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Murine Gammaherpesvirus 68 *ORF52* Encodes a Tegument Protein Required for Virion Morphogenesis in the Cytoplasm▽

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The tegument, a semiordered matrix of proteins overlying the nucleocapsid and underlying the virion envelope, in viruses in the gamma subfamily of *Herpesviridae* is poorly understood. Murine gammaherpesvirus 68 (MHV-68) is a robust model for studying gammaherpesvirus virion structure, assembly, and composition, as MHV-68 efficiently completes the lytic phase and productively infects cultured cells. We have found that MHV-68 *ORF52* encodes an abundant tegument protein conserved among gammaherpesviruses. Detergent sensitivity experiments revealed that the MHV-68 ORF52 protein is more tightly bound to the virion nucleocapsid than the ORF45 tegument protein but could be dissociated from particles that retained the ORF65 small capsomer protein. ORF52, tagged with enhanced green fluorescent protein or FLAG epitope, localized to the cytoplasm. A recombinant MHV-68 bacterial artificial chromosome mutant with a nonsense mutation incorporated into *ORF52* exhibited viral DNA replication, expression of late lytic genes, and capsid assembly and packaging at levels near those of the wild type. However, the MHV-68 *ORF52*-null virus was deficient in the assembly and release of infectious virion particles. Instead, partially tegumented capsids produced by the *ORF52*-null mutant accumulated in the cytoplasm, containing conserved capsid proteins, the ORF64 and ORF67 tegument proteins, but virtually no ORF45 tegument protein. Thus, ORF52 is essential for the tegumentation and egress of infectious MHV-68 particles in the cytoplasm, suggesting an important conserved function in gammaherpesvirus virion morphogenesis.

Virion morphogenesis among the herpesviruses is a multistep process. Nucleocapsid assembly and packaging of the viral DNA occurs in the nucleus, and nascent nucleocapsids are thought to bud into the cytoplasm in an envelopment/de-envelopment process through the nuclear inner and outer envelopes (29). The nucleocapsids are transported, in association with primary tegument proteins, through the cytoplasm to a distinct site of virion assembly. Transmission electron microscopy (TEM) studies have indicated that the major tegumentation and envelopment process occurs here, as the nascent nucleocapsids associate with electron-dense tegument/glycoprotein densities conjunct to Golgi apparatus-derived vesicular membranes (29, 30, 46). Tegumentation results in a "wrapping" of the nascent nucleocapsid and budding into the lumen of the cytoplasmic compartments, forming nearly complete virions within the lumen. These particles then egress in a manner resembling exocytosis, with fusion of the vesicular and plasma membranes and release of virions into the extracellular space. As different tegument proteins are seemingly involved in a number of steps post-nucleocapsid assembly, identifying the role of a particular tegument protein in virion morphogenesis necessarily involves study of when and where during the egress process the protein associates with nascent virions. While the nuclear steps are thought to involve conserved tegument proteins, such as herpes simplex virus 1 (HSV-1) UL34 and UL31 (29) and their gammaherpesvirus homologues, ORF67/BFRF1 (17) and ORF69/BFLF2 (15), the cytoplasmic stages of egress appears to involve additional tegument proteins unique to each subfamily of *Herpesviridae* (16, 29, 30). Though several tegument proteins are conserved in all herpesviruses, a large proportion of the tegument protein density is apparently composed of proteins unique to each subfamily of herpesvirus (4, 6, 23, 30, 32, 55).

In gammaherpesviruses, the functions of many of these tegument proteins are virtually unexplored. Several studies have recently identified the protein constituents of the virions of gammaherpesviruses, including murine gammaherpesvirus 68 (MHV-68), Epstein-Barr virus (EBV), Kaposi's sarcoma-associated herpesvirus (KSHV), and rhesus monkey rhadinovirus (RRV), by mass-spectrometric analyses (4, 6, 23, 31, 32, 37, 55). MHV-68 is a facile model for studying the gammaherpesvirus lytic phase (33, 41, 44, 51, 52), particularly virion structure, composition, and morphogenesis (6, 42, 48). Detergent sensitivity experiments and comparative genomics data suggest that a number of uncharacterized virion proteins are tegument proteins. The functions of these MHV-68-specific tegument proteins can be studied by mutagenesis, taking advantage of herpesvirus genomes cloned as bacterial artificial chromo-

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somes (BACs) (1, 5, 10, 40, 41). Analysis of mutants null for genes encoding tegument proteins, with phenotypes for the assembly and egress of virions or for the immediate-early phases of infection of a naïve cell, promises to unravel the functional roles of these gammaherpesvirus-specific tegument proteins. Indeed, one such protein, encoded by MHV-68 *ORF45*, is essential for gene expression in the immediate-early phase of infection (21). In KSHV, the ORF45 tegument protein is capable of binding IRF7 and preventing its translocation to the nucleus, thus inhibiting the secondary response to type I interferon signals (56, 57).

This paper explores the function of a gamma subfamily-specific protein, encoded by MHV-68 *ORF52*, in the lytic phase of infection. To study this protein, we took advantage of a model for the gammaherpesvirus lytic cycle, MHV-68, that is capable of completing the lytic program and productively infecting cultured cells without chemical or genetic induction (43, 48, 52). MHV-68 ORF52 is an abundant virion protein (6), as are its homologues in KSHV (with which it shares 28% identity) (55), EBV (BLRF2; 40% identity) (23, 38), and RRV (32). In MHV-68, *ORF52* is expressed abundantly as a 0.4-kb transcript with true-late kinetics, activated after viral DNA replication (2, 12, 28). Transposon mutagenesis of the MHV-68 genome cloned as a BAC indicates that *ORF52* is essential to virus replication (41). The EBV gene product homologue, BLRF2, is a highly immunogenic protein, a target for antibody responses in acute EBV infection and a useful biomarker for lytic EBV infection (14, 19, 35). However, the function of the ORF52/BLRF2 family of proteins is unknown. Here, we report that the MHV-68 ORF52 tegument protein is essential to complete virion morphogenesis and egress in the cytoplasm.

MATERIALS AND METHODS

Viruses and cells. Wild-type (WT) MHV-68 was originally obtained from the American Type Culture Collection (VR1465). Working stocks of WT MHV-68 BAC virus and the revertant of 52S (52R) were generated by transfecting BAC DNAs (400 ng) into 293T cells with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) and incubation in Opti-MEM I (Gibco, Grand Island, NY) medium without antibiotics, according to the manufacturer's recommendations, and collecting supernatant after 3 days. Viral titers were determined by plaque assay with BHK-21 cells as previously described (measured in PFU) (51), and viral DNA was quantified by SYBR green quantitative PCR (q-PCR). BHK-21, NIH 3T3, and 293T cells were cultured in complete Dulbecco's modification of Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

PCR, cloning, and plasmids. An enhanced green fluorescent protein (EGFP) fusion expression plasmid containing full-length MHV-68 *ORF52* was generated by PCR amplification from genomic herpesvirus DNA and cloning in frame into pEGFPC1 (Clontech, Mountain View, CA) with primers 1 and 2 (Table 1). Two N-terminal FLAG-tagged MHV-68 *ORF52* expression constructs were similarly generated by cloning *ORF52* into pFLAG-CMV2 (Sigma-Aldrich, St. Louis, MO) at BglII and XbaI sites with primers 3 and 2 and by cloning *ORF52* into the cytomegalovirus immediate-early promoter-driven pcDNA5/TO vector (Invitrogen) at KpnI and XbaI sites with primers 4 and 2) to generate *FLAG-ORF52*. Plasmids were transfected into NIH 3T3 murine fibroblasts or human 293T cells with Lipofectamine 2000, according to the manufacturer's recommendations, for localization and complementation experiments.

Generation of MHV-68/BAC *ORF52*-null and revertant viruses. The *ORF52*-null MHV-68(BAC) plasmid (52S) was generated by allelic exchange in *Escherichia coli*, following procedures described previously (22). The BAC sequence was inserted between nucleotides (nt) 1838 and 1839 of the viral genome without disrupting any known genes (1, 22, 48). To construct a shuttle plasmid, a 700-bp fragment spanning *ORF52* and flanking sequences was generated by a two-step PCR with a proofreading DNA polymerase and primers 5, 6, 7, and 8, in which triple nonsense and frameshift open reading frame (ORF) mutations and a HindIII site were introduced into *ORF52* (nt 71325 to 71343). This PCR fragment cloned into the NsiI-SphI sites of ampicillin (Amp)-resistant plasmid pGS284 (a gift of Greg Smith, Northwestern University) harbored in *E. coli* GS111, sequenced, and used as the donor strain for allelic exchange with recipient strain GS500 (*recA*$^+$), harboring chloramphenicol (Cam)-resistant MHV-68/BAC. Cointegrates were isolated in Cam (34 μg/ml) and Amp (95 μg/ml), resolved by 14 h growth at 32°C in Cam alone, and negatively selected against retention of unintegrated shuttle plasmid on plates with LB and Cam and lacking NaCl, supplemented with 5% sucrose. Colonies were screened for Cam resistance and Amp sensitivity, and the incorporation of the mutation in *ORF52* determined by PCR with primer pairs 1 and 2, 5 and 6, and 9 and 10, followed by HindIII digestion and sequencing. To generate a WT revertant of 52S, designated 52R, a 1,000-bp fragment of WT *ORF52* was cloned into pGS284 with primers 9 and 10, cointegrated with GS500 bacteria harboring 52S, and resolved. BAC DNAs for MHV-68/BAC, 52S, and 52R clones were purified by a midi prep kit (QIAGEN, Valencia, CA).

