Five unique open reading frames of infectious laryngotracheitis virus are expressed during infection but are dispensable for virus replication in cell culture

Jutta Veits, Thomas C. Mettenleiter and Walter Fuchs

Institute of Molecular Biology, Friedrich-Loeffler-Institutes, Federal Research Centre for Virus Diseases of Animals, D-17493 Greifswald – Insel Riems, Germany

The chicken alphaherpesvirus infectious laryngotracheitis virus (ILTV) exhibits several unique genetic features including an internal inversion of a conserved part of the unique long genome region. At one end, this inversion is preceded by a cluster of five open reading frames (ORFs) of 335–411 codons, designated ORF A to ORF E, that are not present in any other known herpesvirus genome. In this report we analysed expression of these genes and identified the corresponding viral RNA and protein products. Northern blot analyses showed 3'-coterminal transcripts of ORFs A and B, and monocistronic mRNAs of ORFs C and D. ORF E is part of a 3'-coterminal transcription unit that includes the conserved glycoprotein H and thymidine kinase genes. Monospecific antisera obtained after immunization of rabbits with bacterial fusion proteins allowed detection of the protein products of ORF A (40 kDa), ORF B (34 kDa), ORF C (38 and 30 kDa), ORF D (41 kDa) and ORF E (44 kDa) in ILTV-infected cells. For functional analyses, five virus recombinants possessing deletions within the individual ORFs and concomitant insertions of a reporter gene cassette encoding green fluorescent protein were generated. All virus mutants were replication competent in cell culture, but exhibited reduced virus titres or plaque sizes when compared to wild-type ILTV. These findings indicate that the ILTV-specific ORF A to ORF E genes might be important for virus replication in the natural host organism.

INTRODUCTION

Infectious laryngotracheitis is an economically important respiratory disease of chickens (Bagust & Guy, 1997). The causative agent (ILTV; gallid herpesvirus 1) has been classified as a member of the Alphaherpesvirinae subfamily of the Herpesviridae (Minson et al., 2000). ILTV possesses a class D herpesvirus genome (Fig. 1a) consisting of a unique long region (UL), and a unique short region (US) which is flanked by inverted repeat sequences (IR, TR) permitting formation of two isomers (Leib et al., 1987; Roizman & Pellet, 2001). The ILTV genome is ca. 155 kbp in size, and has been almost completely characterized by DNA sequencing (reviewed in Fuchs et al., 2000). Most of the identified open reading frames (ORFs) within the ILTV genome exhibit significant homologies to genes identified in the genomes of other avian and mammalian alphaherpesviruses, including Marek’s disease virus (MDV-1; Tulman et al., 2000), varicella-zoster virus (VZV; Davison & Scott, 1986), equine herpesvirus type 1 (EHV-1; Telford et al., 1992), and herpes simplex virus type 1 (HSV-1; McGeoch et al., 1988). Therefore, most of the gene designations originally introduced for HSV-1 (McGeoch et al., 1988; Roizman & Knipe, 2001) were also adopted for ILTV. However, phylogenetic analyses of conserved genes and deduced gene products revealed a considerable evolutionary distance of ILTV from all known mammalian, but also from other avian, alphaherpesviruses (Johnson & Tyack, 1995; McGeoch et al., 2000). Consequently, ILTV was subclassified as the hitherto only member of the new alphaherpesvirus genus Infectious laryngotracheitis-like viruses (Minson et al., 2000).

