Cidofovir Inhibits Genome Encapsidation and Affects Morphogenesis during the Replication of Vaccinia Virus[▽]

Desyreé Murta Jesus,¹ Lilian T. Costa,³ Daniela L. Gonçalves,³ Carlos Alberto Achete,^{3,4}
Marcia Attias,² Nissin Moussatché,^{1,5} and Clarissa R. Damaso^{1*}

Laboratório de Biologia Molecular de Vírus¹ and Laboratório de Ultraestrutura Hertha Meyer,² Instituto de Biofísica Carlos Chagas Filho, and Engenharia Metalúrgica e de Materiais,⁴ Universidade Federal do Rio de Janeiro, Rio de Janeiro, and Divisão de Metrologia de Materiais, Inmetro, Duque de Caxias,³ Brazil, and Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida⁵

Received 24 May 2009/Accepted 26 August 2009

Cidofovir (CDV) is one of the most effective antiorthopoxvirus drugs, and it is widely accepted that viral DNA replication is the main target of its activity. In the present study, we report a detailed analysis of CDV effects on the replicative cycles of distinct vaccinia virus (VACV) strains: Cantagalo virus, VACV-IOC, and VACV-WR. We show that despite the approximately 90% inhibition of production of virus progeny, virus DNA accumulation was reduced only 30%, and late gene expression and genome resolution were unaltered. The level of proteolytic cleavage of the major core proteins was diminished in CDV-treated cells. Electron microscopic analysis of virus-infected cells in the presence of CDV revealed reductions as great as 3.5-fold in the number of mature forms of virus particles, along with a 3.2-fold increase in the number of spherical immature particles. A detailed analysis of purified virions recovered from CDV-treated cells demonstrated the accumulation of unprocessed p4a and p4b and nearly 67% inhibition of DNA encapsidation. However, these effects of CDV on virus morphogenesis resulted from a primary effect on virus DNA synthesis, which led to later defects in genome encapsidation and virus assembly. Analysis of virus DNA by atomic force microscopy revealed that viral cytoplasmic DNA synthesized in the presence of CDV had an altered structure, forming aggregates with increased strand overlapping not observed in the absence of the drug. These aberrant DNA aggregations were not encapsidated into virus particles.

Vaccinia virus (VACV) is the prototypical member of the *Poxviridae*, a family of large DNA-containing viruses. During infection, a cascade of temporally regulated viral gene expression occurs exclusively in the cell cytoplasm, where viral DNA replication takes place. DNA replication is essential for the onset of the intermediate and late steps of viral gene expression (37). VACV morphogenesis is a complex process that starts within the virus factories, or viroosomes, in parallel with the late stage of gene expression. Crescent-shaped virus membranes evolve into immature spherical particles (IV) that subsequently progress to form brick-shaped mature virions (MV) (reviewed in reference 8). Virus assembly and maturation are complex processes requiring telomere resolution of newly replicated DNA (20, 36, 40), genome encapsidation (6, 22, 50), and the proteolytic processing of major structural proteins (38, 51). For the past decade, several reports have analyzed in detail these numerous steps of VACV morphogenesis, unraveling the role of distinct virus late proteins in the progression of viral particle formation (reviewed in reference 8).

Cantagalo virus (CTGV) is a strain of VACV isolated from pustular lesions on cows in Brazil (10). Similar outbreaks of vaccinia-like viruses have been reported frequently over the past 8 years (14, 39, 48). Interestingly, the majority of these

vaccinia viruses circulating in the wild in Brazil bear a striking similarity to the Brazilian vaccine strain used for systematic vaccination during the eradication campaign, which was produced in Rio de Janeiro (19) and called strain IOC (10). This similarity raises the interesting possibility that the circulating vaccinia viruses represent feral derivatives of IOC or of a closely related ancestor. Little is known about the sensitivity of these novel vaccinia viruses to antiviral compounds. In the absence of an active smallpox vaccination campaign, the spread of these vaccinia viruses in the wild, the prevalence of cowpox infections in Europe and elsewhere (39), and the occurrence of complications from smallpox vaccination (52) make the need for effective antipoxvirus treatment a worldwide concern.

Cidofovir (CDV), an acyclic pyrimidine phosphonate analogue, has shown a potent antiviral effect on several poxvirus infections (4, 15, 44, 46). Recently, we have reported the efficacy of CDV in inhibiting the replication of the Brazilian VACV strains CTGV and IOC (26). The mechanism of action of CDV on reactions catalyzed by the VACV DNA polymerase has been studied *in vitro*. CDV is not a chain-terminating analogue but drastically slows chain extension and inhibits the 3′–5′ exonuclease proofreading activity of the enzyme (34). In addition, templates containing CDV cause inhibition of DNA elongation (33). Differences between the effects of CDV on human cytomegalovirus (55) and VACV enzymes have been observed, but overall it has been widely accepted that CDV acts by inhibiting the process of virus DNA replication. Moreover, most CDV-resistant VACV strains contain mutations in

* Corresponding author. Mailing address: Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho, 373, CCS, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil. Phone: 55 (21) 2562-6510. Fax: 55 (21) 2280-8193. E-mail: damasoc@biof.ufrj.br.

[▽] Published ahead of print on 2 September 2009.

the catalytic domain or in the 3'-5' exonuclease domain of the DNA polymerase (2, 5, 28, 45).

Despite the consensus regarding mechanisms of action, the effects of CDV on the stages of the VACV replicative cycle have never been analyzed. We report here that although CDV led to approximately 90% inhibition of VACV progeny production, we observed only 30% inhibition of DNA replication and normal levels of postreplicative virus gene expression. However, the encapsidation of DNA into virus particles and the proteolytic processing of the major core proteins were inhibited in CDV-treated cells, leading to an impairment of virus morphogenesis. These effects on virus assembly are an indirect result of a primary effect of CDV on VACV DNA synthesis. Atomic force microscopy (AFM) analysis revealed that virus DNA isolated from the cytoplasm of CDV-treated cells formed aggregates of highly entangled and intertwined DNA molecules that were not observed in cytoplasmic viral DNA isolated from untreated cells. In addition, these DNA aggregates were not detected in encapsidated virus genomes isolated from particles purified from untreated or CDV-treated cells. Our data suggest that incorporation of CDV into VACV DNA during the replication process may lead to aberrant DNA structures, which are less able to be packaged into virus particles.

MATERIALS AND METHODS

Cells and viruses. BSC-40 cells were grown in monolayer cultures at 37°C in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Invitrogen), 500 U/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, and 2.5 µg/ml amphotericin B (Fungizone). VACV strains CTGV isolate CM-01, IOC clone A1-1-1 (10), and WR were available in the laboratory's collection; they were routinely propagated, and their titers were determined by plaque assays in BSC-40 cells, as described previously (13). VACV mutant *Cts53* was kindly provided by Richard Condit, University of Florida, and was propagated in BSC-40 cells at 31°C (24).

CDV and antibodies. CDV was generously provided by Gilead Sciences Inc. (Foster City, CA). The drug was prepared as a 16 mM stock solution in phosphate-buffered saline (PBS) and was stored at -20°C.

The rabbit antiserum raised against VACV-WR structural proteins has been described elsewhere (12). Mouse anti-A18 was provided by Richard Condit, University of Florida, Gainesville. Rabbit anti-A24 and anti-J6 were made available by Stewart Shuman, Sloan-Kettering Institute, New York. Rabbit anti-D1, anti-D12, anti-D8, anti-E1, and anti-J3 were a gift from Edward Niles, State University of New York, Buffalo. Rabbit anti-F10, anti-H1, anti-F17, anti-H5, and anti-I1 were provided by Paula Traktman, Medical College of Wisconsin, Milwaukee. Rabbit anti-p4a/4a (A10), anti-I7, and anti-p4b/4b (A3) were a gift from Dennis Hruby, Oregon State University, Corvallis.

