Identification of transcripts and protein products of the UL31, UL37, UL46, UL47, UL48, UL49 and US4 gene homologues of avian infectious laryngotracheitis virus

Dorothee Helferich, Jutta Veits, Thomas C. Mettenleiter and Walter Fuchs

Institute of Molecular Biology, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald-Insel Riems, Germany

In the present study, the transcription and protein expression of seven genes of infectious laryngotracheitis virus (ILTV) were investigated: UL31 and UL37 possess homologues in all known avian and mammalian herpesviruses, whereas UL46–UL49 and US4 are only conserved in most alphaherpesviruses. A peculiarity of the ILTV genome is the translocation of UL47 from the unique long region to a position upstream of US4 within the unique short region. Northern blot analyses revealed that all of the analysed genes were transcribed most abundantly during the late (c) phase of replication, but the only true late (c2) gene was UL47. Using monospecific rabbit antisera, the protein products of all of the genes could be detected and localized in ILTV-infected cells. Considerable amounts of the UL31, UL47 and UL48 gene products were found in the cell nuclei, whereas the other proteins were restricted largely to the cytoplasm. Like the respective tegument proteins of other herpesviruses, the UL37 and UL46–UL49 gene products of ILTV were incorporated into virus particles, whereas the UL31 protein and the glycoprotein encoded by US4 (gG) were not detectable in purified virions. It was also demonstrated that the UL48 protein of ILTV is able to activate an alphaherpesvirus immediate-early gene promoter, which is also a typical feature of other US4 homologues. Taken together, these results indicate that the functions of all of the investigated ILTV proteins are related to those of their homologues in other alphaherpesviruses.

INTRODUCTION

Infectious laryngotracheitis virus (ILTV, Gallid herpesvirus 1) causes infectious laryngotracheitis, a worldwide respiratory disease of chickens (Guy & Bagust, 2003). ILTV has been classified as a member of the subfamily Alphaherpesvirinae of the family Herpesviridae and, because of its phylogenetic distance from all previously characterized alphaherpesviruses, is the only current member of the genus Iltovirus (Davison et al., 2005; Johnson & Tyack, 1995; McGeoch et al., 2000). However, recent DNA sequence analyses of the psittacid herpesvirus 1 (PsHV-1) genome has revealed considerable similarities to ILTV (Thureen & Keeler, 2006). An almost-complete DNA sequence of the ILTV genome, which has been assembled from several published sequence fragments (GenBank accession no. NC_006623) is 148 687 bp in size and contains 76 genes (Thureen & Keeler, 2006). However, the palindromic nucleotide sequence of a recently discovered second origin of viral DNA replication (GenBank accession no. AM238250) has not yet been included.

ILTV and PsHV-1 possess type D herpesvirus genomes (Roizman & Pellet, 2001) consisting of long and short unique regions (U1, U3) and of inverted repeat sequences (IRs, TRs) flanking the U5 region (Fig. 1a). In both genomes, gene content and arrangement are related to that found in members of the other alphaherpesvirus genera, Varicellovirus, Simplexvirus and Mardivirus (Davison et al., 2005). However, ILTV and PsHV-1 exhibit several features absent from other herpesvirus genomes, e.g. six genus-specific open reading frames (ORFs), one of them duplicated in ILTV (Thureen & Keeler, 2006; Ziemann et al., 1998a, b). Furthermore, a conserved gene cluster that includes the homologues of herpes simplex virus type 1 (HSV-1) UL22–UL44 is inverted in ILTV and PsHV-1 genomes, and the UL47 homologue is translocated from the U1 to the U5 genome region (McGeoch et al., 1988; Thureen & Keeler, 2006; Wild et al., 1996; Ziemann et al., 1998a). However, except for the absence of UL16 in ILTV and UL48 in PsHV-1 (Fuchs & Mettenleiter, 1999; Thureen & Keeler, 2006), none of the genes conserved in all other avian and mammalian alphaherpesviruses is missing.

Many conserved herpesvirus proteins are required for capsid formation, DNA replication and encapsidation (Roizman & Knipe, 2001; Roizman & Pellet, 2001) or nuclear egress, which involves the UL31 and UL34 proteins (Fuchs et al.,
Fig. 1. (a) Map of the ~150 kbp DNA genome of ILTV, which consists of long (U_L) and short (U_S) unique regions and inverted repeat sequences (IR_S, TR_S) flanking the U_S region. The analysed ORFs (pointed rectangles) and the corresponding viral transcripts (dotted arrows) are indicated, partly in enlarged sections. Bold vertical arrows mark the inversion of a conserved gene cluster within the U_L region and the translocation of UL47 to the U_S region. (b) pcDNA3-derived plasmids for in vitro transcription and translation of ILTV genes and for protein expression in eukaryotic cells, containing heterologous promoters (P_T7, P_SP6, P_HCVM-IE) and polyadenylation signals (A_BGH). (c) In pGex and pET23 plasmids for prokaryotic expression, the viral ORFs, or parts of them, were fused to the GST gene or to a T7-Tag sequence, respectively. The codon ranges of the investigated ILTV genes, as well as relevant restriction sites, in (b) and (c) are indicated (artificial sites in parentheses).