Commensurate with this classification, the ILTV genome exhibits some unique characteristics with respect to gene content and arrangement (Fig. 1a). These include the absence of a UL16 homologue (Fuchs & Mettenleiter, 1999), and the translocation of the UL47 gene from the UL to the US region (Wild et al., 1996; Ziemann et al., 1998a). Furthermore, the ILTV genome differs from that of HSV-1 and most other alphaherpesviruses by an internal inversion within the US region which encompasses the conserved UL22 to UL44 genes (Ziemann et al., 1998a). A similar inversion ranging from UL27 to UL44 was also found in the genome of pseudorabies virus (PrV; Ben-Porat et al., 1983). At one end of the inverted genome part of ILTV, between the conserved UL22 and UL45 genes, an origin of viral DNA replication (OrL) and five unique ORFs consisting of 335–411 codons were identified (Ziemann et al.,
Two other clusters of presumably ILTV-specific ORFs were found at the left genome end (Johnson et al., 1997), and at the right end of the UL genome region (Ziemann et al., 1998b). However, only the two genes of the latter group, UL0 and UL[−1], have been shown to be expressed into proteins. Remarkably, both proteins were predominantly localized in the nucleus of ILTV-infected cells, and their amino acid sequences were shown to exhibit...
significant homologies to each other, indicating an ancient duplication event (Ziemann et al., 1998b). In contrast, the predicted ORF A to ORF E gene products, although related in size, share no detectable similarities in their amino acid sequences. Database searches also revealed no significant homologies to other viral or cellular proteins, nor conserved sequence motifs pointing to possible functions or localizations within cells and virions (Ziemann et al., 1998a).

However, initial RNA analyses indicated that the ILTV-specific ORF A to ORF E genes might be expressed during virus infection (Ziemann et al., 1998a).

To confirm these results, additional Northern blot analyses of RNA from ILTV-infected cells with gene- and strand-specific probes for each of the five ORFs were performed in the present study. Furthermore, ORFs A to E were expressed as bacterial fusion proteins which were used for preparation of monospecific antisera after rabbit immunization. The antisera permitted identification of the protein products of the five ORFs in ILTV-infected cells. To investigate possible functions of ORFs A to E, respective gene deletion mutants of ILTV had to be generated. Such approaches are hampered by the narrow in vitro host range of ILTV, which replicates only in primary chicken cells and, much less efficiently, in a chicken hepatoma cell line (Bagust & Guy, 1997; Schnitzlein et al., 1994). Since infectious full-length clones of the ILTV genome are also not available, only few virus recombinants, possessing deletions in the nonessential thymidine kinase (UL23), dUTPase (UL50) and UL10 genes, have been described previously (Fuchs & Mettenleiter, 1999; Fuchs et al., 2000; Lüscher et al., 2001; Okamura et al., 1994; Schnitzlein et al., 1995). In the present study, we isolated five novel ILTV recombinants exhibiting deletions of ORF A, B, C, D or E, and expressing enhanced green fluorescent protein (EGFP). The in vitro growth properties of these ILTV mutants were compared to those of the parental wild-type virus strain.

METHODS

Viruses and cells. All generated virus recombinants were derived from the pathogenic ILTV strain A489 (obtained from D. Lüttkicken, Intervet Int. BV, Boxmeer, NL) and propagated in primary chicken embryo kidney (CEK) cells (Fuchs & Mettenleiter, 1996). A chicken hepatoma cell line (LMH; Kawaguchi et al., 1987) was used for transfection experiments and plaque assays which were performed as described earlier (Fuchs et al., 2000).

In vitro transcription and translation, and constitutive protein expression in eukaryotic cells. ORFs A to E were amplified individually from genomic ILTV DNA by PCR with Pfx DNA-polymerase (Invitrogen), and custom-made primers which were deduced from the published DNA sequence (GenBank accession no. Y14300; Ziemann et al., 1998a). The PCR products representing nucleotides 7103–8337 (ORF A), 8267–9377 (ORF B), 9359–10385 (ORF C), 10470–11603 (ORF D) or 11670–12921 (ORF E) of the DNA sequence contained artificial restriction sites at their ends to facilitate directed insertion into the multiple cloning site of pcDNA3 (Invitrogen), which permits in vitro transcription of sense and antisense RNAs from the flanking T7 and SP6 promoters, as well as constitutive protein expression in eukaryotic cells under control of the human cytomegalovirus (HCMV) immediate early gene promoter. Insert fragments were sequenced using T7 and SP6 promoter primers (New England Biolabs), and the plasmids were used for calcium phosphate-mediated transfection (Graham & van der Eb, 1973) of LMH cells, for in vitro transcription of 32P-labelled cRNA (T7/SP6 transcription kit, Roche), and for in vitro translation (TNT coupled reticulocyte lysate system, Promega) in the presence of [35S]methionine. Radiolabelled in vitro translation products were separated by discontinuous SDS-PAGE in Mini-Protein II cells (Bio-Rad). The gels were treated with Amplify (Amershams), dried and exposed to X-ray films at −70 °C.

