DNA sequence of the UL6 to UL20 genes of infectious laryngotracheitis virus and characterization of the UL10 gene product as a nonglycosylated and nonessential virion protein

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The 24 kbp KpnI restriction fragment A from the unique long genome region of infectious laryngotracheitis virus (ILTV, gallid herpesvirus-1) has been sequenced. The analysed region contains 14 open reading frames sharing homology with conserved alphaherpesvirus genes. Arrangement of the UL6 to UL20 homologues of ILTV is almost identical to that found in the herpes simplex virus type 1 genome. As in other herpesviruses the UL15 gene consists of two exons and is expressed from a spliced mRNA. However, the UL16 gene, which is usually localized within the intron sequence of UL15, is not conserved at this position of the ILTV genome. Another unique feature is the absence of any putative N-glycosylation motifs within the deduced ILTV UL10 gene product, which is the homologue of the conserved herpesvirus glycoprotein M. After preparation of a monospecific antiserum, two distinct UL10 proteins with apparent molecular masses of 36 and 31 kDa were identified in ILTV-infected cells as well as in purified virions. None of these UL10 gene products is modified by N- or O-linked glycosylation. Isolation of a green fluorescent protein-expressing UL10 deletion mutant of ILTV revealed that this gene is not required for virus replication in cell culture.

Introduction

The molecular analysis of the genome of infectious laryngotracheitis virus (ILTV, gallid herpesvirus-1) is of particular interest for two reasons. First, infectious laryngotracheitis is an economically important respiratory disease of chickens, and prevention might be substantially improved by the development of genetically engineered vaccines (Bagust & Guy, 1997). Second, our knowledge of ILTV might contribute to a more profound understanding of herpesvirus evolution. Previous studies indicated that, although classified as a member of the Alphaherpesvirinae subfamily of the Herpesviridae (Roizman, 1996), ILTV exhibits a considerable phylogenetic distance to all other mammalian and avian herpesviruses investigated so far (Griffin & Boursnell, 1990; McGeoch & Cook; 1994; Johnson & Tyack, 1995). On the other hand, early DNA analyses demonstrated that ILTV possesses a typical alphaherpesvirus class D genome consisting of a long (UL) and a short (US) unique region, which is flanked by inverted repeat (IR, TR) sequences (Leib et al., 1987; Johnson et al., 1991; Roizman, 1996).

Up to now, ca. 90 kbp of the 155 kbp ILTV genome have been analysed by DNA sequencing (Fig. 1a). The characterized genome parts include the entire US region, which encodes a cluster of conserved alphaherpesvirus glycoproteins (Wild et al., 1996), and the adjoining inverted repeat sequences encoding the ILTV homologue of the major immediate early protein ICP4 of herpes simplex viruses (Johnson et al., 1995). The DNA sequences of the left and right ends of the UL genome region (Fuchs & Mettenleiter, 1996; Johnson et al., 1997) indicated a collinear arrangement of conserved alphaherpesvirus genes also within this part of the genome. As in the completely sequenced type D genomes of varicella-zoster virus (VZV; Davison & Scott, 1986) and equine herpesvirus-1 (EHV-1; Telford et al., 1992), the UL region of ILTV is apparently fixed in the opposite orientation to the prototypic isoemer of the herpes simplex virus type 1 (HSV-1) E genome.
Fig. 1. Genome structure of ILTV. (a) The herpesvirus type D genome of ILTV consists of a unique long (UL) and a unique short (US) region, which is flanked by inverted repeat (IR, TR) sequences. Previously sequenced segments are shaded, and locations of KpnI restriction fragments as well as selected genes are indicated. The depicted viral inserts of cosmids pCI-X12, -SB27, -E19 and -S28 were used for sequence analysis of the genomic DNA fragment KpnI A. (b) Enlarged map of the analysed genome part with KpnI and EcoRI (short vertical bars) restriction sites. Nucleotide numbers refer to GenBank entry AJ131832. The identified ORFs (pointed rectangles) are named according to their homologues in HSV-1. Identified viral mRNAs are indicated by dotted arrows. For mRNA detection (Fig. 2), the labelled cRNA probes A–D were transcribed in vitro from plasmids pILT-E42 and -E38, respectively. The synthetic oligonucleotide primers U15-R (nt 7229–7247) and U15-F (reverse of nt 10182–10164) were used for reverse transcription, amplification and sequencing of the spliced UL15 mRNA (Fig. 3). (c) Plasmid pBl-SX3.8 contains the authentic UL10 gene of ILTV. An EcoRV–PvuII fragment comprising UL10 codons 164–342 was replaced by a GFP expression cassette in pBl-ΔUL10G, which was used for generation of ILTV recombinants. For prokaryotic expression, an EcoRI fragment containing the 3′-terminal codons 320–393 of the UL10 ORF was fused to the GST gene in pGEX-UL10.

