man (OMIM) and Medline databases. Gene cellular subcompartments or functional pathways were assigned to a specific class within each category. This classification process brought genes having similar roles into close proximity within each category’s Excel spreadsheet (see information file 56 in the supplemental material), allowing us to immediately appreciate if they were part of extensive cellular pathways. Functional maps were generated by either modifying existing maps on the Gene Microarray Pathway Profiler (GenMAPP) website (http://www.genmapp.org/) or by creating new maps by using GenMAPP software (24) in combination with the Kyoto Encyclopedia of Genes and Genomes database (KEGG, http://www.genome.ad.jp/kegg/) and literature searches. Microarray images and raw data are available on the SMD website, SAM analysis Excel files and functional grouping Excel files are included as six information files in the supplemental material.

Immunofluorescence analysis. For γ tubulin (1 y tub1) and immediate-early protein 1 and 2 (IE1/IE2) double staining, cells were fixed in cold methanol for 5 min at −20°C, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBS) for 20 min on ice, incubated in blocking buffer (1% fetal calf serum in PBS) for 10 min at room temperature, and stained with an anti-y tub1 monoclonal antibody (MAb; 1:10,000, clone GTU-88, Sigma), followed by a Texas red-conjugated goat anti-mouse antibody (1:100; Vector Laboratories, San Bruno, Calif.). After three washes with PBS, cells were incubated with normal mouse immunoglobulin G (1:10; Catlg, Burlingame, Calif.) for 30 min at room temperature before being stained with a fluorescein isothiocyanate (FITC)-conjugated anti-IE1/IE2 antibody (1:600; MAB810F; Chemicon, Temecula, Calif.) and the Cy5-conjugated DNA wash (Invitrogen) (27), or 10 mM hydroxyurea (HU; Sigma) was added 30 min prior to infection with HCMV with Huh7 (obtained from Dr. Stephen Bruno, Eugene, Oreg.). For ppUL44, pp150, and pUL43 staining, infected cells were fixed with 1% paraformaldehyde in PBS for 15 min at room temperature, per-
meabilized, and blocked as described above and then stained with MAbs to ppUL44 (1:500; Goodwin Institute, Plantation, Fla.), pp150 (MAB 36-14), or pUL43 (MAB 20392), followed by an FITC-conjugated goat anti-mouse antibody (1:100; Vector Laboratories). Cellular DNA was highlighted with propidium iodide (Molecular Probes). For ppUL44 and bromodeoxyuridine (BrdU) double staining, cells were labeled with 50 μM BrdU (Sigma) for 1 h at 37°C with 5% CO₂, washed with PBS, fixed with 1% paraformaldehyde for 30 min at room temperature, and incubated with 2 M HCl for 1 h at 37°C. Cells were then washed twice with borate buffer (pH 8.5) and three times with PBS, permeabilized, blocked, and stained with anti-ppUL44 and Texas red anti-mouse antibodies as before. After being washed, the cells were labeled with an FITC-conjugated anti-BrdU antibody (1:100; Molecular Probes) for 1 h at room temperature. Coverslips were mounted with Fluoroguard AntiFade Reagent (Bio-Rad, Hercules, Calif.) before being analyzed on an Olympus BX60 epifluorescence microscope equipped with ×60 or ×100 phase-contrast objective lenses. Images were collected with a Hamamatsu ORCA-100 digital camera and Image Pro Plus 4.0 software (MediaCybernetics, Silver Spring, Md.). Digitized images were stored, electronically colorized, and overlaid for evaluation of two- and three-color experiments.

Transmission electron microscopy. Cells were fixed in 2% glutaraldehyde in PBS, postfixed with 1% osmium tetroxide for 1 h, blocked with 1% aqueous uranyl acetate, dehydrated through graded ethanol steps, and embedded in PolyBed (Polysciences). Thin sections (70 nm) were then prepared, stained with 1% uranyl acetate and 1% lead citrate, and viewed on a Phillips CM12 microscope.

RESULTS AND DISCUSSION

Trends in cellular gene expression at late times during CMV infection and impact of viral chemokine receptor expression.

To identify cellular genes modulated by CMV at late times postinfection, we compared the transcriptional profiles of HFs infected with either wild-type CMV strain AD169varATCC (AD) or RV101 (RV), a mutant virus derived from AD169varATCC exhibiting full replication potential despite the complete absence of US27 and US28 expression (12). We used this particular mutant to avoid potential contribution of adventitious mutations that appear to be carried in US28 mutants that exhibit growth defects. Transcriptional profiling was performed according to the scheme outlined in Fig. 1A. Cy5-labeled cDNAs from HFs infected with either AD or RV for 50, 72, or 98 h were combined with Cy3-labeled cDNA from confluent uninfected HFs used as a reference (29) and hybridized to 12 replicate 42,000 spot human cDNA arrays. After normalization of the spot ratio values, usable data were selected according to an established set of filtering criteria (84). The percentage of qualified spots identified on each array ranged from 44 to 83%, with a mean of 65% ± 11% (Table 1). The data from replicate arrays were organized into four groups to facilitate analysis, three with the 4 arrays at 50, 72, or 98 hpi and one with the 12 arrays from the combined time points (50-72-98 hpi). Each group contained a common set of spots that passed filtering criteria on all arrays contained in that group. These sets consisted of 20,123 spots from the 50-hpi time point (48% of total spots), 14,856 (35%) spots from the 72-hpi time point, 21,940 (52%) spots from the 98 hpi-time point, and 11,942 (29%) spots from the combined 50-72-98 hpi-time points. In order to maximize the usable data in the analysis, two arrays containing <50% qualified spots (AD 72 hpi 1 and RV 72 hpi 1) were excluded from the combined time point group. In this way, 10 replicates composed the 50-72-98 hpi group, and this group had 14,126 (34% of total spots) qualified spots.

To identify genes significantly regulated at late times of CMV infection, we applied SAM one-class analysis to the set of array replicas spanning a 50- to 98-hpi time course. This statistical procedure evaluates the consistency and certainty of gene expression patterns and has eclipsed simple fold-cutoff methods of analysis because it offers an assessment of significance of microarray data (96). Initially, SAM analysis was used to evaluate the combined 50-72-98-hpi set of 14,126 spots with the aim of identifying genes that were consistently modulated throughout late times of infection. Figure 1B (left) shows the plot generated by this analysis. Each datum point in this graph represented a comparison of the observed relative difference value (actual data) plotted against the chance relative difference value (denoted as “expected” in the figure and calculated by permuting the data) for each spot in the set. The distribution of the data along a sigmoidal curve revealed that the observed differences in the expression levels of a large number of genes greatly exceeded the differences expected by chance, with transcript levels showing the strong impact of viral infection on host cells. We selected a conservative false discovery rate (FDR) value of 0.0045 for the combined 50-72-98-hpi data, which was the maximum stringency allowed and indicated that >99.996% of the differences we detected were authentic (see information file S1, FDR and delta values). A total of 5,658 of 14,126 spots, >40% of the data set, were altered at late times, with 1,983 (14%) upmodulated and 3,675 (26%) downmodulated by viral infection (see information file S1, significant spots, in the supplemental material). This dramatic impact of virus infection was also observed when individual time points were analyzed, showing that a wide range of cellular genes were altered by CMV infection at late times during infection no matter how the analysis was completed. The FDR value for each set of arrays was always set to the most conservative value available for the size of the data set, 0.03 for 50-hpi data, 0.01 for 72-hpi data, and 0.01 for 98-hpi data (see information files S2, S3, and S4, FDR and delta values). This approach yielded a >99.97% likelihood that 1,595 spots across the four 50-hpi arrays, 3,177 spots across the four 72-hpi arrays, and 3,596 spots across the four 98-hpi arrays represented authentic changes based on maximally stringent analysis (see information files S2, S3 and S4, significant spots, in the supplemental material). Due to fewer replicates, the number of spots significantly altered by viral infection corresponded to 8, 21, and 16% of the qualified spots within the 50-, 72-, and 98-hpi data sets, respectively. More than 75% of the SAM-significant spots at any of the three late time points were contained in the combined 50-72-98-hpi set and thus exhibited similar levels of transcriptional change (increase or decrease) across all time points. Few SAM-significant spots (1% at 50 hpi, 3% at 72 hpi, and 3% at 98 hpi) showed altered levels at only one time point, due mostly to poor spot quality at other time points. Taken together, these statistical analyses all indicated that large numbers of cellular genes were significantly modulated by CMV infection over the period from 50 to 98 hpi.

Expression levels of US28, a signaling G-protein-coupled receptor, have been shown to be elevated at late times of infection (12). To evaluate the contribution of US28 signaling to the host cell response during the time of maximal expression, we compared the transcriptional profiles of AD and RV virus-infected cells by using SAM two-class (unpaired) analysis. Given the consistency of changes at all late time points in
FIG. 1. (A) Flow chart of microarray data generation and analysis. Cy5-labeled cDNA from infected HFs and Cy3-labeled cDNA from uninfected HFs were mixed and hybridized to replicate 42,000 spot human cDNA arrays to generate data that was normalized and filtered by the SMD and subjected to statistical analysis by SAM software. (B) SAM one-class analysis (left) and SAM two-class (unpaired) analysis (right) of the 14,126 data set from the combined 50-72-98-hpi time points. For each spot, the observed SAM score is plotted against the expected SAM score. The middle line intersecting the origin in each graph represents observed equals expected score values, whereas the additional lines define the upper and lower significance threshold specified by the lowest allowed FDR parameter (96). Significantly regulated spots are shaded gray. Gray spots in the upper right quadrant are upmodulated, and gray spots in the lower left panel are downmodulated. (C) Functional categories (right column) represented in the 1,983 SAM-significant upmodulated (white bars) and 3,675 SAM-significant downmodulated (gray bars) spots from the 50-72-98-hpi data set. The series of bars indicates the distribution of total spots (All spots), unnamed (All unknown spots), and named (All known genes), as well as the distribution of the named genes into functional groups based on their principle role according to the literature. Functional groups include soluble factors and cell surface receptors (Factors Receptor), cytoskeleton-extracellular matrix-adhesion (Cytosk-ECM-Adh), nucleic acid metabolism (Nucl Acid metab), protein metabolism (Protein metab), vesicles and intracellular transport (Vesicles transport), enzymes (Enzyme), transcription factors (Transcr factor), intracellular signaling (Signaling), cell cycle (Cell cycle), other (Other), GTPases (GTPase), lipid metabolism (Lipid metab), immune system (Imm system), and apoptosis (Apoptosis). The categories are ranked according to the total number of spots or genes in each (left column).
the combined data set, this method was initially applied to the 14,126 spot data set, comparing the results of the five replicate AD virus- and five replicate RV virus-infected cell arrays where >50% of spots were qualified. Figure 1B (right) shows the plot generated by this analysis. The distribution of the data fell along a line representing observed equals expected score values, with few deviations. This analysis showed that genes were not expressed differently in AD and RV virus-infected cells. No FDR values of <43 were available for this analysis, indicating that even spots appearing slightly outside the significance threshold had a relatively high (43%) likelihood of being false (see information file S5; FDR and delta values, in the supplemental material). Thus, we concluded that these spots were unlikely to represent true positives and that cellular gene expression was not perceptively influenced by US27 or US28. A comparison of the transcriptional profiles generated by each virus at each time point, conducted by using two arrays per virus per time point, also failed to reveal any differences (data not shown). It is important to consider that the viruses we compared exhibited equivalent replication in HFs (12) and that previous studies (12, 90, 98) have used some US28 mutant viruses that exhibit a variety of growth defects that appear to be unrelated to the disruption of the US28 gene and US28 signaling (12). Growth defects may have been more important than US28 expression in some of the reported differences ascribed to US28. No matter how our data were analyzed, either with replicates from each time point or total combined AD and RV virus-infected cell arrays, a US28 mutant virus that retained normal growth properties did not impact the cellular transcriptional profile any differently than control virus.