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2002a; Klupp et al., 2000; Muranyi et al., 2002; Reynolds et al., 2001). After de-envelopment at the outer nuclear membrane, the tegument is formed around the nucleocapsids in the cytoplasm and they acquire their final envelopes by budding into Golgi-derived vesicles and are then released by exocytosis (Mettenleiter, 2002). Although these steps of virion maturation and egress also seem to be common features of all herpesviruses including ILTV (Granzow, et al., 2001; Guo et al., 1993), only a fraction of the incorporated proteins is conserved in all subfamilies. Among them are the UL36 and UL37 proteins, which interact with each other to form the inner layer of the tegument (Desai, 2000; Desai et al., 2001; Fuchs et al., 2004; Klupp et al., 2001, 2002). Other major tegument proteins of HSV-1, like those encoded by UL46–UL49, are restricted to alphaherpesviruses (Roizman & Knipe, 2001). Whereas UL48 is crucial for productive replication of HSV-1 (Mossman et al., 2000; Weinheimer et al., 1992), pseudorabies virus (PrV) (Fuchs et al., 2002b) and Equid herpesvirus 1 (EHV-1) (von Einem et al., 2006), it is dispensable for the highly cell-associated varicella-zoster virus (VZV) and Marek’s disease virus (MDV) (Cohen & Seidel, 1994; Dorange et al., 2002). In MDV, but not in other alphaherpesviruses, UL49 is also considered to be essential for replication (Dorang et al., 2002), and, at least in PrV, deletion of UL47 detectably affects virion maturation (Kopp et al., 2002). The UL48 proteins are also important for the onset of replication in newly infected cells, as they reinforce transcription of viral immediate-early genes and, therefore, have been designated x-transducing factors (x-TIFs) (Batterson & Roizman, 1983; Campbell et al., 1984; Fuchs et al., 2002b; Misra et al., 1994; Morishita et al., 1993). The UL46 and UL47 proteins of HSV-1 modulate this regulatory function (McKnight et al., 1987; Zhang et al., 1991).

Although homologues of these tegument proteins are encoded by ILTV, none of them has been investigated. Up to now, besides the seven iltivirus-specific proteins (Veits et al., 2003a; Ziemann et al., 1998b), the ILTV homologues of only six conserved glycoproteins (gB, gC, gG, gI, gM and gN) have been identified, using monoclonal antibodies (mAbs) or monospecific antisera (Fuchs & Mettenleiter, 2003b; Poulsen & Keeler, 1997; Veits et al., 2003b).

As the gG gene US4 has been predicted to form a co-terminal transcription unit with the translocated UL47 gene of ILTV (Wild et al., 1996), it was further investigated in the present study. The major aim, however, was a primary characterization of the ILTV homologues of herpesvirus gene products involved in virion maturation. Therefore, the sizes and expression kinetics of the mRNAs of UL31, UL37, UL46, UL47, UL48 and UL49 were determined by Northern blot analyses using gene- and strand-specific probes. The respective ORFs were also expressed in bacterial fusion proteins for rabbit immunization and the obtained antisera were used to identify and localize the viral protein products in infected cells, and, if present, in purified virions of ILTV. Furthermore, the α-TIF function of the UL48 gene product of ILTV and the influence of other tegument proteins were investigated.

METHODS

Viruses and cells. The pathogenic ILTV strain A489 (obtained from D. Lütticken, Boxmeer, The Netherlands) was propagated in primary chicken embryo kidney (CEK) cells, as described previously (Fuchs & Mettenleiter, 1996). A chicken hepatoma cell line (LMH) (Kawaguchi et al., 1987; Schnitzlein et al., 1994) was used for transfection with expression plasmids. Cells were grown as monolayers in minimum essential medium (MEM; Invitrogen) supplemented with 10% fetal calf serum (FCS; Invitrogen) at 37°C and maintained at the same temperature in MEM containing 2–5% FCS after infection or transfection. For plaque assays, the virus inoculum was removed 2 h after infection and cells were overlaid with medium containing 6 g methyl cellulose l−1.

Construction of expression plasmids. The ORFs of ILTV under investigation were cloned into pcDNA3 (Invitrogen), which permits constitutive expression in eukaryotic cells under the control of the human cytomegalovirus (HCMV) immediate-early gene promoter–enhancer complex (P<sub>HCMV</sub>−IE) and the bovine growth hormone polyadenylation signal (A<sub>BGH</sub>), as well as in vitro transcription and translation from the promoters of the bacteriophages T7 and SP6 (Fig. 1b). Additionally, the viral ORFs, or parts of them, were inserted into vectors of the pGex-4T (Amersham) or pET23 (Novagen) series to express fusion proteins with glutathione S-transferase (GST) or with a T7-Tag peptide in Escherichia coli (Fig. 1c).

