Impact of 2-Bromo-5,6-Dichloro-1- β -D-Ribofuranosyl Benzimidazole Riboside and Inhibitors of DNA, RNA, and Protein Synthesis on Human Cytomegalovirus Genome Maturation

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Herpesvirus genome maturation is a complex process in which concatemeric DNA molecules are translocated into capsids and cleaved at specific sequences to produce encapsidated-unit genomes. Bacteriophage studies further suggest that important ancillary processes, such as RNA transcription and DNA synthesis, concerned with repeat duplication, recombination, branch resolution, or damage repair may also be involved with the genome maturation process. To gain insight into the biochemical activities needed for herpesvirus genome maturation, 2-bromo-5,6-dichloro-1- β -D-ribofuranosyl benzimidazole riboside (BDCRB) was used to allow the accumulation of human cytomegalovirus concatemeric DNA while the formation of new genomes was being blocked. Genome formation was restored upon BDCRB removal, and addition of various inhibitors during this time window permitted evaluation of their effects on genome maturation. Inhibitors of protein synthesis, RNA transcription, and the viral DNA polymerase only modestly reduced genome formation, demonstrating that these activities are not required for genome maturation. In contrast, drugs that inhibit both viral and host DNA polymerases potentially blocked genome formation. Radioisotope incorporation in the presence of a viral DNA polymerase inhibitor further suggested that significant host-mediated DNA synthesis occurs throughout the viral genome. These results indicate a role for host DNA polymerases in genome maturation and are consistent with a need for terminal repeat duplication, debranching, or damage repair concomitant with DNA packaging or cleavage. Similarities to previously reported effects of BDCRB on guinea pig cytomegalovirus were also noted; however, BDCRB induced low-level formation of a supergenomic species called monomer+ DNA that is unique to human cytomegalovirus. Analysis of monomer+ DNA suggested a model for its formation in which BDCRB permits limited packaging of concatemeric DNA but induces skipping of cleavage sites.

The *Herpesviridae* family of viruses include several significant human pathogens, including herpes simplex virus type 1 (HSV-1) and -2, varicella-zoster virus, Epstein-Barr virus, Kaposi's sarcoma-associated herpesvirus, and human cytomegalovirus (HCMV). These viruses have large (130- to 235-kb) double-stranded linear DNA genomes that circularize shortly after infection (22, 38, 39, 49). Viral DNA is replicated to form large concatemers of head-to-tail-linked genomes. In a process termed genome maturation, the concatemeric DNA is packaged into capsids and cleaved to produce encapsidated unit length genomes (6, 8, 27, 36, 38, 49, 52, 66).

Genome maturation is highly conserved among the herpesviruses, but little is known about the mechanisms or the machinery involved. Striking similarities to the DNA packaging mechanisms of certain large double-stranded DNA bacteriophages exist, in particular, λ , T3, T4, and T7 (14). In both bacteriophages and herpesviruses, procapsids self-assemble around a protein scaffold. A unique vertex of the procapsid contains portal proteins arranged in a ring to form the entry portal for DNA packaging. In phages, translocation of the DNA into capsids and DNA cleavage are carried out by a

two-subunit enzyme called terminase (12). A functionally equivalent herpesvirus terminase has been postulated to be similarly comprised of two subunits. In HCMV, the terminase subunits are pUL89 and pUL56. These proteins or their homologs in other herpesviruses have been shown to associate with one another (1, 7, 25, 28, 29), bind in vitro to packaging sequences (3, 13), and possess in vitro nucleolytic (13, 50) and ATPase (25, 51) activities (the latter is a requirement for DNA translocation in bacteriophages).

Additional clues to their activities have come from the study of compounds that interfere with genome maturation. These include the halogenated benzimidazoles 2-bromo-5,6-dichloro-1- β -D-ribofuranosyl benzimidazole riboside (BDCRB) and 2,5,6-trichloro-1- β -D-ribofuranosyl benzimidazole riboside (TCRB) and a structurally distinct sulfonamide, BAY38-4766. Resistance mutations to BDCRB and TCRB map to the HCMV terminase genes *UL89* and *UL56* (31, 61), while mutations within the murine cytomegalovirus (MCMV) terminase genes *M89* and *M56* confer resistance to BAY38-4766 (15). How these compounds exert their inhibitory effects and the precise roles of the presumed terminase subunits in vivo remain to be elucidated.

Although the components of the herpesvirus genome maturation machinery are beginning to unfold, the biochemical, enzymatic, and molecular aspects remain poorly understood. In phages T4 and T7, cleavage is enhanced by RNA transcrip-

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tion through the cleavage site (10, 16), and in phages T3 and T7, cleavage creates terminal redundancies (direct repeats) (20, 21, 32) by a process that appears to require the viral DNA polymerase (63). Analogous duplication of terminal repeats has been suggested for HSV-1 (19, 62) and recently confirmed by studies using guinea pig cytomegalovirus (GPCMV) (44). Resolution of branches and DNA repair may also play an important role in preparing regions of the concatemeric substrate to be suitable for packaging.

To investigate the roles of DNA, RNA, and protein synthesis in HCMV genome maturation, we took advantage of the reversible nature of BDCRB inhibition to uncouple concatemer synthesis from genome maturation (11). Infection in the presence of BDCRB allowed accumulation of viral concatemeric DNA while preventing further maturation. Subsequent removal of the BDCRB allowed the maturation of the existing concatemeric DNA into new viral genomes to proceed. The addition of inhibitors of DNA, RNA, and protein synthesis during this time window permitted evaluation of their effects specifically on genome maturation. In addition, analysis of terminal structures on HCMV replicative intermediates revealed striking similarities to effects previously observed during BDCRB treatment of GPCMV (43).

MATERIALS AND METHODS

Viral culture and drug treatments. HCMV strain AD169 was propagated in human foreskin (24) or embryonic lung (MRC-5, ATCC CCL-171) fibroblast cells using Eagle's minimal essential medium supplemented with 10% fetal calf serum. BDCRB was a gift from John Drach and Leroy Townsend; aphidicolin, cycloheximide, actinomycin D, 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole riboside (DRB), α -amanitin, and phosphonoformic acid (PFA) were purchased from SIGMA; ganciclovir (GCV) was a gift of Mark Prichard; and 9-(2-phosphonylmethoxyethyl)guanine (PMEG), 9-(2-phosphonylmethoxyethyl)adenine (PMEA), and (S)-9-(3-hydroxy-2-phosphononylmethoxypropyl)adenine (HPMPA) were gifts from Norbert Bischofberger (Gilead Sciences).

