The location and nucleotide sequence of the thymidine kinase gene of bovine herpesvirus type 1.2

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On the basis of their restriction endonuclease digestion patterns, four Australian bovine herpesvirus type 1 (BHV-1) isolates were classified as belonging to the BHV-1.2a subtypes. The thymidine kinase (TK) genes of all four BHV-1.2a isolates were located on a 3.5 kb SalI restriction fragment. This is in contrast to North American and European BHV-1.1 isolates whose TK genes are contained on a 2-6 to 2.8 kb SalI fragment. The restriction fragments containing the TK genes were cloned into phagemid vectors and their sequences determined using the dideoxynucleotide chain termination method. The BHV-1.2a isolates possessed identical TK gene sequences, which differed from previously published TK sequences for the LA and 6660 BHV-1.1 strains. In addition to five single base alterations, there were six separate base insertions which resulted in two major frameshifts which spanned an area of 72 amino acids or 20% of the expressed TK gene product. The predicted amino acid sequence exhibited a higher degree of similarity to other herpesvirus TKs, suggesting that previously published TK gene sequences may have been incorrect. The present nucleotide sequence and corresponding amino acid composition reinforces previous observations concerning regions of herpesvirus TK amino acid conservation and should assist in future studies into the evolution and functional domains of herpesvirus TKs.

Introduction

Bovine herpesvirus type 1 (BHV-1), the aetiological agent of infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis (IPV), is a globally disseminated virus of cattle. The virus, a member of the virus subfamily Alphaherpesvirinae, is responsible for severe respiratory, reproductive, neonatal, dermal and neurological disease in cattle. The virus can be rapidly spread among immunologically susceptible animals and is of greatest economic concern in intensive livestock situations such as dairy herds and beef feedlots. A serological survey conducted in Australia in 1967 demonstrated that 74% of Australian beef cattle herds and 30% of cattle had serological evidence of recent or past infection with BHV-1 (St. George et al., 1967).

Restriction endonuclease (RE) profiles and clinical manifestations have been used to classify BHV-1 strains further into one of five subtypes, types 1.1 (IBR-like), 1.2a and 1.2b (IPV-like) and 1.3a and 1.3b (encephalitic forms) (Metzler et al., 1985, 1986; Engels et al., 1986/87). Restriction endonuclease maps for several strains have been prepared (Mayfield et al., 1983; Engels et al., 1986/87). As is the case in many other herpesviruses, the BHV-1 genome encodes a viral thymidine kinase (TK) which does not appear to be essential for viral replication in vitro or in vivo (Weinmaster et al., 1982; Kit, 1985; Mittal & Field, 1989). It does, however, appear to play an important role in pathogenicity. Several herpesviruses, including herpes simplex type 1 (HSV-1), BHV-1 and pseudorabies virus (PRV) strains with defective TK genes have demonstrated a lowered pathogenicity in vivo (Field & Wildy, 1978; Kit et al., 1985a, b; Kit & Kit, 1986). The ability to attenuate otherwise virulent viruses by chemically or genetically altering the functioning of their viral TK genes has been an important avenue of vaccine development (Kit & Qavi, 1983; Kit et al., 1985a; Kit & Kit, 1986). The nucleotide sequences have
been published for the TK genes of equine herpesvirus type 1 (EHV) (Robertson & Whalley, 1988), HSV-1 (Wagner et al., 1981), HSV-2 (Swain & Galloway, 1983), varicella-zoster virus (VZV; Davison & Scott, 1986), marmoset herpesvirus (MarHV; Otsuka & Kit, 1984), Epstein–Barr virus (EBV; Baer et al., 1984), herpesvirus saimiri (Honess et al., 1989), Marek’s disease virus (MDV) and the herpesvirus of turkeys (HVT) (Scott et al., 1989).

The locations and nucleotide sequences of the TK gene of both North American and European BHV-1.1 strains have also been reported (Kit & Kit, 1986; Bello et al., 1987; Mittal & Field, 1989). Comparison of the TK nucleotide sequence of the BHV-1.1 LA and 6660 strains showed significant sequence variation between the two viruses (Mittal & Field, 1989).