To continue our investigations of the ILTV genome, we sequenced part of the UL region located between the characterized UL5 (Fuchs & Mettenleiter, 1996) and UL21 (Ziemann et al., 1998a) genes. This region includes the ILTV homologue of the UL10 gene of HSV-1, which was shown to encode a nonessential virion glycoprotein, gM (Baines & Roizman, 1993). The gM gene remained the last of the so far described alphaherpesvirus glycoprotein genes to be detected in the ILTV genome, since homologues of the genes encoding gB (Griffin, 1991), gC (Kingsley et al., 1994), gD, gE, gG, gl, gp60 which is a positional homologue of gl (Wild et al., 1996), gK (Johnson et al., 1997), gl (Fuchs & Mettenleiter, 1996), gH and gN (Ziemann et al., 1998a) were already described (Fig. 1a). However, only the gB, gC, gG and gp60 gene products of ILTV were identified and characterized up to now (Kongsuwan et al., 1993a,b; Kingsley et al., 1994; Poulsen & Keeler, 1997). To identify the predicted gM protein of ILTV, a part of the UL10 ORF was expressed in E. coli, and a rabbit antiserum was generated against the recombinant protein. The antiserum was used to detect the gM protein in Western blots of ILTV-infected cell lysates.

(McGeoch et al., 1988). However, recent sequence analyses revealed several unique features of the ILTV genome. One of them is a large internal inversion within the UL region, which includes the UL22 to UL44 gene homologues (Ziemann et al., 1998a), and another is the translocation of a UL47 homologous gene to the US region (Wild et al., 1996). Even more salient was the identification of a considerable number of apparently expressed open reading frames (ORFs) which are presumably ILTV-specific, since they exhibit neither structural nor positional identity to any other known herpesvirus genes. A set of five unique ORFs was found to be clustered adjacent to a functional origin of replication between the conserved UL22 and UL45 genes (Ziemann et al., 1998a). Two other ILTV-specific genes, which are located at the right end of the UL region (Fuchs & Mettenleiter, 1996; Ziemann et al., 1998b), are related to each other but not to the ICP0 genes found at a similar position within other alphaherpesvirus genomes (Everett et al., 1993).
prepared against the immunoprotein and tested in different immunochemical assays. Furthermore, a UL10-negative ILTV mutant was generated.

Methods

■ Virus and cells. A pathogenic ILTV strain (obtained from D. Lütstücke, Boxmeer, NL) was propagated in primary chicken kidney cells as described earlier (Fuchs & Mettenleiter, 1996). Transfection experiments were performed with the chicken hepatoma cell line LMH (Kawaguchi et al., 1987), which was maintained in minimum essential medium supplemented with 10% foetal calf serum. For plaque assays, ILTV-infected LMH cells were overlaid with medium containing 0.8% methyl cellulose.

■ Cosmid and plasmid cloning of ILTV DNA. Viral DNA was prepared from infected chicken kidney cells as described previously (Fuchs & Mettenleiter, 1996), partially digested with Sau3A I, and dephosphorylated at the 5’ ends. The DNA was then ligated to BamH I-digested vector SuperCos I, packaged in vitro into λ phage particles (Gigapack III XL), and used for infection of E. coli strain XL-I Blue MR (all purchased from Stratagene). In another approach, bacteria were transformed with EcoRI-digested ILTV DNA which had been ligated into plasmid pBS(−) (Stratagene). DNA was prepared from the obtained cosmids and plasmid clones (Qiagen plasmid kit, Qiagen) and characterized by restriction analyses, Southern blot hybridizations and sequencing of the insert termini with vector-specific T3 and T7 primers.

DNA sequencing. The entire sequence of the 24 kbp KpnI fragment A of ILTV DNA was determined using cosmids pCI-E19, -S28, -SB27 and -X12 (Fig. 1a). Starting from the characterized flanking regions (Fuchs & Mettenleiter, 1996; Ziemann et al., 1998) and from the known internal sequences of plasmid-cloned EcoRI fragments of ILTV DNA (pLT-E38, -E42, -E46; Fig. 1b), custom-made primers (GibcoBRL) were derived. Sequencing reactions were performed with the Thermo Sequenase cycle sequencing kit (Amersham) and [α-35S]dATP (ICN) for labelling. The products were separated on denaturing 5% polyacrylamide gels containing 7 M urea in Tris–borate electrophoresis buffer, which were then dried and exposed to X-ray film (X-OMAT AR, Kodak). The obtained sequence data were assembled and analysed with the GCG software package in UNIX version 9.1 (Devereux et al., 1984).

■ Northern blot analyses. Total RNA was prepared from non-infected chicken kidney cells and from cells harvested 10 h after ILTV infection at an m.o.i. of 5. The RNA was separated in agarose gels, transferred to nylon membranes and hybridized with 32P-labelled RNA probes as described previously (Fuchs & Mettenleiter, 1996). Strand-specific probes were transcribed in vitro from plasmids pLT-E38 and -E42 (Fig. 1b) with T3 and T7 RNA polymerases, respectively.