TABLE 1. Primers used in this study

Primer	Sequence (5′ to 3′)	Position[a]
1	ggccAGATCTgcgatgatgatggcgtccaaaaagcct	71364
2	ggccTCTAGA**tta**ttcatgatcatgtctgtgtctt	70958
3	ttttAGATCTgcgtccaaaaagcctgataaaacc	71361
4	ttggGGTACCcc *atggactacaaagacgatgacgacaag*	71361
5	tgatAAGCTT**tcaattaatcaa** gtcaaggaagtagaaaggctt	71325
6	tgac**ttgattaattga** AAGCTTatcaggctttttggacgccat	71343
7	tgacAGATCTcctggtcgtggttctttttttgatgtgtgcc	71693
8	ggccATGCATtattcatgatcatgtctgtgtct	70959
9	tgctATGCATgcttgggcacaaagga	71836
10	tgcaAGATCTctttggatacgacccac	70827

[a] 5′ nucleotide position in the MHV-68 genome (GenBank accession NC_001826). Boldface indicates stop codons, italics indicate FLAG codons, and uppercase letters indicate restriction enzyme sites.

Restriction fragment and Southern blot analyses of MHV-68/BAC clones. BAC DNAs were digested for 12 h at 37°C with restriction enzyme HindIII or EcoRI, electrophoresed on a 1× Tris-acetate-EDTA–0.8% agarose gel, and analyzed by ethidium bromide staining. Restriction fragments were matched with predicted MHV-68/BAC genomic fragments by Gene Construction Kit software (Textco BioSoftware, West Lebanon, NH). DNA fragments in gels were transferred to Hybond N+ nitrocellulose membranes (Amersham Pharmacia Biotech) by Southern blotting, UV-cross-linked, probed, and washed by using standard protocols (22). The probe, a 700-bp PCR product in the *ORF52* locus (primers 2 and 7), was generated with a random priming kit using [α-^{32}P]dCTP (RediPrime II, Amersham Biosciences, Piscataway, NJ) and solid-phase cleanup using a MicroSpin G-25 minicolumn (Amersham Pharmacia Biotech). Radioactivity was detected on a STORM imager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

52S transfection and complementation. Phenotyping and complementation of 52S by transfection was as follows. WT MHV-68/BAC, 52S, and 52R BAC DNAs (300 ng/well) plus empty vector (100 ng/well) or 52S plus plasmid DNA expressing *FLAG-ORF52* (100 ng/well) were transfected into subconfluent 293T cells with Lipofectamine 2000 reagent (Invitrogen), incubated in Opti-MEM I medium (Gibco) without antibiotics, according to the manufacturer's recommendations, with medium changed after 12 h. At intervals posttransfection, supernatant was collected and cleared of any debris by centrifugation at 1,500 × *g*, for a plaque assay (51) and to quantify released viral DNA by q-PCR, and cells were lysed in 1× passive lysis buffer (PLB) (Promega, Madison, WI) to analyze viral protein expression and viral DNA replication. Cultures were observed at 6 days posttransfection for cytopathic effect (CPE).

Infection with 52C virus. Complemented 52C virus was obtained from supernatants of 293T cells cotransfected with 52S and *FLAG-ORF52*. 52C virus could be propagated on *ORF52*-expressing 293T cells that had been transfected one day prior to 52C infection with *FLAG-ORF52* expression plasmid (293T/f52 cells). Viral DNA in 52C viral stocks was quantified by q-PCR, and infectivity per genome copy was estimated by a limiting dilution series on 293T/f52 cells scoring for CPE at 1, 2, 5, and 6 days postinfection (d.p.i.), measurement of replicated viral DNA by q-PCR, and Western blotting to detect viral late antigens. All cotransfections were screened for inadvertent reversion by attempted propagation on 293T and BHK-21 cells. The reversion rate was <1 PFU/10^8 viral DNA copies. MHV-68/BAC and 52R viruses were estimated to have a ratio of 35 ± 6

genome copies/infectious unit (PFU), and 52C virus had a ratio of 450 ± 90 genome copies/infectious unit. For infections, NIH 3T3, 293T cells, or 293T/f52 cells were inoculated with 0.1 or 10 infectious units/cell of MHV-68/BAC, 52R, and 52C viruses, and inocula were removed after 6 h. At intervals postinfection, viral DNA was purified from supernatants and cells and quantified by q-PCR, and viral protein was analyzed by Western blotting.

Real-time q-PCR. Transfected and infected 293T cells were analyzed for viral DNA replication and release into the supernatant in a duplicate 96-well format. Viral DNA was isolated from supernatants clarified by centrifugation at 1,500 × *g* by using a scaled DNeasy protocol (QIAGEN) or from cell pellets in 1× PLB by rapid liquid N_2 freeze–37°C thaw once, incubation with 1 ng/μl proteinase K (Sigma-Aldrich) at 37°C for 1 h, extraction in 25:24:1 phenol-chloroform-isoamyl alcohol, precipitation in 70% ethyl alcohol at −20°C for 12 h, a cold 70% ethyl alcohol wash, and resuspension in 1× PCR buffer. Duplicate 25-μl q-PCRs with 5% (by volume) of supernatant DNA, 25% (by volume) of extracted cellular DNA, or MHV-68/BAC DNA standards were analyzed using a Platinum SYBR green q-PCR kit (Invitrogen) and primers within MHV-68 *ORF57* (sense, 5′-ACTGAAACCTCGCAGAGGTCC-3′; antisense, 5′-GCACGGTGCAATGTGTCACAG-3′). q-PCR using the iQ SYBR green supermix PCR kit (Bio-Rad, Hercules, CA) or *ORF65* primers (sense, 5′-GTCAGGGCCCAGTCCGTA-3′; antisense, 5′-TGGCCCTCTACCTTCTGTTGA-3′) yielded comparable sensitivity and reproducibility. All q-PCRs were performed with a DNA Engine Opticon II instrument (MJ Research/Bio-Rad) and viral copy numbers analyzed with Opticon and Microsoft Excel software calibrated to standards ranging from 8 to 1.6×10^7 MHV-68/BAC genome copies.

Terminal repeat assay. Total viral DNA was purified by q-PCR (see above) at 1, 3, 4, and 6 days posttransfection from 293T cells transfected with MHV-68/BAC DNA, 52S, or 52S-plus-*FLAG-ORF52* cotransfection scaled to a 24-well format. Extracted DNA was resuspended in 1× NE buffer no. 2 (New England Biolabs, Ipswich, MA), HindIII digested, separated by 1× Tris-acetate-EDTA–0.8% agarose gel electrophoresis, and Southern blotted as describe above. The Southern blot was probed with a 0.8-kbp double-stranded DNA fragment (nt 117560 to 118314) PCR-amplified from an MHV-68 genomic region adjacent to the terminal repeat (51).

Viral particle isolation and detergent sensitivity assays. Viral particles were purified from the supernatants of MHV-68/BAC-transfected and 52S/*FLAG-ORF52*-cotransfected 293T cultures by discontinuous sucrose density gradient ultracentrifugation as previously described (6). Intracellular viral particles were released from pelleted 52S-transfected 293T cells at 6 days posttransfection by rapid liquid N_2 freeze–37°C thaw once in 20 mM Tris, pH 7.8, and 50 mM NaCl (TN buffer), supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Particle preparations were cleared of insoluble debris by centrifugation at 3,500 × *g*, and ultracentrifuged through a two-step 25% and 50% sucrose cushion at 42,000 × *g* in an SW41Ti rotor (Beckman, Palo Alto, CA) at 4°C for 2 h with slow acceleration and braking. The visible band at the interface was collected, pelleted at 89,000 × *g*, and resuspended in TN buffer for electron microscopy, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and Western blot analyses. Detergent sensitivity assays were performed as previously described (6). For dissociation of ORF52, virions (2,000 PFU) were left untreated or treated with detergents for 15 min at 37°C and pelleted in a tabletop centrifuge at 4°C and 20,000 × *g* for 45 min. Detergents used were as follows: radioimmunoprecipitation A buffer (RIPA buffer) containing 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, and 1 mM EDTA; HiNa medium, containing 250 mM NaCl, 20 mM Tris-HCl, pH 7.8, 2% NP-40, and 1 mM EDTA; and TxNP medium, containing 10 mM Tris-HCl, pH 8.5, 4% NP-40, 2% Triton X-100, 0.15% SDS, and 1 mM EDTA.

Antibodies, immunoblotting, and immunofluorescence. Polyclonal antibodies against MHV-68 ORF26, ORF45, ORF52, ORF62, ORF65, and ORF67 proteins were generated by cloning ORFs in frame into pET30b+ vector (Novagen), and for generating anti-ORF52 polyclonal serum, a full-length *ORF52* PCR fragment was also cloned into the pQE70 (QIAGEN) bacterial expression vector. Briefly, for each antiserum, protein expression in log-phase *E. coli* strain BL21 was induced with IPTG (isopropyl-β-D-thiogalactopyranoside; 1 μg/ml) for 2 to 4 h, bacteria were lysed, and His-tagged viral protein was batch isolated on Ni-nitrilotriacetic acid agarose beads (QIAGEN), washed, and eluted according to the manufacturer's instructions. The quality of purified His-ORF52 was determined by SDS-PAGE with Coomassie blue detection and Western blotting and detection with anti-(penta-)His monoclonal antibody (QIAGEN) and anti-MHV-68 serum. Individual bacterially expressed proteins were used to immunize New Zealand White rabbits (Covance, Denver, PA) to generate polyclonal antisera. Anti-gp150 and anti-MHV-68 sera were a kind gift of James Stewart (University of Liverpool, United Kingdom) and have been described previously (42, 52). Mouse monoclonal antibody M2 against a FLAG epitope was purchased from Sigma (St. Louis, MO). For Western blotting, after primary antibody incubation, anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated with horseradish peroxidase (Sigma) was used as a secondary antibody. Antigens were detected by chemiluminescence (ECL+PLUS; Amersham Pharmacia Biotech) with a STORM imager and analyzed by ImageQuant. The indirect immunofluorescence assay was performed as described previously (22), with either M2 monoclonal antibody followed by anti-mouse IgG conjugated with Cy3 (Amersham Pharmacia Biotech) or anti-ORF65 or anti-p115/Golgi apparatus (Santa Cruz Biotechnology, Santa Cruz, CA) antisera followed by Cy3-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) secondary antibody. Chromatin was stained with DAPI (4′,6′-diamidino-2-phenylindole), slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA), and images were captured by an epifluorescent Leica DMIRB microscope with a mounted MagnaFire SP charge-coupled-device camera (Olympus, Melville, NY) and Adobe Photoshop software.