FIGE. DNA samples were prepared and analyzed by field inversion gel electrophoresis (FIGE) essentially as previously described (38). DNA species were visualized by ethidium bromide staining and UV light and then transferred to Nytran nylon membranes (Schleicher & Schuell) and hybridized to specifically detect HCMV DNA using 32 P-labeled probes as previously described (38).

Terminal restriction analysis. Restriction analyses of FIGE-separated DNAs were performed as previously described (39). Briefly, concatemeric, monomer+, and 230-kb DNA forms were cut from FIGE gels, digested in situ with HindIII or EcoRI overnight, extracted from the agarose using the Qiaex agarose extraction kit (QIAGEN), and digested again with HindIII and EcoRI. Restriction fragments were separated on 0.6% agarose gels, transferred to Nytran nylon membranes (Schleicher & Schuell), and hybridized as described previously (38) using 32 P-labeled probes derived from pON227 to detect long-arm-terminal fragments, pON2333 to detect short-arm-terminal fragments, or pON226 to detect a sequence-containing fragments (pON226 was derived from pON208 [41] and contains the HCMV *a* sequence).

[32 P]orthophosphate uptake. Cells were infected with HCMV at a multiplicity of infection (MOI) of 2 and incubated for 3 days. The medium was then removed and replaced with fresh medium containing either no drug, 20 μ g/ml HPMPA, or 20 μ g/ml PMEG. After 8 h of incubation, the medium was removed and replaced with low-phosphate medium (4) containing the same compounds plus 5 μ Ci ml $^{-1}$ [32 P]orthophosphate (Amersham). After an additional 24 h of incubation at 37°C, agarose plugs containing total cell DNA were prepared and separated by FIGE as described above. The concatemeric DNA was excised from the FIGE gel, digested with EcoRI, separated on a 0.6% agarose gel, and visualized by autoradiography of the dried gel.

[3 H]thymidine incorporation. Cells were seeded into 96-well tissue culture plates at a density of 500 cells per well. After the cells were allowed to attach for 18 h, the medium was removed and replaced with medium containing inhibitors at various concentrations. Six hours later, 0.8 μ Ci [3 H]thymidine (New England Nuclear) was added to each well. Total incorporated counts were determined 24 h later by harvesting the cells using a PhD cell harvester (model 200A;

Cambridge Technologies, Inc.) and measuring the counts that were retained on the filters using a scintillation counter. Each drug concentration was analyzed in triplicate. Percent inhibitions were calculated by dividing the mean of the three counts measured for each drug at each concentration by the mean of the counts for the three no-drug controls.

RESULTS

Inhibition of genome maturation by BDCRB is reversible. BDCRB inhibition of HCMV genome maturation was previously reported to be reversible (11). To confirm this observation, cells were infected with HCMV at an MOI of 3 in the presence of 10 μ M BDCRB and incubated for 3 days before removal of the BDCRB by washing. Replicate cultures were subsequently incubated for an additional 1, 2, or 3 days either without BDCRB or with additional 10 μ M BDCRB until cells were harvested on days 4, 5, or 6 postinfection (p.i.). Replicate control cultures were incubated with no BDCRB from the time of infection and harvested on day 3 or day 6 p.i. The DNA samples were separated by FIGE, transferred to a nylon membrane, and hybridized to detect replicative forms of HCMV DNA. As expected, incubation without BDCRB for either 3 or 6 days resulted in the synthesis of concatemeric DNA, which underwent maturation to form abundant genomic-length (230-kb) DNA (Fig. 1A). On day 3 p.i., BDCRB-treated cells contained concatemeric DNA but virtually no 230-kb DNA (Fig. 1A), confirming previous reports that BDCRB effectively blocks genome maturation without inhibiting concatemer synthesis (61). After removal of BDCRB, restoration of the maturation process was evidenced by the abundant appearance of 230-kb DNA on days 4, 5, and 6, which was blocked in cultures in which BDCRB was maintained (Fig. 1A). Also consistent with previous reports (31, 61), a slower-migrating species, termed monomer+, was observed in response to BDCRB treatment; curiously, upon BDCRB removal and restoration of genome maturation, the formation of monomer+ DNA increased and it was nearly as abundant as 230-kb DNA (Fig. 1A). These results therefore demonstrate that the maturation-inhibitory effects of BDCRB are reversible. They further suggest that a time window after BDCRB removal can be used to assess the impact of other inhibitory compounds on genome maturation.

Protein synthesis is not required for genome maturation. BDCRB is believed to exert its effects by inhibition of functions performed by the HCMV terminase. Although the previous experiment demonstrated that the inhibitory effects of BDCRB are reversible, it did not address the possibility that BDCRB irreversibly inactivates terminase and that resumption of genome maturation may depend on the replacement of inactivated proteins by de novo protein synthesis. To address this question, replicate cultures were infected in the presence of BDCRB for 3 days, washed, and then incubated with the protein synthesis inhibitor cycloheximide before being harvested on day 4, 5, or 6. Inhibition of protein synthesis by cycloheximide had only a modest effect on genome maturation, as indicated by the relatively abundant formation of 230-kb genomes compared to those in cultures incubated without drug during the same time windows (Fig. 1A). This effect was reproducible in a second similar experiment (Fig. 1B). To ensure that the dose of cycloheximide used was sufficient to effectively inhibit protein synthesis, the same dose was added to HCMV-