The present study was undertaken to extend the sequence data available on the TK gene of different BHV-1 subtypes, to determine the degree of sequence variation occurring within a given subtype and to generate sequence information applicable to the development of a BHV-1 vaccine suitable for use in Australia. For initial analysis, four Australian BHV-1.2a virus isolates were chosen. All four BHV-1.2a isolates were distinguishable from one another on the basis of PstI and XhoI RE profiles.

**Methods**

**Cells.** A primary culture of bovine testis (BTs) cells, established from explant tissue of a BHV-1-seronegative calf, was employed for virus isolation and replication. Cells were propagated in Eagle’s MEM supplemented with 10% heat-inactivated foetal bovine serum, 100 unit/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone and 25 mM-sodium bicarbonate. Cells were maintained at 37 °C in a closed environment.

**Virus.** All viruses, with the exception of the BHV-1 V155 strain, were isolated and maintained at this laboratory. The V155 strain was generously provided by Dr T. St. George (CSIRO Division of Tropical Animal Production, Indooroopilly, Queensland, Australia) at passage level 18. The virus was a vaginal isolate obtained from an animal suffering from vaginitis and was isolated in Victoria in 1964 as previously described (Snowdon, 1964). The 88/86602, Q3932 and Q5710 BHV-1 isolates were recovered from nasal swabs of animals suffering from respiratory tract infection. The three respiratory isolates were obtained from separate disease outbreaks occurring in geographically distant locations of southeast and central Queensland in 1988 and 1986.

**Virus purification and DNA extraction.** BTs cells were grown to confluence in 850 cm² roller flasks. Flasks were decanted and inoculated with the respective virus isolate at an m.o.i. of 1 0 p.f.u./cell. Viral DNA was liberated and purified from infected cells 48 h post-infection by a modification of the method of Dorman et al. (1985). Recovered viral DNA was quantified on a TKO mini-fluorimeter (Hoefer Scientific Instruments) and adjusted to a concentration of 0.5 mg/ml in sterile water.

**Restriction endonuclease analysis.** Viral DNA was digested with selected restriction endonucleases as recommended by the manufacturer (Boehringer Mannheim). Digested DNA was separated by overnight electrophoresis at 20 V in 0.6% agarose gels, employing Tris–Borate–EDTA electrophoresis buffer (89 mm-Tris, 89 mm-boric acid, 2 mm-EDTA pH 8.0).

**Southern transfer and hybridization.** Electrophoretically separated DNA fragments were transferred onto Hybond-N nylon membranes (Amersham) by a modification of the method of Southern (1975). Transferred DNA was fixed to the nylon membrane by u.v. cross-linking. Fragments harbouring the respective BHV-1 TK genes were located by hybridization with a 20-mer oligonucleotide herpesvirus consensus probe. The sequence for this probe (5' GAGCCCAGACCGTGCACTGAGG 3') was kindly provided by Dr J. M. Whalley (Macquarie University, Sydney, Australia) and was synthesized on a Milligen 7500 DNA synthesizer. The oligonucleotide was end-labelled with [γ-32P]ATP using a 5'-terminal kinase labelling kit as instructed by the manufacturer (BRESATEC). Hybridization was performed in Hybri-Ease chambers (Hoefer Scientific Instruments) at 64 °C under conditions of high stringency (Maniatis et al., 1982). Autoradiography was carried out at −70 °C for 3 to 16 h using Agfa-Gevaert Curix-RPI X-ray film and intensifying screens. The M, of RE fragments carrying the TK gene was determined by comparing the resultant autoradiographs to u.v.-photographed, ethidium bromide-stained gels containing BstEI-digested lambda lambda size standards.

