Inducible Gene Expression from African Swine Fever Virus Recombinants: Analysis of the Major Capsid Protein p72

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A method to study the function of individual African swine fever virus (ASFV) gene products utilizing the Escherichia coli lac repressor-operator system has been developed. Recombinant viruses containing both the lacI gene encoding the lac repressor and a strong virus late promoter modified by the insertion of one or two copies of the lac operator sequence at various positions were constructed. The ability of each modified promoter to regulate expression of the firefly luciferase gene was assayed in the presence and in the absence of the inducer isopropyl β-D-thiogalactoside (IPTG). Induction and repression of gene activity were dependent on the position(s) of the operator(s) with respect to the promoter and on the number of operators inserted. The ability of this system to regulate the expression of ASFV genes was analyzed by constructing a recombinant virus inducibly expressing the major capsid protein p72. Electron microscopy analysis revealed that under nonpermissive conditions, electron-dense membrane-like structures accumulated in the viral factories and capsid formation was inhibited. Induction of p72 expression allowed the progressive building of the capsid on these structures, leading to assembly of ASFV particles. The results of this report demonstrate that the transferred inducible expression system is a powerful tool for analyzing the function of ASFV genes.
surfaces of previously accumulated electron-dense membrane structures. These viral membranes become polyhedral structures which evolve toward the generation of virus particles.

MATERIALS AND METHODS

Cells and viruses. Vero cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. The ASFV strain BAV17 was propagated and titrated as previously described (26). Virus infections were carried out with DMEM containing 2% fetal bovine serum. Recombinant virus vA72 was grown in the presence of 1.25 mM IPTG, rabbit anti-mouse immunoglobulin G (IgG) and goat anti-rat Ig, were obtained from Dako and Nordic, respectively.

Plasmid construction. (i) pU104GUSREP. A 122-bp DNA fragment containing the promoter sequence of the early ASFV gene U104 and a 1.3-kb XbaI site was generated by PCR using the primers 5′-GGGCGAATTCGTCGACACTTTAAGTG and 5′-GGGCGTCTAGATGTAGTTGATATTTACAAA, which contain EcoRI and XbaI restriction endonuclease sites at their 5′ ends, respectively. The PCR product was digested with EcoRI and XbaI and was cloned into EcoRI- and XbaI-digested p119.10T to generate the DNA fragment containing the 5′ end of the lacI gene of E. coli. The 5′ end was then inserted into pU104-REP with Klenow enzyme from pRSV-1 plasmid (32), by using the primers 5′-GGGCGAATTCGTCGACACTTTAAGTG and 5′-GGGCGTCTAGATGTAGTTGATATTTACAAA (the former contains HindIII and XbaI sites at the 5′ end). The synthetic PCR fragment was cut with HindIII and MluI and was cloned into pRSV-1 digested with HindIII and MluI to generate pRE. A 1.2-kb XbaI fragment from pRE containing the complete lacI coding sequence was cloned into p119.10T digested with XbaI, generating the plasmid pU104REP. Plasmid pU104REP10T was obtained by cloning a Klenow enzyme-treated 1.3-kb pU104REP fragment into p119.10T digested with HindIII and treated with Klenow enzyme, generating the plasmid pU104REP10T. This fragment contains the CD2 coding sequence, the translation initiation codon of the ASFV B646L gene encoding the protein p72, was obtained by PCR from purified virus DNA by using the primers 5′-GGGCGAATTCGTCGACACTTTAAGTG and 5′-GGGCGGATCCATGGCATCAGGAGGAG and 5′-GGGCGGATCCTAGCTGACCATTGGGCCG were used as primers to obtain a 422-bp PCR DNA fragment corresponding to the 5′ end (400 bp) of the B646L coding sequence (the primers include, respectively, BamHI and BglII restriction sites at their 5′ ends). The PCR product was digested with BamHI and BglII and inserted into BamHI-linearized p72.71, producing the plasmid pF12-p72.71. A 524-bp Smal-to-XbaI fragment treated with Klenow enzyme from pF12-p72.71 was cloned into pF12 digested with HindIII and treated with Klenow enzyme, generating the plasmid pF72P. A 3.3-kb fragment by digestion with Smal and Sall endonucleases and treatment with Klenow enzyme from pF72GAL10T (28) was cloned into Sall-linearized and Klenow enzyme-treated pF72GUS, producing the transfer vectors pF72P.I, pF72P.II, and pF72P.III, where the p72-lacZ chimeric gene was inserted in the same or in the opposite transcriptional orientation as the B646L gene, respectively.