FIG. 1. Impact of metabolic inhibitors on HCMV genome maturation. (A) Cells were infected with HCMV at an MOI of 3 and treated with a variety of drug regimens and then harvested on the days p.i. indicated above each lane. Intracellular DNA species were separated by FIGE, transferred to nylon membranes, and hybridized with probe pON227 to specifically detect HCMV DNA forms. Control cultures to demonstrate the efficacy of BDCRB treatment were incubated in the absence of drug (Φ ; harvested on days 3 and 6 p.i.), or in the presence of 10 μ M BDCRB (harvested on day 3 p.i.). We conducted reversal experiments in which replicate cultures were first treated from days 0 to 3 p.i. with 10 μ M BDCRB and then washed to remove the BDCRB and incubated with either medium alone (Φ) or medium containing 10 μ M BDCRB, 50 μ g/ml cycloheximide (CH), 5 μ g/ml actinomycin D (ACTD), 200 μ g/ml PFA, or 10 μ g/ml aphidicolin (APH) until harvest on days 4, 5, or 6 p.i. (B) Additional inhibitors were tested using essentially the same protocol as described above. Controls were treated with no drug or with 10 μ M BDCRB and harvested on day 3 p.i. Reversals were treated with 10 μ M BDCRB for days 1 to 3 p.i. and then washed and incubated with medium alone (Φ) or medium containing 10 μ M BDCRB, 10 μ g/ml aphidicolin, 1.5 μ g/ml HPMPA, 10 μ g/ml PMEG, 50 μ g/ml PMEAs, 200 μ g/ml PFA, 50 μ g/ml GCV, 50 μ g/ml CH, 5 μ g/ml actinomycin D, 2 μ g/ml α -amanitin (α -AMT), or 17 μ g/ml DRB, until harvest on day 5. (C) Replicate infected cultures of those shown in panel B were treated with the same doses of the indicated drugs from the time of infection until harvesting on day 3 p.i. Arrows indicate the positions of concatemeric (concat.), monomer+ (M⁺), and 230-kb DNA forms.

infected cells from the time of infection until harvest on day 3 p.i. Viral DNA was undetectable in cells treated for 3 days with cycloheximide, whereas large amounts of HCMV DNA were present in untreated, HCMV-infected control cells (Fig. 1C). Therefore, when present at the early stages of infection, the dose of cycloheximide used was sufficient to block DNA synthesis, presumably by blocking the translation of immediate early proteins.

These experiments demonstrate that *de novo* protein synthesis is not required for resumption of genome maturation subsequent to BDCRB removal. Thus, BDCRB does not irreversibly damage the genome maturation machinery.

RNA polymerase II activity is not required for genome maturation. In phages T4 and T7, cleavage is significantly enhanced by RNA transcription through the cleavage site (10, 16). To determine the importance of RNA synthesis in the HCMV genome maturation process, actinomycin D was added to infected cells after BDCRB removal. Actinomycin D potentially inhibited genome maturation (Fig. 1A and B); however, this known DNA interchelator has pleiotropic effects: in addition to inhibiting transcription, it has been reported to inhibit DNA synthesis (9) as well as an ATPase activity necessary for *in vitro* packaging of phage T3 DNA (42). Hence, its effects on HCMV genome maturation could stem from alterations in DNA topology, inhibition of DNA synthesis, or inactivation of a packaging-associated ATPase. To address this concern, more-specific RNA polymerase II inhibitors, α -amanitin and DRB, were similarly assayed. Neither drug had a significant effect on maturation (Fig. 1B). The ability of the same doses of α -amanitin and DRB to indirectly prevent DNA synthesis when added at the time of infection provided evidence that the drugs were able to inhibit transcription at the doses used (Fig. 1C).

From these results, we conclude that RNA polymerase II-mediated transcription is not required for HCMV genome maturation.

Synthesis by the viral DNA polymerase is not required for genome maturation. In bacteriophages T3 and T7, direct terminal repeats are duplicated during cleavage and the T7 DNA polymerase has been implicated in the duplication process (63). Recent evidence also suggests that in herpesviruses that have terminal repeats, a similar duplication occurs (44). To determine if synthesis by the viral DNA polymerase plays a role in HCMV genome maturation, the viral DNA polymerase inhibitor PFA was tested in the BDCRB reversal assay. The relatively abundant formation of 230-kb DNA demonstrated that PFA had only a modest effect on genome formation (Fig. 1A and B). This implied that viral-DNA polymerase activity was not required for DNA cleavage. To strengthen this conclusion, other specific inhibitors of the viral DNA polymerase, HPMPA (1.5 μ g/ml), PMEA (adefovir; 50 μ g/ml), and GCV (50 μ g/ml), were tested. The relatively abundant formation of 230-kb DNA in the presence of HPMPA and PMEA was again consistent with the PFA results, indicating that viral-DNA polymerase activity is not required (Fig. 1B). As before, addition of these drugs at the time of infection confirmed that each drug fully inhibited viral-DNA synthesis at the doses used (Fig. 1C).

Consistent with a previous report by Biron et al. (11), preliminary titration of GCV in BDCRB reversal assays indicated

TABLE 1. Effects of DNA polymerase inhibitors on cellular-DNA synthesis

Treatment	Dose (μ g/ml)	[³ H]thymidine incorporation (% relative to that of untreated cells)
No drug	NA ^a	100
PFA	200	82
HPMPA	10	84
PMEA	150	46
GCV	50	83
PMEG	20	1
Aphidicolin	10	3

^a NA, not applicable.

that relatively low doses of GCV (up to 20 μ g/ml) did not impair HCMV genome maturation (data not shown). Although the 50- μ g/ml dose of GCV used in the experiment shown in Fig. 1B failed to fully inhibit genome maturation, it did substantially reduce the amount of 230-kb DNA, more so than the other viral-DNA polymerase inhibitors (PFA, PMEA, and HPMPA) (Fig. 1B). While GCV is a selective inhibitor of viral-DNA synthesis, much of this selectivity is derived from the fact that it must first be phosphorylated by the HCMV pUL97 protein kinase before host kinases can complete the di- and triphosphorylation steps (33). GCV-triphosphate, however, is only modestly selective for viral versus host DNA polymerases (35). Indeed, the dose used in our experiment was 100-fold above the 50% inhibitory concentration reported for GCV inhibition of uninfected cells that had been engineered to express HSV-1 thymidine kinase, another kinase that can phosphorylate GCV (2). Thus, it is probable that in the experiments described here, intracellular levels of GCV-triphosphate were sufficient to inhibit both viral and host cell DNA polymerases.

Host DNA polymerase activities may be involved in HCMV genome maturation. The results discussed above for PFA, HPMPA, and PMEA indicated that synthesis by the viral DNA polymerase is not required for genome maturation, while the results with GCV suggested the possibility that host DNA polymerases might play a role, either during normal infection or by substituting for viral DNA polymerase when its activity is blocked by specific inhibitors. To investigate this possibility, potent inhibitors of both viral and host polymerases, PMEG and aphidicolin, were tested in the reversal assay. Both compounds were highly potent in inhibiting the formation of 230-kb DNA (Fig. 1A and B).

