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N-Terminal Gag Domain Required for Foamy Virus Particle Assembly and Export

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Among the *Retroviridae*, foamy viruses (FVs) exhibit an unusual way of particle assembly and a highly specific incorporation of envelope protein into progeny virions. We have analyzed deletions and point mutants of the prototypic FV *gag* gene for capsid assembly and egress, envelope protein incorporation, infectivity, and ultrastructure. Deletions introduced at the 3′ end of *gag* revealed the first 297 amino acids (aa) to be sufficient for specific Env incorporation and export of particulate material. Deletions introduced at the 5′ end showed the region between aa 6 and 200 to be dispensable for virus capsid assembly but required for the incorporation of Env and particle egress. Point mutations were introduced in the 5′ region of *gag* to target residues conserved among FVs from different species. Alanine substitutions of residues in a region between aa 40 and 60 resulted in severe alterations in particle morphology. Furthermore, at position 50, this region harbors the conserved arginine that is presumably at the center of a signal sequence directing FV Gag proteins to a cytoplasmic assembly site.

Spumaretroviruses are one subfamily of retroviruses with only one viral genus, the foamy viruses (FVs), while all other known retroviruses belong to the subfamily of orthoretroviruses (39). Major differences in the replication strategies between orthoretroviruses and spumaretroviruses have been described previously (30, 39). The capsid protein Gag is one of the determinants of the FV aberrant replication pathway. FV Gag lacks the typical cleavage products and motifs that have been characterized in orthoretroviruses, such as matrix (MA), capsid, and nucleocapsid proteins, which are the eventual protease cleavage products of the Gag precursor (48). Furthermore, a major homology region (present in orthoretroviral capsid proteins) and cystein-histidine boxes (present in orthoretroviral nucleocapsid proteins) are not found in FV Gag proteins (31, 48). Instead, only a 3-kDa peptide is cleaved by the FV protease from the C terminus of the Gag precursor, when infectious viruses bud from the cell (12). This cleavage appears to be essential for correct particle assembly and viral infectivity (8, 54). Moreover, the C terminus of FV Gag proteins bears sequences, glycine-arginine (GR) boxes, which have been shown to bind nucleic acids (47, 52) and transfer the Gag protein of FVs from primate origin to the nucleus of infected cells (44).

FVs reverse transcribe their (pre-)genomic RNA to a large extent in the virus-producing cell (32, 42, 53). This leads to genomic DNA, which may represent the virion nucleic acid that is relevant for infection (42, 53). However, large amounts of virion RNA and reverse transcription occurring early after infection have also been described previously (4, 42, 53). Since reverse transcription late in the replication cycle depends on Gag protein expression (19), this feature of FVs is probably also one consequence of the unusual structure of Gag.

FV Gag proteins are not myristoylated at the N terminus, and like the morphotype B/D betaretroviruses, FV particles assemble in the cytoplasm (11). However, in sharp contrast to orthoretroviruses, wild-type FV capsids strictly require the presence of cognate Env protein for budding from cellular membranes (11, 27, 36, 50). Thus, wild-type FV Gag proteins expressed on their own do not form virus-like particles (VLPs). Particular motifs in Env located in the cytoplasmic N terminus of the leader peptide (LP) are essential to facilitate capsid export and probably make contact with Gag (14, 27, 28, 51). The FV Env LP has a type 2 transmembrane topology and is an integral part of the virion (27, 28). While specific LP residues, which are likely involved in the Env-Gag interaction, have been described previously (28), little is known about the respective Gag domains. Biophysical studies suggested that the N terminus of Gag harbors such motifs (14, 51). To date, neither genetic nor biochemical studies support this hypothesis.

The 71-kDa prototypic foamy virus (PFV) Gag protein consists of 648 amino acid (aa) residues. To get a better understanding of the function of PFV Gag, we studied the expression, cleavage pattern, ability to support the export of particulate viral material, infectivity, and ultrastructure of a variety of mutants.

MATERIALS AND METHODS

Cells. BHK/LTR(PFV)lacZ, 293T, and HT1080 human fibroblastoid cells were grown in Dulbecco's modified minimal essential medium supplemented with 5 to 10% fetal calf serum and antibiotics as described previously (5, 28, 45).

Recombinant DNA. Established methods in molecular biology were used for the work with recombinant DNA (2, 43). The DNA sequences of plasmids generated by recombinant PCR (20) were determined in the amplified parts to

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verify the presence of the desired alterations and to exclude further nucleotide exchanges. (A detailed description of the generation of the molecular clones can be found online at http://www.uni-wuerzburg.de/virologie/Cartellieri/PFVgag.html.)

The wild-type *env* gene expression plasmids pczHFVenvEM02 and pCenv1, as well as the *pol* gene expression plasmid pCpol2 and the pMD9 vector, have been described previously (11, 18, 29). The infectious PFV molecular clone pcHSRV2 (32) and the *gag* expression plasmid pCZIgag4 served as basic constructs for further cloning procedures.

In pCZIgag4, the PFV *gag* gene was inserted into the pCZI expression vector (18) downstream of the human cytomegalovirus (CMV) enhancer/promoter and CMV intron A. This plasmid is structurally identical to the previously described pCZIgag2 (18). However, in pCZIgag4, the splice acceptor site of the *pol* mRNA in *gag* was mutated from TTAG to CTCG. Furthermore, the *pol* gene ATG start codon was changed to CTG, and *pol* sequences downstream of the *gag* gene stop codon were deleted. To establish pCZIgag4, a 6.58-kb NotI/BamHI fragment derived from pCZIgag2 was ligated with 1.19-kb pCZIgag2 BamHI/NsiI and 0.43-kb pHSRV2-M67 (22) NsiI/SwaI fragments and a 0.36-kb SwaI/NotI-digested PCR product. This amplimer was generated using pHSRV2-M54 template (9). The *pol* gene expression plasmid pCZIpol1 was made by inserting the complete PFV *pol* gene excised as a 3.5-kb SmaI/NdeI fragment from the prokaryotic expression plasmid pET15Bpol-wt (21) into the NotI-digested pCZI vector downstream of CMV intron A by blunt-end ligation.

To facilitate specific deletions of the PFV *gag* gene in the context of the infectious molecular clone, NheI restriction sites were introduced into pcHSRV2 at the aa positions 7/8, 16/17, and 123/124. This led to the pcHSRV2 derivatives M84 (VE7/8AS), M85 (L17S), and M86 (S123A), respectively. The aa alterations generated through this procedure are indicated in brackets and do (in the case of M85) or do not (in the cases of M84 and M86) significantly change the replication competence of HSRV2 (32) (see below). The alterations leading to an NheI cleavage site at aa positions 123/124 were also introduced into the *gag* gene expression vector pCZIgag4, which resulted in plasmid pMC48.

The 3′ *gag* deletion mutants were generated in the pCZIgag4 or pMC48 backgrounds, leading to the plasmids pMC53/ΔC594, pMC52/ΔC544, pMC51/ΔC482, pMC50/ΔC310, pMC60/ΔC297, and pMC61/ΔC276, which truncated the *gag* open reading frame (ORF) after the indicated aa position. Deletions in *gag* further upstream of position 276 were introduced into pCZIgag4, resulting in pMC62/ΔC212, pMC63/ΔC180, pMC64/ΔC166, and pMC65/ΔC133.

Deletions at the 5′ end of *gag* were created in the pCZIgag4 background. This led to plasmids pMC1/Δ7-78, pMC11/Δ7-92, pMC3/Δ7-125, pMC4/Δ7-179, and pMC12/Δ7-199, which are deleted in the N-terminal part of the *gag* ORF between the indicated positions. The plasmids pMC6/Δ16-78, pMC13/Δ16-92, pMC8/Δ16-125, pMC9/Δ16-179, and pMC14/Δ16-199 bear deletions in the *gag* ORF downstream of position 15.

In addition, deletion mutants at the 5′ end of *gag* were constructed in the context of the pcHRSV2 proviral clone, resulting in constructs M87 (Δ7-47), M88 (Δ7-78), and M89 (Δ7-199). The pcHSRV2 derivatives M85 and M86 were modified to obtain the *gag* deletions M90 (Δ16-47), M91 (Δ16-78), M92 (Δ16-199), and M93 (Δ123-199).