RNA analyses. CEK cells were infected with ILTV at an m.o.i. of 5 p.f.u. per cell and incubated in the presence of 100 μg cycloheximide ml−1 for 6 h (x), in the presence of 250 μg phosphonoacetic acid ml−1 for 16 h (β), or in the absence of any drugs for 6 h (y1) and 16 h (y2) at 37 °C. Total RNA of infected and uninfected cells was prepared (Chomczynski & Sacchi, 1987), separated in denaturing agarose gels and hybridized with radiolabelled cRNAs (see above) as described previously (Fuchs & Mettenleiter, 1996).

Expression of fusion proteins in bacteria and antisera preparation. Parts of ORFs A to E (Fig. 1b) were inserted into plasmid vectors of the pGex-4T (Amersham) or pET-23 (Novagen) series, which permit expression of fusion proteins with glutathione S-transferase (GST), or with a T7-tag peptide in E. coli. Protein expression was induced as recommended by the manufacturers of the vectors. The viral DNA-fragments were recloned from plasmids pILT-S3.1 or pILT-S3.2, which contain a genomic 13 862 bp SalI-fragment of ILTV inserted in opposite orientations into pUC19 (Fig. 1a; Ziemann et al., 1998a). Prior to ligation, non-compatible restriction fragment overhangs were blunt-ended by treatment with Klenow polymerase. After cloning of a 1453 bp Hpal/Xhol-fragment representing codons 69–377 of ORF A into the Small/XhoI double-digested vector pGex-4T1, a 60 kDa GST-fusion protein could be isolated. Codons 4–163 of ORF B were expressed as a 20 kDa T7-fusion protein which was obtained after insertion of a 479 bp Sphl/Xhol-fragment of ILTV DNA into the EcoRI/Xhol-cleaved plasmid pET-23b. A 1397 bp NcoI/Xhol-fragment containing the entire ORF C of ILTV was inserted into the EcoRI/Xhol-digested vector pET-23a to obtain a 40 kDa T7-fusion protein. An 18 kDa T7-fusion protein containing the 5′-terminal part of ORF D (codons 1–126), preceded by 30 bp of originally noncoding DNA was expressed after insertion of a 407 bp MluI/HinII-fragment into the EcoRI/Ndel-cleaved vector pET23c. Finally, a 1446 bp Ndel/Xhol-fragment including codons 20–411 of ORF E was inserted into the SalI/Xhol double-digested vector pET-23b. The 42 kDa T7-fusion protein obtained, as well as the other expression products, were purified after SDS-PAGE of bacterial cell lysates, and used for rabbit immunization as described previously (Fuchs et al., 2002). Sera collected before and after immunization were analysed.

Western blot analyses and immunofluorescence (IF) tests. CEK cells were infected with ILTV at an m.o.i. of 2 and incubated at 37 °C for 3, 6, 9, 12 or 24 h. LMH cells were harvested 48 h after calcium phosphate transfection with pcDNA3 expression plasmids. Lysates of infected or transfected, and of uninfected control cells, were separated by SDS-PAGE (ca. 106 cells per lane), and transferred to nitrocellulose filters (Trans-Blot SD cell, Bio-Rad). Blots were incubated with monospecific rabbit antisera, or a mouse monoclonal antibody (MAb) against glycoprotein C (Veits et al., 2003), at dilutions of 1:1000–1:5000, and binding of peroxidase-conjugated species-specific secondary antibodies (Dianova) was detected by chemiluminescence as described previously (Fuchs & Mettenleiter, 1999). For indirect IF tests LMH or CEK cells were fixed with methanol and acetone (1:1) 2 or 3 days after either plasmid-transfection or ILTV-infection at low m.o.i., and subsequently incubated with antisera or the MAb (dilutions 1:100), and fluorescein-conjugated...
secondary antibodies as described earlier (Ziemann et al., 1998b). After chromatin counterstaining with propidium iodide, samples were analysed either by conventional fluorescence microscopy (Diaphot 300, Nikon), or by confocal laser-scan microscopy (LSM 510, Zeiss).