Electron microscopy. Stages of viral assembly and particle morphology were determined by cryo-electron microscopy as previously described (6, 54). Cryo-electron microscopy micrographs of viral particles embedded in vitreous ice were recorded at 100 kV in a JEOL JEM1200 electron cryomicroscope (JEOL Ltd., Tokyo, Japan), at a magnification of ×30,000 with an electron dosage of ~6 electrons/Å. For thin-section TEM, 293T cells were transfected with MHV-68/BAC or 52S or were cotransfected with the 52S/*FLAG-ORF52* expression construct, collected at 6 days posttransfection in phosphate-buffered saline, fixed in 2% electron microscopy-grade glutaraldehyde (Ted Pella, Redding, CA) in phosphate-buffered saline at 4°C for 12 h, postfixed in 1% OsO_4, dehydrated, and embedded in Epon. Approximately 60- to 70-nm-thin sections were stained with 2% uranyl acetate and 0.3% lead citrate and examined at 80 kV on a JEOL JEM-100CX electron microscope (47).

Mass spectrometry. Proteins in purified viral particles were separated by denaturing SDS-PAGE (10%), stained with SYPRO Ruby (Molecular Probes, Eugene, OR), excised, and digested in gel with mass spectrometry-grade modified trypsin Gold (Promega), and peptides were extracted (6, 39, 50) for mass and fragment ion analyses by micro-liquid chromatography with tandem mass spectrometry (μ-LC/MS-MS) using a quadrupole time-of-flight hybrid mass spectrometer with data-dependent acquisition (QSTAR XL; MDS Sciex, Concord, ON, Canada). LC/MS-MS-generated peptide product ion mass spectra were displayed by integral Analyst QS software and then matched to the predicted MHV-68 proteome with Mascot software (Matrix Science, Boston, MA). Detection of ORF64 in WT MHV-68 virions was confirmed by μ-LC/MS-MS with peptide matching by using Sequest software as previously described (6, 50).

RESULTS

MHV-68 *ORF52* encodes a capsid-associated tegument protein. As members of the ORF52/BLRF2 protein family have been identified by mass spectrometry as abundant components of virions in a number of gammaherpesviruses, including MHV-68 (6), KSHV (4, 55), EBV (23), and RRV (32), we postulated that they could be involved in virion morphogenesis or modulate virion infectivity. We first sought to understand the packaging of the MHV-68 ORF52 protein into virion particles. Virion-associated ORF52 protein appears predominantly as an abundant 21-kDa protein (Fig. 1A), in agreement with the majority of peptides matching the protein detected by LC/MS-MS (6). As the capsid proteins in MHV-68 have been identified (6) and are homologous to the conserved herpesvirus capsid proteins (48), we reasoned that ORF52 is likely a component of either the tegument or envelope compartments of the mature virion. We then determined the detergent sensitivity of ORF52 in virions and compared it to those of known capsid (ORF26 and ORF65), tegument (ORF45), and envelope glycoproteins (glycoprotein 150) (6). Under treatment with 2% Triton X-100, ORF52 is primarily found in the detergent-resistant fraction, which includes the capsid proteins and a portion of the ORF45 tegument protein found in virions (Fig. 1A). The detergent-sensitive fraction, on the other hand, con-

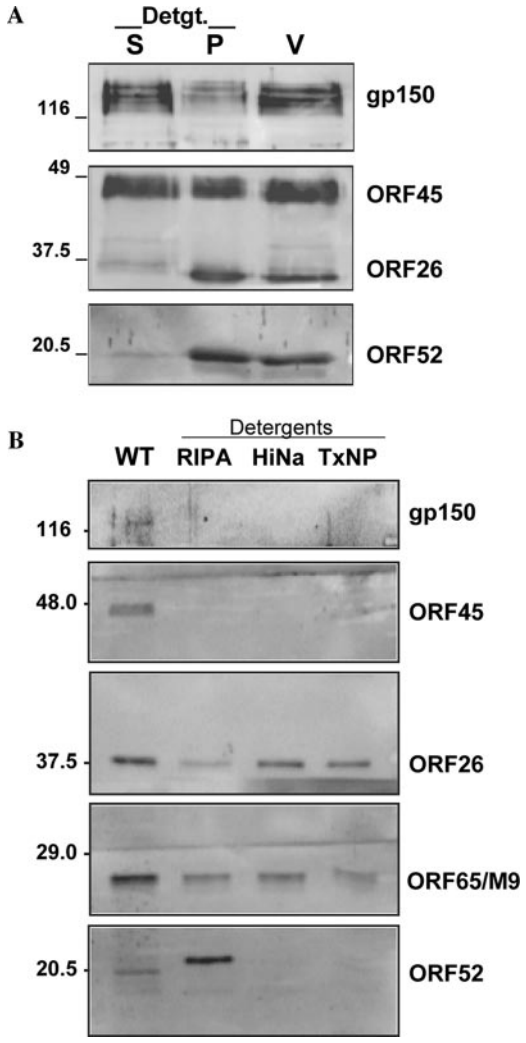

FIG. 1. MHV-68 *ORF52* encodes a capsid-associated tegument protein. (A) Identification of ORF52 virion protein. Virions (V, approximately 5,000 PFU) were incubated with 2% Triton X-100 and pelleted. Supernatant (S) and pellet (P) were removed, denatured in Laemmli buffer, and separated on a 13% SDS-polyacrylamide gel. Western blots are incubated with polyclonal antisera generated in New Zealand White rabbits to recombinant viral proteins glycoprotein 150 (upper panel), ORF45 (middle panel, top row), ORF26 (middle panel, bottom row), and ORF52 (lower panel). Molecular mass markers, in kDa, are shown. Detgt., detergent. (B) Dissociation of ORF52 protein from the capsid. Virions were left untreated (WT) or treated with indicated detergents for 15′ at 37°C and pelleted in a tabletop centrifuge at 4°C, 20,000 × *g* for 45 min. Detergents were RIPA buffer, 2% high-salt NP-40 (HiNa), or 2% Triton X-100 plus 4% NP-40 (TxNP). Western blots are incubated with polyclonal antisera generated in New Zealand White rabbits to the recombinant viral proteins glycoprotein 150, ORF45, ORF26, ORF65/M9, and ORF52. Molecular mass markers, in kDa, are shown.

tains much of the ORF45 protein and almost all of the gp150 present in virions. Thus, ORF52 represents a tegument protein that is more tightly bound to the capsid than the ORF45 tegument protein is.

Furthermore, changing the detergents for treatment of extracellular MHV-68 virions resulted in complete dissociation of ORF52 protein from the capsid fraction (Fig. 1B). Nonionic detergent combined with high-salt extraction of extracellular MHV-68 virions (250 mM NaCl with 2% NP-40) could dissociate ORF52 (and ORF45) from the pelleted phase, which retained the integral ORF26 triplex protein and ORF65, a capsid protein that decorates the outside of nucleocapsids (26, 31, 53). A combined 4% NP-40, 2% Triton X-100, and 0.15% SDS treatment also removed outer tegument and ORF52 but allowed retention of ORF65. However, ORF45 protein was completely removed from the capsid by treatment with RIPA buffer, while ORF52 was selectively retained, with a slightly slower mobility. These results suggest that ORF52 is more tightly bound to the capsid than ORF45 and that differing detergent treatment conditions allow for selective removal of either ORF45 alone or both ORF45 and ORF52.

Thus, ORF52 can be classified as a capsid-associated tegument protein, as opposed to an outer tegument protein, like ORF45, according to these results. The EBV BLRF2 protein (19 kDa), as well as the RRV ORF52 protein (15 kDa), is a virion protein with low detergent sensitivity, suggesting conservation of capsid-associated packaging of this family of proteins among different gammaherpesviruses (23, 32).

MHV-68 ORF52 protein localizes to the cytoplasm. As the subcellular localization of tegument proteins is inherently related to their functions (29, 30), we studied the subcellular localization of gammaherpesvirus ORF52 family proteins in murine fibroblasts permissive to viral replication by expressing them as EGFP fusion proteins and by immunofluorescence. The MHV-68 EGFP-ORF52 fusion protein localizes predominantly to distinct punctae in the cytoplasm (Fig. 2c). In many transfected cells, there is also a diffuse cytoplasmic localization, consistent with localization of transfected FLAG-ORF52 protein (Fig. 2b). This pattern partially colocalizes with p115, a cytoplasmic marker for compartments derived from the Golgi apparatus and vesicles in the secretory pathway (Fig. 2c and f). The polyclonal anti-ORF52 serum that we generated provided insufficient specificity in the immunofluorescence assay. Therefore, to study the localization of ORF52 in the context of infection, we infected cells expressing EGFP-ORF52 with WT MHV-68. The distinct localization pattern observed in MHV-68 EGFP-ORF52 transfectants is preserved in cells infected with WT MHV-68 at a high multiplicity of infection (i.e., 2) (Fig. 2g and h). Thus, the cytoplasmic localization of ORF52 protein is reminiscent of viral interaction with the secretory pathway or cytoskeletal components.