The above results suggested a possible correlation between the extent to which a compound inhibits cellular-DNA synthesis and its ability to inhibit genome maturation. To more quantitatively assess this, we added the same concentrations of each drug to uninfected subconfluent fibroblast cells and determined their effects on cellular-DNA synthesis by measuring [³H]thymidine incorporation. Incorporated counts were used to calculate the percent inhibition of cellular-DNA synthesis relative to that of cells that received no drug. As anticipated, the selective inhibitors of the viral DNA polymerases PFA, HPMPA, and PMEA exhibited relatively modest effects on host DNA synthesis, resulting in synthesis levels 46 to 84% of those of untreated cells, whereas PMEG and aphidicolin were potent inhibitors of host DNA polymerases, reducing synthesis to 1 to 3% of those of untreated cells (Table 1). GCV exhibited

very modest inhibition (83%); however, this was anticipated in uninfected cells, as in the absence of pUL97, the GCV presumably remained largely unphosphorylated. Thus, with the explicable exception of GCV, the ability of a compound to inhibit host DNA synthesis correlates well with its ability to impair genome maturation. It should be noted, however, that PMEG and aphidicolin also inhibit the viral DNA polymerase. Hence, although inhibition of host DNA polymerases is necessary to impair genome maturation, it may not be sufficient; inhibition of both viral and host polymerases may be required.

Host DNA polymerases may mediate nucleotide incorporation into viral DNA during late stages of infection. Given the evidence that terminal repeats on herpesvirus genomes are duplicated during the cleavage process (19, 44, 62) and that similar events in bacteriophage T7 appear to involve DNA synthesis (63), the above results presented the intriguing possibility that DNA synthesis that is associated with genome maturation may be localized to the terminal repeat regions of the viral DNA.

An experiment was devised to determine if radiolabeled nucleotides are incorporated into viral DNA in the presence of viral-DNA polymerase inhibitors and, if so, if the incorporation is broadly distributed or restricted to regions near the terminal repeats. Three replicate flasks of cells were infected with HCMV at an MOI of 2 and incubated for 3 days to allow accumulation of concatemeric DNA. The medium was then replaced with either medium alone, medium containing 20 μ g/ml HPMPA, or medium containing 20 μ g/ml PMEG and incubated an additional 8 h to ensure entry of the drugs. The medium was then replaced with low-phosphate medium containing the same treatments plus [32 P]orthophosphate. After incubation for a further 24 h, intracellular concatemeric DNA was isolated by FIGE, digested with EcoRI, separated by agarose electrophoresis, and visualized by autoradiography of the dried gel.

A relatively high dose of HPMPA was used to ensure effective inhibition of the viral DNA polymerase. Although HPMPA reduced the amount of incorporation compared to that of the no-drug control, incorporation of radioactive nucleotides into viral concatemeric DNA continued to a significant degree (Fig. 2). The pattern of incorporation was essentially identical to that of the control, indicating that incorporation in the presence of HPMPA was not restricted to terminal repeat regions but rather was generally distributed throughout the HCMV genome (Fig. 2). In contrast, PMEG totally blocked all incorporation (Fig. 2), suggesting that the incorporation that occurred in the presence of HPMPA may have been mediated by host rather than viral DNA polymerase.

BDCRB alters the HCMV genome terminal structure. HCMV has a class E genome structure (48) that consists of long and short arms, each consisting of unique long and unique short regions bordered by two different sets of inverted repeats designated *b* and *c* sequences (Fig. 3). One to several reiterated copies of a terminal repeat called the *a* sequence are found at long-arm ends and in an inverted orientation at the junctions that lie between the long and short arms (L/S junctions) (56). Initially, it was believed that, like HSV-1, all HCMV genomes have just one copy of the HCMV *a* sequence at their short-arm ends. However, in studies characterizing the

FIG. 2. Nucleotide incorporation into HCMV DNA in the presence of DNA polymerase inhibitors. Cells were mock infected or infected with HCMV at an MOI of 2 and incubated for 72 h. From 72 to 80 h p.i., the cultures were incubated in the presence of no drug (Φ), 20 μ g/ml HPMPA, or 20 μ g/ml PMEG. From 80 to 96 h p.i., the cultures were incubated with the same drugs in labeling medium containing [32 P]orthophosphate. DNA was prepared at 96 h p.i. and separated by FIGE. Concatemeric DNA was excised, digested with EcoRI, separated by agarose gel electrophoresis, and visualized by autoradiography of the dried gel. Uninfected confluent cell cultures (U) were similarly analyzed in the absence of drugs. The positions of molecular size markers are indicated to the left.

heterogeneity of HCMV genomic termini, Tamashiro and Spector observed 7.1- and 6.5-kb HindIII Q short-arm-terminal fragments. Hybridization with an *a* sequence-specific probe revealed that the 7.1-kb fragment contained an *a* sequence but that the 6.5-kb fragment did not (58). Thus, a proportion of HCMV genomes lack an *a* sequence at their short-arm ends. This feature is analogous to the genomic structure of GPCMV, in which roughly half the genomes have one copy of a 1-kb terminal repeat at each end, while the remaining genomes lack a terminal repeat at the right end (26). In a recent study, we designated GPCMV genomes that lack the right-end repeat as type I and genomes that have one repeat at the right end as type II (43). We propose to apply the same nomenclature to the short-arm-terminal structure of the HCMV genome and use the designations Q₁ for the 6.5-kb terminal HindIII fragment derived from type I genomes and Q₂ for the 7.1-kb fragment derived from type II genomes (Fig. 3).

FIG. 3. HCMV genome structure. Long and short arms are comprised of unique long (U_L) and unique short (U_S) regions flanked by inverted copies of the b sequence (gray boxes) and c sequence (open boxes). One to several reiterated copies of the a sequence are found at left, or long-arm ends (indicated by a_n) and in inverted orientation (indicated by a'_m) at a region designated the L/S junction where the long and short arms join. One or no copies of the a sequence are found at the right or short-arm end. Details of the termini are enlarged below. For simplicity, one a sequence (black box) is shown at the long-arm end, resulting in a 4.0-kb *EcoRI* W fragment. At the short-arm end, genomes designated type I lack an a sequence, resulting in a 6.5-kb *HindIII* Q_1 fragment, whereas genomes designated type II have one a sequence copy, resulting in a 7.1-kb *HindIII* Q_2 fragment (58). Thick bars indicate sequences used as hybridization probes that are cloned in the indicated plasmids.

