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Sequence Analysis of the Genome of Porcine Lymphotropic Herpesvirus 1 and Gene Expression during Posttransplant Lymphoproliferative Disease of Pigs

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The porcine lymphotropic herpesvirus 1 (PLHV-1), the first gammaherpesvirus of pigs, has been detected at a high prevalence in healthy pig populations. A porcine gammaherpesvirus has also been detected at high copy numbers in animals suffering from posttransplant lymphoproliferative disease (PTLD). While human PTLD is a EBV-associated complication following clinical transplantation, porcine PTLD is a disease recently described in pigs undergoing experimental allogeneic hematopoietic stem cell transplantation. Here we demonstrate that PLHV-1 and the virus present in porcine PTLD are indistinguishable, and present the characterization of 73 kbp of the genome of PLHV-1. We identified homologs of cellular genes, including a putative G protein-coupled receptor (GCR) as well as a viral homolog of the bcl-2 oncogene (v-bcl-2) and show significant transcription of these genes as well as of several other PLHV-1 genes in lymph nodes of a PTLD-affected pig. These data indicate that PLHV-1 is active during PTLD and may be involved in the etiology of this lymphoproliferative disease.

Key Words: porcine lymphotropic herpesvirus; post-transplant lymphoproliferative disease; xenotransplantation.

INTRODUCTION

Human herpesviruses cause morbidity and mortality in patients who receive allotransplants. Disease can be induced either by reactivation of latent virus in the recipient or through transmission of the herpesvirus from donor-derived cells or organs (Tolkoff-Rubin and Rubin, 1998). Similarly, during xenotransplantation, it is possible that animal herpesviruses might be transferred to a recipient and possibly cause disease (Chapman *et al.,* 1995). Xenotransplantation research is focused on the use of pigs as donors of organs for human transplantation. Therefore, attempts are presently made to breed pathogen-free pigs for xenotransplantation. For this purpose, valid monitoring methods as well as comprehensive knowledge of porcine microorganisms is needed (Günzburg and Solmons, 2000).

A search for unknown porcine herpesviruses recently identified two novel gammaherpesviruses of pigs (porcine lymphotropic herpesviruses 1 and 2 [PLHV-1 and PLHV-2]). These viruses are widely distributed with high prevalence among domestic and feral pigs (Ehlers *et al.,* 1999; Ulrich *et al.,* 1999). Nucleotide sequence analysis of the DNA polymerase (DPOL) genes of the PLHVs indicated that both viruses are closely related to the alcelaphine herpesvirus 1 (AlHV-1), a herpesvirus of ruminants and the causative agent of fatal malignant catarrhal fever (MCF) in cattle (Ulrich *et al.,* 1999). Although no disease is currently attributable to PLHV infection in pigs under natural conditions, a porcine gammaherpesvirus was recently reported to be associated with experimental posttransplant lymphoproliferative disease (PTLD) in miniature swine following allogeneic hematopoietic stem cell transplantation (Huang *et al.,* 2001). The porcine PTLD syndrome reported by Huang *et al.* closely resembles human PTLD. In particular, both syndromes involve B cell lymphoproliferation and high mortality. Furthermore, while the human PTLD is strongly linked to the presence of Epstein-Barr virus (EBV) (Paya *et al.,* 1999; Ferry and Harris, 1994), markedly elevated levels of a gammaherpesvirus have been observed with the porcine PTLD. Because of the above similarities, PTLD in miniature swine has been discussed as a large animal model for human PTLD (Huang *et al.,* 2001).

To investigate the molecular mechanisms by which EBV and PLHV might be involved in the production of PTLD, it is necessary to know the genes that both viruses possess. While EBV has been completely sequenced, data for PLHV have been limited to the DPOL and glycoprotein B (gB) genes. In this study we isolated 73 kbp of novel PLHV-1 genomic DNA which encompasses open reading frames (ORF) 03 to 52 as well as flanking

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sequences. Furthermore, we demonstrate that PLHV-1 and the PTLD-associated porcine herpesvirus are indistinguishable and are transcribed in PTLD-affected pigs.

RESULTS

Determination of novel PLHV-1 sequences

Novel PLHV-1 sequences were obtained by PCR-based genome walking from the splenic DNA of a domestic pig which was PCR-positive for PLHV-1 but PCR-negative for PLHV-2 (Ulrich *et al.,* 1999). The genome walking approach was chosen because neither a lytical cell culture system nor a permanent cell line harboring latent PLHV-1 is available, limiting experimental work on PLHV-1 to primary organ and tissue materials (Ehlers *et al.,* 1999; Ulrich *et al.,* 1999). The PCR products were sequenced at both ends and verified by BLAST analysis. To confirm the organization of the PCR fragments, new overlapping PCRs were designed using the initial sequence data and the confirmatory products were sequenced in both orientations. When sequencing progressed into nonconserved regions, BLAST verification of the deduced sequences was not possible. Genomic DNAs derived from pigs free of PLHV were used as negative control templates in such cases to ensure the viral origin of the sequences. Using this approach, 73.200 bp of PLHV-1 genomic DNA sequence was obtained and its contiguity demonstrated by performing seven overlapping long-distance-PCRs which generated amplicons of 10.8–13.4 kbp in length (Fig. 1a and c). The overall G+C content (37.9%) and the CpG dinucleotide frequency (ratio observed/expected 0.38) of the 73 kbp were found to be very low.

Coding capacity and gene arrangement of PLHV-1

The conserved genes of gammaherpesviruses are arranged in a common block organization (Simas and Efstathiou, 1998; Schultz, 1998). The 73 kbp region of PLHV-1 presented here comprises ORFs 03 to 52 and therefore includes the entire first and second conserved gene block of this herpesvirus subfamily and the beginning of the third (Fig. 1, Table 1).

Within the first conserved gene block, ORFs for a viral FGAM-synthetase (ORF 03), major DNA binding protein (ORF 06), transport protein (ORF 07), glycoprotein B (gB; ORF 08), DNA polymerase (ORF 09), and two conserved ORFs with unknown function (ORF 10 and 11) were identified. In addition, two ORFs not conserved among the gammaherpesviruses, E4/BALF1$_h$ and A5/BILF1$_h$, were also identified (Fig. 1). Within the second conserved gene block (ORFs 17–50), genes encoding a thymidine kinase (TK, ORF 21), glycoprotein H (gH, ORF 22), major capsid protein (MCP, ORF 25), terminase (ORFs 29a,b), glycoprotein M (gM, ORF 39), helicase (ORF44), and glycoprotein L (gL, ORF 47) genes as well as two immediate early genes (ORFs 45 and 50a,b) were identified. Within the third conserved gene block, ORF 52 and the partial sequence of ORF53 were identified. Between the second and the third conserved gene block, three nonconserved ORFs were identified and designated A6/BZLF1$_h$, A7/BZLF2$_h$, and A8/BLLF1$_h$. Outside the conserved gene blocks, eight unique ORFs were identified which showed no significant homologies to known herpesvirus genes.

