

Characterization of the replication origin (Ori_S) and adjoining parts of the inverted repeat sequences of the pseudorabies virus genome

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The DNA sequence of a 2·4 kbp fragment located in the internal and terminal inverted repeat sequences of the pseudorabies virus genome determined in this study closes a gap between the previously described genes for the ICP4 and ICP22 homologues. The novel sequence contains no conserved herpesvirus open reading frames. Northern blot and cDNA analyses revealed a viral immediate-early transcript of 1·8 kb, which is spliced by the removal of two small introns close to its 5' end and which presumably represents the mRNA of the downstream open reading frame encoding the ICP22 homologue. Upstream of the transcribed region, an imperfect set of three directly repeated sequences was identified. Each of them contains a complementary pair of the alphaherpesvirus origin-binding protein recognition motif GTTCGCAC, spaced by AT-rich sequences. *In vitro* studies confirmed that the DNA fragment analysed includes a functional origin of viral DNA replication.

Pseudorabies virus (PrV, suid herpesvirus-1) is the causative agent of Aujeszky's disease in pigs and is also highly pathogenic for many other mammals, excluding higher primates and humans (Wittmann & Rziha, 1989). PrV is classified as a member of the genus *Varicellovirus* of the subfamily *Alphaherpesvirinae* of the *Herpesviridae*. It contains a class D genome (Roizman, 1996) of about 150 kbp consisting of a long (U_L) and a short (U_S) unique region, the latter of which is flanked by extended inverted repeat (IR_S, TR_S) sequences (Ben-Porat & Kaplan, 1985; Fig. 1*a*).

Whereas the entire genomic sequences of the alphaherpesviruses varicella-zoster virus (VZV; Davison & Scott, 1986),

herpes simplex viruses-1 and -2 (HSV-1 and -2; McGeoch *et al.*, 1988; Dolan *et al.*, 1998), equine herpesviruses-1 and -4 (EHV-1 and -4; Telford *et al.*, 1992, 1998) and bovine herpesvirus-1 (BHV-1; Schwyzer & Ackermann, 1996) are known, the nucleotide sequence of PrV DNA has not yet been determined completely. As far as has been investigated, the gene content and arrangement of the PrV genome appear to be widely similar to that of other mammalian alphaherpesviruses, and therefore the nomenclature was adapted from that of HSV-1 (Roizman & Sears, 1996). The genomic collinearity to HSV-1 and most other alphaherpesviruses is only interrupted by an internal inversion extending from the gB (UL27) gene to the gC (UL44) gene of PrV (Ben-Porat *et al.*, 1983; Bras *et al.*, 1999). Whereas the U_S genome region of PrV has been characterized completely, some sequence gaps remain to be closed within the U_L region, which is apparently fixed in the opposite orientation to the prototypic isomer of the HSV-1 genome (Mettenleiter, 2000).

Within the IR_S and TR_S sequences flanking the U_S region, two PrV genes encoding the homologues of the HSV-1 immediate-early proteins ICP4 (IE180) and ICP22 (RSp40) have been identified and latency-associated transcripts were detected in the antiparallel orientation to the IE180 mRNA (Cheung, 1989, 1991; Vlcek *et al.*, 1990; Zhang & Leader, 1990). Between the two conserved genes, a short stretch of genomic DNA, located at the right end of the internal copy of the 7·7 kbp *Bam*HI fragment 5 (Fig. 1*b*), remained uncharacterized. Since earlier studies indicated the presence of an origin of viral DNA replication within the IR_S and TR_S sequences of PrV (Ben-Porat & Veach, 1980), it was speculated that this regulatory element might reside within the uncharacterized DNA sequences.