**Cloning of BHV-1 TK gene.** The plasmid vector pUC8, phagemid vectors pBluescript KS+ (Stratagene Cloning Systems) and pTZ18U (Pharmacia LKB Biotechnology) and the epicurian, K-12 derived Escherichia coli host strain XL1-Blue (Stratagene Cloning Systems) were utilized for the cloning of the BHV-1 TK genes. Viral DNA from each BHV-1 strain was digested with either HindIII or SalI and electrophoretically separated on a low melting temperature agarose gel (Pharmacia LKB Biotechnology). DNA fragments of M, corresponding to those detected in the Southern blots were excised from the gel and ligated into either pUC8, pBluescript KS+ or pTZ18U as previously described (Burns & Beacham, 1983). The rubidium chloride–calcium chloride method of Kushner (1978) was employed for transformation. Recombinant E. coli clones carrying the respective TK genes were identified by slot blotting of Birnboim & Doly (1979) minipreparations (Bio-Rad) and hybridization with the labelled TK consensus probe as described above. The TK genes of all BHV-1 strains used in this study were cloned in both orientations.

**Construction of nested deletions.** The cloned TK gene of BHV-1 strain 88/86602 was digested with SalI and PstI, excised and subcloned into the SalI- and PstI-digested phagemid vector pBluescript KS+ as described above. A set of double-stranded unidirectional nested deletions was constructed using a commercial kit (Pharmacia LKB Biotechnology) in accordance with the manufacturer’s instructions. The recombinant phagemid was linearized by digestion with KpnI and SalI to allow unidirectional digestion of the TK gene with exonuclease III.

**Preparation of ssDNA from phagemid clones.** Single-stranded DNA was obtained from clones essentially as described by the supplier of the M13KO7 helper phage (Pharmacia LKB Biotechnology). Recovered DNA pellets were resuspended in sterile water and quantified by electrophoretic analysis.

**DNA sequencing.** BHV-1 TK genes were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1977). Single-stranded DNA was used as template in all reactions. In an effort to minimize band compressions, all sequencing was performed in a two-stage labelling then termination reaction using a cloned T7 DNA
polymerase (Tabor & Richardson, 1987) and 7-deaza-dGTP (Pharmacia LKB Biotechnology).

The 17-base universal primer (Pharmacia LKB Biotechnology) was employed for sequencing the nested deletion subclones of Q3932. The nucleotide sequence of Q3932 was then used to design a set of 12 oligonucleotide primers for sequencing the three remaining BHV-1 TK genes. Of these oligonucleotide primers, six were used for sequencing the positive strand and the six remaining primers were used for sequencing the complementary strand. Primers were located 150 to 200 bases apart, allowing for an overlap of between 50 and 100 bases. All TK genes were sequenced in both orientations at least twice. All cloned genes were sequenced in both orientations at least once under strong denaturing conditions (20% formamide at 55 to 60 °C).

Computer analysis of DNA sequences. The nucleotide sequences were compiled with the assistance of a sonic digitizer (International Biotechnologies). Sequence comparison and amino acid predictions were performed using IBI Pustell DNA Sequence Analysis software (IBI) and DNASIS sequence analysis software (Pharmacia).

Results

Restriction endonuclease analysis

The BamHI, EcoRI, HpaI and HindIII restriction endonuclease profiles of BHV-1 isolates V155, 88/86602, Q3932 and Q5710 were consistent with those reported earlier for other Australian vaginal and respiratory BHV-1 virus isolates (Brake & Studdert, 1985) and supports their classification as BHV-1.2a subtypes. By comparing the RE profiles produced by digestion with either PstI or XhoI, it was possible to differentiate all four individual BHV-1 isolates (Fig. 1). The areas of significant RE fragment variation occurred between fragments of length 1-93 kb and 7-24 kb.

The TK gene location of BHV-1.2a subtypes

Results of hybridization experiments performed with the radiolabelled TK consensus probe indicated that the TK gene of the Q3932 BHV-1.2a isolate was located on the following restriction fragments: 21.2 kb HindIII, 6-7 kb KpnI, 3-6 kb PstI, 3-5 kb Sall, 2-2 kb XhoI and 0-965 kb BamHI. The BHV-1.2a isolates 88/86602, Q5710 and V155 yielded Sall and PstI hybridization results similar to that obtained with Q3932. Although the TK consensus probe hybridized specifically with a 2-2 kb XhoI fragment of V155, the probe hybridized to a 1-26 kb XhoI RE fragment from both the 88/86602 and Q5710 isolates.

