LOSS OF THYMIDINE KINASE ACTIVITY DUE TO A BASE DELETION IN A CANDIDATE VACCINE STRAIN OF BOVINE HERPESVIRUS 2

R. GAUT, J.T. MAY*

School of Microbiology, La Trobe University, Bundoora, Victoria, 3083 Australia

Received October 31, 1996; revised January 30, 1997

Summary. – The nucleotide (nt) sequence of the thymidine kinase (TK) gene (a 918 nt long coding region) of two TK-deficient (TK$^-$) strains of bovine herpesvirus 2 (BHV-2) was determined. The candidate vaccine strain C290BU5, which was no longer able to cause disease, was found to have an A deletion after nt 887 of the TK gene with a predicted change of His 296 to Pro, altering the last 10 amino acids (aa) and extending the gene by another 34 aa. The strain which still caused disease, C290BU3, had a T insertion after nt 16 causing a predicted chain termination after only 16 aa.

Key words: bovine herpesvirus 2; candidate vaccine strain; thymidine kinase

BHV-2 (bovine mammillitis virus) causes an ulcerative disease of teats and udder or can cause an unusual generalized infection. No commercial vaccine exists against this disease which is a veterinary problem in many countries and may reduce milk yields (Smee and Leonhard, 1994). As the virus contains a TK gene we have attempted to produce a BHV-2 TK$^-$ live attenuated vaccine (Orders *et al.*, 1995). TK$^-$ herpesvirus vaccines have been successful against other animal herpesviruses although the loss of TK activity in these vaccines does not necessarily mean that they are avirulent for all animal species, e.g. TK$^-$ pseudorabies virus can still cause fatal infections in rabbits (Shibata *et al.*, 1991).

Two BHV-2 TK$^-$ strains were isolated after BHV-2 strain CSIRO290 (C290) was grown in bromodeoxyuridine-resistant bovine kidney cells in the presence of 100 μg/ml of bromodeoxyuridine (Orders *et al.*, 1995; Handley *et al.*, 1996). One isolate designated BHV-2 C29OBU5 was TK$^-$ (3% of wild-type activity) and produced low levels of DNA during infection of bovine cells. This strain did not cause skin disease in guinea pigs and was used to vaccinate these animals against wild-type BHV-2 (Orders *et al.*, 1995). However, another TK$^-$ isolate designated BHV-2 C29OBU3 while having similar levels of TK activity and lower DNA production, still caused skin infections in guinea pigs similarly to wild-type virus with normal TK activity. Thus the lack of TK activity did not always correspond to the inability of BHV-2 to cause disease in guinea pigs. Similar findings have also been reported for TK$^-$ herpes simplex viruses (HSV) in mice (Isumi and Stevens 1988). A single base change in the TK gene reduced the TK activity to 1% of that of the wild-type but had no effect on the neurovirulence of this HSV mutant (Tanka *et al.*, 1993).

To determine the precise changes in the BHV-2 TK gene which could account for the change in virulence, the TK gene of both BHV-2 TK$^-$ viruses was sequenced.

Virus DNA was isolated by the NaI method described previously (May *et al.*, 1990; Sheppard and May, 1989), but only low levels of virus DNA were isolated from both TK$^-$ strains (Orders *et al.*, 1995). In the case of C29OBU3 TK gene, the isolated virus DNA was digested with *Sal*I and cloned into the plasmid pUC18

*Corresponding author.

Abbreviations: aa = amino acid; BHV-2 = bovine herpesvirus 2; HSV = herpes simplex virus; nt = nucleotide; TK = thymidine kinase; TK$^-$ = TK-deficient

