Herpesvirus of turkey reconstituted from bacterial artificial chromosome clones induces protection against Marek’s disease

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INTRODUCTION

Herpesvirus of turkey (HVT) or Meleagrid herpesvirus 1 is a naturally occurring, non-pathogenic alphaherpesvirus that was originally isolated from domestic turkeys in the late 1960s (Kawamura et al., 1969; Witter et al., 1970). As a member of the genus Mardivirus (Fauquet et al., 2005), HVT is antigenically related to Marek’s disease virus (MDV), the causative agent of the highly contagious neoplastic Marek’s disease (MD) in chickens. The antigenic relatedness between HVT and MDV has recently been determined (Afonso et al., 2001). The 160 kbp-long genome is estimated to contain at least 99 functional genes, many of which have homologues in MDV and other herpesviruses. HVT lacks 16 genes that are present in MDV, but has 13 genes for which there are no MDV homologues, including vNr-13, the first Bcl-2 homologue found in an alphaherpesvirus (Afonso et al., 2001).

Because of the strict cell-associated nature of MDV, the vaccines derived from serotypes 1 and 2 are available only as ‘wet’ vaccines that comprise suspensions of viable, infected cells. HVT vaccine was also originally developed as a ‘cell-associated’ wet vaccine. However, because of the distinct ability of HVT to produce cell-free virus in infected tissue culture (Witter et al., 1970), HVT vaccine is also available in a cell-free ‘dry’ lyophilized form that is used widely in countries where the cold storage necessary for the viability of wet vaccines is a problem. HVT is also used as a vector for generating recombinant vaccines and HVT expressing protective genes of avian pathogens such as Newcastle disease virus, Infectious bursal disease virus, infectious laryngotracheitis virus, Avian leukosis virus and Eimeria have been developed (Bublot & Sharma, 2004).

The complete genome sequence of the FC126 strain of HVT has recently been determined (Afonso et al., 2001; Kingham et al., 2001). The 160 kbp-long genome is estimated to contain at least 99 functional genes, many of which have homologues in MDV and other herpesviruses. HVT lacks 16 genes that are present in MDV, but has 13 genes for which there are no MDV homologues, including vNr-13, the first Bcl-2 homologue found in an alphaherpesvirus (Afonso et al., 2001). A detailed understanding of the functions of
the HVT-encoded genes is very important to elucidate the unique biological features of HVT as well as to develop HVT-based vectors with enhanced efficacy and versatility.

Analyses of gene functions by manipulation of herpesvirus genomes have been revolutionized by the development of bacterial artificial chromosome (BAC) technology and BAC clones containing full-length genomes of several herpesviruses including MDV have been constructed (reviewed by Zelnik, 2003). In this paper, we describe the construction of full-length HVT BAC (pHVT) clones and show that infectious viruses recovered from these clones are indistinguishable from the wild-type HVT (WTHVT) in plaque morphology. We also show that pHVT-derived viruses, in spite of the differences in growth kinetics in vitro, were as effective as WTHVT in inducing protection against virulent MDV. This is the first report on the construction of BAC clones of HVT and will provide the opportunity for rapid manipulation of the viral genome to identify the molecular determinants associated with the unique features of HVT and for development of more efficient and versatile recombinant vaccine vectors.

**METHODS**

**Viruses and cells.** WTHVT FC126 strain (ninth duck embryo fibroblast passage stock), obtained from the Avian Disease and Oncology Laboratory (ADOL), East Lansing, MI, USA, was used for the construction of the BAC. The very virulent RB-1B strain of MDV originally obtained from Dr K. A. Schat, Cornell University, USA (Petherbridge et al., 2004) was used as the challenge virus in protection studies. All viruses were propagated in primary chicken embryo fibroblasts (CEF) prepared from 10-day-old specific-pathogen-free embryos.

