Short Communication

Correspondence
Hanns-Joachim Rziha
achim.rziha@tue.bfav.de

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The genus Parapoxvirus (PPV) of the family Poxviridae includes the species parapoxvirus ovis (Orf virus; ORFV), parapoxvirus bovis 1 (Bovine papular stomatitis virus; BPSV), parapoxvirus bovis 2 (Pseudocowpox virus; PCPV) and Parapoxvirus of red deer in New Zealand (PVDVNZ) (Robinson & Mercer, 1995). BPSV infection usually leads to mild and transient inflammatory lesions on the muzzles of calves, except for lesion manifestations on the udders of lactating cows (Inoshima et al., 2001; Kuroda et al., 1999; Mayr & Büttner, 1990). Due to genetic heterogeneity, molecular classification of PPV remains problematic and still relies on the source of the virus isolate (Büttner & Rziha, 2002; Gilray et al., 1998; Rafii & Burger, 1985; Robinson et al., 1982, 1987). So far, most information on the molecular biology and sequence data has been obtained from the New Zealand ORFV strain NZ2 (Fleming et al., 1993; Haig & Mercer, 1998; Mercer et al., 1996, 1997; Robinson & Lyttle, 1992; Sullivan et al., 1995a, b). Sequence data from PPV other than ORFV are very sparse. A partial sequence of the major envelope gene has been suggested for discrimination between BPSV and ORFV (Inoshima et al., 2001). As in all poxviruses, the ORFV genome is composed of a conserved central region and variable genomic termini containing genes not required for in vitro growth (Fleming et al., 1993; Mercer et al., 1995, 1996; Rziha et al., 2000; Sullivan et al., 1995a). Factors determining species- and strain-specific properties and influencing virulence, pathogenesis and host range of poxviruses are encoded in the terminal parts of the viral genome (Alcamí & Koszinowski, 2000; Alcamí & Smith, 1995; Smith et al., 1997). Potential ORFV virulence genes include an interferon-resistance gene, which is a homologue of the vaccinia virus (VACV) E3L gene (Haig et al., 1998; McCluskey et al., 1998), and a functional dUTPase (Cottone et al., 2002). The right end of the ORFV genome shows greater variability than the left end (Mercer et al., 1995) and is composed of genes that are not found in other poxviruses including a viral interleukin (IL)-10 (Fleming et al., 1997, 2000; Imlach et al., 2002), a factor (GIF) inhibiting granulocyte macrophage colony stimulating factor and interleukin-2 activity (Deane et al., 2000; Haig et al., 1996), and a new member of the vascular endothelial growth factor (VEGF) family (Haig & Mercer, 1998; Lyttle et al., 1994), designated VEGF-E (Meyer et al., 1999). The latter mediates angiogenesis in vitro and in vivo and is therefore thought to be responsible for the induction of proliferative bloody lesions (Meyer et al., 1999; Ogawa et al., 1998; Savory et al., 2000; Wise et al., 1999). Originally, VEGF-E was detected in two independent New Zealand ORFV isolates NZ2 and NZ7, which showed substantial sequence difference (Lyttle et al., 1994) not affecting

Relatedness and heterogeneity at the near-terminal end of the genome of a parapoxvirus bovis 1 strain (B177) compared with parapoxvirus ovis (Orf virus)

H.-J. Rziha,1 B. Bauer,1 K.-H. Adam,1 M. Röttgen,1 R. Cottone,1 M. Henkel,1 C. Dehio2 and M. Büttner1

1Federal Research Centre for Virus Diseases of Animals, Institute of Immunology, Paul-Ehrlich-Straße 28, D-72076 Tübingen, Federal Republic of Germany
2Division of Molecular Microbiology, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland

The present study provides for the first time an extended investigation of individual genes located at the near-terminal right end of the genome of parapoxvirus bovis 1, Bovine papular stomatitis virus (BPSV) strain B177 and Orf virus (ORFV). Comparison of the respective DNA sequences of ORFV strain D1701 (9-9 kbp) and BPSV B177 (7-7 kbp) revealed a very similar organization of closely related genes transcribed in a rightward orientation. The most salient findings of this study were: (i) the absence of the ORFV-specific vascular endothelial growth factor (VEGF-E) gene in the BPSV isolate; (ii) the presence of an interleukin-10 (IL-10) orthologue; and (iii) the detection of three new genes encoding ankryrin-repeat-containing polypeptides. These results not only contribute to potential improvements of future molecular differentiation between the parapoxvirus species, but also shed new light on different pathobiologies among parapoxviruses.

