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The African Swine Fever Virus Nonstructural Protein pB602L Is Required for Formation of the Icosahedral Capsid of the Virus Particle▽

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African swine fever virus (ASFV) protein pB602L has been described as a molecular chaperone for the correct folding of the major capsid protein p72. We have studied the function of protein pB602L during the viral assembly process by using a recombinant ASFV, vB602Li, which inducibly expresses the gene coding for this protein. We show that protein pB602L is a late nonstructural protein, which, in contrast with protein p72, is excluded from the viral factory. Repression of protein pB602L synthesis inhibits the proteolytic processing of the two viral polyproteins pp220 and pp62 and leads to a decrease in the levels of protein p72 and a delocalization of the capsid protein pE120R. As shown by electron microscopy analysis of cells infected with the recombinant virus vB602Li, the viral assembly process is severely altered in the absence of protein pB602L, with the generation of aberrant "zipper-like" structures instead of icosahedral virus particles. These "zipper-like" structures are similar to those found in cells infected under restrictive conditions with the recombinant virus vA72 inducibly expressing protein p72. Immunoelectron microscopy studies show that the abnormal forms generated in the absence of protein pB602L contain the inner envelope protein p17 and the two polyproteins but lack the capsid proteins p72 and pE120R. These findings indicate that protein pB602L is essential for the assembly of the icosahedral capsid of the virus particle.

African swine fever virus (ASFV), the sole member of the *Asfarviridae* family (17), is a large enveloped virus that causes a highly lethal disease in domestic pigs. The viral genome is a double-stranded DNA molecule of 170 to 190 kbp that encodes more than 150 polypeptides (32). The complex ASFV particle, with an icosahedral morphology and a diameter of 200 nm, consists of an internal DNA-containing nucleoid coated by a thick protein layer designated the core shell. This coat is in turn enwrapped by an inner envelope and an outer icosahedral capsid (5, 7, 12). The extracellular virion contains an additional external membrane acquired by budding through the plasma membrane (9). About 50 proteins compose the different domains of the viral particle (11, 19).

The virus assembly process occurs within cytoplasmic areas designated viral factories, which are close to the microtubule organizing center (22). These virus assembly sites are surrounded by a vimentin cage and recruit mitochondria and cellular chaperones (13, 22, 30). Virus morphogenesis is thought to begin with the recruitment of endoplasmic reticulum (ER) cisternae, which are then transformed to give rise to precursor viral membranes (5, 31). The envelope precursors develop into icosahedral structures by the progressive assembly of the capsid layer, formed by protein p72, in an ATP- and calcium-dependent process (7, 14, 20). Simultaneously, the core shell domain is formed under the inner envelope, and finally, the viral DNA and nucleoproteins are packaged and condensed to form the nucleoid (3, 7, 10).

During the last few years, our laboratory has established the role of several ASFV proteins in the morphogenetic process by using recombinant viruses that inducibly express structural proteins. Thus, the structural membrane protein p54 was shown to be involved in the recruitment of ER membranes to the viral factory (28). Also, protein pE120R was identified as the virus component responsible for the transport of intracellular viruses from the assembly sites to the plasma membrane (6). On the other hand, the absence of the viral polyprotein pp220, which constitutes, together with polyprotein pp62, the core shell domain, provokes the assembly of empty capsids (4), while the repression of protein p72, the major capsid protein, leads to the generation of aberrant "zipper-like" structures consisting of a core shell-like domain flanked by ER cisternae (3, 20). In addition, the essentiality of the proteolytic processing of polyproteins pp220 and pp62 was demonstrated by the finding that, in the absence of the polyprotein-processing proteinase, noninfectious aberrant particles containing an acentric nucleoid are generated (1).

Given the complexity of the virus morphogenetic process, it is likely that, in addition to the structural proteins of the virus, other nonstructural, virally encoded proteins, or even cellular proteins, might play essential functions related to the assembly process. These proteins may include molecular chaperones to facilitate the folding of the structural components, thus allowing correct protein-protein interactions to occur during the construction of the viral particle, as has been described for other viruses (8, 16, 25). In relation to this, it has been reported

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that the ASFV-encoded protein pB602L, also named CAP80, functions as a chaperone for the folding of the major capsid protein p72 (15). This conclusion was based on the finding that coexpression of pB602L protein with p72 prevents the aggregation of the major capsid protein in the transfected cells. Also, a transient association between both proteins was detected in ASFV-infected cells.

In the present work, we have examined the function of protein pB602L during the infection to ascertain whether the protein is required for the assembly of the virus particle. To this end, we used a recombinant ASFV inducibly expressing the pB602L protein to see the consequences of the repression of gene B602L on virus morphogenesis. We show that the ASFV protein pB602L is a late nonstructural protein, which, unexpectedly and in contrast with protein p72, is excluded from the viral factory. Repression of the pB602L protein severely alters the viral assembly pathway, with the generation of "zipper-like" structures similar to those found in cells infected under restrictive conditions with the recombinant virus vA72 inducibly expressing the capsid protein p72. The dilemma raised by the different localizations of proteins p72 and pB602L in relation to the function of the latter protein is discussed.

MATERIALS AND METHODS

Cells and viruses. Vero cells were obtained from the American Type Culture Collection and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS). The ASFV strain BA71V adapted to grow in Vero cells and vGUSREP, a BA71V-derived recombinant virus that expresses the *Escherichia coli lac* repressor, have already been described (18, 20).

Antibodies. The monospecific rabbit polyclonal sera against the structural proteins pp220/p150, p37, pp62/p35, pE120R, and p72 as well as the rat anti-p54 antibody and the mouse monoclonal antibodies 17LD3 against protein p72 and 17KG12 against protein p17 have been described previously (26, 28, 33–35). To prepare an antibody against protein pB602L, the complete open reading frame (ORF) B602L was amplified by PCR from the EcoRI B restriction fragment of ASFV DNA, using oligonucleotides 5′-AACTCAGATCTATGGCAGAATTTA ATATTGATGAGCTTCTC and 5′-ATCTCTGAATTCTTACAATTCTGCTT TTGTATATAAAATTTC containing BglII and EcoRI restriction sites (underlined), respectively, and cloned in pRSETB. The pRSETB-B602L plasmid was expressed in *E. coli*, and the recombinant protein fused to a six-His tag was purified according to the procedure of the manufacturer (QIAGEN, Inc.). The antibody against the purified recombinant protein was raised in rabbits.

Construction of plasmid pIND3.B602L. For inducible ASFV gene expression, the intermediate transfer vector pIND3 was used. This plasmid vector is essentially identical to pIND1 (6), except that the distance between the synthetic late promoter *p72.4* and the core sequence of the *E. coli lac* operator in the inducible promoter *p72.I** is of 2 bp instead of 6 bp. This reduced distance between operator and promoter results in a stronger repression of gene expression (20). This transfer vector also contains the *lacZ* gene under the control of the strong late ASFV promoter *p72* (20) and two multiple cloning sites to allow the cloning of the target gene and the corresponding upstream and downstream flanking sequences.