**Construction of HVT BAC.** Construction of the BAC clone was carried out by insertion of the bacterial miniF plasmid into the US region of HVT, essentially using the method described for the construction of MDV BAC (Schumacher et al., 2000; Petherbridge et al., 2004). For the construction of the transfer vector with homologous sequences to regions of the HVT genome, 2-0 kbp and 2-7 kbp fragments flanking the HVT US2 gene were amplified by PCR using appropriate primers (Table 1). The PCR products amplified from total DNA extracted from HVT-infected cells were digested with appropriate restriction enzymes (EcoRI, PacI and BamHI) and cloned into the pGem-T vector (Promega) linearized with EcoRI and BamHI to create the pHVTDS vector. The 7-3 kbp PacI fragment containing the miniF plasmid released from the plasmid pHAI (kindly provided by Dr M. Messerle, Ludwig-Maximilians-Universität, Munich, Germany) was cloned into the pHVTDS vector to generate the pHVTDS–pHA1 recombination transfer vector. BAC clones of HVT were generated following the procedures described previously (Petherbridge et al., 2003, 2004) and by Schumacher et al. (2000). Briefly, primary CEF cultures were co-transfected with approximately 2 μg FC126 genomic DNA and 10 μg pHVTDS–pHA1 DNA. Genomic DNA extracted from these CEF cultures after four rounds of selection in medium containing mycophenolic acid, xanthine and hypoxanthine were electroporated into *Escherichia coli* DH10B cells. Chloramphenicol-resistant colonies containing high molecular mass DNA were tested for infectivity after transfection into CEFs using lipofectamine (Invitrogen).

**Analysis of the DNA.** HVT DNA preparations from WTHVT or pHVT BAC clones were analysed by restriction digestion with appropriate enzymes. For Southern hybridization, DNA was separated by 1-0 % agarose gel electrophoresis, transferred to membranes and probed with DIG-labelled probes (Roche Diagnostics) specific for gpt (Petherbridge et al., 2004) and glycoprotein B (gB) using

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**Table 1. Sequences of oligonucleotides used in PCR and Southern blotting**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Details</th>
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<tbody>
<tr>
<td>US2 left sense*</td>
<td>5’-GATgattcACATCGGGCCAGCTTCCGCC-3’</td>
<td>pHVTDS 2-0 kb left region</td>
</tr>
<tr>
<td>US2 left antisense*</td>
<td>5’-ATAGTCGACTtaattaaATGGACCTGAGTGTGGAAT-3’</td>
<td>pHVTDS 2-7 kb left region</td>
</tr>
<tr>
<td>US2 right sense*</td>
<td>5’-ATCGCATGCtaattaaACTAATATGGGCAACCCAC-3’</td>
<td></td>
</tr>
<tr>
<td>US2 right antisense*</td>
<td>5’-ATCGgattcTGGGCACTCTAGTTGATTATT-3’</td>
<td>KanR gene for gB deletion</td>
</tr>
<tr>
<td>gB Kan sense†</td>
<td>5’-ATATCGTTGAGAGCCCACAAATTTTATTTTTAAGTTCTTTT</td>
<td>KanR FRT cassette for gB deletion</td>
</tr>
<tr>
<td>gB Kan antisense†</td>
<td>5’-CTGATATCGTTGAGAGCCCAAAATTTTATTTTTAAGTTCTTT</td>
<td></td>
</tr>
<tr>
<td>gB Kan_FRT sense†</td>
<td>5’-ATATTGAGAGCCCAAAATTTTATTTTTAAGTTCTTT</td>
<td></td>
</tr>
<tr>
<td>gB Kan_FRT antisense†</td>
<td>5’-ATGACTGTTGAGAGCCCAAAATTTTATTTTTAAGTTCTTT</td>
<td></td>
</tr>
<tr>
<td>gB revertant sense</td>
<td>5’-CTGCGCGTTTCCGAGTTTGG-3’</td>
<td>gB gene for construction of revertant virus</td>
</tr>
<tr>
<td>gB revertant antisense</td>
<td>5’-TGACCCTCTTGGTACCGTG-3’</td>
<td></td>
</tr>
<tr>
<td>gpt sense</td>
<td>5’-ATGACGCAAGAAATACCATGTC-3’</td>
<td>gpt probe</td>
</tr>
<tr>
<td>gpt antisense</td>
<td>5’-ATGACGCAAGAAATACCATGTC-3’</td>
<td></td>
</tr>
<tr>
<td>gB sense</td>
<td>5’-ACACTGCGATGACATTTTTATCATCCGTCT-3’</td>
<td>gB probe</td>
</tr>
<tr>
<td>gB antisense</td>
<td>5’-AGCGAAAGTAGTAGTCGGGG-3’</td>
<td></td>
</tr>
</tbody>
</table>

