IPTG-dependent vaccinia virus: identification of a virus protein enabling virion envelopment by Golgi membrane and egress

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ABSTRACT

A novel method has been developed to study the functional roles of individual vaccinia virus gene products that is neither limited by the possible essentiality of the target gene nor by the availability of conditional lethal mutants. The system utilises the *E.coli* lac repressor protein, the operator sequence to which it binds and the specific inducer IPTG. It allows the generation of recombinant viruses in which the expression of any chosen gene, and hence virus replication, can be externally controlled. In principle, this system is broadly applicable to the functional analysis of genes in any large DNA virus. This approach has demonstrated that the gene encoding the 14 kDa membrane protein of vaccinia virus is non-essential for the production of infectious intracellular virus particles, but essential for the envelopment of intracellular virions by Golgi membrane and for egress of mature extracellular viral particles. This is the first vaccinia virus protein shown to be specifically required for these processes. *In vivo* this system may prove useful as a means of attenuating recombinant vaccinia virus vaccines by preventing virus spread without reducing the amount of the foreign antigen expressed in each infected cell. Attenuation of other live virus vaccines may be developed in a similar way.

INTRODUCTION

Vaccinia virus is the most extensively studied orthopoxvirus, a group of large DNA viruses that replicate in the cytoplasm of infected cells (1). The cytoplasmic site of virus replication dictates that many of the enzymes required for virus transcription and DNA replication are encoded by the virus. Nucleotide sequence data are now available for the majority of the vaccinia virus genome (strain WR) and the analysis of these data have revealed a large number of potential open reading frames (ORFs). Although many virus enzymes and several structural proteins have been mapped to specific loci on the virus genome (1), the majority of the predicted ORFs have no function assigned to them. The classical genetic approach to study gene function in poxviruses depends largely on conditional lethal mutants (2). The most significant limitation of this approach is that mutants may only be isolated for genes encoding functions essential for virus replication in tissue culture. An alternative approach that is limited to the study of non-essential genes, is to delete or inactivate specific genes by insertional mutagenesis and to determine the consequences on virus replication *in vitro* and *in vivo*.

In this report we describe the a method, illustrated in Figure 1, that is applicable to the functional study of both essential and non-essential genes which is based on the use of the lac repressor/operator system (3). Previously, recombinant vaccinia viruses have been constructed that inducibly express exogenous (4,5) or endogenous genes (5). Inducible control was obtained by placing the protein coding region of the target gene downstream of a late vaccinia virus transcriptional start site and one or two copies of the lac operator sequence. The lac I repressor gene is placed under the control of the constitutive vaccinia virus 7.5K (6) promoter and expressed from the same recombinant virus. The repression/induction levels were dependent upon the proximity of the operator to the RNA start site and the number of copies of the operator (5). With our vectors, target gene expression was repressed by 97 or 99.9% with one or two copies, respectively, of the lac operator sequence(s) located immediately downstream of the transcriptional initiation site of the promoter. Derepression at any time after infection can be obtained by the addition of the specific inducer isopropyl-β-thiogalactoside (IPTG) to the culture medium. In this report the utility of the inducible expression in vaccinia virus is illustrated as a method to study endogenous vaccinia virus gene function. A recombinant vaccinia virus was constructed which expressed the virus 14 kDa membrane protein only in the presence of IPTG. Analysis of the growth of the resultant virus shows that the 14 kDa protein is non-essential for the production of infectious intracellular virus particles, but, surprisingly, is essential for the envelopment of these particles by Golgi membrane and for egress of mature particles from the cell. This represents a significant step forward in our understanding of the complex process of poxvirus morphogenesis.

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MATERIALS AND METHODS

Cells and viruses

TK$^-$143 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS). Viruses were propagated and titrated in human TK$^-$143 in DMEM containing 2% FBS and 5mM IPTG as previously described (5). Recombinant viruses were generated according to established protocols (7). Selection of recombinant virus containing the selectable marker gene xanthine guanine phosphoribosyl transferase (Ecogpt) was carried out as previously described (8,9) and in addition the selective medium was supplemented with 5mM IPTG.