Functional analysis of cellular gene expression at late times of infection. Genes whose expression was altered by viral infection in the combined 50-72-98-hpi data set relative to uninfected HFs were categorized according to functional activity, cellular localization site, or metabolic pathway. According to the SOURCE database (http://source.stanford.edu; see Materials and Methods), 3,210 spots were found to represent 2,227 functionally characterized genes (983 spots were represented more than once on each array), with 2,448 spots representing either expressed sequence tags (ESTs) or genes that had not been annotated. Based on known properties listed in SOURCE, as well as in literature searches, each gene was assigned into one functional category shown in Fig. 1C (see information file S6 in the supplemental material). The five categories with the greatest numbers of genes, soluble factors and cell surface receptors (14%), cytoskeleton-extracellular matrix-adhesion (13%), nucleic acid metabolism (10%), protein metabolism (10%), and vesicle and intracellular transport (9.6%) accounted for more than half of the genes whose expression was altered by infection. Of the 2,227 functionally characterized genes, a smaller percentage (927; 42%) was upmodulated, and a greater percentage (1,300; 58%) was downmodulated. Genes belonging to most of the functional categories showed similar distributions; however, the cytoskeleton-extracellular matrix-adhesion, vesicles and intracellular transport, GTPase, and apoptosis categories contained even greater percentages (>68%) of downmodulated genes, and the nucleic acid metabolism, enzyme, cell cycle, and immune system categories contained greater percentages of upmodulated genes (Fig. 1C). Our finding that 2,448 ESTs and 2,227 named genes out of a survey of ca. 14,000 host genes were modulated by CMV infection contrasts with a previous published analysis (16). Although past work focused on early times of infection comparing infectious virus and UV-inactivated virus particles, a dramatic drop in numbers of modulated genes from 900 to 650 was reported to occur between 24 and 48 hpi. Our preliminary analysis had revealed no such drop (S. Watanabe, M. B. Eisen, P. O. Brown, and E. S. Mocarski, Abstr. 24th International Herpesvirus Workshop, Cambridge, abstr. 15.019, 1999). Comparison of previously published 48 hpi data (16) with the analysis reported here is difficult due to differences in the application of statistical analysis.

Relationships between the genes in a category were queried by using OMIM and literature searches. Genes within the same category showing similar transcriptional changes were often found to encode proteins that were functionally related and could be organized into classes within each category (see information file S6, column class). Certain categories (cytoskeleton-extracellular matrix-adhesion, GTPase, enzyme, cell cycle, and nucleic acid metabolism) contained a large proportion of genes in functional relationships that were diagrammed by using GenMAPP software in combination with the KEGG database. Although most of the maps were assembled by using the SAM-significant genes from the combined 50-72-98-hpi set, we added several SAM-significant genes from single time points when they were excluded from the combined set because spots representing the gene did not qualify at other time points. We found that the pathways emerging from these analyses were more valuable than a list of genes to illustrate the impact of the virus on the host cell transcriptome during the late phase of infection. Gene maps are shown in Fig. 2 to 5. Being microarray-derived, all changes described in the present study refer exclusively to transcript levels. In addition to transcriptional changes, other cellular mechanisms affecting translation, posttranslational modifications, protein stability, and activity affect the amount and functionality of the products of the genes displayed on the maps.

Cytoskeleton-, extracellular matrix-, and adhesion-related genes. Expression of cytoskeleton-extracellular matrix-adhesion transcripts showed the greatest level of downmodulation of any category (Fig. 1 and Table 2). The 299 genes in this category were assigned to four classes, with 110 genes (37%)

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**TABLE 1. Number and percentage of spots passing the filtering criteria on each array**

| Slide print no. | Slide name | Good spots (n) | Good spots (%) |
|----------------|------------|----------------|---------------|
| SHDB 156       | AD 50 hpi 1 | 29657          | 71            |
| SHDB 154       | AD 50 hpi 2 | 28860          | 69            |
| SHDB 163       | AD 72 hpi 1 | 20060          | 48            |
| SHDB 165       | AD 72 hpi 2 | 26189          | 64            |
| SHDB 167       | AD 98 hpi 1 | 27774          | 66            |
| SHDB 170       | AD 98 hpi 2 | 27866          | 66            |
| SHDB 157       | RV 50 hpi 1 | 32131          | 77            |
| SHDB 155       | RV 50 hpi 2 | 27573          | 66            |
| SHDB 164       | RV 72 hpi 1 | 18458          | 44            |
| SHDB 166       | RV 72 hpi 2 | 22045          | 53            |
| SHDB 168       | RV 98 hpi 1 | 29769          | 71            |
| SHDB 169       | RV 98 hpi 2 | 34657          | 83            |

*a* Arrays with percentages of good spots of <50% that were excluded from the combined 50-72-98-hpi data set.
TABLE 2. Statistically significant cytoskeleton-, extracellular matrix-, and adhesion-related genes consistently upmodulated (positive fold change) or downmodulated (negative fold change) across late times of infection

| Category and full gene name | Gene designation | Fold change | Subclass |
|----------------------------|------------------|-------------|----------|
| Adhesion molecules         |                  |             |          |
| A disintegrin and metalloprotease domain 12 (melrin α) | ADAM12 | -2.3 | Cell-cell |
| A disintegrin and metalloprotease domain 19 (melrin β) | ADAM19 | -1.6 | Cell-cell |
| Cadherin 11, type 2, OB-cadherin (osteoblast) | CDH11 | -3.5 | Cell-cell |
| Cadherin 12, type 2 (N-cadherin 2) | CDH12 | 2.1 | Cell-cell |
| Cadherin 13, H-cadherin (heart) | CDH13 | 1.9 | Cell-cell |
| Cadherin 2, type 1, N-cadherin (neuronal) | CDH2 | -2.8 | Cell-cell |
| Cadherin 5, type 2, VE-cadherin (vascular epithelium) | CDH5 | -1.4 | Cell-cell |
| CD164 antigen, sialomucin | CD164 | -1.5 | Cell-cell |
| CD33 antigen (gp80) | CD33 | -1.6 | Cell-cell |
| CD68 antigen | CD68 | -2.1 | Cell-cell |
| Discoidin domain receptor family, member 1 | DDR1 | 1.7 | Cell-cell |
| Fracture callus 1 homolog (rat) | FDC1 | -2.1 | Cell-cell |
| Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 4 | MLLT4 | 2 | Cell-cell |
| Myeloid/lymphoid or mixed-lineage leukemia; translocated to, 7 | MLLT7 | 2.5 | Cell-cell |
| Neogenin homolog 1 (chicken) | NEO1 | 2.3 | Cell-cell |
| Neural cell adhesion molecule 1 | NCAM1 | 3.4 | Cell-cell |
| Neural cell adhesion molecule 2 | NCAM2 | 1.9 | Cell-cell |
| Protein tyrosine phosphatase, receptor type, M | PTPRM | -1.4 | Cell-cell |
| Proteocadherin 1 (cadherin-like 1) | PCDH1 | -1.5 | Cell-cell |
| Proteocadherin β5 | PCDH5 | 1.6 | Cell-cell |
| Proteocadherin γ subfamily C, 3 | PCDHGC3 | -6.6 | Cell-cell |
| Thrombospondin 1 | THBS1 | -19.1 | Cell-cell |
| Thrombospondin 2 | THBS2 | -6.7 | Cell-cell |
| Thrombospondin 3 | THBS3 | -1.8 | Cell-cell |
| Thy-1 cell surface antigen | THY1 | -1.7 | Cell-cell |
| CD44 antigen (homing function and Indian blood group system) | CD44 | -6.9 | Cell-matrix |
| CD47 antigen (Rh-related antigen, integrin-associated signal) | CD47 | -1.4 | Cell-matrix |
| Similar to triple functional domain (PTPRF interacting) | LOC115557 | 1.5 | Cell-matrix |
| Integrin β4-binding protein | ITGB4BP | -1.9 | Integrin |
| Integrin, α1 | ITGA1 | -1.3 | Integrin |
| Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor) | ITGA2 | -7.3 | Integrin |
| Integrin, α3 (antigen CD49C, α3 subunit of VLA-3 receptor) | ITGA3 | -1.8 | Integrin |
| Integrin, α4 (antigen CD49D, α4 subunit of VLA-4 receptor) | ITGA4 | -3.2 | Integrin |
| Integrin, αE (antigen CD103, human mucosal lymphocyte antigen 1) | ITGAE | 1.4 | Integrin |
| Integrin, αV (vitronectin receptor, α polypeptide, antigen CD51) | ITGAV | -2.5 | Integrin |
| Integrin, β1 (fibronectin receptor, β polypeptide) | ITGB1 | -6.2 | Integrin |
| Integrin, β5 | ITGB5 | -1.6 | Integrin |
| Integrin, β8 | ITGB8 | -3.7 | Integrin |
| Integrin, β-like 1 (with EGF-like repeat domains) | ITGBL1 | -1.4 | Integrin |
| LIM and senescent cell antigen-like domains 1 | LIM51 | -3.2 | Integrin |
| Transmembrane 4 superfamily member 2 | TM4SF2 | 2 | Integrin |
| Transmembrane 4 superfamily member 7 | TM4SF7 | -2.1 | Integrin |
| Armadillo repeat gene deletes in velocardiofacial syndrome | ARVC | 1.6 | Integrin |
| Ems1 sequence | EMS1 | -1.6 | Integrin |
| Bullous pemphigoid antigen 1, 230/240 kDa | BPAG1 | -2.1 | Integrin |
| Breast cancer anti-estrogen resistance 1 | BCAR1 | -1.4 | Integrin |
| Paxillin | PXN | -2.4 | Integrin |
| Protein tyrosine kinase 2β | PTK2B | 1.8 | Integrin |
| Gap junction protein, α1, 43 kDa (connexin 43) | GJA1 | -2.4 | Integrin |
| Gap junction protein, α7, 45 kDa (connexin 45) | GJA7 | -1.5 | Integrin |
| Gap junction protein, β3, 31 kDa (connexin 31) | GJB3 | 2 | Integrin |
| Angiomotin like 1 | AMOTL1 | -1.6 | Integrin |
| Angiomotin like 2 | AMOTL2 | -2.1 | Integrin |
| Claudin 3 | CLDN3 | -2.9 | Integrin |
| Claudin 4 | CLDN4 | -3.1 | Integrin |
| Claudin 8 | CLDN8 | 1.6 | Integrin |
| Junctional adhesion molecule 1 | JAM1 | 3 | Integrin |
| Activated leukocyte cell adhesion molecule | ALCAM | -1.4 | Integrin |
| Catenin (cadherin-associated protein), α2 | CTNN2 | -1.6 | Integrin |
| CD151 antigen | CD151 | -2.5 | Integrin |
| Cerebral cell adhesion molecule | LOC51488 | 1.9 | Integrin |
| Cysteine and histidine-rich domain (CHORD)-containing 1 | CHOROC1 | -1.6 | Integrin |
| FAT tumor suppressor homolog 1 (Drosophila) | FAT | 2.4 | Integrin |
| Heparan sulfate proteoglycan 2 (perlecian) | HSPG2 | -1.9 | Integrin |
| Immunoglobulin superfamily containing leucine-rich repeat | ISLR | -2.2 | Integrin |
| KISS-1 metastasis-suppressor | KISS1 | -1.4 | Integrin |
| L1 cell adhesion molecule | LICAM | -1.5 | Integrin |
| Mesothelin | MSLN | 1.6 | Integrin |
| Neurexin 2 | NRXN2 | 4.9 | Integrin |
| Neurofascin | NFASC | -1.5 | Integrin |
| Ninjurin 2 | NINJ2 | 1.4 | Integrin |
| Poliovirus receptor | PVR | -1.4 | Integrin |
| Syndecan 1 | SDC1 | -2.2 | Integrin |