Except for US4, all of the analysed ORFs were amplified from virion DNA by PCR with P<sub>Bgl</sub> I DNA polymerase (Invitrogen) and custom-made primers (MWG Biotech), which were deduced from the published DNA sequence of the ILTV genome (Thureen & Keeler, 2006) and supplemented by artificial restriction sites to facilitate cloning (Table 1). After digestion with the indicated enzymes, the obtained PCR products were inserted into the similarly cleaved plasmids pcDNA3 (all genes), pGex-4T1 (UL31, UL37 and UL49) or pET23a (UL46–UL48). For cloning of UL46, the vectors were digested with B<sub>amHI</sub> instead of B<sub>glII</b>, as these enzymes generate compatible fragment ends. As UL37 proved to be longer than predicted from published DNA sequences (see Results and Discussion), two PCR products of this ORF were generated and cloned into pcDNA3 (Fig. 1b). However, for prokaryotic expression of UL37, only the shorter product encoding aa 1–904 was used (Fig. 1c).

The US4 gene of ILTV was recloned from plasmid pILT-K16, which contains a 5165 bp K<sub>pnI</k> fragment of genomic ILTV DNA, in pBS(−) (Stratagene). After trimming of the plasmid by double digestion with EcoRI and N<sub>Hil</n>, Klenow treatment and religation, the complete US4 ORF could be isolated within a 1565 bp EcoRI–Hp<sub>rol</sub> fragment containing nt 128587–130149 of the ILTV genome (Thureen & Keeler, 2006). This fragment was inserted into pcDNA3 that had been digested with EcoRI and EcoRV (Fig. 1b). For prokaryotic expression, a 1085 bp X<sub>hol</sub>–EcoN<sub>1</n> fragment of pILT-K16 containing codons 33–293 of the US4 gene was isolated and recloned in the X<sub>hol</sub>/Tth1111 doubly digested vector pGex-4T1 (Fig. 1c). As the bacterial fusion proteins containing the complete UL31, UL34 and UL47 ORFs were barely expressed, the respective plasmids had to be modified. After double digestion with N<sub>Hil</n> and X<sub>hol</n> and religation, pGex-IUL31 retained codons 1–186 of UL31. Plasmids pET-UL46 and pET-IUL47 contained codons 1–332 and 1–163 of the respective viral ORFs and were obtained after SacI/Xhol digestion of the original expression plasmids (Fig. 1c). In the described cloning experiments, non-compatible fragment ends were blunt-ended by

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Table 1. Oligonucleotide primers for PCR amplification of the UL31, UL37 and UL46–UL49 genes of ILTV

Nucleotide positions refer to GenBank accession no. NC_006623 (Thureen & Keeler, 2006). Reverse-strand sequences are indicated by ‘r’. Additional nucleotides are printed in lower case, restriction sites are underlined and initiation and stop codons are shown in italics.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide position</th>
<th>Sequence</th>
<th>Restriction site</th>
<th>Product size (bp)</th>
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<tbody>
<tr>
<td>IUL31-F</td>
<td>51470–51489 (r)</td>
<td>cagatccTCTGATATGCTGACCTTCCG</td>
<td>BamHI</td>
<td>1080</td>
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<tr>
<td>IUL31-R</td>
<td>50426–50443</td>
<td>cactggCGGTAGTGCAAACACGC</td>
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<tr>
<td>IUL37-F</td>
<td>65870–65887 (r)</td>
<td>cagatccGAAACCATGCGGGCCATC</td>
<td>BamHI</td>
<td>2732, 3130</td>
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<tr>
<td>IUL37-Ra</td>
<td>63169–63191</td>
<td>cagccGGCGCGCAATAACTTAAATACAGTACCATTGACATTAC</td>
<td>NotI</td>
<td></td>
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<tr>
<td>IUL37-Rb</td>
<td>62773–62793</td>
<td>cagccGCCTGATATGCAAACACGC</td>
<td>NotI</td>
<td></td>
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<tr>
<td>IUL46-F</td>
<td>21878–21895</td>
<td>cagatcTGCCCTAAACATGACCAGGC</td>
<td>BglII</td>
<td>1731</td>
</tr>
<tr>
<td>IUL46-R</td>
<td>23574–23593 (r)</td>
<td>cactggATGGCGATTTACTGTAAC</td>
<td>XhoI</td>
<td></td>
</tr>
<tr>
<td>IUL47-F</td>
<td>126616–126638</td>
<td>cagatccATGAGCTTGGCCATCGATTAC</td>
<td>EcoRI</td>
<td>1890</td>
</tr>
<tr>
<td>IUL47-R</td>
<td>128467–128487 (r)</td>
<td>cagatccGCTATTCCGATTTCCGGCG</td>
<td>NotI</td>
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<td>IUL48-F</td>
<td>20686–20704</td>
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<td>875</td>
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<tr>
<td>IUL49-R</td>
<td>20572–20595 (r)</td>
<td>cactggTGTATCAAAATATGCGGAGAACAC</td>
<td>XhoI</td>
<td></td>
</tr>
</tbody>
</table>

Klenow treatment. All plasmid constructs were characterized by DNA sequence analysis of the insert fragments using a Thermo Sequenase Cycle Sequencing kit (USB) and vector- as well as ILTV-specific primers.