For the generation of plasmid pIND3.B602L, a synthetic DNA fragment of 794 bp containing the nucleotide sequence from positions −10 to +784 relative to the translation initiation codon of the B602L ORF was obtained by PCR, using the EcoRI B fragment as a template and the oligonucleotides 5′-ACTC TCTAGAATGGCAGAATTTAATATTG and 5′-CAGGTGTCAAGCTTGGT ATCTGCACAC, which contain XbaI and HindIII restriction sites (underlined), respectively. Plasmid pIND3.B602L.FI was generated by inserting the XbaI- and HindIII-digested PCR fragment into the XbaI- and HindIII-linearized plasmid pIND3. A synthetic fragment of 751 bp containing the nucleotide sequence from the adjacent B385R ORF was obtained by PCR, using the EcoRI B fragment as a template and the oligonucleotides 5′-CTCATCGGTACCAAATTCTGCCAA ATCTTAGAG and 5′-GTCCCCAACTGCGGCCGCGGATTTCTACCAAG, with KpnI and NotI restriction sites (underlined), respectively. The PCR fragment was inserted into the KpnI and NotI sites of plasmid pIND3.B602L.FI to obtain the final transfer vector pIND3.B602L.

Generation of recombinant virus vB602Li. Recombinant virus was generated essentially as previously described (27), with minor modifications. Briefly, Vero cells were transfected with linearized plasmid pIND3.B602L and infected with virus vGUSREP in the presence of different concentrations of isopropyl-β-D-thiogalactopyranoside (IPTG). At 72 h postinfection (hpi), the cells were harvested and the recombinant virus vB602Li was isolated by sequential rounds of plaque purification in the presence of 250 μM IPTG, a concentration which was found to be optimal for the production of the recombinant virus.

Plaque assay. Vero cell monolayers, in six-well plates, were infected with recombinant virus vB602Li or parental BA71V. After 2 h, the inoculum was removed and the cells were overlaid with Dulbecco's modified Eagle's medium containing 0.55% Noble agar and 3% FCS in the presence or absence of 250 μM IPTG. Five days later, the medium was removed and the monolayers were stained with 1% crystal violet.

One-step virus growth curves. Vero cell monolayers, in 24-well plates, were infected with 5 PFU per cell of recombinant vB602Li or parental BA71V. After 2 h of adsorption, the cells were incubated in medium supplemented with 2% FCS. IPTG (250 μM) was added immediately after the adsorption period as indicated above. Infected cells with their culture supernatants were harvested at different times postinfection, and titers were determined by a plaque assay in the presence of 250 μM IPTG.

Western blotting. Preconfluent Vero cell monolayers were either mock infected or infected with BA71V or recombinant vB602Li virus at a multiplicity of infection of 10 PFU per cell in the presence or absence of 250 μM IPTG. The cells were lysed at 12 hpi in Laemmli sample buffer, and equivalent amounts were electrophoresed in sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose as described previously (7). Protein detection was performed with peroxidase-conjugated antibodies and the enhanced chemiluminescence system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunoprecipitation analysis. Vero cells were mock infected or infected with BA71V or vB602Li virus at a multiplicity of infection of 4 PFU per cell in the presence or absence of 250 μM IPTG. The cells were labeled for 10 min at 14 hpi with 1 mCi of [^{35}S]methionine-[^{35}S]cysteine (Promix in vitro cell labeling mixture; Amersham Pharmacia Biotech) per ml. The cells were lysed with immunoprecipitation buffer (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% sodium deoxycholate, 1% IGEPAL [(octylphenoxy)polyethoxyethanol] CA-630, 0.1% SDS) supplemented with protease inhibitors (complete EDTA-free cocktail; Roche), and the lysates were centrifuged at 14,000 rpm for 15 min. The soluble extracts were immunoprecipitated with the monoclonal antibody 17LD3 against protein p72 immobilized on protein A-Sepharose (Sigma). As an internal control, each of the anti-p72 antibodies was mixed with the anti-pp62 antibody. Proteins were resolved by SDS-12% polyacrylamide gel electrophoresis and detected by autoradiography.

Northern blot analysis. Total RNA from Vero cells mock infected and infected with the parental BA71V virus or with vB602Li virus in the presence or absence of IPTG was prepared at 12 hpi by the TRIzol method (Invitrogen). The RNAs were fractionated on a formaldehyde-agarose gel, transferred to a nitrocellulose membrane, and hybridized as described previously (29), with ^{32}P-labeled oligonucleotides specific for ORFs B646L (5′-TAAGCTTGTTTCCCAA GGTGGGGG-3′) and B602L (5′-GCGTTTCTTCGGATATTTCAGTAGAGG G-3′).

Indirect immunofluorescence. For immunofluorescence assays, preconfluent Vero cells grown on coverslips were mock infected or infected with BA71V or recombinant vB602Li virus at a multiplicity of 1 PFU per cell in the presence or absence of 250 μM IPTG. At 14 hpi, the cells were fixed with 3% paraformaldehyde at room temperature for 15 min and then incubated with 1% Triton X-100 in phosphate-buffered saline (PBS) at room temperature. After 15 min, the coverslips were blocked for 30 min with blocking buffer (2% FCS, 1% bovine serum albumin, 40 mM glycine in PBS). The cells were then sequentially incubated for 30 min with primary and the corresponding secondary antibodies diluted with blocking buffer. Finally, the coverslips were mounted on glass slides with Mowiol/Dabco and DAPI (4′,6′-diamidino-2-phenylindole) to stain the DNA in nuclei and virus factories. Preparations were examined with a Bio-Rad Radiance 2000 confocal laser-scanning microscope. Images were processed using Adobe Photoshop software.

Electron microscopy. For conventional Epon section analysis, Vero cells were infected with 10 PFU per cell and fixed at 18 hpi with 2% glutaraldehyde in PBS for 1 h at room temperature. Postfixation treatment was carried out with 1% OsO_4 and 1.5% $K_3Fe(CN)_6$ in H_2O at 4°C for 30 min. Samples were dehydrated with acetone and embedded in Epon according to standard procedures. For

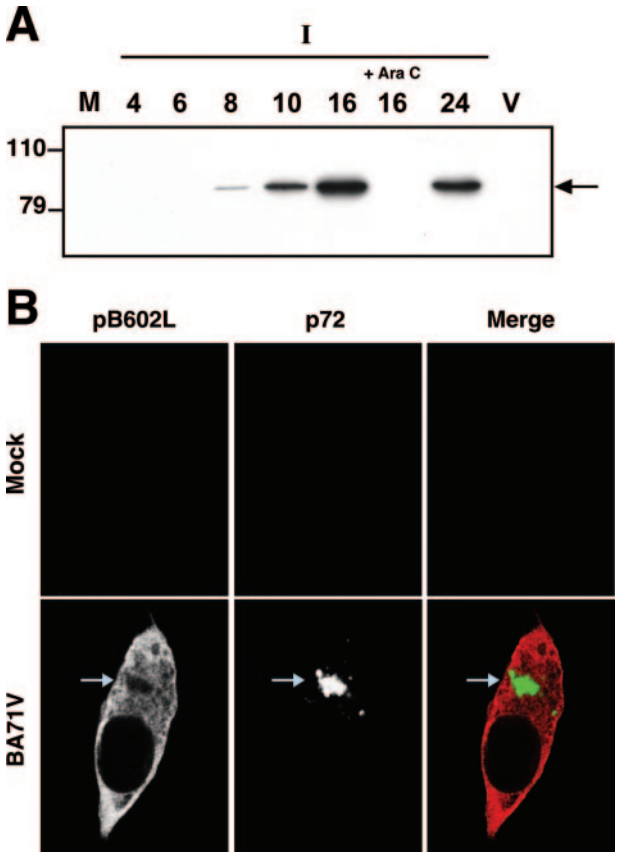

FIG. 1. (A) Expression of protein pB602L in ASFV-infected Vero cells. Western blot analysis with anti-pB602L antibody was carried out for mock-infected cells (M) or ASFV-infected cells (I) harvested at different times postinfection. Results obtained with cells infected for 16 h in the presence of cytosine arabinoside (+Ara C) and with 2 μg of highly purified ASFV particles (11) (V) are also shown. The band corresponding to protein pB602L is indicated. (B) Immunofluorescence detection of protein pB602L in Vero cells infected with ASFV. Mock-infected (Mock) or ASFV-infected (BA71V) Vero cells were fixed at 12 hpi and double labeled with anti-pB602L antibody and anti-p72 monoclonal antibody, which were detected with Alexa 594 goat anti-rabbit immunoglobulin G and Alexa 488 goat anti-mouse immunoglobulin G, respectively. The viral factory labeled with anti-p72 antibody is indicated by an arrow.