Generation of recombinant viruses. Recombinant viruses were generated as previously described (45). The structures of all the recombinant viruses described in this report were confirmed by DNA hybridization analysis (data not shown). The runs of seven or more consecutive thermolytic (T) residues in the coding strand are signals for mRNA 3′-end formation (2, 3). Thus, to minimize the risk of obtaining nontranscriptional distal ends which may be recognized by the virus genome, genes for the 3′-end formation of ASFV mRNAs were placed in transfer vectors. The positions of these signals in the viral genome of ASFV recombinants generated in this report are indicated in Fig. 1, 2A, and 3A.

(iii) pINDp72.4-gusA and pINDp72.4-gal(i). A synthetic DNA fragment of 478-bp containing the lacI sequences, was inserted into the XbaI site of a single BamHI-digested fragment of pUC119. This fragment contains the CD2 coding sequence was obtained by insert-
against p72, a goat anti-rat Ig, and protein A-gold complexes (15 nm; BioCell Research Laboratories, Cardiff, United Kingdom).

For preembedding immunolabeling, cells were processed as previously described (53). In general, Vero cells were permeabilized with 4% of the bacterial toxin streptolysin O (Sigma)/ml and fixed with 4% paraformaldehyde for 5 min on ice. Cells were sequentially incubated with a rat serum against p72 or a mixture of monoclonal antibodies (17L-D3 and 19B.A2), anti-p72 (48), and protein A-gold complexes (5 nm), and were postfixed in 1% glutaraldehyde. Finally, cells were stained with 1% OsO4–1.5% K3Fe(CN)6 for 60 min, followed by 1% magnesium uranyl acetate for 60 min, and were processed for conventional Epon embedding.

Specimens were viewed with a JEOL 1010 or a JEOL 1200× electron microscope.

RESULTS

Generation of an ASFV recombinant expressing the lac repressor protein. In order to incorporate into ASFV the inducible expression system based on the E. coli lac operon, we first generated a recombinant virus expressing the lac repressor protein. This virus was obtained by cloning the lacI gene into a transfer vector downstream of the promoter of the ASFV U104L early gene (2). The resulting virus, vGUSREP, possesses the genomic structure shown in Fig. 1, where the coding sequences of lacI and a reporter gene, gusA, are inserted into the nonessential TK gene (45) of the virus strain BA71V. The gusA gene, which encodes GUS, allowed for the selection and purification of vGUSREP by blue staining of the recombinant plaques with the enzyme substrate X-Gluc as previously shown (28). The presence of functional repressor protein in lacI-infected cells was confirmed by gel retardation analysis of a radiolabeled DNA fragment containing the core 21-bp operator O2 sequence (data not shown).

Generation of vGUSREP-derived recombinant viruses with hybrid virus promoters containing the lac operator sequence.

To test if the repressor protein was able to control the gene expression from ASFV recombinants, we generated different hybrid virus promoters by fusing the operator sequence to a virus late promoter. One or two copies of the operator O2 were placed at different locations between the strong promoter p72.4 (28) and the coding sequence of the firefly luciferase gene. This would permit us to ascertain the influence of the position of the operator relative to the promoter and of the number of operators on the degree of the repression and induction of gene activity. Another construct, lacking the operator, was generated as a positive control of the p72.4 promoter activity. All these chimeric genes (a general scheme is shown in Fig. 2B) were inserted into the nonessential region corresponding to the CD2 gene (46). DNA hybridization and PCR analysis were carried out to confirm the genomic structures of the recombinant viruses thus obtained, vA2, vA3, vA4, vA5, and vA6 (data not shown). The genomic structure of vA3, which is generally applicable for all of these, is shown in Fig. 2A.

Vero cells were infected with the recombinants described above at 20 PFU per cell, and the luciferase activity was determined at 24 hpi in the presence or absence of IPTG (Fig. 2B). The spacing between the operator and the transcription initiation site was found to be important for the level of repression. Distances of 8 (vA3) or 2 (vA5) bp resulted in ex-
pression levels of 6 and 1%, respectively. The presence of a second operator reinforced the tightness of repression. Two tandem operators located 8 (vA4) or 2 (vA5) bp from the RNA start site allowed levels of luciferase activity less than 0.4% of the control level. The level of IPTG-induced luciferase expression is also dependent on the number of the operators and on their location. Thus, the gene activity in cells infected with viruses containing one operator placed at 8 (vA4) or 2 (vA5) bp reached values of 75 and 37%, respectively. On the other hand, the activity in cells infected with viruses containing tandem operators was only about 6%. Similar induction/repression rates of luciferase expression were obtained for all recombinants at different multiplicities of infection (MOI) (0.1 to 20 PFU per cell) (data not shown).