*Sequences of restriction sites introduced for cloning are shown in lower case.
†Nucleotides homologous to the UL27 flanking region are underlined.
Plaque assay. The rates of strain and pHVT viruses culture dishes seeded with $2 \times 10^6$ p.f.u. of each of the viruses was inoculated onto 60 mm tissue-virus. For this, a 3-kbp PCR fragment containing the pHVT-3 sequence and FRT-spectinomycin cassette was amplified from this insert at the end of the gB coding region. Finally, a 4-kbp cassette from the pCR8 vector (Invitrogen) with flanking FRT sites was inserted at the end of the gB coding region. Amplified by PCR from the pACYC177 plasmid (NEB) using the AgB Kan sense and AgB Kan antisense primers (Table 1), which contained an additional 50 nt of homology with the region flanking the HVT UL27 gene to allow recombination, was electroporated into the competent cells. 

We also constructed an identical gB deletion mutant of pHVT3 by Red mutagenesis (Yu et al., 2000; Lee et al., 2001) using EL250 cells (kindly provided by Dr N. Copeland, NCI Frederick, MD, USA) by inserting a KanR cassette flanked by FRT sites, amplified using oligonucleotides AgB Kan_FRT sense and AgB Kan_FRT antisense (Table 1) from the plasmid pKD13 (Datsenko & Wanner, 2000). This AgB mutant, after excision of the KanR cassette by the expression of FLP recombinase using 0.2% arabinose, was used for the construction of the revertant virus. For this, a 3-0-kbp fragment of HVT gB (Tulman et al., 2000) with 350–450 bp homology at each end was amplified by PCR and cloned into the pKD13 vector (Invitrogen). A spectinomycin cassette from the pCR8 vector (Invitrogen) with flanking FRT sites was inserted at the end of the gB coding region. Finally, a 4-5-kbp PCR product containing the whole gB ORF with flanking homologous sequence and FRT-spectinomycin cassette was amplified from this vector and electroporated into EL250 competent cells containing pHVT-3AgB to induce recombination. BAC DNA from revertant clones resistant to spectinomycin and chloramphenicol was transfected into primary CEFs using lipofectamine.

Comparison of the rates of in vitro growth of the WtHVT strain and pHVT viruses

Plaque assay. The rates of in vitro growth of the viruses were studied on CEFs by counting the p.f.u. at various time points. Briefly, 100 p.f.u. of each of the viruses was inoculated onto 60 mm tissue-culture dishes seeded with $2 \times 10^6$ CEFs and incubated at 38°C. At 0, 12, 24, 48, 72, 96 and 120 h after inoculation, the infected cells were trypsinized and serial 10-fold dilutions were added in triplicate onto the six-well plates of CEFs. The titres of the virus at each time point were calculated after 4 days from the number of plaques compared with those at the zero time point was plotted against hours post-infection for each of the three viruses.