Virus purification. BSC-40 cells grown in 150-mm-diameter dishes were infected with the indicated viruses at a multiplicity of infection (MOI) of 5. At time zero, 65 µM CDV or PBS (control) was added to the cultures. At 24 h postinfection, the monolayers were harvested, and intracellular virus was purified by centrifugation through a 36% sucrose cushion and sedimentation in a 25-to-40% sucrose gradient, as described previously (11). Banded virions were aspirated and washed in 10 mM Tris-HCl (pH 7.5). The number of particles was determined, on the assumption that 1 A₂₆₀ unit corresponded to 1.2 × 10¹⁰ particles per ml. The virus yield was assayed as described previously (13).

Analysis of virus DNA accumulation by slot blot assay. Confluent BSC-40 cells grown in 35-mm-diameter dishes were infected with the indicated viruses at an MOI of 5 in the presence or absence of 65 µM CDV and were harvested at multiple times postinfection. Cells were resuspended in TLD buffer (10× SSC [1.5 M NaCl, 0.15 M sodium citrate] [pH 7.0], 1 M ammonium acetate), and samples (40 µl) were applied in triplicate under a vacuum to nylon membranes (Hybond-N; GE Healthcare, Piscataway, NJ) using a slot blot apparatus. DNA was then denatured in situ as described previously (42). The HindIII D fragment of the VACV-WR genome was used as a probe after nick translation labeling with [α -³²P]dCTP (Perkin-Elmer Life Sciences, Waltham, MA), and hybridization was carried out for 18 h at 43°C in a formamide-containing solution. The

blots were washed three times at 56°C in 0.1× SSC-1% sodium dodecyl sulfate (SDS) and were exposed to X-ray film as described elsewhere (12).

For the analysis of DNA content, suspensions of purified viruses corresponding to 1.5, 3, and 6 µg of protein were applied in triplicate to replica membranes. The Hybond-N membrane was processed for DNA detection essentially as described above, and the nitrocellulose membrane (Hybond-C; GE Healthcare) was used for Western blot assays with an antiserum to VACV proteins. Data were quantitated by densitometry analysis of the scanned autoradiograms using the image processing and analysis program Scion Image (Beta Release 4; Scion Corporation).

Genome resolution assay. Confluent 60-mm-diameter dishes of BSC-40 cells were infected with CTGV or VACV-IOC at 5 PFU per cell and then incubated in the absence or presence of 65 µM CDV or 45 µM isatin- β -thiosemicarbazone (IBT; provided by R. Condit, University of Florida). Cells were harvested at 18 h postinfection, and DNA was isolated essentially as described previously (13). Samples were digested with 10.5 U of BstEII (Invitrogen) for 3 h at 50°C, resolved in 1% agarose gels, and transferred to Hybond-N membranes. The blots were hybridized as described above, using nick-translated pBD6 (35), provided by M. Merchlinsky, U.S. Food and Drug Administration, Bethesda, MD.

Protein pulse-labeling. Cells seeded in 35-mm-diameter dishes were infected at an MOI of 5 in the presence or absence of 65 µM CDV. At various times postinfection, cells were pulse-labeled with 80 µCi/ml [³⁵S]methionine-cysteine (Perkin-Elmer Life Sciences) for 30 min in methionine-free MEM (Invitrogen) containing 65 µM CDV or PBS (control). The monolayers were resuspended in an SDS-containing sample buffer, and protein samples were resolved by electrophoresis on SDS-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) gels, followed by autoradiography.

For pulse-chase assays, cells grown in 35-mm-diameter dishes were infected (MOI = 5) and either left untreated or treated with 65 µM CDV or 100 µg/ml rifampin (Sigma, St. Louis, MO). Thirteen hours after infection, cells were pulsed with 120 µCi/ml [³⁵S]methionine-cysteine (Perkin-Elmer Life Sciences) in methionine-free MEM (Invitrogen) for 45 min at 37°C. Samples were harvested immediately in an SDS-containing buffer (pulse) or were incubated with a 100-fold excess of unlabeled methionine for 3 or 5 h (chase). Protein samples were resolved by SDS-PAGE, followed by autoradiography.

Immunoblot analysis of viral proteins. Protein samples from infected cells resolved by SDS-PAGE were transferred to Hybond-C membranes and processed for the immunoblot assay essentially as described previously (13). In some assays, samples of purified virus (15 µg) were solubilized in an SDS sample buffer, resolved by SDS-PAGE, and transferred to Hybond-C membranes as described above. The blots were probed with primary antibodies diluted as follows, for the following times: 1:500, for 20 h (anti-D1, anti-D12, and anti-J6); 1:500, for 1 h (anti-D8 and anti-p4b/4b); 1:1,000, for 18 h (anti-A12, anti-A24, anti-E1, anti-F17, anti-H5, anti-I1, anti-I7, and anti-J3); 1:1,000, for 1 h (anti-F10, anti-H1, and anti-p4a/4a); 1:3,000, for 1 h (anti-VACV); and 1:5,000, for 1 h (anti-A18). The bound antibodies were detected using horseradish peroxidase-conjugated anti-rabbit or anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA), followed by incubation with Luminol Western blot reagent (Santa Cruz Biotechnology), as suggested by the manufacturer.

Transmission electron microscopy. Cells seeded in 60-mm-diameter dishes were infected at 5 PFU per cell, incubated or not with 65 µM CDV, and processed for transmission electron microscopy at 20 h postinfection as described previously (10). Briefly, monolayers were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 18 h at 4°C and were postfixed in 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer for 60 min at room temperature. After a wash, the samples were dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with 5% uranyl acetate and lead citrate and were observed under a Zeiss 900 (Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro) or Morgagni 268 FEI (Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro) electron microscope. For purified-virus analysis, 2.5 × 10¹⁰ particles were centrifuged for 3 min at 13,000 × g, and the pelleted viruses were processed essentially as described above.

DNA preparation for AFM analysis. Cells (100-mm-diameter dishes) were infected at an MOI of 5 and incubated in the absence or presence of 65 µM CDV. At 24 h postinfection, cytoplasmic DNA was isolated as described previously (18), with modifications. All steps of the procedure were performed at 4°C without shaking and vortexing, and by using wide-bore pipette tips. Briefly, cells were washed in isotonic buffer (150 mM NaCl, 20 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) and incubated in hypotonic buffer (10 mM Tris-HCl [pH 8.0], 10 mM KCl, 5 mM EDTA [pH 8.0]) for 10 min at 4°C, followed by an additional 10 min in the presence of 1% Triton X-100 and 40 mM β -mercaptoethanol. Postnuclear extracts were obtained and centrifuged at 18,000 × g for 60 min at

FIG. 1. Inhibition of progeny production by CDV. (A) BSC-40 cells were infected with CTGV at an MOI of 1 or 5. At time zero, CDV at the indicated concentrations was added to the culture medium. After 24 h of infection, the cells were harvested for virus titration by a plaque assay. (B) One-step growth assay in the presence of CDV. BSC-40 cells were infected with CTGV at an MOI of 5 in the absence or presence of 65 μ M CDV. At the indicated time points, cell monolayers were harvested for virus titration by a plaque assay.

4°C. The pellet was resuspended very gently in 10 mM Tris-HCl (pH 8.0), and the mixture was adjusted to 5 mM EDTA (pH 8.0), 280 mM β -mercaptoethanol, 0.5 mg/ml of freshly prepared proteinase K (Invitrogen), and 5% Sarkosyl. After incubation at 54°C for 2 h, nucleic acid (500 μ l) was extracted twice with phenol-chloroform in 15-mm-diameter vials for 16 h in an orbital shaker at 80 rpm. Ethanol-precipitated pellets were resuspended in 10 mg/ml DNase-free RNase and incubated for 30 min at 37°C, followed by treatment with 0.5 mg/ml proteinase K for 2 h at 54°C. DNA was gently reextracted, first with phenol-chloroform and then with chloroform for 16 h at 80 rpm, and was then ethanol precipitated. The DNA pellet was gently resuspended in autoclaved MilliQ water (resistivity, 18.2 M Ω cm). To obtain DNA from purified virus, particles were resuspended in 10 mM Tris-HCl (pH 8.0) and processed essentially as described above.

Immediately before use, 12.5 mM MgCl₂ was added to DNA samples, and 5 μ l of a 5-ng/ μ l DNA solution was deposited on freshly cleaved mica. After incubation for 5 min at room temperature, the material was rinsed with MilliQ water, gently dried under an N₂ flow, and used for AFM imaging (9).