Point mutations were introduced at the *gag* N terminus in pcHSRV2. The mutants M94 (L17A), M95 (I36A), M96 (W45A), M97 (R50A), M98 (L66A), M99 (L12A), M100 (I20A), M101 (L56A and L58A), M102 (D60A), M103 (W44A), M104 (P65A), M105 (P69A), M106 (P89A), and M107 (DELED140-144GALAG) were obtained by recombinant PCR.

Mutagenesis of a pChatul3 (42) feline FV (FFV) *gag* fragment resulted in pChatul3 (gagW38A). To raise antibodies against FFV, the *gag* and *pol* ORFs of the FFV clone pChatul1 (42) were amplified and inserted into the pRsetA bacterial expression vector (Invitrogen) in frame to the N-terminal His tag. Protein expression was induced in BL21(DE3) cells (Novagen), and recombinant proteins were purified by Ni^{2+} chelate chromatography under denaturing conditions, as described previously (21). An N-terminal fragment of the FFV *env* gene-specifying LP sequences was amplified from pczFFVenv (35) and inserted into pMAL-C2 (New England Biolabs). Recombinant protein was affinity purified from transformed *Escherichia coli* TB1 cultures on amylose matrix according to the instructions of the manufacturer (New England Biolabs). Purified proteins were used to generate rabbit antisera at a commercial facility (Eurogentec).

Transfections and analysis of viral infectivity. The day before transfection, 293T cells were seeded at a density of 1.6×10^6 cells/6-cm dish. The cells were transfected with a total of 6 μg plasmid DNA per dish using Polyfect essentially as described by the manufacturer (QIAGEN). Transcription from the CMV promoter was induced by the addition of 10 mM sodium butyrate for 8 h on the day after transfection. For the vector transfer analysis of subgenomic 5′ *gag* mutants, the cells were cotransfected with pMD9, pCpol2, and pCenv1. Constructs modified at the 3′ end were analyzed after cotransfection of 293T cells with pMD9, pCZIpol1, and the *env* gene EM02 expression plasmid. The supernatant was harvested 48 h after transfection, and viral infectivity was determined following filtration through 0.45-μm-pore-size filters (Schleicher & Schuell) on HT1080 cells by flow cytometry (18) or on BHK/LTR(PFV)lacZ cells by a blue cell assay (45) in case of proviral constructs. In addition, cell-bound infectivity was measured. To do this, the cells were detached from the plastic support 48 h after transfection, resuspended in 4 ml Dulbecco's modified minimal essential medium, and subjected to three cycles of freezing and thawing (at −80°C and 37°C). After clearance by low-speed centrifugation and filtration through 0.45-μm filters, the infectivity was determined as described above. The titration assays were performed at least three times.

Protein detection. Cellular lysates and virus-containing supernatants were prepared and analyzed by immunoblotting, as described previously (34), with polyclonal rabbit antisera raised against the PFV Gag C terminus (1, 16); the PFV Env LP (28); the recombinant FFV Gag, Pol, and Env LP; and the mouse monoclonal antibodies (MAbs) SGG1, directed against PFV Gag (18), 15E10 and 3E11, directed against PFV Pol (21), and P3E10, directed against the PFV Env surface (SU) subunit (6) as detailed in the figure legends. After reaction with secondary anti-rabbit or anti-mouse antibodies (Dako), the blots were developed using ECLplus reagent (Amersham). The chemiluminescence signals were either monitored by exposure to X-ray films (Agfa Curix 100 NIF) or digitally recorded using an LAS-3000 imager (Fujifilm).

Ultrastructural analysis. 293T cells were transfected with plasmids, harvested 48 h after transfection, and processed for electron microscopy (EM) as described previously (25).

RESULTS

Analysis of C-terminal Gag truncations. The 3′ PFV *gag* deletions were introduced into the pCZIgag4 vector as shown in Fig. 1A. The constructs were analyzed for the generation of Gag protein following transient cotransfection of 293T cells together with plasmids expressing Pol and Env proteins and the MD9 vector (Fig. 1C). All deletion constructs gave rise to detectable levels of Gag proteins in lysates from transfected cells (Fig. 1C), with the expected higher electrophoretic mobility than wild-type Gag. The ΔC133 construct was an exception, probably because the Gag protein expressed from this construct was too short to be clearly detected by the SGG1 Gag MAb. However, this was not further investigated. The analysis of particulate material concentrated from the cell-free supernatant of transfected cells revealed the release of Gag proteins up to the truncation construct Δ297 (Fig. 1C, lane 14).

FV wild-type capsids require the presence of envelope protein for cellular export. It is theoretically possible that cellular egress of C-terminally truncated Gag becomes Env independent. However, when particulate material from the supernatants was analyzed for FV Env proteins, bands specific for the precursor ($gp130^{Env}$), the SU subunit ($gp80^{SU}$), and the LP ($gp18^{LP}$) were detected in Gag-positive samples (Fig. 1D and E). Intracellular expression of the respective Env proteins was detected in all lanes (Fig. 1D and E). Furthermore, the sole transfection of cells with the *gag* deletion constructs did not result in the export of particulate material (Fig. 1B).

The perturbation of Gag multimerization domains in constructs smaller than pMC60ΔC297 could certainly mimic the deletion of a Gag-Env interaction domain. Furthermore, the deletion of the Gag L domain (Fig. 1A) can significantly impair PFV particle release (33, 46). However, some of the truncated Gag variants behaved like full-length proteins with respect to the specificity of PFV particle egress. Therefore, a domain responsible for the interaction with Env is probably located within the first 297 aa of Gag.