Assay of HVT genome copy numbers by quantitative PCR (qPCR). DNA was prepared by phenol extraction (Sambrook & Russell, 2001) from infected CEFs harvested at 0, 12, 24, 48, 72, 96 and 120 h after inoculation. HVT genome copies per 10 000 CEFs were quantified using real-time PCR, taking the mean value for duplicate wells for each test sample. The method used was essentially as described previously, using a duplex PCR to detect both the virus gene and the host overtansferring gene (Baigent et al., 2005), with the following modifications. The primers and probe used were specific for the HVT gene sORFI as published previously by Islam et al. (2004). The standard curve for the sORFI reaction, for calibration of HVT genome copy number, was prepared using a dilution series of pHVT BAC3 DNA. The fold increase in HVT genome copies per 10 000 cells compared with those at the zero time point was plotted against hours post-infection for each of the three viruses.

The ability of the viruses to produce cell-free virus from infected CEFs was also compared. For this, tissue-culture supernatants were collected 4 days after infection from CEFs infected with 100 p.f.u. WtHVT or pHVT virus stocks. After centrifugation at 450 g for 5 min to remove any cells, 10-fold dilutions were added to duplicate wells of fresh CEFs in a six-well plate. Tenfold dilutions of cell lysates obtained after four rapid freeze–thaw cycles were also titrated similarly on duplicate wells of CEFs. The virus titres were determined from infected cells 4 days after titration.

Immunological detection of HVT plaques. HVT plaques were detected by immunohistochemical staining with a polyclonal antiserum collected from birds infected with the HPRS-16 strain of MDV. CEF monolayers were fixed in acetone–methanol, blocked for 1 h with 5% newborn calf serum in PBS and incubated with a 1:1000 dilution of the antiserum at room temperature for 1 h. After washing the cell sheets three times in PBS containing 0.1% Tween 20, the cells were left at room temperature for 1 h with 1:500 dilution of anti-chicken–horseradish peroxidase conjugate (Sigma). After further washing, the cells were incubated for 1 h at room temperature in a developing solution of 3-amino-9-ethylcarbazole (Sigma), diluted to a final concentration of 0.2 mg ml$^{-1}$ in 0.1 M sodium acetate (pH 4.8) with 0.015% hydrogen peroxide, and the mean virus titres were calculated from the number of plaques. HVT plaques were also detected by immunofluorescence staining with the above chicken antiserum and anti-chicken–fluorescein isothiocyanate conjugates (Sigma).

In vivo protection studies. Protection experiments were carried out in 1-day-old specific-pathogen-free Rhode Island Red chicks, maintained at the Poultry Production Unit of the Institute for Animal Health. All experiments were carried out in separate rooms of the experimental animal house as per UK Home Office guidelines. Chicks were randomly divided into groups of 15 and vaccinated intramuscularly with a total of 5000 p.f.u. of either WtHVT, pHVT3 or pHVT4 in two doses at 1 and 7 days of age. A further group was inoculated with non-infected CEFs as a negative control. At 13 days of age these birds were then infected intra-abdominally with 1000 p.f.u. RB-1B virus. The birds were inspected regularly and all the birds that died during the experiment or were killed at the end of the trial were evaluated for gross and histological lesions by necropsy. Cumulative survival rates were used to assess the protective efficacy of each vaccine virus.

RESULTS

Construction of BAC clones containing the complete HVT genome

Transfection of WtHVT DNA together with pHVTDS–pHA1 DNA into CEFs and subsequent growth in selection medium showed evidence of virus replication indicating the presence of recombinant viruses. These infected cells were seeded onto fresh CEFs and grown in the presence of selection medium for a further four passages. Electroporation of DNA from these CEFs into E. coli DH10B cells produced several chloramphenicol-resistant colonies. Two of these colonies that contained high molecular mass
extrachromosomal DNA were selected for further analysis. Transfection of DNA from these two clones (pHVT3 and pHVT4) into primary CEFs produced HVT-specific plaques that were visible from about 72 h, demonstrating their infectivity. The morphology and size of the plaques were indistinguishable from those of wT HVT plaques (Fig. 1). BamHI–EcoRI digestion of the DNA isolated from pHVT clones 3 and 4 showed identical restriction patterns, indicating that the two clones were indistinguishable from each other at the molecular level (Fig. 2). The pattern and size of the restriction fragments were also identical to the expected digestion pattern predicted from the full-length genome sequence of HVT (GenBank accession nos AF282130 and AF291866), indicating that the pHVT BAC clones contained the full complement of the HVT genome.