AFM image acquisition and analysis. All images were acquired with a Nanoscope IIIA MultiHead AFM (Digital Instruments, Santa Barbara, CA), operating in dynamic mode under ambient conditions, with 46% relative humidity, using AC240 cantilevers (Veeco, Woodbury, NY) with a spring constant of 2 N/m and a resonance frequency of $\sim$ 70 kHz. The nominal tip radius was <10 nm. AFM image analysis was performed using JPK Image Processing software.

RESULTS

Inhibition of CTGV and VACV-IOC progeny production by CDV. We have previously shown that CDV inhibited CTGV and VACV-IOC replication in low-MOI (0.001) infections of BSC-40 cells. We have also shown that as much as 632 μ M CDV was not toxic to the cells after 48 h of treatment (26). In the present study, we elevated the MOI in order to evaluate the stages of the virus cycle. We first examined the effects of different concentrations of CDV on the production of virus particles in BSC-40 cells after 24 h of infection. Figure 1A shows that the drug gradually inhibited CTGV progeny yield, reaching a plateau of $>90\%$ inhibition at an MOI of 5 and 65 μ M CDV. Because the effect of CDV has not been regularly analyzed with elevated MOIs, no correlation with previously published data could be inferred. Next, we performed a one-step growth assay and observed that 65 μ M CDV progressively inhibited CTGV titers after 8 h of infection, reaching approximately a 10-fold reduction at 24 h (Fig. 1B). Similar kinetics of virus production and levels of yield inhibition in the presence

of CDV were obtained for VACV-IOC (data not shown). This effect did not revert with the removal of CDV from cell culture and was not intensified by further addition of the drug to the culture medium at 8 h of infection (data not shown). Therefore, we chose the concentration of 65 μ M CDV for all subsequent experiments.

CDV effects on viral DNA replication, genome resolution, and protein synthesis. CDV is reported to inhibit the activity of VACV DNA polymerase, and thus, it is suggested that viral DNA replication is probably the main target of the drug (34). Therefore, we examined viral DNA accumulation throughout infection in the presence of CDV. Cells were infected with CTGV, treated with 65 μ M CDV, and harvested at the indicated times postinfection for slot blot hybridization. Figure 2A shows that viral DNA accumulated with the progression of infection in untreated infected cells. Interestingly, the presence of CDV during infection inhibited viral DNA synthesis slightly, reaching a reduction of nearly 30% at 24 h postinfection (Fig. 2A), although virus yields were inhibited $>90\%$ under identical experimental conditions (Fig. 1). Similar results were obtained when we analyzed the time course of accumulation of VACV-IOC DNA during infection in the presence of CDV (data not shown). Increasing concentrations of CDV did not alter these results (data not shown).

Genome resolution is a crucial step for the completion of the VACV replicative cycle. As a result of the DNA replication process, concatemeric genome structures are formed and resolved into monomeric molecules with the aid of the virus resolvase A22. The single genome unit is then packaged into the virus particle (20, 36, 40). To investigate whether CDV treatment inhibited virus telomere resolution, total DNA was extracted from infected BSC-40 cells and analyzed by Southern blot hybridization. Figure 2B shows that in untreated cells, CTGV and VACV-IOC genomes produced distinct restriction patterns, generating bands of approximately 3.1 kb and 2.1 kb, respectively, representative of resolved telomeres. These patterns differed from the 1.3-kb fragment that has been reported for VACV-WR as representative of the monomeric genome (36). The presence of 65 μ M CDV during infection did not

FIG. 2. Effects of CDV on viral DNA replication and genome resolution. Cells were infected with CTGV (A and B) or VACV-IOC (B) at 5 PFU per cell and were treated with 65 μ M CDV. (A) At the indicated times postinfection, extracts of CTGV-infected cells were processed for slot blot hybridization to a 32 P-labeled HindIII D fragment. Numbers resulting from the densitometric analysis (arbitrary units) are averages for six independent assays performed in triplicate. (B) At 18 h postinfection, total DNA of CTGV- or VACV-IOC infected cells was extracted, digested with BstEII, and analyzed by Southern blotting using a 32 P-labeled telomeric region probe of VACV-WR DNA. Arrows indicate nonresolved concatemeric fragments. Arrowheads indicate resolved genome fragments. M, mock-infected cells; CT, infected cells not treated with CDV; CDV, infected cells treated with CDV; IBT, infected cells treated with IBT.

inhibit genome resolution, in contrast with treatment with 45 μ M IBT, which blocks VACV late gene expression and impairs telomere resolution (13, 36). In IBT-treated cells, but not in CDV-treated cells, unresolved concatemers accumulated, as indicated by the detection of fragments of approximately 6.2 kb (CTGV) and 4.2 kb (VACV-IOC).

We then decided to investigate in detail the progression of the postreplicative stage of virus infection. Analysis of viral protein synthesis by SDS-PAGE is a suitable approach to evaluating the stages of the virus cycle. Infected cells were treated with 65 μ M CDV and were pulse-labeled with [35 S]methionine at the indicated times postinfection. Samples were then analyzed by SDS-PAGE. In untreated cells infected with CTGV (Fig. 3A) or VACV-IOC (data not shown), viral proteins were initially detected at 4 h, and the synthesis of late polypeptides progressed at approximately the same rate until 24 h postinfection. In the presence of CDV, similar protein profiles were detected throughout infection, revealing that the synthesis of viral polypeptides was not affected by the drug.

These results were validated by four independent assays for CTGV, VACV-IOC, and the prototypical strain VACV-WR. Experiments were carried out in triplicate in the presence of 65 μ M CDV, and cells were harvested simultaneously at 24 h postinfection for virus yield determination, DNA slot blot hybridization assay, and Western blot analysis of viral late proteins (data not shown). The results confirmed the data presented so far and indicate that although VACV DNA replication was moderately inhibited in the presence of 65 μ M CDV, late gene expression progressed successfully and could not account for approximately 90% inhibition of virus titers. Therefore, subsequent stages of the VACV cycle should be affected by CDV.

CDV inhibits the proteolytic processing of major core proteins. The proteolytic cleavage of some VACV core proteins is required for successful virus morphogenesis, and its failure arrests the transition from IV to MV (8, 37, 38, 51). To eval-

uate the effect of CDV on the processing of the precursors p4a and p4b, we performed pulse-chase assays in cells infected with CTGV (Fig. 3B) and VACV-IOC (data not shown) in the absence or presence of 65 μ M CDV. At 13 h postinfection, the cells were pulse-labeled with [35 S]methionine for 45 min (pulse); then they were chased for an additional 3 or 5 h. In control infections, cleavage of p4a and p4b into the mature forms 4a and 4b was clearly detected after 3 h of chase. However, when virus infection proceeded in the presence of CDV, the precursors still accumulated during the chase period, and the detection of the mature proteolytic products 4a and 4b was severely reduced (Fig. 3B). Longer chase periods generated similar results (data not shown). The proteolytic processing of the VACV-WR major core proteins was also inhibited by CDV treatment, as detected by the inhibition of p4a and p4b cleavage and diminished accumulation of the mature forms 4a and 4b, relative to cleavage and accumulation in untreated cells, in Western blot assays (data not shown). As a negative control (Fig. 3C), pulse-chase assays were carried out in CTGV-infected cells treated with rifampin, which inhibits VACV proteolytic processing (38, 51). Although the effect of CDV was not as dramatic as the action of rifampin, the pulse-chase assay clearly indicated that CDV affected the proteolytic processing of core protein precursors and that virus assembly could be compromised.