In order to verify this, we cloned the 2·4 kbp *Bam*HI–*Sal*I fragment 5B and the overlapping *Kpn*I fragments I and K (Fig. 1*a*) from virion DNA of PrV strain Ka (Kaplan & Vatter, 1959) into plasmid pBS (–) (Stratagene). After digestion with suitable restriction enzymes, the inserts of the independently cloned plasmids pBS-BS5B-1 and -2 (Fig. 1*c*) were shortened unidirectionally by treatment with exonuclease III and religated (nested-deletion kit, Pharmacia). Deletion plasmids were sequenced (Thermo Sequenase cycle-sequencing kit, USB) with

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The GenBank accession number of the nucleotide sequence reported in this paper is AJ251976.

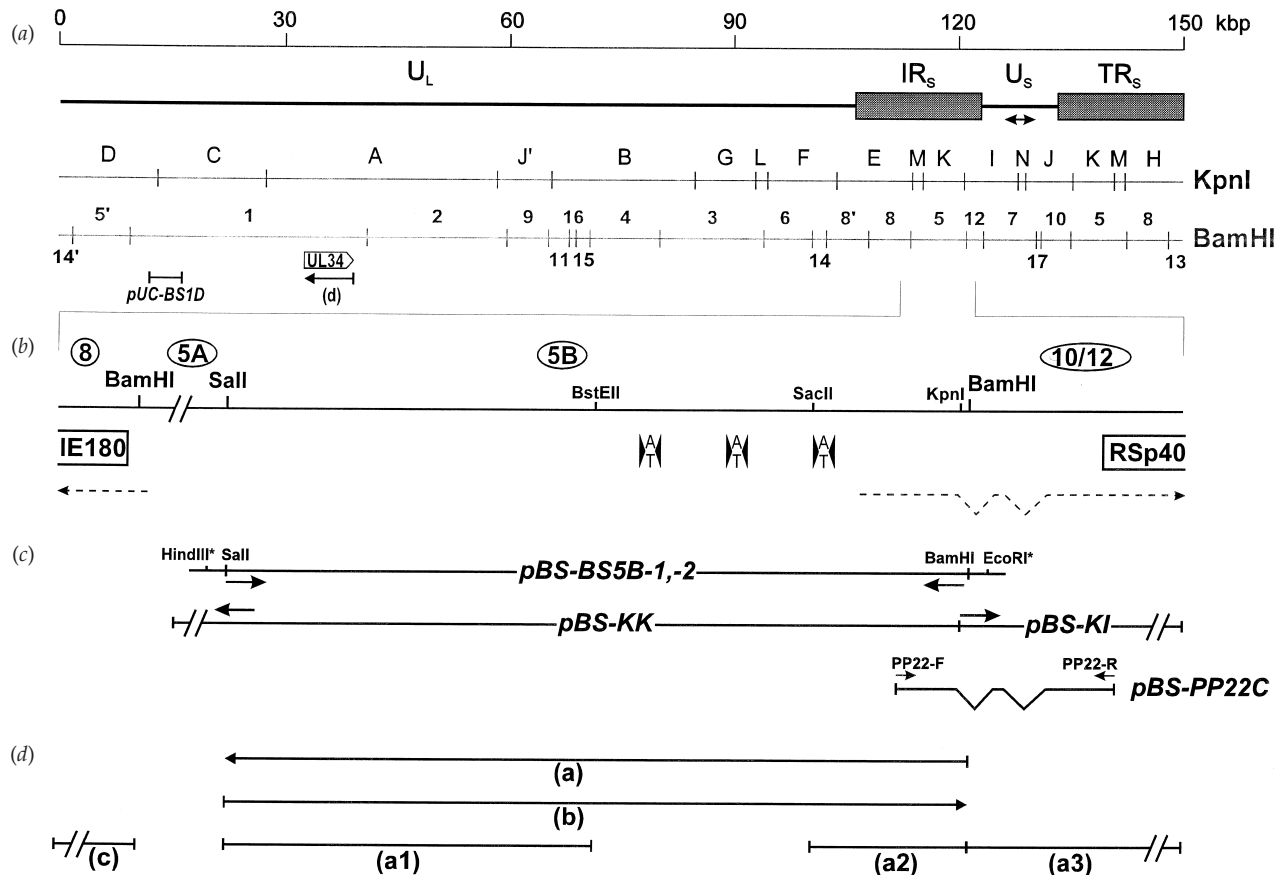


Fig. 1. (a) Physical map of the PrV genome consisting of a long (U_L) and a short (U_S) unique region. Inverted repeat sequences (IR_S , TR_S) permit isomerization (\leftrightarrow) of the U_S region. *KpnI* and *BamHI* restriction fragments and relevant plasmids are indicated. (b) Enlarged map of the genome analysed with the restriction sites used and *BamHI* fragment numbers. mRNAs identified (dotted arrows) and the corresponding ORFs (open and pointed rectangles) are shown. Arrowheads mark three pairs of the OBP-recognition sequence GTTGAC, each spaced by AT-rich DNA sequences. (c) Plasmids used for determination of the DNA sequence. After digestion with the restriction enzymes indicated and thionucleotide-protection (*) of one site, sets of unidirectionally shortened deletion plasmids were generated. Arrows denote the directions of DNA sequencing. The oligonucleotide primers PP22-R and -F were used to amplify the RSp40 cDNA fragment cloned in pBS-PP22C. (d) Strand-specific, ^{32}P -labelled RNA probes for Northern blot analyses were synthesized (*In vitro* transcription kit, Stratagene) from pBS-BS5B and from the plasmid-cloned UL34 gene (arrows). The isolated *BamHI* fragments 8 and 12, as well as subfragments of *BamHI* fragment 5 (bars), were ^{32}P -labelled after random priming (Rediprime system, Amersham). Designations of the probes refer to Fig. 2 and the text.