Continued on following page
TABLE 2—Continued

| Category and full gene name | Gene designation | Fold change | Subclass |
|-----------------------------|------------------|-------------|----------|
| Syndecan 3 (N-syndecan)     | SDC3             | –2.6        |          |
| Transforming growth factor, β-induced, 68 kDa | TGFBI | –5.8 |          |
| Trophomin associated protein (fastin) | TROAP | –2.5 |          |
| Type I transmembrane protein F114 | FN14 | –2.9 |          |
| TYRO3 protein tyrosine kinase | TYRO3 | 1.6 |          |
| Vinculin | VCL | –2 |          |
| Extracellular matrix        |                  |             |          |
| Collagen, type I, α1        | COL1A1           | –6.5        | Collagen |
| Collagen, type I, α2        | COL1A2           | –7.7        | Collagen |
| Collagen, type III, α1 (Ehlers-Danlos syndrome type IV) | COL3A1 | –13 | Collagen |
| Collagen, type IV, α1       | COL4A1           | –8.7        | Collagen |
| Collagen, type IV, α2       | COL4A2           | –3.5        | Collagen |
| Collagen, type IV, α5 (Alport syndrome) | COL5A5 | –3.3 | Collagen |
| Collagen, type IV, α6       | COL4A6           | –1.5        | Collagen |
| Collagen, type IX, α2       | COL9A2           | 1.9         | Collagen |
| Collagen, type V, α1        | COL5A1           | –3.9        | Collagen |
| Collagen, type V, α2        | COL5A2           | –2.2        | Collagen |
| Collagen, type V, α3        | COL5A3           | –2.2        | Collagen |
| Collagen, type VI, α1       | COL6A1           | –10.4       | Collagen |
| Collagen, type VI, α2       | COL6A2           | –4.2        | Collagen |
| Collagen, type VI, α3       | COL6A3           | –25.6       | Collagen |
| Collagen, type VII, α1      | COL7A1           | –5.6        | Collagen |
| Collagen, type VIII, α2     | COL8A2           | 1.4         | Collagen |
| Collagen, type XI, α2       | COL11A2          | 1.7         | Collagen |
| Collagen, type XII, α1      | COL12A1          | 1.3         | Collagen |
| Collagen, type XIV, α1 (undulin) | COL14A1 | –2.6 | Collagen |
| Collagen, type XV, α1       | COL15A1          | –2.8        | Collagen |
| Collagen, type XVI, α1      | COL16A1          | –6.4        | Collagen |
| Procollagen (type III) N-endopeptidase | PCOLCN3 | 1.9 | Collagen |
| Procollagen C-endopeptidase enhancer | PCOLCE | –2.9 | Collagen |
| Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 | PLOD2 | –2 | Collagen |
| Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3 | PLOD3 | –1.8 | Collagen |
| Procollagen-proline, 2-oxoglutarate 4-dioxygenase α I | P4HA1 | –4 | Collagen |
| Procollagen-proline, 2-oxoglutarate 4-dioxygenase α II | P4HA2 | –2.9 | Collagen |
| Serine (or cysteine) proteinase inhibitor, clade H, member 2 | SERPINH2 | –5.6 | Collagen |
| A disintegrin-like and metalloprotease with thrombospondin type 1 motif | ADAMTS1 | –11.4 | Enzyme |
| A disintegrin-like and metalloprotease with thrombospondin type 1 motif | ADAMTS2 | –2.5 | Enzyme |
| Exostoses (multiple) 1     | EXT1             | –4          | Enzyme   |
| Fibroblast activation protein, α | FAP | –3.3 | Enzyme   |
| Glucosamine (N-acetyl)-6-sulfatase (Sanfilippo disease IID) | GNS | –1.6 | Enzyme   |
| Glucoronicidase, β         | GUSB             | 1.8         | Enzyme   |
| Heparan sulfate (glucosamine) 3-O-sulfotransferase 3A1 | HSST3A1 | –2.8 | Enzyme   |
| Heparan sulfate (glucosamine) 3-O-sulfotransferase 3B1 | HSST3B1 | –4.2 | Enzyme   |
| Heparan sulfate (glucosamine) 3-O-sulfotransferase 4 | HSST4 | –2.2 | Enzyme   |
| Heparan sulfate 2-O-sulfotransferase 1 | HSST1 | –1.4 | Enzyme   |
| Heparan sulfate 6-O-sulfotransferase 1 | HSST6 | 2.2 | Enzyme   |
| Hyaluronan synthase 2      | HAS2             | –1.4        | Enzyme   |
| Duroindoxin, α-L            | IDUA             | 3.5         | Enzyme   |
| Lysyl oxidase               | LOX              | –11.4       | Enzyme   |
| Lysyl oxidase-1             | LOXL1            | –2.4        | Enzyme   |
| Lysyl oxidase-2             | LOXL2            | –3.4        | Enzyme   |
| N-Decarboxylase/V-sulfotransferase (heparan glucosaminy) 1 | NDS1 | –2.7 | Enzyme   |
| Suppression of tumorigenicity 14 (colon carcinoma, matrigel, epithin) | ST14 | 1.5 | Enzyme   |
| Uronyl-2-sulfotransferase   | UST              | –3.5        | Enzyme   |
| Xylosylprotein β1,4-galactosyltransferase, polypeptide 7 | B4GALT7 | –1.3 | Enzyme   |
| Basigin (OK blood group)    | BSG              | –1.4        | MMP      |
| Matrix metalloproteinase 1 (interstitial collagenase) | MMP1 | –1.5 | MMP      |
| Matrix metalloproteinase 11 (stromelysin 3) | MMP11 | –1.8 | MMP      |
| Matrix metalloproteinase 17 (membrane inserted) | MMP17 | 1.5 | MMP      |
| Matrix metalloproteinase 2 (gelatinase A) | MMP2 | –5 | MMP      |
| Matrix metalloproteinase 24 (membrane inserted) | MMP24 | –1.8 | MMP      |
| Matrix metalloproteinase 3 (stromelysin 1, progelatinase) | MMP3 | –5.2 | MMP      |
| Reversion-inducing-cysteine-rich protein with kazal motifs | RECK | –5.9 | MMP      |
| Tissue inhibitor of metalloproteinase 1 | TIMP1 | –3.1 | MMP      |
| Tissue inhibitor of metalloproteinase 2 | TIMP2 | –5.9 | MMP      |
| Tissue inhibitor of metalloproteinase 3 | TIMP3 | –13.7 | MMP      |
| Biglycan                    | BGN              | –3          |          |
| Capillary morphogenesis protein 2 | CMG2 | –1.7 |          |
| Cartilage associated protein | CRTAP | –3.3 |          |
| Cartilage linking protein 1 | CRTL1 | –1.7 |          |
| Cartilage oligomeric matrix protein | COMP | –1.8 |          |
| Chondroitin sulfate proteoglycan 6 (bamacan) | CSGP6 | 1.7 |          |