CTGV and VACV-IOC morphogenesis is arrested in the presence of CDV. The progression of the morphogenetic process may be monitored by transmission electron microscopy. Therefore, to gain insight into the generation of CTGV and VACV-IOC particles, infected cells were treated with 65 μ M CDV for 20 h and processed for transmission electron microscopic analysis. In the absence of CDV, we observed clusters of MV and IV in the cytoplasm of cells infected with CTGV (Fig. 4A) or VACV-IOC (Fig. 4B). When CDV was present during infection, we detected a reduced number of MV and a large number of immature forms (Fig. 4C and D) compared with

FIG. 3. Effects of CDV on viral protein synthesis and the proteolytic processing of p4a and p4b. BSC-40 cells were infected with CTGV at an MOI of 5 in the absence (control) or presence of 65 μ M CDV. (A) At the indicated times, cells were pulse-labeled with [35 S]methionine for 30 min, and samples were analyzed by 10% SDS-PAGE, followed by autoradiography. Arrowheads indicate viral late proteins. (B) At 13 h postinfection, cells were pulse-labeled with [35 S]methionine and either harvested immediately (P) or chased for 3 or 5 h. Samples were analyzed by SDS-PAGE as described for panel A. (C) Pulse-chase assay of cells treated with 100 μ g/ml rifampin (Rif). M, mock-infected (with [+]/without [-] CDV). Molecular size markers (in kilodaltons) are indicated on the right.

those in untreated cells. Analysis of several ultrathin sections revealed that the immature particles produced in CDV-treated cells were represented by regular-shaped IV, IV with nucleoids (IVN) (Fig. 5A), several dense spherical particles, and many aberrant forms (Fig. 5B). Most of these abnormal particles were IV with an irregular contour, double-layered membranes, or anomalous distribution of the internal electron-dense material (Fig. 5B to F). We also observed several structures resembling DNA crystalloids with an internal striated aspect (Fig. 5G and H). In some sections, we detected abnormal circular membrane elements distinct from the typical crescents associated with the periphery of the viroosomes (Fig. 5I).

Quantitative analysis of approximately 30 random fields in three independent assays revealed that in the presence of CDV, the numbers of MV were reduced nearly 2.4-fold and 3.5-fold in CTGV- and VACV-IOC-infected cells, respectively, from those in untreated cells (Table 1). In parallel, the number

of total immature particles increased approximately 3.2-fold in CDV-treated cells infected with either virus. Although IVN were more abundant in the presence than in the absence of CDV, the ratio of IVN to IV decreased in CDV-treated cells. In untreated cells, we detected nearly 1.55 IVN per 10 IV for both viruses. However, in the presence of CDV, the ratio was approximately 0.9 IVN per 10 IV.

Analysis of CTGV virions isolated from CDV-treated cells.

To further investigate the effect of CDV on the formation of virus particles, we purified virions from CTGV-infected cells that were either left untreated or treated with CDV for 24 h, and we examined the particles for morphology, yield, protein, and DNA content. To analyze particle morphology, purified viruses were processed for transmission electron microscopy. Figure 6 shows that control virus comprised mainly mature forms (Fig. 6A), whereas virus purified from CDV-treated cells (henceforth referred to as CDV-treated virus) consisted of a few mature forms and several immature particles (Fig. 6B), strengthening the results shown in Fig. 4 and 5. Abnormal IV with an irregular contour, as well as dense particles, represented most of the immature forms detected in distinct assays (Fig. 6B). As indicated in Fig. 6, CTGV-infected cells treated with 65 μ M CDV produced 86% fewer infectious particles than untreated cells, leading to a 9.2-fold reduction in the ratio of infectious particles to total virus particles.

Samples of control and CDV-treated viruses were equalized by protein concentration and resolved by SDS-PAGE, followed by Coomassie-blue staining (Fig. 7A). Despite the differences observed in virus forms in the presence of CDV, the protein profiles were quite similar, except that the accumulation of mature forms of the core proteins 4a and 4b appeared less intense in CDV-treated virus than in control virus (Fig. 7A). To examine the presence of specific membrane and core proteins, we analyzed 16 viral proteins by Western blotting. The results shown in Fig. 7B revealed no major differences between control and CDV-treated viruses, with the notable exception of the major core proteins p4a and p4b, which showed larger amounts of the precursor forms in the CDV-treated virus than in the control virus. This effect was particularly evident for p4b.

The encapsidation of viral DNA in control and CDV-treated virus particles was quantitated by slot blot hybridization. Equal amounts of protein of purified viruses were applied to duplicate membranes. One replica was hybridized to a radiolabeled VACV HindIII D fragment DNA probe, while the other was processed for Western blot analysis to detect VACV structural proteins, used as a loading control. As shown in Fig. 7C, the DNA content of CDV-treated virus was clearly reduced from that of control particles, revealing 64.4% inhibition as measured by densitometric analysis of distinct assays. Similarly, assays performed with purified VACV-IOC and the prototypic strain VACV-WR indicated that DNA encapsidation in the CDV-treated virus was inhibited by 65.3% and 67.7%, respectively, compared with control particles (data not shown). Together, our data demonstrate that genome encapsidation and the morphogenesis process were inhibited by CDV during virus infection. In addition, the results suggest that the effect of the drug represents a general mechanism of action not restricted to the Brazilian VACV strains.

FIG. 4. Analysis of CTGV and VACV-IOC morphogenesis in the absence or presence of CDV. Cells were infected at 5 PFU per cell in the absence (A and B) or presence (C and D) of 65 μ M CDV. At 20 h postinfection, cells were processed for transmission electron microscopy. (A and C) Representative infection with CTGV; (B and D) representative infection with VACV-IOC. Vi, virosomes; Nu, nucleus. Bars, 2 μ m.

CDV does not directly impair virus morphogenesis. The inhibitory effect of CDV on two important processes of virus assembly could indicate a direct action of the drug on VACV morphogenesis, or it could reflect an indirect result of a primary effect of CDV on a previous stage of the virus replication cycle. To address this question, we evaluated the effect of CDV on VACV progeny production after release from morphogenesis arrest by rifampin treatment. In the presence of rifampin, cells infected with VACV accumulate virosomes but do not produce immature particles, due to a defect in the formation of the D13-containing external lattice of IV (58). After rifampin release, virus assembly is fully accomplished within 3 h (50). As outlined in Fig. 8A, infection proceeded in the presence of rifampin for 12 h, at which time cells were incubated with rifampin-free medium either without drugs or in the presence of 65 μ M CDV or 40 μ g/ml cytosine arabinoside (AraC; an inhibitor of DNA replication), or both. At the indicated time points after rifampin release, cells were harvested for virus titration. As a control, cells received fresh medium containing rifampin for similar periods. The results in Fig. 8B show that

the addition of CDV after rifampin removal did not impair the resumption of virus yield, clearly suggesting that CDV does not directly affect virus morphogenesis. Similar results were obtained when AraC or a combination of CDV and AraC was added to the cells.

As an alternative experimental strategy, we performed shift-down assays with the temperature-sensitive mutant of VACV *Cts53*, which has been characterized previously as defective in late protein synthesis but competent in DNA replication (24). Cells were infected with *Cts53* under nonpermissive conditions (40°C) for 12 h and were then shifted to permissive conditions (31.5°C) in the presence of CDV or AraC, or both. At 7, 12, or 15 h postshift, cells were harvested for virus yield determination. As in the rifampin release assay, CDV did not inhibit the recovery of progeny production at the permissive temperature compared with that of cells shifted down in the absence of drugs (data not shown).

Together, these data strongly suggest that the inhibitory effects of CDV on VACV genome encapsidation and proteolytic processing were not caused by a direct action of the drug on virus

FIG. 5. Detailed analysis of CTGV and VACV-IOC morphogenesis in the presence of CDV. Representative images of CTGV- and VACV-IOC infected cells treated with 65 μ M CDV are shown. (A, D, E, and G) CTGV-infected cells; (B, C, F, H, and I) VACV-IOC-infected cells. (A) Regular-shaped IV and IVN. (B) Cluster of aberrant immature forms: dense spherical particles (De) and IV with irregular contours (black arrows), some with the inner electron-dense material detached from the viral membrane (black arrowheads). (C) Higher magnification of aberrant particles. (D) Abnormal heart-shaped and incompletely formed particles (open arrows). (E and F) Aberrant spherical particles with partial or complete double-layer membranes (open arrowheads). (G and H) Amorphous inclusions of a multilayered structure resembling crystalloids were frequently observed in infected cells treated with CDV (white arrows). (I) Fragments of irregular membranes vesiculated around small virosomes (white arrowheads). Bars, 200 nm.

morphogenesis. Instead, they resulted from a primary effect of CDV at an earlier stage of infection, probably DNA synthesis, leading to major defective phenotypes at the later stage of virus assembly. This is particularly interesting in light of the fact that

CDV only slightly inhibited VACV DNA replication and did not affect genome resolution or late gene expression.