the vector-specific M13 universal or reverse primers. Both strands of the DNA fragment investigated were analysed completely and proved to be complementary at all positions. To confirm the adjacent localization of the genomic *BamHI* fragments 5 and 10 or 5 and 12 (Fig. 1*b*), the terminal part of pBS-KI (Fig. 1*c*) was also sequenced. Finally, by sequencing of pBS-KK (Fig. 1*c*) with PrV-specific custom primers (Gibco BRL), an overlap with characterized upstream sequences was generated. Data were assembled and analysed by using the GCG software package in UNIX version 10.0 (Devereux *et al.*, 1984).

The PrV DNA sequence obtained (GenBank accession no. AJ251976) is 2462 bp in length and exhibits a G + C content of 69.5%, which is slightly below the mean percentage of 73% (Ben-Porat & Kaplan, 1985). The 2396 bp inserts of the two

plasmids analysed (pBS-BS5B-1 and -2) proved to be identical. At both ends, the novel sequence overlaps with previously described DNA sequences of the same PrV strain (Ka). Nucleotides 1–39 correspond to the reverse of the first bases of a sequence of the PrV IE180 gene region (Vlcek *et al.*, 1990; GenBank accession no. M34651) and nucleotides 2424–2462 are identical to bases 1–39 of *BamHI* fragment 10 (Zhang & Leader, 1990; GenBank accession no. D00676).

The DNA fragment characterized contains several open reading frames (ORFs) of up to 315 codons (not shown). However, the deduced translation products share no homologies with known herpesvirus proteins. Furthermore, these ORFs are not preceded by TATA box-like elements and putative mRNA polyadenylation signals (AATAAA) are completely absent.