Homology and possible function of nonconserved PLHV-1 genes

Structural homologs of PLHV-1 FGAM-synthetase (ORF 03) are found at the same position in alcelaphine herpesvirus 1 (AlHV-1), equine herpesvirus 2 (EHV-2), herpesvirus saimiri (HVS), and herpesvirus ateles (HVA). There is also considerable homology of PLHV ORF 03 to the ORF 75, which is located at the right-hand ends of several rhadinovirus genomes, including AlHV-1, EHV-2, and Kaposi sarcoma herpesvirus (KSHV) and the left end of the EBV genome (ORF BNRF1).

The E4/BALF1$_h$ gene which was identified in PLHV-1 has no counterpart in AlHV-1, but homologs are present in EHV-2 and EBV (genes E4 and BALF1, respectively). BALF1 was reported to be a viral homolog (v-bcl-2) of the mammalian bcl-2 oncogene encoding for a functional apoptosis inhibitor (Marshall *et al.,* 1999). Amino acid alignments revealed a uniform distribution of homology of PLHV-1 E4/BALF1$_h$ to human bcl-2, EBV BALF1, BHRF1, and EHV-2 E4. The homology of E4/BALF1$_h$ is not significantly higher to the BH domains of BALF1 or the other proteins (data not shown). Interestingly, like EBV BALF1 and EHV-2 E4, the predicted v-bcl-2 protein of PLHV-1 lacks a putative transmembrane domain at the C terminus which is in contrast to the human and other gammaherpesvirus bcl-2 genes (Marshall *et al.,* 1999; Bellows *et al.,* 2000).

The PLHV ORF A5/BILF1$_h$ is related to ORF A5 of AlHV-1, ORF E6 of EHV-2 and ORF BILF1 of EBV. The A5/BILF1 homologs all encode for proteins with seven hydrophobic domains which show weak homology to the ORF 74 gene products of KSHV and other gammaherpesviruses. In KSHV, the ORF 74 gene product was determined to represent a functional CXC chemokine receptor with homology to the human IL-8R (Arvanitakis *et al.,* 1997; Rosenkilde *et al.,* 1999).

The PLHV ORF A6/BZLF1$_h$ shows only very low homologies to the positional counterparts of AlHV-1 (ORF A6), EBV (ORF BZLF1), and HHV-8/KSHV (ORF K8) (Lin *et al.,* 1999). PLHV ORF A7/BZLF2$_h$ was most closely related to ORF A7 of AlHV-1 and ORF BZLF2 of EBV. The BZLF2 ORF was reported to encode a glycoprotein which is required for the entry of EBV into B cells via HLA-DR (Spriggs *et al.,* 1996). PLHV ORF A8/BLLF1$_h$ is most closely related to ORF A8 of AlHV-1 and ORF BLLF1