Analysis of VACV DNA structure by AFM. To gain insight into the VACV genome produced in the absence or presence

TABLE 1. Quantitation of CTGV and VACV-IOC particles resolved by electron microscopy

Strain and treatment	No. (%) of particles ^a					Total
	Mature	Immature			Dense	
		Total immature ^b	IV	IVN		
CTGV						
Control	2,263 (79.24)	593 (20.76)	486 (81.96)	93 (15.68)	14 (2.36)	2,856
CDV	790 (32.78)	1,620 (67.22)	1,305 (80.56)	145 (8.95)	170 (10.49)	2,410
VACV-IOC						
Control	1,976 (73.87)	699 (26.13)	573 (81.97)	105 (15.02)	21 (3.00)	2,675
CDV	694 (20.62)	2,671 (79.38)	2,015 (75.44)	253 (9.47)	403 (15.09)	3,365

^a Approximately 30 random fields in three independent assays were analyzed, and the particles were counted. For mature particles and total immature particles, numbers in parentheses are percentages of total particles. For IV, IVN, and dense particles, numbers in parentheses are percentages of total immature particles.
^b Total immature particles correspond to the sum of IV, IVN, and dense particles. The total number of immature particles is significantly different ($P < 0.05$) from the total number of mature particles.

of CDV, we evaluated the structure of viral DNA by AFM, which has been used extensively to elucidate conformational changes of nucleic acids (32). Because CDV inhibited DNA encapsidation into virus particles, we chose to initiate our analysis by investigating total viral DNA generated during infection and isolated from the cytoplasm of infected cells. Cytoplasmic DNA sedimented from postnuclear supernatants was composed of virus core DNA as well as virosome-derived DNA, as detected by analysis of cytoplasmic DNA isolated from rifampin-treated infected cells (D. M. Jesus and C. R. Damaso, unpublished data). Therefore, cytoplasmic DNA from untreated or CDV-treated cells infected with VACV-WR for 24 h was gently isolated and prepared for AFM imaging. All images were acquired using Mg^{2+} to adsorb the DNA onto the mica substrate (9).

Figure 9 shows that cytoplasmic DNA from untreated infected cells formed highly organized DNA networks (Fig. 9A), which were observed in all images obtained from distinct DNA preparations (detail in Fig. 9B). In addition, stretched segments of DNA were noted (Fig. 9A). Network structures have been reported frequently in studies with different types of DNA (small or long, linear or circular molecules), and DNA concentration and size are important factors. Networks are formed during sample deposition onto the substrate under several experimental conditions, particularly with the use of bivalent cations, which act as positively charged bridges between the negatively charged mica substrate and negatively charged DNA strands as well as between DNA strands. Therefore, DNA networks do not represent an array of concatemers but consist of an arrangement of layers of tangled DNA strands

FIG. 6. Analysis of purified CTGV particles isolated from cells treated with CDV. CTGV particles were purified from cells infected for 24 h in the absence (A) or presence (B) of 65 μ M CDV. Pelleted virions were processed for transmission electron microscopy. (A) Typical brick-shaped mature particles purified from control cells. (Inset) Higher magnification of a mature particle. (B) Few brick-shaped forms and aberrant spherical particles were present in virus samples purified from CDV-treated cells. Bars, 200 nm. An analysis of total particle production and virus yield is tabulated below the micrographs.

FIG. 7. Protein and DNA contents of purified CTGV particles isolated from cells treated with CDV. (A) Protein samples (35 μ g) were analyzed by 12% SDS-PAGE and Coomassie blue staining. (B) Protein samples (10 μ g) of purified CTGV were analyzed by Western blotting using antibodies raised against the indicated proteins. Gene functions are given in parentheses. (C) Samples of purified CTGV were applied to nylon membranes and hybridized to a 32 P-labeled HindIII D fragment (left) or applied to a nitrocellulose membrane and immunoblotted using an antiserum against VACV-WR structural proteins (right). Representative images are shown. CT, purified virus isolated from untreated cells; CDV, purified virus isolated from cells treated with 65 μ M CDV.

overlapping each other when placed on proper substrates (31, 41, 54, 56). In our assays, sample dilution decreased the formation of networks (data not shown). However, it also reduced the probability of finding a significant number of single DNA molecules and then observing representative DNA structures.

Similar network structures, along with stretched segments of DNA, were also observed in images of cytoplasmic DNA from infected cells treated with CDV (Fig. 9C). However, besides the formation of DNA networks, we also observed DNA aggregates with disordered arrangements and increased entanglements, showing intense and disorganized strand overlapping and interwinding (Fig. 9D to H). Aggregated DNA structures were observed only in cytoplasmic DNA from infected cells treated with CDV and were repeatedly detected in independent DNA preparations. DNA strands forming organized networks in untreated and CDV-treated samples presented similar heights and widths, ranging from 0.6 nm to 0.8 nm and 20 nm to 30 nm, respectively. However, aggregated structures of viral DNA from CDV-treated cells revealed increased heights, ranging from 4 to 5 nm (Fig. 9D and G) or from 1.5 nm to 2 nm and sometimes 6 nm (Fig. 9E, F, and H), suggesting intense strand overlapping. These measurements indicate structural

changes in the DNA associated with unusual aggregated forms instead of the classical network formation.

The cell-fractionation procedure used to isolate VACV DNA from the cytoplasm probably leads to cosedimentation of mitochondria as well. Thus, as controls, we analyzed cytoplasmic DNA obtained from uninfected cells that were either left untreated or treated with CDV. We rarely detected DNA in either sample after several imaging attempts (Fig. 9I and J), supporting the conclusion that the DNA images acquired from samples of infected cells corresponded to viral DNA. To evaluate whether the DNA aggregates were encapsidated into virus particles during morphogenesis, we analyzed DNA extracted from purified virions isolated from cells that were either left untreated or treated with CDV. Figures 9K and L show that the two samples of DNA extracted from purified virus particles had similar and unaltered structures of characteristic DNA networks. Aberrant DNA aggregates were not detected in distinct assays. Therefore, our data indicate that viral DNA synthesized in the presence of CDV had an altered structure, forming aggregations that were not encapsidated into

FIG. 8. Effect of CDV on virus yield after release from rifampin (RIF)-induced arrest of morphogenesis. Cells were infected with VACV-WR at 5 PFU per cell in the presence of 100 μ g/ml RIF. At 12 h postinfection, cells were washed extensively, and the medium was replaced with a RIF-free medium, either without drugs or containing either 65 μ M CDV, 40 μ g/ml AraC, or both. At 0, 9, 12, or 15 h post-RIF release, cells were harvested for virus titration by a plaque assay. As a control, cells were incubated with a RIF-containing medium at time zero after RIF release. (A) Diagram of the assay. (B) Results are expressed as means from two assays.

virus particles but remained in the cytoplasm of infected cells treated with CDV.

DISCUSSION

CDV is one of the most promising and effective anti-orthopoxvirus drugs (15). Several *in vitro* studies with purified DNA polymerase of human cytomegalovirus and VACV (33, 34, 55), and *in vivo* studies with CDV-resistant viruses (2, 5, 28, 45) have pointed to virus DNA replication as the main target for CDV action. However, no detailed analysis of the effect of CDV on the VACV replicative cycle has ever been reported. In this study, we evaluated the VACV cycle in BSC-40 cells infected at an MOI of 5 in the presence of CDV. Interestingly, although we detected 1-log-unit inhibition of virus titers, DNA replication was reduced by only 30%, and no significant effect on genome resolution or postreplicative protein synthesis was observed. However, a virus MOI as low as 0.001 leads to DNA synthesis inhibition levels of >90% (26). These contrasting effects dependent on the virus MOI may possibly be explained in light of previous data from Magee and coworkers (33) showing that CDV, a non-chain-terminating analogue, inhibited VACV DNA polymerase more severely when incorporated

into the template strand, mostly affecting secondary rounds of DNA synthesis. Thus, the outcome of this effect on viral DNA replication is expected to be more pronounced when the virus MOI is very low and few copies of the input genome are available for the onset of DNA synthesis.