■ Reverse transcription and PCR amplification of viral mRNA. RNA of ILTV-infected and noninfected cells (5 µg each) was hybridized with 2.5 pmol of each primer U15-R (nt 7229–7247) and incubated for 1 h at 42 °C with 200 U reverse transcriptase (SuperScript II, GibcoBRL). After 15 min incubation at 70 °C template RNA was digested with RNases H and T1 for 30 min at 37 °C. Aliquots of the cDNAs, or 10 ng of genomic ILTV DNA as control, were amplified by PCR with 20 pmoles of primers U15-R and U15-F (reverse of nt 10164–10182), 2.5 mmol of each dNTP and 1 U Deep Vent DNA polymerase (New England Biolabs). An initial denaturation step for 1 min at 97 °C was followed by 35 cycles of 95 °C and 55 °C for 30 s each, and 72 °C for 3 min (Primus 96 Thermocycler, MWG Biotech). After phenol extraction and ethanol precipitation, the PCR products were treated with Klenow polymerase and polynucleotide kinase, purified from agarose gels (Qiagen gel extraction kit, Qiagen), cloned into Smal-digested plasmid pBS(−), and analysed by DNA sequencing.

■ Generation of a UL10-negative ILTV mutant. To obtain a transfer plasmid for deletion of the ILTV UL10 gene the green fluorescent protein (GFP) expression vector pEFP-N1 (Clontech) was doubly digested with BgII and BanHI and religated to remove the multiple cloning site located between the GFP reading frame and the preceding human cytomegalovirus (HCMV) immediate early gene promoter. The modified expression cassette was chosen as a 1581 bp Ascl–AflII fragment, blunt-ended with Klenow polymerase and inserted into the polylinker region of the Smal-digested plasmid pbLueScript SK(−) (Stratagene). In the same vector, a 3831 bp SpeI–XhoI fragment of the ILTV genome was subcloned from cosmid pCI-E19. From the resulting plasmid pBl-SX3,8 (Fig. 1c), a 1973 bp SpeI–EcoRV fragment and a 1324 bp Poul–XhoI fragment were subsequently recloned in the novel GFP expression vector, which had been treated with BanHI, Klenov enzyme and SpeI, or EcoRV and XhoI, respectively. The final plasmid, pBl-UL10G (Fig. 1c), carries a 534 bp deletion of ILTV DNA sequences (nt 15103–15636) representing UL10 codons 164–342, which are replaced by the GFP reporter gene. Subconfluent monolayers of LMH cells were transfected with pBl-UL10G (ca. 10 µg per 105 cells) by calcium phosphate coprecipitation (Mammalian transfection kit, Stratagene). After 24 h the inoculum was removed, the cells were infected with ILTV at an m.o.i. of 1, and further incubated at 37 °C until complete lysis occurred. Serial dilutions of the progeny virus and plaque assays were performed to determine the ratio between GFP-expressing recombinants and wild-type ILTV, which was monitored in a fluorescence microscope between 2 and 5 days after infection. The ILTV recombinants were then isolated by limiting dilutions of the transfection progeny on primary chicken kidney cells grown in microtitre plates. The procedure was repeated until the selected virus populations appeared homogeneous, and a single GFP-expressing isolate designated ILTV-∆UL10G was further analysed.

■ Southern blot analyses. Restriction fragments of ILTV DNA (1 µg per lane) were separated in 0.7% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham) by standard procedures. The blots were incubated overnight in 720 mM NaCl, 80 mM NaHPO4, 4 mM EDTA, 1% SDS, 0.5% low-fat milk, 0.5 µg/ml denatured herring sperm DNA at 62 °C. After 6 h, the probes were added, which had been prepared by labelling (Rediprime system, Amersham) of plasmid DNA with [α-32P]dCTP (ICN). Blots were washed at 62 °C twice with 2 x SSC (300 mM NaCl, 30 mM sodium citrate), 0.5% SDS, and three times with 0.1 x SSC, 0.5% SDS for 30 min each, and finally exposed to X-ray film.

■ Prokaryotic expression, preparation of antiserum and Western blot analysis. A 414 bp EcoRI fragment encoding the C terminus (aa 320–393) of the predicted UL10 protein was recloned from pBl-SX3,8 into expression vector pGEX-4T2 (Pharmacia). An expected 37 kDa fusion protein was expressed and purified as described (Fuchs et al., 1996). A rabbit was immunized four times at 2 week intervals by intramuscular injection of 100 µg of the fusion protein emulsified in mineral oil. Sera collected before and after immunization were analysed. For that purpose, lysates of ILTV-infected (m.o.i. of 5, 24 h after infection) and noninfected chicken kidney cells were separated on discontinuous SDS–polyacrylamide gels (Laemmli, 1970) and electrotransferred to nitrocellulose filters (TransBlot cell, Bio-Rad). The blots were subsequently incubated for 1 h each with 5% low-fat milk, rabbit antiserum and peroxidase-conjugated secondary antibodies (Dianova), all diluted in TBS-T (10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.25% Tween 20).
After repeated washing, antibody binding was visualized by luminescence (ECL Western blot detection system, Amersham) and recorded on X-ray film.