Plasmid constructions

The plasmid vector used to delete the 14K gene, pPR42, was constructed in two steps. First, a 2.1 kb DNA fragment containing the Ecogpt gene fused to the vaccinia virus p7.5 promoter was obtained by digestion of the plasmid pGpt07/14 (8). After treatment with Klenow enzyme the fragment was cloned into the EcoRV site of pE17 (10). The resulting plasmid, pPR41, was digested with BamHI, treated with Klenow enzyme and ligated to a 0.4 kb DraI DNA fragment from pE17 containing the sequences located immediately upstream of the 14K coding region. The resulting plasmid, pPR42, contains the sequences flanking the 14K gene but lacks the gene coding region except for the 3' 49 nucleotides.

Electron microscopy

At different times post-infection cell cultures infected with WR 32-7/14K at a multiplicity of infection (MOI) of 2 plaque forming units (PFU)/cell were processed for electron microscopy as previously described (11). Samples were visualised using a Joel Mod. JEM 100CX electron microscope.

RESULTS

The approach to study the function of endogenous vaccinia virus genes is outlined in Figure 1. First, the protein coding region of target gene gene is obtained by PCR reaction and cloned into a plasmid vector (in this case pPR34) downstream of the inducible late promoter. This gene, together with the lac I repressor gene expressed from the vaccinia virus 7.5K promoter, is then introduced into the TK gene locus of vaccinia by transfection and selection of TK$^-$ recombinants (7). The resultant intermediate virus contains two copies of the target gene, one in the natural position in the virus genome and the second in the TK locus under IPTG-inducible control. In the next step, the endogenous copy of the gene is destroyed by insertional mutagenesis (Figure 1b) using the dominant selectable marker Ecogpt (8,9), while the expression of the inducible gene is turned on by the addition of IPTG.

To test the suitability of this approach we generated a virus containing a unique inducible version of a gene encoding a virus structural protein. The gene selected encodes a 14 kDa protein expressed late during viral infection (12,13), and has temporal regulation of transcription comparable to that of our inducible promoter (5). This protein forms part of the viral membrane, is a target for neutralising monoclonal antibodies and was thought to play a major role in viral penetration (14). WR 32-7/14K (5) is an intermediate recombinant virus (Figure 1) that contains the original 14K gene plus a copy of the 14K coding region under the control of the late hybrid 4b/op promoter in the TK gene locus, and was constructed as previously described (5). Immunoblotting with a monoclonal antibody against the 14 kDa protein demonstrated that expression was repressed by greater than 95% (5). The original 14K gene of this virus was then deleted by transfection of WR 32-7/14K infected cells with a plasmid (pPR42) containing the Ecogpt fused to the viral