These results observed with CDV do not seem to represent general effects of inhibitors of DNA synthesis. Adenosine at approximately 30 μ M inhibits VACV DNA accumulation by 30%, similarly to CDV, but reduces virus yield by only 20% (29, 30). Nonetheless, other inhibitors of DNA synthesis, especially other acyclic nucleosides, should be further investigated to address this issue in detail.

Therefore, our data clearly indicate that, under the present conditions of infection, the effects of CDV extended beyond simply an impairment of VACV DNA synthesis. In fact, we detected the inhibition of genome encapsidation and proteolytic processing of the precursors p4a and p4b, ultimately leading to the impairment of virus morphogenesis. The nonstringent conditions of infection used in this work (90% yield inhibition) allowed a small percentage of the virus population to progress through the replicative cycle. This has certainly contributed to the detection of all stages of virus morphogenesis in CDV-treated cells. However, the results of morphometric analysis were statistically supported and indicated that the number of MV decreased significantly when CDV was present, concurrent with an increase in the number of immature forms of CTGV. Abnormal IV, including dense spherical particles, were consistently observed, resembling aberrant forms described during an infection with VACV mutants defective in the expression of I7, D6, A32, A4, A10, J1, I6, G7, and A13. Some of these mutants are deficient in the proper formation of IV (3, 6, 7, 17, 22, 23, 25, 27, 47, 50, 53). Importantly, DNA crystalloids were also detected in cells infected in the presence of CDV, and the ratio of IVN to total IV decreased. Correspondingly, the DNA content of purified VACV particles isolated from CDV-treated cells was approximately 35% of that detected in virions purified from untreated cells.

Our data indicate that essential processes of VACV morphogenesis were affected in CDV-treated cells in a fashion that could be correlated with the overall inhibition of VACV replication. However, we also show that CDV did not directly impair VACV morphogenesis when added after virus DNA had accumulated to sufficient levels. Therefore, our results suggest that the presence of CDV in the DNA strands probably led to the later defects observed in VACV morphogenesis.

The encapsidation of virus DNA is an essential step prior to the maturation of IV into MV (reviewed in reference 8). The process is not well understood, and few viral proteins have been reported to have a role in genome packaging. When A32 expression was repressed, or in I6-conditional lethal mutants, similar phenotypes were observed (6, 22). Abnormal IV possessing a distorted membrane contour and an asymmetrical distribution of the internal electron-dense material were reported. DNA crystalloids and numerous dense spherical particles were also detected, observations similar to our findings for CDV-treated cells. Virus particles had normal protein content and failed to encapsidate DNA. In addition, late protein synthesis occurred at normal levels, genome resolution was not impaired, and the proteolytic processing of major virus core proteins was inhibited (6, 22). These features were also ob-

FIG. 9. Analysis of viral DNA conformation by AFM. (A to H) DNA was extracted from the cytoplasm of cells infected with VACV-WR in the absence (A and B) or presence (C to H) of 65 μM CDV. (G and H) High-magnification images of panels D and E, respectively, pointing out increased entanglements and disorganized strand overlapping and intercrossing. (I and J) Images of cytoplasmic DNA from mock-infected cells either left untreated (I) or treated with 65 μM CDV (J). (K and L) DNA was extracted from purified VACV-WR particles isolated from cells that were either left untreated (K) or treated with 65 μM CDV (L). Stars indicate networks of viral DNA that formed on the mica surface. White arrowheads point out stretched segments of DNA. Black arrowheads indicate some of the residual contaminants of the extraction procedure, which are commonly detected during image scanning. Arrows indicate DNA aggregates and distortions observed in cytoplasmic DNA from infected cells treated with CDV. All DNA samples (5 ng/ μl) were incubated with 12.5 mM Mg^{2+} , deposited onto a mica surface, and imaged in air. Representative amplitude images acquired in dynamic mode are shown. Bars, 0.5 μm .

served in the presence of CDV. A13 is a membrane protein also apparently involved in DNA encapsidation. Repression of A13 expression led to the appearance of DNA crystalloids and abnormal circular membrane structures surrounding the viro-somes (50), resembling the structures shown in the present work.

It is tempting to hypothesize that CDV may act by affecting DNA structure and thus preventing genome packaging. Little is known about the relevance of DNA structure for the accurate encapsidation of viral genomes. Studies of the DNA packaging process are mostly reported for double-stranded DNA bacteriophages, herpes simplex virus type 1, and adenovirus, focusing mainly on *cis*-acting sequences and viral packaging proteins (21, 43, 57). However, it has been shown that the HSV-1 *pac1* sequence adopts novel DNA structures essential for its recognition by the packaging protein U_L28 (1). A model for VACV DNA encapsidation in which the monomeric genome bound to I6 protein would be encapsidated into A32-containing IV was proposed by Grubisha and Traktman (22). I6 is a telomere-binding protein that recognizes extrahelical bases at the hairpin termini of the VACV genome. A suitable DNA structure is essential for the formation of a complex between telomere-binding proteins and the hairpin termini (16). Therefore, it is plausible to suppose that alterations in DNA structure induced by the incorporation of nucleoside analogues could compromise the interaction of DNA with DNA-binding proteins and the subsequent encapsidation of the viral genome.

Alterations in DNA structure caused by the incorporation of CDV have not been reported previously. Nevertheless, it has been shown that the absence of the deoxyribose sugar ring in another acyclic nucleoside phosphonate, tenofovir, makes it more flexible than regular nucleoside analogues, and its incorporation at the end of primers induces conformational changes in tenofovir and, consequently, in primer structure (49).

AFM has proved to be a powerful tool for the study of morphological changes in DNA structure, such as the formation of knots, kinks, supercoils, and bends (32). Here, our AFM images revealed crucial differences in the structure of VACV DNA isolated from the cytoplasm of untreated or CDV-treated cells. In untreated cells, cytoplasmic viral DNA formed organized network structures of tangled strands, which were likely to occur due to the long VACV genome size, approximately 68 μ m (8). The formation of DNA networks and the mesh size in AFM have been shown to be dependent on the concentration/type of bivalent cations, the type of solid substrate, the concentration of DNA, and the size of DNA. Under similar conditions of sample preparation, the formation of regular networks of oligonucleotides requires around 125 μ g/ml of DNA, whereas for plasmids and linear lambda DNA (48.5 kb), approximately 70 ng/ml and 20 ng/ml have been used, respectively (9, 31, 41, 54, 56). Therefore, based on its size, the use of VACV DNA at 5 ng/ml would be expected to form networks. It is worth noting that the formation of networks does not imply that virus DNA molecules are concatamers. In fact, the VACV genome was successfully resolved into monomers under our experimental conditions, as shown by Southern blot assays.

DNA networks were also observed in viral DNA obtained from the cytoplasm of infected cells treated with CDV. Nev-

ertheless, these samples also contained DNA aggregates with increased, disorganized entanglements and strand interwinding. These aggregates remained unpackaged in the cytoplasm of infected cells, since the encapsidated genome of viral particles purified from CDV-treated cells formed a normal DNA network indistinguishable from that of control DNA under our assay conditions. The DNA aggregations represent important conformational distortions in DNA structure, but we cannot exclude the occurrence of other minor, subtle conformational changes in DNA, not detected by our experimental approach. However provocative, these initial data do not permit a causative association between the alterations of DNA structure and the inhibition of viral DNA encapsidation during CDV treatment. In addition, we cannot exclude the possibility that the slight inhibition of DNA replication by CDV could lead to minor defects in late protein synthesis, below the level of detection by pulse-labeling assays, that could affect virus morphogenesis in an important way. However, the encouraging AFM evidence supports the previous hypothesis, which is worth further investigation.