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| Category and full gene name | Gene designation | Fold change | Subclass |
|-----------------------------|------------------|-------------|----------|
| Decorin                     | DCN              | −1.5        |          |
| Dermatan sulfate proteoglycan 3 | DSPG3         | 1.3         |          |
| EGF-containing fibulin-like extracellular matrix protein 1 | EEFMP1        | −1.7        |          |
| EGF-containing fibulin-like extracellular matrix protein 2 | EEFMP2        | −5.4        |          |
| Fibrillin 2 (congenital contractual arachnodactyly) | FBN2           | −5.8        |          |
| Fibronectin 1               | FN1              | −19.7       |          |
| Fibulin 1                   | FBLN1            | −3.6        |          |
| Fibulin 2                   | FBLN2            | −4.3        |          |
| Glypican 1                  | GPC1             | −3          |          |
| Inter-α (globulin) inhibitor H4 | ITIH4         | 1.9         |          |
| Laminin receptor 1 (ribosomal protein SA, 67 kDa) | LAMR1        | −1.7        |          |
| Laminin, α4                 | LAMA4            | −4.7        |          |
| Laminin, β1                 | LAMB1            | −1.7        |          |
| Laminin, γ1 (formerly LAMB2) | LAMC1            | −2          |          |
| Lumican                     | LUM              | −21         |          |
| Matrix Gla protein           | MGP              | −4          |          |
| Microfilibr-associated glycoprotein-2 | MAGP2        | −3          |          |
| Microfilibr-associated protein 4 | MFA4         | −1.6        |          |
| Mucin, 4, tracheobronchial   | MUC4             | 1.4         |          |
| Proline arginine-rich end leucine-rich repeat protein | PRELP         | −4.3        |          |
| Secreted protein, acidic, cysteine-rich (osteonectin) | SPARC         | −14.5       |          |
| Serine (or cysteine) proteinase inhibitor, clade E, member 2 | SERPINE2      | −10.9       |          |
| Sparse/osteonectin/cow and kazal-like domains proteoglycan (testican) | SPOCK        | −3.3        |          |
| Surfactant, pulmonary-associated protein B | SFTPB        | −2.3        |          |
| Tenasin C (hexabrachion)     | TNC              | −11.3       |          |
| Tissue factor pathway inhibitor 2 | TFFP12       | −1.7        |          |
| Trefol factor 1              | TFF1             | −1.5        |          |
| Tuftelin 1                   | TUFT1            | 1.8         |          |
| Major cytoskeletal fiber     |                  |             |          |
| Intermediate filament protein syncofilin | SYNOILIN     | −1.6        | Intermediate filaments |
| Keratin 13                   | KRT13            | 1.7         | Intermediate filaments |
| Keratin 18                   | KRT18            | 1.6         | Intermediate filaments |
| Keratin 19                   | KRT19            | −6.4        | Intermediate filaments |
| Keratin 5                    | KRT5             | 1.6         | Intermediate filaments |
| Keratin, hair, basic, 5      | KRTHB5           | 1.5         | Intermediate filaments |
| Keratin, hair, basic, 6 (monilethrix) | KRTHB6       | 1.7         | Intermediate filaments |
| Neurofilament 3 (150-kDa medium) | NEF3         | 1.7         | Intermediate filaments |
| Periplakin                   | PPL              | 2           | Intermediate filaments |
| Plectin 1, intermediate filament-binding protein, 500 kDa | PLEC1        | −4.8        | Intermediate filaments |
| Vimentin                     | VIM              | −6.2        | Intermediate filaments |
| Actin binding LIM protein 1  | ABL1M1           | 1.6         | Microfilaments actin |
| Actin filament-associated protein | AFAP         | −2.4        | Microfilaments actin |
| Actin-related protein 2/3 complex, subunit 1A, 41 kDa | ARPC1A        | −1.6        | Microfilaments actin |
| Actin-related protein 2/3 complex, subunit 1B, 41 kDa | ARPC1B        | −2.4        | Microfilaments actin |
| Actin-related protein 2/3 complex, subunit 4, 20 kDa | ARPC4        | −2.1        | Microfilaments actin |
| Actin-related protein 2/3 complex, subunit 5, 16 kDa | ARPC5        | −2          | Microfilaments actin |
| Actin, α2, smooth muscle, aorta | ACTA2        | −4.8        | Microfilaments actin |
| Actin, β                     | ACTB             | −4.4        | Microfilaments actin |
| Actin, γ2, smooth muscle, enteric | ACTG2        | −3.5        | Microfilaments actin |
| Actinin, α4                  | ACTN4            | −2          | Microfilaments actin |
| Adducin 2 (β)                | ADD2             | 1.7         | Microfilaments actin |
| Adducin 3 (γ)                | ADD3             | −1.9        | Microfilaments actin |
| B-cell CLL/lymphoma 7A       | BCL7A            | 1.5         | Microfilaments actin |
| Calcium/calmodulin-dependent serine protein kinase (MAGUK family) | CASK         | −1.6        | Microfilaments actin |
| Caldesmon 1                  | CALD1            | −7          | Microfilaments actin |
| Calponin 2                   | CN2N             | −2.7        | Microfilaments actin |
| Calponin, 3, acide           | CN3              | −3.3        | Microfilaments actin |
| Capping protein (actin filament) muscle Z-line, α1 | CAPZA1       | −2.2        | Microfilaments actin |
| CD2-associated protein       | CDZAP            | 1.9         | Microfilaments actin |
| Chaperonin containing TCP1, subunit 4 (6) | CCT4        | 2           | Microfilaments actin |
| Chaperonin containing TCP1, subunit 8 (6) | CCT8        | 1.7         | Microfilaments actin |
| Coactosin-like 1 (Dictyostelium) | COTL1       | −2.8        | Microfilaments actin |
| Corinon, actin-binding protein, 1A | CORO1A       | 2.3         | Microfilaments actin |
| Corinon, actin-binding protein, 1C | CORO1C       | −2.3        | Microfilaments actin |
| Drebin 1                     | DBN1             | −1.5        | Microfilaments actin |
| Enabled homolog (Drosophila)  | ENAHE            | −3          | Microfilaments actin |
| FERM, RhoG EF (ARHGEF) and pleckstrin domain protein 1 | FARP1        | −2.5        | Microfilaments actin |
| Filamin A, α (actin-binding protein 280) | FLNA        | −3.1        | Microfilaments actin |
| Filamin C, γ (actin-binding protein 280) | FLNC        | −2.8        | Microfilaments actin |
| Gelsolin (amyloidosis, Finnish type) | GSN          | −1.5        | Microfilaments actin |
| LIM and SH3 protein 1        | LIM1             | −1.8        | Microfilaments actin |
| Microtubule-actin crosslinking factor 1 | MACF1       | −1.8        | Microfilaments actin |
| Mocsin                       | MSN              | −3          | Microfilaments actin |
| Nebulin                      | NEB              | −1.8        | Microfilaments actin |

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TABLE 2—Continued

| Category and full gene name* | Gene designation | Fold change | Subclass |
|-----------------------------|------------------|-------------|----------|
| Kinesin 14 (arginine, serine, proline-rich) | KIF14 | 1.5 | Microtubules |
| Kinesin family member 14B | KIF14B | 1.9 | Microtubules |
| Kinesin family member 15A | KIF15A | 2.1 | Microtubules |
| Kinesin family member 15B | KIF15B | 2.1 | Microtubules |
| Kinesin family member 15C | KIF15C | 2.1 | Microtubules |
| Kinesin family member 15D | KIF15D | 2.1 | Microtubules |
| Kinesin family member 15E | KIF15E | 2.1 | Microtubules |
| Kinesin family member 15F | KIF15F | 2.1 | Microtubules |
| Kinesin family member 15G | KIF15G | 2.1 | Microtubules |
| Kinesin family member 15H | KIF15H | 2.1 | Microtubules |
| Kinesin family member 15I | KIF15I | 2.1 | Microtubules |
| Kinesin family member 15J | KIF15J | 2.1 | Microtubules |
| Kinesin family member 15K | KIF15K | 2.1 | Microtubules |
| Kinesin family member 15L | KIF15L | 2.1 | Microtubules |
| Kinesin family member 15M | KIF15M | 2.1 | Microtubules |
| Kinesin family member 15N | KIF15N | 2.1 | Microtubules |
| Kinesin family member 15O | KIF15O | 2.1 | Microtubules |
| Kinesin family member 15P | KIF15P | 2.1 | Microtubules |
| Kinesin family member 15Q | KIF15Q | 2.1 | Microtubules |
| Kinesin family member 15R | KIF15R | 2.1 | Microtubules |
| Kinesin family member 15S | KIF15S | 2.1 | Microtubules |
| Kinesin family member 15T | KIF15T | 2.1 | Microtubules |
| Kinesin family member 15U | KIF15U | 2.1 | Microtubules |
| Kinesin family member 15V | KIF15V | 2.1 | Microtubules |
| Kinesin family member 15W | KIF15W | 2.1 | Microtubules |
| Kinesin family member 15X | KIF15X | 2.1 | Microtubules |
| Kinesin family member 15Y | KIF15Y | 2.1 | Microtubules |
| Kinesin family member 15Z | KIF15Z | 2.1 | Microtubules |

* Genes are listed in alphabetical order within each subclass.

encoding major cytoskeletal fibers, 96 genes (32%) encoding extracellular matrix components, 79 genes (26%) encoding cell adhesion molecules, and 14 genes (5%) encoding miscellaneous cytoskeletal gene products. A very high proportion (84%) of transcripts encoding extracellular matrix-related gene products, including 30 encoding collagens and collagen-altering enzymes, 20 encoding extracellular matrix-modifying enzymes, and 11 encoding matrix metalloproteinases (MMP) or associated regulators, were consistently downmodulated by viral infection, with seven types of collagens, fibronectin 1, laminin α4 and γ1, MMP2, MMP3, and two tissue inhibitors of metalloproteinases (TIMP2 and 3) among the transcripts reduced.
>10-fold (Table 2). Three-quarters of the genes encoding cell adhesion molecules, including 28 mediators of cell-cell and cell-matrix adhesion, 14 integrin subunits, and 15 components of various kinds of junctions, were suppressed by infection, with two thrombospondins (THBS1 and -2), integrin β1, and integrin α2 among the transcripts reduced >5-fold (Table 2). Two-thirds of the genes in the major cytoskeletal fiber class, including 64 microfilament-related genes, 35 microtubule-related genes, and 11 intermediate filament-related genes, were also markedly reduced during infection, with transgelin, caldesmon 1, vimentin, and two tropomyosins (TPM2 and -4) among the transcripts reduced >5-fold (Table 2).

Forty-one genes from this category were assembled in a gene map that depicts the downmodulation of signaling pathways linking integrins to the actin cytoskeleton by viral infection (Fig. 2). Expression levels of 6 of 18 (33%) known integrin α subunits (α1, α2, α3, α4, αE, and αV) and of 4 of 8 (50%) known integrin β subunits (β1, β4, β5, and β8) were altered by infection: eight subunits were consistently downmodulated, whereas two (αE and β4) were upmodulated. These data revealed a major negative impact of infection on mediators of cell-extracellular matrix matrix attachment, a finding consistent with the virus-induced decline in the substrate adhesion properties of the infected cell (3, 104). In agreement with this notion, several genes encoding integrin-binding partners, such as fibronectin, laminins, and collagens, as well as genes encoding the major focal adhesion-localized proteins vinculin, α-actinin 4, talin 1, and paxillin, were strongly downmodulated. In addition, three main members of the rho family of GTPases that control the formation of stress fibers and focal adhesions (rhoA), lamellipodia (rac1), and filopodia (cdc42), together with eight mediator proteins links these GTPases to the actin modifying proteins (PAK2 and PAK3, ROCK1, LIMK1, phosphatidylinositol-4-phosphate 5-kinase [PI(4)PSK], WASP family verprolin-homologous protein 2 [WAVE2], and myosin light chain phosphatase [MYPT] regulatory subunits 1 and 2), were also downmodulated. Thus, expression of a broad range of integrin-to-actin bridging gene products appeared to be negatively impacted by infection, a finding consistent with a broad disruption of integrin binding and signaling responses during infection. Most actin modifiers, which are the targets of integrin signaling, including filamin A and C, gelsolin, transgelin, moesin, the capping protein muscle Z line (capZ1), four of the six subunits composing the actin-related protein 2/3 complex (arp1A, -1B, -4, and -5), and the assembler of the spectrin-actin lattice, adducin 3, were consistently downmodulated, together with the regulatory type of myosin light chain (i.e., MLC2), controlling the assembly of actin-myosin filaments, two calponin 1 homologs (calponins 2 and 3), and four actomyosin regulatory proteins (tropomyosins 2 and 4, tropomodulin 3, and caldesmon). Finally, transcripts for three of the six known actin isoforms were also downmodulated by infection.

These findings demonstrated that the genes encoding proteins involved in the assembly and disassembly of actin fibers were downmodulated by CMV at late times. Such changes, along with a similar impact on all three major rho family GTPases and their downstream effectors, are likely to disrupt the organization of the cytoplasmic microfilaments and to strongly impair the formation of stress fibers, filopodia, and lamellipodia, eventually leading to the rounded, enlarged cell cytopathic effect observed at late times of infection. The same alterations, combined with the reduction in cellular adhesion transcript levels, would also predictably affect the potential for...
infected cells to migrate, weakening GTPase-mediated mechanisms on one hand and diminishing adhesiveness on the other hand.

