Analysis of the Role of Vaccinia Virus H7 in Virion Membrane Biogenesis with an H7-Deletion Mutant

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Essential vaccinia virus genes are often studied with conditional-lethal inducible mutants. Here, we constructed a deletion mutant lacking the essential H7R gene (the ΔH7 mutant) with an H7-expressing cell line. Compared to an inducible H7 mutant, the ΔH7 mutant showed a defect at an earlier step of virion membrane biogenesis, before the development of short crescent-shaped precursors of the viral envelope. Our studies refine the role of H7 in virion membrane biogenesis and highlight the values of analyzing deletion mutants.

Vaccinia virus (VACV) is a member of the Poxviridae family of large cytoplasmic DNA viruses (1). The linear genome of VACV contains approximately 200 genes categorized into early, intermediate, and late classes (2–4). Early genes are transcribed immediately following viral entry, while intermediate and late genes (collectively referred to as postreplicative genes) are transcribed after viral DNA replication. Postreplicative genes are transcribed and translated in viral factories (5), where assembly of virions takes place. Virion assembly goes through a series of intermediate stages discernible by electron microscopy (EM) (reviewed in reference 6). Electron-dense viroplasms composed of viral core proteins appear first; this is followed by the development of crescent-shaped membranes at the periphery of viroplasms. Crescent membranes are stabilized by an external scaffold composed of D13 proteins (7, 8). Spherical immature virions (IV) form when crescent membranes engulf part of the viroplasm. IV undergo additional transformations, including encapsidation of the viral genome and removal of the D13 lattice, before they become infectious mature virions (MV).

The origin and biogenesis of crescent membranes are among the least understood aspects of poxvirus biology, although many of the viral proteins involved in this process have been identified. F10 (9, 10), A11 (11), H7 (12), L2 (13), and A6 (14) participate at an early step in virion membrane biogenesis, while A14 (15, 16) and A17 (17, 18) participate at a later step, after viral membrane precursors are acquired. These proteins, as well as other proteins essential for viral replication, have been studied mostly with inducible or temperature-sensitive mutants that have a conditional defect in the specific gene (19, 20). While these mutants have proven valuable for uncovering gene functions, their phenotypes could be affected by leaky expression under nonpermissive conditions, leaving ambiguity about the gene’s exact point of action. A case in point is the inducible H7 mutant, which developed short crescent membranes under nonpermissive conditions (12). However, the formation of these short crescent membranes may nevertheless require H7, since a minute amount of H7 protein may be present under nonpermissive conditions to provide the necessary function for the formation of these membranes. These uncertainties could be resolved by studying deletion mutants constructed with appropriate complementing cell lines. However, deletion mutants have been reported for only a small number of essential VACV genes, all of which are of the early class (D4 [21], A8, A23 [22], and L2 [23]). It is uncertain whether the functions of postreplicative genes such as that for H7 could be complemented by cell lines, as the time (late) and location (factories) of their transcription and translation are different from those of cellular or VACV early genes. To gain further understanding of the role of H7 in virion membrane biogenesis, we constructed an H7-deletion mutant.

Establishment of a cell line that stably expresses VACV H7 protein. As H7 is essential for VACV replication, a prerequisite for the construction of an H7-deletion VACV is the establishment of a complementing cell line that stably expresses H7. To achieve optimal expression of H7 in mammalian cells, the H7 coding sequence was codon optimized for human cell expression, chemically synthesized (GenScript), and cloned into mammalian expression vector pCDNA3.1 (Invitrogen). Two micrograms of the expression vector was transfected into B5-C-1 cells (ATCC CCL-26) with Lipofectamine (Invitrogen). After 48 h, the transfected cells were plated into a new dish at ~15% confluence and underwent drug selection with medium containing 250 μg/ml of G418 (Invitrogen). Colonies of cells that survived 10 days of selection were individually picked up and transferred to 96-well plates. They were then screened by immunofluorescence analysis and Western blotting with a monoclonal antibody (MAb) against H7. Anti-H7 MAb 25E2 was developed from mice immunized with purified recombinant H7 protein, in a manner similar to that which we described previously (24). It specifically recognized the H7 protein and did not cross-react with any cellular proteins in Western blot or immunofluorescence assays (Fig. 1A and C). A cell line that showed the highest level of H7 expression (referred to here as BSC-H7 cells) was chosen for all subsequent experiments. Immunofluorescence analysis showed that all of the BSC-H7 cells...
in the monolayer were positive for staining with anti-H7 MAb and that H7 proteins were distributed throughout the cytoplasm (Fig. 1B), similar to the H7 protein distribution in VACV-infected cells (Fig. 1C) (12). BSC-H7 cells duplicated slightly faster than the parental cells, indicating that constitutive expression of H7 protein had no deleterious effect on cell growth.