The effect of IPTG concentration on induction of luciferase expression was studied in cells infected with recombinant viruses vA3 and vA5. A stepwise increase of the IPTG concentration in the medium up to 1.25 mM gradually increased the gene activity (Fig. 2C). Thus, the transferred inducible expression system will allow the quantitative regulation of expression for a target gene. Moreover, maximum levels of gene activity were obtained with an IPTG concentration of 1.25 mM, which has no effect on infectious virus yield or on plaque formation (see below).

Construction of an ASFV recombinant inducibly expressing the p72 structural protein. An application of the lac operator-repressor system described above would be the study of the function of ASFV genes through their conditional expression. To test this, we inducibly expressed the B646L gene, which encodes the major capsid protein p72 (18, 37). For this purpose, an operator was inserted between the gene and its promoter into the vGUSREP genome. Since p72 is a major structural protein (13, 57) expressed during the late phase of the infection cycle, we constructed transfer vectors allowing high levels of gene induction. To this end, and based on the results described above (Fig. 2B), we inserted the operator sequence 8 bp downstream from the transcriptional start point in the recombinant vGUSREP. The purification of the resulting recombinant virus was carried out in the presence of IPTG in order to allow the expression of p72 in the presence of the repressor. Thus, the recombinant virus vA72 was obtained by using the transfer plasmid pINDp72.I (Fig. 3A). In this construct, the chimeric gene p72-lacZ is inserted in the transcriptional orientation opposite that of the B646L gene.

vA72 is an IPTG-dependent recombinant virus. The ability of recombinant virus vA72 to form plaques under repression or induction conditions was studied. Plaques obtained on vA72-infected cells in the presence of IPTG were smaller than those obtained after infection with the parental BA71V virus (Fig. 3B). This result was not due to toxicity of the inducer, which at 1.25 mM had no effect on plaque formation by the parental virus (Fig. 3B), but to an incomplete induction of the protein p72 (see below). The number of plaques formed in the absence of the inducer was strongly reduced (40- to 50-fold). One-step virus growth curves of vA72 showed that the virus titers do not increase over time in the absence of IPTG, remaining about 3 log units below the titers obtained with the parental virus, BA71V (Fig. 3C). Under permissive conditions, the infectious virus yield of vA72 increased during the infection, but the maximal levels observed were lower, by 0.5 log units, than those found after BA71V infections. We have also tested the ability of vA72-infected cells maintained for different times under nonpermissive conditions to produce infectious virus after the addition of inducer. As shown in Fig. 3C, virus titers increased sharply after the addition of inducer at 16 hpi. When the inducer was added later, virus production increased more slowly. Interestingly, the later the time of IPTG addition, the lower the final virus yield obtained, indicating that restoration of infectivity depends on the time of p72 induction.

Synthesis of the protein p72 is IPTG-dependent. To test whether the expression of protein p72 was dependent on the presence of IPTG, we labeled infected cells with [35S]methionine from 16 to 17 hpi. Similar protein profiles were obtained with parental BA71V and recombinant vA72 viruses in the presence and absence of the inducer, with the exception that a
protein band with an electrophoretic mobility of about 115 kDa, representing the β-Gal enzyme, was present on vA72-infected cells (Fig. 4A). Since GUS protein comigrates with protein p72, it was not possible to discern in this assay the effect of IPTG on the induction of p72.

Therefore, to analyze the expression of p72, cell lysates were immunoprecipitated with a p72-specific antibody. As shown in Fig. 4B, a strong reduction of p72 levels was observed in the absence of the inducer. A densitometric quantification of the autoradiography showed that p72 expression in cells infected with vA72 in the absence and in the presence of IPTG was 5 and 60%, respectively, of that obtained in cells infected with the parental virus, BA71V. Immunoprecipitation with a serum against the ASFV polyprotein pp220 (anti-p37/p44). The immunoprecipitated polypeptides are shown.