**GTPases and related genes.** Transcripts encoding GTPase category gene products involved in vesicle transport were also strongly downmodulated and extended beyond those functions involved in integrin-to-actin signaling (Fig. 3). The 112 genes in this category were assigned to one of six classes, with 32 genes (29%) encoding rho-related proteins, 26 genes (23%) encoding arf-related proteins, 16 genes (14%) encoding G-protein-related proteins, 14 genes (12.5%) encoding ras-related proteins, and seven genes (6%) encoding ran-related proteins. Forty-nine of these were assembled in the map, which depicts the main members of the six major GTPase families. Expression levels of 11 main G-protein family members were altered by infection, with eight subunits, including two Gα subunits (Goα2 and -12), three Gβ subunits (Gβ1, -4, and -2-like), and three Gγ subunits (Gγ10, -11, and -12), consistently downmodulated. Three Gα subunits (Gaolf, Go13, and Go14) showed upmodulation. Heterotrimeric G proteins play an important role in transducing signals derived from the activation of cell surface receptors by various hormones, neurotransmitters, chemokines, and sensory stimuli. Based on the observed downmodulation of most Gα and all Gβ and Gγ transcripts that scored in the analysis, we would predict that the sensitivity of infected cells to signals conveyed via G-protein-coupled receptors would be reduced. This downmodulation may contribute to the failure of pUS28 to have a detectable impact on host gene expression at late times of infection.

Expression levels of nine ras family members were altered: seven (R-ras2/TC21, R-ras3/M-ras, ralB, rap1A, ragA, ragB, and GEM/KIR) were consistently downmodulated, and two (K-ras and rasD) were consistently upmodulated (Fig. 3). Ras family proteins are part of complex signaling networks, controlling cellular proliferation and survival, vesicle trafficking, cytoskeletal organization, and integrin function. The observed persistent downmodulation of multiple ras family members would be predicted to impair these cellular functions. In particular, the downmodulation of R-ras2/TC21 and rap1A, which have been shown to be potent activators of integrins in fibroblasts (54), was consistent with a broad inhibitory effect on integrin functions, in remarkable agreement with the loss-of-adhesion phenotype observed with the cytoskeleton-extracellular matrix-adhesion-related genes. The expression levels of nine major representatives of the rho family were modified during infection: eight (rhoA, rhoB, rhoC, rac1, cdc42, TC10B/ rhoJ, rhoI, and rnd3/rhoE) were downmodulated, and one (rnd2/rhoN) was upmodulated. Although rhoB, rhoC, and TC10B/rhoJ regulate the assembly of focal adhesions and actin stress fibers in a way similar to rhoA, the rnd proteins rnd1, rnd2, and rnd3 control rounding of cells by countering some of the rhoA-regulated responses (74, 106). Consistent with the results obtained from the analysis of the cytoskeleton-extracellular matrix-adhesion category, the upmodulation of rnd2, together with the downmodulation of rhoB, rhoC, and TC10B/rhoJ, pointed toward the destabilization of adhesion structures and the rounding of the infected cell. Expression levels of 25 genes encoding main representatives of the two GTPase families involved in intracellular control of vesicle trafficking, the arf and the rab families, were also modified in infected cells (Fig. 3). Five of these genes encoded arf family members: two (arf6 and arf11) were consistently downmodulated, and three (arf3, arf4, and arf8) were upmodulated. Arf1 helps maintain normal Golgi body architecture (61) and, together with arf3, may interact with vesicle-tethering factors associated with the Golgi apparatus (75). The modification of arf1 and arf3 expression levels in the infected cell could indicate a viral strategy to exploit Golgi function for processing viral glycoproteins and for virion envelopment (28, 47). The persistent downmodula-
tion of arf6, which is involved with macropinocytosis at the cell periphery, as well as in cell migration and wound healing through activation of rac1 (27), may therefore have negative effects not only on endocytic pathways but also on cell shape and motility. This alteration was consistent with the observed negative impact of infection on the expression of several actin cytoskeleton-related genes (Fig. 2). Transcript expression levels of 20 of the 60 (33%) known human rab family members were altered during infection: 13 (rab1a, -1b, -2a, -3a, -6a, -7, -13, -18, -21, -23, -31, -34, and -40b) were consistently downmodulated, and seven (ray/rab1C and rab2L, -8, -20, -27a, -32, and -38) were upmodulated by infection. These data showed a strong impact of CMV infection on this essential family of vesicle traffic regulators, suggesting a possible role for several rabs in the final steps of virion morphogenesis and egress. The apparent negative impact on rab7 (important for endosome transport and phagosome maturation) (30, 45), rab18 (involved in apical endocytosis and recycling) (63), and rab34 (required for efficient macropinocytosis) (92) suggested global inhibition of the endocytic process. This hypothesis was supported by the consistent downmodulation of arf6 (see above) and by the expression pattern of five genes encoding interactors of rab5, an essential regulator of membrane traffic kinetics in the early endocytic pathway (17) (see information file S6, GTPases, class Rab family, in the supplemental material). Four of these genes were consistently downmodulated and encoded proteins involved either in signal transmission from the active rab5 to the membrane docking apparatus (rabaptin-5, rabex-5, and RIN2) or in endosome fusion (early endosome antigen 1). Interestingly, the fifth and only upmodulated gene encoded the rab5 interactor tuberin, whose main function is to negatively regulate rab5 activity in endocytosis (109).

Transport mechanisms involving the Golgi apparatus also seemed to be negatively affected, based on the downmodulation of arl1 (see above) and of rab1a, rab1b, rab2a, and rab6a, which are required to form and maintain normal Golgi cisternae and to regulate standard and retrograde transport between the endoplasmic reticulum (ER) and the Golgi body (64, 95, 108).

Three of the six consistently upmodulated rabs transcripts encode proteins involved in melanosome transport and morphogenesis (rab8a, rab38, and rab27a). Although rab38 is necessary to target the melanin biosynthesis enzyme tyrp1 to endstage melanosomes (60), rab8 plays a role in melanosome trafficking (20) and rab27a is required for the recruitment of myosin Va to melanosomes, which then stimulates the microtubule-mediated transport of melanosomes to the tips of dendrites in melanocytes and their transfer to adjacent keratinocytes (48). Since fibroblasts do not possess melanosomes, it is tempting to speculate that the observed transcriptional upmodulation of these genes might be part of a virus-controlled strategy to promote its maturation and release through the specific vesicle traffic mechanisms active in melanocytes.

Lastly, the downmodulation of rab13, a structural and functional regulator of tight junctions (65), was in optimal agreement with our results pointing to cell rounding, whereas the upmodulation of rab32, which participates in synchronizing the mitochondrial fission process (5), was particularly interesting in connection with the observed disruption of the reticular mitochondrial network in CMV-infected HFss (66). Altogether, these data are the first to highlight GTPases and related genes as major targets of transcriptional modulation at late times during CMV infection.

**Nuclear DNA-encoded mitochondrial genes.** The enzyme category had the most broadly upmodulated transcript levels (Fig. 1 and 4). The 146 genes in this category were assigned to one of five classes, with 55 genes (38%) encoding mitochondrial functions, 39 genes (27%) associated with carbohydrate metabolism, 11 genes (7%) involved in detoxification, 8 genes (5%) participating in the synthesis and/or utilization of glutathione, and 33 genes (23%) representing miscellaneous enzymes. Mitochondrial functions stood out as being the largest class in this category and had a remarkably high proportion of upmodulated genes (91%). Fifty-one genes from this class and two genes from the carbohydrate metabolism class were assembled in a map depicting the mitochondrial oxidative phosphorylation (oxphos) and the related biochemical pathways involved in oxidation of fatty acids (β-oxidation cycle), catabolism of amino acids, and transport of NADH, acetyl coenzyme A (CoA), and ATP (Fig. 4). Strikingly, all 24 genes encoding components of the electron transport chain complexes I to IV, representing one-third of the known electron transport chain subunits, were upmodulated by infection. Half of the 18 genes that are part of the ATP synthase complex and 8 genes involved in the assembly, biosynthesis, or activity regulation of specific components of the oxphos (coenzyme Q7 [CoQ7], cytochrome c synthase, ATPase inhibitory factor 1, chaperone ABCI, F1 complex assembly factor 1 and 2, and cytochrome c oxidase 15 [COX15] and COX17) were consistently upmodulated during infection. Altogether, these data revealed a very strong positive effect of CMV infection on the expression levels of multiple genes related to ATP synthesis, which has not been observed previously. This impact likely results in enhanced energy production that benefits infection. Transcripts for four (short and medium chain acyl-CoA dehydrogenases, 3-ketoacyl-CoA thiolase, and 3-hydroxyacyl-CoA dehydrogenase) of the six standard enzymes in the β-oxidation cycle and one (enoyl-CoA isomerase) of the three auxiliary enzymes involved in the oxidation of unsaturated fatty acids were also upmodulated. Interestingly, the only downmodulated enzyme in the β-oxidation cycle was the short chain enoyl-CoA hydratase, which reverses the direction of the cycle by catalyzing the opposite reaction of the long chain enoyl-CoA hydratase. Both the electron transfer flavoprotein (ETF), which transfers the electrons generated by the β-oxidation cycle to complex II of the oxphos system, and the glutaryl-CoA dehydrogenase, which transfers electrons generated from the degradation of the amino acids lysine, hydroxylysine, and tryptophan to the ETF, were upmodulated. These findings demonstrated that the entire mitochondrial system involved in respiration, as well as the degradation of fatty acids and the utilization of amino acid catabolism products, was consistently upmodulated at late times of infection, eventually leading to an increased exploitation of the fatty acid deposits of the cell in order to produce ATP. Further support for this hypothesis was provided by the consistent downmodulation of the key mediators of the fatty acid nonsynthetic pathway (citrate synthase, citrate transporter, and ATP citrate lyase), which use the acetyl-CoA produced by the β-oxidation cycle to store energy for later use instead of synthesizing ATP for immediate con-
Expression levels of nine genes belonging to four transport systems, one importing NADH from the cytosol and three exporting ATP from the inner mitochondrial matrix, were also modified by infection. The cytoplasmic and mitochondrial isoforms of glutamate oxaloacetate (GOT1 and GOT2, respectively) and the mitochondrial malate dehydrogenase (part of the NADH transport system), the fibroblast isoform of adenine nucleotide translocator (ANT2), and the ubiquitous isoform of creatine kinase (part of the ATP transport system) were upmodulated, whereas liver ANT3 and sarcomeric creatine kinase were downmodulated. VDAC1, which forms voltage-gated pores in the outer mitochondrial membrane to allow diffusion of small hydrophilic molecules and adenine nucleotides in normal situations and of cytochrome c during apoptosis was also downmodulated, whereas the low-abundance VDAC2 isoform that inhibits mitochondrion-mediated apoptosis when overexpressed (22) was upmodulated. Taken together, these data show that the vast majority of the genes composing the two main mitochondrial energy production systems and their associated substrate import and product export mechanisms were consistently upmodulated. In contrast, the genes mediating the first three steps of the pathway leading to the reconstitution of the fatty acids pools were downmodulated. These findings indicate that increased mitochondrial energy production is important at late times during CMV infection and suggest that fatty acid catabolism may dominate over neosynthesis, leading to the depletion of cellular fat stores during infection.

CMV infection has been shown to increase the mitochondrial size (71), to disrupt the reticular mitochondrial networks (66), to interfere with the mitochondrial proapoptotic pathways (40, 41, 66, 87), and to stimulate mitochondrial DNA synthesis (37). Our results reveal that CMV infection dramatically and coordinately induces the expression of most mitochondrial ATP-production system components, possibly resulting in the intensification of high-energy phosphate production in order to sustain elevated energy demands of viral replication.