Northern blot analyses. CEK cells were infected with ILTV at an m.o.i. of 5 p.f.u. per cell and incubated at 37°C for 6 h in the presence of 100 µg cycloheximide ml⁻¹ (for detection of immediate-early or α gene expression), for 20 h in the presence of 250 µg phosphonoacetic acid ml⁻¹ (for detection of early or β gene expression), or for 6 and 20 h without any drugs [for late or γ gene expression (γ6 and γ20)]. Total RNA from infected and uninfected cells was prepared (Chomczynski & Sacchi, 1987), and 5 µg RNA per lane was separated in denaturing 0.7% agarose gels, transferred to nylon membranes and hybridized with radiolabelled cRNAs, as described previously (Fuchs & Mettenleiter, 1996). These cRNAs were transcribed with SP6 RNA polymerase (Promega) from the pcDNA3 expression plasmids of the major immediate-early gene promoter of PrV (Vlcek et al., 1990). This construct was derived from pBl-GFP (Fuchs & Mettenleiter, 1999), 5′-methyl-dC-methyl-dG-methyl-dA-methyl-dT 1 : 1000. After methylation the total RNA from infected and uninfected cells was prepared (Chomczynski & Sacchi, 1987), and 5 µg RNA per lane was separated in denaturing 0.7% agarose gels, transferred to nylon membranes and hybridized with radiolabelled cRNAs, as described previously (Fuchs & Mettenleiter, 1996). Five micrograms of virion proteins or lysates of ~10⁶ infected, transfected or uninfected cells per lane were separated by SDS-PAGE, transferred to nitrocellulose filters and incubated with antibodies, as described previously (Fuchs & Mettenleiter, 1999). The obtained rabbit antisera were used at dilutions of 1 : 50 000 to 1 : 200 000, and a mouse mAb against ILTV glycoprotein gC (Veits et al., 2003a) was diluted 1 : 1000.

In vitro translation. The analysed genes were transcribed from the respective pcDNA3 expression plasmids with T7 RNA polymerase, translated in a cell-free system (TNT Coupled Reticulocyte Lysate System; Promega) from the pcDNA3 expression plasmids of UL31, UL37 and UL46–UL49 (Fig. 1b). Unlike these plasmids, pcDNA-IUS4 contained not only the analysed ORF, but also parts of the adjacent US5 gene (Fig. 1b). Therefore, a derivative of this plasmid was used from which these sequences had been removed by deletion of two XbaI fragments of 144 and 845 bp (Fig. 1b).

Antiserum preparation. After transformation of bacteria with the pGex-4T1 or pET23a constructs of the investigated ILTV genes (Fig. 1c), expression of fusion proteins was induced as recom- mended by the manufacturers of the vectors. In all cases, abundant proteins exhibiting the approximate expected molecular masses were detected after SDS-PAGE of bacterial cell lysates, and were isolated and used for immunization of rabbits, as described previously (Fuchs et al., 2002a). Sera collected before and after immunization were analysed.

Western blot analyses. CEK cells were infected with ILTV at an m.o.i. of 2 and incubated for various times at 37°C. LMH cells were harvested 48 h after transfection with pcDNA3 expression plasmids by calcium phosphate co-precipitation (Graham & van der Eb, 1973). Virions of ILTV were sedimented from the supernatants of infected CEK cells and purified by centrifugation through sucrose step gradients, as described previously (Fuchs & Mettenleiter, 1999). Five micrograms of virion proteins or lysates of ~10⁶ infected, transfected or uninfected cells per lane were separated by SDS-PAGE, transferred to nitrocellulose filters and incubated with antibodies, as described previously (Fuchs & Mettenleiter, 1999). The obtained rabbit antisera were used at dilutions of 1 : 50 000 to 1 : 200 000, and a mouse mAb against ILTV glycoprotein gC (Veits et al., 2003a) was diluted 1 : 1000.

Immunofluorescence tests. For indirect immunofluorescence tests, CEK cells were grown on coverslips, infected with ILTV at a low m.o.i. (~0.001 p.f.u. per cell) and incubated for 24 h under Methocel-containing medium. The cells were fixed with acetone for 20 min at ~20°C, dried and subsequently incubated with the rabbit antisera or the gC-specific mAb (diluted 1 : 100) and Alexa Fluor 488-conjugated secondary antibodies (Invitrogen), as described previously (Ziemann et al., 1998b). After chromatin counterstaining with propidium iodide, fluorescence was analysed in a confocal laser scanning microscope (LSM 510; Zeiss).