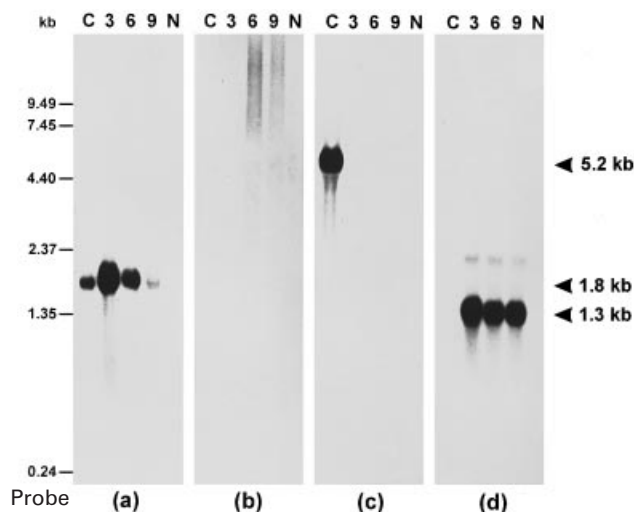


Fig. 2. Northern blot analysis of total RNA (10 µg per lane) from PrV-infected (m.o.i. = 10) and uninfected (N) PSEK cells. Cells were harvested 6 h after infection in the presence of 100 µg/ml cycloheximide (C) or after 3, 6 and 9 h in the absence of inhibitors. RNA was separated in formamide-containing 0.8% agarose gels and blots were hybridized with the probes indicated in Fig. 1 (a, d). Sizes of RNA markers and of the viral RSp40 (1.8 kb), IE180 (5.2 kb) and UL34 (1.3 kb) mRNAs are given.

In order to investigate transcription of this part of the virus genome, porcine kidney (PSEK) cells were infected with PrV at an m.o.i. of 10 and incubated in the presence or absence of 100 µg/ml cycloheximide as an inhibitor of protein synthesis. Total RNA was prepared and Northern blot analyses were performed essentially as described previously (Fuchs & Mettenleiter, 1996). With ³²P-labelled cRNA probes of pBS-BS5B (Fig. 1d), a PrV-specific RNA of 1.8 kb was identified (Fig. 2a), whereas no defined viral transcripts from the opposite strand were detectable (Fig. 2b). The 1.8 kb RNA was shown to be polyadenylated, since it was bound by oligo(dT)-coupled magnetic beads (Dynal) (data not shown). Blot hybridization with labelled *Bam*HI fragment 12 (probe a3, Fig. 1d) as well as with pBS-BS5B subfragment a2 but not with a1 (Fig. 1d) revealed that the transcript detected represents an mRNA of the RSp40 gene of PrV (data not shown). The RSp40 mRNA was described previously not to be expressed in the presence of cycloheximide (Zhang & Leader, 1990). However, our results demonstrate expression of the 1.8 kb RNA under immediate-early conditions, although it becomes more abundant at early times after infection in the absence of drugs (Fig. 2a). In contrast, the major immediate-early mRNA of IE180 (Fig. 2c) accumulates only in the presence of cycloheximide, indicating that transcription of this gene is rapidly down-regulated after the onset of virus replication. Other transcripts of PrV, e.g. that of UL34 (Fig. 2d) or UL23 (thymidine kinase, not shown), were not found in cycloheximide-treated cells, demonstrating that the inhibition of viral protein synthesis was sufficient in our experiments and that the discrepancy from earlier results might be due to the different cell line used or to

a higher sensitivity of our hybridization probes. Thus, RSp40 of PrV, like the homologous ICP22 of HSV-1 and BHV-1 (Roizman & Sears, 1996; Schwyzer *et al.*, 1994), can be expressed from an immediate-early RNA and is possibly required for efficient transcription of early and late viral genes.