In recent studies, we characterized the effects of BDCRB on GPCMV (43). In contrast to its effects on HCMV, BDCRB did not significantly impact the formation of approximately unit length GPCMV genomes. These genomes, however, were abnormal. At their right ends they were exclusively type II, while at their left ends they were heterogeneously truncated, missing 2.7 to 4.9 kb of left-end sequences (43). This led us to consider whether the approximately genome length HCMV DNA formed in the presence of BDCRB is, despite the quantitative difference, qualitatively similar to the abnormal genomes formed by GPCMV. We therefore sought to characterize the terminal structures of the genome length HCMV DNAs that were formed in the presence of BDCRB and other inhibitory compounds. Replicates of the DNA samples used in the experiment shown in Fig. 1A were separated on replicate FIGE gels, and for each time point and drug treatment, concatemeric, monomer+, and 230-kb DNA samples were excised from the gels. Following restriction with both *HindIII* and *EcoRI*, the DNA fragments were separated by agarose electrophoresis, transferred to nylon membranes, and hybridized with probes to specifically detect long-arm- or short-arm-terminal fragments. Sequences cloned in pON227 were used to detect the 4.0-kb *EcoRI* W long-arm-terminal fragment, and sequences in pON2333 were used to detect short-arm-terminal *HindIII* Q_1 and Q_2 fragments (Fig. 3). Because terminal sequences are also found at L/S junctions, junction fragments with molecular sizes of 10.3 and 10.7 kb were predicted to hybridize to all probes used in these studies.

Consistent with studies by Tamashiro and Spector (58) and our previous studies of the HCMV terminal structure (38), the pON2333 probe detected two *HindIII* short-arm-terminal fragments having molecular weights consistent with those of Q_1 and Q_2 (Fig. 4A). To provide additional confirmation, the membrane was rehybridized with a probe consisting of pON226, a plasmid that contains only the HCMV a sequence (Fig. 3). The pON226 probe hybridized to the ~7-kb fragment

presumed to be Q_2 , indicating that it contains an a sequence, but failed to hybridize to the ~6.5-kb fragment presumed to be Q_1 , indicating that, as anticipated, it lacks an a sequence (Fig. 4A).

HCMV concatemeric DNA formed in the presence of BDCRB exhibited a complete loss of Q_1 fragments with essentially no change in Q_2 fragments. Curiously, removal of BDCRB did not result in the restoration of Q_1 fragment formation (Fig. 4A) despite clear evidence that genome maturation had resumed (Fig. 1A). Similarly, 230-kb intracellular DNA formed in the absence of BDCRB contained both the Q_1 and Q_2 fragments (Fig. 4B), and treatment with BDCRB followed by its removal resulted in resumed production of both virion and 230-kb DNAs that contained Q_2 but lacked Q_1 fragments (Fig. 4B). Thus, as observed for concatemeric DNA, restoration of genome maturation upon BDCRB removal did not restore the formation of genomes with type I termini. The paucity of virion and 230-kb DNAs formed in the continued presence of BDCRB made analysis difficult; however, a lengthy exposure confirmed the absence of the Q_1 fragment from 230-kb DNA formed in the continuous presence of BDCRB 6 days p.i. (Fig. 4C).

In order to determine if, like GPCMV, HCMV genomes produced in the continuous presence of BDCRB are truncated at the long-arm end, the small amount of 230-kb DNA formed after 6 days of incubation with BDCRB (Fig. 1A) was analyzed and compared to virion DNA produced in the absence of BDCRB. The latter exhibited the expected W, Q_1 , and Q_2 terminal fragments, as well as the 10.3- and 10.7-kb junction fragments (Fig. 4C). Consistent with the observations described above, the 230-kb DNA produced in the presence of BDCRB appeared to contain junction and Q_2 fragments while lacking Q_1 fragments; long-arm W terminal fragments, however, were undetectable (Fig. 4C).

Collectively, these results indicate that although the amount of 230-kb DNA formed in the presence of BDCRB is greatly

FIG. 4. Effects of BDCRB on the termini of HCMV DNA forms. Concatemeric, monomer+, and 230-kb DNAs were excised from FIGE gels containing replicate samples of those used in the experiment shown in Fig. 1A. Each DNA sample was digested with EcoRI and HindIII, separated by agarose gel electrophoresis, transferred to nylon membranes, and sequentially hybridized with probes derived from plasmid pON2333 to detect short-arm-terminal fragments, plasmid pON227 to detect long-arm-terminal fragments, or plasmid pON226 to detect α sequence-containing fragments (for probe details, see Fig. 3). (A) Concatemeric DNAs; (B) 230-kb DNAs formed after removal of BDCRB; (C) 230-kb DNAs formed after 6 days in the continuous presence of BDCRB; (D) monomer+ DNA (M^+) formed on day 4 p.i., 1 day after the removal of BDCRB. Numbers above lanes indicate days p.i. that samples were harvested; V, extracellular virion DNA produced in the absence of BDCRB; Φ , DNAs prepared after 3 days of culture in the absence of BDCRB; V6 (B), extracellular virion DNA prepared from culture supernatants of cells analyzed in the adjacent lane (i.e., cultured with BDCRB from days 1 to 3 and without BDCRB from days 3 to 6). The positions of molecular size markers are indicated to the right of each figure.

reduced in comparison to that which is formed when GPCMV is treated with BDCRB, qualitatively, BDCRB's effects on the two viruses are similar. In both cases, BDCRB modifies the genome maturation process such that right ends are exclusively type II, having one copy of the terminal repeat, and left ends are truncated.