Glycosidase treatment of virion proteins. ILT and pseudorabies virions were sedimented from the supernatants of infected cells, and further purified by centrifugation through step-gradients of 30, 40, and 50% sucrose (Klupp et al., 1992a). The particles collected from the interface between 40% and 50% sucrose were first treated with neuraminidase (1 mM/µg protein) in 50 mM sodium acetate (pH 5.2), 4 mM CaCl₂ for 2 h at 37 °C and then sedimented for 1 h at 45 000 r.p.m. in a Beckman TLA45 rotor. For treatment with β-galactosidase (0.1 mM/µg) the pellet was resuspended in 20 mM Tris–phosphate (pH 7.4), and incubation with β-galactosidase F (20 mM/µg) was performed in 50 mM potassium phosphate (pH 7.2), supplemented with 50 mM EDTA and 0.5% CHAPS (all enzymes were purchased from Boehringer Mannheim). After 20 h at 37 °C, proteins (ca. 5 µg per lane) were denatured for 5 min at 56 °C in sample buffer (Laemmli, 1970) containing 2% SDS and 5% β-mercaptoethanol, and subjected to electrophoresis and Western blot analysis as described above.

Results and Discussion

Sequence of KpnI fragment A of ILTV DNA

Physical mapping of the ILTV genome (Johnson et al., 1991) revealed that the largest KpnI restriction fragment A is localized within the right part of the U₁ region. We sequenced this fragment using cosmids which contain overlapping, randomly cloned ILTV DNA fragments (Fig. 1a). The sequence was verified by subsequent analysis of at least two independent clones for every part of the analysed genome region with ILTV-specific custom primers. To minimize band compressions and nonspecific termination, all sequence reactions were performed with a thermostable DNA polymerase at 72 °C in the presence of 7-deaza dGTP. Therefore, only very few sequence ambiguities had to be resolved by reverse-strand analysis. The total length of the assembled DNA sequence (GenBank accession no. AJ131832) is 24 300 bp, and no differences were found between the duplicate clones used for characterization. The novel sequence overlaps with described parts of the ILTV genome at both ends. From position 1 to 22 the sequence corresponds to the 5’ upstream region of UL21 (Ziemann et al., 1998a; GenBank no. Y14301), and from position 24 140 to 24 300 the sequence overlaps with the UL5 gene (Fuchs & Mettenleiter, 1996; GenBank no. X97256). The KpnI fragment A itself is 24 124 bp in size, and contains 44.9% G and C residues, which is in good agreement with the predicted overall G+C content of ILTV DNA of 45% (Plummer et al., 1969).

Localization and putative functions of conserved genes

The newly determined ILTV DNA sequence contains 14 ORFs sharing homologies with characterized alphaherpesvirus genes (Table 1) whose arrangement (Fig. 1b) is almost perfectly collinear to that found in HSV-1, VZV, EHV-1, bovine herpesvirus-1 (BHV-1) and pseudorabies virus (PrV) DNA (Davison & Scott, 1986; McGeoch et al., 1988; Telford et al., 1992; Vlcek et al., 1995; Klupp et al., 1992b; Dijkstra et al., 1997b). Within the analysed genome region no unique genes

<table>
<thead>
<tr>
<th>ORF</th>
<th>Gene product/function</th>
<th>HSV-1 (x)</th>
<th>VZV (x)</th>
<th>EHV-1 (x)</th>
<th>EBV (γ)</th>
<th>HCMV (β)</th>
<th>HSV-1/ VZV</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL20</td>
<td>Intrinsic membrane protein/virus egress</td>
<td>22.5</td>
<td>24.9</td>
<td>25.7</td>
<td>ND</td>
<td>ND</td>
<td>22.8</td>
</tr>
<tr>
<td>UL19</td>
<td>Major capsid protein</td>
<td>37.6</td>
<td>38.9</td>
<td>41.0</td>
<td>29.7</td>
<td>30.0</td>
<td>53.2</td>
</tr>
<tr>
<td>UL18</td>
<td>Capsid protein</td>
<td>32.9</td>
<td>32.4</td>
<td>33.5</td>
<td>ND</td>
<td>ND</td>
<td>43.9</td>
</tr>
<tr>
<td>UL17</td>
<td>DNA cleavage and packaging</td>
<td>27.6</td>
<td>28.0</td>
<td>28.6</td>
<td>ND</td>
<td>ND</td>
<td>35.7</td>
</tr>
<tr>
<td>UL15</td>
<td>DNA cleavage and packaging</td>
<td>49.5</td>
<td>49.1</td>
<td>50.0</td>
<td>38.4</td>
<td>37.2</td>
<td>61.8</td>
</tr>
<tr>
<td>UL14</td>
<td>?</td>
<td>27.6</td>
<td>25.5</td>
<td>25.4</td>
<td>ND</td>
<td>ND</td>
<td>30.4</td>
</tr>
<tr>
<td>UL13</td>
<td>Protein kinase</td>
<td>26.7</td>
<td>26.1</td>
<td>30.8</td>
<td>25.0</td>
<td>21.6</td>
<td>32.5</td>
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<td>UL12</td>
<td>Alkaline nuclease</td>
<td>34.0</td>
<td>31.9</td>
<td>35.1</td>
<td>24.6</td>
<td>31.7</td>
<td>34.5</td>
</tr>
<tr>
<td>UL11</td>
<td>Myristylated membrane protein/virus egress</td>
<td>27.5</td>
<td>24.3</td>
<td>38.5</td>
<td>ND</td>
<td>ND</td>
<td>32.0</td>
</tr>
<tr>
<td>UL10</td>
<td>Intrinsic membrane protein (gM)</td>
<td>29.6</td>
<td>28.4</td>
<td>27.9</td>
<td>24.0</td>
<td>24.0</td>
<td>30.1</td>
</tr>
<tr>
<td>UL9</td>
<td>Origin-binding protein</td>
<td>38.6</td>
<td>38.6</td>
<td>40.3</td>
<td>ND</td>
<td>ND</td>
<td>46.5</td>
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<tr>
<td>UL8</td>
<td>Component of helicase–primase complex</td>
<td>22.2</td>
<td>28.4</td>
<td>26.4</td>
<td>ND</td>
<td>ND</td>
<td>29.6</td>
</tr>
<tr>
<td>UL7</td>
<td>?</td>
<td>26.0</td>
<td>28.4</td>
<td>29.8</td>
<td>ND</td>
<td>ND</td>
<td>34.0</td>
</tr>
<tr>
<td>UL6</td>
<td>DNA cleavage and packaging</td>
<td>36.4</td>
<td>38.7</td>
<td>38.1</td>
<td>27.2</td>
<td>25.3</td>
<td>42.2</td>
</tr>
</tbody>
</table>
Table 2. Properties of the identified ILTV genes