Cell cycle- and cellular DNA replication-related genes. Consistent with data in the literature (10, 32, 34, 52, 83, 107), both cell cycle and nucleic acid metabolism categories were strongly and positively affected by infection (Fig. 1 and 5). The functional genomics approach used here allowed us not only to expand the number of known cell cycle- and cellular DNA replication-related genes but also to uncover systematic differences in gene expression among cell cycle and DNA replication categories (Fig. 8A and B). The most striking result was the significant upmodulation of the genes encoding the DNA replication enzymes DNA polymerase (POLA), DNA polymerase (POLB), DNA polymerase (POLH), DNA ligase (LIGA), and DNA ligase (LIGB) during CMV infection. This upmodulation was consistent with the observed increase in DNA synthesis and the accumulation of DNA replication intermediates during CMV infection (35). The upmodulation of DNA polymerases was also correlated with the downmodulation of the genes encoding DNA repair enzymes, such as DNA ligase (LIGC) and DNA repair protein (DPC). These findings suggest that CMV infection may disrupt DNA repair mechanisms in order to enhance DNA replication and to sustain the high level of viral DNA synthesis required for efficient viral replication.

The functional genomics approach used here also allowed us to identify a number of genes encoding proteins involved in the regulation of cell cycle progression and DNA replication. For example, the upmodulation of the genes encoding cyclin-dependent kinases (CDK1, CDK2, CDK4, CDK6) and their regulatory subunits (CDC25A, CDC25B) was consistent with the observed upmodulation of cell cycle-related genes during CMV infection. These findings suggest that CMV infection may disrupt the normal regulation of cell cycle progression and DNA replication, which may contribute to the observed increase in viral DNA synthesis and the accumulation of DNA replication intermediates during CMV infection.

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replication-related genes upmodulated by CMV but also to gain a better understanding of how these functions may be linked and have a concerted impact on the cell. The 113 genes in the cell cycle category were assigned to one of five classes, with 42 genes (37%) associated with the G2/M phase, 27 genes (24%) involved in general growth control, 19 genes (17%) associated with the G1 or G1/S phases, 13 genes (11%) associated with the S phase, and 12 genes (11%) related to p53 or pRb activities. The 229 genes in the nucleic acid metabolism category were divided into three classes: RNA metabolism (103 genes, 45%), DNA metabolism (101 genes, 44%), and nucleotide metabolism (25 genes, 11%). In the cell cycle category, the S-phase class contained the highest percentage of upmodulated genes (85%), followed by the p53-pRb-related class (75%) and the G2/M phase class (64%). In the nucleic acid metabolism category, the DNA metabolism class stood out as having the largest percentage of upmodulated genes (66%), followed by the mostly downmodulated nucleotide metabolism (42% upregulated) and RNA metabolism (38% upregulated) classes. These data revealed a very strong positive effect of CMV infection on the expression of multiple S-phase, G2/M-phase, and DNA activity regulators, probably all of

**FIG. 5.** GenMAPP of virus-induced gene expression changes affecting cell cycle and DNA synthesis. (A) Cell cycle phases G1, S, G2, and M are depicted by blue arrows, with genes modulated by infection listed underneath. (B) DNA synthesis functions in the prereplication complex (left) and replication fork machinery (right) regulated by infection. The same scheme as shown in Fig. 2 is used with the addition of inhibitory relationships denoted by a ↓. Ink 4 a/c contains ink a, b, and c; HDAC 2/10 contains the histone deacetylases 1, 2, 3, 4, 5, 6, 7A, 8, 9, and 10; E2F 2/6 contains E2F 2, 3, 4, 5, and 6; and Apc 1/11 contains apc 1, 2, 4, 5, 6, 7, 10, and 11.
which are required for efficient viral replication. Interestingly, DNA and RNA metabolism gene transcript levels were affected in opposite ways by viral infection, suggesting that genes involved in DNA metabolism might be preferentially transcribed during viral infection. A total of 112 genes from both categories were organized into the maps shown in Fig. 5A and B, which depict cell cycle control and DNA synthesis functions, respectively. A total of 78% of the genes present on the maps and altered by infection were upmodulated. Expression levels of several G1/S- and S-phase regulators were modified by infection: while both cyclin D1 and the associated cyclin-dependent kinase 6 (CDK6) were strongly downmodulated, the G1 and S cyclin-CDK complex inhibitors ink4d, kip1, kip2, and cip2 were consistently upmodulated. Transition through the G1/S boundary is normally promoted by the activity of the cyclin D1-CDK4 or cyclin D1-CDK6 complexes and is normally inhibited by members of the ink, kip, and cip families of CDK inhibitors. The observed expression pattern of these key regulators, therefore, was indicative of a virus-induced cell cycle arrest in G1, as proposed elsewhere (14, 26, 62). The downmodulation of the CDK2, -3, -4, and -6 inhibitor cip1 and of the G1/S transition inhibitor growth arrest-specific 1 (GAS1), together with the upmodulation of cyclin A, cyclin G1, the cyclin E- and cyclin A-CDK2 complex activator cdc25a, the transcription factor E2F1, and the E2F1 dimerization partner DP2, however, suggested a transition to an S-phase-like environment, a possibility consistent with earlier studies (49, 81, 86). The induction of cyclin A transcript levels at late times of infection, in particular, was reported previously (49), although this comparison must be made to nonstimulated confluent HFs and not to serum-stimulated cells (83). The upmodulation of nine genes encoding core components of the prereplication complex (all six minichromosome maintenance genes [MCM2 to -7] and three of the six origin recognition complex genes [ORC1L, -3L, and -4L]) and of 10 genes encoding core components of the DNA replication system (DNA polymerase α [POLA], α prime 1 [PRIM1], the processivity factor proliferating cell nuclear antigen [PCNA], two of the three subunits of replication factor A [RPA2 and RPA3], and four of the five subunits of replication factor C [RFC1 to -4]) further supported this hypothesis. Finally, the expression of several functions that interact with the DNA synthesis complex was altered by infection: the prereplication complex loading inhibitor geminin, the origin activation stimulator cdc7, the POLA-recruiting factor cdc45L, the DNA repair polymerases β [POLB] and ε [POLE], and the E1A-binding protein p300 (EP300), which facilitates the DNA repair functions of PCNA, were all upmodulated, whereas the inhibitor of the initiation step in DNA replication deleted in oral cancer 1 (DOCI) and four additional DNA polymerases (polymerase α2 [POLA2], the regulatory subunit of polymerase δ [POLD2], and the two proofreading polymerases ζ [POLZ] and κ [POLK]) were downmodulated. In agreement with other reports (10, 107), these data indicate a broad enhancement in the expression of cellular DNA synthesis machinery and related components, possibly reflecting a need for their presence to support viral DNA replication. In this regard, the twofold upmodulation of DNA topoisomerase II α (TOP2A), an enzyme that catalyzes the relaxation of supercoiled DNA and the catenation and decatenation of circular DNA and is essential for CMV replication (8), may contribute to the proposed directional rolling-circle model of CMV genome replication (67). Altogether, these results further extend and deepen our understanding of the well-established dysregulation of multiple G1, S, and DNA replication genes (34, 67), unveiling a complex system of virus-induced events potentially aimed at optimizing viral replication rates.

Multiple components of two fundamental cell cycle control mechanisms, the pRb and p53 systems, were also affected by infection. In addition to E2F1 and DP2, eight pRb interacting partners were upmodulated: five pRb-binding proteins (RBBP2, -4, -5, -6, and -8), two histone deacetylases (HDAC1 and -11) and prohibitin, which is a protein that represses E2F-mediated transcription (102) and, interestingly, also impacts the assembly of mitochondrial respiratory chain complexes (73). Two other genes encoding pRb-binding proteins, the Abelson murine leukemia viral oncogene homolog 1 (ABL1) and the E2F1 partner DP1, were downmodulated. Expression levels of six p53-related genes were modified; four were upmodulated (p53 itself, tumor protein p53-binding protein 2 [TP53BP2], apoptosis-stimulating protein of p53 1 [ASPP1], and protein phosphatase 2C α [PP2Cα]), and two were downmodulated (mouse double minute homolog 2 [mdm2] and ataxia telangiectasia mutated [ATM]). p53-mediated transcription of proapopotic genes is activated by ATM as a result of DNA damage, is enhanced by TP53BP2 and ASPP1, and is inhibited by mdm2 and PP2Cα. Thus, the persistent upmodulation of p53 and the activators TP53BP2 and ASPP1, coupled with the downmodulation of the inhibitor mdm2, might result in the induction of apoptosis were it not for the concurrent downmodulation of the stimulator ATM and the upmodulation of the inhibitor PP2Cβ. These results complement and extend data in previous reports (49), revealing a multifaceted effect of CMV infection on the expression of several pRb- and p53-related genes and emphasizing the ability of this virus to simultaneously induce an S-phase-like environment in cells, achieve replication of the viral genome, and avoid the onset of apoptosis triggered by the checkpoint effectors.