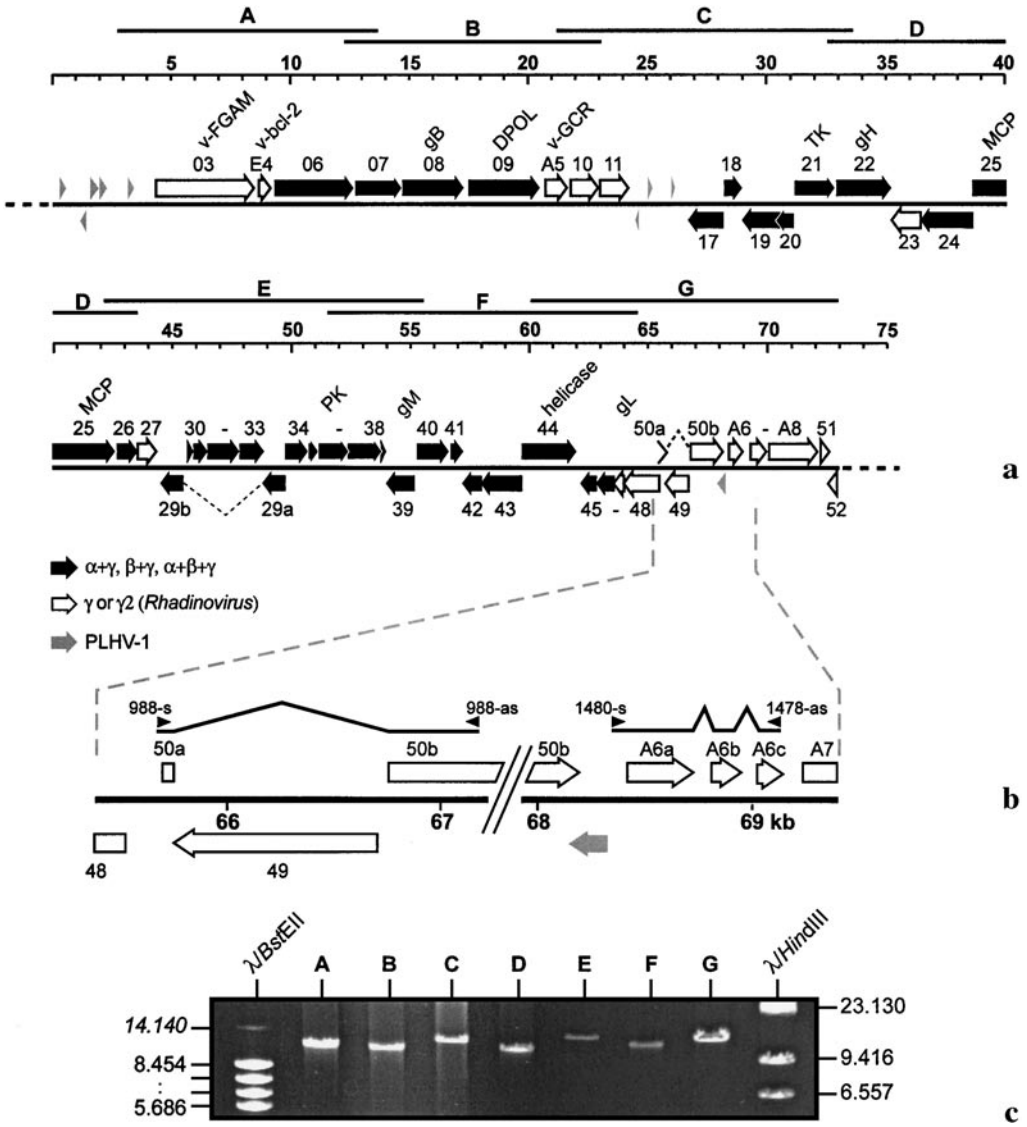

FIG. 1. Map of the open reading frames of the 73-kbp genome stretch of PLHV-1. (a) ORFs are shown as arrows. Description of ORFs and abbreviations are given in Table 1. Black arrows indicate ORFs found in two or all three subfamilies of the herpesviruses. Open arrows symbolize ORFs described only for gammaherpesviruses and grey arrows symbolize ORFs specific for PLHV-1. Black lines designated A to G symbolize the genome stretches covered by each of the seven long-distance PCRs. A scale in kbp is given above the genome. Base 1 is the first base of the genome stretch so far determined by genome walking. This position is not identical with the beginning of the long unique region (LUR) of PLHV-1, which has not yet been determined. (b) Magnification of the ORF 50-ORF A6 region. The results of the cDNA analysis for the spliced ORFs 50 and A6 are shown by black lines above the ORFs. Primer pairs 988 and 1478 are indicated as arrowheads. (c) Gel electrophoresis of the long-distance PCR fragments A–G as depicted schematically in part "a" of this figure. Second-round PCR products were loaded on a 0.7% agarose gel in 2× TBE. λ/*Bst*EII or λ/*Hin*dIII DNA was used as molecular markers as indicated. The cohesive ends of the λ/*Bst*EII marker were not completely separated in order to achieve an additional band of 14.140 bp.

(gp350/220) of EBV. BLLF1 has been reported to mediate attachment of EBV to B cells via the CD21/CR2 receptor (Tanner *et al.,* 1987) and to induce T cell apoptosis (Tanner and Alfieri *et al.,* 1999).

PLHV-1 is closely related to AIHV-1 and other ruminant gammaherpesviruses

At the amino acid level, almost all PLHV-1 genes are closely related to those of the Gammaherpesvirinae, in particular AIHV-1 (Table 1). Phylogenetic analysis of concatenated amino acid sequences of the conserved ORFs 06, 08, 09, 29, and 44 (4015aa) placed PLHV-1 close to AIHV-1 (Fig. 2a). This was also observed in parsimonial analysis (program Protpars) and in analyses of individual proteins with both algorithms (data not shown). In addition, phylogenetic analysis of concatenated DPOL and gB sequences only (360aa), in which partial gB and DPOL sequences of additional gammaherpesviruses (Chmielewicz *et al.,* 2001) were included, revealed PLHV-1 as similarly close to AIHV-1, ovine herpesvirus 2

TABLE 1

Potential PLHV-1 ORFs and Homologs to AIHV-1, EBV, and HVS

PLHV-1			AIHV-1		EBV		HHV-8/KSHV		
ORF	Pol.	Size (aa)	% id	ORF	% id	ORF	% id	ORF	Description
3	+	1378	32.1	3	—	—	—	—	Tegument protein. v-FGAM-synthase
E4/BALF1	+	178	—	—	20.1	BALF1	—	—	v-bcl-2
6	+	1126	56.7	6	43.2	BALF2	47.4	6	Major DNA binding protein (MDBP)
7	+	675	49.1	7	37.9	BALF3	43.1	7	Transport protein
8	+	876	56.3	8	45.6	BALF4	46.7	8	Glycoprotein B (gB)
9	+	1004	63.3	9	55.3	BALF5	57.5	9	DNA polymerase (DPOL)
A5/BILF1	+	325	31.1	A5	24.3	BILF1	—	—	G-protein coupled receptor (v-GCR)
10	+	401	28.5	10	25.0	RAJILF1	§	10	
11	+	409	41.9	11	29.3	RAJILF2	24.5	11	
17	−	500	46.9	17	37.0	BVRF2	40.8	17	Capsid protein
18	+	261	44.4	18	30.7	BVRF1.5	40.7	18	
19	−	549	50.9	19	36.6	BVRF1	44.1	19	Tegument protein
20	−	275	39.2	20	31.7	BXRF1	40.9	20	
21	+	580	40.4	21	35.8	BXLF1	29.1	21	Thymidine kinase (TK)
22	+	778	38.4	22	32.2	BXLF2	27.1	22	Glycoprotein H (gH)
23	−	398	42.0	23	34.4	BTRF1	33.3	23	
24	−	736	51.4	24	41.0	BcRF1	42.2	24	
25	+	1372	67.9	25	55.9	BcLF1	56.7	25	Major capsid protein (MCP)
26	+	304	60.9	26	50.2	BDLF1	48.7	26	Capsid protein
27	+	294	39.6	27	22.1	BDLF2	30.1	27	
29	−	683	62.5	29	52.4	BDRF1	54.1	29	Cleavage and/or packaging protein
29b		*379		29b		(BDRF1)		29b	Cleavage and/or packaging protein. Exon II
30	+	79	35.4	30	20.3	BDLF3.5	26.8	30	
31	+	206	44.8	31	34.2	BDLF4	40.2	31	
32	+	453	33.3	32	24.3	BGLF1	26.7	32	Viral DNA cleavage/packaging protein
33	+	339	42.1	33	45.6	BGLF2	32.7	33	
29a		*304		29a		(BGRF1)		29a	Cleavage/packaging protein. Exon I
34	+	326	46.6	34	33.3	BGLF3	37.0	34	
35	+	151	34.2	35	28.2	BGLF3.5	26.5	35	
36	+	453	42.1	36	30.9	BGLF4	26.3	36	Kinase (PK)
37	+	485	61.6	37	45.3	BGLF5	42.3	37	Alkaline exonuclease
38	+	63	44.8	38	33.3	BBLF1	34.5	38	
39	−	378	61.7	39	48.4	BBRF3	43.1	39	Glycoprotein M (gM)
40	+	455	30.6	40	24.2	BBLF2	24.2	40	Helicase–primase complex component
41	+	162	24.7	41	23.5	BBLF3	23.4	41	Helicase–primase complex component
42	−	266	45.5	42	35.8	BBRF2	36.5	42	
43	−	567	62.3	43	50.7	BBRF1	53.6	43	Capsid protein
44	+	782	63.9	44	51.4	BBLF4	54.6	44	Helicase
45	−	223	32.3	45	29.4	BKRF4	27.4	45	
46	−	251	65.3	46	55.6	BKRF3	53.8	46	Uracil–DNA–glycosidase
47	−	142	33.6	47	26.7	BKRF2	27.5	47	Glycoprotein L (gL)
48	−	498	29.6	48	§	BRRF2	20.1	48	
49	−	309	—	—	23.0	BRRF1	22.9	49	
50	+	*510	33.4	50	20.8	BRLF1	24.1	50	R transactivator homologue
50a		*14		50a		—		50a	R transactivator homologue. exon I
50b		496		50b		—		50b	R transactivator homologue. exon II
A6/BZLF1	+	172	22.8	A6	§	BZLF1	§	K8	Zta (ZEBRA) transcription factor in EBV
A6/BZLF1a		101		—		BZLF1a		K8a	Exon I
A6/BZLF1b		35		—		BZLF1b		K8b	Exon II
A6/BZLF1c		36		—		BZLF1c		K8c	Exon III
A7/BZLF2	+	234	31.2	A7	28.0	BZLF2	—	—	gp 42 in EBV (B cell fusion)
A8/BLLF1	+	725	23.0	A8	21.9	BLLF1	—	—	gp350/220 in EBV (B cell attachment)
51	+	115	—	—	—	—	—	—	
52	−	136	36.9	52	29.0	BLRF2	28.0	52	