ORFs are named according to their HSV-1 homologues. Locations of coding regions and putative transcription signals refer to GenBank sequence no. AJ131832, and patterns located on the reverse strand are marked by (r). The expected minimum transcript sizes were evaluated from the distances between TATA- and poly(A) signals and compared to the viral RNAs detected by Northern blot analysis. Note that UL15 consists of two exons (UL15A and UL15B). NA, Not analysed.

<table>
<thead>
<tr>
<th>ORF</th>
<th>Codons</th>
<th>Location</th>
<th>TATA box</th>
<th>Poly(A) signal</th>
<th>Transcript size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UL19</td>
<td>1403</td>
<td>1032–5243</td>
<td>844–850</td>
<td>6440–6444</td>
<td>&lt; 5/6/NA</td>
</tr>
<tr>
<td>UL18</td>
<td>319</td>
<td>5427–6386</td>
<td>5365–5371</td>
<td>6440–6444</td>
<td>&lt; 1/1/NA</td>
</tr>
<tr>
<td>UL15B</td>
<td>395</td>
<td>7655–6468(r)</td>
<td>6453–6449(r)</td>
<td>2/6/NA</td>
<td></td>
</tr>
<tr>
<td>UL17</td>
<td>717</td>
<td>7723–9876</td>
<td>7674–7680</td>
<td>10155–10159</td>
<td>&lt; 2/5/NA</td>
</tr>
<tr>
<td>UL15A</td>
<td>369</td>
<td>11129–10023(r)</td>
<td>11421–11415(r)</td>
<td>2/1/NA</td>
<td></td>
</tr>
<tr>
<td>UL14</td>
<td>253</td>
<td>10963–11724</td>
<td>10877–10883</td>
<td>13001–13005</td>
<td>&lt; 1/5/NA</td>
</tr>
<tr>
<td>UL13</td>
<td>465</td>
<td>11580–12977</td>
<td>11515–11521</td>
<td>13001–13005</td>
<td>&lt; 1/5/NA</td>
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<td>UL12</td>
<td>526</td>
<td>13157–14737</td>
<td>13085–13091</td>
<td>14883–14887</td>
<td>&lt; 1/5/15</td>
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<tr>
<td>UL11</td>
<td>80</td>
<td>14626–14868</td>
<td>14485–14491</td>
<td>14883–14887</td>
<td>&lt; 0/4/0/5</td>
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<td>UL10</td>
<td>393</td>
<td>16126–14945(r)</td>
<td>16434–16428(r)</td>
<td>14923–14919(r)</td>
<td>&lt; 1/5/15</td>
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<td>UL9</td>
<td>892</td>
<td>16075–18753</td>
<td>15877–15883</td>
<td>21372–21376</td>
<td>&lt; 5/5/7 + 3/5</td>
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<tr>
<td>UL8</td>
<td>795</td>
<td>18746–21133</td>
<td>18690–18696</td>
<td>21372–21376</td>
<td>&lt; 2/7/2/9</td>
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<td>UL7</td>
<td>358</td>
<td>22257–21181(r)</td>
<td>22348–22342(r)</td>
<td>21125–21121(r)</td>
<td>&lt; 1/2/NA</td>
</tr>
<tr>
<td>UL6</td>
<td>713</td>
<td>24175–22034(r)</td>
<td>24263–24257(r)</td>
<td>21125–21121(r)</td>
<td>&lt; 3/1/NA</td>
</tr>
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</table>

of ILTV could be detected between the conserved ORFs. However, one conserved ORF of the above-listed mammalian alphaherpesviruses, but also of beta- and gammaherpesviruses, like HCMV (Chee et al., 1990) and Epstein–Barr virus (EBV, Baer et al., 1984), is apparently absent from the corresponding position in the ILTV genome. In HSV-1, this gene was named UL16, and its product was identified as a capsid-associated virion protein which is dispensable for virus replication in vitro (Nalwanga et al., 1996). Therefore, absence of a corresponding ORF from the ILTV genome would be likely, but in analogy to UL47 (Wild et al., 1996), the UL16 gene of ILTV might also reside at an as yet unknown position.