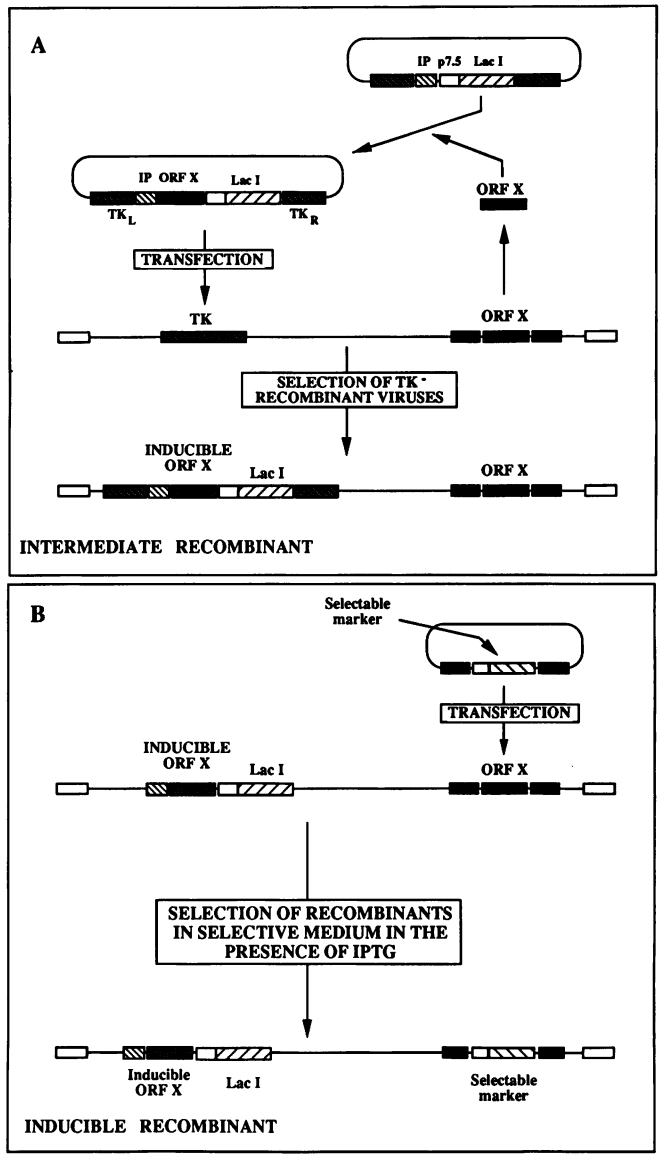

Figure 1. Generation of recombinant viruses containing inducible endogenous genes. A) A copy of the coding region of the gene under study (ORF X) is produced by PCR and cloned downstream of a vaccinia virus hybrid inducible promoter (IP). The resultant plasmid contains the lac repressor gene (Lac I) fused to the viral promoter p7.5 and inducible promoter fused to ORF X flanked by sequences from the thymidine kinase (TKL and TKR) gene of vaccinia virus. This plasmid is used to transfect cell cultures infected with vaccinia virus and a TK$^-$ recombinant virus is selected on TK$^-$ cells in the presence of BudR. This intermediate recombinant virus contains the original copy of the target gene, the lac repressor gene, and the ORF X under the control of the inducible promoter. B) The original copy of gene X is replaced by a dominant selectable marker. A plasmid that contains a selectable marker gene (Ecogpt) driven by a vaccinia virus promoter, and sequences from the flanking region of the original ORF X, is transfected into cells infected with the intermediate recombinant virus. The final recombinant virus (Inducible recombinant) is selected in the presence of medium selective for the dominant selectable gene and containing the inducer IPTG.

promoter p7.5K and flanked by sequences identical to those flanking the 14K ORF within the viral genome. Selection of recombinant viruses containing the Ecogpt gene was performed by plating out the viral progeny in selective medium containing mycophenolic acid, xanthine, hypoxanthine (8,9) and IPTG. Three plaque purifications were performed in selective medium. Southern blot analysis (data not shown) confirmed that the insertion of the selectable marker had resulted in the expected deletion of the target gene leading to the generation of the virus WR 32-7/Ind 14K which contains a single inducible copy of the 14K gene (Figure 2a).

