Construction and properties of a turkey herpesvirus recombinant expressing the Marek’s disease virus homologue of glycoprotein B of herpes simplex virus

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A herpesvirus of turkeys (HVT) recombinant containing a 3.9 kbp fragment of Marek’s disease virus (MDV) DNA encoding MDV glycoprotein B (gB), stably integrated into the thymidine kinase (TK) gene of HVT, has been constructed. The replication of the recombinant in chick embryo fibroblasts (CEF) was comparable to that of wild-type HVT. The recombinant expressed authentic MDV gB and its processed forms (110K, 65K and 48K) in CEF as shown by immunoblotting using an MDV-specific anti-peptide serum. Northern blot analysis showed that MDV gB mRNA was transcribed from MDV promoter sequences flanking the MDV gB open reading frame and also from the HVT TK promoter. However, the level of replication of the recombinant in vivo appeared to be lower than wild-type HVT as shown by the titres of HVT antibodies determined by ELISA. Pathogenicity tests showed that the recombinant was safe and did not cause microscopic or gross Marek’s disease lesions or other abnormalities. The results suggest that HVT has potential as a vector for recombinant vaccines.

Introduction

Herpesvirus of turkeys (HVT) has been used extensively as a vaccine against Marek’s disease (Witter et al., 1970) and is usually administered to day-old chicks. The relatively fast growth rate of HVT in chick embryo fibroblasts (CEF) compared to Marek’s disease virus (MDV) and the production of infectious virus that can be lyophilized are two important factors that have contributed to its wide use as a vaccine. There is much interest in developing safe and effective recombinant poultry vaccines in view of the adverse side-effects of many vaccines such as those against Newcastle disease and infectious bronchitis. So far, it has been demonstrated that fowlpox virus can be used as a vector for expressing antigens of Newcastle disease virus and infectious bursal disease virus (Boursnell et al., 1990; Taylor et al., 1990; Bayliss et al., 1991). Previous studies have shown that herpesviruses also have potential as vectors and reports of the expression of foreign genes using varicella-zoster virus (Lowe et al., 1987), herpes simplex virus (HSV) (Shih et al., 1984) and pseudorabies virus (Thomsen et al., 1987) have been published. It has also been shown that the MDV homologue of the US2 gene of HSV is a non-essential gene which has potential for expression of foreign genes in MDV (Cantello et al., 1991). However, the replication of the recombinant in vivo was not reported. In this paper we have investigated the potential of using the thymidine kinase (TK) gene of HVT as a site for expression of foreign genes. We report on the construction of an HVT recombinant that expresses MDV gB and the properties of this recombinant.

Methods

Virus and cells. The FC126 strain of HVT (Witter et al., 1970) was propagated in CEF as described (Ross et al., 1975). Selection of TK− virus was carried out in the presence of 1 μM of the nucleoside analogue 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-methyluracil (FMAU) (Schat et al., 1984). The highly oncogenic strain of MDV, RB1B (Schat et al., 1982), was used as a source of MDV gB.

Plasmids. The plasmid pTK1B (Scott et al., 1989; Scott, 1990) containing a 3.2 kbp HindIII fragment of HVT cloned in pUC13 was the source of the TK gene and flanking sequences which comprise most of the gH gene 3' of TK. A 3.9 kbp EcoRI–SalI fragment of MDV (strain RB1B) DNA containing the gB gene and flanking sequences which had been cloned in pUC13 (MS.B27; Ross et al., 1989) was the source of MDV gB.