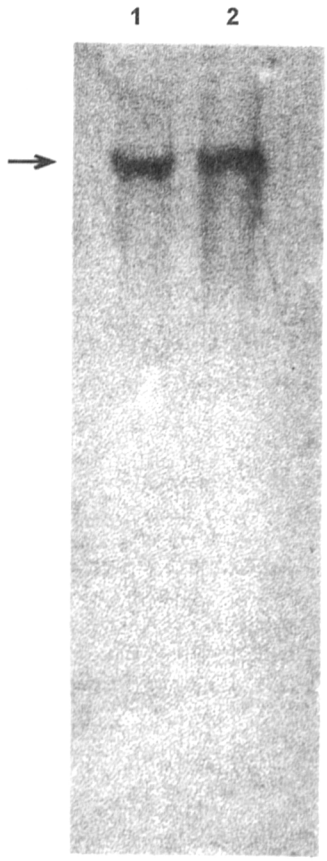

Fig. 1
Southern blot hybridization *Hind*III-digested BHV-2 TK⁻ DNA
Digoxin-labelled 0.5 kb *Pst*I-*Sph*I region of BHV-2 TK gene was used as the probe. *Hind*III-digested C290BU5 DNA (lane 1) and C290BU3 DNA (lane 2) were electrophoresed, blotted to nitrocellulose and hybridized with the probe. Labelling of the probe, hybridization conditions and detection were performed according to the manufacturer of the digoxin labelling kit (Boehringer-Mannhein). The arrow indicates the position of the 11.7 kb *Hind*III fragment.

as described previously (Sheppard and May, 1989). The gene was located within a *Sal*I 2.6 kb region and had an identical restriction endonuclease profile with that reported previously (Sheppard and May, 1989). However, with the candidate vaccine strain C29OBU5, the TK gene was difficult to isolate after *Sal*I digestion of the same amount of virus DNA with even ten-fold more restriction endonuclease than previously used. As both virus strains have initially been isolated after passage in cells transfected with a linearized plasmid containing a modified TK gene (Orders *et al.*, 1995; Handley *et al.*, 1996), it was possible that the virus TK gene of strain C29OBU5 could contain an additional gene insert at the *Pst*I site of the TK gene through homologous recombination between the replicating virus DNA and plasmid DNA.

Hybridization studies using the 0.5 kb *Pst*I-*Sph*I region of the TK gene as a probe (Sheppard *et al.*, 1989) showed that 70% of the hybridization occurred with the 8.0 kb *Sal*I DNA fragment and minor hybridizations with the 6.3 kb, 4.3 kb and 2.6 kb *Sal*I DNA fragments. However, the hybridization studies also indicated that the TK gene was still located in the 11.7 kb *Hind*III region of DNA of the two TK⁻ strains (Fig. 1). This suggested that no large DNA insertion had occurred within the TK gene of C29OBU5 strain. Primary digestion with *Hind*III followed by a *Sal*I digestion of the virus DNA resulted in exclusive hybridization with only 2.6 kb DNA fragment. The TK gene of this strain was cloned into the plasmid pUC18 after double digestion with *Hind*III and *Sal*I and found to be present in the 2.6 kb *Sal*I region. The TK gene also had an identical restriction profile to the wild type BHV-2. Thus the *Hind*III region containing the TK gene (consisting of a sequence of the 3.7, 2.6 and 1.7 kb *Sal*I fragments) of C29OBU5 DNA was prone to partial *Sal*I digestion. Various combinations of these three different *Sal*I DNA fragments generated by partial *Sal*I digestion (except for the two fragments outside the 2.6 kb TK region) would account for the sizes of the TK gene detected in the previous hybridization studies. A partial digestion would also account for an additional DNA fragment of 6.3 kb seen previously in *Sal*I DNA fragment profiles of C290BU5 DNA as compared to that of wild-type BHV-2 DNA (Orders *et al.*, 1995). The other partially digested DNA fragments were of a size that would not be distinguished from completely digested *Sal*I fragments using the ethidium bromide staining method (Maragos *et al.*, 1986).

When cloned into the pUC18 plasmid the TK gene (and subclones of the TK gene) was sequenced using the Sequenase 2 Kit (Amersham) using both the M13 universal primer and the M13 reverse primer. The previously published BHV-2 TK gene sequence concerned strain BHM-1 (Sheppard and May, 1989; GeneBank accession number D0053). No TK⁻ strain had any gene or part of a gene insertion within the TK gene (Orders *et al.*, 1995) as determined by sequencing the whole gene. The sequences of TK genes of both TK⁻ strains were identical to the published sequence except for the following changes.

For both TK⁻ strains, C 523 was deleted and a C was inserted after nt 525 thus changing aa 175 from Pro to Arg. However, this alteration would make the aa sequence of the BHV-2 TK identical with 17 other herpesviruses, all of which have an Arg in a similar position (Remond *et al.*, 1995), and may be an error in the original sequencing. Also for both TK⁻ strains, there were changes in the nt sequence, namely a deletion of C 715, and an insertion of a C after nt 713, which preserved Leu 238 but changed aa 239 from Leu to Val. This Val is different from the predominant Phe or the occasional Leu in that position (Remond *et al.*, 1995), and may represent a difference in the BHV-2 strains BHM-1 and C290.