Note. Pol., polarity; ORF, orientation; % id, percent identity to the corresponding PLHV-1 ORF; *, splice sites experimentally confirmed; §, identity values below 20%.

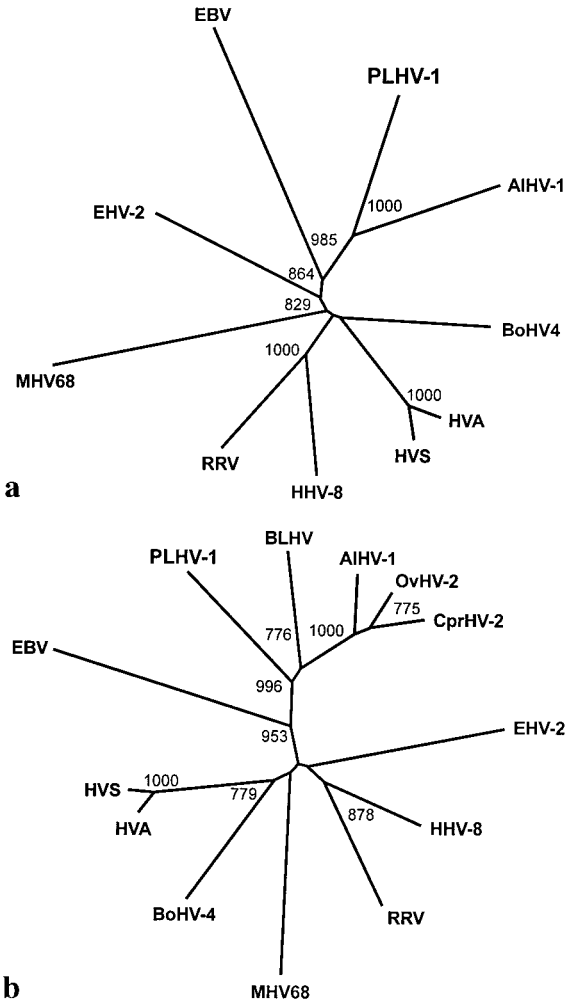

FIG. 2. Phylogenetic relatedness of PLHV-1. Phylogenetic trees were constructed with the neighbor/joining method, using concatenated multiple amino acid sequence alignments of conserved regions of (a) ssDNABP (ORF06), gB (ORF 08), DPOL (ORF 09), terminase (ORF 29), and helicase (ORF 44) and (b) gB and DPOL. The PLHV-1 amino acid sequences were deduced from the nucleotide sequence determined in this study. Amino acid sequences of other gammaherpesviruses were obtained from GenBank (accession numbers in Methods). The bootstrap values are indicated at the branches of the tree. Low bootstrap values were found for BoHV-4, EHV-2, and MHV68, indicating uncertain branching. These values are not included in the figure.

(OvHV-2), bovine lymphotropic herpesvirus (BLHV), and caprine herpesvirus 2 (CprHV-2). These five viruses form a separate clade as shown in Fig. 2b.

PLHV-1 is associated with PTLD in pigs and is genetically stable

A porcine gammaherpesvirus has been detected at high copy numbers in miniature swine presenting an early form of PTLD (Huang *et al.,* 2001). We compared the complete gB sequence of this herpesvirus (GenBank accession number AY044427) with the PLHV-1 gB gene reported in this paper and found them to be identical. We also sequenced more than 1.5 kb of the gB gene and the entire noncoding region between ORF 11 and 17 from a virus-positive blood sample (#1187) of another miniature swine (#13801) suffering from PTLD as well as ORFs 40–50 from a lymph node sample (#1328) of a third PLHV-1 positive PTLD-affected swine (#13271) (data not shown). All sequence alignments revealed nucleotide identities in excess of 99.9%, demonstrating that the PTLD-associated gammaherpesvirus and PLHV-1 are essentially indistinguishable. High sequence identity (>99.9%) was also detected in PLHV-1 sequences amplified from German pigs, indicating PLHV-1 as a genetically stable virus.

PLHV-1 genes are transcribed in lymph nodes of PTLD-affected pigs

We examined the transcription of a number of key PLHV genes in the lymph nodes of an immunosuppressed PTLD-affected miniature swine (#13813) as well as two untreated healthy miniature swine (#14265 and #14268). Transcripts of PLHV-1 ORF 03, ORF E4/BALF1$_h$, ORF 08 (gB), ORF A5/BILF1$_h$, ORF 29, ORF 45, ORF50, and ORF A7/BZLF2$_h$. were detected in the PTLD-affected animal, but not in the healthy pigs (Fig. 3, Table 2).

