Implication of the product of the bovine herpesvirus type 1 UL25 gene in capsid assembly

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The UL25 ORF of bovine herpesvirus type 1 (BHV-1) was demonstrated recently to represent a gene encoding a 63 kDa viral protein. To investigate the role of this gene in virus replication, a BHV-1 UL25 deletion mutant was constructed. Although the UL25 mutant synthesizes late viral proteins and viral DNA, it fails to produce virus progeny in cells that do not express the UL25 gene, demonstrating that the UL25 protein is essential for the replicative cycle of BHV-1. Moreover, Southern blotting analyses of HindIII-digested DNA from infected non-complementing cells probed with the leftward terminal fragment of the BHV-1 linear genome revealed that the cleavage of the viral DNA produced is not impaired. However, the packaging of this cleaved DNA is compromised severely, since only few full C capsids were observed in infected non-complementing cells by transmission electron microscopy.

During the replication process, cleavage and packaging of viral DNA are tightly linked functions at least requiring the following seven genes in HSV-1: UL6, UL15, UL17, UL25, UL28, UL32 and UL33 (Poon & Roizman, 1993; Taus et al., 1998; McNab et al., 1998; Tengelsen et al., 1993; Addison et al., 1990; Lamberti & Weller, 1996, 1998; al-Kobaisi et al., 1991). Viruses mutated in one of these genes synthesize near-wild-type levels of viral DNA but fail to cleave and package concatemeric DNA into capsids, resulting in an accumulation of B (intermediate) capsids in infected cells, with the exception of the UL25 mutant, which was shown to cleave newly synthesized viral DNA (McNab et al., 1998). Moreover, in contrast to the latter study where no packaging of matur DNA was observed, Stow (2001) reported that a significant amount of DNA synthesized by the UL25 mutant was packaged stably into capsids.

To propagate a BHV-1 deletion mutant with a potentially lethal mutation in the UL25 gene to study the function of this gene, it was necessary to develop a complementing cell line expressing the UL25 protein in trans. For this purpose, RK13 cells (rabbit kidney cells, ATCC CCL-377) were stably transfected with 2 μg ScaI-linearized pRetroTET-OFF/UL25, encoding the complete UL25 ORF under the control of a tetracycline-regulated promoter (Fig. 1). A selected cell line grown in the absence of doxycycline, a tetracycline derivative compound (Gossen et al., 1995), expressed a 63 kDa protein that reacted specifically with the UL25 antisera (results not shown; Desloges et al., 2001). This polypeptide represents the full-length product of UL25, since its size correlated with that of the protein observed in BHV-1-infected cells (Desloges et al., 2001). This cell line, named RK13/UL25+, was used subsequently as a complementing host cell for the construction of a UL25 deletion mutant.

The UL25-encoding sequence is located at positions 62398–60602 of the BHV-1 genome. To genetically engineer a BHV-1 UL25 deletion mutant, we constructed the plasmid pKS/58-63kAUL25/β-gal+ from pKS/BHV58-63kApa-Bam, by replacing the Sall fragment encoding aa 86–393 of the UL25 protein by a β-Gal reporter gene cassette (Fig. 1). In this way, the deletion introduced into UL25 should not alter the expression of neighbouring genes. The plasmid pKS/58-63kAUL25/β-gal+ was linearized and used with BHV-1 viral DNA to co-transfect RK13/UL25+ cells grown in the absence of doxycycline in the presence of the chromogenic compound X-Gal. A β-Gal-positive mutant virus, designated BHV-1ΔUL25, was plaque-purified five times on RK13/UL25+ cells before virus production.

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The known, complete sequence of the wild-type BHV-1 genome allowed us to determine the restriction profiles that were expected for the mutated genome. We compared the HindIII restriction profiles of mutant versus BHV-1 DNA-purified, infected cell lysates. No divergence between the patterns observed and that expected for the mutant genome was noted (results not shown). These findings demonstrate that the β-Gal reporter gene cassette interrupts, as intended, the UL25-encoding sequence of the virus mutant.