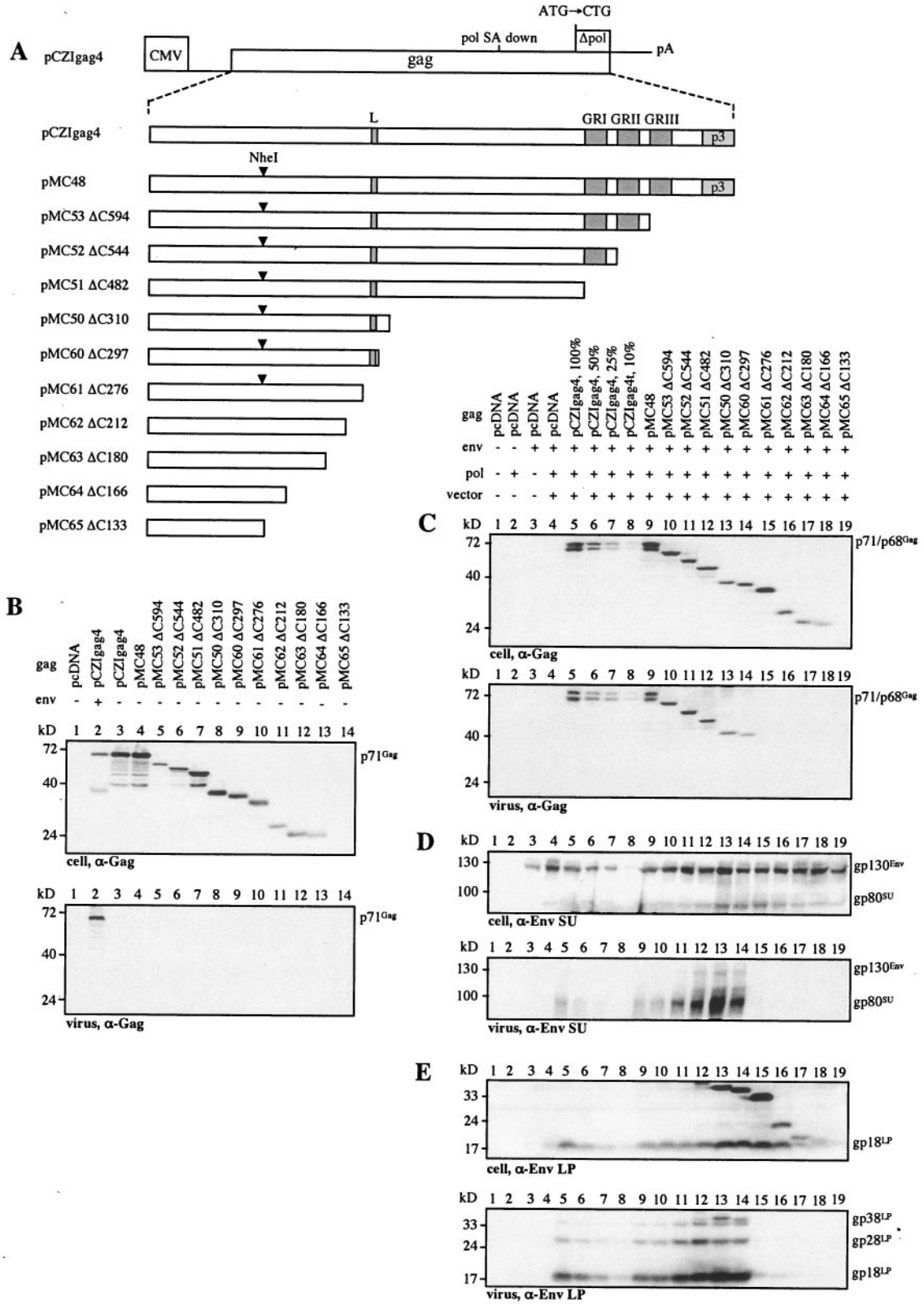


FIG. 1. Schematic representation and expression profile of 3′ *gag* gene deletion constructs generated in a subgenomic expression vector. (A) Constructs. The splice acceptor (SA) site for the *pol* mRNA and the *pol* gene start codon were mutated, and *pol* gene sequences downstream of the *gag* stop codon were deleted. The locations of the late assembly domain (L), identified recently (33, 46), of the three glycine-arginine-rich boxes (GRI to GRIII) and the p3 peptide that is cleaved from the Gag C terminus are shown. Some constructs were made in the pMC48 background that bears mutations leading to an NheI restriction site (vertical arrows). (B to E) Cells were transfected with the *gag* constructs

We also analyzed the infectivity of the 3′ *gag* deletion constructs by determination of their ability to support pMD9 vector transfer after cotransfection of 293T cells with *env* (EM02) and *pol* (pCZIpol1) constructs. With respect to the wild-type pCZIgag4 plasmid, the introduction of the NheI restriction site at aa positions 123/124 in pMC48 insignificantly affected vector transfer via the supernatant (data not shown). Deletion of GR box III in construct ΔC594 (Fig. 1A) resulted in a drop of vector titer from that of the wild type to about 5% (data not shown). The further deletion of GR box II in construct ΔC544 resulted in an additional drop, making vector transfer rates hardly detectable. All further *gag* deletions did not specify proteins that were able to support any vector transfer (data not shown). In general, our results on vector transfer by the 3′ *gag* deletions in a subgenomic background are in good agreement with a previous report where such deletions were investigated in a proviral context (47).

Analysis of 5′ *gag* mutants. To investigate the role of the Gag N terminus in particle release, we generated deletion mutants at the 5′ end of *gag* in the pCZIgag4 background. The deletions were introduced downstream of aa 6 or 15 and extended to aa positions 79, 93, 126, 180, and 200, as shown in Fig. 2A. 293T cells were transfected with the recombinant molecular clones together with the pMD9 vector, pCenv1, and pCpol2. The expression of steady-state Gag protein levels was analyzed by immunoblot. As shown in Fig. 2B, all Gag deletion constructs showed cellular expression levels comparable to that of the pcziGag4 wild-type plasmid, and the sizes of the identified Gag species reflected the introduced deletions. When the 5′ deletion constructs were analyzed for particle export, only cells transfected with the wild-type Gag construct gave rise to detectable amounts of extracellular protein, as depicted in Fig. 2B, lane 2. Similar to the 3′ deletion constructs, the 5′ mutants were also incompetent to produce VLPs in the absence of Env (data not shown). The determination of infectivity revealed that all *gag* mutants were unable to support pMD9 vector transfer (data not shown).

To analyze whether the defect in cellular egress of the N-terminal deletion mutants could be rescued by wild-type Gag, 293T cells were cotransfected with plasmids specifying deletions downstream of aa 6 together with pCZIgag4 and pCenv1. As shown in Fig. 2C, the constructs in question gave rise to comparable levels of cellular expression. All shortened Gag proteins were detected in the supernatants of transfected cells together with wild-type Gag, provided that Env was coexpressed (Fig. 2C). This indicated that although the mutants have lost the ability to associate with Env or promote Env-independent particle egress, they retained the ability to associate with full-length Gag proteins. Interestingly, even constructs pMC4 and pMC12, which were deleted between aa positions 6 and 180 and 6 and 200, respectively, gave rise to proteins that were clearly identified in particulate material from the supernatants of transfected cells. In these two mutants, the coiled-coil region that was identified to be important for Gag-Gag interactions in PFV is lost (49).

Some of the 5′ *gag* deletions were also introduced into the proviral pcHSRV2 context, as shown in Fig. 3A. In addition, we analyzed the pcHSRV2 mutants with the introduced NheI restriction site M84 (VE7/8AS), M85 (L17S), and M86 (S123A) for protein expression, particle export, and infectivity. As depicted in Fig. 3B, all plasmid clones gave rise to comparable levels of intracellular protein expression. Interestingly, M89 (Δ7-199), M92 (Δ16-199), and M93 (Δ123-199) were not able to give rise to processed Gag proteins, despite the generation of similar amounts of Pol (Fig. 3B and data not shown). Only the wild-type construct, M84, and M86 supported the export of viral particles (Fig. 3B, lanes 2, 3, and 7). The determination of the infectivity revealed analogous findings. The supernatants and cellular lysates of cells that were transfected with the wild-type plasmid, M84, and M86 were of equal infectivity (Fig. 3C). A lack of protease-mediated C-terminal Gag cleavage of N-terminal Gag deletions indicated severe structural alterations in the capsid which may either mask the protease cleavage site or prevent Pol particle incorporation. A similar finding has been previously reported for FFV (14). The results obtained with proviral clones corroborated the findings on the subgenomic constructs and, furthermore, pointed to individual residues, such as those mutated in M85, that could potentially be critical in FV capsid egress. This prompted a closer inspection of PFV point mutants at the Gag N terminus.

Analysis of *gag* point mutations. To investigate Gag domains, which may be involved in particle egress, we first looked for residues that are conserved among different FV Gag protein N termini. The rationale is based on the finding that FV Env proteins of different origin are capable of cross-pseudotyping FV capsids (D. Lindemann and D. Westphal, unpublished observation). This pseudotyping is more effective the closer the different FVs are evolutionary related. As shown in Fig. 4, motifs with prolines, (iso-)leucines, tryptophans, and acidic residues (D and E) were among the most conserved aa identified in the N-terminal region of Gag proteins between FVs of different origins. Proviral constructs were established in which these individual residues were changed to alanines (Fig. 5). For control purposes, we mutated the conserved arginine at position 50 to alanine (M97). The R50A virus has been reported to be deficient in correct capsid assembly and in the export of particulate material (7). 293T cells were transiently transfected with the plasmids and cellular lysates, and extracellular virus was analyzed for PFV protein expression (Fig. 6A to C). All mutants generated approximately equal amounts of Gag protein and were competent to allow Gag processing (Fig. 6A). Cleavage of Gag protein was already indicative of *pol* gene expression, since the FV protease is encoded within the *pol* ORF. When the protein lysates were specifically analyzed for

together with (C to E) or without (B) *pol* and *env* expression constructs and pMD9 vector. Cellular lysates (cell) and particulate material from the supernatant (virus) were analyzed by immunoblot with anti-Gag (α-Gag) (B and C) and anti-Env (α-Env) SU (D) mouse MAbs (SGG1 and P3E10, respectively) and rabbit antiserum against the Env LP (E). In lanes 5 to 8, the lysates from cells and viral sediment derived from the transfection of 293T cells with wild-type pCZIgag4 were diluted as indicated to get an estimate of the sensitivity of the assay. The 3′ *gag* deletion constructs up to aa 297 gave rise to proteins that were specifically exported from transfected cells when wild-type *env* was coexpressed. When the *env* construct was omitted from the transfection cocktail (B), proteins specified by all constructs were disabled for cellular egress.