immunoelectron microscopy, the cells were fixed at 24 hpi with 8% paraformaldehyde and 0.2% glutaraldehyde in 120 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)], 50 mM HEPES, 4 mM $MgCl_2$, 20 mM EGTA, pH 6.9, for 2 h at room temperature. The fixative was then removed and a solution of 8% paraformaldehyde in 60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9, was added. Finally, the fixed cells were prepared for cryosectioning and immunolabeling as previously described (21).

RESULTS

Expression and immunolocalization of ASFV pB602L protein in infected cells. To study whether protein pB602L plays a role in the ASFV morphogenetic process, we first determined the expression of the protein and its localization in Vero cells infected with the ASFV BA71V strain. For these studies, we generated an antibody against the purified recombinant pB602L protein, as described in Materials and Methods. As can be seen in Fig. 1A, this antibody specifically recognized a band of 80 kDa, which was first detected at 8 hpi and accumulated up to 24 hpi. This band was not detected in the presence of cytosine arabinoside, an inhibitor of viral DNA replication and late transcription, indicating that the pB602L protein is a late protein, in agreement with the results obtained by Irusta et al. (23) for macrophages infected with ASFV Malawi Lil 20/1. As previously described (15, 23), the estimated size of protein pB602L (80 kDa) is greater than its theoretical size (67.9 kDa), which might be due to an anomalous mobility in polyacrylamide gels or to posttranslational modifications. As also shown in this figure, the protein is not a structural component of the virus particle. The finding that protein pB602L is expressed at late times postinfection is in line with a function related to the virus assembly process.

To examine the intracellular localization of protein pB602L, mock-infected and ASFV-infected Vero cells were fixed at 12 hpi and analyzed by immunofluorescence with the anti-pB602L antibody and with an antibody against the capsid protein p72 to identify the viral factories. As shown in Fig. 1B, no background signal was observed with these antibodies in mock-infected cells. In infected cells, the protein pB602L was distributed throughout the cytoplasm but was excluded from the viral factory. Identical results were obtained when the cells were fixed and analyzed at 8 and 10 hpi (not shown). The absence of pB602L protein from the virus assembly sites could be due to a rapid exclusion of the newly synthesized protein or, alternatively, to the cytoplasmic synthesis of pB602L, outside the factory. The finding that proteins p72 and pB602L are localized in different compartments at postinfection times when virus morphogenesis is under way is intriguing and raises questions as to how the protein could perform a role related to the p72 protein.

Inducible expression of protein pB602L by recombinant virus vB602Li. The function of protein pB602L in relation to virus morphogenesis was studied in more detail by using a recombinant ASFV, vB602Li, which inducibly expresses protein pB602L, to see the consequences of gene B602L repression on the viral assembly process. In the vB602Li virus, the expression of gene B602L is controlled by the *E. coli lac* operator/repressor system (Fig. 2A). For this, the BA71V-derived vGUSREP recombinant virus, which constitutively expresses the *E. coli lac* repressor, was modified by replacing the original promoter of gene B602L with an inducible promoter, *p72.I** (20).

To test the inducer dependence of the recombinant vB602Li virus, a plaque assay was performed in the presence and in the absence of 250 μM IPTG. In the presence of the inducer, the numbers and the sizes of the plaques were similar for both parental and recombinant viruses, while in its absence, the plaque number for recombinant vB602Li was dramatically reduced (not shown).

To see whether the plaque phenotype of vB602Li is related to the IPTG-dependent expression of protein pB602L, mock-infected and BA71V- or vB602Li-infected cells were analyzed by Western blotting. As can be seen in Fig. 2B, the protein pB602L band was not detectable in the absence of IPTG, while under permissive conditions, the levels of expression for the protein were comparable to those obtained with the parental virus BA71V. These results demonstrate that the expression of

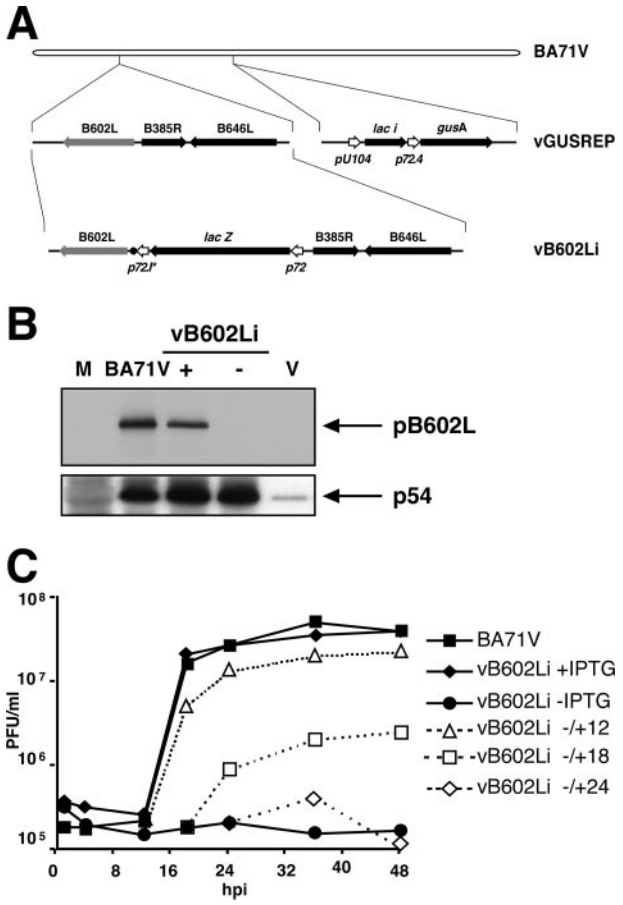

FIG. 2. (A) Genomic structure of the ASFV recombinant vB602Li. The recombinant virus vB602Li was obtained from vGUSREP, a BA71V-derived recombinant virus, which contains the *lac* repressor-encoding gene *lacI* inserted into the nonessential thymidine kinase locus (20). In the vB602Li virus, the promoter of gene B602L was replaced by an inducible promoter, *p72.I**. Gene B646L codes for the capsid protein p72. (B) Inducible expression of protein pB602L. Vero cells were either mock infected (M) or infected with parental BA71V or recombinant vB602Li virus in the presence (+) or absence (−) of 250 μM IPTG. At 12 hpi, the cells were lysed and analyzed, along with 2 μg of purified ASFV particles (V), by immunoblotting them with antibodies against protein pB602L and protein p54 (control). The positions of the detected proteins are indicated. (C) One-step growth curves of vB602Li virus. Vero cells were infected with 5 PFU of vB602Li per cell in the presence or absence of 250 μM IPTG. At the indicated times postinfection, the total virus titer of each sample was determined by a plaque assay with Vero cells in the presence of the inducer. Parental BA71V infections were also titrated as a control. Recombinant vB602Li virus was also grown under restrictive conditions for 12, 18, or 24 h and then induced with 250 μM IPTG. At different times after induction, the infectious virus was titrated as described above.

pB602L protein can be strongly repressed in the inducible virus.