Another common feature of the ICP22 mRNAs of HSV-1 and BHV-1 is the removal of introns from their 5'-untranslated sequences (Roizman & Sears, 1996; Schwyzer *et al.*, 1994; Wirth *et al.*, 1991). It appears likely that the 1.8 kb RSp40 mRNA of PrV is similarly spliced, since we localized its 5' end within *Bam*HI fragment 5 but the predicted initiation codon is at positions 584–586 and the polyadenylation signal is at positions 1782–1787 of the downstream *Bam*HI fragment 10 or 12 (GenBank accession no. D00676; Zhang & Leader, 1990). To test this hypothesis, we tried to characterize the 5' end of the detected mRNA by PCR amplification of oligo(dC)-tailed cDNA (5' RACE system, Gibco BRL), which was prepared from polyadenylated RNA obtained 3 h after PrV infection (m.o.i. = 10) of PSEK cells. In repeated experiments with an oligo(dG) primer and the antisense primer PP22-R (reverse of nt 588–610 of D00676; Fig. 1c), we were not able to detect defined amplification products, indicating premature termination of cDNA synthesis at various positions. However, with primers PP22-F (nt 2260–2292 of GenBank accession no. AJ251976; Fig. 1c) and PP22-R, a 498 bp cDNA fragment could be amplified reproducibly and plasmid-cloned (pBS-P22C, Fig. 1c). Sequencing of pBS-P22C revealed the absence of two internal 121 and 155 bp fragments of genomic PrV DNA (nt 2410 of AJ251976 to nt 107 of D00676 and nt 161–315 of D00676). The 5' and 3' ends of both introns and the surrounding nucleotides fit the consensus sequences of eukaryotic splice-donor and splice-acceptor sites (Breathnach & Chambon, 1981). Although the precise 5' end of the RSp40 mRNA and possible further post-transcriptional modifications remain to be determined, we identified the next well-conserved TATA-box motif (TATATAT) at positions 2000–2006 of the novel DNA sequence. The functions of the extended mRNA and intron sequences upstream of the RSp40 coding region remain unclear.

Most alphaherpesvirus origins of DNA replication contain the conserved recognition sequence GTTCGCAC of the viral origin-binding protein (OBP) (Elias & Lehman, 1988; Koff & Tegtmeyer, 1988). The DNA sequence of *Bam*HI–*Sal*I fragment 5B of the PrV genome contains three complementary pairs of this motif at positions 1407–1414 and 1457–1464, 1687–1694 and 1737–1744 and 1966–1973 and 2016–2023 (Fig. 1b). The 42 nucleotides between the two predicted OBP-binding sites of each pair contain between 86 and 89% A and T residues, which corresponds to the base compositions in the central regions of other alphaherpesvirus origins located at related positions within the IR_S and TR_S sequences flanking the U_S genome regions of HSV-1 and -2, VZV, EHV-1 and BHV-1 (Ori_S) (Baumann *et al.*, 1989; Lockshon & Galloway, 1988; Schwyzer *et al.*, 1994; Stow &

McMonagle, 1983; Stow & Davison, 1986) or within the U_L regions of HSV-1 and -2 and PrV (Ori_L) (Lockshon & Galloway, 1988; Klupp *et al.*, 1992). In contrast to the situation in other alphaherpesvirus genomes, the AT-rich sequences of the predicted Ori_S of PrV are poorly palindromic. Whereas in the IR_S and TR_S sequences of HSV-1, EHV-1 and VZV, only single Ori_S elements were found, they are duplicated in HSV-2 and BHV-1 DNA. In the case of PrV, three directly repeated copies of the putative Ori_S domain were found close to each other. These are presumably the consequence of ancient duplication events, since nucleotides 1353–1630 share sequence identities of 88.5 and 86.2% with the following nucleotides 1631–1911 and 1912–2172, respectively.