Transfection and isolation of recombinants. Infectious DNA was extracted from purified HVT nucleocapsids using proteinase K and SDS as described previously (Lee et al., 1980). Usually 1 μg of full-length genomic HVT DNA was cotransfected with 1 to 5 μg linearized...
plasmid DNA using lipofectin (20 μg per 5 cm dish containing 1 x 10⁶ cells) as described by the manufacturers (Gibco BRL). In the work reported here we used sub-confluent, pre-formed monolayers of CEF seeded 16 h previously for transfection. The efficiency of transfection was 100 to 200 p.f.u./lag HVT DNA. When c.p.e. was extensive, transfected cultures were harvested and cell-free virus obtained by sonication in phosphate-buffered sucrose solution containing glutamate and albumin (SPGA) as described by Calnek et al. (1970). Virus progeny was cultured in the presence of FMAU to select for TK- virus. Individual plaques were picked, amplified in the presence of FMAU, sonicated in SPGA and subjected to two further cycles of plaque purification in the presence of the drug. Recombinants were distinguished from spontaneous TK- mutants by Southern blot analysis of viral DNA and hybridization to an MDV gB probe under stringent hybridization conditions that preclude hybridization to HVT DNA (Ross et al., 1981).

**Hybridization.** Southern and Northern blot hybridizations were carried out using standard procedures (Sambrook et al., 1989).

**Hybridization probes.** The plasmid MS.B27 was digested with Sall/EcoRI and the 3.9 kbp fragment containing MDV gB was separated from the vector by electrophoresis in agarose. The fragment was purified using GeneClean (Stratech) and labelled with [α³²P]dCTP by nick translation using commercially available reagents (Amersham). Similarly, the 3.2 kbp fragment containing HVT TK and part of gH was separated from the pUC13 vector by digestion with HindIII, purified and labelled as above.

For Northern blot hybridization, the plasmid containing HVT TK was digested with DraIII/HindIII and the fragment comprising the first 500 bases of TK was used as a hybridization probe. The MDV gB probe was obtained by labelling a 1-19 kbp fragment obtained by digesting MS.B27 with SspI. This enzyme cuts MDV gB at positions 572 and 1759 and the fragment used for hybridization comprises sequences from 67 bp upstream of the ATG of gB up to 1120 bp downstream of the ATG (Ross et al., 1989).

**Immunoblotting.** Expression of MDV gB in infected CEF was demonstrated by immunoblotting using an MDV-specific anti-peptide serum as described previously (Ross et al., 1989).

**Measurement of HVT antibodies by ELISA.** HVT antigens were obtained by sonication of 10⁸ HVT-infected CEF in 1 ml PBS. The antigen preparations were usually diluted 1/100 to 1/200 and wells were coated with approximately 100 μg of antigen in 100 μl overnight. The wells were then saturated with 100 μl of 5% skimmed milk for 1 h. After washing with TBST (0-1% Tween 20 in Tris-buffered saline pH 7-4), wells were treated with dilutions of chicken serum in TBST for 60 min, washed in TBST and treated with alkaline phosphatase-conjugated goat anti-chick IgG serum (Sigma) diluted 1/1000 in TBST. Bound conjugate was detected using p-nitrophenyl phosphate substrate as described previously (Mockett et al., 1987).

**Pathogenicity experiments.** Day-old Rhode Island Red chicks (HPRS RIR) susceptible to Marek’s disease (HPRS RIR) were inoculated intramuscularly (i.m.) with 5000 p.f.u. of recombinant virus and were observed for a period of up to 19 weeks in high security accommodation. At the end of the experiment, they were killed and examined for the presence of gross and microscopic lesions (Biggs & Milne, 1972).

**Results**

**Construction of HVT recombinant**

The construction of the recombinant HVT is outlined in Fig. 1. The strategy adopted involved the construction in the first instance of a recombinant plasmid in which the