**Construction of an H7-deletion virus.** BSC-H7 cells were then used for the construction of an H7-deletion mutant. A DNA fragment consisting of *Escherichia coli* β-glucuronidase (GUS) flanked by ~500 bp of sequences preceding and following the H7 open reading frame (ORF) was assembled by PCR as described previously (25). The DNA was transfected into BSC-H7 cells that had been infected with wild-type (WT) VACV WR. After plaque purification of GUS-expressing viruses on BSC-H7 cells, recombinant viruses that had GUS regulated by the P11 late promoter in place of the H7 ORF and the H7 promoter (Fig. 2A; nucleotides 93428 to 93901 of WT VACV WR) were readily isolated. The recombinant virus (referred to here as the ΔH7 mutant) was confirmed to be free of WT VACV by PCR amplification of H7R flanking sequencing and Western blotting with anti-H7 MAb (shown later).
FIG 3 Construction and characterization of iH7/GFP, an IPTG-inducible H7R mutant of VACV. (A) Schematic representation of the iH7/GFP genome. iH7/GFP was constructed through homologous recombination of vT7LacO1 and a PCR product containing green fluorescent protein (GFP), the T7 promoter, and the E. coli lac repressor flanked by H6R and H7R sequences. The recombination resulted in the replacement of the native promoter of the H7 gene (nucleotides 93428 to 93901 of the WT VACV WR genome) with an IPTG-inducible T7 promoter and the simultaneous insertion of a GFP cassette between the H6R gene and the T7 promoter. LacO, lac operator; T7 pol, bacteriophage T7 RNA polymerase; LacI, E. coli lac repressor; P75, T7 promoter; IRES, the internal ribosomal entry site from encephalomyocarditis virus. (B) IPTG-dependent synthesis of H7 by iH7/GFP. BS-C-1 cells were infected with iH7/GFP at an MOI of 5 PFU/cell in the absence or presence of 100 μM IPTG. Cells were harvested at 12 h p.i. and analyzed by Western blotting with MAbs against H7, D8, and Hsp70. The values to the left are molecular sizes in kilodaltons. (C) Plaque morphology. BS-C-1 cells were infected with iH7/GFP in the presence or absence of 100 μM IPTG for 48 h. Cells were fixed and stained with crystal violet. (D) One-step growth curve analysis. BS-C-1 cells were infected at an MOI of 5 PFU/cell with iH7/GFP in either the presence or the absence of 100 μM IPTG. After 0, 12, 24, and 48 h, infected cells were harvested and virus titers were determined by plaque assay in the presence of 100 μM IPTG.

On BS-C-1 cells, the ΔH7 mutant did not form any plaques or increase its titer over 48 h of infection in a one-step growth curve analysis, which is consistent with the idea that H7 is essential (Fig. 2B and C). The replication defect can be rescued by transfecting a plasmid containing H7 (data not shown), indicating that the defect was due solely to the deletion of the H7 gene. On BSC-H7 cells, the plaque size and replication kinetics of the ΔH7 mutant were similar to those of WT VACV (Fig. 2B and C).

As reported previously (12), endogenous viral H7 was expressed postreplicatively by WT VACV (Fig. 2D). H7 protein was detected by Western blotting at 6 h postinfection (p.i.), and its level continued to increase afterward, similar to the expression kinetics of another VACV late protein D8 (Fig. 2D). In contrast, H7 protein expressed by BSC-H7 cells was at its highest level prior to infection with the ΔH7 mutant and its level gradually decreased as the infection progressed. This is consistent with a shutdown of host protein synthesis resulting from VACV infection. Nevertheless, throughout the infection, the H7 protein level in BSC-H7 cells was similar to the peak level of virus-encoded H7 protein expressed by WT VACV at later stages of infection.