To examine the effect of protein pB602L repression on virus replication, one-step growth curve analyses were carried out. Figure 2C shows that, under permissive conditions, the virus titers of recombinant virus vB602Li were essentially the same as those obtained with the parental BA71V virus. In contrast, under restrictive conditions, vB602Li production was reduced

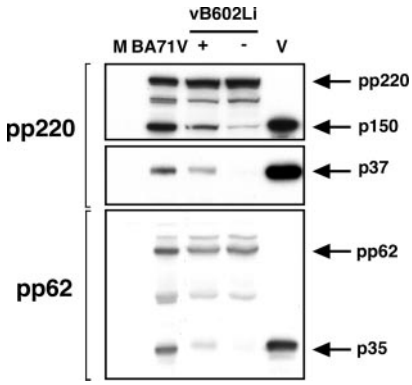

FIG. 3. Polyprotein processing requires the expression of protein pB602L. Polyprotein processing was analyzed by Western blotting with antibodies against polyprotein pp220 and its mature products p150 and p37 and polyprotein pp62 and its mature product p35 in the extracts used in the experiment whose results are shown in Fig. 2B. M, mock-infected cells; BA71V, cells infected with parental BA71V; + and −, cells infected with recombinant vB602Li virus in the presence and absence, respectively, of 250 μM IPTG. A sample of 2 μg of highly purified ASFV (V) was also analyzed. The positions of the detected proteins are indicated.

by more than 2 log units at all postinfection times examined. We also tested the capacity of the recombinant virus grown under restrictive conditions for different time periods to produce infectious virus upon addition of the inducer. As can be seen in this figure, the recovery of virus production was dependent on the time of the induction. Thus, when IPTG was added at 12 hpi, the virus yield approached that obtained under permissive conditions, but when the inducer was added at 18 or 24 hpi, the maximal titers were strongly reduced. This may be due to anomalies in the viral processes that become more pronounced as the period in the absence of the protein is prolonged. The results for the plaque assays and the one-step growth curve analyses indicate that recombinant virus vB602Li is an IPTG-dependent lethal conditional mutant. This is the first ASFV recombinant generated that inducibly expresses a nonstructural protein.

Polyprotein processing is inhibited in the absence of protein pB602L. Proteolytic processing of the ASFV polyproteins pp220 and pp62 is an essential maturational process required for the correct assembly of the viral core (1, 3). Previous results obtained with other inducible ASFV recombinants indicated that the inhibition of polyprotein processing is a very sensitive biochemical indicator of various alterations in the morphogenetic process (3, 28). We therefore investigated whether the processing of polyproteins pp220 and pp62 was also impaired in cells infected with the recombinant virus vB602Li under restrictive conditions. As shown in Fig. 3, an inhibition of the processing of polyprotein pp220 to its mature products p150 and p37 and of polyprotein pp62 to its product p35 was observed in the absence of pB602L protein. This finding suggested that, as in the case of other inducible viruses, the morphogenetic process might also be altered in cells infected with the vB602Li virus under nonpermissive conditions.

Immunofluorescence microscopy of vB602Li-infected cells. To further investigate the phenotype of the recombinant vB602Li virus, we analyzed by immunofluorescence the local-

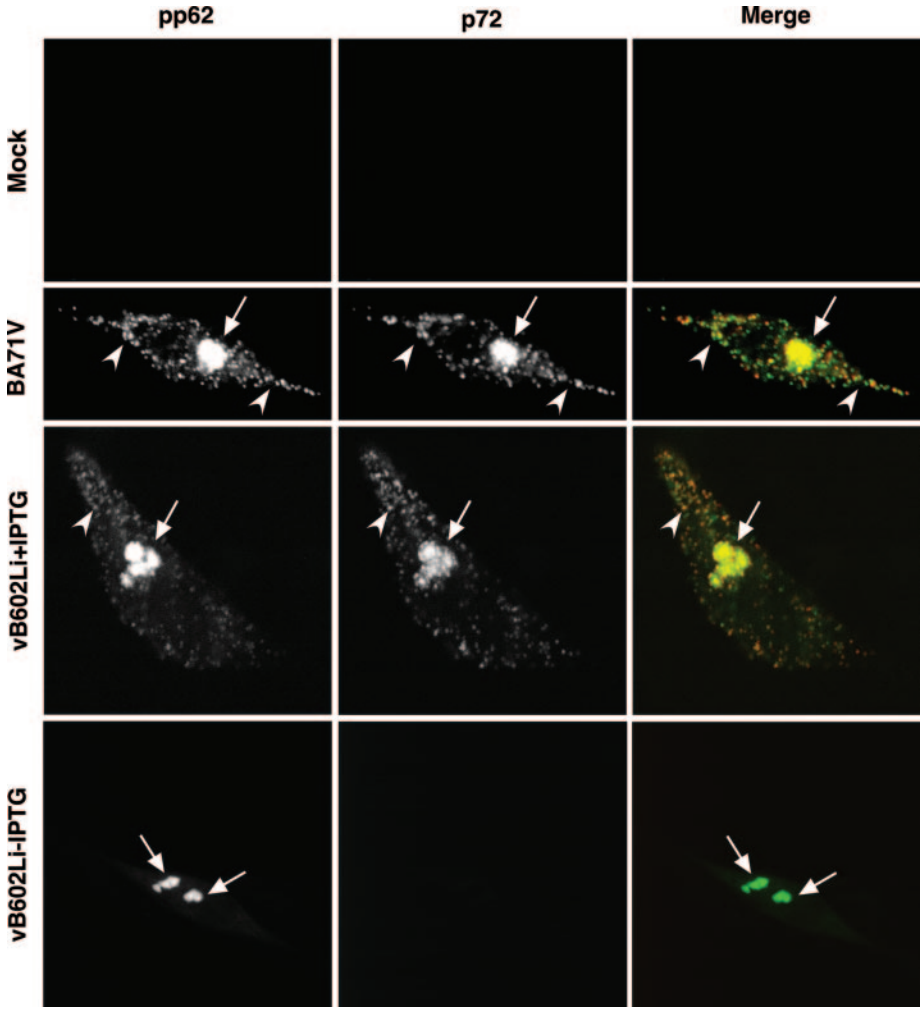

FIG. 4. Localization of pp62 and p72 proteins in cells infected with vB602Li virus. Vero cells mock infected or infected with parental BA71V or with recombinant vB602Li virus in the presence or absence of 250 μM IPTG were fixed at 14 hpi and double labeled with rabbit anti-pp62 antibody and mouse anti-p72 monoclonal antibody. Labeling was revealed with Alexa 488 goat anti-rabbit immunoglobulin G and with Alexa 594 goat anti-mouse immunoglobulin G, respectively. The viral factories and virions spread throughout the cytoplasm are indicated by arrows and arrowheads, respectively. The localization of polyprotein pp62 at the viral factory in cells infected with vB602Li virus in the absence of IPTG was confirmed by DAPI staining of the viral DNA in nonconfocal microscopy analysis (not shown).