Monomer+ DNA contains short-arm termini but lacks long-arm termini. A striking feature of the halogenated benzimidazoles is the formation of a novel replicative intermediate termed monomer+ due to its apparent molecular size of 270 kb, slightly greater than a 230-kb monomer (31, 60, 61). This size coincides with the size predicted for a genome with one extra short arm, or a "short-long-short" structure. Formation

of such a structure might result if BDCRB induces occasional cleavage site skipping. For example, we have proposed that concatemers are packaged starting with their short-arm ends and that cleavage normally occurs after one genome, comprised of a short arm followed by a long arm, has entered the capsid (Fig. 5A) (40). If the normal cleavage site is skipped and packaging continues, an additional short arm would be packaged before the next cleavage site is encountered. Cleavage at this site would produce a 270-kb DNA having a short-long-short structure (Fig. 5C). This model predicts that monomer+ DNA should contain short-arm termini but lack long-arm termini. Our analysis of monomer+ DNA confirmed this prediction. Monomer+ DNA was found to contain junctions and

FIG. 5. Models for genome maturation in the absence or presence of BDCRB. HCMV concatemers are illustrated, with short arms represented by open boxes and long arms represented by lines. A normal genome consists of one long arm and one short arm. (A) Normal cleavage in the absence of BDCRB. Starting with a short-arm concatemer end, packaging proceeds until one full short arm and one full long arm have entered the capsid. Cleavage then occurs at the left end of the long arm, releasing a normal genome within the capsid. (B) Premature cleavage in the presence of BDCRB. Packaging occurs as described for panel A, but cleavage occurs prior to the normal cleavage site, releasing a left-end-truncated genome within the capsid. (C) Monomer+ cleavage in the presence of BDCRB. Packaging occurs as described for panel A but continues past the normal cleavage site until an additional sort arm is packaged. Cleavage then occurs at the internal cleavage site located at the left end of the short arm, releasing a short-long-short monomer+ molecule within the capsid.

short-arm-terminal Q_2 fragments. Consistent with the observations described above, monomer+ DNA lacked Q_1 fragments. Notably, however, monomer+ DNA contained almost no detectable long-arm-terminal W fragments (Fig. 4D).

DNA, RNA, and protein synthesis inhibitors do not alter terminal structures. Terminal analyses were also performed on replicate DNA samples formed during inhibitor treatments in the experiment shown in Fig. 1A. While all time points were analyzed, for simplicity, only representative time points are shown (Fig. 6). The results essentially mirrored those observed for BDCRB alone; in no case did an inhibitory compound have any additional effects on either long-arm or short-arm termini. However, these results served to confirm and strengthen the findings described for BDCRB above. Two hundred thirty-kilobase DNA that formed in the presence of cycloheximide or PFA after BDCRB removal exhibited normal levels of W and

Q_2 ends but lacked Q_1 ends (Fig. 6A). Monomer+ DNA from the same cells similarly exhibited Q_2 and lacked Q_1 short-arm ends but was strikingly devoid of long-arm W ends (Fig. 6B). The fact that the 230-kb DNA shown in Fig. 6A was excised from the same lane of the same gel, yet contains W fragments, serves to assuage concerns that the monomer+ DNA may not have been cleanly segregated from 230-kb DNA in these experiments. Concatemer DNA contained Q_2 but lacked Q_1 fragments, despite incubation for 3 days without BDCRB (Fig. 6C). Consistent with our earlier findings (38), concatemer DNA was devoid of long-arm W fragments in the absence or presence of BDCRB or other drug treatments (Fig. 6C and data not shown).

DISCUSSION

Herpesvirus genome maturation is a complex process that shares many similarities with genome maturation and capsid morphogenesis of the large double-stranded DNA bacteriophage. The foremost player in this process is terminase, an enzymatic complex hypothesized to function both as a DNA translocase, moving concatemer DNA into recipient capsids, and as a sequence-specific nuclease, cleaving the DNA at precise locations to produce mature, packaged genomes. The HCMV proteins pUL89 and pUL56 are thought to comprise the principle components of terminase and are therefore presumed to carry out the translocation and cleavage functions. Several ancillary activities have also been implicated in the maturation process. For example, the duplication of terminal repeats during cleavage suggests the possible involvement of DNA nicking and/or polymerase activities, and the instability of capsids packaged by HSV-1 alkaline nuclease mutants suggests that debanching, DNA recombination, or DNA repair activities may be important for stable DNA packaging (36, 47, 53). Enzymes mediating such activities may well comprise accessory components of terminase; however, as an active terminase complex has not been isolated or characterized, the precise composition and biochemical activities of herpesvirus terminases remain speculative.

This project sought to shed light upon the biosynthetic and biochemical activities involved with the genome maturation of HCMV. Using the inhibitor BDCRB, concatemers were allowed to accumulate, while their cleavage to form unit length genomes was inhibited. Genome formation was restored following removal of BDCRB, and the impact of metabolic inhibitors on this process was assessed. De novo protein synthesis was not necessary for genome maturation, as evidenced by the efficient formation of unit length genomes in the presence of the protein synthesis inhibitor cycloheximide. This result is fully consistent with a previous observation that cycloheximide does not significantly impair genome maturation by HSV-1 (18). That study used a temperature-sensitive protease mutant of HSV-1 in which genome maturation is impaired at 39°C but restored upon shifting the infected cells to 31°C. Our studies, however, used BDCRB to inhibit genome maturation. Based on the observation that certain amino acid changes within pUL56 and pUL89 confer resistance to BDCRB (31, 61), this drug has been proposed to act by inhibiting terminase functions. Our finding that restoration of genome maturation is not sensitive to cycloheximide indicates that BDCRB does not

FIG. 6. Effects of inhibitors on the termini of HCMV DNA forms. Replicates of samples shown in Fig. 1A that were treated with inhibitors after BDCRB removal were analyzed as described for Fig. 4. (A) Two hundred thirty-kilobase DNA formed in the presence of cycloheximide (CH) or PFA; (B) monomer+ (M^+) DNA formed in the presence of cycloheximide (CH) or PFA; (C) concatemeric DNA formed in the presence of no drug (Φ), cycloheximide, actinomycin D (ACTD), PFA, or aphidicolin (APH). V, extracellular virion DNA produced in the absence of BDCRB (used as standards). The positions of molecular size markers are indicated to the right of each figure.

irreversibly bind to or otherwise damage protein components of the genome maturation machinery, such as pUL89 and pUL56, but rather, the functionality of the inhibited proteins must be rapidly restored upon BDCRB removal.

The second activity that we investigated was the role of RNA polymerase. These studies were inspired by reports that for phage T4 and T7, transcription through the cleavage site significantly enhances cleavage site utilization (10, 16). Knowing that protein synthesis was not required for efficient genome maturation, we reasoned that sensitivity to RNA polymerase inhibitors would indicate a role for RNA transcription beyond that of production of mRNAs for protein synthesis; however, the RNA polymerase II inhibitors α -amanitin and DRB had little effect on the amount of 230-kb DNA formed, suggesting that RNA polymerase II activity has no significant role in the HCMV genome maturation process.