As known from other herpesviruses, the ORFs 29 (terminase) and ORF 50 (rta homologue) are spliced genes. For the detection of transcripts of these genes in PLHV-1-infected lymph nodes, primers were used which flanked the predicted splice sites. The sizes of the ORF 29 (primers 989-s and 990-as) and the ORF 50 (primers 988-s and 988-as, Fig. 1b) PCR products were as expected for spliced transcripts and verified by sequencing (data not shown).

The first ORF analysis of the 73-kbp genome stretch of PLHV-1 showed that ORF A6/BZLF1$_h$ was significantly shorter (104 aa) than its positional counterparts in AlHV-1 (210 aa) and EBV (245 aa), resulting in a 487-bp gap between the predicted ORFs A6/BZLF1$_h$ and A7/BZLF2$_h$. As EBV ORF BZLF1 is a spliced gene consisting of three

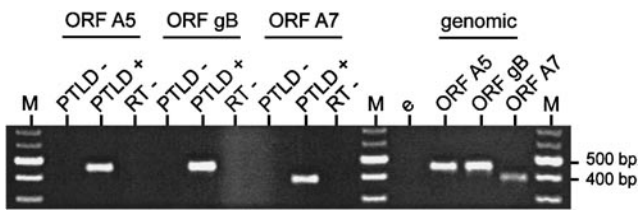

FIG. 3. PLHV gene expression in normal and PTLD affected tissues. As an example, some of the RT-PCR results are shown for the genes indicated above the left part of gel. Lanes are labeled for the template cDNA: (PTLD−) = RNA/cDNA prepared from a lymph node of a non-treated, healthy pig; (PTLD+) = RNA/cDNA prepared from a lymph node of a PTLD-affected pig, (RT−) indicates "no reverse transcriptase" control, RNA prepared from the same PTLD-affected pig. As a positive control, the right part of the gel shows the PCR results with genomic DNA from a lymph node of a PTLD-affected pig as template. (M), 100 bp ladder (Promega), (e), empty lane.

TABLE 2

PLHV-1 Genes Transcribed in a PTLD-Affected Pig

ORF		PIG #13813 PTLD	PIG #14265 unaffected	PIG #14268 unaffected
E4/BALF1	v-bcl-2	+[a]	—[b]	—[b]
3	Tegument	+	—	—
8	Glycoprotein B	+	—	—
A5/BILF1	GCR	+	—	—
29	Terminase	+	n.d.	n.d.
45	IE protein	+	—	—
50a, b	IE protein	+	—	—
A7/BZLF2	Glycoprotein	+[c]	—[c]	—[c]

Note. a, b, No RT controls were in all cases negative. n.d., PCR not done.

[a] RT-PCR results from the lymph nodes of a PTLD pig.

[b] RT-PCR results from the lymph nodes of nontreated, healthy pigs.

[c] Results were identical with two different primer pairs, see Materials and Methods section.

exons, we performed RT-PCR analysis for PLHV-1 to determine whether additional exons were encoded within the 487-bp gap. Lymph node RNA from a PTLD-affected pig (#14203) was used for cDNA preparation. Sequence analysis of different PCR products which were shorter than predicted from the genome sequence revealed that ORF A6/BZLF1$_h$ consists of three exons (Fig. 1b). The longest spliced transcript of this ORF was detected with primers 1480-s and 1478-as. The amplimer contains the initial ATG and ends 12 bp upstream of a stop codon which is followed by a polyadenylation signal. From these data, a coding capacity of 172 aa can be deduced. As A6/BZLF1$_h$ analysis is part of an ongoing project on PLHV-1 transcription, RACE analysis will be carried out to confirm this result.

DISCUSSION

This study provides the complete sequence of a major part of the genome of PLHV-1, the first porcine gammaherpesvirus to be described. The region sequenced spans approximately 73 kbp and comprises more than 60 ORFs (Table 1). Sequence analysis of more than 10 kbp including several noncoding regions of the gammaherpesvirus DNA amplified from three different PTLD-affected miniature swine and of PLHV-1 DNA amplified from healthy German domestic pigs showed, in comparison with the PLHV-1 73 kbp sequence, more than 99.9% identity. This indicates the general genetic stability of PLHV-1 and shows, together with the marked increase of PLHV-1 copies in PTLD-affected miniature swine (Huang *et al.,* 2001), the association of PLHV-1 with PTLD.

Experimental porcine PTLD presents with symptoms very similar to human PTLD, which has a close association with EBV (Huang *et al.,* 2001). Interestingly, four PLHV-1 genes not conserved among the Gammaherpesvirinae (ORFs E4/BALF1$_h$, A5/BILF1$_h$, A7/BZLF2$_h$, and A8/BLLF1$_h$) might be involved in the pathological similarities between PLHV-1-associated PTLD in pigs and EBV-associated PTLD in humans and are discussed below.

The E4/BALF1$_h$ gene of PLHV-1 encodes a v-bcl-2 homologue from the same genomic position as EHV-2 (ORF E4) and EBV (BALF1). Viral bcl-2 genes are hypothesized to confer evasion from the immune system and to promote tumorigenesis by preventing apoptosis as a response to virus infection (Thompson, 1995). EBV BALF1 represents, in addition to BHRF1, the second protein of EBV with this function (Marshall *et al.,* 1999). E4/BALF1$_h$, E4, and BALF1 lack a putative C terminal transmembrane domain which is in contrast to, e.g., the ORF16-encoded v-bcl-2 of HVS, HHV-8/KSHV, and BoHV-4, as well as BHRF1 of EBV or human bcl-2 and bax. Future experiments are required to determine whether these three viral bcl-2 genes are functional. The completion of the sequencing of PLHV will reveal whether this virus also encodes a second v-bcl-2-like EBV BHRF1. The putative PLHV-GCR (A5/BILF1$_h$) and the corresponding GCR of AIHV-1, EHV-2, and EBV (A5, E6, and BILF1, respectively) show weak homology to the ORF 74 gene products of KSHV and other rhadinoviruses. The ORF74 gene product of KSHV has been found to encode a functional homolog of human IL-8R and to behave as an oncogene in KSHV-mediated tumors by constitutively activating cell-signalling pathways to induce transformation and angiogenesis (Arvanitakis *et al.,* 1997; Bais *et al.,* 1998). We have also sequenced the porcine IL-8R gene and compared it with the PLHV-GCR. No greater homology was found between porcine IL-8R and the PLHV-GCR as compared to other mammalian GCRs (M. Goltz and B. Ehlers, paper in preparation). These results may indicate that the GCR gene was present in an ancestor of PLHV-1, AIHV-1, EHV-2, and EBV at a time before the cospeciation of mammals and their herpesviruses. Future functional studies are required to elucidate the role of the putative PLHV-GCR in the pathogenesis of PTLD. Because the mechanisms by which gammaherpesviruses can induce lymphoproliferative conditions are not precisely defined, it will be interesting to examine the biological functions of the novel PLHV v-bcl and GCR as this might provide further insight into the mechanisms of proliferation as well as transformation.