**Effect of p72 repression on virus morphogenesis.** To analyze the effect of p72 repression on virus morphogenesis, we performed electron microscopy studies of infected cells. In general, the virus-induced structures present in vA72-infected cells in the presence of IPTG (Fig. 5A) were similar to those found in parental BA71V-infected cells (4-6). Assembling virions and irregular and parallel arrangements of membrane-like structures were observed in the replication areas. A recent report has shown that ASFV particles assemble from these viral membranes, which become polyhedral structures after capsid formation on their convex surfaces (4). Interestingly, the analysis of vA72-infected cells in the absence of the inducer showed no production of polyhedral viral structures but a strong accumulation of unusual membranous structures (Fig. 5B). These structures, which have been referred to as “zipper-like” (5, 6), consist of one pair of parallel and extended viral envelopes bound by a thick protein layer structurally similar to the core shell of the virus particle (4) (Fig. 5B). A close inspection revealed two types of zipper-like structures. One of them, referred to as “single,” is formed by a copy of the core shell (Fig. 5B, insert a), while a second one, referred to as “double,” is composed of two copies (Fig. 5B, insert b). Altogether, both zipper-like structures represent a minor proportion of the virus structures induced in the replication areas of parental BA71V (5, 6) or vA72-infected cells under permissive conditions (Fig. 5A). However, while the single structures were frequently detected (Fig. 5A), the double structures were rarely seen.

**Effect of p72 expression on virus morphogenesis.** To examine the effect of p72 expression on the structures seen in the replication areas constituted under nonpermissive conditions, we analyzed vA72-infected cells maintained during 16 h in the absence of the inducer and then incubated for different periods with IPTG. Major ultrastructural changes were observed in the assembly sites from 4 h postinduction onward (Fig. 5C and 6). Expression of p72 led to the formation of polyhedral structures from the previously accumulated membranous structures either on single (Fig. 6A) and double (Fig. 6B) zipper-like structures or on normal viral envelopes (Fig. 6C). A high-magnification analysis revealed that this transformation was concomitant with the appearance of a new layer about 7 nm thick on the external surfaces of the membrane-like structures (Fig. 6). We conclude that this layer corresponds to the viral capsid, as deduced by the regular array of subunits composing it (Fig. 6A), as well as by its effect on the virus shape.

Interestingly, capsid formation gave rise to different assembling virions depending on the type of precursor membranous structures. Thus, typical polyhedral virions were assembled from normal viral membranes (Fig. 7A). On the other hand, capsid formation on double zipper-like structures seemed to lead to separation between the two copies of the core shell, thus resulting in the assembly of apparently normal particles (Fig. 7B). These observations are in good accordance with the restoration of vA72 infectivity observed after the addition of the inducer at 16 hpi (Fig. 3C). Finally, capsid formation on single zipper-like structures led to the generation of a subpopulation of assembling virions morphologically distinct from normal particles. This type of intermediate form, rarely observed in infections with normal ASFV, incorporated two membrane envelopes encompassing the core shell (Fig. 7C and E). These double-enveloped particles likely represent aberrant forms of ASFV. However, we cannot discard the possibility that these viral forms eventually evolve to normal virus by segregation of the innermost envelope, as is suggested in Fig. 7C2. Additionally, we detected double-enveloped virions with an electron-dense nucleoid (Fig. 7D). Whether this nucleoid has a composition identical to that of normal virus particles remains to be answered.

To verify that capsid formation was a consequence of p72 expression, we performed immunogold labeling on ultrathin sections of vA72-infected cells with specific antibodies. Viral factories of cells infected for 16 hpi in the absence of inducer were poorly labeled (Fig. 8A1). In contrast, in infected cells induced for a 8-h period beginning at 16 hpi, strong labeling was detected in the replication areas (Fig. 8A2) on zipper-like structures and polyhedral virus particles.

**Ultrastructural localization of p72 in the virus particle.** Recently, Cobbold et al. (19) have proposed that p72 is externally and internally located in the intracellular virus particles, pe-
ripherally bound to both surfaces of the viral envelope. However, the ultrastructural studies described in the present report clearly indicate that the capsid is built exclusively on the outer surface of the viral envelope. To analyze this apparent contradiction, the precise localization of p72 in the virus structure was determined by preembedding labeling experiments with infected cells permeabilized with streptolysin O (as described in Materials and Methods). For this purpose, we infected Vero cells with either the parental virus, BA71V, or recombinant vA72 virus under permissive conditions.