Altogether, the data showed that the H7 protein ectopically expressed by BSC-H7 cells is fully functional for VACV replication, even though its expression kinetics are different from those of viral H7.

Effect of H7 gene deletion on virion membrane biogenesis. Next, we studied the effect of H7 gene deletion on virion morphogenesis by transmission EM. An isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible H7 (iH7) mutant (Fig. 3) constructed in a manner similar to that described previously (12) was included as a control. In BS-C-1 cells infected with iH7 for 20 h in the absence of IPTG, electron-dense viroplasmic inclusions (labeled V in Fig. 4) accumulated in the cytoplasm and short crescent membranes (arrows in Fig. 4B) were included as a control. In BS-C-1 cells infected with iH7/GFP in either the presence or the absence of 100 μM IPTG. After 0, 12, 24, and 48 h, infected cells were harvested and virus titers were determined by plaque assay in the presence of 100 μM IPTG.

Effect of H7 gene deletion on the stability of selected VACV proteins. A11 is an essential factor in viral membrane biogenesis. Its stability in infected cells was greatly affected by repression of L2 expression, which reduced A11 protein to a nearly undetectable level (26). To assess whether H7 could affect membrane biogenesis partly by modulating A11 function, we determined the A11 protein level in ΔH7 mutant-infected cells. As a control, the levels of a few selected early and late proteins, including MV membrane protein L1, were also determined. Repression of H7 expression...
was previously shown to reduce the L1 protein level (12). BS-C-1 cells were infected with iH7 in the presence (A) or absence (B) of IPTG or with the ΔH7 mutant at an MOI of 1 PFU/cell for 20 h (C and D). The cells were fixed and prepared for transmission EM. Panel D is a higher magnification of the boxed area of panel C. Note the short membranes (arrows) at the peripheries of viroplasm inclusions in panel B but not in panels C and D. N, nucleus; V, viroplasm.

FIG 4 The ΔH7 mutant shows a defect at an earlier step of virion membrane biogenesis than the IPTG-inducible H7 mutant (iH7). BS-C-1 cells were infected with either WT VACV or the ΔH7 mutant at 10 PFU/cell. At 10 and 24 h p.i., the levels of various viral proteins were determined by Western blotting with specific antibodies (Fig. 5). As expected, no H7 protein was detected in BS-C-1 cells infected with the ΔH7 mutant, while it was present in cells infected with WT VACV. At 10 h p.i., L1 could be detected in WT VACV-infected cells but not in ΔH7 mutant-infected BS-C-1 cells. A11 and VACV early protein E3 were present at similar levels in cells infected with either virus, while late proteins D8 and D13 were present at a lower level in ΔH7 mutant-infected BS-C-1 cells than in WT VACV-infected cells. At 24 h p.i., L1 protein was present at a higher level in WT VACV-infected cells but remained barely detectable in ΔH7 mutant-infected BS-C-1 cells. In contrast, a substantial amount of A11 protein was detected in ΔH7 mutant-infected BS-C-1 cells, although its level was lower than that in WT VACV-infected cells. Overall, the data showed that while H7 gene deletion greatly reduced the L1 protein level, it had no effect on the A11 protein level as late as 10 h p.i. The relatively minor effect of H7 gene deletion on the A11 protein level at 24 h p.i. was not specific to the A11 protein, as the levels of a few other viral proteins were reduced similarly.
FIG 6 Effect of H7 gene deletion on intracellular localization of VACV proteins. BHK cells were infected with the ΔH7 mutant or WT VACV for 8 h and processed for fluorescence microscopy as described previously (25). Cells in panels A to E were stained with MAbs against the indicated proteins, while cells in panels F to I were stained with both anti-A11 MAb 10G11 (IgG2b [25]) and a MAb against A10 (BG3, IgG2a [25]), A13 (11F7, IgG2a [28]), or D8 (AB12, IgG1 [24]). The latter were further stained with isotype-specific secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 594. The areas of the cell within the white boxes are enlarged and shown in more detail in the last column. The white arrows in panels C, D, and E point to staining around viroplasmic inclusions. F, viral factory.
Effect of H7 gene deletion on the localization of VACV proteins to viral factories. Recent studies showed that repression of A6 expression caused a defect in A11 localization to viral factories (25). In addition, repression of A6, A11, or L2 caused a defect in the localization of many MV membrane proteins to viral factories (14, 23, 25). We thus assessed the effect of H7 gene deletion on the localization of viral proteins by immunofluorescence analysis of ΔH7 mutant-infected BHK cells (ATCC CCL-10) as previously described (25). Viral factories, defined in fluorescence microscopy as cytoplasmic areas with DNA staining, were present in all ΔH7 mutant-infected cells (Fig. 6). However, there were many DNA-free "holes" in the factories and they could all be stained with an antibody against viral core protein A10 (Fig. 6A). This indicates that these "holes" were actually filled with viroplasmic inclusions composed of viral core proteins, which had been shown to accumulate in viral factories when H7 expression was repressed (12).