Construction of an *ORF52*-null mutant. In order to understand the function of *ORF52* in the MHV-68 lytic phase, we constructed an *ORF52*-null mutant virus in an MHV-68/BAC system. We have established an MHV-68/BAC system in the laboratory and have successfully mutated a number of MHV-68 genes by allelic exchange, including *ORF31* and *ORF45* (21, 22), as well as analyzing the genetic requirements for almost all known and suspected MHV-68 genes by signature-tagged transposon mutagenesis (41). Although *ORF52* was identified as being essential for productive virus replication in cultured cells, insertion of the 1.2-kbp Mu transposon may have disrupted the expression of genes proximal to the *ORF52* locus. Therefore, we introduced a stop codon into all three leftward-reading ORFs, and a HindIII site, near the 5′ end of the predicted *ORF52* gene (Fig. 3A). The mutated site, nt 71325 to 71343 in the MHV-68 genome, also eliminates the codon for Met-13, which is a possible secondary initiation codon for an *ORF52*-encoded protein (48). Viral BAC

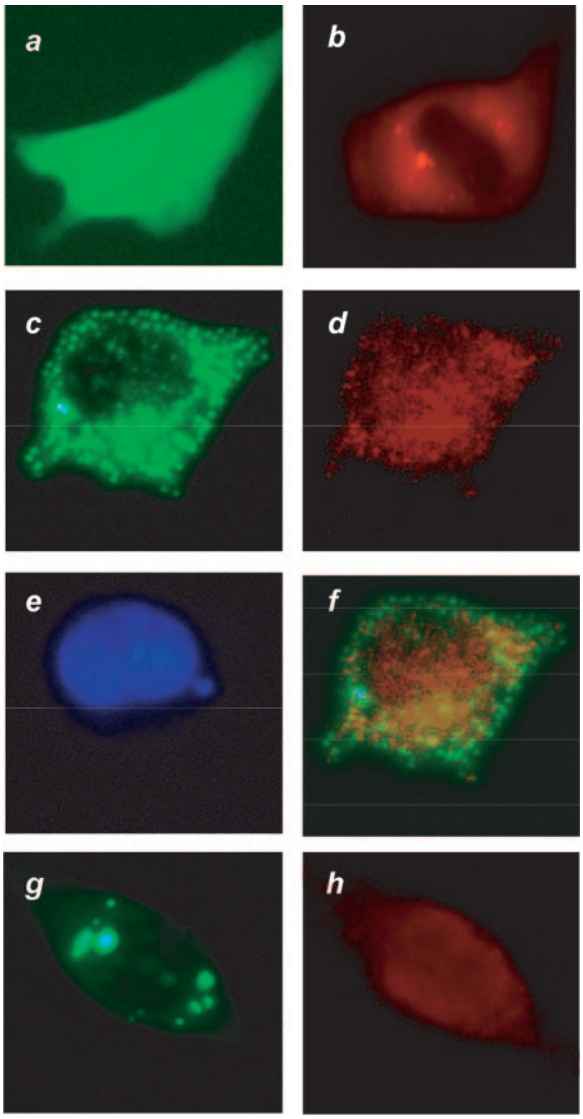

FIG. 2. Localization of MHV-68 ORF52 protein. NIH 3T3 cells were transfected with tagged expression constructs encoding full-length predicted fusions of viral ORF proteins, fixed, and imaged by fluorescence microscopy or fixed and permeabilized for indirect immunofluorescence assay. Representative images are shown. Localizations of EGFP alone (a), FLAG-ORF52 by immunofluorescence assay (b), and MHV-68 EGFP-ORF52 (c) are shown. Images from an immunofluorescence assay with anti-FLAG monoclonal antibody and anti-mouse/Cy3 secondary antibody (b) or anti-p115/Golgi apparatus and anti-rabbit/Cy3 secondary antibody (d) are shown. DAPI staining (e) and a merge (f) of fixed transfected cells in panels c and d are also shown. NIH 3T3 cells expressing MHV-68 EGFP-ORF52 infected 1 day posttransfection with WT MHV-68 (multiplicity of infection, 2) (g), probed 24 hours postinfection by immunofluorescence assay with polyclonal anti-ORF65 antibody and anti-rabbit/Cy3 secondary antibody (h).

DNA from selected recombinants was examined by restriction analysis and Southern blotting, confirming the mutation in *ORF52* (Fig. 3B). A mutant MHV-68/BAC clone (Fig. 3B, clone 1) exhibiting insertion of the enzyme site and the expected 11-kbp and 4-kbp bands on the Southern blot and an otherwise WT MHV-68/BAC restriction fragment pattern analyzed with two restriction enzymes (HindIII and EcoRI) was designated MHV-68/BAC/*ORF52-stop* or simply 52S. The revertant 52R was generated from the MHV-68/BAC/*ORF52-stop* clone by allelic exchange, eliminating the null mutation and replacing it with a WT copy of the *ORF52* gene.

52S is deficient in the release of infectious viral particles but can be rescued by expression of FLAG-ORF52. To identify the stage in the lytic phase at which *ORF52* is required, we transfected 52S (BAC), WT MHV-68 (WT/BAC), and 52R (BAC) viral DNAs into mammalian cells and screened for their ability to establish productive infection. It has been shown previously that transfecting WT MHV-68 (or WT/BAC) genomic DNA into cultured fibroblasts is sufficient to induce viral replication and the production of infectious extracellular virus (7, 52). Whereas transfecting WT MHV-68 (WT/BAC) or the revertant, 52R, resulted in high levels of CPE in mammalian cell cultures, transfecting 52S did not (Fig. 4A). This result is consistent with the classification of *ORF52* as an essential gene by transposon mutagenesis (41). It is not surprising that 52S transfection does not result in the efficient release of infectious virus particles that are capable of forming plaques on susceptible BHK cells (Fig. 4B). The specificity of this requirement to the function of *ORF52* was established by the complete rescue of the mutant virus by reversion (52R) (Fig. 4A and B).

To determine whether the mutation in *ORF52* specifically prevents the release of infectious particles, supernatants from WT/BAC or 52S transfections or from the 52S cotransfection with a plasmid exogenously expressing *FLAG-ORF52* (52C) were normalized for viral DNA content and viral capsid antigens recoverable in particles (Materials and Methods). Infection at low viral DNA copy numbers (0.1 genome copy/cell) with supernatant containing the putative complemented 52C virions resulted in a marked increase in the production of late lytic antigens in cells transiently expressing *FLAG-ORF52*, in comparison to infection of naive 293T cells (Fig. 4C). Infection with supernatants from 52S transfectants alone does not result in the expression of late lytic antigen, whether *FLAG-ORF52* is supplied exogenously or not. Infection of naive 293T cells with 52C supernatants (0.1 genome copy/cell) does not result in the efficient release of virions from the cells (as measured by viral DNA released into the culture supernatant) unless *FLAG-ORF52* is expressed in the cells (293T/f52 cells) (Fig. 4D). Infections with WT/BAC and 52R supernatants result in efficient release of virions, as expected, on 293T cells (Fig. 4D) and on 293T/f52 cells (not shown). The *trans*-complementation of 52S by cotransfection with the *FLAG-ORF52* expression plasmid does not rescue the plaque-forming ability of released virus particles, indicating that inadvertent reversion did not occur in the complementation experiment (not shown).

These results suggest that release of virions is recovered in cotransfection of 52S BAC DNA and *FLAG-ORF52*. Infecting cells exogenously expressing *FLAG-ORF52* with this complemented virus (52C) rescues its ability to propagate in tissue culture and provides a source of extracellular, complemented 52C virions for further analyses. The 52S clone, however, does not result in high levels of CPE, suggesting a severe attenuation in the lytic phase. The implication is that *ORF52* is required for the release of infectious virions capable of subsequent rounds of infection.