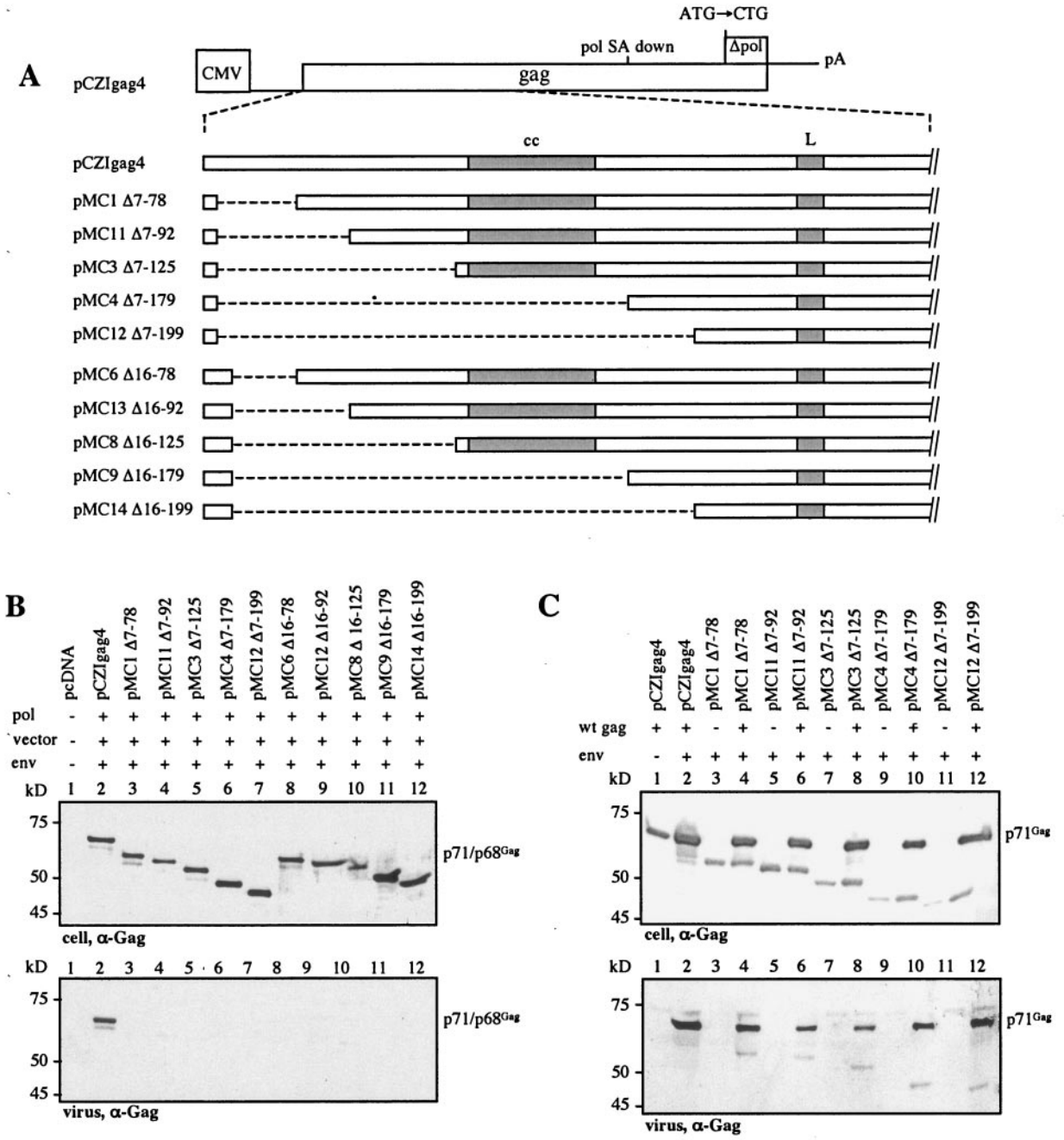


FIG. 2. Schematic representation and expression profile of 5′ *gag* gene deletion constructs in the subgenomic pCZIgag4 expression plasmid. (A) Constructs. Abbreviations are as defined in the legend of Fig. 1. The location of the coiled-coil domain (cc) implicated in Gag-Gag interactions (49) is indicated. The mutant plasmids were deleted in the region specifying Gag residue 7 or 16 at the N terminus and the indicated residues at the C terminus. (B and C) Protein detection in lysates and cell-free virus from the supernatant of transfected 293T cells. In B, the cells were cotransfected with the *gag* constructs and wild-type *env* and *pol* expression plasmids plus pMD9 vector DNA. While Gag protein synthesis was detected in lysates (cell), only the undeleted *gag* plasmid gave rise to particulate material that could be sedimented from the supernatant (virus). In C, we investigated whether the deleted constructs specified proteins that could be detected in the supernatant fraction when wild-type (wt) *gag* (pCZIgag4) was coexpressed.

Pol protein species, an expression pattern similar to that of the wild type was revealed (data not shown).

Mutants M85 (L17S), M96 (W45A), M97 (R50A), and M101 (L56A and L58A) were found incompetent for capsid egress from transfected cells (Fig. 6A, lanes 20 and 23 to 25). In addition, M103 (W44A) and M104 (P65A) were severely hampered in particle export which was reduced to less than 25% compared to that of wild-type Gag (Fig. 6A, lanes 11 and 22). The deficiency in capsid release of these mutants was not due to a lack of Env expression, as shown in Fig. 6B and C.