Transactivation studies. To test whether the UL46–UL49 gene products of ILTV were able to regulate alphaherpesvirus gC gene expression, LMH cells grown in six-well plates were co-transfected with the respective pcDNA3 constructs and a reporter plasmid encoding enhanced green fluorescent protein (EGFP) under control of the major immediate-early gene promoter of PrV (Vlcek et al., 1990). This construct was derived from pBl-GFP (Fuchs & Mettenleiter, 1999) by deletion of a 649 bp KpnI–Eco47III fragment containing the HCMV immediate-early promoter, followed by insertion of a KpnI–BamHI fragment representing nt 135454–136878 of the PrV genome (GenBank accession no. BK001744) (Klupp et al., 2004). In addition to the resulting plasmid pPIE180-GFP, a promoter deletion plasmid (pAP-GFP), an expression plasmid for PrV UL48 (pcDNA-PUL48) (Fuchs et al., 2002b) and pcDNA3 were used as controls. Cells were incubated with 1 µg of each plasmid DNA and FuGene HD transfection reagent (Roche) for 48 h at 37°C. Cells were trypsinized and resuspended in 100 µl PBS. Undiluted and diluted (1:10) samples were transferred to 96-well plates and fluorescence was quantified using an image analyser (FLA-3000; Waltham, MA).
Identification of ILTV proteins

RESULTS AND DISCUSSION

Sequence analyses of the UL37 and UL31 genes of ILTV strain A489

The DNA sequences of the amplified and cloned UL46–UL49 and US4 genes of ILTV strain A489 proved to be identical to the published sequence of the ILTV genome (Thureen & Keeler, 2006). For UL46, UL48 and UL49 this was not surprising, as the corresponding part of the genome sequence originates from the same virus strain. In contrast, the previously published UL31 and UL37 sequences had been determined from the Australian ILTV vaccine strain SA-2, and, as expected, several alterations were found in our PCR products. These discrepancies could be confirmed by cloning and bidirectional sequencing of two independently created amplification products of each ORF. The 1064 bp DNA sequence of the UL31 gene region of ILTV A489 (GenBank accession no. AM083948) exhibited 11 nt exchanges, which led to 6 aa substitutions and an additional stop codon. Hence, our UL31 ORF consisted of only 315 codons, whereas the UL31 gene of ILTV strain SA-2 is predicted to be 335 codons in size (Thureen & Keeler, 2006). The 5747 bp DNA sequence of the UL37 gene region of ILTV A489 (GenBank accession no. AM083949) contained 58 nt changes and nine single nucleotide deletions or insertions, resulting in 32 aa substitutions and an enlargement of the ORF from 891 to 1023 codons. This was verified by the observation that only in cells transfected with a full-length expression plasmid (pcDNA-IUS4; Fig. 1b) was a protein similar in size to that detectable in ILTV-infected cells found, whereas the protein expressed from a plasmid containing the originally proposed 891 bp ORF (Thureen & Keeler, 2006) appeared to be significantly smaller (results not shown).

The UL31 and UL37 gene products of other herpesviruses such as HSV-1 and PrV have been shown to play crucial roles during viral egress (Desai et al., 2001; Fuchs et al., 2002a; Klupp et al., 2001; Reynolds et al., 2001). It is likely that the homologous ILTV proteins possess similar important functions and therefore it appears unlikely that the respective ORFs of ILTV strains A489 and SA-2 differ in frame usage and stop codon positions. The monospecific antisera that are now available (see below) might be used to elucidate whether the UL31 and UL37 gene products of individual ILTV strains really exhibit different sizes.

Transcriptional analysis of the UL31, UL37, UL46–UL49 and US4 genes of ILTV

Northern blot analyses were performed to identify the mRNAs of the predicted ILTV genes and to determine their transcription kinetics (Fig. 2). For detection of viral immediate-early (α) and early (β) RNAs, infected cells were incubated in the presence of inhibitors of protein synthesis or DNA replication, respectively. Late (γ) transcripts were further differentiated by the use of total RNA samples prepared after 6 (γ6) or 20 (γ20) h incubations without any drugs. The blots were incubated with gene- and strand-specific antisense cRNA probes, which had been transcribed from pcDNA3 expression plasmids of the individual ORFs (Fig. 1b). As pcDNA-IUS4 contained unwanted parts of the US5 gene, a truncated plasmid obtained after XbaI digestion and religation was used for these studies. An antisense cRNA probe of the expression plasmid pRc-ICP4 (Fuchs et al., 2000), which detects the described 4.5 kb mRNA of the immediate-early protein ICP4 of ILTV (Ziemann et al., 1998b), was used as a control.