Using the GCG program ‘Gap’, the predicted ILTV proteins were compared pairwise with their homologues to determine the percentage of positionally conserved, identical amino acid residues (Table 1). The results of these studies clearly confirmed the classification of ILTV as an alphaherpesvirus, since related proteins of beta- and gammaherpesviruses exhibited much lower degrees of identity, if detectable at all. However, comparison of conserved proteins of different mammalian alphaherpesviruses to each other, for example that of HSV-1 to that of VZV, in most cases revealed significantly greater homologies than that found with the ILTV gene products (Table 1). Only few sequence data are available from the corresponding genome regions of other avian alphaherpesviruses. The predicted products of UL19 of turkey herpesvirus (SWISS-PROT no. Q86528), and UL9 of Marek’s disease virus (Wu et al., 1996) share only 37-3% and 40% of identical amino acids with the respective ILTV proteins, demonstrating that there is no close relationship between these viruses. Thus, in agreement with profound phylogenetic studies (McGeoch & Cook, 1994), our results confirm that ILTV should be considered as the only member of an alphaherpesvirus genus which evolved separately from the mammalian and other avian members of this herpesvirus subfamily.

Except for UL7 and UL14, gene functions were assigned to all HSV-1 counterparts of the newly identified ORFs of ILTV (Table 1; Roizman & Sears, 1996). In addition to their overall homologies, several of the predicted ILTV proteins contain distinct domains, indicating a similar virion localization or role during virus replication. For example, the deduced UL20 gene product of ILTV contains four extended hydrophobic regions which could represent transmembrane domains (not shown). The UL11 protein might be membrane-anchored by myristic acid, since an N-myristylation site was identified at amino acids 2–7 with the GCG program ‘Motifs’. Using the same program, a consensus sequence of serine/threonine protein kinases was found within the UL13 gene product of ILTV (aa 219–231). Within the predicted origin binding protein (UL9) both an ATP/GTP binding site (aa 102–109) and a leucine zipper (aa 171–193) are conserved.

Transcriptional analysis

In Table 2 the precise locations and sizes of the identified ILTV ORFs are listed together with the positions of putative transcription signals. Assuming usage of the first in-frame...
ATG codons, several of the adjacent ORFs overlap by up to 224 nucleotides (Table 2). TATA box-like elements which fit the consensus sequence TATA\textsuperscript{A}/AT\textsuperscript{A} (Breathnach & Chambon, 1981) with maximally one mismatch could be identified upstream of all predicted genes, whereas putative polyadenylation signals (AATAAA; Wickens, 1990) were not found behind every ORF (Table 2). This finding indicates that UL6 and 7, UL8 and 9, UL11 and 12, UL13 and 14, as well as UL18, 19 and 20, are presumably expressed from 3'-coterminal sets of transcripts. The calculated minimum sizes of the expected viral mRNAs are listed in Table 2, but the addition of poly(A) tails has to be considered. Until now, the apparent transcript sizes were determined only for one segment of the analysed genome part by Northern blot analyses (Fig. 2). Total RNA harvested 16 h after ILTV infection of cultured chicken kidney cells was separated, blotted and hybridized with \textsuperscript{32}P-labelled, strand-specific cRNA probes A, B and C (Fig. 1b). RNA standards are indicated on the left, and the sizes of the detected viral UL10, UL9, UL8.5 and UL8 mRNAs are given in Table 2.

![Fig. 2. Northern blot analysis. Total RNA of ILTV-infected (m.o.i. of 5, 16 h post-infection) and noninfected (n.i.) chicken kidney cells was separated in 1% formaldehyde-0.8% agarose gels, transferred to nylon membranes and hybridized with the \textsuperscript{32}P-labelled, strand-specific cRNA probes A, B and C (Fig. 1b). RNA standards are indicated on the left, and the sizes of the detected viral UL10, UL9, UL8.5 and UL8 mRNAs are given in Table 2.](image)

The detected viral RNAs of 1-9 and 0-5 kb (Fig. 2). The additional faint signal at 4 kb probably represents a read-through transcript of the upstream UL14 and UL13 genes. In accordance with the absence of complementary ORFs, with probe D (Fig. 1b) no viral RNAs could be identified (not shown).

The highly conserved UL15 protein, which is required for cleavage and encapsidation of the viral genome, was shown to be expressed from spliced mRNAs in HSV-1 and other herpesviruses (Baines & Roizman, 1992). To verify that the homologous ILTV gene has a similar structure, and to determine the precise splice junction between exons UL15A and UL15B, a part of the UL15 mRNA from ILTV-infected cells was reverse-transcribed and amplified with synthetic primers U15-F and U15-R (Fig. 1b), and reverse-transcribed RNAs of ILTV-infected (lane 2) and noninfected (lane 3) chicken kidney cells were amplified by PCR with primers U15-R and U15-F (Fig. 1b). DNA markers (M) and PCR products were separated in a 0.8% agarose gel. Sizes were determined by DNA sequencing. Sequencing of the amplified UL15 cDNA revealed a large deletion between nt 10023 and 7655 of genomic ILTV DNA. Both intron–exon boundaries match the consensus sequences of eukaryotic splice donor and acceptor sites, respectively (underlined and shaded nucleotides). Amino acid positions of the predicted UL15 protein are indicated.

