Structure and properties of a herpesvirus of turkeys recombinant in which US1, US10 and SORF3 genes have been replaced by a lacZ expression cassette

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In the process of generating an insertional mutant of herpesvirus of turkeys (HVT) expressing lacZ at the protein kinase (PK) locus, we isolated a recombinant which contained an intact PK gene but the short unique regions US1, US10 and SORF3 had been deleted and replaced by the lacZ cassette. Moreover, the virus contained duplicate copies of gD, gI and gE in an opposite orientation flanking lacZ, US2 and PK which were contiguous. These results are of interest in relation to the flexibility of the short unique segment (US) and of the inverted repeats flanking US of the alphaherpesviruses. The recombinant expressed β-galactosidase and was genetically stable in vitro and in vivo. Chickens inoculated with the virus developed antibodies to HVT antigens and to β-galactosidase but the replication of the recombinant in vivo was impaired in comparison to parental HVT as shown by a reduction in the proportion of infected lymphocytes.

Herpesvirus of turkeys (HVT) is serologically related to Marek's disease virus (MDV) and has been used extensively as a vaccine against Marek's disease (Witter et al., 1970). Recent studies have shown that the short unique segment (US) regions of MDV and HVT encode proteins homologous to herpes simplex virus (HSV) US1, US2, US3, US6, US7, US8 and US10 but that one protein (SORF3) has no counterpart in mammalian herpesviruses (Brunovskis & Velicer, 1995; Sakaguchi et al., 1992; Zelnik et al., 1993). In addition, the US of MDV encodes three unique proteins (SORF1, SORF2 and SORF4) (Brunovskis & Velicer, 1995). It has also been reported that US10 and thymidine kinase (TK) of HVT are non-essential genes (Morgan et al., 1992; Ross et al., 1993) but it is not known whether other genes mapping in US are also dispensable. We have demonstrated in this study that US1, US10 and SORF3 of HVT are not essential for virus replication.

A 3-7 kbp EcoRV–EcoRI (EE1) fragment of HVT DNA, containing the protein kinase (PK) gene and adjacent sequences (Zelnik et al., 1993) was subcloned into SmaI- and EcoRI-digested pUC18. The pUC18-EE1 plasmid obtained was digested with SmaI and XhoI to generate a linearized plasmid having a deletion in PK into which a lacZ expression cassette could be inserted. The plasmid pCMVβ (Clontech), which contains the lacZ gene of Escherichia coli under the control of the cytomegalovirus (CMV) immediate early promoter, was first digested with EcoRI and the ends were repaired using Klenow enzyme. The plasmid was then digested with SalI and the 4.5 kbp fragment ligated into the XhoI/SmaI-digested pUC18-EE1 plasmid to generate the transfer plasmid pEE1dPK-B1 (Fig. 1).

Monolayers of chick embryo fibroblasts (CEF) were transfected with a mixture of 2-5 μg of the transfer plasmid linearized by digestion with HindIII and 1 μg of purified infectious HVT DNA using lipofectin as described previously (Ross et al., 1993). Cultures were treated with X-Gal (0.2 mg/ml) when plaques appeared, usually after 4–5 days. Blue plaques (foci of rounded cells) were picked, cultured in CEF and plaque-purified three times. One stock of recombinant virus which produced 100% blue plaques (HVTdUS) was used throughout in this study. Its replication in CEF was not significantly different from the parental HVT (wt-HVT) and titres of cell-associated virus approaching 5-5 x 10^6 p.f.u./ml (0-1 p.f.u./cell) were readily obtained after 5 days.

Analysis of the structure of the recombinant by Southern blotting confirmed that the lacZ expression

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cassette was integrated into Us. This is clear from the hybridization results obtained with probe XS1 (which spans part of the 3’ end of PK and the 5’ end of gD) (Fig. 1d), which showed that the BamHI A (30 kbp) fragment of wt-HVT was replaced by two smaller fragments in the recombinant and that the 7.3 kbp EcoRI fragment of wt-HVT was enlarged to 10.4 kbp in the recombinant. However, the fact that the XS1 probe hybridized to two BamHI fragments and also to two SmaI fragments in the recombinant indicated that the recombinant contained two copies of the sequence located in different parts of Us. Hybridization to the lacZ probe (Fig. 1d) confirmed that the increase in size of the 7.3 kbp EcoRI fragment of wt-HVT to the 10.4 kbp fragment in the recombinant was due to insertion of the expression cassette (3 kbp fragment derived from pCMVβ). However, the lacZ probe hybridized to the 7.7 kbp SmaI fragment of the recombinant but not to the 4.7 kbp fragment which hybridized to the XS1 probe (Fig. 1d). Since these results indicated that the recombinant had a complex structure, we decided to use PCR analysis to investigate the site of integration of the lacZ cassette in the HVT genome.