To verify that the engineered mutant was indeed incapable of expressing the UL25 gene product, we performed
Western blotting analyses of protein extracts from RK13 cells infected for different periods of time with either BHV-1 or BHV-1UL25. As shown in Fig. 2(A), extracts infected with BHV-1 revealed the presence of an abundant 63 kDa protein, which was detected specifically with the UL25 antiserum, accumulating in cells from 12 to 18 h post-infection (p.i.). In contrast, the UL25 antiserum detected no protein in extracts from BHV-1UL25-infected RK13 cells, thus demonstrating that the virus mutant was incapable of expressing the UL25 protein in RK13 cells (Fig. 2A). This protein was absent in lysates prepared in the presence of phosphonoacetic acid (PAA), a DNA synthesis inhibitor, confirming further our previous findings that BHV-1 UL25 belongs to viral genes of the y2 class, since its expression is dependent strictly on viral DNA synthesis (Desloges et al., 2001).

To investigate the consequence of the UL25 defect on the replicative cycle of BHV-1, virus production was examined by establishing one-step growth curves of both BHV-1ΔUL25 and BHV-1 in non-complementing RK13 as well as in complementing RK13/UL25+ cells. Cells were infected synchronously with one or the other virus at an m.o.i. of 5 and then harvested at different times p.i., after which the total number of virus particles present either extracellularly or intracellularly in each sample was determined by titration assays, as described previously (Desloges & Simard, 2003). BHV-1 was capable of replicating efficiently in both RK13 and RK13/UL25+ cells, reaching similar maximal virus yields at 21 h p.i. (Fig. 2B). In contrast, BHV-1ΔUL25 could only replicate in RK13/UL25+ cells, as no progeny was produced in RK13 cells (Fig. 2B). These results demonstrate that the UL25 gene product is necessary for virus replication in non-complementing cells, as was observed also with an HSV-1 UL25 deletion mutant (Ali et al., 1996). In addition, the virus yield obtained with BHV-1ΔUL25 on RK13/UL25+ cells was similar to that of BHV-1 virus yield recovered from either cell line, indicating that the RK13/UL25+ cell line was capable of fully complementing the UL25 defect of the mutant virus. These results demonstrate that the amount of the UL25 protein within the complementary cell line is sufficient to allow replication of BHV-1ΔUL25 to BHV-1 levels, indicating that no mutations in other essential genes had been introduced during the recombination event giving rise to the mutant.

To identify the step at which the UL25 deletion mutant replication aborts in RK13 cells, we examined whether the mutation compromised the expression of late viral gene products. For this purpose, the expression of the BHV-1 ε gene trans-inducing factor protein encoded by the UL48 gene, a late gene (Misra et al., 1994; Desloges et al., 2001), was analysed. At different times p.i., protein lysates were prepared from BHV-1ΔUL25- and BHV-1-infected RK13 cells and analysed by immunoblot for the presence of the UL48 gene product using a BHV-1-specific UL48 antiserum directed against an Escherichia coli-expressed T7 tag–UL48...
fusion protein. The antiserum detected a 50 kDa protein in lysates collected at 18 h p.i. from both BHV-1UL25- and BHV-1-infected RK13 cells (Fig. 2C). Moreover, the production of the UL48 gene product was abrogated by PAA treatment (Fig. 2C, + PAA), confirming further that UL48 is a γ2 gene. Thus, these results demonstrate that the UL25 defect does not impair the expression of viral genes of the γ2 class or, consequently, the expression of genes of the earlier classes (α, β1, β2 and γ1). These results also demonstrate indirectly that the mutation does not compromise viral DNA synthesis, since genes whose expression is dependent strictly on viral DNA synthesis (such as UL48) are expressed efficiently.