Nucleotide sequence analysis of BHV-1.2a TK gene

A total of 18 clones, each representing progressive unidirectional deletions from the 5' end of the TK gene of BHV-1.2a strain Q3932, were selected from approximately 3 × 10^3 recombinants generated by the nested deletion protocol. Each nested deletion subclone was selected on the basis of size by electrophoretic separation on a 0-8% agarose gel and represented a progressive deletion of between 200 and 250 bp. The nucleotide sequence of each clone was determined and the sequence aligned on the basis of sequence overlap between successive clones. The nucleotide sequence of approximately 2-8 kb of the 3-5 kb Sall fragment harbouring the Q3932 TK gene was determined (complete data not shown). This area spans the region between the Sall RE recognition sites of the TK gene sequence of the BHV-1.1 LA strain (Kit & Kit, 1986).

The nucleotide sequence and predicted amino acid composition of the BHV-1.2a Q3932 TK gene and flanking 3' and 5' regions is presented in Fig. 2. This region corresponds to the sequence of the BHV-1.1 6660 TK gene published by Mittal & Field (1989) and allows for direct comparison of BHV-1.1 and BHV-1.2a TK protein coding regions. No nucleotide sequence variation was observed between the three BHV-1.2a viruses examined. Comparison of the sequence of Q3932 with that published for the BHV-1.1 6660 virus revealed five base alterations and six base insertions (Fig. 2). The first base alteration occurs at nucleotide -144, a position outside of the protein coding region, and involves an A→G substitution. The remaining base alterations all occur within the proposed protein coding region and occur at positions 164 (G→T substitution), 600 (G→A; Fig. 3), 675 (A→G; Fig. 5) and 835 (A→G).
In addition to the base alterations indicated above, six bases not present in the sequence of Mittal & Field (1989) were revealed (Fig. 4 and 5). These included the insertion of a guanosine at positions 235, 351, 762 and 777, as well as the insertion of a cytosine at positions 239 and 674. This resulted in the insertion of two additional amino acids.
Thymidine kinase gene sequence of BHV-1.2

Fig. 5. Areas of the BHV-1.2a Q3932 TK gene where a second limited frameshift (encompassing 40 amino acids) was created by the insertion of three bases. (a) Sequences were obtained for both strands. The frameshift was initiated by the insertion of a C residue at position 674 (solid arrow). The substitution of a G residue for an A residue (open arrow) occurs at position 675. (b) A G residue has been inserted at positions 762 and 777. The base substitutions and insertion and the proposed frameshift are based on the published BHV-1.1 TK sequence of Mittal & Field (1989).

Discussion

In this study we have compared RE profiles and nucleotide sequences of the TK genes of four Australian BHV-1.2a isolates. Although the nucleotide sequences of the TK genes of all four BHV-1.2a isolates were identical, significant variation was observed when they were compared to BHV-1.1 TK gene sequences.

Although RE profiles of the four BHV-1 isolates studied indicated that all were of the BHV-1.2a subtype, each virus had distinctive PstI and XhoI RE profiles. As all were from chronologically and geographically distinct disease outbreaks, it is reasonable to assume that they are representative of the predominant BHV-1 subtype associated with disease outbreaks in Australia. The discovery of distinctive PstI RE profiles in BHV-1 isolates is consistent with the recent observations of Whetstone et al. (1989). RE profiles obtained with XhoI suggest that this enzyme may also be useful in identifying genome variation within a given subtype.