The third activity that we investigated was the role of DNA synthesis. The genomes of many herpesviruses have direct terminal repeats, and several studies have observed duplication of these terminal repeats in association with the cleavage process (19, 44, 62). If this phenomenon is a genuine duplication of the

repeats (and not the result of degradation of intervening genomes within concatemers), some form of DNA synthesis associated with the genome maturation process seems to be necessary. Indeed, bacteriophage T7 provides a precedent. The 160-bp terminal repeat of T7 appears to be duplicated during cleavage, as single copies of the repeat are found within concatemers, but two copies (one at each end of the genome) are present on mature genomes (20, 32). Omission of T7 DNA polymerase from in vitro cleavage/packaging assays results in the formation of T7 genomes that are normal at one end but unusually truncated (missing sequences) at the other (63). Furthermore, protein linkage studies suggest that the T7 DNA polymerase may be a component of the T7 terminase complex: while it does not bind directly to the terminase subunits, yeast two-hybrid analyses indicate that T7 DNA polymerase can interact with a small protein that also has the capacity to interact with the T7 terminase large subunit (5).

We therefore sought to evaluate the impact of various DNA synthesis inhibitors on HCMV genome maturation. We observed that inhibitors selective for the viral DNA polymerase, including PFA, HPMPA, and PMEA, only moderately reduced

the amount of 230-kb DNA formed. This finding is fully consistent with a study by Church et al. which used the HSV-1 temperature-sensitive protease mutant to demonstrate that the HSV-1 DNA polymerase inhibitor acyclovir only modestly impairs (~20 to 40%) HSV-1 genome maturation (17). These and our findings therefore indicate that DNA synthesis by the viral DNA polymerase is not a critical requirement for genome maturation by either HCMV or HSV-1. However, as also noted by Church et al. (17), the significance of the modest impairment is difficult to evaluate. While it may suggest a minor ancillary role for the viral DNA polymerase, such as repeat duplication or repair, the inhibitory compounds used must also impair concatemer synthesis during the treatment window and hence may reduce the amount of genome maturation by reducing the amount of substrate concatemer available.

In contrast to the compounds that specifically inhibit the viral DNA polymerase, compounds such as aphidicolin and PMEG, which potentially inhibit both viral and host DNA polymerases, virtually eliminated genome formation. We therefore hypothesized that some form of DNA synthesis is important for genome maturation. While it is possible that this function is strictly mediated by a host DNA polymerase, it is also possible that viral DNA polymerase normally serves this function but that, in the presence of viral-DNA polymerase-specific inhibitors, host DNA polymerases can substitute for this function. We further hypothesized that if the cleavage-associated synthesis is specifically involved in the duplication of terminal repeats, incorporation of radioactive nucleotides into viral DNA should occur selectively within terminal repeat sequences when viral DNA polymerase is inhibited, but that if synthesis occurs throughout the genome, as might be expected for concatemer synthesis or randomly distributed repair processes, incorporation would not be localized to specific genomic regions. These hypotheses were tested by evaluating radioisotope incorporation into viral DNA under conditions in which concatemer DNA was first allowed to accumulate but subsequent synthesis by the viral DNA polymerase was blocked by the addition of HPMPA. To our surprise, significant amounts of incorporation occurred in a pattern that was not localized to terminal sequences but rather was distributed throughout the genome. That in a parallel experiment the broad-spectrum DNA polymerase inhibitor PMEG was able to fully block all incorporation suggests that host enzymes may be responsible for the incorporation that occurred in the presence of HPMPA. While this result is consistent with synthesis by host enzymes in association with DNA damage repair or branch resolution, the possibility remains that DNA synthesis in association with repeat duplication occurs but is either in the minority, compared to a separate broadly distributed synthesis, or is initiated at terminal repeats by the cleavage process but extends considerable distances into the concatemer.

In previous studies, we observed that BDCRB treatment of GPCMV selectively abolished the formation of type I termini on both concatemer and 230-kb DNAs and resulted in the formation of 230-kb DNAs that were heterogeneously truncated at their left ends (43). We therefore sought to determine the impact of BDCRB as well as other metabolic inhibitors on the terminal structure of HCMV replicative DNA forms. As was observed for GPCMV, BDCRB selectively inhibited the

formation of type I termini on HCMV concatemeric DNA as well as intracellular 230-kb and monomer+ DNAs. Curiously, this effect was not reversible. Even genomes formed 3 days after BDCRB removal (when genome maturation had clearly been restored) remained exclusively type II. In addition, monomer+ DNA did not decrease after BDCRB removal but in fact increased significantly (Fig. 1A). The reason for the irreversibility of either effect is not clear; however, we note that for GPCMV, abolition of type I genomes occurs at doses well below the drug's 50% inhibitory concentration (43), and for HCMV, monomer+ formation occurs at similarly low doses (61). Thus, it is possible that washing removes sufficient BDCRB to restore cleavage but that residual low levels of intracellular BDCRB are sufficient to promote monomer+ formation and inhibit type I genome formation.

In contrast to GPCMV, BDCRB dramatically reduces the quantity of HCMV 230-kb DNA that is formed (Fig. 1 and reference 61), making analysis of this species difficult. Even so, extended hybridization exposures revealed the presence of junctions and short-arm type II ends on 230-kb DNA formed in the continuous presence of BDCRB, with the apparent absence of long-arm ends. Thus, it appears that, despite differences in the quantities produced, GPCMV and HCMV 230-kb DNAs formed in the presence of BDCRB are qualitatively similar: in both cases, right ends are exclusively type II (type I ends are lacking), while left ends show evidence of truncation.

Consequently, the model that we proposed to explain the effects of BDCRB on GPCMV genome maturation may also be applied, with modification, to HCMV (Fig. 5). In the case of GPCMV, BDCRB was proposed to induce premature cleavages just prior to completion of the packaging of each genome, resulting in the relatively abundant formation of slightly left-end-truncated genomes (reference 43 and Fig. 5B). In the case of HCMV, approximately genome length DNA is formed in the presence of BDCRB but is much less abundant. That this DNA is analogous to the left-end-truncated genomes formed by GPCMV was confirmed by the observation that it completely lacks left-end-terminal W fragments (Fig. 4C). Hence, premature cleavage, as illustrated in Fig. 5B, may also occur with HCMV but is infrequent. We propose that concatemer packaging at a similarly low frequency continues beyond the region of premature cleavage and even beyond the cleavage point appropriate for normal genome formation. An additional 40-kb short arm is packaged and cleavage occurs at the next cleavage site encountered, giving rise to the short-long-short structure that we propose for monomer+ DNA (Fig. 5C).