Quite surprisingly, the majority (50 genes, 61%) of the 82 cell cycle genes whose expression was modified by infection (Fig. 5A) were related to the G1/M phase, highlighting the likely importance of functions associated with this part of the cycle for CMV infection. Expression levels of the G2/M transition regulators wee1, myt1, cyclin B2, CDK1, cdc28 kinase regulatory subunit 2 (CKS2), polo-like kinase 1 (pkl1), peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (pin1), protein phosphatase 1α (PP1A), and the PP1A regulatory subunit 7 (PP1R7) were upmodulated. Entry into mitosis relies upon the properly timed activation of the cyclin B-CDK1 complex. The activity of this complex is controlled by the antagonistic actions of wee1 and myt1 kinases, which suppress CDK1 activity, and the cdc25B and cdc25C phosphatases, which promote CDK1 activation (79). In turn, cdc25C activation in prophase is mediated by phosphorylation through the cyclin B-CDK1-mediated feed-forward loop, by the PP1A-mediated removal of an inhibitory phosphate on serine 216, by a p110-catalyzed peptidyl-proline isomerization, and by the plk1-mediated phosphorylation of serine 198, which induces the translocation of cdc25C into the nucleus (94). The consistent upmodulation of all of these regulators revealed a very strong
positive effect of CMV infection on G2/M transition control mechanisms, possibly leading to the stimulation of infected cell progression toward a mitosis-like state. Additional evidence supporting this hypothesis was provided by the observation that a large proportion (41 genes, 82%) of the 50 altered G2/M-related genes encoded proteins specifically involved in the implementation of M-phase events. More than half (22 genes, 54%) of these genes encode proteins implicated in the correct formation of the mitotic spindle and of stable spindle-chromosome attachments. These included six genes controlling the centromere and kinetochore creation process, 10 genes required for centrosome and mitotic spindle functions, and six components of the spindle assembly checkpoint (SAC) (Fig. 5A). Five of the six centromere and kinetochore genes were upregulated and encoded proteins involved in marking a chromosomal region for centromere formation (centromere protein A [CENPA]), in the assembly of the kinetochore complex (suppressor of G2 allele of SKP1 [SGT1]), in the attachment of the kinetochore to microtubules (cdc1A), and in general centromere activation (centromere protein F [CENPF] and ZW10 interactor [zwint]). The only downmodulated gene in this group, centromere protein C1 (CENPC1), encodes a protein required for the inactivation of one centromere and the creation of a functionally monocentric chromosome. Eight of the ten centromere and mitotic spindle genes were upmodulated and encoded proteins important for centrosome integrity (NIMA-related kinase 2 [NEK2]), duplication (centrin 3), and maturation into a microtubuleorganizing center (centrosomal protein 1 [cepl]), for the nucleation of microtubule assembly at the spindle poles (γ tub1), for the regulation of the number of microtubule ends in the spindle (katanin p60 and p80), and for the positioning (kinasin family member 4A [kif4a]) and segregation (mitotic centromere-associated kinesin [MCAK]) of mitotic chromosomes. The two downmodulated genes encoded nuclear mitotic apparatus protein 1 (numa1), which is essential for the organization and stabilization of spindle poles and checkpoint with forkhead and ring finger domains (CHFR), which monitors centrosome separation and retards entry into mitosis in the absence of this event. The observed global upmodulation of numerous genes encoding proteins that regulate the assembly and functionality of the mitotic spindle was unexpected and prompted us to consider the possible progression of the infected cells into a metaphase-like state. The downmodulation of CENPC1, CHFR, and numa1 expression, however, suggested the presence of abnormalities in this process, as did the consistent upmodulation of six genes encoding the SAC components budding uninhibited by benzimidazoles 1 (bub1), mitotic arrest-deficient 2A and 2B (mad2A and mad2B), monopolar spindle 1-like (mps1LT/TTK), aurora A, and CENPE. In cells containing disrupted or mispositioned spindles, mps1LT/TTK recruits active CENPE at the kinetochores. This is followed by the association of mad1 and mad2 and by the mad2-mediated inhibition of the anaphase promoting complex/cyclosome (APC/C), which effectively blocks mitotic cells in metaphase (1). The upmodulation of these key genes hinted at the presence of an active SAC-like process in infected cells. This change, along with the upmodulation of aurora A, which is associated with centrosome amplification and multipolar spindles (111), and of NEK2, which induces splitting of centrosomes and dispersal of centrosomal material (36), suggested a damaging effect of infection on centrosome structure. The expression levels of three APC/C core components were modified in infected cells, with one (cdc27/apc3) downmodulated and two (apc7 and cdc23/apc8) upmodulated. Two APC/C regulatory factors, cdc20 and snk/plk-akin kinase (sak), were also upmodulated. The APC/C ubiquitin ligase complex is activated by cdc20 and controls exit from mitosis by targeting B-type cyclins and securin (also called pituitary tumor-transformation gene 1 [PTTG1]) for degradation. The destruction of securin stimulates the activity of separase (also called extra spindle poles-like 1 [espl1]), a caspase-like protease, which triggers sister chromatid separation by cleaving the scc/rad21 subunit of the cohesin complex (43). Both cohesin and condensin complexes ensure the correct segregation of replicated chromosomes by organizing chromosomes into highly compact mitotic structures (condensin) and by keeping sister chromatids together until they split at anaphase (cohesin). Expression levels of securin, separase, two of the four cohesin subunits (scc/rad21 and structural maintenance of chromosomes 3 [SMC3]), and one of the five condensin subunits (chromosome associated protein E [CAPE]) were upmodulated, whereas the expression of PTTG1-interacting protein (PTTG1-IP), which facilitates securin nuclear translocation, was downmodulated. These data indicate a substantial increase in transcript levels of chromosome management factors, suggesting their importance during the late phase of infection, possibly to properly organize the mass of newly replicated viral DNA, which can reach 4,000 copies/infected cell (69). Finally, four genes encoding NIMA-related kinases (NEK4, -6, -7, and -9) and four genes encoding septins (septin2/gedd5, septin4/pmtl2, septin7/cdc10, and septin9/msf) were transcriptionally altered in infected cells and all except septin4/pmtl2 were downmodulated. Elimination of NEK9 results in spindle abnormalities and chromosomal misalignments (80), whereas inhibition of NEK6 arrests cells in M phase and triggers apoptosis (110). The consistent downmodulation of NEK9 could therefore negatively impact the process of spindle assembly, in agreement with the observed alterations in the expression of spindle-morphogenesis-related genes. The downmodulation of NEK6 and of septin2/gedd5 and septin9/msf, whose depletion results in the accumulation of binucleated and cells arrested during cytokinesis (93), also suggested that exit from a mitosis-like environment might be impaired. Altogether, these results show a global and consistent increase in the transcript levels of numerous cell cycle regulators and DNA replication machinery components, indicating that the ability to control cell cycle phases and functions is important for the successful completion of CMV replication.

**Time-dependent induction of a mitosis-like state during CMV infection and association with CMV replication components.** The persistent transcriptional upmodulation of a very large number of M-phase genes suggested that CMV-infected cells might proceed through a mitosis-like state even though cellular DNA replication is blocked. To investigate this process further, uninfected or AD-infected HFs (MOI of 10) were subjected to immunofluorescence analysis for γ tub1, a protein known to localize at centrosomes, mitotic spindle microtubules, and spindle poles (58) and whose expression levels were consistently upmodulated by infection. Cellular DNA was detected by Hoechst 44432 dye staining, and infected cells were highlighted by immunolabeling with an MAb to the viral nu-
clear antigens IE1/IE2. In uninfected interphase cells, the IE1/IE2 antigens were absent and γtub1 was diffusely cytoplasmic with a concentration in one or two spots corresponding to the centrosomes (Fig. 6A). In infected interphase-like cells, the IE1/IE2 antigens were confined to nuclei bounded by an intact nuclear membrane (Fig. 6C), and γtub1 was observed in both cytoplasm and at centrosomes (Fig. 6B). Unlike uninfected interphase cells, however, several of these cells contained more than two γtub1-labeled centrosomes, usually positioned adjacent to the nucleus (Fig. 6B and D). These supernumerary centrosomes likely originated from aberrations in some or all of the mechanisms controlling centrosome duplication, maturation, and separation. Thus, CMV infection appears to be associated with centrosomal injuries, a finding consistent with early observational reports (18, 39, 71). The data obtained from functional genomic analysis, however, allows us to understand how the complex interplay between CMV and the cell cycle machinery may be affecting the functioning of the centrosome and to speculate which genes might be involved in the generation of the observed injuries. The upmodulation of NEK2, aurora A, and mps1L/TTK in infected cells may be directly related to this process, given that overexpression of these gene products in mammalian cells results in aberrant centrosome duplication (31, 36, 111). The product of the upmodulated gene PP1A is also a good candidate as an effector function, because this protein localizes at centrosomes during mitosis (7) and modulates NEK2 kinase activity (46). The microtubule organizing center, where centrosomes normally reside, is where final steps of viral particle production occur, likely as the ultimate site of virion tegumentation and envelopement (82). Moreover, this structure is visible as a cytoplasmic inclusion and functions as a gathering site for cellular organelles, dense bodies, virions, and other virus-like particles at late times postinfection (88). Thus, the increased expression levels of several genes whose products are involved in governing the functionality of the microtubule organizing center, such as centrin 3, cep1, plk1, and cdk1, might be part of a viral strategy to gain control of this structure.

In uninfected mitotic cells, γtub1 was associated with the two spindle poles and the surface of the aster microtubules (Fig. 7A), and chromosomal DNA was condensed into a highly ordered metaphase plate (Fig. 7B and C), as expected (58). In infected nonnucleated cells, the localization of γtub1 revealed the presence of two types of spindles: bipolar with γtub1 associated with each pole and with the aster microtubules (Fig. 7D, upper left cell) and bipolar or multipolar with γtub1 localized at each pole, at additional pole-like structures around the aster, and distributed along the aster microtubules (Fig. 7D, lower right cell, L, and M). In these cells, chromosomal DNA appeared condensed in bulks, which were either concentrated in the same area occupied by the spindle (Fig. 7F and I) or dispersed throughout the entire cell volume (Fig. 7K). Consistent with the loss of nuclear integrity during mitosis, the IE1/IE2 antigens were dispersed throughout the cell body (Fig. 6G, L, and M). Depending on the orientation of the cell on the coverslip, the previously reported (57) association of the IE1 protein with chromosomal DNA was more (Fig. 7J) or less evident (Fig. 7G). No matter the spindle type, chromosomal

FIG. 6. Abnormal centrosome numbers in nucleated CMV-infected cells. (A to D) Uninfected or CMV-infected HFs stained with an anti-γtub1 MAb (red) and a FITC-conjugated MAb to the viral nuclear proteins IE1/IE2 (green). (A) γtub1 localization in a representative uninfected interphase cell; (B to D) γtub1 (B) and IE1/IE2 (C) localization in a representative infected nucleated cell and a B+C merged image (D). Arrows point at the two additional centrosomes in the infected cell. Magnification, ×1,000.
DNA bulks were never correctly organized in a metaphase plate typical of normal mitosis. Thus, the observed phenotype of these nonnucleated cells and of uninfected mitotic cells were similar in that both contained γ-tub1-labeled spindles and condensed chromosomal DNA; however, the localization of γ-tub1, the number of spindle poles, and the appearance and intracellular distribution of the condensed DNA in the nonnucleated cells were clearly different from those observed in normal mitosis. This phenotype revealed that pathological events had occurred during their formation, and therefore these infected cells were denoted “pseudomitotic.”

Pseudomitosis has been previously noted in CMV-infected cultures (11) but never subjected to direct study in relation to viral gene expression during the late phase of infection. The percentage of IE1/IE2-positive pseudomitotic cells observed in AD-infected HF cultures in three independent experiments peaked at 72 hpi (33% of cells), increasing from ca. 2% at 24 hpi to 16% at 50 hpi (Fig. 8A). Interestingly, the proportion of pseudomitotic cells appeared to decline to 20 to 29% (mean value, 25%) by 98 hpi. Background mitosis in mock-infected confluent cultures was very low, i.e., 0.2% at 24 h, 0.05% at 50 h, 0.03% at 72 h, and <0.005% at 98 h (data not shown), indicating that virus infection was modulating cell behavior. The vast majority of the pseudomitotic cells in infected cul-

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FIG. 7. Abnormal mitotic figures in nonnucleated CMV-infected cells. (A to M) Uninfected or CMV-infected HFs stained with an anti-γ-tub1 MAb (red), an FITC-conjugated MAb to the viral nuclear proteins IE1/IE2 (green) and Hoechst 44432 to detect cellular DNA (blue). (A to C) γ-tub1 (A) and cellular DNA (B) localization in a representative uninfected mitotic cell and A + B merged image (C); (D to I) γ-tub1 (D), cellular DNA (E), and IE1/IE2 (G) localization in a representative infected nonnucleated cell; phase-contrast image of the same cells (H), D + E merged image (F), and F + G + H merged image (I). (J and K) IE1/IE2 (J) and cellular DNA (K) localization in a representative infected nonnucleated cell; (L and M) IE1/IE2 (green) and γ-tub1 (red) localization in representative infected nonnucleated cells. Magnification, ×1,000.
tures were IE1/IE2 positive (between 97% at 24 hpi and 100% at 98 hpi), indicating that the induction of the pseudomitotic features was strictly associated with infection. Pseudomitosis, therefore, developed in an apparent transient fashion during the late phase of viral infection with a peak at 72 hpi. Altogether, these results showed that, as predicted from the microarray data, CMV infection was associated with the initiation of mitotic events in the host cell and with the generation of abnormalities in the processes of centrosome duplication, spindle assembly, and chromosome condensation.