***ORF52* is not required for MHV-68 DNA replication.** In order to understand the requirement for ORF52 protein to com-

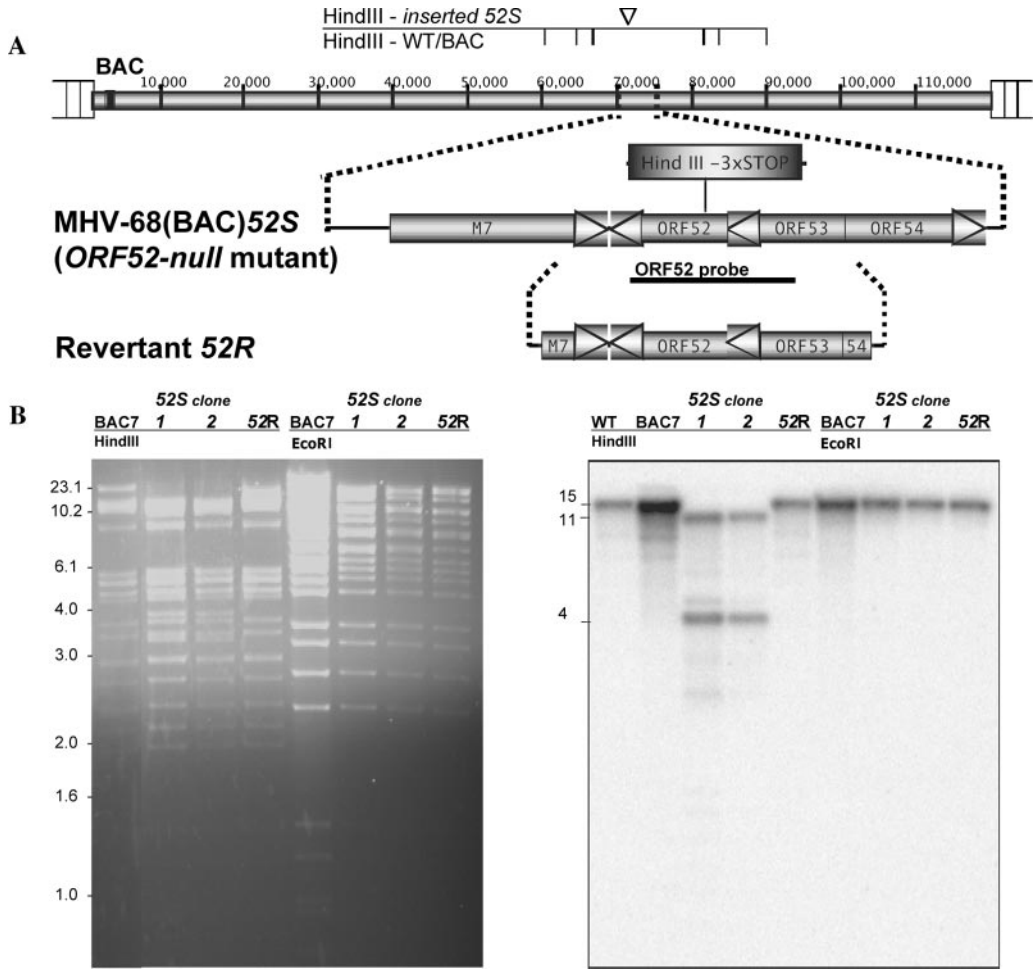

FIG. 3. Construction of an *ORF52*-null virus (52S) and its revertant (52R). (A) Cointegration and resolution of targeting constructs with MHV-68/BAC. A HindIII restriction site and stop codons in all three left-reading ORFs were introduced into a 700-bp fragment of the MHV-68 *ORF52* locus, amplified by two-step PCR with a proofreading DNA polymerase, cloned into shuttle plasmid pGS284, and sequenced. Plasmid was cointegrated with WT MHV-68 cloned as a BAC in *recA*$^+$ GS500 bacteria and resolved, and clones containing the insertion were screened by PCR and restriction digestion. Putative positive clones were further amplified, BAC DNA purified, and used for restriction digestion and Southern blot analyses and transfection of cell cultures. The positive BAC/*ORF52*-null mutant clone (clone 1 in panel B) is designated 52S here. A 1,000-bp fragment of WT *ORF52* was cloned into pGS284, cointegrated with G500 bacteria harboring 52S, resolved, and screened to derive the revertant BAC/*ORF52R* virus, designated 52R. (B) Restriction fragment and Southern analyses of 52S, 52R, and WT(BAC7) clones. BAC DNA was digested with two restriction enzymes: HindIII, to identify the inserted HindIII site in mutant clones and loss of HindIII site in revertant clones, and EcoRI, an unaltered site. Digested DNAs analyzed by ethidium bromide staining (left) and Southern blotting (right) probed with a 700-bp PCR product in the *ORF52* locus as a probe (right inset). Molecular mass markers, in kDa, are shown.

plete the lytic phase of MHV-68 infection, we determined whether *ORF52* is required for viral DNA replication. MHV-68 genome replication, like DNA replication by other herpesviruses, is thought to proceed through a rolling circle mechanism with subsequent cleavage of concatemers with different numbers of terminal repeats, followed by packaging into assembled capsids (11, 20, 36, 51). We examined viral DNA replication and processing by use of a terminal repeat assay (51). The accumulation of DNA concatemers with different lengths of terminal repeats indicates that input BAC DNA clones were replicated upon transfection with 52S BAC DNA, similar to the case with 52S-plus-*FLAG-ORF52* cotransfection and WT/BAC transfection (Fig. 5A). Thus, *ORF52* is required neither for viral DNA replication nor for cleavage of viral DNA concatemers, a prerequisite for packaging the DNA into nucleocapsids.

We also examined the requirement for an intact *ORF52* for viral DNA replication in infected cells. We isolated complemented MHV-68 virions containing an *ORF52*-null genome (52C virions) by transfecting 52S BAC DNA into complementing 293T cells expressing *FLAG-ORF52* (293T/f52 cells), harvesting supernatants, and determining titers by viral DNA quantification and limiting dilutions on 293T/f52 cells. Viral DNA replication was detected in cultured NIH 3T3 fibroblasts infected with 52C virus, resulting in an accumulation of viral DNA (Fig. 5B). q-PCR analysis showed that intracellular viral DNA content increased by approximately two orders of magnitude, indicating active replication of the MHV-68 genome. By 96 h postinfection, viral DNA increased from 1.7×10^3 copies/10^4 cells to 5.4×10^5 copies/10^4 cells in WT MHV-68-infected cells, from 1.4×10^3 copies/10^4 cells to 4.4×10^5

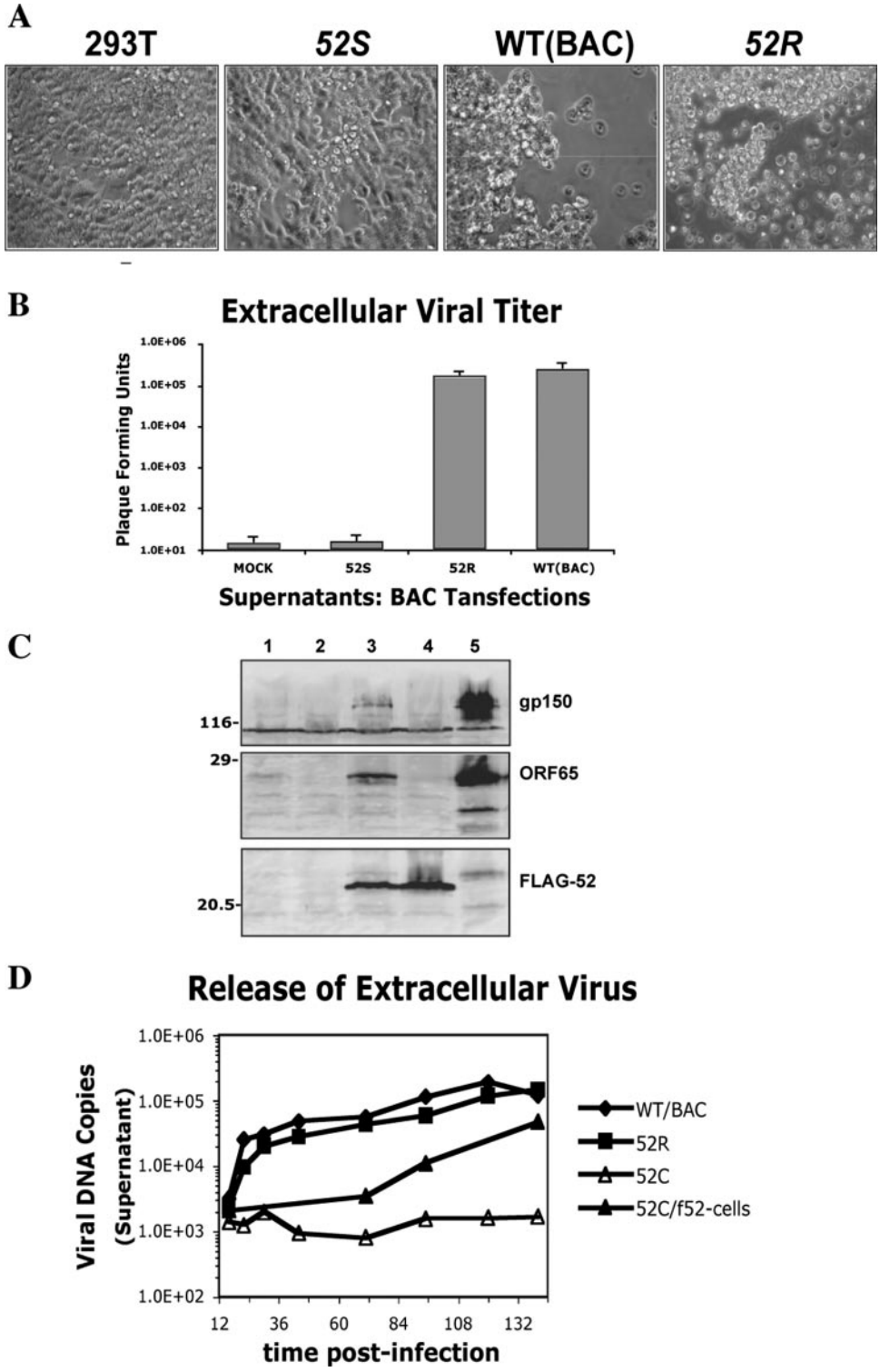

FIG. 4. *ORF52* is required for the production of infectious extracellular progeny. (A) 52S virus does not produce significant CPE in transfected cells. 52S, WT(BAC) MHV-68, and 52R BAC DNAs were transfected with high efficiency into 293T cells and imaged after 6 days by light microscopy. 293T, mock-transfected cells. (B) Supernatants of 52S-transfected cultures do not contain plaque-forming virus. Supernatants from BAC-transfected 293T cultures 6 days posttransfection were cleared of large debris by centrifugation (15,000 × *g*), used to infect BHK cells, and overlaid with methylcellulose media for the plaque assay. Plaques were scored in duplicate. (C) Propagation of 52S by providing FLAG-ORF52 in *trans*. Supernatants from 52S transfection (lanes 2 and 4), 52C cotransfection (52S plus a *FLAG-ORF52* expression construct; lanes 1 and 3), or WT/BAC transfection (lane 5) were collected as described for panel B and used to infect either untransfected 293T cells (lanes 1, 2, and 5) or complementing 293T/f52 cells expressing *FLAG-ORF52* (FLAG-52; lanes 3 and 4). Total cell lysates solubilized 4 d.p.i. were probed by Western blotting with anti-gp150, anti-ORF65 polyclonal serum, and anti-FLAG (M2) monoclonal antibody. Molecular mass markers, in kDa, are shown. (D) Recovery of released viral DNA in culture supernatants by propagation of 52S on cells expressing *FLAG-ORF52.* WT/BAC, 52R, and 52C supernatants (see description for panel B) were normalized for viral DNA content by quantitative PCR and used to infect 293T cells or 293T cells expressing *FLAG-ORF52*. At time points indicated postinfection (in hours), supernatants were clarified and viral DNA was isolated and quantified by SYBR green q-PCR with primers for MHV-68 *ORF57*.