As mentioned above, previous studies have pointed to the process of DNA replication as the target for the inhibitory activity of CDV. On the other hand, our data revealed that CDV only slightly affected DNA replication during infection but that instead, virus morphogenesis was significantly impaired. These apparently contradictory findings regarding the mechanism of action of CDV could be reconciled by our results showing an important inhibition of virus DNA encapsidation. These effects, however, resulted from a primary action of CDV on DNA synthesis. Therefore, in sum, we suggest that the incorporation of CDV into DNA molecules by the viral DNA polymerase would be the first step toward the downstream effect of the drug on virus morphogenesis, possibly related to alterations of DNA structure and subsequent impairment of DNA encapsidation. Further analyses are in progress to investigate this model in detail and to evaluate whether the occurrence of aberrant DNA aggregations is directly involved in the inhibition of DNA encapsidation during CDV treatment.

ACKNOWLEDGMENTS

We thank Gilead Sciences, Inc. (Foster City, CA), for providing CDV and R. Condit, E. Niles, S. Shuman, P. Traktman, D. Hraby, and M. Merchlinsky for the mutant virus, antisera, and recombinant DNA clone. We also thank Richard Condit and Richard Moyer for invaluable comments and critical review of the manuscript, Januário Cabral and Narcisa Cunha-e-Silva for helpful discussions, Lucio Caldas for help with electron microscopy, Thaís Souto-Padron for the use of the Morgagni 268 electron microscope, and Ademilson Bizerra for technical assistance.

This work was partly supported by grants from CNPq, FAPERJ, and IFS to C.R.D. D.M.J. was a recipient of a fellowship from CNPq.

REFERENCES

- Adelman, K., B. Salmon, and J. D. Baines. 2001. Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. *Proc. Natl. Acad. Sci. USA* **98**:3086–3091.
- Andrei, G., D. B. Gammon, P. Fiten, E. De Clercq, G. Opdenakker, R. Snoeck, and D. H. Evans. 2006. Cidofovir resistance in vaccinia virus is linked to diminished virulence in mice. *J. Virol.* **80**:9391–9401.
- Ansarah-Sobrinho, C., and B. Moss. 2004. Role of the I7 protein in proteolytic processing of vaccinia virus membrane and core components. *J. Virol.* **78**:6335–6343.