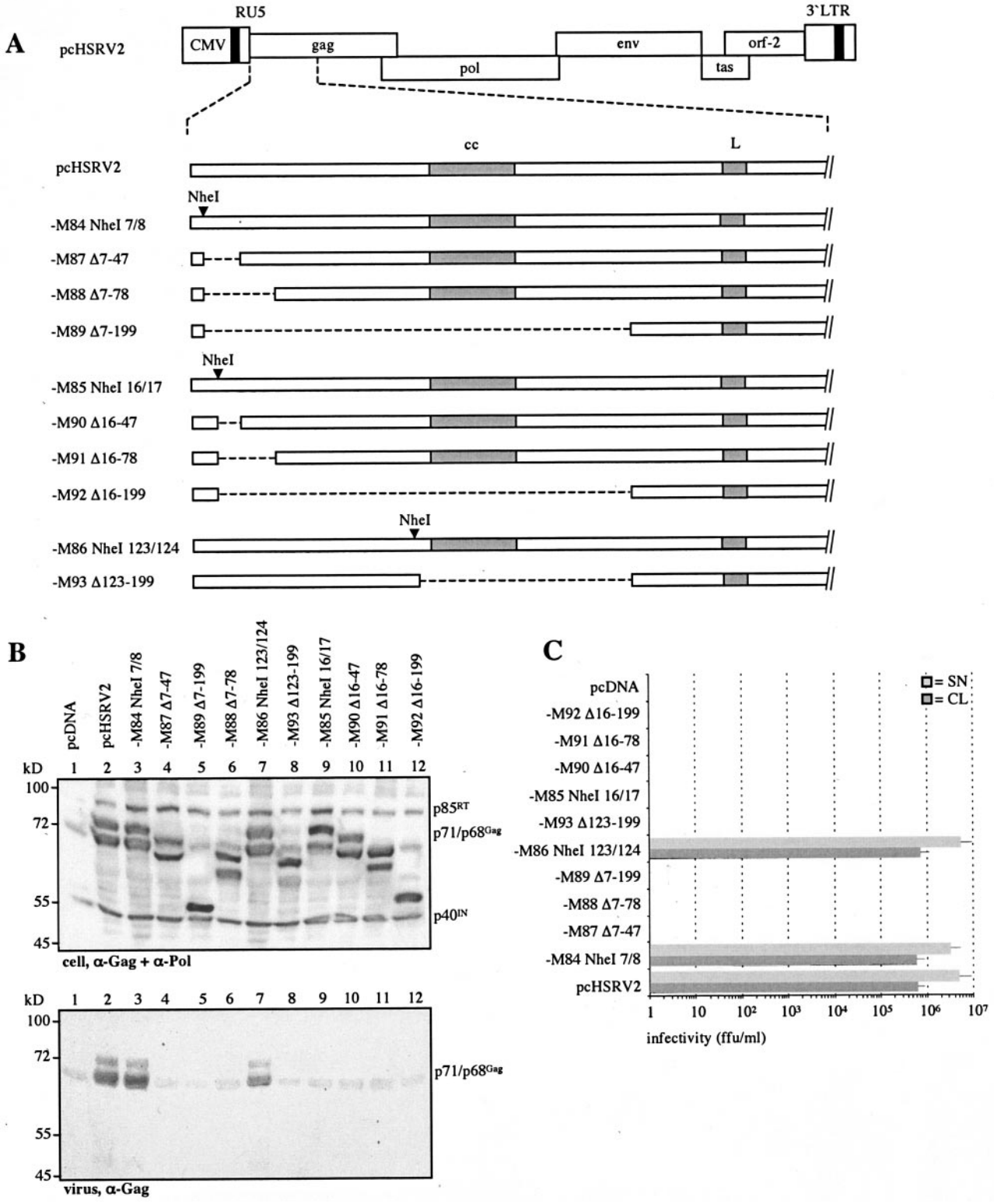


FIG. 3. Analysis of mutations in the proviral context. (A) Genome organization, deletion constructs in the 5′ *gag* region, and introduction of NheI restriction sites (vertical arrows) in the proviral clone pcHSRV2. LTR, long terminal repeat; RU5, repeat and unique regions of the long terminal repeat. The locations of the *gag*, *pol*, and *env* genes and of the accessory PFV ORFs (*tas* and *orf-2*) are indicated. The other abbreviations used are as described in the legends of Fig. 1 and 2. (B and C) Determination of cellular protein expression, cellular export, and infectivity of the proviral mutants. In B, 293T cells were transfected with the plasmids, and proteins in lysates from transfected cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and incubated with Gag-specific (SGG1) and Pol-specific (15E10 and 3E11) MAbs (cell). The export of particulate material was observed from cells transfected with the wild-type pcHSRV2 construct M84 and M86, as detected by staining proteins extracted from sedimented material in the supernatant with the anti-Gag MAb (virus). In C, the infectivity in focus-forming units (ffu) per milliliter was determined in cellular lysates (CL) and the supernatant (SN) from transfected cells by a blue cell assay on BHK/LTRlacZ cells (44).

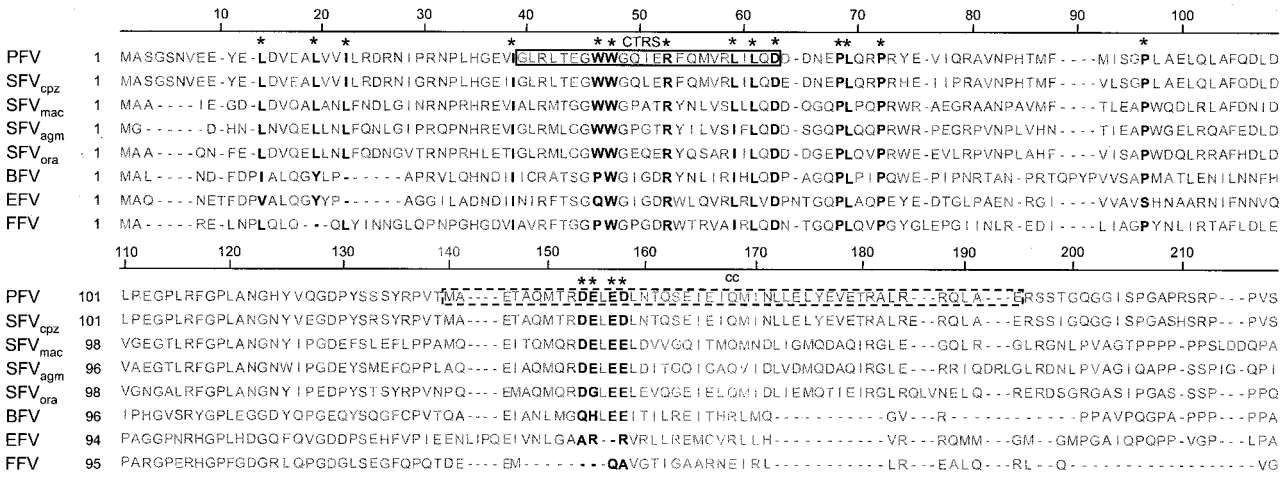


FIG. 4. Alignment of FV Gag sequences using the program MegAlign of the DNAStar software package. The sequences were derived from GenBank (PFV accession number NC001736; SFVcpz, from chimpanzee, accession number U04327; SFVmac, from macaque, accession number X54482; SFVagm, from African green monkey, accession number M74895, SFVora, from orangutan, accession number AJ544579; bovine foamy virus [BFV] accession number U94514; equine foamy virus [EFV] accession number AF201902; FFV accession number AJ564746). Conserved residues are in boldface type. The proposed PFV Gag CTRS and the coiled-coil domain (cc) are indicated. Residues mutated in this study are marked by an asterisk.

Mutants M96, M97, M101, and M103 cluster in the Gag region between aa 40 to 60. The mutation in M85 (L17S), however, is located far upstream. Therefore, we created M94 (L17A), in which the conserved leucine was changed to alanine. The analysis of this mutant revealed its full competence in capsid export (Fig. 6A, lane 21). This finding does not support the hypothesis that the Gag region around aa 20 is important for Gag-Env or Gag-Gag interactions.

The determination of the infectivity of the point mutants corroborated the results on particle export (Fig. 6D). The mutants that gave rise to detectable virus in the supernatant were infectious, and in the case of mutants deficient in detectable virus export, no infectivity could be measured in the indicator cell assay. However, some mutants that showed normal particle egress, as determined by the detection of Gag in the supernatant, produced infectious virus titers lower than those of the wild type. The analysis of M99 (L12A), M95 (I36A), M104 (P65A), M98 (L66A), and M107 (DELED140-144GALAG) revealed the generation of infectious viral titers that were reduced by 1 or 2 orders of magnitude (Fig. 6D). Most prominent was the reduction in the generation of infectious virus by M94 (L17A) and M103 (W44A), which gave rise to titers reduced by 5 and 6 orders of magnitude, respectively (Fig. 6D).

The analysis of the other mutations of prolines and acidic residues located further downstream in Gag (M105 and M106) and conserved among the FVs of primate origin (Fig. 4) revealed that they produced infectious titers after transient transfection of 293T cells that were identical to those of the wild type (Fig. 6D).

Analysis of FFV Gag. The results obtained so far were compatible with the view that a domain in Gag between aa 40 and 60 is involved in the specificity of FV capsids to interact with cognate Env LPs. To further support this assumption, we mutated tryptophan residue 38 in FFV Gag to alanine (Fig. 7A). The W38 in FFV Gag corresponds to W45 in PFV Gag (Fig. 4). The PFV M96 (W45A) mutation was normal in cellular Gag expression and cleavage. However, no capsid protein was detected in the supernatant from cells transfected with M96 (Fig. 6A, lane 23). The alteration of FFV Gag W38 to A in the context of the full-length proviral clone pChatul3 also resulted in a deficiency in export of capsid protein (Fig. 7B). Reaction of the FFV blot with Env- and Pol-specific antisera supported this finding (data not shown). However, pChatul3 (gagW38A) was also defective in normal Gag cleavage (Fig. 7B). In PFV Gag, cleavage is not a condition for particle export (11, 24, 53). How FFV behaves in this respect is not known. This obviously limits conclusions on the role of FFV Gag W38 in the specificity of the interaction with the autologous Env LP.