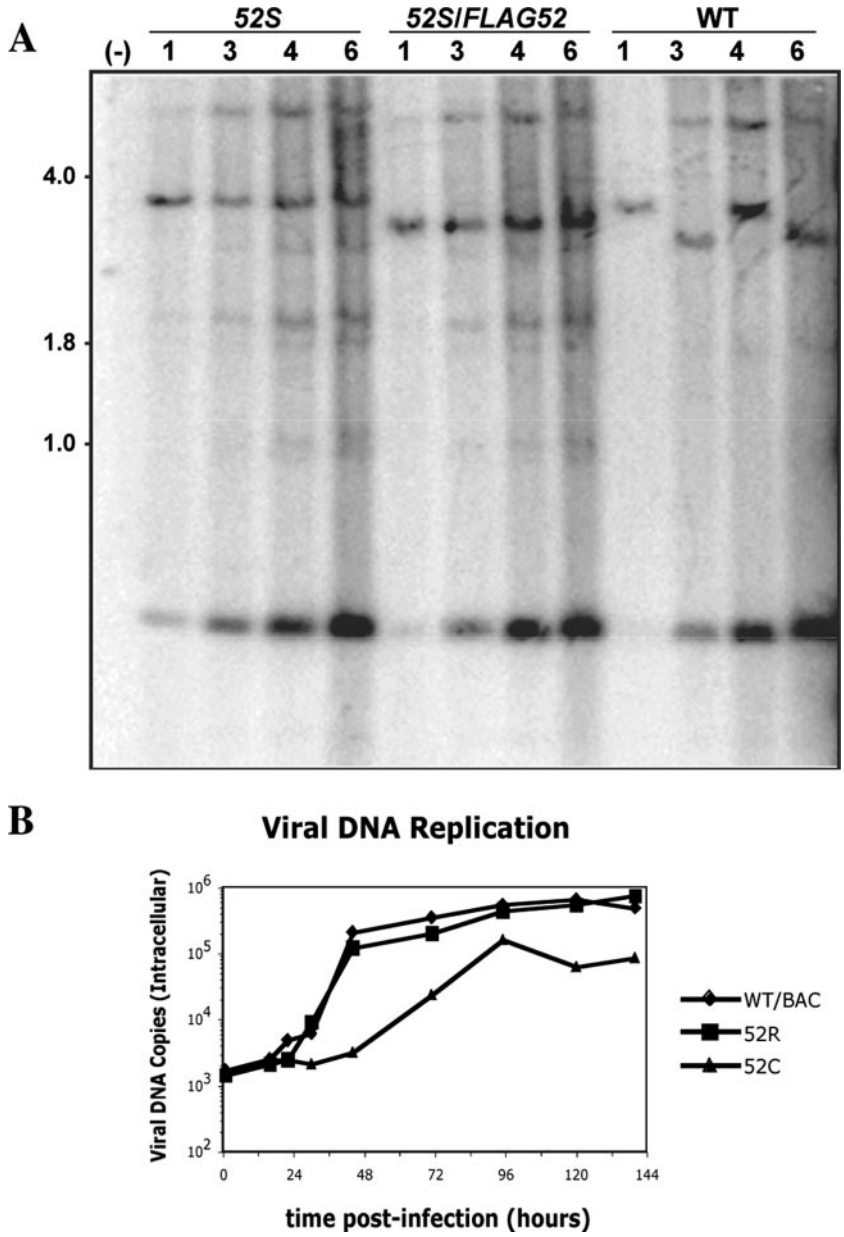

FIG. 5. Replication of viral DNA in 52S transfection and 52C infection. (A) Terminal repeat assay. Total DNA was purified from 293T cultures transfected with 52S, 52S-plus-*FLAG-ORF52* (FLAG52), or WT/BAC DNA at the indicated time points. BAC DNA was HindIII digested. Southern blots were probed with a PCR-amplified probe to the MHV-68 genomic region adjacent to the terminal repeat, specifying relative replication and terminal repeat cleavage variants. Molecular mass markers, in kDa, are shown. (B) Viral DNA replication measured after infection of NIH 3T3 fibroblasts with WT/BAC, 52R, or 52C *trans*-complemented virus. Total intracellular DNA purified and viral genome copy numbers were quantified by SYBR green q-PCR with primers for MHV-68 *ORF57*. Copy numbers are averages of two 96-well-format experiments and duplicate q-PCRs.

copies/10^4 cells in 52R-infected cells, and from 1.7×10^3 copies/10^4 cells to 1.6×10^5 copies/10^4 cells in 52C-infected cells. We noted that viral DNA replication in cells with the *ORF52*-null virus infection reached a lower final level of DNA replication than WT/BAC (by about fourfold). The difference may be attributed to the lack of efficient release of virions, effectively blocking a second round of infection, and the resulting further amplification of viral genomes observed in WT/BAC-infected cell cultures.

This phenotype contrasts sharply with the lack of extracellular viral DNA accumulation (Fig. 4D), indicating that while 52C infection results in entry and viral DNA replication, rerelease of viral DNA-filled virions is minimal. As viral DNA is replicated, it can be presumed that expression of immediate-early, early, and early-late (γ-1) genes required for viral DNA replication and progression through the lytic phase are not significantly affected by the loss of ORF52 protein.

***ORF52* is not required for lytic gene expression.** The expression of viral lytic proteins was examined in cells transfected with 52S BAC DNA by probing Western blots with polyclonal

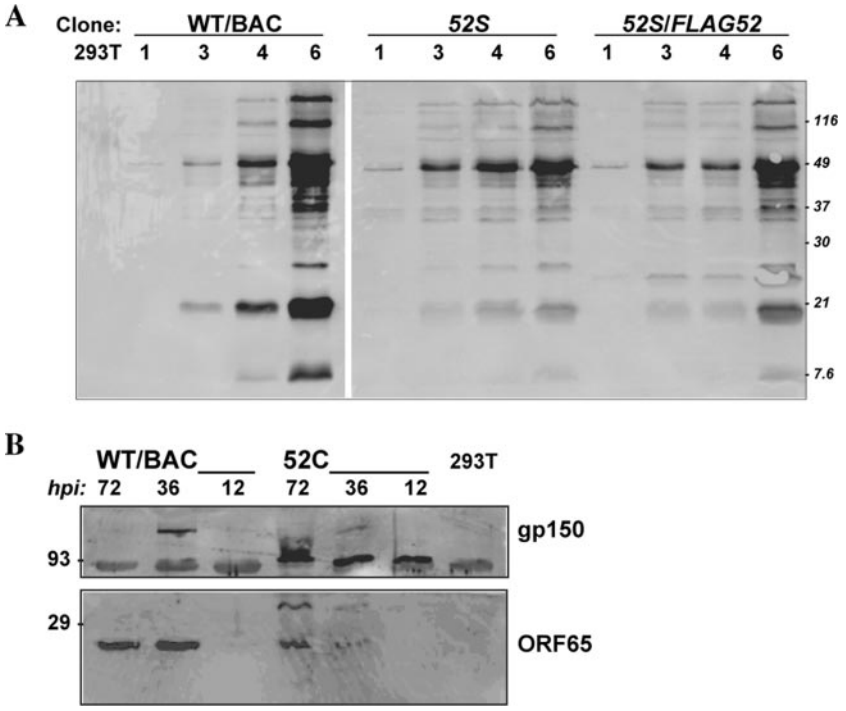

FIG. 6. Expression of lytic proteins in the absence of *ORF52*. Molecular mass markers, in kDa, are shown. (A) 52S exhibits high-level expression of lytic proteins. 293T cell cultures were transfected with WT(BAC), 52S, or 52S-plus-*FLAG-ORF52* expression construct (FLAG52) and harvested in PLB at time points posttransfection. Western blots probed with polyclonal anti-MHV68 antisera. (B) Expression of late proteins measured after infection of NIH 3T3 fibroblasts with WT/BAC or 52C *trans*-complemented virus or infection with mock-transfected 293T supernatant. Cell lysates were solubilized at the indicated time points postinfection in Laemmli buffer, separated by SDS-PAGE, Western blotted, and probed with anti-ORF26 (capsid) and anti-gp150 (glycoprotein) polyclonal antisera. hpi, hours postinfection.

anti-MHV-68 serum (Fig. 6A). In cells infected with 52C virus, the lytic phase progressed at least to the production of late lytic antigens, including virion proteins such as ORF65 (capsid) and gp150 (envelope glycoprotein) (Fig. 6B). Though lytic antigen expression does not fully reach WT levels in 52S-transfected (Fig. 5A and 6A) and 52C-infected (Fig. 5B and 6B) cultures, qualitatively, *ORF52* is not required for the initiation of late gene expression or for the viral life cycle to proceed to a measurable accumulation of lytic proteins. Similar to viral DNA replication, the lack of further rounds of infection in 52S-transfected and 52C-infected cultures may account for the apparently slower progression. Still, without a functional *ORF52* gene, there were significant levels of both viral DNA replication and expression of late lytic genes. Notably, viral DNA replication is required for the efficient expression of a subset of lytic genes (γ-2, or true-late genes) encoding virion structural proteins (12, 28). As a true-late gene product, the ORF52 protein likely functions late in the lytic phase, directly or indirectly, in either virion morphogenesis or release.

52S is deficient in a cytoplasmic stage of virion assembly and release. To better understand the nature of the lytic phase arrest caused by the null mutation in *ORF52*, we examined the ultrastructure of WT/BAC-, 52S-, and 52S-plus-*FLAG-ORF52*-transfected cells by thin-section TEM. Virion assembly and egress were examined by noting the existence of nascent viral particles at each stage of virion maturation, according to current models (29, 30), beginning with capsid formation and packaging in the cell nucleus, nuclear egress of nascent nucleocapsids into the cytoplasm to associate with tegument and envelope densities, and budding of complete virions into Golgi apparatus-derived compartments for egress via the cellular secretory pathway.

In WT/BAC, 52S, and 52S-plus-*FLAG-ORF52* transfections, herpesvirus capsid species were observed in cell nuclei, consistent with progression to a late stage of the lytic phase (29, 31). Empty or A capsids, as well as B capsids containing a scaffolding protein, and packaged DNA-filled C capsids were visible (Fig. 7, lower panels). In addition, at least the C capsid species in 52S-transfected cells are apparently capable of egress through the nuclear envelope into the cytoplasm. Partially tegumented nucleocapsids associated with electron-dense protein structures could be found juxtaposed to cytoplasmic membranous compartments in 52S-transfected cells (Fig. 7, 52S panel). However, we failed to find fully enveloped virions within cytoplasmic vesicles, or virions associated with or in the extracellular space adjacent to the cellular plasma membrane, as observed for WT/BAC-transfected and complemented (52S-plus-*FLAG-ORF52*) transfected cells (Fig. 7).