**Characterization of HVT reconstituted from BAC DNA**

Having demonstrated the ability of the two pHVT clones to reconstitute infectious virus in CEFs, we determined whether the growth curves of the reconstituted viruses from the BAC clones were comparable to that of the parent wT HVT. For this, primary CEFs were infected with 100 p.f.u. of either wT HVT or one of the two pHVT-derived viruses. The number of plaques produced at various days post-infection was determined by seeding 10-fold dilutions of virus-infected cells on fresh CEFs and examining for virus plaques. The rate of virus replication measured by qPCR for genome copy numbers (Fig. 3a) and plaque titration (Fig. 3b) showed that pHVT3 virus replicated at a higher rate than wT HVT and pHVT4. The growth rate measured by virus plaques peaked at 96 h, whereas the genome copy numbers remained high even at 120 hours post-infection. Since HVT is capable of producing cell-free virus in tissue culture, we also examined whether pHVT could produce cell-free virus in infected CEFs. Titration of the culture supernatant and cell lysates from infected CEFs showed that the two pHVT clones were capable of producing cell-free virus, although the levels were lower than those of wT HVT (Fig. 3c).
Mutagenesis of pHVT clone for deletion of gB sequences

In order to examine the amenability of the pHVT clones to BAC mutagenesis, we chose to construct a gB deletion mutant (pHVT3ΔgB) by using a one-step mutagenesis procedure to replace the gB gene with a KanR gene by homologous recombination and selecting for chloramphenicol- and kanamycin-resistant colonies. BamHI–EcoRI digestion of the DNA extracted from one of these clones showed a very similar restriction pattern to that of the donor pHVT3 BAC, indicating that the molecular integrity of the genome was not affected by the mutation. The appearance of an extra band of 12.5 kb and the loss of two bands of 7.2 and 7.0 kb (Fig. 2), resulting from the loss of an EcoRI site, is in agreement with those calculated after insertion of the KanR gene in the gB coding sequence.

Southern blotting hybridization of the BamHI digest of the DNA from the pHVT3, pHVT4 and pHVT3ΔgB clones with the gpt probe identified a single 14 kb band in all lanes except the lane with wtHVT DNA. (b) The same membrane reprobed with the DIG-labelled gB probe, showing a single 25 kb band in all lanes except that with pHVT3ΔgB DNA. The corresponding size (kb) of the DNA ladder of EcoRI–HindIII-digested λ DNA is shown.

We also carried out Southern hybridization to confirm the authenticity of the deletion of gB from the pHVT3ΔgB clone. For this, the gpt probe was stripped off the membrane, which was then hybridized with a DIG-labelled gB probe generated by PCR using specific primers (Table 1). As expected, a single band of the predicted size of about 25 kb was detected in the DNA from the two pHVT BAC clones as well as in the virus stocks reconstituted from these clones. A similar-size band was also detected in the DNA samples from wtHVT-infected cells. However, no signals were obtained on pHVT3ΔgB DNA (Fig. 4b), confirming the
deletion of gB from this clone. In order to check the effect of the deletion of gB on virus replication, DNA prepared from pHVT3 and pHVT3<sup>DgB</sup> clones was transfected onto fresh CEFs and incubated at 37°C for 3–4 days. HVT plaques, the specificity of which was confirmed by positive immunofluorescence staining with a polyclonal anti-MDV serum, could readily be detected in cells transfected with pHVT3 DNA from 48 hours post-infection (not shown). Compared with this, no plaques were visible in the cells transfected with pHVT3<sup>DgB</sup> DNA. However, single cells that expressed HVT proteins were demonstrated by immunofluorescence, indicating that, in the absence of gB, HVT is unable to spread from cell to cell. This was also demonstrated using a second pHVT3AgB mutant prepared with a Kan<sup>R</sup> cassette flanked by FRT sites. To confirm that this defect in cell-to-cell spread was due to the deletion of gB, we constructed a revertant virus in which the deleted region was replaced with the UL27 gene by recombination. Transfection of the DNA from the revertant BAC clone produced virus plaques (data not shown).