In order to verify the function of PrV Ori_S *in vitro*, rabbit kidney (RK13) cells were co-transfected (lipofectamine plus reagent, Gibco BRL) with pBS-BS5B (Fig. 1c) and the control plasmid pUC-BS1D (Fig. 1a), which contains a randomly chosen 2 kbp *SalI* fragment of the PrV genome cloned in pUC19 (New England Biolabs). One day after transfection, half of the cell sample was infected with PrV at an m.o.i. of 5 and harvested 8 h later. DNA of infected and uninfected cells was prepared as described previously (Fuchs & Mettenleiter, 1996) and doubly digested with *EcoRI* and *HindIII* to release the cloning vectors pBS (-) (3.2 kbp) and pUC19 (2.7 kbp). Digestion with modification-specific restriction enzymes followed by electrophoretic separation and Southern blot hybridization with ^{32}P -labelled pUC19 allowed discrimination between Dam-methylated input plasmids and newly synthesized DNA (Fig. 3). After PrV infection, about a third of the pBS-BS5B DNA was resistant to cleavage by the methylation-dependent enzyme *DpnI* and, consequently, cleavable by *DpnII*, which is inhibited by Dam-methylation. In contrast, the control plasmid pUC-BS1D remained completely sensitive to *DpnI* and resistant to *DpnII* (Fig. 3, left two lanes). Plasmid replication was not detectable in uninfected cells, indicating that viral proteins are required for initiation. Furthermore, it was confirmed (Fig. 3, right part) that no unique elements of the vector pBS (-) are responsible for autonomous DNA replication of the cloned *BamHI-SalI* fragment 5B. The minimum sequence requirement of PrV Ori_S and the influence of the consensus sequence copy number on the efficiency of viral DNA replication remain to be tested.

In summary, we have closed one of the gaps within the genomic DNA sequence of PrV and confirmed the close phylogenetic relationship between mammalian alphaherpesviruses (McGeoch & Cook, 1994). RNA splicing and expression kinetics of the ICP22 homologue of PrV, as well as the adjacent position of the replication origin Ori_S , were very similar to the situation found in other members of this subfamily. However, the high copy number of three Ori_S elements in each of the inverted repeat sequences, together with the presence of two additional replication origins within the U_L region (Klupp *et al.*, 1992; Kupersmidt *et al.*, 1991), appear to be structural characteristics of the PrV genome.

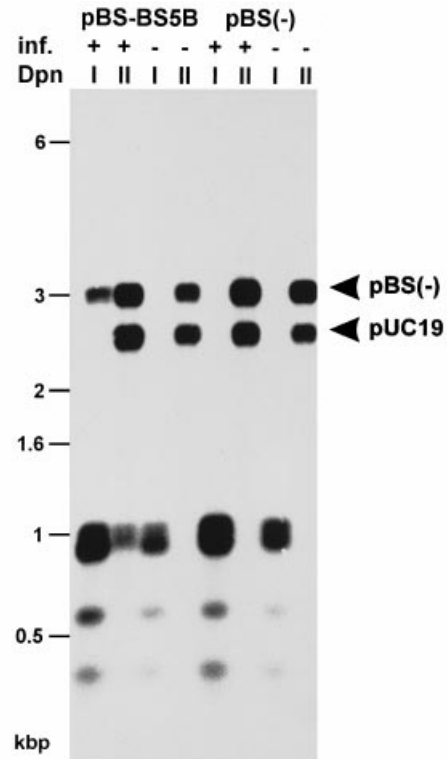


Fig. 3. For DNA replication assays, monolayers of RK13 cells grown in 6-well plates were transfected with 1 μ g per well each of pBS-BS5B and pUC-BS1D or pBS (-) and pUC-BS1D (see Fig. 1a, c) and, after 24 h, half of the cell sample was infected (inf.) with PrV at an m.o.i. of 5. DNA of infected (+) and uninfected (-) cells was doubly digested with *EcoRI* and *HindIII* followed by treatment with either *DpnI* or *DpnII* as shown. After separation in a 0.7% agarose gel, the DNA was transferred to nylon membrane (Hybond-N+, Amersham) and hybridized with ^{32}P -labelled (Rediprime system, Amersham) plasmid pUC19. Sizes of DNA markers and the detected vectors pBS (-) and pUC19 are indicated.

Possibly, these could be among the prerequisites that enable PrV to replicate faster and in a broader range of host cells and animal species than most of its cognates.

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