The A7/BZLF2$_h$ and A8/BLLF1$_h$. genes are likely to affect the cell tropism of PLHV. The protein deduced from the A7/BZLF2$_h$ ORF is related to the BZLF2 glycoprotein of EBV which was reported to form a ternary complex with the glycoproteins gH and gL. In this complex, BZLF2 is essential for the entry of EBV into B cells via HLA-DR (Spriggs *et al.,* 1996; Wang and Hutt-Fletcher, 1998). In AIHV-1, the homologous A7 seems to be required for AIHV-1 pathogenicity in rabbits (Handley *et al.,* 1995). A8/BLLF1$_h$ is the homolog of A8 of AIHV-1 (Ensser *et al.,*

1997) and BLLF1 (gp350/220) (Jackman *et al.,* 1999). The latter protein is required for the attachment of EBV to B cells via the complement receptor molecule CD21 (Tanner *et al.,* 1987; Haan *et al.,* 2000) and was reported to induce apoptosis of T cells (Tanner and Alfieri, 1999). Although a functional analysis of the respective EHV-2 and AlHV-1 ORFs has not been reported, a B cell tropism has also been observed for EHV-2 (Drummer *et al.,* 1996). The tropism of AlHV-1 in its natural host, the wildebeest, is not known. However, the closely related OvHV-2, like AlHV-1 a causative agent of MCF in cattle, was found in B cells of its natural host, the sheep (Baxter *et al.,* 1997). There are two lines of evidence which indicate that PLHV-1 also has B cell tropism. First, oligoclonal proliferation of cells of the B lineage by a factor of approximately 10^2 was observed in lymph nodes in immunosuppressed pigs suffering from PTLD and the amount of PLHV-1 genome copies concomitantly rose by a factor of 10^5 (Huang *et al.,* 2001). Second, we demonstrate in the tissue of a PTLD-affected pig, the transcriptional activity of two ORFs (A7/BZLF2$_h$ and A8/BLLF1$_h$) (Table 2) which encode proteins putatively responsible for entry into B cells (Table 1). Taken together, these data are strongly suggestive of PLHV-1 infecting B cells.

According to amino acid sequence comparisons (Table 1) and phylogenetic analyses (Fig. 2), the partial genome of PLHV-1 has a gene content and arrangement very similar to the ruminant rhadinovirus AlHV-1. In addition, the low G+C content of 37% and the strong suppression of the CpG dinucleotide frequency is indicative for a rhadinovirus (Ulrich *et al.,* 1999). A characteristic feature of the lymphocryptoviruses is the presence of several genes coding for nuclear antigens (EBNA genes), which are involved in the maintenance of EBV latency (Masucci and Ernberg, 1994). No such homologs were identified in PLHV-1. Taken together, these data suggest that PLHV-1 should be classified as a rhadinovirus. However, four ORFs of PLHV-1 (E4/BALF1$_h$, A5/BILF1$_h$, A7/BZLF2$_h$, and A8/BLLF1$_h$) question this conclusion. All four ORFs have homologs in EBV (lymphocryptovirus). Three of them have homologs in AlHV-1 (rhadinovirus) and one of them in EHV-2 (rhadinovirus). No such ORFs have been identified in other completely sequenced rhadinoviruses. A likely explanation might be a common ancestor of PLHV-1, AlHV-1, EHV-2, and EBV, which diverged earlier from a common ancestor of the primate rhadinoviruses, thus separating PLHV-1 (and AlHV-1, EHV-2) from the other rhadinoviruses and linking them evolutionaryly closer to EBV. However, for a final classification, the complete PLHV-1 genome sequence is needed.

PLHV-1 DNA was abundant in peripheral blood mononuclear cells and lymph nodes of pigs affected with PTLD (Huang *et al.,* 2001). Now we observed that immediate–early (e.g., ORFs 45, 50) and late (e.g., major capsid protein, gB, terminase) PLHV-1 genes as well as the genes coding for a GCR and a v-bcl-2 are actively transcribed in PTLD-affected pigs but were undetectable in healthy pigs (Table 2). Presently, we do not precisely know whether only the number of cells supporting PLHV transcription is increased or, in addition, the transcription per cell is increased. However, the disproportionate rise in PLHV DNA (Huang *et al.,* 2001) suggests the latter possibility. In EBV-associated human PTLD, although the majority of malignant lymphoid proliferations are thought to be associated with latent EBV, it has been claimed that lytic activity of EBV might also be associated with the initiation of the disease process (Rea *et al.,* 1994a, 1994b; Montone *et al.,* 1996). Taken together, these data support the hypothesis that PLHV-1 is involved in the etiology of PTLD.

PLHV-1, being possibly causative of porcine PTLD, raises a concern with respect to the use of porcine cells and tissues in xenotransplantation. Although humans and pigs are only very distantly related species (Murphy *et al.,* 2001), the transmission of PLHV-1 from porcine cells and tissues to human xenotransplant recipients and subsequent adaptation might be possible, especially as transplant recipients are likely to be immunosuppressed. Furthermore, several herpesviruses have successfully been transmitted between different species which are sometimes only distantly related. This is exemplified by pseudorabiesvirus which infects not only pigs but also dogs and cats (Kelley and Ratcliffe, 1983; Hagemoser *et al.,* 1980), and OvHV-2 which is well adapted to its natural host, the sheep, but causes MCF with high mortality not only in cattle but also in pigs (Loken *et al.,* 1998 and M. Ackermann, S. Albini, and B. Ehlers, manuscript in preparation). The close relationship of PLHV-1 to AlHV-1 (Table 1, Fig. 2) and other MCF viruses (Fig. 2b) like OvHV-2 is an indication that PLHV-1 is potentially pathogenic not only for pigs but also for immunosuppressed transplant recipients. Therefore, elimination of PLHV-1 may be mandatory for the use of pigs in xenotransplantation. This requires knowledge about how PLHV is transmitted to offspring and the identification of the sites of latency. Completion of the PLHV-1 sequence will reveal candidate latency-associated genes and elucidate the presence of other genes potentially involved in the pathogenicity of PLHV-1. Furthermore, comparison of the gene content and molecular pathogenesis of PLHV-1 and EBV with respect to their capability to induce lymphoproliferative conditions will answer the question whether PLHV-1 in pigs may be a suitable large animal model system for human PTLD.