We next sought to determine whether the appearance of pseudomitotic cells was dependent upon cellular or viral DNA replication. AD-infected HFs were either left untreated or maintained in the presence of 1 mM HU (to block only cellular DNA synthesis), 10 mM HU (to block cellular and viral DNA synthesis) (6, 15, 59), or 300 µg of PFA/ml (to block only viral DNA synthesis) (97, 99) before the cells were processed for immuno fluorescence analysis with anti-α-tub1 and anti-IE1/IE2 antibodies. As expected, treatment with these drugs did not have any effect on IE1/IE2 expression, and similar, almost-uniform infection based on IE1/IE2 positive cells was observed in all cultures. The percentage of IE1/IE2-positive pseudomitotic cells observed at 72 hpi ranged from a mean of 31% (six independent experiments) in untreated cultures to a mean of 25% (three independent experiments) in 1 mM HU-treated cultures to a mean value of 2% (three independent experiments) in 10 mM HU-treated cultures to a mean value of 2% (five independent experiments) in PFA-treated cultures (Fig. 8B). The strong reduction in the percentage of pseudomitotic cells observed in conditions in which viral DNA synthesis was inhibited but not where cellular DNA synthesis alone was blocked demonstrated that the appearance of pseudomitotic cells followed and was dependent on initiation of viral DNA replication during infection.

To determine whether the viral replication cycle progressed normally in pseudomitotic cells, infected cultures were subjected to immunofluorescence analysis for the viral delayed early protein ppUL44 (68, 105), the early-late protein pp150 (82), and the true-late protein pUL43 (2). At 50, 72, and 98 hpi, the vast majority of pseudomitotic cells expressed ppUL44; however, three distinct localization patterns were evident: diffuse throughout the whole-cell volume (Fig. 9A), diffuse and concentrated in large aggregates mostly positioned at the center of the cell (Fig. 9B), and diffuse and concentrated in one or two globular masses that appeared to be at the opposite poles of the cell (Fig. 9C). At 72 hpi, both pp150 and pUL43 were expressed and diffused throughout the whole-cell volume; in some cells pp150 appeared to be concentrated in punctate aggregates located approximately at the center of the cell (not shown). In nucleated cells present in the same cultures, all three antigens showed the expected distribution consistent with viral replication, i.e., associated with the nuclear replication compartments for UL44 (76) and condensed in a cytoplasmic structure adjacent to the nucleus for pp150, as well as for pUL43 (2, 82) (not shown). The expression of these antigens indicated that virus infection in pseudomitotic cells was proceeding beyond the immediate-early phase and was associated with different antigen localization patterns compared to nucleated cells. Therefore, the normal trafficking and function of these viral proteins appeared to be affected by the intracellular environment.

To establish whether CMV DNA replication was proceeding in pseudomitotic cells, sites of viral DNA synthesis were labeled by BrdU incorporation (Fig. 9D). Although cellular DNA replication does not proceed under the conditions used here (26), any possible contribution from cellular DNA replication was controlled by infecting and subsequently maintaining quiescent HFs in the presence of 1 mM HU, a treatment...
FIG. 9. Progression of CMV replication cycle in pseudomitotic cells. (A to C) ppUL44 (green) and cellular DNA (red) localization in three representative infected pseudomitotic cells; (D) BrdU (green) incorporation and y tub1 (red) localization in a representative infected pseudomitotic cell; (E and F) transmission electron microscopy of a representative infected pseudomitotic cell. The rectangle in panel E shows the area magnified in panel F. Arrows indicate viral DNA encapsidation structures; arrowheads indicate events of partial or complete enclosure of capsids in vesicular structures. Ch, condensed mitotic chromosomes; Mt, mitochondria. Magnification: E, ×3,000; F, ×17,000.
that prevents cellular DNA synthesis without any impact on viral replication (26). BrdU was added to the culture medium at 71 hpi for 1 h before the cells were processed for immunofluorescence analysis with anti-BrdU and anti-tyb1 antibodies. In almost every pseudomitotic cell, BrdU was incorporated in numerous minute dots, most of which were clustered near the cell center (Fig. 9D). A minor population of dots were also scattered throughout the cell volume and, in some cells, appeared to amass in a few areas at the cell periphery (not shown). As expected, in the vast majority of the nucleated cells present in the same cultures BrdU was incorporated in large nuclear domains corresponding to the viral replication compartments (76). These results revealed the presence of newly synthesized viral DNA at novel structures within the pseudomitotic cells, suggesting that CMV genome replication was proceeding despite the disruption of nuclear structures and the mitosis-like intracellular organization of these cells.

To confirm and extend these results, pseudomitotic cells from thin sections of AD-infected HF cultures at 72 hpi were monitored by transmission electron microscopy for the presence of capsids and their assembly intermediates (Fig. 9E and F). Numerous A, B, and C capsid types were found in pseudomitotic cells; consistent with the incorporation of BrdU, the majority of these capsids were grouped in a central region also containing dense bodies, mitochondria, bulks of condensed DNA, and a multitude of vesicles and vesicle-like structures (Fig. 9E); in addition, isolated capsids were also observed in the cell periphery. Higher magnification (17,000-fold) revealed the presence of multiple capsids connected with a thin fibrillar filament partially contained within the capsid and partially protruding from it (Fig. 9F, arrows). The occurrence of these figures, which are consistent with viral DNA encapsidation events (44) in pseudomitotic cells, suggested the occurrence of viral replication and viral particle assembly processes. In addition, some capsids appeared partially or completely surrounded by vesicular structures (Fig. 9F, arrowheads); these formations were morphologically indistinguishable from the virion envelopment intermediates observed in the cytoplasmic viral fields of infected nucleated cells (88) (data not shown), suggesting that at least part of the particle envelopment process was proceeding in pseudomitotic cells. Thus, notwithstanding the striking intracellular environment alterations occurring in pseudomitotic cells, including the absence of a nuclear membrane, key steps of the viral life cycle were still progressing.

These findings reveal that CMV infection is associated with the upmodulation of a substantial number of mitosis-related genes and with the appearance of transient pseudomitotic features that peak around 72 hpi and affect approximately one-third of the infected HF at late times postinfection; induction of these features is strictly linked to viral DNA replication and does not appear to be inhibitory for the progression of the viral life cycle. It is important to note that we have used the very common ATCC variant of CMV strain AD169 (AD169var ATCC) for these studies and that we have observed viral strain and variant differences in the percentage of cultured cells that undergo pseudomitosis (L. Hertel and E. S. Mocarski, unpublished data), suggesting that particular viral genes control this process. Previous studies on pseudomitosis and CMV infection have focused mainly on the analysis of virus-induced chromosomal damages in infected cells (35) and very little on the mechanisms behind the generation of these abnormal cells. Our data are the first to show that an entire array of functionally related genes normally involved in the implementation of mitotic functions is coordinately upmodulated by CMV during the late phase of infection, strongly suggesting that the appearance of pseudomitotic cells in infected cultures is the consequence of a specific virus-controlled program. The initial stages of this program are likely to occur during the immediate-early phases of infection, when cyclin B1 begins to be expressed (26, 49, 81), but the climax is probably reached much later, as indicated by the dramatic and sudden increase in the number of pseudomitotic events from 24 to 50 hpi. Given that the induction of chromosomal damage occurs early and requires virion penetration but not de novo viral protein synthesis (33), whereas the appearance of pseudomitotic cells depends upon or is a consequence of viral DNA replication, we speculate that the implementation of the mitosis-related gene expression program and the generation of genotoxic events might occur independently. The exact mechanisms linking the fulfillment of this viral strategy to control the cellular mitotic functions and the appearance of pathological mitotic figures in infected cultures remain to be elucidated. We suspect, however, that the actual development of pseudomitosis is a result of errors in the correct execution of the viral program. If this were not the case, then pseudomitosis should have reached the totality of infected cells in our experiments, where infections are carried out at a high MOI giving one-step growth conditions and with confluent cells held in Go.

In cultured cells, pseudomitosis accompanied and did not appear to inhibit the progression of the viral cycle, suggesting that some component of this process promotes replication at late stages of infection. The purpose of this CMV-induced impact on the cell remains a mystery. Our current hypothesis is that the viral functions may coordinately and consistently target specific cellular factors that are needed to perform particular functions required for the completion of the viral replication cycle. Several mitotic proteins upmodulated during infection are normally involved in controlling chromosome condensation, cohesion, and separation and may therefore be used by CMV to modify the compaction of cellular DNA and the structure of nuclear chromatin (53), possibly in order to increase the volume available to accommodate newly replicated viral genomes or to segregate the two DNA populations (cellular and viral). Other mitotic factors are known to modulate the structure and function of microtubules, thus potentially becoming useful tools for the rearrangement of the microtubule organizing center and the reestablishment of microtubular networks at late times postinfection (78), when they are likely to be needed for trafficking of newly assembled virions out of the cell. Finally, CDK1 has been shown to phosphorylate nuclear lamins (77) and to trigger the depolymerization of the nuclear lamina, followed by the dissolution of the nuclear envelope. The activity of this kinase might therefore be exploited by CMV for the modification of the nuclear membrane in order for virions to leave the nucleus, eventually in association with specific viral gene products, as has been suggested for protein kinase C acting in conjunction with late viral gene products (70).

Altogether, data presented here demonstrate the value of comprehensive functional genomic analysis in revealing and
dissecting cellular events that are important in viral replication. The remarkable impact of this virus on the infected cell appears to extend well beyond an activation of cell cycle-regulated cyclins to mimic the S phase of the cell cycle and make host cell transcription and DNA replication functions available to benefit the virus. CMV dysregulation extends beyond an impact on early cell cycle-regulated functions to effect an environment that mimics mitosis. Such a broad and concerted process likely provides benefit well beyond viral DNA replication to the latter steps in replication, including virion maturation. Although cellular DNA replication does not occur in cells that support CMV replication, cellular gene expression is dramatically dysregulated and gives rise to structures normally associated with cell division.

ACKNOWLEDGMENTS

We acknowledge Tom Jones for providing mutant virus, William Britt for providing anti-pp150 antibody (MAB 56-14), Derrick Dagan for providing anti-pp65 antibody (MAB 23932), and the staff of the Stanford Microarray Database for assistance with data analysis. We thank Patrick Brown, Michael Eisen, and Shinya Watanabe for their initial efforts that provided guidance to the experiments described here. We thank James McDonald and the members of the SAM-software E-mail list (sam-software@yahoogroups.com) for useful suggestions on SAM usage and Noshan Temi for providing informatics support to perform spot list intersections. We are very grateful to Naïsa Ghori for outstanding assistance with electron microscopy and to John Boothroyd, Karla Kirkegaard, and Christine Martens for critical reading of the manuscript.

This study was supported by U.S. Public Health Service grants AI50153 and AI33852.

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