All probes detected defined viral transcripts (Fig. 2) exhibiting the approximate expected molecular masses (Table 2). The given minimum transcript sizes were calculated from the positions of initiation codons, or putative transcription initiation [TATA(A/T)A(A/T)] and mRNA polyadenylation signals (AATAAA) in the available DNA sequence (Thureen & Keeler, 2006). In ILTV, as in other herpesvirus genomes, many ORFs are not directly followed by polyadenylation signals, but organized in 3’-co-terminal transcription units (Fig. 1a). Therefore, it was inevitable that several of the gene-specific probes used additionally detected co-terminal transcripts of upstream genes. Thus, the UL47 and UL48 mRNAs were also detected by the US4 and UL46 probes, respectively (Table 2, Fig. 2). For the same reason, the UL31 and UL49 probes detected the upstream transcripts of UL32 and UL49.5, respectively, which were not further analysed in the present study (Table 2, Fig. 2). Furthermore, several probes reacted with unexpectedly large viral transcripts (Fig. 2), which might have resulted from occasional skipping of polyadenylation signals. The hybridization reactions of the UL31- and UL32-specific transcripts were too weak for photographic reproduction (results not shown). This was in agreement with subsequent protein analyses, which also indicated very limited expression of UL31 (see below).

As described previously (Ziemann et al., 1998b), the ICP4 mRNA of ILTV accumulates in the absence of de novo protein synthesis (α gene) and is downregulated after the onset of viral protein expression (Fig. 2). All of the other analysed mRNAs became more abundant during the late (γ) phase of the virus replication cycle (Fig. 2). However, overexposure of the blots revealed that traces of UL46 and US4 transcripts were also present under immediate-early (α) conditions and that inhibition of viral DNA replication (β) did not completely prevent transcription of UL31, UL37, UL46, UL48, UL49 and US4 (not all signals visible in Fig. 2). In contrast, UL47 proved to be a true late (γ) gene and was transcribed at detectable levels only after overnight incubation of ILTV-infected cells in the absence of any inhibitors (Fig. 2). Thus, the transcription kinetics of the investigated ILTV genes seemed to be slightly different from those of...
Fig. 2. For Northern blot analyses, CEK cells were infected with ILTV at an m.o.i. of 5 and incubated for 6 h in the presence of cycloheximide (for α gene expression), for 20 h in the presence of phosphonoacetic acid (for β gene expression), and for 6 (γ6) and 20 (γ20) h without drugs (for γ gene expression). Total RNA from infected and uninfected (N) cells was separated, transferred to nylon membranes and hybridized with 32P-labelled antisense cRNAs of the ICP4, UL37, UL46, UL47, UL48, UL49 and US4 genes. Sizes (kb) of RNA standards and the detected viral mRNAs are indicated.
their homologues in HSV-1, where for example UL47 has been described as a γ1 gene, UL31 represents a γ2 gene and neither UL46 nor US4 transcripts are detected during the immediate-early phase of replication (Roizman & Knipe, 2001). Up to now, it is unclear, whether these different expression kinetics correlate with distinct functions of the conserved alphaherpesvirus genes in HSV-1 and ILTV.

Identification and localization of protein products

After cloning in the eukaryotic expression vector pcDNA3 (Fig. 1b), all of the analysed ILTV genes could be transcribed and translated in a cell-free system, which does not permit extensive post-translational modifications. Consistently, in most cases SDS-PAGE of the radiolabelled in vitro translation products indicated molecular masses close to those that were calculated from the deduced amino acid sequences (Table 2). For UL31 and UL37, the agreement with our newly determined sequences of ILTV A489 confirmed the observed alterations compared with other published DNA sequences (see above). However, the apparent molecular masses of the UL47 and US4 in vitro translation products were significantly different from those that were calculated from the deduced amino acid sequences, although there was no evidence for sequence alterations. In these cases, unusual protein structures or charge distributions may lead to an aberrant electrophoretic mobility.

Preparation of monospecific rabbit antisera against bacterial fusion proteins permitted the detection of the UL31, UL37, UL46–UL49 and US4 proteins of ILTV by Western blot analyses of cells transfected with the pcDNA3 expression plasmids (results not shown), as well as of infected cells (Fig. 3). Increasing amounts of the viral proteins were found from 9 (UL46, UL49) or 12 h after infection, whereas uninfected cells showed no specific reactions (Fig. 3). The specificity of the obtained Western blot signals was further confirmed by their absence from blots incubated with the respective pre-immune sera (results not shown).

Furthermore, the apparent masses of the major viral UL31, UL37, UL48 and UL49 gene products were only slightly different from those of the respective in vitro translation products (Table 2). To verify such minor size alterations, parallel analyses on the same gels would be required. However, such studies were hampered by the different intensities of radioactive and chemiluminescence signals, and only the UL47- and US4-specific antisera were suitable for detection of their target proteins by immunoprecipitation from metabolically labelled infected cells (results not shown).

As the apparent molecular masses of the viral gene products detected in plasmid-transfected cells were similar to those found in infected cells, only the latter are listed in Table 2.