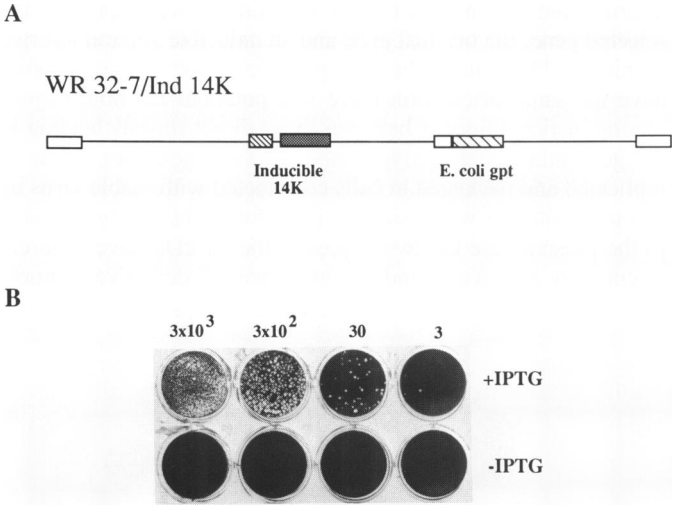

Figure 2. A) Genomic structure of the recombinant virus WR 32-7/Ind 14K.The genome of the recombinant virus WR 32-7/Ind 14K contains a single copy of the 14K ORF under the transcriptional control of the inducible late promoter p4b/Op, and the lac I repressor gene (Lac I) under the control of the viral promoter p7.5. The original 14K ORF has been replaced by the selectable marker gene (Ecogpt) driven by the constitutive viral promoter p7.5. B) Plaque size phenotype of WR 32-7/Ind 14K. Monolayers of human TK^- cells grown in 24 well plates were infected with WR 32-7/Ind 14K with the indicated amount of infective particles. Cultures were maintained in either the presence or absence of the specific inducer IPTG. At 48 hours post-infection the media was removed from the wells and the monolayers were stained with 0.1% crystal violet in 20% ethanol.

The growth of WR 32-7/Ind14K in human TK^-143 cell monolayers in the presence or absence of IPTG (Figure 2b) demonstrated that plaque formation was dependent upon the presence of IPTG in the medium. Similarly, infection of cell cultures at low multiplicity (0.01 PFU per cell) with WR 32-7/Ind14K yielded 1000 fold less virus at 72 h post-infection if IPTG was omitted from the culture medium. In parallel infections with the parental virus, WR 32-7, there was no significant difference in virus yield when the infections were carried out with, or without, IPTG (data not shown). These results strongly indicated that the 14K gene is essential for viral replication in a multi-step growth curve. However, when similar experiments were conducted using a higher multiplicity of infection (MOI) (2 PFU per cell), viral growth in both IPTG-treated and untreated cultures showed identical kinetics and final virus titres (Figure 3a). This surprising result was in apparent contradiction to that obtained in the multiple step growth curves. To further investigate this phenomenon the production of extracellular virus was examined either in the presence or absence of IPTG. The results (Figure 3b) show a 95% reduction in the titres of extracellular virus when the expression of the 14 kDa protein was repressed.

During infection with vaccinia virus the great majority of infectious progeny remains inside the infected cell and is termed intracellular naked virus (INV). However, a small fraction of viral particles become wrapped in two layers of Golgi-derived membrane and then migrate to the cell surface where fusion of the outermost virus membrane with the plasma membrane occurs, resulting in the release of extracellular enveloped virus (EEV) from the cell (11). Although both the INV and the EEV forms of the virus are infectious (15), the latter contains an extra envelope and associated proteins, and is thought to play a major role in viral dissemination *in vivo* (16, 17). The genes encoding two proteins of the extracellular envelope have been sequenced