![Fig. 3. Analysis of the ILTV UL15 mRNA. (a) Genomic ILTV DNA (lane 1), and reverse-transcribed RNAs of ILTV-infected (lane 2) and noninfected (lane 3) chicken kidney cells were amplified by PCR with primers U15-R and U15-F (Fig. 1b). DNA markers (M) and PCR products were separated in a 0.8% agarose gel. Sizes were determined by DNA sequencing. (b) Sequencing of the amplified UL15 cDNA revealed a large deletion between nt 10023 and 7655 of genomic ILTV DNA. Both intron–exon boundaries match the consensus sequences of eukaryotic splice donor and acceptor sites, respectively (underlined and shaded nucleotides). Amino acid positions of the predicted UL15 protein are indicated.](image)

Identification of the UL10 gene products of ILTV

The deduced UL10 gene product of ILTV consists of 393 amino acids (Table 2) with a predicted molecular mass of 43-1 kDa and an isoelectric point of 10-12, and it possesses homologues in all herpesvirus subfamilies (Table 1). All described UL10 homologues exhibit characteristics of multiply

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inserted membrane proteins containing six to eight hydrophobic domains and a more hydrophilic C-terminal region (Lehner et al., 1989). Hydrophilicity profiles of the predicted ILTV protein were determined with the GCG program (Lehner et al., 1996). Within the respective ILTV gene product the cysteine residue is present at position 51, but no consensus motifs for the addition of N-linked glycans could be detected within the entire amino acid sequence. To verify whether this might be a particular feature of the investigated virus isolate, we cloned and sequenced the UL10 genes of six additional field and vaccine strains of ILTV after PCR amplification of viral DNA. Sequence comparison revealed few differences, but none of the deduced proteins specified an N-glycosylation site (data not shown).

The UL10 gene products of several herpesviruses, including HSV-1, PrV, EHV-1, BHV-1, HCMV and EBV were already characterized as glycosylated virion proteins, and were therefore designated as gM (Lehner et al., 1989; Baines & Roizman, 1993; Dijkstra et al., 1996; Pilling et al., 1994; Wu et al., 1998; Lake et al., 1998). To identify the homologous protein of ILTV, we expressed the hydrophilic C-terminal part of the UL10 gene (aa residues 320–393) as a glutathione S-transferase (GST) fusion protein in E. coli (Fig. 1c). A rabbit antiserum raised against this fusion protein was tested in Western blot analyses and was shown to recognize two proteins with apparent molecular masses of 36 and 31 kDa in ILTV-infected cells (Fig. 4a, upper panel). These proteins were not detected by the respective preimmune serum, and the specific reactions of the anti-UL10 serum could be completely abolished by preincubation with the bacterial UL10 fusion protein, but not with other GST-fused expression constructs (data not shown). After metabolic labelling of ILTV-infected chicken cells with [35S]methionine, both the 36 and 31 kDa UL10 gene products were also detectable by immunoprecipitation, and indirect immunofluorescence reactions of the anti-UL10 serum in infected cells, which had been fixed and permeabilized with a 1:1 mixture of methanol and acetone, revealed local accumulations of the detected proteins in the cytoplasm, which might represent virion-containing Golgi-derived vesicles (data not shown). Finally, the presence of the ILTV gM homologue in sucrose-gradient-purified virion preparations could be demonstrated (Fig. 4b, upper panel).

Two forms of the UL10 gene product of ILTV could be identified reproducibly. These might represent differentially processed proteins, or result from initiation of translation at different sites. The latter possibility is supported by the detection of presumably two distinct UL10 mRNAs (Fig. 2a). Besides the predicted initiation codon, there are additional in-frame ATG codons at positions 20, 89 and 97 of the UL10 ORF. The calculated molecular masses of the deduced proteins are 43.1, 40.8, 33.6 or 32.6 kDa, respectively. Furthermore, there are putative signal peptidase cleavage sites (von Heijne, 1986) behind the first and second hydrophobic domains at positions 39 and 114 of the predicted protein. Cleavage would reduce the molecular masses to 38.6 or 30.7 kDa, which best correspond to the electrophoretic mobilities of the two detected proteins (Fig. 4). However, an aberrant electrophoretic behaviour of highly hydrophobic proteins, as implicated by in vitro translation studies of the UL10 homologue of PrV (Dijkstra et al., 1996), should also be considered.