Ultrastructural analysis of export-defective PFV Gag mutants. 293T cells were transfected with the recombinant plasmids, which were unable to export capsids, and processed for ultrastructural analysis by EM. We first analyzed the deletion constructs at the 5′ end of *gag* in the subgenomic expression background (Fig. 2A) after cotransfection of cells with *env*- and *pol*-expressing plasmids and the pMD9 vector. As depicted in Fig. 8A, the *gag* construct with the largest deletion (pMC12/Δ7-199) directed the expression of protein that assembled into viral capsid structures. Such capsids were also observed upon examination of cells transfected with constructs bearing smaller *gag* deletions (pMC3/Δ7-125 and pMC11/Δ7-92 in Fig. 8B and C, respectively). However, the 5′ *gag* deletion construct pMC1/Δ7-78 gave rise to intracellular particulate material assembling into tubular structures (Fig. 8D). Tubular structures have been noted previously, for instance, in orthoretroviral assembly-defective *gag* mutants (25). Since all of the N-terminally deleted capsid proteins were deficient in cellular egress (Fig. 2), the morphological findings are compatible with the view that a motif in Gag upstream of aa 92 is involved in the recognition of Env LP.

We examined the mutants with altered individual residues in the context of the provirus. In cells transfected with M85

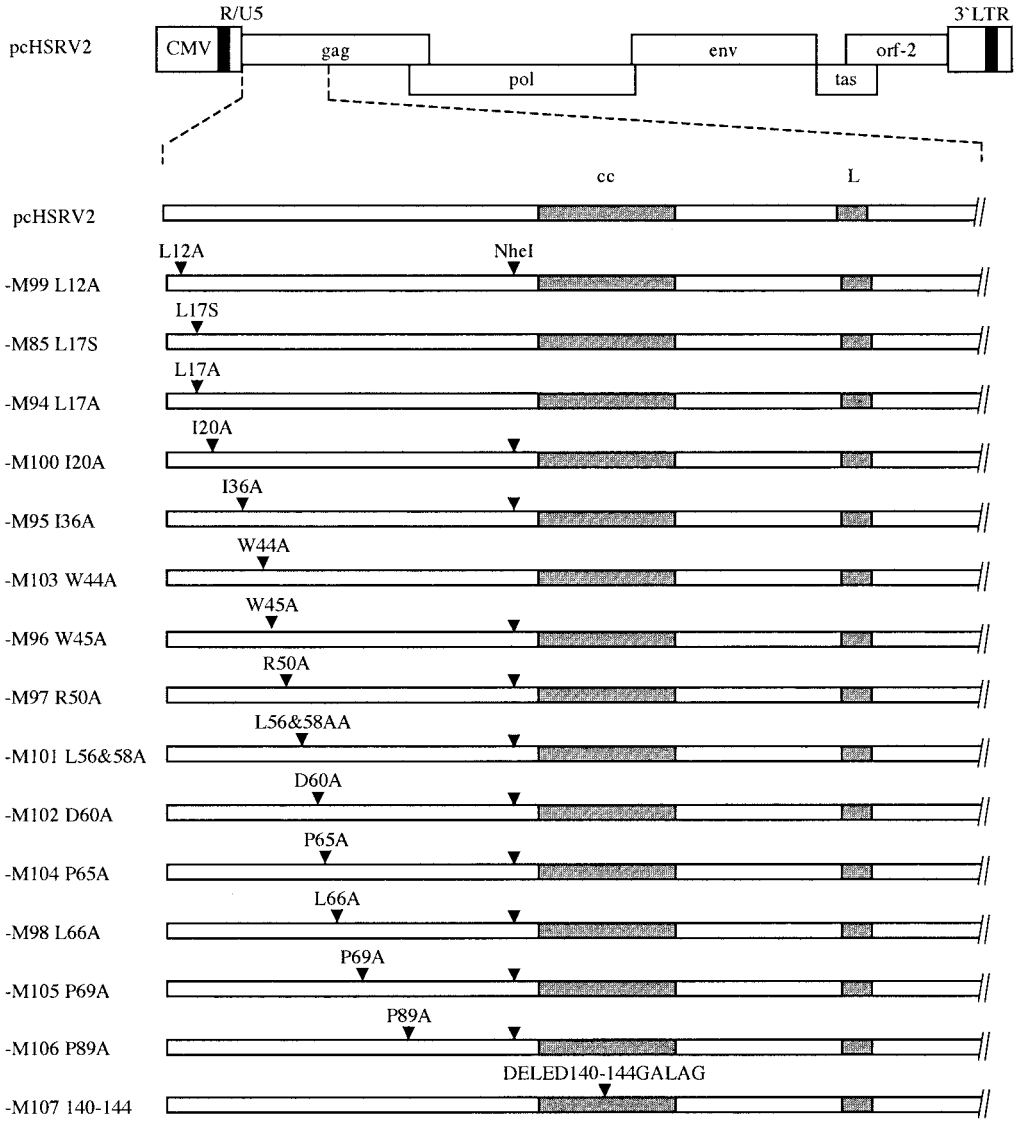
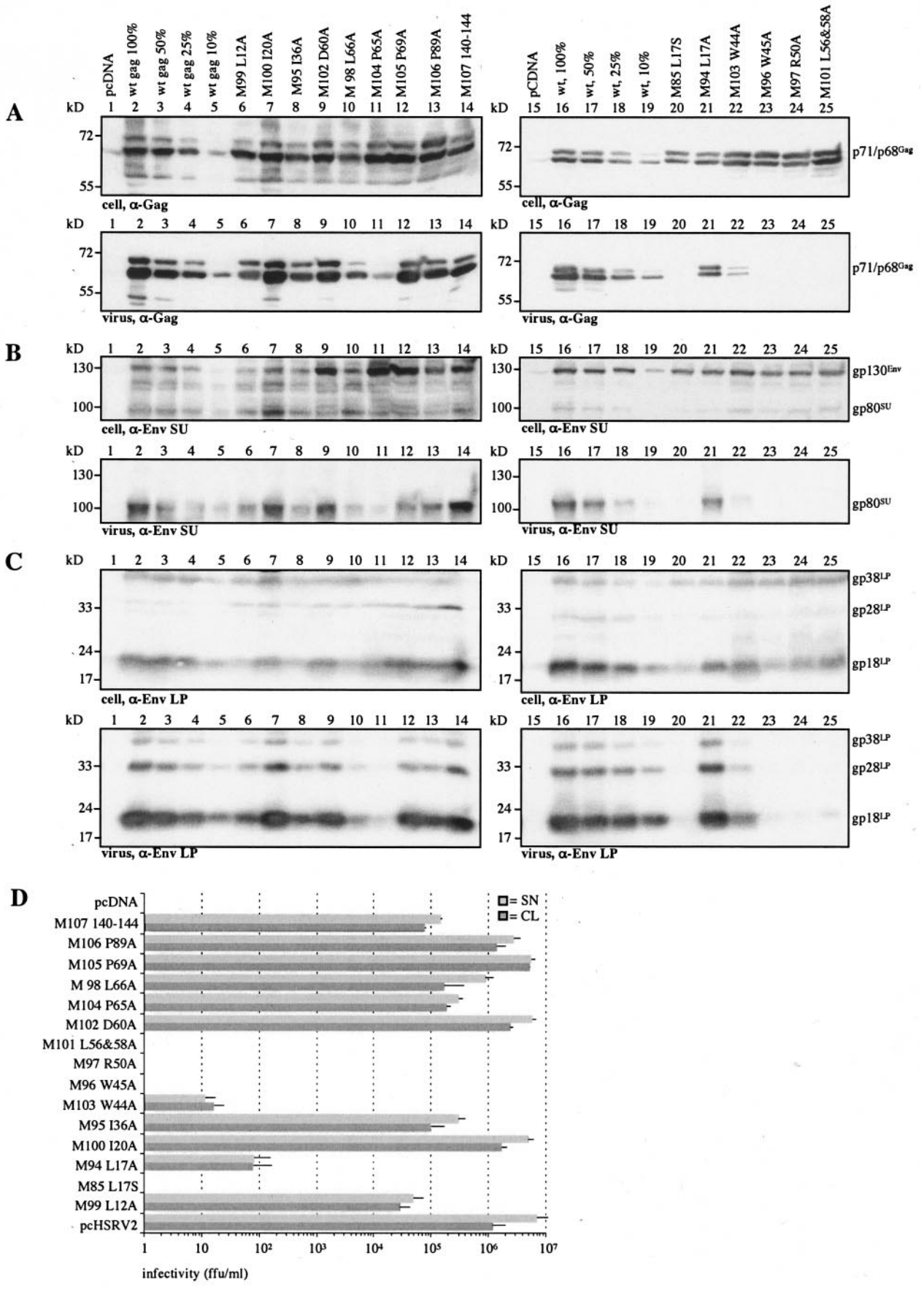


FIG. 5. *gag* point mutations leading to the indicated aa alterations in the proviral context of pcHSRV2. Some plasmids were created in the M86 background, which does not change the phenotype compared to the wild-type pcHSRV2 plasmid (Fig. 3). Abbreviations are as described in the legends of Fig. 1 and 3.