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**Fig. 1.** (a) Construction of transfer plasmid. The 3.2 kbp HindIII fragment of HVT DNA containing the TK gene and flanking sequences was cloned in pUC13. The recombinant plasmid obtained (pTKoligo) was digested with DraIII and an oligonucleotide containing an EcoRV site was inserted to generate the plasmid pTKoligo. The DraIII site is located within TK, 530 bp from the 5′ end of the HindIII fragment. The EcoRI–SalI fragment of MDV DNA containing the MDV homologue of HSV gB and flanking sequences was isolated from the recombinant plasmid pMDVgB by digestion with EcoRI and SalI followed by electrophoresis in agarose gels. The 3.9 kbp fragment was end-repaired using Klenow and T4 DNA polymerases, and ligated to EcoRV-digested pTKoligo to generate the transfer plasmid pTKgB. (b) Construction and structure of recombinant HVT. pTKgB was linearized with SalI and co-transfected with infectious HVT DNA. TK- virus was selected using the nucleoside analogue FMAU. Recombinant virus was identified by hybridization to MDV gB and was plaque-purified. (i) Structure of wild-type HVT genome showing location of the 24.8 kbp B fragment and the 3.2 kbp HindIII fragment that contains the TK gene and flanking sequences. (ii) Structure of linearized pTKgB showing insertion of MDV sequences into TK and the presence of BamHI and HindIII sites in MDV gB. (iii) Structure of part of the TK region of recombinant HVT showing that insertion of the MDV sequence into TK results in the introduction of one BamHI site and one HindIII site within the original BamHI B fragment of HVT. Fragment sizes are in kbp. H, HindIII; B, BamHI; D, DraIII; E, EcoRI; S, SalI.
HVT recombinant

TK gene of HVT was disrupted by insertion of a synthetic oligonucleotide containing an EcoRV cloning site that would allow insertion of a wide range of blunt-ended foreign DNAs. To prevent the fortuitous synthesis of unwanted fusion proteins, the cloning site was flanked by translation stop codons in all six reading frames. Two complementary oligonucleotides, synthesized commercially, were annealed to produce the following double-stranded adaptor:

3' TCCAATTGATGATATAGATTGATTGATC 5'
5' TTAACTACTAGATATCTAACTAACTAGAGG 3'

The adaptor was ligated to a DraIII digest of the pTK1B plasmid and recombinant plasmids were identified by hybridization to end-labelled oligonucleotide. One plasmid (pTKoligo) was used subsequently for insertion of the MDV gene. Insertion of the oligonucleotide and foreign genes at the DraIII site of HVT TK is expected to disrupt the TK gene without affecting the HVT homologue of UL24 of HSV which is encoded by the complementary strand and which overlaps the 5' end of TK (Scott, 1990).

The plasmid containing the gB gene of MDV was digested with EcoRI/SalI and the 3-9 kbp fragment containing gB was separated from the pUC13 vector by electrophoresis in low melting point agarose and purified using Geneclean. After end repair using the Klenow fragment of DNA polymerase and T4 DNA polymerase, the 3-9 kbp fragment was ligated to EcoRV-digested and dephosphorylated TK transfer plasmid (pTKoligo) as described (Sambrook et al., 1989). Recombinant plasmids containing the gB gene of MDV were identified by hybridization to MDV gB. One clone (pTKgB clone 6) was selected for insertion into HVT by homologous recombination. Restriction enzyme analysis of pTKgB showed that the MDV gB open reading frame (ORF) and the TK ORF were in the same orientation (not shown).

The recombinant plasmid pTKgB DNA was linearized by digestion with SalI (a unique site located in the polylinker region of the pUC13 vector) and 5 to 10 μg was cotransfected with 1 μg of infectious genomic HVT DNA using lipofectin (BRL) as described in Methods. Several clones of TK− virus were isolated using FMAU and were plaque-purified three times in the presence of the drug.

Southern blot analysis of genomic DNA obtained from these isolates showed that five out of six isolates contained the entire 3-9 kbp MDV sequence within the TK gene of HVT and that one isolate (no. 134) was a spontaneous mutant. Fig. 2(a) shows that the recombinant HVT contained a 3-8 kbp HindIII fragment that hybridized strongly to MDV gB, and a 3-2 kbp HindIII fragment that hybridized less strongly. In contrast, the spontaneous TK− mutant and the original wild-type HVT did not hybridize to MDV gB. Hybridization of the same blot (after eluting the MDV...
gB probe) to the HVT TK probe (Fig. 2b) showed that all three virus preparations contained a 3-2 kbp fragment that hybridized strongly to HVT TK but that the recombinant contained an additional 3-8 kbp fragment. It was noted that the hybridization signal to this fragment is weak in comparison to the 3-2 kbp band, consistent with the fact that only 530 bp of the 3-8 kbp HindIII fragment in the recombinant are TK sequences, whereas the majority of the 3-2 kbp fragment is TK. Further evidence for insertion of MDV sequences into HVT TK was obtained from analysis of BamHI digests of the virus DNA. The MDV gB probe (Fig. 3a) hybridized to two fragments of 18 and 11 kbp in digests of the recombinant, whereas the HVT TK probe (Fig. 3b) hybridized to a 24-8 kbp fragment in BamHI digests of wild-type HVT and mutant virus, but to two fragments in the recombinant. The 11 kbp fragment hybridized strongly whereas the 18 kbp fragment hybridized weakly. These results are consistent with the insertion of the 3-9 kbp MDV gB sequence into the TK gene of HVT as depicted in Fig. 1(b).