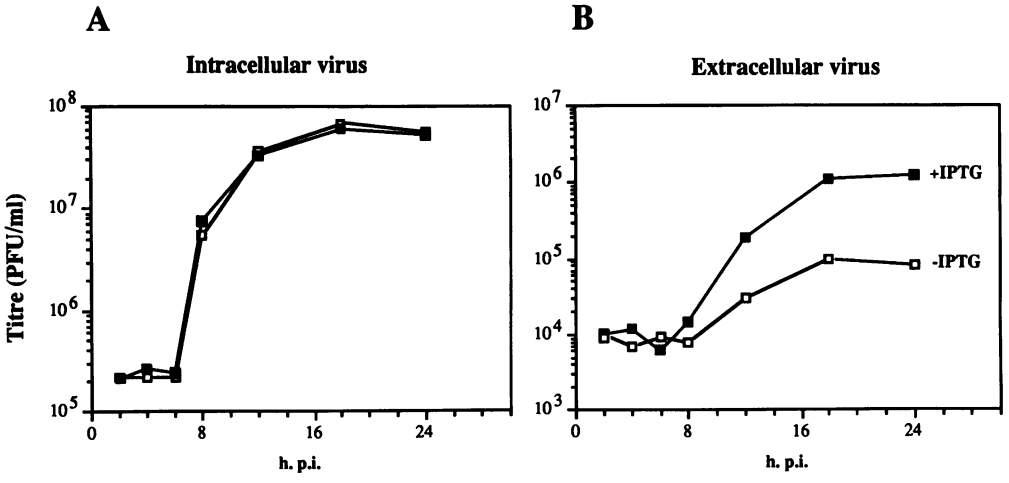

Figure 3. Growth curves of WR 32-7/Ind 14K. Human TK^- cells were infected with 2 plaque forming units (PFU) per cell with the recombinant virus WR 32-7/14K either in the absence (■) or in the presence (□) of the inducer IPTG. At the indicated times post-infection (p.i.) samples were taken and titrated by plaque assays on fresh TK^- cells. A) Production of intracellular virus. Infected cells were washed twice in phosphate buffered saline, and then collected by scraping followed by centrifugation (1000g, 5 min). After resuspension in PBS the intracellular virus was released by three freeze-thaw cycles and sonication. B) Production of extracellular virus. Infected cell supernatants were collected. Cell debris was removed by centrifugation (1000g, 5 min) and the supernatants used for the plaque assay.

(18, 19), but other components and the proteins responsible for virion envelopment and egress have not been identified.

The results described above indicate that the 14K protein is not required for the production of INV but is essential for the generation of EEV. This was further investigated by electron microscopy. This analysis confirmed that the expression of the 14K gene is neither required for the appearance of viral factories nor for the formation of INV (Figure 4a and b show representative sections of cells infected with WR 32-7/ind 14K in the presence or absence of IPTG). However, after an extensive search we failed to detect the presence of either wrapped intracellular virions or cell-associated EEV in cultures maintained in the absence of IPTG, indicating the inability of virions formed in the absence of the 14 kDa protein to interact with the Golgi apparatus. In contrast, IPTG-induced expression of 14 kDa protein during infection restores the interaction of INV with Golgi-derived membranes leading to the formation of abundant EEV (Figure 4c-f). These findings are in agreement with the results obtained by titration of the viral progenies and allow the conclusion that the 14 kDa protein is essential for envelopment of INV by Golgi membrane and for their subsequent egress from the cell.

DISCUSSION

The construction of vectors allowing inducible expression of genes inserted into the vaccinia virus genome has enabled the design of a novel strategy to study the functional role of viral genes. The first step (Figure 1) requires the generation of an intermediate recombinant virus containing two copies of the selected gene, the original gene and an inducible version inserted within the TK locus of the viral genome. If the two gene copies have the same orientation there is a potential for homologous recombination to occur between these direct repeats leading to the generation of defective genomes. Such genomes might be replicated and packaged in cells co-infected with viable virus by complementation with essential gene functions acting in trans. In the present case the two copies of the 14 kDa have different orientation and we found no evidence of defective genome