(L17S), we observed membrane-associated plaques or an electron-dense thickening of the plasma membrane that was presumably caused by viral capsid protein (Fig. 8E). Analogous structures have been observed previously in orthoretroviral systems and were interpreted as early budding arrests (25). Transfection of cells with plasmid M94, in which residue 17 was changed from L to A, also led to the detection of these electron-dense membrane-associated plaques (data not shown), in addition to intracellular capsids and fully enveloped particles (Fig. 8F and G). The examination of cells transfected with M103 (W44A) resulted in the detection of asymmetrical capsid structures with a completely closed shell, of which the wall was thickened to approximately one-third of the circumference (Fig. 8H). No capsids were observed in cells transfected with M96 (W45A). In these samples, we saw the thickening of the plasma membrane described above for M85. A representative example is shown in Fig. 8I. The membrane-associated plaques were also seen in cells transfected with the double mutant M101 (L56A and L58A) (data not shown). However, more prominent was the observation of amorphous material located in the cytoplasm as shown in Fig. 8J. The structures observed upon transfection of cells with M101 were significantly smaller than assembled capsids in cells transfected with the unmutated plasmid (Fig. 8K). Mutant M97 (R50A) had a phenotype that was indistinguishable from that of M101 (data not shown).

DISCUSSION

The replication strategy of FVs occupies a functional niche between orthoretroviruses and hepadnaviruses (39). The way FVs assemble reflects their unique position. FV capsids do not


A
pcDNA
wt gag 100%
wt gag 50%
wt gag 25%
wt gag 10%
M99 L12A
M100 I20A
M95 I36A
M102 D60A
M 98 L66A
M104 P65A
M105 P69A
M106 P89A
M107 140-144
pcDNA
wt, 100%
wt, 50%
wt, 25%
wt, 10%
M85 L17S
M94 L17A
M103 W44A
M96 W45A
M97 R50A
M101 L56&58A
kD
cell, α-Gag
virus, α-Gag
p71/p68Gag
B
cell, α-Env SU
virus, α-Env SU
gp130Env
gp80SU
C
cell, α-Env LP
virus, α-Env LP
gp38LP
gp28LP
gp18LP
D
= SN
= CL
pcDNA
M107 140-144
M106 P89A
M105 P69A
M 98 L66A
M104 P65A
M102 D60A
M101 L56&58A
M97 R50A
M96 W45A
M103 W44A
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M100 I20A
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M85 L17S
M99 L12A
pcHSRV2
infectivity (ffu/ml)

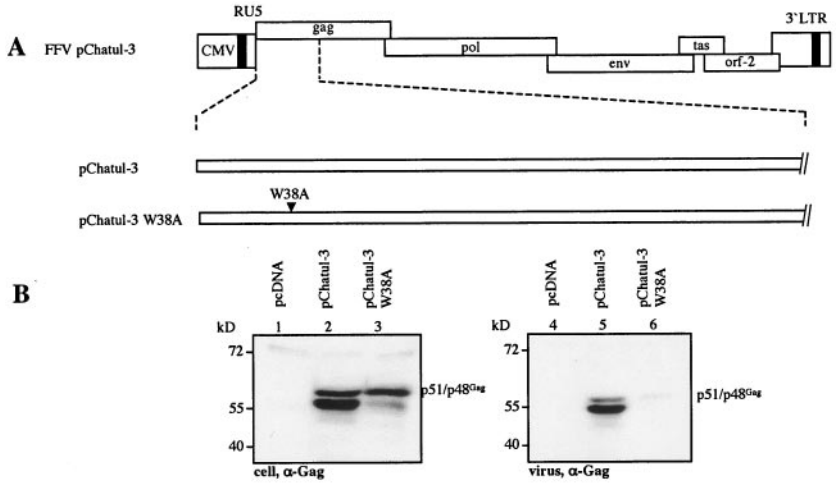

FIG. 7. Gag residue W38 appears to be involved in FFV particle egress. (A) Genome organization of the wild-type infectious molecular FFV clone pChatul3 and the *gag* mutant W38A. (B) Immunoblot of cellular lysates and cell-free particulate material from the supernatant of 293T cells that were transfected with the indicated plasmids and allowed to react with Gag antiserum.

form extracellular VLPs (11). Instead, they require the presence of cognate glycoprotein for cellular egress (27, 36). Moreover, while replicating virus induces massive syncytium formation as one key feature of the cytopathic effect in adherent cells, the expression of *env* alone is tolerated and syncytia are rare (37). However, coexpression of *gag* induces quantitative cell surface expression of Env and consequently the typical cytopathic effect formation (37). In other words, in PFV, Env is required for Gag (capsid) export and Gag is required for quantitative Env translocation to the surface of cells. These findings argue for a highly specific interaction between Env and Gag.

When we investigated the basis for this interaction, a surprising feature and topology of the mature Env glycoprotein were uncovered (28). Instead of being composed of two subunits, the SU linked to the transmembrane (TM) protein, as usually found in orthoretroviruses, FV glycoproteins initially have a type III transmembrane topology, and the mature Env is made up of three proteins. A membrane-spanning LP and SU and a membrane-spanning TM are cleaved from one precursor by cellular enzymes (6, 13, 27). From mutagenesis studies, it was suggested that the cytoplasmic part at the very N terminus of LP probably makes contact with the viral capsid, since the alteration of one or two conserved tryptophan residues in this location of LP abolishes the envelopment of FV capsids by cognate glycoprotein (28, 51).

The structural basis of the FV Gag-Env interaction is largely unknown, as are domains in Gag that specifically participate. Cryoelectromagnetic studies were suggestive of a matrix layer between the capsid and viral membrane resembling orthoretroviral MA proteins (51). Biophysical studies indicated that recombinant FFV Gag residues 1 to 154 specifically bind in vitro to an FFV LP motif of 30 aa (51). Furthermore, the deletion of FFV *gag* sequences comprising aa 4 to 23 up to aa 4 to 97 in the proviral context led to a severe defect in particle egress and rendered the mutants noninfectious (14). Here, we attempted to gain some additional information on FV Gag by the investigation of deletion and point mutants for particle export and morphology.

Analyses of the deletion constructs proved to be highly instructive. Deletions from the 3′ end demonstrated that the first 297 aa of Gag are sufficient to assemble into particulate material that is specifically exported from the cell by cognate Env and can be centrifuged through sucrose. The inability of the ΔC297 mutant to egress from cells in the absence of Env revealed that Gag does not possess an inhibitory motif in the C-terminal half that can be deleted to render FV particles similar to orthoretroviral capsids in terms of budding.

All deletions at the Gag N terminus were defective in export of particulate material. However, the capsid morphology of the larger deletions from Δ7-92 up to Δ7-199 was similar to that of wild-type virus. The ultrastructural analysis of a smaller deletion (Δ7-78) revealed a severe morphological alteration. This is a strong argument in favor of a Gag-interacting domain located upstream of residue 92. Therefore, we investigated mutants of individual residues conserved among FVs in this region. All mutants that were identified to be defective in Env-specific particle export also showed severe alterations in capsid morphology.