The DNA sequences of D1701 (9979 nucleotides) and of B177 (7702 nucleotides) are deposited in GenBank under accession nos.AY186732 and AY186733, respectively.
functional activity (Meyer et al., 1999; Ogawa et al., 1998; Wise et al., 1999).

Due to a rearrangement of terminal sequences, the highly attenuated ORFV strain D1701 contains two copies of the VEGF-E and IL-10 genes (Cottone et al., 1998; Rziha et al., 1999). Recently, it was suggested that BPSV, PCPV and PVNZ do not harbour an ORFV-like IL-10 gene (Fleming et al., 2000). Data on the presence of the VEGF-E gene in PPV other than ORFV do not exist. Various DNA hybridization experiments have indicated the absence of VEGF-E homologous sequences in the genome of the BPSV isolate B177. This virus isolate was obtained from a typical non-proliferative BPS lesion from a calf muzzle by inoculation of foetal bovine kidney cells. Before plaque purification and isolation of viral DNA, B177 was propagated a maximum of three times in the bovine kidney cell line BK-KL3A (Cottone et al., 1998). Southern blot hybridization using 32P-labelled probes was performed as described by Cottone et al. (1998). The plasmid pVEGF was used as a probe specific for the D1701 VEGF-E gene (Meyer et al., 1999), which shows 90 % nucleotide identity to that of ORFV strain NZ2 (Mercer et al., 2002). The probe specific for the ORFV strain NZ7 was obtained from plasmid pSB161 (generously provided by S. Fleming, University of Otago, New Zealand). Using various stringencies of annealing conditions, neither the probe specific for the D1701-type nor the NZ7-type VEGF-E gene reacted with any DNA restriction fragment from BPSV B177, although under relaxed conditions the NZ7-type-specific probe detected the D1701 counterpart (data not shown). In contrast, genes adjacent to and upstream of D1701 VEGF-E (e.g. F9L or F10L; Fig. 1) could be detected easily with B177 DNA (data not shown). Only very weak hybridization was found with probes covering the D1701 ANK-2 and ANK-3 genes (Fig. 1B).

Missing or weak hybridization signals could result from different G+C content or could be due to different genes located in the genomic termini of different PPV, which might allow adaptation to different hosts (Gassmann et al., 1985; Inoshima et al., 2001; Robinson & Mercer, 1995; Wittek et al., 1980). So far, from ORFV NZ2, contiguous sequences upstream of the F10L gene have not been reported. We cloned and sequenced those parts of the ORFV D1701 and BPSV B177 DNA shown in Fig. 1 essentially as reported by Cottone et al. (1998). DNA sequencing of the cloned 15·6 kbp HindIII fragment D of D1701 was achieved with a transposon-generated system (Template Generation System, Finnzymes Oy) to obtain overlapping DNA subfragments. The sequences of the cloned D1701 fragment HindIII–H' and the B177 PstI fragments cloned in pV4-1, pV8-94 and pV8-152 (Fig. 1) were obtained by primer walking. The DNA sequence across the HindIII or PstI sites was verified by sequencing smaller

![Fig. 1](image-url)

Fig. 1. (A) Restriction map of D1701 DNA, which has been updated to that published recently (Cottone et al., 1998). The inverted terminal repeat (ITR) region is depicted as well as the map location of some selected genes, with arrowheads pointing to their orientation. (B) Enlargement of the HindIII fragment H' and part of fragment D showing the potential genes as explained in the text. (C) The sequenced part of BPSV B177 was obtained from plasmids pV4-1, pV8-94 and pV8-152 and the predicted open reading frames are shown. Some selected restriction sites are indicated (H, HindIII; P, PstI; S, SalI). Positions of potential early promoters (flags) and the early transcription stop motif T5NT (filled square) are marked.
restriction fragments overlapping these sites. The DNA sequence of BPSV B177 showed the presence of the late F10L and F9L genes, as described for D1701 and NZ2 (Mercer et al., 1995; Rziha et al., 1999). Both genes displayed high DNA and amino acid identity to the ORFV counterparts (Table 1). Most sequence diversity was found in the N-terminal part of the F10L gene, which was missing an alanine-rich stretch of 20 amino acids present in the BPSV B177 protein. The VACV F10L and F9L orthologues, therefore, appear to be conserved among all poxviruses. Whereas the F10L gene encodes a serine/threonine protein kinase, which is essential for the regulation of VACV morphogenesis (Betakova et al., 1999), the function of F9L is still unknown. Deletion of F9L did not result in a viable D1701 virus (H.-J. Rziha & M. Henkel, unpublished data), possibly indicating its indispensability for ORFV multiplication.