MATERIALS AND METHODS

Sample collection

Blood samples were collected from commercial pig herds and spleens from a slaughterhouse in Brandenburg, Germany. Spleens from pigs of commercial herds

in Spain were kindly provided by M. Domingo (Universidad de Barcelona, Spain). Lymph node samples from experimental MGH MHV-inbred miniature swine were collected at the Transplantation Biology Research Unit (TBRC), Massachusetts General Hospital, Harvard Medical School, Boston, MA.

Induction of PTLD

MGH MHV-inbred miniature swine were conditioned 2 days prior to allogeneic peripheral blood stem cell transplantation with thymic irradiation and *in vivo* T cell depletion and treated daily with cyclosporine beginning 1 day prior to transplantation and continuing for 30 to 60 days as described earlier (Huang *et al.,* 2001). On the occurrence of clinical signs of PTLD, the animals were sacrificed and organ samples collected.

Genome walking

PLHV-1 sequence information was generated by genome walking followed by sequencing of the PCR products. Genome walking was carried out as described earlier for the PLHV DNA polymerase locus (Ulrich *et al.,* 1999). In brief, spleen samples of a Spanish pig (#056) PCR-positive for PLHV-1 (0.1–1 genome per cell) but PCR-negative for PLHV-2 (Ulrich *et al.,* 1999) as well as two PLHV-1-negative pigs (#054, Spain and #504, Germany) as negative controls were used for DNA preparation with the QIAamp Tissue kit (Qiagen, Chatsworth, CA). Genome walking was performed by Genexpress GmbH (Berlin, Germany) using the Universal GenomeWalkerTM Kit (Clontech Laboratories, Palo Alto, CA) (Siebert *et al.,* 1995a,b). Six restriction enzymes were used for generating batches of digested genomic DNA which were ligated to adaptors. PLHV amplimers were generated by nested PCR using adaptor- and PLHV-specific primers.

Sequence determination

PCR fragments obtained by genome walking were sequenced using BigDye terminator chemistry and ABI 377 or ABI 3100 automated sequencers (Applied Biosystems, Foster City, CA). Additional overlapping PCRs were performed for sequence completion with 100 ng of porcine DNA using AmpliTaq Gold (Applied Biosystems) under the following conditions: 12 min activation at 95°C, 45 cycles with 20 s at 95°C, 30 s at primer-dependent annealing temperature (usually 55–65°C) and 2–3 min at 72°C followed by final extension at 72°C for 15 min. PCR products were purified from remaining primers and dNTPs using Microspin S-300 HR or S-400 HR columns (Amersham Biosciences) and sequenced as described above. The sequence was determined double-stranded, with a 7.5-fold redundancy on average. Each basepair was determined from at least two PCR amplimers generated with different primers.

Nucleotide and protein sequence analysis

The sequence files were assembled with the AutoAssembler software (Applied Biosystems). ORF prediction and initial analysis was performed with MacVector (Version 7.0, Oxford Molecular Group). BLAST searches were performed at the NCBI database, and GAP program from the GCG package (Devereux *et al.,* 1984) was used for calculating similarity and identity values.

ORF designation

Genome orientation and nomenclature of the PLHV-1 ORFs were adapted to the standard nomenclature of rhadinovirus genomes and the reference virus HVS (Albrecht *et al.,* 1992). The ORFs which are present in PLHV-1 and in only a minority of gammaherpesviruses (AlHV-1, EHV-2, and EBV) were designated as homologs of the most similar gene, present in AlHV-1 or EHV-2 at the same position, and the corresponding EBV gene (for example E4/BALF1$_h$ or A5/BILF1$_h$). The ORFs unique to PLHV-1 having no homologs in databases were not named in order to avoid redesignation after possible detection of additional unique ORFs between the 5′-end of the sequence and the left terminus of the genome.

Multiple sequence alignments and phylogenetic analysis

Multiple sequence alignments of individual proteins were performed and protein pair distances calculated with the clustalW module of MacVector. For phylogenetic analysis, gaps or insertions unique to a certain species were removed from the multiple sequence alignments and the remaining conserved regions concatenated for each individual protein (McGeoch *et al.,* 2000). Finally, the combined sequences were again concatenated to produce a single phylogenetic tree which is based on multiple genes (Searles *et al.,* 1999). The tree was constructed with the PHYLIP program package using the programs Protdist (Dayhoff PAM matrix) and Neighbor or, alternatively, the program Protpars with randomized input of sequences. The tree was statistically evaluated by bootstrap analysis (1000-fold resampling), using the programs Seqboot and Consense (Felsenstein, 1985, 1993).

Nucleotide sequence accession numbers and virus abbreviations

The accession number for the 73-kbp sequence of PLHV-1 presented in this study is AF478169. Abbreviations and accession numbers for the sequences used in comparisons are the following.

Complete genomes: AlHV-1 (alcelaphine herpesvirus 1) (accession no. AF005370); BoHV-4 (bovine herpesvirus 4) (accession no. AF318573); EBV (Epstein-Barr virus) (accession no. X00784); EHV-2 (equine herpesvirus 2) (accession no. U20824); HHV-8 (human herpesvirus 8) (accession no.

U75698); HVS (herpesvirus saimiri or saimiriine herpesvirus 2 [SaHV-2]) (accession no. X64346); HVA (herpesvirus ateles or ateline herpesvirus 3 [AtHV-3]) (accession no. AF083424); MHV-68 (murine gammaherpesvirus 68) (accession no. U97553); RRV (rhesus monkey rhadinovirus) (accession no. AF029302).

DPOL and gB sequences: BLHV (bovine lymphotropic herpesvirus) (DPOL, accession no. AF327830; gB, accession no. AF327832); CprHV-2 (caprine herpesvirus 2) (gB and DPOL, accession no. AF283477); OvHV-2 (ovine herpesvirus 2) (DPOL, accession no. AF327831; gB, accession no. AF327833).