Table 2. Properties of the viral gene products

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<th>Property</th>
<th>ORF (codons)</th>
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<tr>
<td>mRNA size (kb):</td>
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<tr>
<td>Expected</td>
<td>&gt;1.07 (&gt;2.85)</td>
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<tr>
<td>Detected</td>
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<td>35.4*</td>
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<tr>
<td>Cytoplasm</td>
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Identification of ILTV proteins

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resulted from proteolytic cleavage, premature termination or initiation of translation at internal ATG codons (Fig. 3).

In several alphaherpesviruses, such as HSV-1 and PrV, the UL37 and UL46–UL49 gene homologues have been shown to encode tegument proteins (Mettenleiter, 2002; Roizman & Knipe, 2001). Presumably, this also applies to ILTV, as the corresponding gene products could be detected in purified virions (Fig. 3). In contrast, the UL31 and US4 proteins of ILTV were only found in infected cells (Fig. 3), which is in agreement with the absence of the corresponding gene products from mature PrV particles (Fuchs et al., 2002a; Rea et al., 1985). Furthermore, our studies confirmed the results of earlier investigations (Kongsuwan et al., 1993), which

**Fig. 3.** For Western blot analyses, CEK cells were infected with ILTV at an m.o.i. of 2 and harvested after 3, 6, 9, 12, 24 and 48 h. Proteins of infected and uninfected (N) cells and of purified virions (V) were separated and analysed with monospecific rabbit antisera against the UL31, UL37, UL46–UL49 and US4 proteins or with a mAb against virion glycoprotein gC. Molecular masses (kDa) of marker proteins and of the detected viral gene products are indicated.
indicated that gG is secreted from ILTV-infected cells (results not shown).

To allow subcellular localization of the individual proteins, indirect immunofluorescence reactions of the monospecific antisera with ILTV-infected chicken cells were analysed by confocal microscopy (Fig. 4). These studies revealed that the UL31 gene product of ILTV is predominantly localized in the cell nucleus and accumulates along the nuclear membrane (Fig. 4). The homologous PrV and HSV-1 proteins exhibit similar nuclear rim localizations, correlating with important functions during egress of viral nucleocapsids from the nucleus (Fuchs et al., 2002a; Reynolds et al., 2001). Like the envelope protein gC and the non-structural US4 gene product, the proposed tegument proteins encoded by UL37, UL46 and UL49 of ILTV accumulated in the cytoplasm of virus-induced syncytia, but were not found in cell nuclei at detectable levels (Fig. 4). This supports the hypothesis that the bulk of the tegument is added to nascent herpesvirus particles in the cytoplasm, prior to or during secondary envelopment of nucleocapsids in the trans-Golgi region (Mettenleiter, 2002). However, considerable proportions of the tegument proteins encoded by UL47 and UL48 of ILTV were localized within the host cell nuclei. As several alphaherpesvirus tegument proteins including the UL48 and UL47 gene products have been shown to be involved directly in regulation of viral gene expression, the intranuclear UL47 and UL48 proteins of ILTV might fulfil similar functions (see below). However, both ILTV proteins were detected in the cytoplasm as well (Fig. 4) and therefore might also be relevant for secondary envelopment of virus particles, as has been shown for the homologous gene products of PrV (Fuchs et al., 2002b; Kopp et al., 2002).

**Fig. 4.** Indirect immunofluorescence analysis of ILTV-infected CEK cells. After 24 h, cells were fixed with acetone and incubated with monospecific rabbit antisera (α-UL31, α-UL37, α-UL46–α-UL49, α-US4) or a gC-specific mAb (α-gC). Binding was detected using Alexa Fluor 488-conjugated secondary antibodies and chromatin was counterstained with propidium iodide. Fluorescence was analysed by confocal microscopy. Bar, 50 μm.
In addition to its incorporation into virus particles, the UL49 gene product of HSV-1 has been shown to traffic independently from infected or transfected cells to surrounding cells (Elliott & O’Hare, 1997). The biological relevance of this phenomenon is still unknown, and our fluorescence analyses of infected cells (Fig. 4) or cells transfected with a UL49 expression plasmid (results not shown) provided no evidence for similar migration of the ILTV protein.

**α-TIF function of the UL48 protein of ILTV**

The UL48 gene products of HSV-1 (VP16, α-TIF) and other alphaherpesviruses have been shown to stimulate transcription of viral immediate-early genes via interaction with cellular transcription factors (Batterson & Roizman, 1983; Campbell *et al.*, 1984; Fuchs *et al.*, 2002b; Misra *et al.*, 1994; Moriuchi *et al.*, 1993). Apparently, the UL46 and UL47 proteins of HSV-1 modulate the transactivation by UL48, and physical interactions with the UL49 protein may also possess regulatory functions (Elliott *et al.*, 1995; McKnight *et al.*, 1987; Zhang *et al.*, 1991). Previous studies have also shown that eukaryotic expression plasmids for either ICP4 or UL48 of ILTV increase the infectivity of naked virion DNA in transfection experiments (Fuchs *et al.*, 2000). This finding indicates that the UL48 protein of ILTV, like its homologues, is important for the onset of viral gene expression. Furthermore, the localization of considerable amounts of the UL48 and UL47 gene products in the nuclei of ILTV-infected cells (Fig. 4) also suggests roles at the level of transcriptional regulation.