4. Baker, R. O., M. Bray, and J. W. Huggins. 2003. Potential antiviral therapeutics for smallpox, monkeypox and other orthopoxvirus infections. *Antivir. Res.* **57**:13–23.
5. Becker, M. N., M. Obraztsova, E. R. Kern, D. C. Quenelle, K. A. Keith, M. N. Prichard, M. Luo, and R. W. Moyer. 2008. Isolation and characterization of cidofovir resistant vaccinia viruses. *Viol. J.* **5**:58.
6. Cassetti, M. C., M. Merchlinsky, E. J. Wolffe, A. S. Weisberg, and B. Moss. 1998. DNA packaging mutant: repression of the vaccinia virus A32 gene results in noninfectious, DNA-deficient, spherical, enveloped particles. *J. Virol.* **72**:5769–5780.
7. Chiu, W. L., and W. Chang. 2002. Vaccinia virus J1R protein: a viral membrane protein that is essential for virion morphogenesis. *J. Virol.* **76**:9575–9587.
8. Condit, R. C., N. Moussatché, and P. Traktman. 2006. In a nutshell: structure and assembly of the vaccinia virion. *Adv. Virus Res.* **66**:31–124.
9. Costa, L. T., M. Kerkmann, G. Hartmann, S. Endres, P. M. Bisch, W. M. Heckl, and S. Thalhammer. 2004. Structural studies of oligonucleotides containing G-quadruplex motifs using AFM. *Biochem. Biophys. Res. Commun.* **313**:1065–1072.
10. Damaso, C. R., J. J. Esposito, R. C. Condit, and N. Moussatché. 2000. An emergent poxvirus from human and cattle in Rio de Janeiro state: Cantagalo virus may derive from Brazilian smallpox vaccine. *Virology* **277**:439–449.
11. Damaso, C. R., and N. Moussatché. 1992. Detection of RNA-protein complex in vaccinia virus core in vitro transcription system. *J. Gen. Virol.* **73**:1243–1249.
12. Damaso, C. R., and N. Moussatché. 1998. Inhibition of vaccinia virus replication by cyclosporin A analogues correlates with their affinity for cellular cyclophilins. *J. Gen. Virol.* **79**:339–346.
13. Damaso, C. R., M. F. Oliveira, S. M. Massarani, and N. Moussatché. 2002. Azathioprine inhibits vaccinia virus replication in both BSC-40 and RAG cell lines acting on different stages of virus cycle. *Virology* **300**:79–91.
14. Damaso, C. R., S. A. Reis, D. M. Jesus, P. S. Lima, and N. Moussatché. 2007. A PCR-based assay for detection of emerging vaccinia-like viruses isolated in Brazil. *Diagn. Microbiol. Infect. Dis.* **57**:39–46.
15. De Clercq, E. 2007. The acyclic nucleoside phosphonates from inception to clinical use: historical perspective. *Antivir. Res.* **75**:1–13.
16. DeMasi, J., S. Du, D. Lennon, and P. Traktman. 2001. Vaccinia virus telomeres: interaction with the viral I1, I6, and K4 proteins. *J. Virol.* **75**:10090–10105.
17. Ericsson, M., S. Cudmore, S. Shuman, R. C. Condit, G. Griffiths, and J. K. Locker. 1995. Characterization of *ts16*, a temperature-sensitive mutant of vaccinia virus. *J. Virol.* **69**:7072–7086.
18. Esposito, J. J., R. C. Condit, and J. F. Obijeski. 1981. The preparation of orthopoxvirus DNA. *J. Virol. Methods* **2**:175–179.
19. Fenner, F., D. A. Henderson, I. Arita, J. Jezek, and I. D. Ladnyi. 1988. Smallpox and its eradication. World Health Organization, Geneva, Switzerland.
20. Garcia, A. D., and B. Moss. 2001. Repression of vaccinia virus Holliday junctions resolvase inhibits processing of viral DNA into unit-length genomes. *J. Virol.* **75**:6460–6471.
21. Grimes, S., P. J. Jardine, and D. Anderson. 2002. Bacteriophage ϕ 29 DNA packaging. *Adv. Virus Res.* **58**:255–294.
22. Grubisha, O., and P. Traktman. 2003. Genetic analysis of the vaccinia virus I6 telomere-binding protein uncovers a key role in genome encapsidation. *J. Virol.* **77**:10929–10942.
23. Heljasvaara, R., D. Rodríguez, C. Risco, J. L. Carrascosa, M. Esteban, and J. R. Rodríguez. 2001. The major core protein (A10L gene) of vaccinia virus is essential for correct assembly of viral DNA into the nucleoprotein complex to form immature viral particles. *J. Virol.* **75**:5778–5795.
24. Hooda-Dhingra, U., C. L. Thompson, and R. C. Condit. 1989. Detailed phenotypic characterization of five temperature-sensitive mutants in the 22- and 147-kilodalton subunits of vaccinia virus DNA-dependent RNA polymerase. *J. Virol.* **63**:714–729.
25. Hu, X., L. J. Carroll, E. F. Wolffe, and B. Moss. 1996. De novo synthesis of the early transcription factor 70-kilodalton subunit is required for morphogenesis of vaccinia virions. *J. Virol.* **70**:7669–7677.
26. Jesus, D. M., N. Moussatché, and C. R. Damaso. 2009. In vitro activity of cidofovir against the emerging Cantagalo virus and the smallpox vaccine strain IOC. *Int. J. Antimicrob. Agents* **33**:75–79.
27. Kane, E. M., and S. Shuman. 1993. Vaccinia virus morphogenesis is blocked by a temperature-sensitive mutation in the I7 gene that encodes a virion component. *J. Virol.* **67**:2689–2698.
28. Kornbluth, R. S., D. F. Smee, R. W. Sidwell, V. Snarsky, D. H. Evans, and K. Y. Hostetler. 2006. Mutations in the E9L polymerase gene of cidofovir-resistant vaccinia virus strain WR are associated with the drug resistance phenotype. *Antimicrob. Agents Chemother.* **50**:4038–4043.
29. Leão-Ferreira, L. R. 1999. Ph.D. thesis. Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.
30. Leão-Ferreira, L. R., R. Paes-De-Carvalho, F. G. De Mello, and N. Moussatché. 2002. Inhibition of vaccinia virus replication by adenosine in BSC-40 cells: involvement of A₂ receptor-mediated PKA activation. *Arch. Virol.* **147**:1407–1423.
31. Liu, Z., Z. Li, H. Zhou, G. Wei, Y. Song, and L. Wang. 2005. Imaging DNA molecules on mica surface by atomic force microscopy in air and in liquid. *Microsc. Res. Tech.* **66**:179–185.
32. Lyubchenko, Y. L. 2004. DNA structure and dynamics: an atomic force microscopy study. *Cell Biochem. Biophys.* **41**:75–98.
33. Magee, W. C., K. A. Aldern, K. Y. Hostetler, and D. H. Evans. 2008. Cidofovir and (S)-9-[3-hydroxy-(2-phosphonomethoxy)propyl]adenine are highly effective inhibitors of vaccinia virus DNA polymerase when incorporated into the template strand. *Antimicrob. Agents Chemother.* **52**:586–597.
34. Magee, W. C., K. Y. Hostetler, and D. H. Evans. 2005. Mechanism of inhibition of vaccinia virus DNA polymerase by cidofovir diphosphate. *Antimicrob. Agents Chemother.* **49**:3153–3162.
35. Merchlinsky, M., C. F. Garon, and B. Moss. 1988. Molecular cloning and sequence of the concatemer junction from vaccinia virus replicative DNA. Viral nuclease cleavage sites in cruciform structures. *J. Mol. Biol.* **199**:399–413.
36. Merchlinsky, M., and B. Moss. 1989. Resolution of vaccinia virus DNA concatemer junctions requires late-gene expression. *J. Virol.* **63**:1595–1603.
37. Moss, B. 2007. *Poxviridae: the viruses and their replication*, p. 2905–2946. In D. M. Knipe, P. M. Howley, D. E. Griffin, R. A. Lamb, M. A. Martin, B. Roizman, and S. E. Straus (ed.), *Fields virology*, 5th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, PA.
38. Moss, B., and E. N. Rosenblum. 1973. Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. *J. Mol. Biol.* **81**:267–269.
39. Moussatché, N., C. R. Damaso, and G. McFadden. 2008. When good vaccines go wild: feral orthopoxvirus in developing countries and beyond. *J. Infect. Developing Countries* **2**:156–173.
40. Moyer, R. W., and R. L. Graves. 1981. The mechanism of cytoplasmic orthopoxvirus DNA replication. *Cell* **27**:391–401.
41. Peng, C., Y. Song, G. Wei, W. Zhang, Z. Li, and W. F. Dong. 2008. Self-assembly of lambda-DNA networks/Ag nanoparticles: hybrid architecture and active-SERS substrate. *J. Colloid Interface Sci.* **317**:183–190.
42. Rempel, R. E., M. K. Anderson, E. Evans, and P. Traktman. 1990. Temperature-sensitive vaccinia virus mutants identify a gene with an essential role in viral replication. *J. Virol.* **64**:574–583.
43. Sheaffer, A. K., W. W. Newcomb, M. Gao, D. Yu, S. K. Weller, J. C. Brown, and D. J. Tenney. 2001. Herpes simplex virus DNA cleavage and packaging proteins associate with the procapsid prior to its maturation. *J. Virol.* **75**:687–698.
44. Smee, D. F., K. W. Bailey, M. Wong, and R. Sidwell. 2001. Effects of cidofovir on the pathogenesis of a lethal vaccinia virus respiratory infection in mice. *Antivir. Res.* **52**:55–62.
45. Smee, D. F., M. K. Wandersee, K. W. Vailey, K. Y. Hostetler, A. Holý, and R. W. Sidwell. 2005. Characterization and treatment of cidofovir-resistant vaccinia (WR strain) virus infections in cell culture and in mice. *Antivir. Chem. Chemother.* **16**:203–211.
46. Stittelaar, K. J., J. Neyts, L. Naesens, G. van Amerongen, R. F. van Laveren, A. Holý, E. De Clercq, H. G. Niesters, E. Fries, C. Maas, P. G. Mulder, B. A. van der Zeijst, and A. D. Osterhaus. 2006. Antiviral treatment is more effective than smallpox vaccination upon lethal monkeypox virus infection. *Nature* **439**:745–748.
47. Szajner, P., H. Jaffe, A. S. Weisberg, and B. Moss. 2003. Vaccinia virus G7L protein interacts with the A30L protein and is required for association of viral membranes with dense viroplasm to form immature virions. *J. Virol.* **77**:3418–3429.
48. Trindade, G. S., F. G. da Fonseca, J. T. Marques, M. L. Nogueira, L. C. N. Mendes, A. S. Borges, J. R. Peiró, E. M. Pituco, C. A. Bonjardim, P. C. P. Ferreira, and E. G. Kroon. 2003. Aracatuba virus: a vaccinia-like virus associated with infection in humans and cattle. *Emerg. Infect. Dis.* **9**:155–160.
49. Tuske, S., S. G. Sarafianos, A. D. Clark, Jr., J. Ding, L. K. Naeger, K. L. White, M. D. Miller, C. S. Gibbs, P. L. Boyer, P. Clark, G. Wang, B. L. Gaffney, R. A. Jones, D. M. Jerina, S. H. Hughes, and E. Arnold. 2004. Structures of HIV-1 RT-DNA complexes before and after incorporation of the anti-AIDS drug tenofovir. *Nat. Struct. Mol. Biol.* **11**:469–474.
50. Unger, B., and P. Traktman. 2004. Vaccinia virus morphogenesis: A13 phosphoprotein is required for assembly of mature virions. *J. Virol.* **78**:8885–8901.
51. Vanslyke, J. K., S. S. Whitehead, E. M. Wilson, and D. E. Hruby. 1991. The multistep proteolytic maturation pathway utilized by vaccinia virus P4a protein: a degenerate conserved cleavage motif within core proteins. *Virology* **183**:467–478.
52. Vora, S., I. Damon, V. Fulginiti, S. G. Weber, M. Kahana, S. L. Stein, S. I. Gerber, S. Garcia-Houchins, E. Lederman, D. Hruby, L. Collins, D. Scott, K. Thompson, J. V. Barson, R. Regnery, C. Hughes, R. S. Daum, Y. Li, H. Zhao, S. Smith, Z. Braden, K. Karem, V. Olson, W. Davidson, G. Trindade, T. Bolken, R. Jordan, D. Tien, and J. Marcinak. 2008. Severe eczema vaccinatum in a household contact of a smallpox vaccinee. *Clin. Infect. Dis.* **46**:1555–1561.
53. Williams, O., E. J. Wolffe, A. S. Weisberg, and M. Merchlinsky. 1999. Vac-

- cinia virus WR gene A5L is required for morphogenesis of mature virions. *J. Virol.* **73**:4590–4599.
54. **Wu, A., Z. Li, L. Yu, H. Wang, and E. Wang.** 2001. Plasmid DNA network on a mica substrate investigated by atomic force microscopy. *Anal. Sci.* **17**:583–584.
55. **Xiong, X., J. L. Smith, and M. S. Chen.** 1997. Effect of incorporation of cidofovir into DNA by human cytomegalovirus DNA polymerase on DNA elongation. *Antimicrob. Agents Chemother.* **41**:594–599.
56. **Ye, J. Y., K. Umemura, M. Ishikawa, and R. Kuroda.** 2000. Atomic force microscopy of DNA molecules stretched by spin-coating technique. *Anal. Biochem.* **281**:21–25.
57. **Zhang, W., J. A. Low, J. B. Christensen, and M. J. Imperiale.** 2001. Role for the adenovirus IVa2 protein in packaging of viral DNA. *J. Virol.* **75**:10446–10454.
58. **Zhang, Y., and B. Moss.** 1992. Immature viral envelope formation is interrupted at the same stage by *lac* operator-mediated repression of the vaccinia virus D13L gene and by the drug rifampicin. *Virology* **187**:643–653.