izations of the viral polyproteins as well as of proteins p72 and pE120R, components of the viral capsid, in cells infected with the inducible virus under permissive or restrictive conditions, using antibodies specific for the different proteins. In cells infected with the parental BA71V virus or with vB602Li virus in the presence of the inducer, the antibodies against proteins pp62 and p72 strongly labeled the viral factories as well as virus particles scattered throughout the cytoplasm (Fig. 4). In the absence of protein pB602L, polyprotein pp62 was exclusively confined to the viral factory, while protein p72 was undetectable. It is noteworthy that under these conditions, no viral particles were detected. When the localization of polyprotein pp220 was examined, the labeling pattern obtained was similar to that of pp62 (not shown). The antibody specific for protein pE120R also labeled viral factories and cytoplasmic virus particles in cells infected with the wild-type virus or with vB602Li virus under permissive conditions (Fig. 5). By contrast, under restrictive conditions, protein pE120R was found in the nucleus, which appeared strongly labeled, and dispersed in the cytoplasm. A diffuse cytoplasmic signal, barely detectable, was obtained with the anti-p72 antibody. Virus morphogenesis appears to be blocked under these conditions, as no virus particles are detected in the cytoplasm of the infected cells.

Electron microscopy of vB602Li-infected cells. To characterize more precisely the stage at which virus morphogenesis is blocked in the absence of protein pB602L, cells infected with vB602Li virus in the presence or in the absence of IPTG were examined by electron microscopy. Under permissive conditions (Fig. 6A), the cytoplasmic viral factories contained, at 18 hpi, envelope precursors and immature and mature icosahedral particles in proportions similar to those found in cells infected with wild-type virus (not shown). When the inducer was omitted, the assembly sites were completely devoid of icosahedral particles, containing instead large numbers of aberrant structures (Fig. 6B). These structures, which are similar to the "zipper-like" forms found in cells infected under restric-

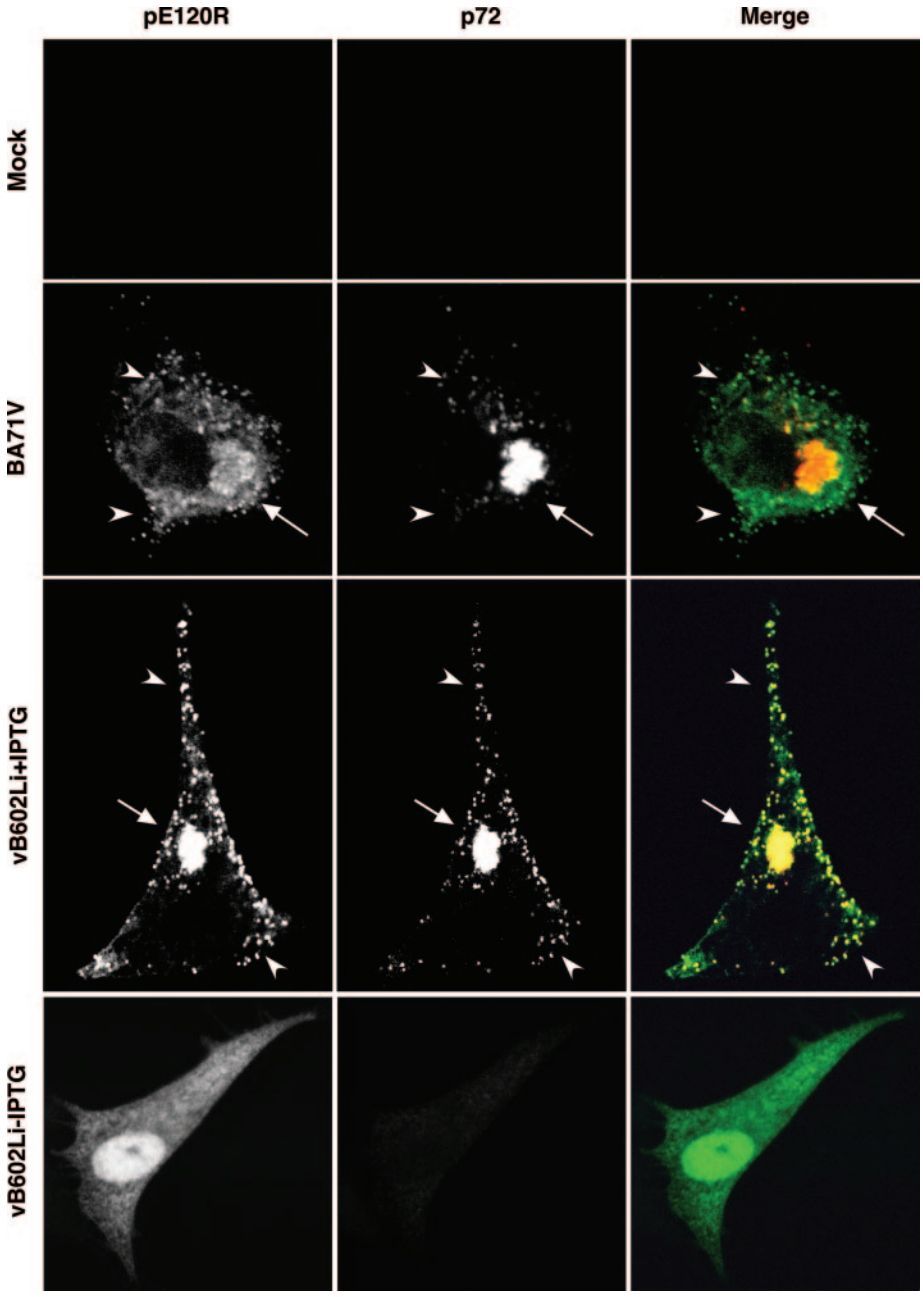

FIG. 5. Localization of proteins pE120R and p72 in cells infected with vB602Li virus. Vero cells mock infected or infected with parental BA71V or with recombinant vB602Li virus in the presence or absence of 250 μM IPTG were fixed at 14 hpi and double labeled with rabbit anti-pE120R antibody and mouse anti-p72 monoclonal antibody. Labeling was revealed with Alexa 488 goat anti-rabbit immunoglobulin G and with Alexa 594 goat anti-mouse immunoglobulin G, respectively. Viral factories and virions spread throughout the cytoplasm are indicated by arrows and arrowheads, respectively.

tive conditions with ASFV recombinant vA72, which inducibly expresses the capsid protein p72 (20), consist of two parallel cisternae bound by either one (Fig. 6C) or two (Fig. 6D) copies of a thick layer symmetrically subdivided by a thin and electron-dense structure. As previously described for the aberrant structures observed in the absence of protein p72, this layer between the two cisternae resembles the virus core shell domain. As can also be seen in Fig. 6C and D, these "zipper-like" structures apparently lack a capsid.