To quantify the block in virion assembly that occurs in the absence of *ORF52*, viral particles in various stages of maturation in electron micrographs were classified and enumerated (Table 2). Particles were classified as nuclear capsids (A, B, and C capsids), cytoplasmic A and B capsids, immature cytoplasmic virions (C capsids in the cytoplasm), or extracellular virions. WT/BAC and 52S transfectants had similar proportions of capsids to total particles in cell nuclei (0.47 and 0.55, respectively). Differences were noted in later stages of virion

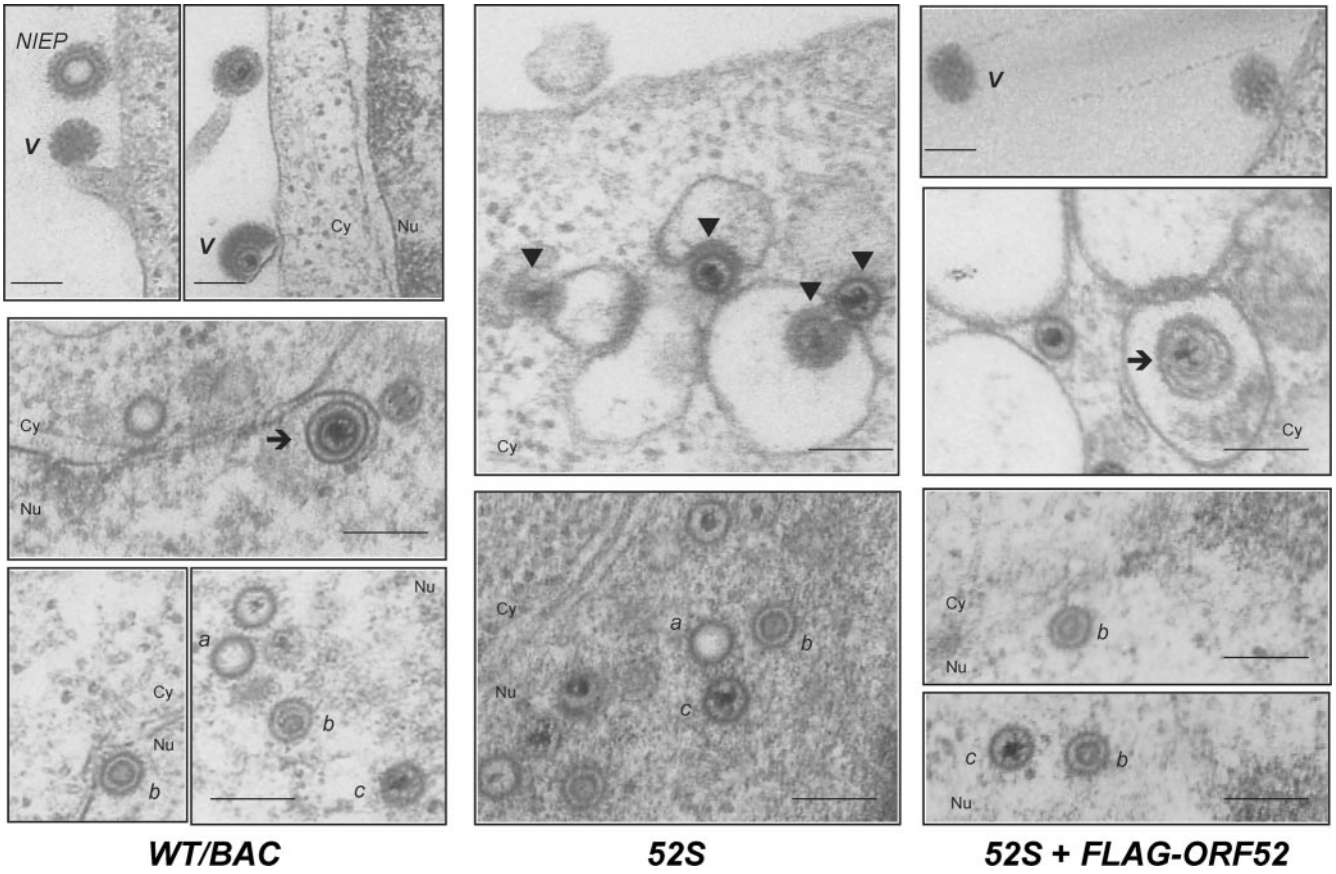

FIG. 7. Thin-section TEM of WT(BAC), 52S, and 52S-plus-*FLAG-ORF52*-transfected 293T cells. Approximately 70-nm-thin sections were examined 6 d.p.i. by TEM. Putative A capsid (a), B capsid (b), and C capsids containing packaged viral DNA core (c) were found in WT(BAC), 52S, and 52S-plus-*FLAG-ORF52*-transfected cell nuclei (Nu). In the cytoplasm (Cy), viral particles include enveloped-tegumented virions in WT(BAC) and 52C transfectants (→) and partially tegumented capsids in 52S (▼). In the extracellular space, WT(BAC) cultures contained putative egressed virions (V) and noninfectious enveloped particles (NIEPs) and 52S-plus-*FLAG-ORF52*-transfected cultures contained virions, while no identifiable virions NIEPs were found in 52S cultures. Bars, 200 nm.

egress, however. Whereas cytoplasmic stages of virion maturation appeared to proceed efficiently in WT/BAC-transfected cells, resulting in egress of virions, 52S-transfected cells exhibited a marked accumulation of immature virions in the cytoplasm, coupled with a complete absence of extracellular virions ($\chi^2 = 2.48$, relative to WT distribution; $P < 0.077$). Of all immature, DNA-containing viral particles in the cytoplasm in 52S transfections, approximately 20% appeared to be closely associated with electron-dense proteinaceous structures and cytoplasmic vesicular membranes, possibly arrested in a late stage of virion maturation (Fig. 7, 52S panel). These observations suggest that ORF52 is required to facilitate tegumentation/envelopment of nucleocapsids at a cytoplasmic stage of virion morphogenesis prior to virion egress.

TABLE 2. Distribution of viral particles

BAC transfection	No. of particles[a]	Fraction of total particles of indicated region			
		Nucleus (all capsids)	Cytoplasm		Extracellular virions[c]
			A and B capsids	Immature virions[b]	
52S	406	0.55	0.14	0.31	0
WT	174	0.47	0.01	0.11	0.41

[a] Total viral and subviral particles ennumerated in 14 TEM images, 7 days posttransfection.
[b] Fraction of immature virions, including C capsids in the cytoplasm of the total number of particles.
[c] Fraction representing fully mature extracellular virions.

Particles assembled in the absence of ORF52 contain conserved capsid proteins and a subset of tegument proteins. Without ORF52 protein, virions do not efficiently egress from the cell. We were unable to purify infectious virions from the supernatants of 52S-transfected cultures, unlike those of WT/BAC or 52C (Fig. 8; see below). We examined the nascent viral particles produced in 52S transfectants by cryo-electron microscopy. Unlike extracellular WT/BAC virions (6), particles released from 52S-transfected 293T cells by freeze-thaw treatment appear to have a structure similar to that of MHV-68 nucleocapsids, possibly with an inner, capsid-associated tegument layer (<15-nm radial thickness) but virtually devoid of envelope and outer tegument layers (Fig. 8A). Virion proteins present in extracellular WT/BAC virions, extracellular complemented 52C virions or 52S intracellular particles were identified by Western blotting (Fig. 8B). 52S particles, as well as 52C and WT/BAC virions, contain the conserved herpesvirus capsid proteins ORF62, ORF26, and ORF65 (6, 20, 31, 54). How-