To investigate these possible functions of the ILTV tegument proteins directly, the pcDNA3 expression plasmids of UL46–UL49 (Fig. 1b) were used for transactivation studies in LMH cells transfected with defined amounts of DNA (Fig. 5). Cells were co-transfected with a reporter plasmid containing the inducible promoter of the major immediate-early protein IE180 of PrV (Vlcek *et al.*, 1990) upstream of the ORF encoding EGFP (pPIE180-GFP). Basic EGFP expression from this construct could be significantly increased by co-transfection with a plasmid encoding the UL48 protein of PrV (pcDNA-PUL48), which has been shown to possess α-transinducing activity (Fuchs *et al.*, 2000).

Fig. 5. For transactivation studies, LMH cells grown in six-well plates were co-transfected with EGFP reporter gene plasmids (pPIE180-GFP, pΔP-GFP or pBl-GFP) and pcDNA3 expression plasmids of UL46, UL47, UL48 or UL49 of ILTV. The empty vector pcDNA3 and an expression plasmid for the UL48 protein of PrV (pcDNA-PUL48) were used as controls. Fluorescence intensities (LAU mm⁻²) were determined after 48 h. The results are shown as means ± SD of four independent experiments.
A similar effect was achieved with pcDNA-IUL48 expressing the UL48 protein of ILTV, which increased fluorescence intensity approximately 7-fold compared with that obtained with the empty expression vector pcDNA3 (Fig. 5). In contrast, the UL48 gene product of ILTV neither induced EGFP expression from a promoterless control plasmid (pAP-GFP), nor enhanced the constitutive reporter protein expression of pBl-GFP (Fig. 5). The basic promoter activity of pPIE180–GFP was not influenced significantly by expression of the UL46, UL47 or UL49 gene product of ILTV. However, co-expression of ILTV UL46, UL47 or UL49 increased EGFP expression induced by UL48 approximately 1.2–1.6-fold compared with that of cells co-transfected with identical amounts of pPIE180, pcDNA-IUL48 and pcDNA3 (Fig. 5). The significance of these minor modulating effects has yet to be verified by more extensive studies considering the efficiency of DNA uptake and expression levels of the individual proteins in transfected cells.

Our results demonstrated that the ILTV UL48 protein, like its HSV-1 and VZV homologues, enhances the activity of immediate-early gene promoters of heterologous alphaherpesvirus species (Campbell & Preston, 1987; Moriuchi et al., 1993). The direct influence of the UL48 gene product of ILTV on the expression of homologous immediate-early genes, such as the described ICP4 gene (Johnson et al., 1995; Ziemann et al., 1998b), remains to be investigated.

Up to now, the biological relevance of the α-TIF activity of ILTV UL48 has been unknown. Several attempts to generate UL48-negative ILTV recombinants have been unsuccessful, indicating essential or at least very important functions of the protein. The UL48 homologues of several other alphaherpesviruses, such as HSV-1, PrV and EHV-1, are also crucial for virus replication, but predominantly due to their structural functions during secondary envelopment of nascent virions (Fuchs et al., 2002b; Mossman et al., 2000; von Einem et al., 2006; Weinheimer et al., 1992). In all herpesviruses tested so far, the UL31 and UL37 proteins are required for nuclear egress of virions or for early tegumentation steps in the cytoplasm, respectively (Desai et al., 2001; Fuchs et al., 2002a; Klupp et al., 2001; Muranji et al., 2002; Reynolds et al., 2001). Therefore, the failure of our attempts to delete the homologous ORFs from the ILTV genome is not surprising. However, we were also unable to isolate UL46- or UL49-negative ILTV mutants, although their homologues are dispensable for replication of PrV and HSV-1 (del Rio et al., 2002; Elliott et al., 2005; Fuchs et al., 2002c; Kopp et al., 2002; Zhang et al., 1991), and in MDV only UL49, but not UL46, possesses essential functions (Dorange et al., 2002). Considering the phylogenetic distance of ILTV from the other alphaherpesviruses (McGeoch et al., 2000), it is conceivable that several conserved genes have retained or acquired more important functions than in other members of this virus subfamily. However, to demonstrate clearly the essential functions of the UL31, UL37, UL46, UL48 and UL49 genes of ILTV, corresponding deletion mutants will have to be isolated in trans-complementing cells, which are not yet available. Therefore, of the investigated genes, only UL47 and US4, which both proved to be non-essential for replication of ILTV in cell culture, have been investigated functionally up to now (see Helferich et al., 2007).

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