As shown in Fig. 8B for the parental virus, gold particles strongly decorated the external layers, i.e., the capsids, of the intracellular virions and "open" virus structures (Fig. 8B1 and B2). Interestingly, labeling was virtually absent from the inner side of the envelope in these open particles. Moreover, most of the labeling associated with the precursor viral envelopes was located only on one of their two faces (Fig. 8B3), probably the side on which the capsid would be assembled. A similar labeling pattern was obtained with the recombinant vA72 virus (data not shown).

These results, together with the ultrastructural analysis of recombinant vA72-infected cells, argue in favor of an exclusively external location of p72 in the intracellular virus particles.

**DISCUSSION**

**Inducible expression of genes from ASFV recombinants.** A system for the inducible expression of genes from ASFV recombinants is presented. This system is based on the binding of the E. coli lac repressor protein to the operator sequence of an inducible promoter and has been previously transferred successfully to regulate the expression of transfected or integrated reporter genes in mammalian cells (for a review, see reference 30) and vaccinia virus-infected cells (1, 27, 36, 43, 47, 62).

The E. coli lacI gene, encoding the repressor protein, was inserted into the virus genome of the ASFV strain BA71V under the transcriptional control of the promoter of the virus early gene U104L, generating the recombinant virus vGUSREP. Gel retardation analysis showed that the repressor was present late in infection and thus was available for regulating any virus late promoter containing the operator se-
FIG. 6. Capsid formation. Shown are ultrathin Epon sections of cells infected with vA72 that were treated with IPTG at 16 hpi during an 8-h period. Single zipper-like structures (A), double zipper-like structures (B), and normal viral membranous structures (C) acquire polyhedral morphology by the progressive formation over their external surfaces of a thin layer of about 7 nm (arrowheads). This layer is the viral capsid, as deduced by the ordered array of individual capsomers composing it (arrows in panel A2). Bars, 50 nm.
sequence. Five different hybrid promoters were inserted into the vGUSREP genome, and the luciferase activity for each construct in the presence and in the absence of the inducer IPTG was determined. The results showed that both the distance between the promoter and the operator and the number of operators inserted were critical for the repression and induction activities. Thus, maximal levels of gene induction were obtained with the recombinants vA3 and vA5, which contained one operator sequence. However, these levels were lower than those obtained with the p72.4 promoter in the control recom-
FIG. 8. Immuno-electron microscopy of protein p72. (A) Lowicryl sections of vA72-infected cells maintained 16 h in the absence of IPTG (A₁) or treated with the inducer at 16 hpi during an 8-h period (A₂). The samples were labeled after embedding with a rat anti-p72 serum, a goat anti-rat Ig, and protein A-gold complexes (15 nm). Note that while in the absence of IPTG the replication areas were poorly labeled, in the presence of the inducer the label strongly increased and was mainly associated with zipper-like structures and polyhedral virus particles (arrows). Bars, 200 nm. (B) BA71V-infected cells permeabilized at 20 hpi with streptolysin O. After brief fixation, the cells were incubated with a mixture of anti-p72 monoclonal antibodies (17L.D3 and 19B.A2), and then with protein A-gold (5 nm). Finally, the cells were processed for conventional Epon embedding, and very thin sections (less than 60 nm) were analyzed. Note that the labeling is usually associated with the outer, but not the inner, surfaces of open virus particles (arrowheads in panels B₁ and B₂) and with one of the two sides of the precursor viral membranes (arrowheads in panel B₃). Bars, 200 (B₁) and 100 (B₂ and B₃) nm.
which most of the membrane-associated p72 was resistant to localization was proposed from trypsin protection assays in corporated by wrapping to the virus structure. Such double faces of an endoplasmic reticulum cisterna which is in-

internal located in the intracellular virus, likely bound to Consistent with this, antibodies to p72 labeled the external capsid on the external surfaces of normal viral envelopes as layers structurally similar to the core shell of the virion (4–6).

zipper-like structures. Both types of viral intermediates consist of vA72-infected cells revealed that repression of protein p72 are presumably needed for normal function, and therefore indicating that the repression of p72 was not complete. How-

repression escape mutants, as has been proposed for condi-

in the presence of IPTG, were most likely produced by these conditions, which were similar in size to those observed inducer was reduced more than 99% compared to that of the natural promoter. In this way, we obtained the expression of the analyzed gene to be adjusted to defined levels. This possibility will further our understanding of the function of ASFV proteins under the control of this inducible system, since the amount of target protein expressed can define the phenotype of the mutant virus.