It should be noticed that, despite these similarities between the phenotypes of the ASFV recombinants vB602Li and vA72, they differ in several aspects. Thus, the viral factories in cells infected with the inducible vA72 virus under restrictive conditions are essentially devoid of membranes (20), while those in cells infected with the recombinant vB602Li contain abundant membranous structures (Fig. 6B). Also, the limiting cisternae of the "zippers" formed with the vB602Li virus are with some frequency not collapsed (Fig. 6B and E), while most of the

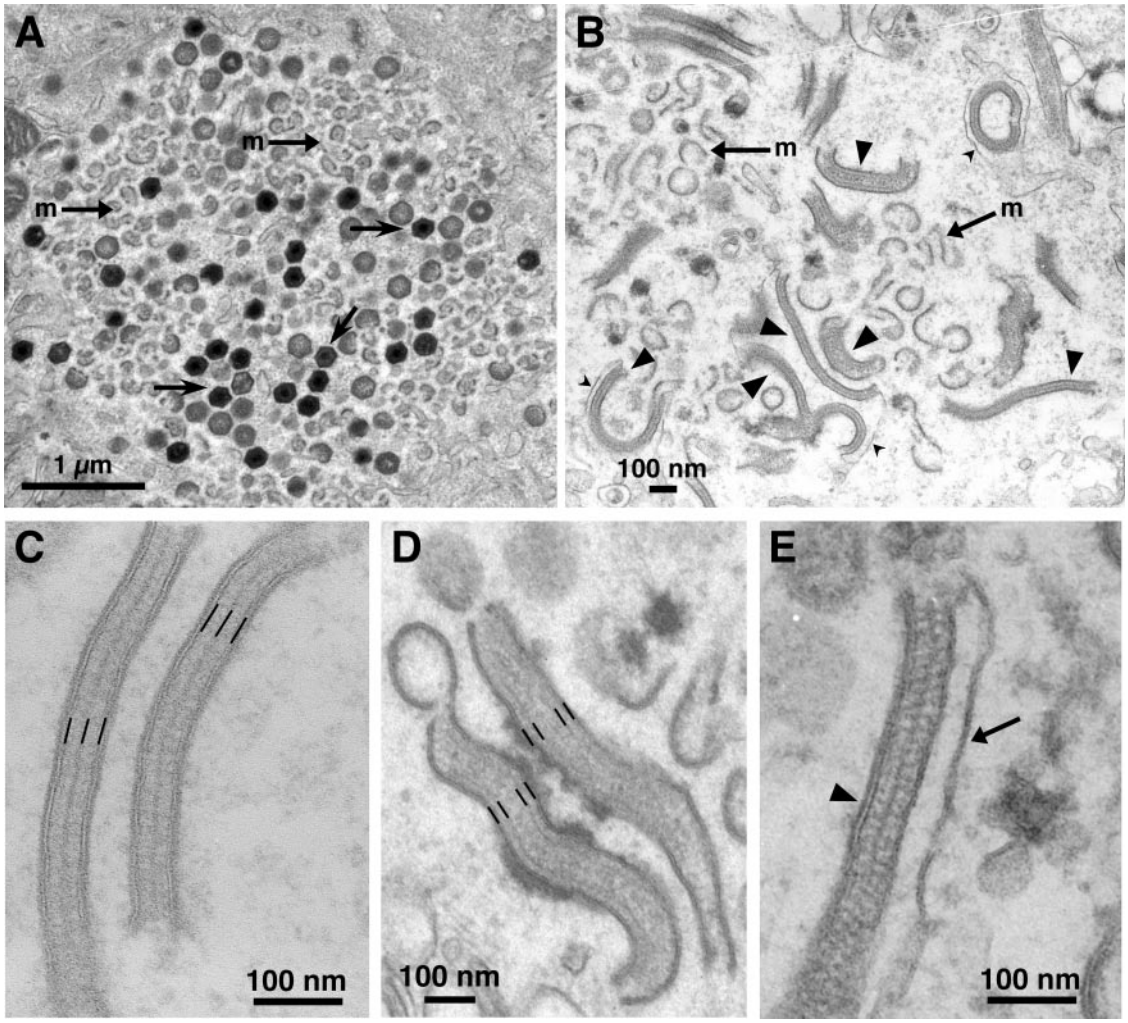

FIG. 6. Electron microscopy of vB602Li-infected cells. Ultrathin Epon sections of vB602Li-infected Vero cells incubated for 18 h in the presence (A) or in the absence (B to E) of IPTG are shown. In the presence of the inducer (A), the assembly sites contain large amounts of membranes (m) as well as immature and mature virions (arrows). Under restrictive conditions (B), the defective factories contain, in addition to membranes (m), abundant "zipper-like" structures (large arrowheads). These aberrant structures consist of two parallel cisternae bound by either one (C) or two (D) copies of a thick layer symmetrically subdivided by a thin and electron-dense structure (parallel lines). With some frequency, the limiting cisternae of the "zippers" are not collapsed (small arrowheads and arrow in panels B and E, respectively). The arrowhead in panel E indicates a collapsed cisterna.

"zippers" generated in cells infected with the vA72 recombinant present collapsed cisternae (20). These differences may suggest that, in addition to protein p72, another viral protein(s) could be affected in the absence of protein pB602L.

To characterize in more detail the abnormal "zipper-like" forms that accumulate at the viral factories under restrictive conditions in cells infected with the recombinant vB602Li virus, we analyzed by immunoelectron microscopy the presence in these structures of marker proteins for the different domains of the normal virus particle. As can be seen in Fig. 7A and B, no significant labeling of the abnormal structures was obtained with antibodies against the capsid proteins p72 and pE120R, confirming that the "zipper-like" forms are devoid of a capsid. By contrast, the antibody against the inner-envelope protein p17 strongly labeled the outer domains of these structures (Fig. 7C), indicating that they are limited by virally modified membranes. The anti-pp220 and anti-pp62 antibodies also strongly labeled the inner domains of these forms (Fig. 7D and E), which would therefore contain the unprocessed polyproteins, as in the case of the "zippers" generated in the absence of p72 (3).

The observations made by electron microscopy indicate that, in the absence of protein pB602L, the ASFV morphogenetic process is drastically altered, probably due to an inability to form an icosahedral capsid on envelope precursors. This would lead to the aberrant assembly of the unprocessed polyproteins pp220 and pp62 on the viral membranes with the generation of the "zipper-like" structures.

Expression of protein p72 in vB602Li-infected cells. As shown above, protein p72 was barely detectable by immunofluorescence analysis in cells infected with the recombinant virus vB602Li under restrictive conditions. To confirm this, we used Western blot analysis as a more quantitative method to assess the levels of the protein under nonpermissive condi-