Several observations support this hypothesis for monomer+ formation. First, a short-long-short structure has a predicted molecular size of 270 kb, precisely that observed for monomer+ DNA based on its migration in pulsed-field gels (61). Second, a short-long-short structure would not contain long-arm-terminal fragments but would contain short-arm-terminal fragments, precisely as observed in our terminal-fragment analyses of monomer+ DNA. Third, by analogy to our findings with GPCMV, monomer+ DNA should be packaged within capsids yet be sensitive to nuclease. In this regard, abundant DNA-containing capsids can be observed by electron microscopy of TCRB-treated HCMV-infected cells (John Drach, personal communication), and by pulsed-field analysis, we have

clearly demonstrated that monomer+ DNA is fully sensitive to nuclease (data not shown). While additional studies will be needed to conclusively demonstrate that monomer+ DNA is packaged within nuclease-permeable capsids, the current results provide strong inferential support for this model.

Why then would BDCRB induce premature cleavages in GPCMV while promoting cleavage site skipping in HCMV? While the answer may lie in subtle differences between the terminase proteins of the two viruses and how they interact with BDCRB, the differences in genome structure also present intriguing possibilities. In the context of the cleavage site skipping model (Fig. 5C), the absence of internal cleavage sites from the GPCMV genome makes two predictions. First, GPCMV should not produce monomer+ DNA. Indeed, we have never observed a monomer+-sized GPCMV DNA species in response to BDCRB treatment (43). Second, GPCMV should instead produce 460-kb dimers, as skipping of the normal cleavage site would require packaging of an additional genome before terminase would encounter the next cleavage site. We have not observed dimer-sized GPCMV DNA species in response to BDCRB treatment (43); however, a dimer may well exceed the packaging capacity of the capsid; thus, packaging may stall or perhaps reverse before the next cleavage site is encountered. While these negative observations are suggestive, more-rigorous support for this model would come from a demonstration that removal of the HCMV internal cleavage site abolishes monomer+ formation. Efforts to construct such a mutant virus are under way.

One weakness of this model is that it also predicts that BDCRB treatment should result in formation of long-arm termini on HCMV concatemers (Fig. 5C). This clearly does not occur. HCMV concatemers are essentially devoid of long-arm termini (Fig. 6C and reference 38) and remain so in the presence of BDCRB (data not shown). Thus, for this model to be correct, either cleavage must occur in such a way that no long-arm termini are formed on concatemers or that, once formed, such termini must be rapidly removed or degraded; indeed, short-arm termini must be restored on concatemer ends as they are detected at unaltered levels in the presence of BDCRB (Fig. 4A). An analogous paradox occurs in our model for BDCRB's interference with GPCMV genome maturation, in that premature cleavage must leave up to 4.9 kb of extra sequences on right concatemer termini (Fig. 5B). Consequently, we proposed a trimming step that restores proper right termini on GPCMV concatemers (43). Such an activity could, in theory, be responsible for the removal of entire long-arm segments from the abnormal HCMV concatemer ends that are predicted by the cleavage site skipping model.

Analysis of termini on HCMV DNA species formed in the presence of metabolic synthesis inhibitors revealed no evidence for specific alterations. Effects on the formation of type I termini, however, could not be assessed as inhibitor treatments were all done in the context of BDCRB reversal, which irreversibly blocked formation of type I termini. Thus, to the extent that these analyses could detect changes in terminal structure, it appears that the inhibition of RNA, DNA, or protein synthesis does not overtly alter terminal structures at the molecular level. These analyses also bear on the hypothesis that DNA synthesis is involved in the duplication of terminal repeats, in that inhibition of DNA synthesis should impair

repeat duplication, perhaps resulting in a loss of termini having multiple *a* sequence reiterations. Unfortunately, reiterated *a* sequences occur only at long-arm termini, which are exclusively found on 230-kb DNA, not on concatemer or monomer+ DNA. Our ability to evaluate alterations in terminal *a* sequence reiteration was therefore restricted to circumstances in which 230-kb DNA was formed. In the presence of cycloheximide and PFA, we observed no alterations to the formation of multiply reiterated terminal *a* sequences (Fig. 6A), even upon extended exposure (not shown). Thus, we can conclude that inhibition of protein synthesis or viral-DNA polymerase activity does not affect *a* sequence reiteration. Similar analyses were conducted on aphidicolin- and actinomycin D-treated samples; however, these compounds impaired 230-kb DNA formation to such an extent that the signal intensities from long-arm-terminal fragments were too weak to be interpreted (data not shown).

Taken together, our results are consistent with a role for host DNA polymerase-mediated DNA synthesis during the HCMV genome maturation process. While repair of randomly distributed gaps, branches, or damaged regions by host damage repair mechanisms seem a reasonable prerequisite to viral-DNA packaging, it is also possible that both viral and host DNA polymerases participate in concatemer synthesis, particularly during later stages of viral-DNA replication, or that host DNA polymerases can take over elongation functions at established replication forks in the event that the viral DNA polymerase is inhibited. While several DNA repair polymerases (e.g., polymerases β , θ , η , ι , and κ) can be eliminated from consideration on the basis of their insensitivity to aphidicolin (23, 34, 37, 59, 64), PMEG and aphidicolin sensitivity do not serve to discriminate between damage repair and concatemer synthesis as potential roles for host DNA polymerases. The DNA polymerases that mediate host genome replication, polymerases α , δ , and ϵ , are each sensitive to aphidicolin and PMEG (30, 45, 65) and are therefore prime candidates if host polymerases play a role in concatemer synthesis. However, polymerases δ and ϵ are also involved in damage repair (54), and the DNA damage repair polymerases ζ , λ , and ϕ are also sensitive to aphidicolin (46, 55, 57). Additional studies, perhaps using RNA interference approaches to selectively inhibit the expression of individual host DNA polymerases, will be needed to confirm a role for host DNA polymerases in viral DNA replication and/or genome maturation and to elucidate their specific functions.

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