Construction of ILTV recombinants. For introduction of single gene deletions and concomitant reporter gene insertions within ORFs A to E of ILTV (Fig. 1c) the plasmids pILT-S3.1 or pILT-S3.2 (Ziemann et al., 1998a) and pBl-GFP (Fuchs & Mettenleiter, 1999) were used. The latter construct contains an EGFP-expression cassette (Clontech) inserted at the Smal site within the polylinker of pBluescript SK(−) (Stratagene). To generate a transfer plasmid for deletion of codons 99–172 of ORF A (Fig. 1c), a 2333 bp BamHI/BstXI-fragment, and a 1056 bp PstI-fragment of the ILTV genome were isolated from pILT-S3.1 and subsequently inserted into pBl-GFP, which had been digested with BamHI and AflII, or PstI, respectively. As in the following experiments, non-compatible restriction fragment ends were blunt-ended by Klenow polymerase. For deletion of codons 2–298 of ORF B (Fig. 1c) a 1328 bp fragment generated by digestion of cloned ILTV DNA with EcoRI and partial cleavage with SalI was inserted into the EcoRI/PstI double-digested vector pBl-GFP. Subsequently, a 4715 bp SalI-fragment of pILT-S3.1 was inserted downstream of the reporter gene cassette. To obtain unique restriction sites for deletion of ORF C, the insert of pILT-S3.2 was truncated by cleavage with XbaI followed by religation. Thereafter, codons 15–278 of ORF C were removed by double-digestion with EagI and Eco47III, and replaced by the EGFP expression cassette contained in a 1623 bp EcoRV/XbaI-fragment of pBl-GFP (Fig. 1c). A transfer plasmid for deletion of codons 126–343 of ORF D (Fig. 1c) was prepared by consecutive insertion of 1100 bp BamHI/NruI- and 1934 bp KpnI/HincII-fragments of ILTV DNA into pBl-GFP, which had been cleaved with BamHI and AflII, or KpnI and EcoRV, respectively. In a similar manner, 1019 bp SalI/NruI- and 2654 SalI/FspI-fragments of the ILTV genome were inserted into pBl-GFP which had been doubly-digested with SalI and EcoRV, or BamHI and SstI. In the resulting plasmid codons 19–357 of ORF E were replaced by the EGFP expression cassette (Fig. 1c). GFP-expressing virus recombinants were obtained after calcium phosphate-mediated transfection (Graham & van der Eb, 1973) of LMH cells with the above described transfer plasmids, virion DNA of ILTV A489, and expression constructs of the ILTV homologues of VP16 and ICP4 which have been shown to increase virion DNA of ILTV A489, and expression constructs of the ILTV 1973) of LMH cells with the above described transfer plasmids, calcium phosphate-mediated transfection (Graham & van der Eb, 1973).

Plaque assays and one-step growth kinetics. For determination of plaque sizes, LMH cell monolayers were infected with ILTV at a low m.o.i. (<0.001). After 2 h, the inoculum was replaced by medium containing 6 g methylcellulose l−1. After 3 days at 37 °C the diameters of 60 plaques of each GFP-expressing virus mutant were determined by fluorescence microscopy. Plaques of wild-type virus were visualized by indirect IF reactions of a MAb against glycoprotein C of ILTV. Average diameters and standard deviations were calculated. One-step growth analyses were performed essentially as described (Fuchs et al., 2000). Briefly, CEK cells were infected at a low m.o.i. of 3, and after 1 h non-penetrated input virus was inactivated by treatment with citric acid (Mettenleiter, 1989). At different times after infection, cells were scraped into the medium, lysed by freezing and thawing, and progeny virus titres were determined by plaque assays on LMH cells. The average results of two independent experiments were plotted.

RESULTS AND DISCUSSION

Identification of in vitro translation products and viral mRNAs of ORF A to ORF E

Since the deduced translation products of ORFs A to E of ILTV exhibit no significant homologies to any known proteins (Ziemann et al., 1998a) we first tested whether stable gene products can be generated in vitro. For this purpose, pcDNA3 expression plasmids containing authentic ORFs A, B, C, D or E preceded by the T7 promoter were transcribed and translated in a cell-free system, and the 35S-labelled products obtained were separated by SDS-PAGE. For each of the five ORFs abundant in vitro translation products were detected, and their apparent molecular masses were very similar to those calculated from the predicted amino acid sequences (Table 1).