**Reconstituted HVT from pHVT clones protects against virulent MDV challenge**

Since HVT is used as a highly efficient vaccine against oncogenic MDV strains, we compared the protective ability of the virus stocks reconstituted from the two pHVT clones with that of <sub>WT</sub>HVT in an experimental MDV-challenge model. The protective efficacies of the vaccine viruses were determined by the cumulative survival rates and gross/histological lesions in vaccinated and unvaccinated chickens experimentally challenged with the highly virulent RB-1B strain of MDV. Evidence of MD could be observed in the unvaccinated control birds from about 4 weeks after infection with the RB-1B strain and nearly 80% of the birds developed MD during the 60-day experimental period (Fig. 5). Post-mortem examination of these birds showed evidence of lymphoid tumours in several visceral organs. Compared with this, none of the birds vaccinated with <sub>WT</sub>HVT or either of the two pHVT-derived viruses showed any evidence of the disease. Tissues from all birds collected at the end of the experiment were also examined for evidence of any histological lesions. These studies further showed that vaccination with either <sub>WT</sub>HVT or the pHVT-derived viruses were effective even against the induction of microscopic lesions, although one bird from the group vaccinated with <sub>WT</sub>HVT showed histological lesions of MD (Fig. 5).

**DISCUSSION**

Since its introduction in the 1970s, HVT has served as an important live vaccine for the prevention of MD. Although other generations of MD vaccines have been introduced to protect against the continuing increase in virulence of MDV (Witter <i>et al.</i>, 2005), HVT vaccines are still being used widely either alone or in combination with other vaccines. Despite its widespread use, little is known about the functions of HVT genes that are associated with the immune responses that control MDV infection. Recent studies using microarray analysis have shown that HVT infection of CEFs has a wide range of effects on cellular homeostasis (Karaca <i>et al.</i>, 2004).

HVT is antigenically related to MDV and induces a persistent viraemic infection with similar infection kinetics (Holland <i>et al.</i>, 1998). However, there are several features that are unique to HVT. For example, (i) HVT is distinct from MDV in its ability to produce cell-free virus in culture, a property made use of in the production of freeze-dried cell-free vaccines, (ii) HVT appears to replicate less efficiently in the skin than does MDV, a phenotype that is thought to be associated with the relatively infrequent transmission of HVT among chickens (Cho & Kenzy, 1975) and (iii) unlike MDV, HVT has the ability to replicate in embryonic tissues (Sharma, 1987), a phenomenon of great significance since the widespread practice of <i>in ovo</i> vaccination for the control of MD. A fundamental understanding of the functions of HVT genes and the molecular basis for the unique features of HVT would be useful in improving the immune responses induced by HVT.

The availability of the complete genome sequence of the FC126 strain of HVT (Afonso <i>et al.</i>, 2001; Kingham <i>et al.</i>, 2001) has further highlighted the importance of the HVT genome as a tool for future vaccine development and evaluation.
Recombinant HVT from BAC clones