Expression of MDV gB by the recombinant

To investigate the expression of MDV gB by the recombinant, extracts of CEF infected with the virus and appropriate controls were denatured and processed for immunoblotting using an anti-peptide serum specific for MDV gB. The results (Fig. 4) show clearly that the three molecular species of MDV gB (110K, 65K and 48K) were detected indicating that MDV gB encoded by the recombinant was processed normally as in MDV-infected cells (Ross et al., 1989).

Transcription of MDV gB mRNA

A Northern blot analysis was carried out to determine whether transcription of MDV gB originated from the TK promoter, from the MDV gB promoter or from both promoters. Fig. 5(a) shows that a probe specific for MDV gB (1-19 kbp SspI fragment described in Methods) hybridized to an RNA transcript approximately 3-0 kb in size in extracts of MDV-infected CEF but to two transcripts, 3-9 and 3-4 kb, in extracts of CEF infected with recombinant virus. RNA from HVT-infected cells failed to hybridize. In contrast, an HVT TK-specific probe derived from the first 500 bases of HVT TK hybridized strongly to a major 3-9 kb transcript in CEF infected with the recombinant and to a 4-3 kb transcript in extracts from HVT-infected CEF (Fig. 5b). These results suggest that MDV gB is transcribed from both the HVT TK and from the MDV gB promoter in cells infected with recombinant virus. The fact that the longer transcript (3-9 kb) contained both TK and MDV gB sequences whereas the smaller (3-4 kb) transcript contained MDV gB sequences support these conclusions. Larger RNA transcripts were noted in CEF infected with recombinant virus but not in CEF infected with HVT. These larger RNAs were detected using both MDV gB and HVT TK probes but appear as faint bands in Fig. 5(a) because the MDV gB probe was weaker than the HVT TK probe. The origin of the larger RNA transcripts noted in extracts from recombinant virus is not clear and is discussed below.

Growth properties and stability of the recombinant

Plaques of recombinant virus in CEF seemed different from those of wild-type HVT and contained a higher proportion of rounded cells. Since gB of HSV has been reported to affect the surface properties of infected cells, it is probable that the expression of MDV gB may have affected the plaque morphology of the HVT recombinant. RB1B plaques are distinct from HVT plaques and consist of rounded cells which tend not to form syncytia. However, it was not always possible to
Fig. 5. Northern blot analysis. Total RNA (30 µg) extracted from CEF infected with wild-type HVT (lanes 1), MDV (lanes 2) or recombinant HVT (lanes 3) virus was separated in 1% denaturing formaldehyde-agarose gels and blotted onto nylon membranes (Amersham International) and hybridized to (a) MDV gB and (b) HVT TK probes. Note presence of two bands, 3.9 and 3.4 kb in recombinant HVT RNA lane in (a) but one main band of 3.9 kb in (b).

identify recombinant virus by plaque morphology in double-blind experiments.

The presence of MDV gB in recombinant virus did not affect the yield of the virus in CEF compared to wild-type HVT. Titres of cell-free virus (obtained by disrupting 4·7 x 10⁶ cells in 1 ml SPGA) reached a peak of 2·2 x 10⁶ p.f.u./ml 48 h after infection. The corresponding titre of parental HVT was 2 x 10⁶ p.f.u./ml.

Similarly, measurement of the titre of infectious virus stored in liquid nitrogen over a period of 6 months did not show significant differences in stability (not shown).