This finding can be explained by assuming that the Env-interacting Gag domain is embedded in a region that is required for correct particle assembly and for cytoplasmic trans-

FIG. 6. Analysis of cellular protein expression, egress of particulate viral material, and infectivity after transient transfection of 293T cells with the pcHSRV2 point mutants. In an immunoblot, cellular lysates (cell) and cell-free particulate material sedimented from the supernatant (virus) were allowed to react with anti-Gag (A) and anti-Env SU (B) MAbs. A rabbit anti-Env LP was used in panel C. In panel D, the generation of infectious cell-free virus (SN) and cell-bound virus (CL) was monitored by the blue cell assay.

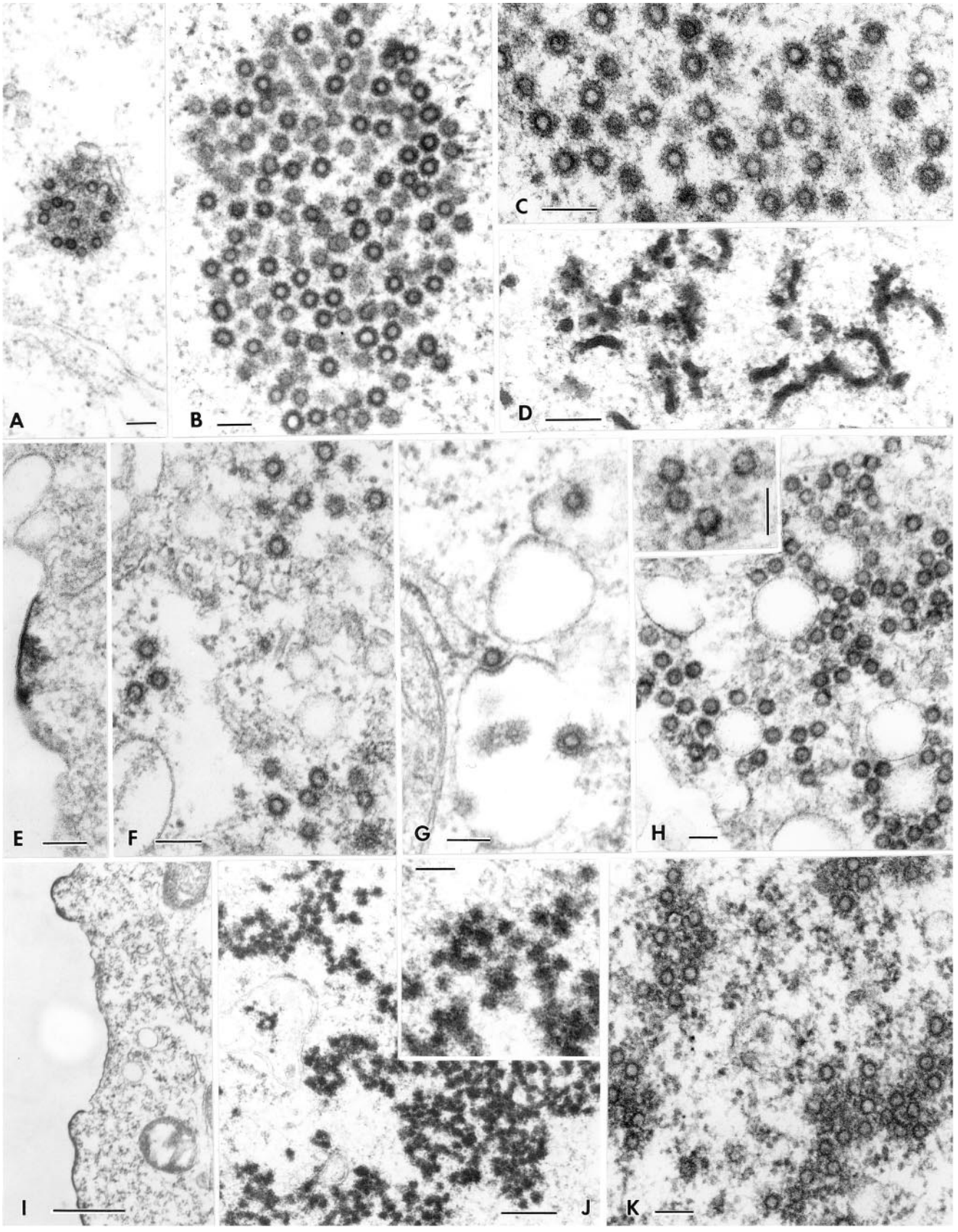

FIG. 8. Ultrastructural analysis of wild-type PFV and *gag* mutants. 293T cells were cotransfected with *env*-, *pol*-, and *gag*-expressing plasmids or with derivatives of the pcHSRV2 infectious molecular clone and processed for EM analysis. (A) pMC/Δ7-199; (B) pMC3/Δ7-125; (C) pMC11/Δ7-92; (D) pMC1/Δ7-78; (E) pcHSRV2-M85 (L17S); (F and G) pcHSRV2-M94 (L17A); (H) pcHSRV2-M103 (W44A); (I) pcHSRV2-M96 (W45A); (J) pcHSRV2-M101 (L56A and L58A); (K) pcHSRV2 (wild type). The bars are 100 nm (B, C, E, F, G, H, insert in H, and K), 200 nm (A, D, and insert in J), and 500 nm (I and J) in length.

port to the site of FV budding. Based on the homology to a region in the Mason-Pfizer monkey virus (MPMV) Gag protein, PFV Gag probably possesses a cytoplasmic targeting/retention signal (CTRS) (7, 51). Arginine residue 50 is at the center of this signal. The complete MPMV CTRS is 18 aa in size (3). The point mutants M103 (W44A), M96 (W45A), M97 (R50A), and M101 (L56 and 58A) were deficient in capsid export and bear alterations within the PFV *gag* region of homology to the MPMV CTRS (Fig. 4). M101 (L56A and L58A) had a morphotype virtually indistinguishable from that of M97 (R50A). Therefore, it is possible that a region encompassing approximately aa 40 to 60 of PFV Gag is required for correct particle assembly, cytoplasmic location, and interaction with Env. The second possibility is that the region between aa 40 and 60 is only essential for correct assembly and direction of capsids to the cytoplasmic site where they can interact with Env. In keeping with this hypothesis, the actual Gag-Env-interacting domain would be located in regions of Gag different from the CTRS motif somewhere upstream of aa 297. By deleting the CTRS motif, the interacting domain could be masked or simply be rendered nonfunctional, because the altered capsid is not transported to the cytoplasmic location where it gets into contact with Env glycoprotein.

Further genetic and biochemical studies are required to define the mechanism of the Gag-Env interaction more precisely. Whether this is a direct interaction, as suggested by biophysical studies (14, 51), or an indirect cellular protein-mediated interaction requires to be investigated further.

It is intriguing to find severe structural Gag changes by mutations leading to alterations of single residues at the PFV Gag N terminus and relatively normal-structured capsids by deleting the complete region from aa 7 to 199. However, this is not without precedent. In human immunodeficiency virus type 1, for instance, minor mutations in the *gag* region specifying the MA protein have been described which resulted in various severe defects in morphogenesis (for a review, see reference 26). On the other hand, large MA deletions still allowed human immunodeficiency virus type 1 particle assembly at an alternate intracellular site (10), and the complete deletion of MA resulted in the generation of replication-competent virus, provided that the long cytoplasmic tail of Env TM was removed (38). Furthermore, different defects have been described for *gag* mutants in the MA domains of beta- and gammaretroviruses (15, 17, 23, 40, 41). Among these were mutants affecting the folding, intracellular transport, and stability of the respective Gag proteins. In particular, the transport and stability issues have not yet been analyzed for our replication-defective mutants. It is therefore conceivable to speculate that similar processes driving the morphogenesis of orthoretroviruses are involved in the assembly of spumaviruses.

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