Table 1. Changes in the nucleotide and amino acid sequences of the BHV-2 TK⁻ strains

Position of aa	5	6	7	16	295	296	297	340
BHV-2 wild type	Leu CTG	Arg CGG	Val GTG		Val GTG	His CAC	Arg CGT	
BHV-2 TK strain C290BU3	Leu CTG	**Leu** CTG	**Gly** GGT	**TAA**	GTG	CAC	CGT	
BHV-2 TK strain C290BU5 (candidate vaccine strain)	Leu CTG	Arg CGG	Val GTG		Val GTG	**Pro** CCC	**Val** GTG	**TAA**

Changes are shown in bold letters.

The major changes in the sequences are indicated in Table 1. In the two TK⁻ strains, an insertion of a nt at the amino end of the protein (C290BU3 strain) or a nt deletion at the carboxy end of the protein (C290BU3, candidate vaccine strain) were detected. The candidate vaccine strain had a deletion of A 887 which would allow the gene to be read up to the stop codon TAA at nt 1022-1024 (Sheppard and May, 1989). This would extend the protein by 34 aa. Strain C290BU3 had a T insertion after nt 16 (in the ATP binding region; May *et al.*, 1989; Remond *et al.*, 1995) which would allow a chain termination at nt 48-50 (TAA) and the protein would be truncated after only 16 aa. This early alteration in the TK gene could affect the expression of the UL24 gene which can overlap herpesvirus TK genes (Griffin and Boursnell, 1990) and could account for the particularly low level of virus DNA produced during the growth of this strain, which still is able to cause the disease in guinea pigs.

With the changes in the TK gene of the BHV-2 TK⁻ strains as determined by sequence analysis, whether the predicted non-production of the protein or extension of the protein (in the candidate vaccine strain) account for the change in the ability to cause disease is unknown. The genes involved in the virulence of BHV-2 remain unidentified, unlike those of extensively studied herpesviruses, such as pseudorabies virus (Gielkens and Peters, 1994).

References

Gielkens ALJ, Peters BPH (1994): Function of Aujeszky's disease virus proteins in virus replication and virulence. *Acta Vet. Hung.* **42**, 227–241.

Griffin AM, Boursnell MEG (1990): Analysis of the nucleotide sequence of DNA from the region of the thymidine kinase gene of infectious laryngotracheitis virus: Potential evolutionary relationship between the herpesvirus subfamilies. *J. Gen. Virol.* **71**, 841–850.

Handley A, Puclin J, May JT (1996): Isolation of a thymidine-kinase deficient bovine herpesvirus 2 that still causes disease. *Aust. Vet. J.* **74**, 158–159.

Izumi KM and Stevens JG (1988): Two thymidine kinase deficient herpes simplex viruses exhibit unexpected virulence properties. *Microb. Pathog.* **4**, 145–153.

Maragos C, Shaw R, May JT (1986): Restriction endonuclease cleavage analysis of DNA from two bovine mammillitis viruses isolated in different parts of Australia. *Aust. Vet. J.* **63**, 372–373.

May JT, Awad M, Reum A, Sheppard M (1990): Bovine mammillitis virus thymidine kinase gene. *Acta Virol.* **34**, 188–192.

Orders G, Handley A, May JT (1995): Bovine herpesvirus 2 with low thymidine kinase activity and virulence. *J. Gen. Appl. Microbiol.* **41**, 371–376.

Remond M, Sheldrick P, Lebreton F, Foulon T (1995): Sequence of the canine herpesvirus thymidine kinase gene: taxon-preferred amino acid residues in the alphaherpesviral thymidine kinases. *Virus Res.* **39**, 341–354.

Sheppard M, May JT (1989): Location and characterisation of the bovine herpesvirus type 2 thymidine kinase gene. *J. Gen. Virol.* **70**, 3067–3071.

Shibata I, Inuba Y, Akashi H (1991): Avirulent ts and thymidine kinase-deficient mutant of Aujeszky's disease virus. *J. Vet. Med. Sci.* **4**, 663–670.

Smee DF, Leonhardt JA (1994): Vaccination against bovine herpes infections in guinea pigs. *Intervirology* **37**, 20–24.

Tanaka S, Toh Y, Mori Y (1993): Molecular analysis of a neurovirulent herpes simplex virus type 2 strain with reduced thymidine kinase activity. *Arch. Virol.* **131**, 61–73.