The system described may be a useful and easy way to study the precise function of structural proteins during ASFV mor-

phogenesis.

Role of the major capsid protein p72. This report shows, by analysis of the B646L gene, which encodes the major capsid protein p72 (16, 18, 19, 37), the utility of the inducible gene expression system. We considered it preferable to maintain the target gene in its original site and insert the lac operator downstream of the natural promoter. In this way, we obtained the vA72 recombinant, which contains a hybrid promoter similar to that directing the luciferase expression in vA3, thus allowing high levels of induction.

The essentiality of the protein p72 was demonstrated by the fact that the mutant virus is IPTG dependent. The yield of vA72 under one-step growth conditions in the absence of the inducer was reduced more than 99% compared to that of the parental BA71V. The very few plaques produced by vA72 in these conditions, which were similar in size to those observed in the presence of IPTG, were most likely produced by lacI repression escape mutants, as has been proposed for condi-

tional-lethal recombinant vaccinia viruses (63). Small amounts of the protein could be detected in the absence of the inducer, indicating that the repression of p72 was not complete. How-

ever, since p72 is a major structural protein, large quantities are presumably needed for normal function, and therefore suppression of most of the synthesis of p72 was sufficient to study its function.

Interestingly, the ultrastructural analysis of replication areas of vA72-infected cells revealed that repression of protein p72 synthesis gives rise to accumulation of either single or double zipper-like structures. Both types of viral intermediates consist of pairs of parallel viral envelopes bound by one or two protein layers structurally similar to the core shell of the virion (4–6).

Synthesis of p72 leads to the progressive building of the capsid on the external surfaces of normal viral envelopes as well as zipper-like structures, which became polyhedral forms. Consistent with this, antibodies to p72 labeled the external surfaces of intracellular virus particles. In this context, Cobbold et al. (19) have recently suggested that p72 is externally and internally located in the intracellular virus, likely bound to both faces of an endoplasmic reticulum cisterna which is incorpor-

ated by wrapping to the virus structure. Such double localization was proposed from trypsin protection assays in which most of the membrane-associated p72 was resistant to the protease. Our ultrastructural and immunocytochemical analyses argue in favor of an exclusively external location of both the capsid layer and protein p72 in the intracellular particles.

Interestingly, the thickness of the capsid, about 7 nm, was found to be similar to that of iridoviruses, approximately 6 to 9 nm (7, 23, 54), but in some conflict with the 13 nm previously reported for ASFV capsomers (15). Thus, our data strengthen the similarities observed between ASFV and iridoviruses in virus shape (15), capsid protein sequences (38, 49), and capsomicer disposition in a closely packed hexagonal array (20, 23, 59, 60).

Very little is known about the mechanism of virion assembly and the protein interactions involved in this process (4, 19, 41). Most probably, the correct assembly pathway requires the temporally regulated presence of all the needed factors in the replication area. In relation to this, the absence of a major structural component of the capsid in vA72-infected cells under nonpermissive conditions likely explains the accumulation of zipper-like structures. The fine analysis of viral intermediates suggested that a certain proportion of the mature virions could be obtained after IPTG addition from double structures, which were rarely found in normal infections. Therefore, these membranous structures may be aberrant forms which would switch to normal ASFV assembly, constituting an alternative morphogenesis pathway originating as a consequence of capsid formation inhibition. In this sense, it has been reported that different agents can induce aberrant structures during the as-

sembly of viruses. Thus, the drug rifampin prevents the forma-

tion of vaccinia virus particles and causes the appearance of characteristic inclusion bodies in the cytoplasm of infected cells. The block can be rapidly reversed by removal of the drug, allowing the assembly of normal vaccinia virus (40, 63).

On the other hand, a subpopulation of ASFV particles with two inner envelopes, some of them with an electron-dense central nucleoid, was observed after induction, most probably developed by capsid acquisition on single zipper-like structures (5, 6). Further experiments must be undertaken in order to determine whether these virions are infectious or not.

In conclusion, we have demonstrated that the E. coli lac operator-repressor system provides a powerful tool for study-

ing the role of ASFV genes involved in the virus assembly, making it possible to correlate molecular with morphogenetic events. Similarly, conditional expression of virus genes would allow for the understanding of transcriptional regulation mech-

anisms, as well as the molecular interactions involved in the virus-host relationship, such as virus infectivity or immune response modulation. Extension of inducible expression to ad-

ditional ASFV genes is in progress.

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