The location of the TK gene on 2-6 to 2.8 kb SalI RE fragments has been reported for the LA BHV-1.1 strain, the Cooper I BHV-1.1 strain (Kit & Kit, 1986; Bello et al., 1987) and the 6660 BHV-1.1 strain (Mittal & Field, 1989). In contrast, the TK gene of the four BHV-1.2a isolates used in this study was located on a 3.5 kb SalI restriction fragment. Despite this variation, the location of the BHV-1.2a TK gene on the BamHI, KpnI, PstI and HindIII RE fragments is similar to that described for the BHV-1.1 LA strain (Kit & Kit, 1986). Similarly, the location of the TK gene of BHV-1.2a V155 and Q3932 isolates on a 2.2 kb XhoI fragment is consistent with that described for the BHV-1.1 LA strain (Kit & Kit, 1986). The finding that the TK genes of both 88/86602 and Q5710 were located on 1.26 kb XhoI RE fragments suggests that intersubtype sequence variation occurs within the TK flanking regions of the BHV-1.2a genome.

The BHV-1.2a genome map of strain 227 prepared by Engels et al. (1986/87) allows the TK gene to be positioned between map units 0.471 and 0.490 of the Q3932 BHV-1.2a isolate and that reported for the BHV-1.1 LA strain (Kit & Kit, 1986). Similarly, the location of the TK gene of BHV-1.2a V155 and Q3932 isolates on a 2.2 kb XhoI fragment is consistent with that described for the BHV-1.1 LA strain (Kit & Kit, 1986). The finding that the TK genes of both 88/86602 and Q5710 were located on 1.26 kb XhoI RE fragments suggests that intersubtype sequence variation occurs within the TK flanking regions of the BHV-1.2a genome.

Several base alterations, insertions and deletions have been identified between the nucleotide sequence of the BHV-1.2a Q3932 isolate and that reported for the BHV-1.1 LA strain. The greatest difference occurred within a region of the herpesvirus TK sequence which appears to be highly conserved. This area has been designated region III (Mittal & Field, 1989) and has been linked to the substrate binding specificity of herpesvirus TK enzymes (Darby et al., 1986). Within this region, the sequence of the 6660 BHV-1.1 strain was found to vary from that of the BHV-1.1 LA
Comparison of the amino acid sequences of the BHV-1.2 TK's reported here with that of other herpesvirus TKs once again introduces a strongly conserved motif not found in the predicted amino acid sequence of either of the BHV-1.1 TK gene sequences. As indicated in Fig. 6, the motif WRXXW can be found in the TKs of all five alphaherpesviruses thus far reported. Indeed, the amino acid sequence WRDGW can be found in both BHV-1.2a and EHV.

The TK sequence conservation displayed among the four BHV-1.2a isolates employed here might suggest that mutational events leading to TK sequence variation among the members of the BHV-1.2a subtype is a rare occurrence. Of the four BHV-1.2a isolates examined, two (V155 and 88/86602) were isolated 27 years apart from geographically distant areas. This apparently strict conservation contrasts with the TK sequence variability reported between BHV-1.1 strains (Kit & Kit, 1986; Mittal & Field, 1989).

The most likely explanation to account for these observed sequencing variations is that the previously reported BHV-1.1 TK sequences were incorrectly determined. The six additional bases reported here all occur in regions where base discrepancies have been reported between the LA and 6660 BHV-1.1 strains, and all occur in regions of high GC content. As neither of the previous papers reported the use of techniques which might resolve base compressions or strongly secondary structure, such as sequencing at elevated temperatures or the use of 7-deaza-GTP, it is possible that the additional bases described here may have been obscured. The strong sequence conservation displayed between the chronologically and geographically distinct BHV-1.2 isolates employed in this study, and the presence of several conserved amino acid motifs not found in the BHV-1.1 TKs, both support this view. However, the absence of BHV-1.1 strains in Australia and the quarantine restrictions placed on the importation of such products into this country prevents side by side comparison to substantiate these assertions.
Similar TK sequence analysis of other BHV-1 subtypes may provide further insight into the TK sequence stability of BHV-1 viruses, which in turn could increase our understanding of the functional domains of other herpesvirus TK genes. Efforts are currently in progress to clone and sequence the TK gene of the BHV-1.3a N569 encephalitogenic strain.

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References


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