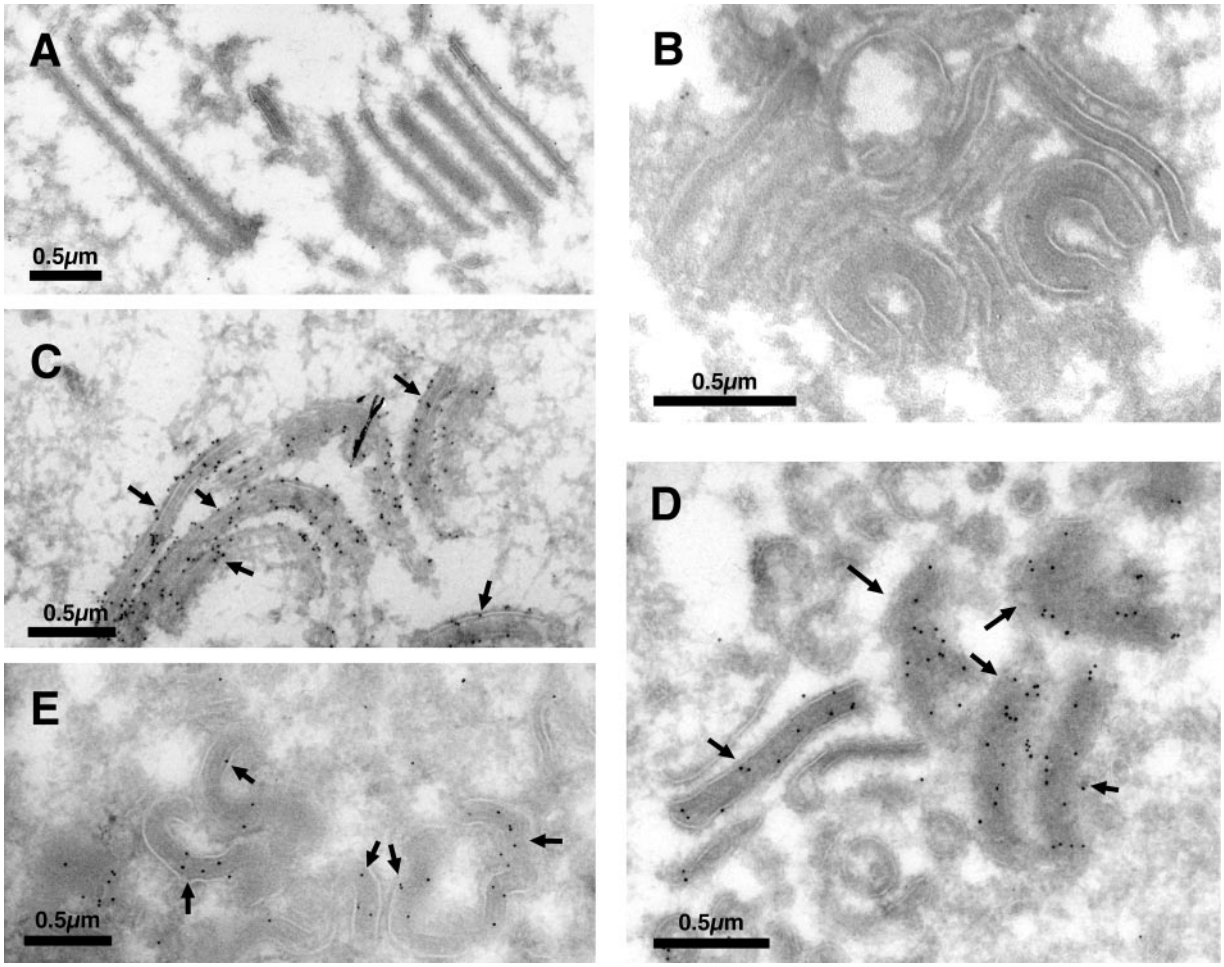

FIG. 7. Immunoelectron microscopy of vB602Li-infected cells. Vero cells were infected with recombinant vB602Li virus in the absence of IPTG. At 24 hpi, the cells were fixed and prepared for cryosectioning. Cryosections were incubated with antibodies against the capsid proteins pE120R (A) and p72 (B), the inner envelope protein p17 (C), and the core shell polyproteins pp220 (D) and pp62 (E), followed by incubation with protein A-gold (10 nm). The arrows indicate significant labeling of the inner envelope and the core shell of the aberrant "zipper-like" structures. No significant labeling was observed with antibodies against the capsid proteins (A and B).

tions. As can be seen in Fig. 8A, and in line with the immunofluorescence data, considerable decreases in the levels of protein p72 were observed in cells infected in the absence of IPTG. To investigate the possibility that the transcription of the p72 gene could be inhibited, we analyzed by Northern blotting the levels of p72 transcripts under restrictive conditions. As shown in Fig. 8C, the p72 probe detected two RNA bands of approximately 5 and 2 kb in the parental BA71V infection. Given the size of the B646L ORF, the 2-kb band most likely corresponds to transcripts of this gene ending at the 9T termination signal immediately downstream (2) (Fig. 8C). The 5-kb band may correspond to a transcript extending from the p72 gene to the strong termination signal (10T) downstream of the B602L gene. These results suggest that with some frequency, the transcriptional machinery bypasses the 9T signal but recognizes the stronger 10T signal. The detection of an RNA band of this size with the B602L probe strongly supports this explanation (Fig. 8C). The 2-kb RNA band was also detected with the same intensity in cells infected with the recombinant vB602Li virus under permissive or restrictive conditions. In these infections, instead of the 5-kb band, an RNA of approximately 3 kb is detected, probably due to transcriptional termination at the 10T signal introduced upstream of the *lacZ* gene in the construction of the recombinant virus. This figure also shows that, as expected, no transcripts of gene B602L were detected in the infection with the recombinant virus under restrictive conditions. Taken together, these findings indicate that the transcription of the p72 gene is not inhibited in the absence of protein pB602L.

To further investigate the expression of protein p72 under restrictive conditions, the cells were pulse-labeled with [^{35}S]methionine-[^{35}S]cysteine for various time periods and, after the pulse, extracts were prepared and immunoprecipitated with the 17LD3 monoclonal antibody against protein p72. The immunoprecipitated proteins were finally separated by electrophoresis in polyacrylamide gels. As shown in Fig. 8B, a band corresponding to protein p72 was clearly detected after a 10-min pulse in cells infected with the parental BA71V virus or the recombinant vB602Li virus under permissive conditions. In contrast, the p72 band was hardly detectable in cells infected