The IV scaffold protein D13 accumulated as inclusions outside the factories (Fig. 6B). A small fraction of the MV membrane proteins A13 and D8 localized to the peripheries of the viroplasmic inclusions in the factories (white arrows in Fig. 6C and D), but the majority localized outside the factories. These results indicate that H7 gene deletion also caused a defect in the proper localization of D13 and MV membrane proteins to viral factories, similar to that in mutants deficient in A6, A11, or L2 (14, 23, 25). However, in contrast to the phenotype of the A6 mutant, A11 localized to viral factories in ΔH7 mutant-infected cells (Fig. 6E), similar to that of cells infected with WT VACV (11). To assess the localization of A11 with respect to viral structural proteins, ΔH7 mutant-infected cells were also doubly stained with antibodies against A11 and another viral protein. A11 did not colocalize with A10 (Fig. 6F), but it colocalized with the small fractions of A13 and D8 that were present in the viral factories (Fig. 6G and H). Overall, the data showed that H7 gene deletion caused a defect in the localization of MV membrane proteins to viral factories but had no effect on A11 protein localization.

Concluding remarks. The success in constructing the ΔH7 mutant demonstrates that an essential postreplicative gene can be deleted from VACV through the use of complementing cell lines, thus expanding the feasibility of this useful technique to all VACV genes. However, we are uncertain about whether this success was partly due to several unique properties of the H7 protein. First, unlike the products of many postreplicative genes, H7 localizes throughout the cytoplasm (12), suggesting that the site of action of H7 is not limited to viral factories. This is perhaps the reason why H7 protein that is ectopically expressed outside viral factories is fully functional for viral replication. Second, H7 protein appears to be quite stable by itself in the absence of other viral proteins and its overexpression does not cause cellular toxicity. Therefore, a high level of H7 protein remains in BSC-H7 cells even at later stages of infection, when translation of host mRNA, including the H7 transcript made by the cell line, is shut down. It remains to be determined whether similar deletion mutants can be made for postreplicative genes that function predominantly in viral factories. These deletion mutants can be very useful for genetic and structure-function studies. They could also potentially be developed into safe nonreplicating vectors for vaccines (27).

Analysis of the ΔH7 mutant furthered our understanding of H7 function, which has been studied previously with an inducible mutant (12). A key phenotypic difference between the ΔH7 and iH7 mutants is the lack of short crescent membranes in ΔH7 mutant-infected cells, indicating that H7 is essential for the development of short crescent membranes and that even a small and undetectable amount of H7 can facilitate short crescent development. This result illustrates the value of deletion mutants in defining gene function. Further analysis of the ΔH7 mutant revealed a defect in the localization of IV scaffold protein D13 and MV membrane proteins to viral factories, similar to the defect caused by repressing the expression of L2, A6, and A11 (14, 23, 25). This extends a correlation between the defect in virion membrane biogenesis and the defect in MV membrane protein localization to viral factories, suggesting that these two processes may occur simultaneously (Fig. 7 shows a model). We also observed a key phenotypic difference between deficiencies in H7 and other proteins involved in membrane biogenesis. While its stability or proper localization was greatly affected by the repression of L2 or A6 (23, 25, 26), the A11 protein remained stable and localized to viral factories in ΔH7 mutant-infected cells. The A11 protein was recently shown to associate with viral membranes but not with cellular membranes (25). The presence of A11 along with a small fraction of MV membrane proteins in the factories of ΔH7 mutant-infected cells suggests that some precursors of viral membranes are developed in the absence of H7. However, we were not able to identify any viral membrane structure by EM. It is possible that viral membranes are present in small amounts or in an unrecognized precursor form.

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REFERENCES

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