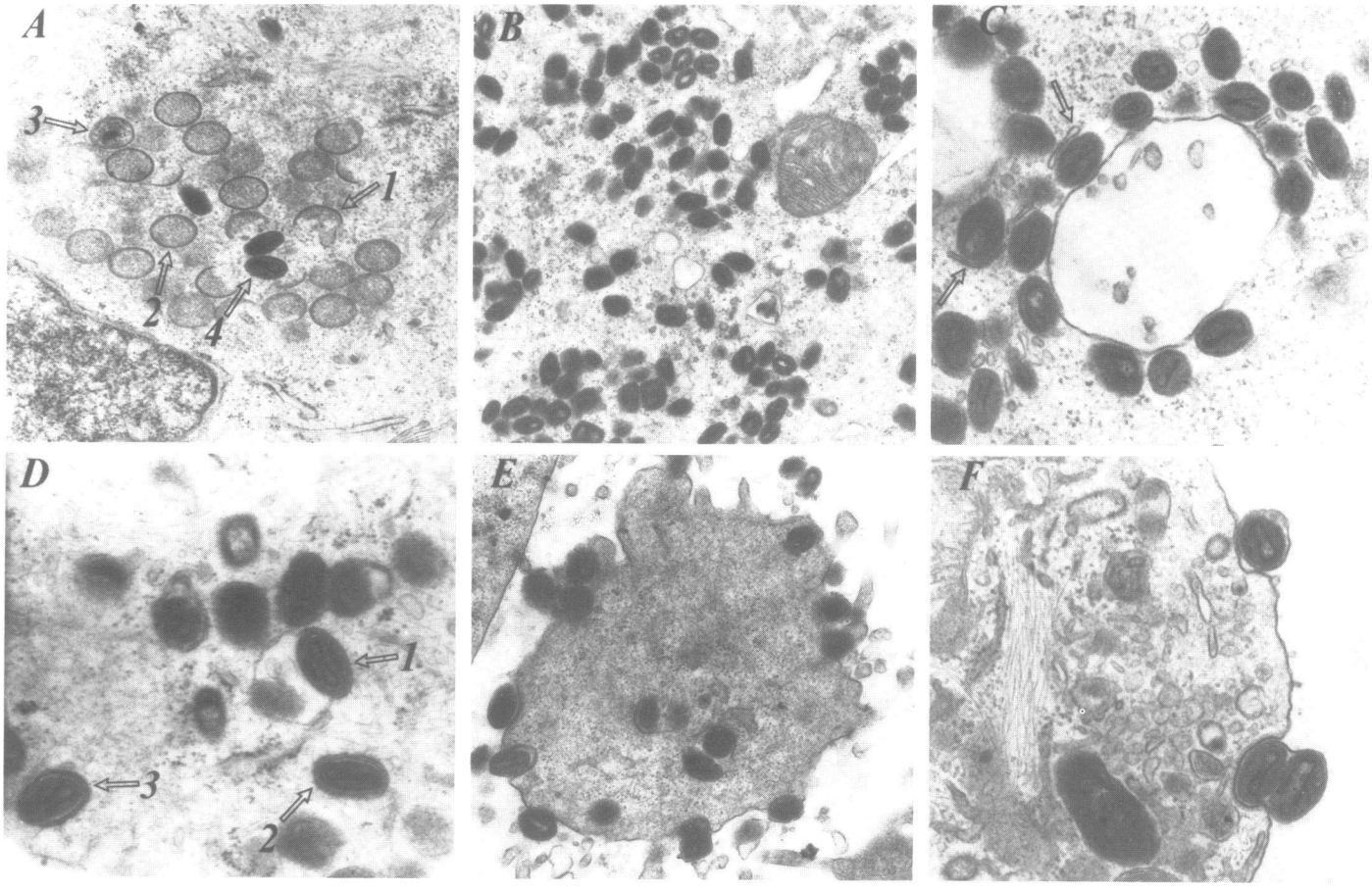

Figure 4. Effect of the induction of the 14K expression on viral morphogenesis. A) Detail of a viral factory formed at 12 h. post-infection in the absence of IPTG showing different stages of viral maturation. 1) Early stage during the formation of a viral crescent. 2) Fully formed viral crescent. 3) Immature viral particle showing the initiation of nucleoprotein condensation. 4) Mature INV. Magnification×24,700. B) Viral factory containing mature INV at 24 h. post-infection in the absence of IPTG. Magnification×17,800. C) Detail of a cell at 24 h. post-infection in the presence of IPTG. The arrows indicate viral particles interacting with Golgi-derived membranes. The interaction of mature INV with multi-vesiculated bodies like the one shown in this panel was frequently observed in cultures treated with the inducer and in WT infected cells. Magnification×35,700. D) Detail of a cell at 24 h. post-infection in the presence of IPTG showing viral envelopment and egress 1) A viral particle wrapped in two layers of Golgi membrane. The outermost membrane appears to cover more than one virion. 2) Virion tightly wrapped in a double membrane. 3) EEV leaving the the cell. The outermost viral membrane is fused to the plasma membrane. Magnification×35,700. E) Cross-section of a microvillus from a cell infected in the presence of IPTG at 24 h. post-infection showing multiple EEV associated with the plasma membrane and membrane-wrapped intracellular virus. Magnification×24,700. F) Detail of a cell at 24 h. post-infection in the presence of IPTG showing EEV associated with the plasma membrane. Two of these particles (lower part of the panel) are enveloped by a single continuous membrane. Magnification×35,700.