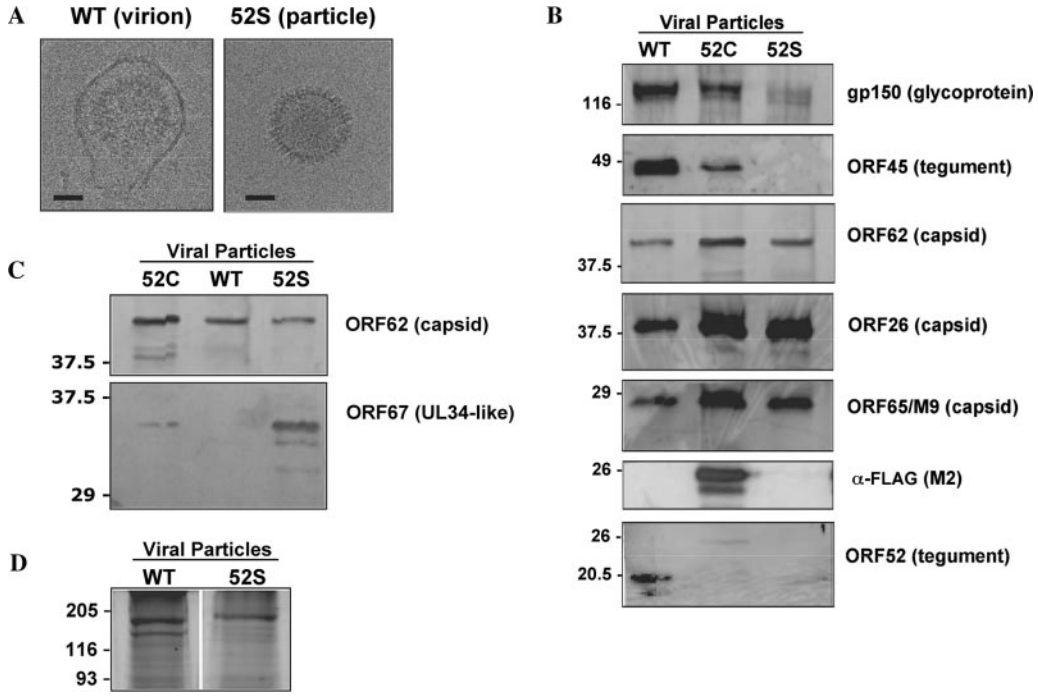

FIG. 8. Protein compositions of 52S particles, WT(BAC) virions, and complemented 52C virions. Viral particles were isolated from the supernatants of WT(BAC)- and 52S-plus-*FLAG-ORF52*-transfected cultures or from 52S-transfected cells. Molecular mass markers, in kDa, are shown. (A) Electron cryomicrograph of WT(BAC) and 52S particles embedded in vitreous ice and imaged at 300 kV on a JEOL JEM1200 (magnification, ×30,000; 6 electrons/Å). Representative images are shown. Bars, 100 nm. (B) Virion protein compositions of WT(BAC), complemented 52C, and 52S particles separated by SDS-PAGE, Western blotted, and probed with polyclonal antisera to virion proteins indicated or anti-FLAG monoclonal antibody (M2). (C) Detection of ORF67 protein in 52S particles. WT(BAC), 52C, and 52S particles separated by SDS-PAGE, Western blotted, and probed with polyclonal anti-ORF67 serum. Capsid protein was detected by anti-ORF62 serum. (D) Identification of ORF25 and ORF64 proteins in WT/BAC virions and 52S capsids by mass spectrometry. Proteins in WT(BAC) and 52S particles were separated by SDS-PAGE for band excision, digestion with trypsin, and extraction of peptides for LC/MS-MS analysis.

ever, the 52S particles do not contain appreciable amounts of ORF45 tegument protein, unlike WT/BAC virions and 52C virions, suggesting that ORF52 is necessary for progression to the step in virion morphogenesis at which ORF45 is incorporated into assembling virus particles. ORF52 protein is present in WT/BAC virions (apparent molecular mass, 21 kDa) and complemented 52C virions (apparent molecular mass with the FLAG tag, 25 kDa) but not 52S particles.

As 52S particles apparently represent an arrested stage in virion morphogenesis prior to efficient incorporation of outer tegument and envelope glycoproteins, we sought to examine the association of primary tegument proteins that transiently associate with the nascent nucleocapsid to facilitate nuclear egress. In MHV-68, the ORF67 protein exhibits limited homology to HSV-1 UL34. ORF67 is more closely related to EBV BFRF1, which has been shown to conserve the nuclear egress function of UL34 and interaction with the UL31 homologue, BFLF2 (encoded by *ORF69* in the MHV-68 genome) (15, 17). At least a portion of 52S particles contain ORF67 protein, while extracellular WT/BAC virions do not (Fig. 8C). 52C virions contain a small amount of ORF67, possibly indicating that this population contains particles that have not fully matured.

Finally, 52S particles and WT/BAC virions were subjected to mass spectrometric analyses to identify high-molecular-mass proteins for which antisera are not available (105- to 270-kDa-mass range) (Fig. 8D). Proteins were separated by SDS-PAGE, SYPRO Ruby stained, and excised for trypsin digestion and extraction of peptides. Peptides were analyzed by LC/MS-MS and identified by using Mascot or Sequest software to search for matches in the predicted MHV-68 proteome (6, 50). Peptides representing the major capsid protein (ORF25) and large tegument protein (ORF64, the HSV-1 UL36 homologue) were identified in both WT/BAC virions and 52S particles (Table 3). These results confirm the presence of all four canonical, conserved gammaherpesvirus capsid proteins (6, 31) in 52S nascent nucleocapsids, providing further evidence that ORF52, while tightly bound to the nucleocapsid, is not required for its formation; moreover, these results suggest that the assembly of ORF64 tegument protein is independent of ORF52.

DISCUSSION

In this paper, we report that MHV-68 *ORF52* encodes a capsid-associated tegument protein essential for tegumentation/envelopment of nucleocapsids in the cytoplasm and subsequent egress from infected cells. *ORF52* is a highly expressed gene with true-late kinetics, activated after viral DNA replication (12, 28). Mutation of *ORF52* in the MHV-68/BAC genome leads to arrest of the lytic phase of infection after viral genome replication, late gene expression, viral DNA cleavage/

TABLE 3. Identification of proteins of a 105- to 270-kDa-mass range in WT and 52S particles

Particle	NCBI accession no.	Predicted mass (kDa)	Protein		No. of tryptic peptide matches	P^a	Most confident peptide match (amino acid positions/sequence)
			Name	Predicted function			
WT	NP_044863	153.3	ORF25	Major capsid protein	2	0.01	158–172/TITSALQFGIDALER[b]
	NP_044902	273.5	ORF64	Large tegument	3	0.05	2391–2423/NPEPCVKENPPGV THDPPLRIQHMEQTVNSSK[c]
52S	NP_044863	153.3	ORF25	Major capsid protein	6	0.001	374–382/VFAIESLQR[b]
	NP_044902	273.5	ORF64	Large tegument	4	0.05	502–507/ALEISK[b]

[a] *P* value of peptide data assignment.
[b] Determined by Mascot software analysis of LC/MS-MS peptide fragment ion spectra collected on a QSTAR mass spectrometer.
[c] Determined by Sequest software analysis of LC/MS-MS peptide fragment ion spectra collected on a LCQ-Deca mass spectrometer.

packaging, and nucleocapsid assembly in the nucleus but prior to complete virion tegumentation and envelopment in the cytoplasm and egress of infectious virions from the cell.

Partially tegumented virion particles isolated from cells transfected with the MHV-68 BAC *ORF52*-null mutant clone (52S) morphologically appear similar to nucleocapsids and contain the four conserved capsid proteins required to form the icosahedral herpesvirus capsid, a structure MHV-68 shares with all other herpesviruses (6, 20, 31, 45, 54). The 52S particles also contain at least one tegument protein found in extracellular virions, the conserved ORF64/UL36 homologue (25). Interestingly, the HSV-1 UL36 protein has been shown to have deubiquitinating activity (24); whether this function is conserved in the ORF64 homologue is not known. Another conserved primary tegument protein thought to be involved in egress of nucleocapsids into the cytoplasm, the ORF67/UL34 homologue (15, 29), was also found in association with 52S particles but not WT MHV-68 virions. ORF67 in MHV-68 infection may mimic UL34, which dissociates from capsids in the cytoplasm and is not present in mature, extracellular alphaherpesvirus virions (29). If so, the loss of *ORF52* may result in a defect in virion maturation prior to this step or inefficient dissociation of ORF67 from C capsids that have egressed into the cytoplasm. Moreover, 52S particles contain no ORF45, a protein less tightly bound to the nucleocapsid than ORF52 is. As such, 52S particles likely represent an arrested stage of virion morphogenesis, at which nascent nucleocapsids associated with ORF67 and ORF64 have egressed from the nucleus into the cytoplasm. Indeed, immature virions accumulate in the cytoplasm of cells transfected with 52S (Table 2). ORF52 protein appears to be required to efficiently progress through the next stage of virion assembly, i.e., the loss of ORF67 from nascent nucleocapsids, association with outer tegument and glycoproteins required for canonical tegumentation and envelopment (wrapping), and egress from the cell. The existence of distinct, layered subdomains within the MHV-68 tegument has been recently suggested by electron cryotomography (W. Dai and Z. H. Zhou, submitted for publication). Consistent with this proposed function as a tegument protein, MHV-68 ORF52 localizes to the cytoplasm of transfected and infected cells. An EGFP-ORF52 fusion protein localized to the cytoplasm in distinct subcellular structures juxtaposed to the Golgi apparatus-derived network.

How exactly ORF52 functions is unclear. Understanding the mechanism of ORF52's function(s) requires a better understanding of the protein's interactions with other viral proteins and cellular machinery involved in the formation and release of virions from the infected cell. Studies have identified alphaherpesvirus tegument proteins that are specifically required for virion morphogenesis in the cytoplasm, such as pseudorabies virus UL37 (25) and UL48 in both pseudorabies virus and HSV-1 (16, 49). Undoubtedly, other tegument proteins are also involved in these processes. For instance, conserved herpesvirus tegument proteins ORF67/UL34, ORF69/UL31, and ORF64/UL36 have been implicated in the egress of nucleocapsids from the nucleus and capsid transport in the cytoplasm (27, 29). In addition, a number of tegument proteins, including ORF52, are unique to each subfamily of herpesvirus and may have distinct roles in the formation and egress of virions. The complete set of tegument proteins encoded by each herpesvirus genome accomplishes a conserved evolutionary function, facilitating egress and entry of nucleocapsids through the cytoplasm. It has been recently suggested that herpesvirus nucleocapsids and tailed DNA bacteriophages may share a common ancestor (3). In such a scenario, tegument proteins represent a subsequent adaptation to coping with another cellular layer, the cytoplasm, that had engulfed the common ancestor's original host, the primordial nucleus. Subsequent structural and genomic divergence has preserved this essential tegument function yet allowed incorporation of divergent proteins with effects on the infectivity of incoming virions, such as the gammaherpesvirus ORF45 (21, 56) and gammaherpesvirus UL41 virion host shutoff protein (34). In this light, ORF52 protein has an apparently central role in tegumentation and egress and thus is an evolutionary link between the gammaherpesvirus proteome and the host cell.

In summary, we have analyzed the function of the ORF52 tegument protein in the MHV-68 model of the gammaherpesvirus lytic phase. The lytic phase of infection among gammaherpesviruses, such as, for example, KSHV, has been implicated in tumor establishment and progression, as well as reactivation and spread of the virus within patients, from tissues initially infected to B cells or lymphatic epithelial cells (8, 9, 13, 18). Understanding the production of virions is important for understanding the transmission and pathogenesis of these pathogens and developing therapeutic interventions in EBV- and KSHV-associated diseases.

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