Primers used for PCR analysis (Fig. 2a) were designed from the sequence of HVT Us reported previously (Zelnik et al., 1993) and were as follows: PK1, 5’ ATGGAAGTAGATGTTGAGTCTTCG; PK2, 5’ CGATATACACGCATTGCCATACAC; PK3, 5’ TTAGACACTGTCAGAGGGTGTGAT. The M13 Forward primer was 5’ CCCAGTCACGACGTTGTA- AAACG. Primer pairs PK1/PK2 were expected to amplify sequences within the PK gene of wt-HVT but not in the transfer plasmid or in the recombinant whereas primer pairs PK1/M13 Forward were expected to amplify sequences present in the transfer plasmid and the recombinant but not in wt-HVT. The results of the PCR analysis shown in Fig. 2(b–d) confirmed that unexpected events had occurred during recombination. Thus an amplified product of 505 bp was generated by PK1 and PK2 when wt-HVT DNA or recombinant HVT DNA was used as template but not the transfer plasmid
Fig. 2. PCR analysis of DNAs derived from wild-type HVT (wt), recombinant virus (rec), recombinant passaged in chickens (rec-p) and from the transfer plasmid pEE1dPK-B1 (tp). The locations of the primers and the expected sizes of the amplified products in bp (underlined) are shown (a). The primers were PK1 (1), PK2 (2), PK3 (3) and M13 Forward (F) as described in the text. The results of electrophoresis of the PCR products obtained using the indicated primer pair combinations are shown (b–e). L, 1 kb DNA ladder.

(Fig. 2b). PK1 and M13 Forward primers produced a PCR product (1390 bp) only when the transfer plasmid was used as template (Fig. 2c). These results showed that sequences mapping within the PK gene had not been deleted from the recombinant and are consistent with the results of Southern blot analysis (Fig. 1d).

To map the integration site of the lacZ sequences and investigate further the structure of the recombinant, viral DNA obtained from the recombinant was subcloned into pBluescript and some of the cloned fragments were partially sequenced (data in EMBL database). Sequencing the ends of the 10.4 kbp EcoRI fragment (Fig. 1c) confirmed that gI was present at both ends (not shown). Similarly, sequencing the 883 bp EcoRV–NotI fragment (Fig. 1c) revealed that the expression cassette was located in the recombinant virus downstream of the HVT US2, its 5' end being adjacent to the 3' end of US2 (not shown). The results indicated further that 510 bp of the CMV promoter present originally in the transfer plasmid were missing in the recombinant. Interestingly, the remaining 315 bp of the CMV promoter was still functional and could drive expression of the lacZ gene efficiently in vitro and in vivo. Sequencing 343 bp of the 4.6 kbp NotI/SmaI subclone mapping at the 3' end of the lacZ gene cassette revealed the presence of lacZ sequences followed by sequences downstream of the
Fig. 3. Mechanism proposed for the generation of the recombinant. (a) Initial integration of transfer plasmid into wt-HVT by double crossing-over recombination as expected. (b) Non-homologous recombination between (a) and wt-HVT in the region spanning the CMV immediate early enhancer and the 5' end of PK. (c) Stable recombinant obtained lacking the 5' PK and part of the CMV immediate early enhancer sequence present originally in the transfer plasmid.