Long-distance PCR

Long-distance PCRs were performed with the Expand Long Template PCR System and the Expand High Fidelity PCR System (Roche Diagnostics, Germany) according to the manufacturer's instructions. Amplimers were obtained by nested PCRs. For the second round reaction, 1 μl of the first PCR reaction was used as template. The following primers were used.

fragment A, first round: 1461-s 5′-CATACCTTGTGCGAGTGAGTGGAC-3′ + 1461-as 5′-ATAGTGACCTGTTAGCGGCGATTC-3′, second round: 1462-s 5′-CACTAACTCCTATTACCCCACATG-3′ + 1462-as 5′-TGAGCTATACAAGCTACCACAGAA-3′, *fragment B,* first round: 1463-s 5′-TCATTCTCAAAGAACAACCTTGCC-3′ + 1463-as 5′-TTTGAGCGGTAGACTCTGGTAGCC-3′, second round: 1464-s 5′-AACCTAATTAAACAGAATTCATCGCA-3′ + 1464-as 5′-AAATGTCCACAAAGACTCCGTCGT-3′, *fragment C,* first round: 1465-s 5′-CAGGTGGAACCCTTACCAGCAGAT-3′ + 1465-as 5′-AGATCCAGATCATGCTCCTTTGCC-3′, second round: 1466-s 5′-ATTATGTCTGATACTCACTGTTGCTA-3′ + 1466-as 5′-TCCAATGTCTTGTGATAATTCCTA-3′, *fragment D,* first round: 1467-s 5′-GAGGTCAATGGTCCAACTAAGG-3′ + 1467-as 5′-AGAACAAATACGAGCGATAAAA-3′, second round: 1468-s 5′-GTCTAAGAGCATCTTCGCTACAC-3′ + 1468-as 5′-CAACTTCCATGATACGGTCAACT-3′, *fragment E,* first round: 696-s 5′-GAACACTAACCATTTCCTACATT-3′ + 837-as 5′-ATCGCTTATCTGTATCACTCTATCGT-3′, second round: 698-s 5′-TACCTTCCCCAACGAGCTTTACA-3′ + 794-as 5′-AGCTTAGTTACGCCTAACACTTGC-3′, *fragment F,* first round: 803-s 5′-AGATCATGAACGATCATTACAA-3′ + 936-as 5′-ATCCATATTGATGTTGGATTG-3′, second round: 776-s 5′-GCCGAGGGTACTTACGCCAACATT-3′ + 964-as 5′-TGTGACATTTTAGATGTCAGTCCC-3′; *fragment G,* first round: 881-s 5′-CTCATATCTAGTCCAGTCGAACAA-3′ + 1218-as 5′-GTAGTCTGGTCCTGTCCGTTTA-3′, second round: 883-s 5′-AACCGTCGTAGCTTCTCAAAATC-3′ + 1181-as 5′-CCTCGTGTCTGGGGTAACTAT-3′.

Preparation of cDNA and PCR

Random primed cDNA was prepared from 1.2 μg of total cellular RNA from lymph node tissue, using the SuperScript First-Strand Synthesis System (Gibco BRL, USA) according to the manufacturer's instructions. Random hexamer oligonucleotides were used to prime the reverse transcriptase to the RNA. PCR was performed with HotStarTaq (Qiagen) according to the manufacturer's instructions. The following primers were used.

ORF E4/BALF1: sense: 5′-GTTAACTTGGCTGGTAAAGCAA-3′, antisense: 5′-CTTGTATTCTAACCATCCACGG-3′; *ORF 03/tegument:* sense: 5′-TTCCCAACTTCAAGATTTACAC-3′, antisense: 5′-GTATTTGCTTGGATTGCTAAAA-3′; *ORF 08/gB:* sense: 5′-CACAAGCGTCATGAGCATG-3′, antisense: 5′-TAACGGTCTTCTCGTCCCTG-3′; *ORF A5/BILF1:* sense: 5′-CAATGGAATTGTCAACTCCTCT-3′, antisense: 5′-TAGCAACAGTGAGTATCAGACATAAT-3′; *ORF 45/IE:* sense: 5′-TTGTGCTTTAATTTTCTTGTTACTG-3′, antisense: 5′-TCGGGTCCTCATGATATTCTTC-3′; *ORF 50/IE:* sense: 5′-CTGACTGCAATTCAATCCAACAT-3′, antisense: 5′-CAAGCATTCTTCCCTGACCTACA-3′; *ORF A7, no1:* sense: 5′-TTCCTCATACAGATGATGTAGGGC-3′, antisense: 5′-CACTGTGAATCTGGGCTTCTTTTA-3′; *ORF A7, no2:* sense: 5′-TTTTGTATTTCCTCATACAGATG-3′, antisense: 5′-GCATAAAACCAGTAACATTTAGA-3′.

The reactions were denatured for 15 min at 95°C, followed by 36 cycles of 10 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The thermal cycling was concluded with an incubation at 72°C for 5 min.

For the detection of spliced transcripts of ORF 29, a nested PCR was performed. Primers 990-s (5′-CAACGGCCACCAACAGATCATCTGA-3′) and 989-as (5′-TTAATTCCAAGAAGACATGGGA-3′) were used for first-round PCR, primers 989-s (5′-TGGCAACTTCACTCACTATCTG-3′) and 990-as (5′-TAGGTTATGTTGCACATCAGAAG-3′) for the second-round PCR. For detection of spliced transcripts of ORF 50 primers 988-s (5′-CTGACTGCAATTCAATCCAACAT-3′) and 988-as (5′-CAAGCATTCTTCCCTGACCTACA-3′) were used. PCR reactions were performed with 1 μl of 1:2 diluted oligo-dT-cDNA using AmpliTaq Gold as described above (Sequence determination) with an annealing temperature of 57 or 58°C and an elongation time of 3 min. For detection of spliced transcripts of ORF A6, cDNA was prepared from total cellular RNA from lymph node tissue, using the RNeasy Mini Kit (Qiagen, Germany). The longest spliced transcript was detected with primers 1480-s (5′-TAAAAGACTAACAAAACATGCACA-3′) and 1478-as (5′-AAATTGAGTATCTGGAAACACTGA-3′). PCR reactions were performed with 1 μl of 1:10 diluted oligo-dT-cDNA using AmpliTaq Gold as described above (Sequence determination) with an annealing temperature of 57°C and an elongation time of 2 min.

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