formation either initially, or after serial passage. If genome instability does occur when genes have the same orientation, this may be overcome by constructing plasmid vectors which contain the inducible promoter and lac I gene in the opposing orientation in the TK locus.

In the second step, the original gene is deleted from the intermediate recombinant by homologous recombination between the virus genome and a plasmid deletion vector. This contains the selectable maker Ecogpt flanked by sequences identical to those flanking the target gene within the viral genome. Our results show that positive selection of recombinants using mycophenolic acid is not affected by the presence in the culture medium of the inducer IPTG. Southern blot analysis demonstrated that the genome of the recombinant virus, WR 32-7/ind 14K, containing a unique inducible copy of the 14K gene, is stable after serial passage in the presence of IPTG (data not shown). WR 32-7/ind 14K shows a IPTG-dependent plaque phenotype (Figure 2b). Spontaneous reversion to an IPTG-independent phenotype is lower than 10^{-4} (data not shown).

The generation of a recombinant virus containing a unique inducible version of the 14K gene has allowed us to carry out a detailed investigation of the role of this protein during the replicative cycle of the virus. The results shown above demonstrate that the 14 kDa protein is not essential for the production of infectious INV. This result is somewhat surprising in light of the previous findings showing that this protein is the target for neutralising monoclonal antibodies (14). However, the dramatic failure to produce EEV in the absence of 14 kDa protein (Figure 3b), and the inability of the INV lacking the 14 kDa protein to interact with the Golgi-derived membranes (Figure 4), clearly demonstrate this protein is essential for the last stages of viral maturation. The failure to form lytic plaques during low MOI infections carried out in the absence of the inducer shows that rapid cell-cell dissemination of vaccinia virus also requires the synthesis of the 14K protein (Figure 2a). The interesting process of viral envelopment by Golgi membranes must initiate by interaction of the protein(s) on the surface of INV with virus or host cell components on the cytoplasmic side of Golgi membrane. This basis of this interaction may now be studied in detail. It is possible that the 14K protein interacts directly or indirectly with the a virus-coded, 37K acylated protein that is present in Golgi membrane (20).

The attenuation of vaccinia virus is desirable if recombinant vaccinia virus vaccines are to find utility as new live vaccines (21). One strategy to achieve virus attenuation is the identification and subsequent deletion of the genes encoding the virus glycoproteins present in the extracellular envelope. The virus described in this communication directly achieves this objective by preventing INV envelopment and the release of EEV. Since the EEV form of vaccinia virus has increased infectivity and enhanced dissemination *in vivo* (14–16), a vaccine defective in EEV formation will be less likely to spread within a vaccinee or cause accidental infections in contacts. Accidental infections alone represented 33.7% of vaccination-related complications (22). Although the virus is likely to have a reduced ability to spread *in vivo*, foreign antigens expressed by such a recombinant vaccinia virus will still be synthesised normally in each infected cell, potentially retaining the immunogenicity of the candidate vaccine. Attenuation of other live virus vaccines may be achieved by a similar approach in which the selected virus gene is expressed either inducibly from within the virus genome, or from a complementing cell line. Stocks of recombinant virus are grown in these cell lines or in the presence of the inducer, and subsequently the vaccine is administered without expression of the selected protein so that only abortive, self-limiting infections are initiated. Such immunogens might combine the safety of dead vaccines with the immunogenicity of live vaccines. Attenuation will not be limited to DNA viruses since the process of reverse genetics now allows manipulation of RNA virus genomes also (23–25).

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