**Short communication**

3'PK CMV 5'PK

\[ m \]

\[ \]

\[ V \]

\[ gE* \]

1 10 SORF3 2 PK gD gI gE

lacZ

\[ i~ \]

2 PK gD gI gE

CMV

XhoI site at the 3' end of the PK gene. These results (not shown) demonstrate that the junction between the 3' end of the lacZ expression cassette and the 3' end of the PK gene was preserved in the recombinant and was as expected from the structure of the transfer plasmid. The sequence of the ends of the 4.5 kbp EcoRI–BglII fragment and of the whole of the 880 bp EcoRV–BglII fragment (Fig. 1c) were also determined (not shown) and confirmed the structure of the recombinant. Taken as a whole, our data suggest the following: (1) the lacZ expression cassette was inserted in the HVT genome downstream of the US20 ORF and oriented towards the inverted short repeat (IR\(_s\)) of HVT (i.e. in the opposite orientation to that expected) leaving the HVT PK intact; (2) the lacZ, US2 and PK cluster was flanked by duplicate copies of gD, gI and gE in opposite orientation, resulting in an enlargement of the inverted repeats [short terminal repeat (TR\(_s\)) and IR\(_s\)] and a reduction in the size of US; (3) US1, US10 and SORF3 had been deleted in the recombinant.

The results of Southern blot hybridization using the ES probe which spans US1, US10 and SORF3 confirmed that these genes were absent from the recombinant (Fig. 1d) and showed further that the recombinant was not contaminated with wt-HVT.

Further support for the structure of the recombinant proposed above was obtained by PCR analysis using PK2 and M13 Forward primers. The results (Fig. 2d) showed that a 2125 bp fragment was amplified only when recombinant DNA was the template and are consistent with the presence of an intact PK gene and of lacZ in opposite orientation in the recombinant. Similarly, a large fragment (5813 bp) was amplified using a combination of PK2 and PK3 primers only when the recombinant DNA was used as template (Fig. 2e).

To examine the replication of the recombinant in vivo, 1-day-old Rhode Island Red (HPRSRIR) chicks were inoculated intramuscularly with 5000 p.f.u. and the proportion of circulating lymphocytes scoring as infectious centres was determined 6 days after infection. The results showed that recombinant virus could be reisolated from 5/6 chickens (mean of 12 p.f.u./10\(^6\) lymphocytes) whereas wt-HVT could be re-isolated from 6/6 chickens (mean of 224 p.f.u./10\(^6\) lymphocytes). It was also demonstrated using an ELISA (Ross *et al.*, 1993) that the chickens inoculated with the recombinant developed antibodies to HVT antigens and to β-galactosidase (not shown). The virus recovered from infected chickens was genetically stable as shown by Southern blotting and PCR analysis (Figs 1 and 2).

The work reported here has shown that US1, US10 and SORF3 of HVT are not essential for virus replication in vitro and in vivo. An unexpected finding in this study is the observation that the PK gene originally targeted for insertion of lacZ was intact and that the lacZ cassette had been integrated in the opposite orientation to that expected and was flanked at its 3' end by copies of gD, gI and gE in opposite orientation to the cluster of these genes present in prototype HVT. At present we are unable to explain the events that generated this recombinant. It is not known whether HVT PK is required for virus replication and whether attempts to generate PK deletion mutants might favour non-homologous recombination. However, this is unlikely since there is evidence that the PK gene of MDV is non-essential (Sakaguchi *et al.*, 1993). PCR analysis of virus produced from individual plaques after the second and subsequent rounds of screening suggested that the recombinant had been generated at an early stage in the selection process. We speculate that double crossing-over recombination between the transfer plasmid and wt-HVT DNA might have occurred as expected during the initial transfection but that subsequently, non-homologous recombination between the recombinant and intact wt-HVT having Us in opposite orientation occurred (Fig. 3). The loss of approximately 480 bp from the potential enhancer region
of the CMV immediate early gene and of sequences at the 5' end of PK adjacent to the CMV enhancer region (present originally in the transfer plasmid) suggests that this region might have been a 'hot spot' where non-homologous recombination occurred. Whatever the mechanism of recombination might be, the genome structure of the recombinant is of interest since it appears to have a much reduced Us and enlarged IRs and TRs. Similar mechanisms could have operated during evolution to produce herpesviruses such as varicella-zoster virus which differs from herpes simplex virus in the location of some homologous genes and in the relative sizes of Us and of IRs and TRs as postulated by Davison & McGeoch (1986).

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References


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