To test for possible glycosylation of the UL10 proteins, gradient-purified ILT virosomes were treated with either N-glycosidase F or neuraminidase and O-glycosidase and subsequently analysed in Western blot with the anti-UL10 serum (Fig. 4b, upper panel). As expected from the known amino acid sequence, no N-linked sugars are present. Incubation with O-glycosidase does not increase the electrophoretic mobility of any form of the UL10 protein. In contrast, analysis of the
same samples with an ILTV-specific hyperimmune serum revealed alterations of several as yet uncharacterized proteins after treatment with either enzyme (data not shown). As an additional control, pseudorabies virions were prepared, treated similarly, and Western blots were incubated with a monoclonal antibody specific for gB. This protein is proteolytically cleaved during processing (Whealy et al., 1990), and besides traces of the unprocessed gBa (110 kDa), the chosen antibody detects the amino-terminal fragment gBb (68 kDa). Mobility shifts after glycosidase treatment revealed that PrV gB is N-glycosylated (Fig. 4b, lower panel).

Up to now, ILTV is the only herpesvirus species shown to generally express a nonglycosylated gM homologue. However, a similar phenomenon was observed in several PrV mutants, which exhibit spontaneous base changes within the conserved N-glycosylation consensus sequence and consequently express nonglycosylated UL10 proteins (Dijkstra et al., 1998). Another membrane protein, the UL49.5 gene product, which is glycosylated in several herpesviruses, was recently demonstrated to form a disulfide-linked complex with the gM protein in PrV, BHV-1 and EBV (Jöns et al., 1998; Lake et al., 1998; Wu et al., 1998). Within the ILTV genome a conserved UL49.5 gene was also identified (Ziemann et al., 1998), and characterization of its product is in progress.

Construction of a UL10-negative ILTV mutant

Although gM is structurally conserved throughout the herpesvirus family, little is known about its function. UL10 deletion mutants could be isolated from HSV-1, PrV and EHV-1 (Baines & Roizman, 1993; MacLean et al., 1993; Dijkstra et al., 1996; Osterrieder et al., 1996), demonstrating that gM is not required for replication of these viruses in cell culture. To investigate whether this is also the case for the homologous protein of ILTV we used a plasmid-cloned viral DNA fragment to substitute UL10 by a GFP expression cassette (pBl-∆UL10G; Fig. 1c). Due to the overlapping UL9 gene (Table 2), only a part of the UL10 ORF comprising codons 164–342 was deleted. As a consequence of this mutation the amino-terminal part including the first three hydrophobic domains of the UL10 protein might still be expressed, whereas the retained 3′-terminal gene fragment lacks an in-frame start codon. To generate an ILTV recombinant, chicken hepatoma cells (LMH) were transfected with the deletion plasmid, and infected with wild-type virus 24 h later. Virus progeny was screened for GFP-expressing recombinants and one of them was plaque-purified and further characterized.

Genomic DNA of the obtained ILTV mutant ΔUL10G and of wild-type virus was digested with EcoRI and analysed by Southern blot hybridization (Fig. 5). As expected, the radio-labelled plasmid pBl-SX3.8 (Fig. 1c) detected four 5·5, 1·1, 1·0 and 0·4 kbp fragments of wild-type ILTV DNA (Fig. 5b). In the genome of ILTV ΔUL10G the sizes of the 5·5 and 0·4 kbp fragments are altered to 5·1 and 2·0 kbp, respectively (Fig. 5b).

This is the expected consequence of the deletion of one, and the introduction of another EcoRI site in the transfer plasmid pBl-∆UL10G (Fig. 1c). Furthermore, a probe derived from the deleted UL10 gene reacted only with wild-type ILTV DNA (Fig. 5c), and a GFP gene probe hybridized only with the 2·0 kbp EcoRI fragment of the ILTV ΔUL10G genome (Fig. 5d). Analysis of the ethidium-bromide-stained gels (Fig. 5e) revealed no other than the described alterations of restriction patterns, and even by over-exposure of the blots no traces of the wild-type genome could be detected in ILTV ΔUL10G DNA. The successful UL10 gene deletion was further confirmed by Western blot analyses. Equal amounts of the ILTV-specific UL0 protein (Ziemann et al., 1998) were detectable 24 h after infection of chicken kidney cells with either wild-type virus, or ILTV ΔUL10G (Fig. 4a, lower panel). In contrast, no UL10 protein was detectable in cells infected with the virus mutant (Fig. 4a, upper panel). Strictly speaking, this result only demonstrates the absence of the C-terminal part of the ILTV UL10 protein, which was used for prokaryotic expression and antiserum preparation (Fig. 1c). Although the 5′-terminal fragment of UL10 is maintained in our virus mutant, it appears unlikely that this fragment, if stably expressed at all, still encodes a functional protein. We therefore conclude that, like its homologues in other alphaherpesviruses, the UL10 gene of ILTV is not required for replication in cell culture.
Preliminary investigations of the *in vitro* growth properties of ILTV ΔUL10G indicated deficiencies with respect to both plaque sizes and virus titres (data not shown). However, *trans* complementing cell lines and virus revertants have to be generated to determine the phenotypic effects of the UL10 gene deletion precisely. Since recent studies demonstrated that gM-negative PrV is attenuated in its natural host, the pig (Dijkstra et al., 1997b), *in vivo* experiments with ILTV ΔUL10G might also contribute to the development of improved ILTV vaccines for chickens.

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