Pathogenicity

To determine the pathogenicity of recombinant virus, groups of seven chickens were inoculated i.m. at 1 day old with 5000 p.f.u. of the virus and were observed for a period of 19 weeks in high security accommodation. None of the chickens died or developed clinical disease during the period of study and no gross or microscopic lesions characteristic of Marek’s disease were found in visceral organs or peripheral nerves on post-mortem (Biggs & Milne, 1972). No lesions were observed in uninoculated controls or in chicks inoculated with the spontaneous TK⁻ mutant or wild-type HVT. These results indicate that recombinant virus is non-pathogenic.

The titres of HVT antibodies determined 4 weeks after inoculation are shown in Table 1. The results show that both the recombinant and the TK⁻ mutant induced HVT antibody in six of seven birds examined although the titres were lower than in chicks inoculated with wild-type HVT.

Discussion

These results demonstrate that it is possible to obtain a stable HVT recombinant containing 3·9 kbp of a foreign (MDV) DNA inserted into the TK gene. Moreover, the MDV gB gene product expressed was authentic, and was processed in a similar manner as in MDV-infected cells giving rise to three molecular forms of gB (110K, 64K and 48K). The growth of the recombinant in vitro was not impaired compared to wild-type HVT and titres of cell-free virus approaching 2 x 10⁶ p.f.u./ml could be obtained.

It is not known whether HVT gB is also synthesized by the recombinant and whether it interacts with MDV gB. However, we have no reason to suspect that HVT gB is not synthesized. We are in the process of developing monoclonal antibodies specific for each gB so that this aspect may be studied. One advantage of an HVT recombinant with two gBs is that it might induce a
broader immune response than conventional HVT. Moreover, the recombinant might be less susceptible to neutralization by HVT maternal antibodies than is conventional HVT, a problem commonly encountered in commercial flocks.

We have obtained evidence that both the HVT TK and MDV gB promoters participated in transcription in cells infected with recombinant virus. The presence of additional RNA transcripts in CEF infected with recombinant virus could be due to the failure of recognition of transcription termination signals in MDV gB or to structural changes introduced by the oligonucleotide. By analogy with the HSV model, the TK gene of HVT is expected to be transcribed as an early gene and MDV gB as a late gene. It is therefore possible that MDV gB is transcribed for a longer period and is more abundant in cells infected with the recombinant virus than in MDV-infected cells. This could be an advantage in developing a strong immune response to MDV gB. However, in our recombinant, the presence of translation stop codons in the synthetic adaptor flanking the MDV gB gene would probably preclude the translation of RNA derived from the TK promoter.

The recombinant virus was genetically stable as shown by Southern blot analysis of viral DNA after two or three passages in chickens (not shown) and was non-pathogenic. Susceptible chickens inoculated with 5000 p.f.u. of recombinant virus did not develop clinical disease or gross or microscopic Marek’s disease lesions upon examination of visceral organs and peripheral nerves at post-mortem. This indicates that the recombinant is safe. It is interesting that replacement of the gB gene of the pathogenic ANG strain of HSV-1 by the corresponding gene from the non-pathogenic KOS strain of HSV-1 has been reported to result in a change in plaque morphology and the loss of neuropathogenicity (Weise et al., 1987). Their results and those of Thompson et al. (1986) led Weise et al. (1987) to speculate that gB could be a determinant of neurovirulence of HSV-1. Our results show that the introduction of MDV gB did not change the non-pathogenic nature of HVT.

The TK- recombinant and the spontaneous TK- mutant appear to replicate less well than wild-type HVT in vivo as indicated by the lower titres of HVT antibodies induced by the TK- viruses (Table 1). Work is in progress to compare the efficacy of the recombinant and TK- mutant to wild-type HVT in protection assays. An investigation of the effect of the route of inoculation of our HVT recombinant on virus replication and on protective immunity in different lines of chickens is also warranted. Our results suggest that HVT has potential as a vector and that it would be worthwhile to investigate the expression of antigens from other avian pathogens in HVT.

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References


HVT recombinant


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