To obtain direct evidence that the UL25 defect does not compromise viral DNA synthesis in RK13 cells, Southern blotting analysis of HindIII digests of total cell DNA isolated from BHV-1UL25-infected RK13 and RK13/UL25+ cells was performed. The HindIII fragment N, representing the leftward terminal region of the BHV-1 linear viral genome, was used as probe (Fig. 1). Probe N hybridized to a major 2-4 kbp and two minor non-well-resolved 14 and 15 kbp fragments present in DNA from BHV-1UL25-infected RK13 cells obtained at 12 and 24 h p.i. (Fig. 3A, lanes 2 and 3). In contrast, no hybridization signal was observed at 0 h p.i. (Fig. 3A, lane 1), demonstrating that the bands observed represented newly synthesized viral DNA. The DNA profile observed at 24 h p.i. of RK13 cells (Fig. 3A, lane 3) was very similar to that observed in BHV-1UL25-infected RK13/UL25+ cells (Fig. 3A, lane 4). The 2-4 kbp bands common to the two profiles co-migrated with the KpnI–HindIII insert of the recombinant plasmid pKS/Nhd, which harbours the complete HindIII fragment N (Fig. 3A, lane 5), demonstrating that these bands represented the leftward terminal 2-4 kbp HindIII fragment N of the BHV-1 linear genome. The sizes of the other two bands correlated to those expected for head-to-tail HindIII genomic fragments (Fig. 1, N–F and N–H head-to-tail fragments are in the range of 14 and 15 kbp, respectively), indicating the presence within infected cell lysates of viral DNA, which was in the form of either immature DNA concatemers or of circular unit-length genomes, or both. Interestingly, the levels of the 2-4 kbp fragment observed were similar in the two cell lines. These findings indicate that DNA replication of BHV-1UL25 is as effective in RK13 cells as in complementing RK13/UL25+ cells. Moreover, the much higher abundance of terminal versus head-to-tail HindIII fragments indicate that mutant viral DNA synthesized in infected cells is processed efficiently into linear unit-length genomes.

To determine whether mutant viral DNA is packaged into
capsids, thin sections of BHV-1UL25-infected RK13 and RK13/UL25+ cells as well as BHV-1-infected RK13 cells, collected at 18 h p.i., were examined by transmission electron microscopy to assess the presence of full (C), intermediate (B) and empty (A) capsids, as described previously (Desloges & Simard, 2003). Significant numbers of all three capsid types were found on sections from both BHV-1-infected RK13 cells (A capsids, 35·7%; B capsids, 16·7%; C capsids, 47·6%) and BHV-1UL25-infected RK13/UL25+ cells (A capsids, 29·6%; B capsids, 51·7%; C capsids, 18·7%). Each of these infected cell samples (Fig. 3B, upper left and right panels, respectively) presented several C capsids surrounded by a bilayer envelope. In contrast, most of the capsids in BHV-1UL25-infected RK13 cells were of types A and B (40·0 and 57·2%, respectively), as illustrated in Fig. 3(B, lower left panel), showing an accumulation of both capsid types in infected cells. The generation of empty A capsids devoid of both DNA and internal structure is believed to result from an abortive packaging event. Abortive packaging has been observed also in mutants of the dsDNA bacteriophage P22, which presents similarities to alphaherpesviruses concerning the DNA packaging and capsid assembly process. In Salmonella cells infected with mutants of phage P22 defective in genes 4, 10 and 26, it was shown that the procapsids are assembled and that viral concatemeric DNA is packaged but that the newly filled capsids are unstable and lose their DNA (Strauss & King, 1984). The phenotype observed in bacterial cells infected with these mutants is an accumulation of empty A capsids, similar to those observed in BHV-1UL25-infected RK13 cells.

However, careful examination of these infected cells revealed the presence of few electron-dense cored C capsids (2·8%). This is demonstrated in Fig. 3(B, lower right panel), in which a complete enveloped C capsid is shown in the extracellular compartment of BHV-1UL25-infected RK13 cells. It was reported previously that the HSV-1 UL25 mutant (KUL25NS) does not package mature DNA (McNab et al., 1998). Recently, Stow (2001) detected successfully low levels of KUL25NS DNA being packaged and retained into capsids. The latter findings are corroborated in the present study by the observation of few full C capsids in electron micrographs of thin sections of BHV-1UL25-infected RK13 cells. However, it appears that these enveloped C capsids were either non-infectious or their number was too low for cytopathogenesis, as no virus was detected in one-step growth curve analyses. Also, it could be that UL25 mutant genomes are packaged efficiently into capsids but are released subsequently and then degraded rapidly, as suggested by Stow (2001).

In summary, we describe the engineering and characterization of a UL25 deletion mutant of BHV-1. Our results show that the mutant is not impaired in its ability to synthesize viral proteins and to replicate and cleave viral DNA into unit-length genomes. Moreover, the mutant is still able to encapsidate viral DNA, albeit at very low levels. Further studies will be required to elucidate more precisely the role played by UL25 in the encapsidation process and especially in the stabilization of the packaged DNA into the capsid.

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