Sequence analysis of BPSV B177 showed the absence of a VEGF-E gene located downstream of the F9L gene (Fig. 1B). Instead, another potential ORF (ORF X) was predicted from the B177 DNA sequence (Fig. 1C, Table 1). However, homology searches using search programs BLASTP, BLASTX, PRODOM and MOTIF did not show significant similarity to any known gene deposited in GenBank or any protein consensus motif. The hybridization results indicated that the VEGF-E gene has not been translocated to another part of the viral genome. Another new VEGF-E variant might be not detectable by the hybridization approaches used, although only the two VEGF-E variants, predominantly the NZ2 or D1701 gene variant, have been detected in a larger number of ORFV isolates (Mercer et al., 2002; H.-J. Rziha & others, unpublished data). Therefore, a standardized proliferation assay was used to test for VEGF-E-specific mitogenic activity (Meyer et al., 1999). Cells were infected with different m.o.i.s of ORFV D1701 or BPSV B177 and the virus-free supernatants were harvested 24 h later. Supernatants from ORFV-infected cells showed at least a three- to fivefold specific stimulation of human umbilical vein endothelial cell (HUVEC) proliferation. In contrast, in three independent assays, supernatants from B177-infected cells did not exhibit mitogenic activity (data not shown), substantiating the absence of a VEGF-E gene in BPSV B177. From the data presented, as well as the finding of additional VEGF-E-negative BPSV (H.-J. Rziha & others, unpublished data), it is intriguing to suppose a link to the BPSV-induced non-proliferating inflammatory disease. Genetic analysis of additional BPSV isolates needs to show whether genuine BPSV are distinguishable from ORFV and PCPV by the absence of VEGF-E combined with a typical DNA restriction pattern (Gassmann et al., 1985; Inoshima et al., 2001; Menna et al., 1979; Mercer et al., 1997; Robinson & Lyttle, 1992; Robinson & Mercer, 1995; Wittek et al., 1980).

The relationship of the ORF3 genes is assumed from the marked amino acids similarity between D1701 ORF3 and the 95 sequenced amino acids of B177 (Table 1). The early ORF3 gene was first described for ORFV NZ2 and NZ7 (Fleming et al., 1991; Fraser et al., 1990) and displays 76-2 % amino acid identity to that of D1701. This gene of unknown function does not show homology to any other poxviral gene and seems to be unique for PPV. Beyond the ORF3 gene, the available ~1000 bases of the D1701 sequence show no homology to that of NZ2 or NZ7 (not shown). Upstream of the F10L gene, three so far unknown genes were found in ORFV D1701 and BPSV B177. Database searches using Pfam alignment showed that all three potential genes contained tandemly arranged stretches of 33–44 amino acids in length characteristic of ankyrin repeats and have been tentatively designated as ANK-1 to ANK-3 (Fig. 1). From the available sequence data, the C-terminal 211 amino acids of the B177 ANK-1 gene beginning with the last of eight ankyrin repeats seen in D1701 indicate considerable similarity to D1701 ANK-1 (Table 1). The ANK-2 and ANK-3 genes are of similar size and contain eight or nine ankyrin repeat motifs, and the deduced amino acid sequences show 62-5 or 66-9 % amino acid similarity,