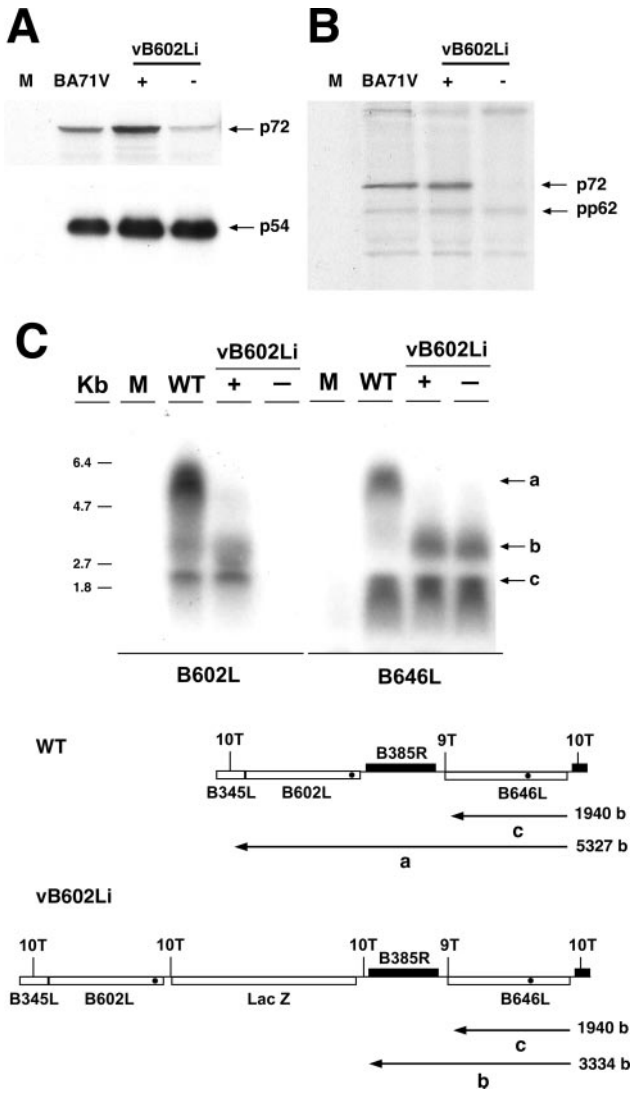

FIG. 8. Expression of protein p72 in vB602Li-infected cells. (A) Protein p72 expression was analyzed by Western blotting with the polyclonal antibody. As a loading control, protein p54 was also analyzed with the anti-p54 antibody. M, mock-infected cells; BA71V, cells infected with the parental BA71V virus; + and −, cells infected with recombinant vB602Li virus in the presence and absence, respectively, of IPTG. The positions of p72 and p54 proteins are indicated by arrows. (B) ^{35}S-labeled extracts from Vero cells mock infected (M) or infected with the parental BA71V virus (BA71V) or with vB602Li virus in the presence (+) or absence (−) of IPTG were immunoprecipitated with the monoclonal antibody 17LD3 against protein p72. As an internal control, the anti-pp62 antibody was mixed with the anti-p72 antibody. The positions of proteins p72 and pp62 are indicated by arrows. (C) Northern blot analysis of RNA prepared from Vero cells mock infected (M) or infected with the parental BA71V virus (WT) or with vB602Li virus in the presence (+) or absence (−) of IPTG. The blots were hybridized with ^{32}P-labeled oligonucleotides specific for ORFs B602L and B646L (p72). The specific bands of B646L are indicated (a, b, and c). The positions of size markers are indicated on the left. The diagram illustrates the arrangement of ORFs B646L and B602L in the parental and recombinant viruses. The *lacZ* gene in the recombinant virus and the various termination signals in the region are also indicated. The black dots represent the oligonucleotides used in the hybridizations. The transcripts detected with the p72 probe (a, b, and c) are shown with their corresponding sizes.

with the recombinant virus in the absence of IPTG. Similar findings were obtained with longer pulses (1 and 4 h) (not shown). These results could be explained by a rapid degradation of the protein during the pulse itself, although the possibility that the synthesis of protein p72 might be affected in the absence of protein pB602L cannot be ruled out. The inability to detect significant amounts of the newly synthesized p72 protein in cells infected with the recombinant vB602Li virus under restrictive conditions reinforces the conclusion that a critical defect in the absence of protein pB602L is the pronounced decrease in the levels of protein p72, which could account for the observed phenotype, characterized by the formation of "zipper-like" structures lacking protein p72.

DISCUSSION

In this work, we have studied the role of the ASFV late protein pB602L in the virus morphogenetic process. Although the protein is not a structural component of the viral particle, it is essential for the assembly of the icosahedral capsid of the virus, as demonstrated in experiments using an ASFV recombinant virus, vB602Li, which inducibly expresses protein pB602L. In the absence of the protein, the virus assembly process is severely altered, with the formation of aberrant "zipper-like" structures instead of icosahedral virus particles. The aberrant forms, as concluded from immunoelectron microscopy data, contain viral proteins of the inner envelope and core shell domains of the virus particle but lack the capsid proteins pE120R and p72. On the other hand, our observations in immunofluorescence experiments, as well as the data obtained by Western blotting and immunoprecipitation analysis, indicate that, in the absence of pB602L protein, the levels of the capsid protein p72 are considerably decreased. This would affect the processes that occur in the normal morphogenetic route, leading to the generation of the same abnormal structures that are formed in cells infected under restrictive conditions with the recombinant vA72 virus which inducibly expresses protein p72 (20). With both inducible viruses, the "zipper-like" forms can be single and double and contain the viral polyproteins pp220 and pp62 in an unprocessed state, underlining the similarity between the "zipper" structures generated with the two viruses. However, certain differences in the phenotypes of the recombinant vB602Li and vA72 viruses are observed. In this regard, it is particularly noteworthy that, in contrast to what was found for the recombinant vA72, the limiting cisternae of the "zippers" generated with the inducible vB602Li virus are with some frequency not collapsed, suggesting that the viral protein(s) involved in the collapsing event of the virus envelope (28) may also be affected in the absence of protein pB602L.

The phenotype of the recombinant vB602Li virus observed when the B602L gene is repressed could be explained by the previously proposed function of pB602L protein as a molecular chaperone for the correct folding of the capsid protein p72 (15). Thus, the low levels of protein p72 found under these conditions, which are not due to an inhibition of transcription, may reflect a lower stability for the protein as a consequence of an altered conformation in the absence of protein pB602L. The degradation of protein p72 under these conditions probably does not involve the proteasome, since, in the presence of

the proteasome inhibitor adamantane-acetyl-(6-aminohexanoyl)$_3$-(leucinyl)$_3$-vinyl-(methyl)-sulfone (24), no protection of protein p72 was observed (not shown). On the other hand, it is noteworthy that the ASFV capsid protein pE120R, which interacts with protein p72 (6, 26) and may possess a nuclear localization signal (L. Martínez-Pomares, Ph.D. thesis, Universidad Autónoma, Madrid, Spain, 1990), is translocated into the nucleus in cells infected with the recombinant vB602Li under restrictive conditions, in contrast with its retention at the viral factory in cells infected in the presence of the inducer. The release of protein pE120R from the factory is probably due to the low levels of p72 protein observed in the absence of protein pB602L.

In contrast with the delocalization of protein pE120R in the absence of pB602L, the two viral polyproteins pp220 and pp62 are retained in the viral factory, probably due to their interaction with the ER precursor membranes to form "zipper-like" structures. This may suggest the existence of mechanisms for the retention of the viral structural proteins at the assembly site. In support of this view are also the observations made with the recombinant ASFV that inducibly expresses protein p54 (28). Thus, under restrictive conditions, in which the viral factory is completely devoid of precursor membranes, several major components of the virus particle, such as the polyproteins (28) and protein p72 (J. M. Rodríguez, unpublished observations), are excluded from the assembly area. This points to a pivotal role for the recruited ER membranes in maintaining these proteins within the virus assembly site by their anchorage to the membranes, which are very abundant in the factory. Protein-protein interactions, such as that of pE120R with p72, might represent additional mechanisms that prevent the escape of the structural proteins from the assembly area. These mechanisms may in fact be key determinants in the construction of the virus particle itself, a process that could be envisaged as a cascade of events initiated by a virally induced modification of the precursor membranes that would trigger the association of protein p72 and the polyproteins to the membranes, with the subsequent interaction of other structural proteins with this morphogenetic intermediate.

Although our findings support a role for protein pB602L related to the capsid protein p72, the different localizations of the two proteins during the infection found in immunofluorescence experiments raise difficulties in understanding how protein pB602L could perform its function as a molecular chaperone of the major capsid protein. However, a transient interaction between the two proteins, as has been described by Cobbold et al. (15), might be undetectable by immunofluorescence experiments, which essentially show the areas of protein accumulation. This brief interaction could take place shortly after the synthesis of the two proteins, either at the viral factory or at the cytoplasm. Alternative explanations may also be considered. Thus, the effect of protein pB602L on protein p72 could be indirect, by, for example, modifying in some way the cytoplasmic environment of the cell or activating cellular chaperones. Further work will be needed to clarify this dilemma.

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