2001) has enabled comparisons between the HVT and MDV genomes to examine the molecular bases for some of the distinct biological features of HVT. For example, the absence of several important MDV-specific genes such as meq and vCXC chemokine or the presence of distinct HVT-specific genes such as the Bcl-2 homologue vNr-13 in the HVT genome might account for some of the distinct features, such as the non-pathogenic phenotype (Afonso et al., 2001). Although such genomic comparisons are valuable, delineation and precise mapping of the functional determinants would require the application of reverse genetic tools for the rapid manipulation of the genomes. The manipulation of large herpesvirus genomes has been facilitated by the development and widespread application of BAC technology (Brune et al., 2000). BAC clones of various strains of MDV have been constructed and used for examining gene function. As a first step in identifying the molecular determinants associated with the unique features of HVT, we have generated BAC (pHVT) clones of the FC126 strain. Infectivity of the pHVT clones was confirmed by the ability to reconstitute the virus from the transfected DNA. The viruses rescued from the two pHVT clones were indistinguishable from the parental virus in plaque morphology (Fig. 1). However, there were differences in the replication rates between the BAC and wild-type viruses (Fig. 3). Virus derived from the pHVT3 clone showed a much higher replication rate compared with WT HVT. On the other hand, pHVT4 virus replicated at a slower rate than WT HVT. These results show that the BAC clones represent the individual genomes present in the WT HVT pool that may differ in biological characteristics. Titration of the culture supernatant and cell lysates from infected CEFs showed that the pHVT clones produced cell-free virus. However, the titres of cell-free viruses were much lower than those produced by WT HVT. Although the reasons for this defect are not clear, it may be related to the loss of US2. We are currently trying to restore US2/SORF3 in the BAC clones to examine this. The pHVT-derived viruses could be passaged several times in vitro, demonstrating that the pHVT clones are stable and could be useful as seed stocks for production of vaccines.

Since HVT is primarily used as a vaccine against MDV, we compared the immunogenicity of viruses derived from the two pHVT clones with that of the FC126 strain of WT HVT. In the virulent RB-1B-challenge infection model, viruses derived from the two pHVT clones induced 100% protection, similar to WT HVT. In comparison, nearly 80% of the unvaccinated birds developed MD during the 60-day experimental period, demonstrating that the viruses derived from the pHVT clones are equally effective as WT HVT in inducing protective immune responses. Birds vaccinated with pHVT were completely protected even against the development of microscopic lesions, further proving induction of strong protective responses against MD.

We also examined the ease with which the pHVT clones could be subjected to mutagenesis techniques. For this, we chose to construct a gB deletion mutant of HVT using the one-step mutagenesis protocol described by Datsenko & Wanner (2000). Using this approach we were able to manipulate the HVT genome and construct the gB deletion mutant pHVT3ΔgB. The deletion of gB from the pHVT3ΔgB clone was confirmed by restriction digestion and Southern blotting (Figs 2 and 4). Under the same transfection conditions in which the pHVT3 DNA produced viral plaques on CEFs, pHVT3ΔgB DNA was unable to produce any viral plaques. However, single cells expressing HVT proteins could be detected by immunofluorescence staining of cells using polyclonal antiserum from MDV-infected birds, indicating that the deletion of gB interfered with the cell-to-cell spread of HVT. This defect in pHVT3ΔgB was rescued using a revertant virus in which UL27 was restored. These results further confirm the essential nature of gB for replication of herpesviruses (Pereira, 1994; Schumacher et al., 2000). HVT encodes other glycoproteins present in MDV, including gB, gC, gD, gE, gH, gL, gK, gL, gM and gN (Kingham et al., 2001). Previous studies have shown that the requirement of glycoprotein complexes for MD replication is distinct from that of other herpesviruses (Osterrieder & Vautherot, 2004). Although most alphaherpesviruses can grow in cell culture in the absence of various glycoproteins, deletion of gE, gL, gM or gN blocks MDV replication. This differential requirement of glycoproteins is suggested to be attributable to the strict cell-associated nature of MDV (Tischer et al., 2002). As HVT can produce cell-free virus in culture, it is possible that the requirements of glycoprotein complexes of HVT are distinct from those of MDV. The availability of the BAC clones provides the opportunity to compare the requirements of individual glycoproteins for HVT replication.

HVT is also used as a vector for expression of heterologous antigens. Recombinant HVT expressing antigens from various avian pathogens has been constructed using conventional recombination approaches (Bublot & Sharma, 2004). The construction of the pHVT clones will considerably speed up the development of new, highly immunogenic, multivalent recombinant HVT containing multiple antigens or cytokines.

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