<table>
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<tr>
<th>Gene</th>
<th>Identity (%)</th>
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<td>Amino acids</td>
<td>Nucleotides</td>
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<td>B177</td>
<td>% G+C</td>
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<td>1491</td>
<td>(211)</td>
<td>(633)</td>
<td>71-0</td>
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<td>555</td>
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<td>573</td>
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<tr>
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<td>520</td>
<td>1560</td>
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<td>1545</td>
<td>67-4</td>
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<tr>
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<td>497</td>
<td>1494</td>
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<td>672</td>
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<tr>
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<td>132</td>
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<td>–</td>
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<td>56-1</td>
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<td>(95)</td>
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<td>81-8</td>
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respectively (Table 1). The lower DNA identity (maximally 65%) between D1701 and B177 of the ANK-2 and ANK-3 gene region explains missing or weak hybridization signals with specific probes. Although the DNA homology of the described genes varies between both viruses, the average G+C content of this BPSV genome seems high, comparable with ORFV (Wittek et al., 1979). The existence of counterparts of the new ANK genes in other PPV is unknown. Apart from the presence of the ankyrin repeat motifs, the three ANK genes showed no similarity to the GIL gene, which is the first reported ankyrin-repeat-containing ORFV gene and maps at the left terminus of the genome (Cottone et al., 1998; Sullivan et al., 1995b). A number of ankyrin-repeat-containing proteins have been identified in various poxviruses, also located at near-terminal genomic regions, and are expressed early in infection. Early expression of the new ANK genes, which was expected from the presence of sequence motifs resembling poxviral early promoters and the canonical early transcription stop motif (Fig. 1), was found for ANK-2 and ANK-3 in D1701-infected cells (data not shown). Numerous prokaryotic and eukaryotic proteins acting as protein linkers use the ankyrin repeats as binding domains for protein–protein interactions, thereby influencing cell-cycle control, cell differentiation and host immunity (Ghosh et al., 1998; Rubtsov & Lopina, 2000). The cowpoxvirus-encoded ankyrin repeat protein gene CHOhr determines cell tropism (Spehner et al., 1988) and functions as an anti-apoptotic gene (Ink et al., 1995). Whether PPV ANK genes can also mediate protein–protein interactions and exert important functions for the tropism or pathobiology of ORFV awaits further investigation. Additionally, it would be interesting to see whether other PPV also display a collinear genomic arrangement of related ANK genes, which might further indicate their importance for the PPV life cycle.

The recently described ORFV IL-10 gene was also detected in ORFV D1701 and BPSV B177 (Fig. 2 and Table 1). Most sequence differences were found in the first 50 amino acids, whereas the final two-thirds of the proteins show the highly conserved IL-10 core motifs (Fig. 2). Signal peptide cleavage sites, identified using program SignalP, could be located in the variable N terminus of the proteins. Remarkably, the IL-10 genes exhibited a noticeably low G+C content.
compared with the flanking sequence (Table 1), supporting earlier suggestions that those genes acquired from the natural host have retained the low G+C content (Fleming et al., 1997, 2000; Lyttle et al., 1994). This was supported by the comparison of amino acid relationships. Homology searches using the bc2 program (EMBL) revealed a higher score of BPSV IL-10 to bovine IL-10 (score 703) over a range of 139 amino acids (aa 52–189) than to ovine IL-10 (score 678). Closer inspection of the amino acid sequences showed that B177 and bovine IL-10 possess identical amino acids at six positions within the conserved part of the protein (Fig. 2, asterisks). At these positions the three ORFV strains exhibited residues that differ from the bovine IL-10 but are identical to the ovine IL-10, explaining the closer relationship of ORFV and ovine IL-10. One of the amino acids exclusively shared by the bovine and BPSV IL-10 is predicted to interact with the IL-10 receptor chain 1 (Fig. 2, dots and asterisks) and might indicate a specific interaction with the bovine IL-10 receptor. Interestingly, the amino acid sequence of the D1701 IL-10 differs in three positions and most notably, a valine has replaced the isoleucine-115 present in all other IL-10 proteins (Fig. 2, triangle). This isoleucine residue can be very critical for retaining stimulatory activity of human IL-10 (Ding et al., 2000). Whether this amino acid exchange might negatively affect the IL-10 activity and contribute to the attenuation of ORFV D1701 remains speculative. Collectively, the presented results do not support the recent suggestion that ORFV, but not BPSV, encodes an IL-10 homologue (Fleming et al., 2000). Based on the B177 IL-10 DNA sequence, a specific PCR could be used to demonstrate the presence of IL-10-specific sequences in a variety of ORFV, BPSV and PCPV (H.-J. Rziha and others, unpublished data). A possible anti-inflammatory property and suppression of an innate early immune response by the IL-10 of other PPV, as shown for inflammatory property and suppression of an innate early Rziha and others, unpublished data). A possible anti-


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