The expression plasmids used for in vitro translation also permitted transcription of labelled antisense cRNAs of ORFs A to E, which were used for identification of the viral mRNAs. In Northern blot analyses of total RNA of CEK cells harvested 16 h after infection with ILTV each of the five probes detected distinct viral transcripts, which were not found in uninfected cells (Fig. 2). The sizes of most of these RNAs were in good agreement with those calculated from DNA sequence positions of putative initiation codons, TATA box elements and polyadenylation signals (Fig. 1b; Table 1) as well as with preliminary RNA analyses (Ziemann et al., 1998a). The transcripts of ORF A and ORF B, as well as that of ORF E and the conserved glycoprotein H (UL22) and thymidine kinase (UL23) genes apparently possess common 3′-ends, whereas ORF C and ORF D are expressed from non-overlapping monocistronic mRNAs (Fig. 1b). However, the detection of two ORF D-specific RNAs (Fig. 2) indicated that transcription of this gene might initiate at different sites. Remarkably, a putative TATA-signal has been identified 23 bp downstream of the first ATG-codon of ORF D (Ziemann et al., 1998a). The detected 1·2 kb RNA might start at this signal whereas the 1·5 kb transcript might permit translation of the entire open reading frame (Fig. 1b). Presumably, the 4·7 kb RNA additionally recognized by the ORF D-specific probe starts at the strong ORF A promoter and results from occasional read-through of the first polyadenylation signal (Fig. 1b). This hypothesis was supported by detection of corresponding hybridization signals after overexposure of the ORF A- and ORF B-probed blots shown in Fig. 2. With several probes, additional signals at ca. 1·7 kb were obtained, which are most likely caused by an unspecific accumulation of degraded viral RNA within the bulky band of the 18S ribosomal RNA.

Transcription kinetics of the five unique ILTV genes were also investigated (Table 1). They revealed that moderate amounts of the ORF A-, ORF C- and ORF E-specific RNAs were produced even if viral protein synthesis was blocked by cycloheximide (Table 1, 2). Whereas transcripts encoding the major immediate early protein ICP4 accumulated
under these conditions, the mRNAs of most other conserved herpesvirus genes of ILTV, including UL22 and UL23, were not detectable (results not shown). Like UL23, but unlike UL22, the five ILTV-specific genes were also transcribed if viral DNA replication was inhibited by phosphonoacetic acid (Table 1, b). However, maximum transcription levels of all five genes were observed in the absence of any drugs. Whereas the mRNAs of ORFs C, D and E were most abundant at 6 h after infection, the transcripts of ORF A and ORF B accumulated at late time-points (Table 1, c1, c2). Identification of the ORF A to ORF E proteins in plasmid-transfected and in ILTV-infected cells

For detection of the predicted viral gene products of ORF A, ORF B, ORF C, ORF D and ORF E parts of the coding sequences were expressed in bacteria, and monoclonal antisera were prepared after immunization of rabbits with the isolated fusion proteins. All sera exhibited specific reactivity in Western blot analyses of LMH cells which had been transfected with the pcDNA3 expression vector.

**Table 1. Properties of the ORF A to E genes and proteins of ILTV**

The expected minimum mRNA sizes (kb) were calculated from the nucleotide positions of putative TATA-box elements or start codons, and polyadenylation signals (Fig. 1b). Viral transcripts were detected by Northern blot analyses with gene-specific antisense cRNAs (Fig. 2). The expected molecular masses (kDa) of the proteins were calculated from the deduced amino acid sequence, whereas the apparent masses of *in vitro* translation products and viral proteins were determined by SDS-PAGE (Fig. 3). The presence or absence, and the relative amounts of viral transcripts and proteins at different times post-infection (p.i.), are indicated by plus and minus signs. For RNA analyses infected cells were also incubated in the presence of 100 µg cycloheximide (CH) ml⁻¹ or of 250 µg phosphonoacetic acid (PAA) ml⁻¹.

<table>
<thead>
<tr>
<th>Gene...</th>
<th>ORF A</th>
<th>ORF B</th>
<th>ORF C</th>
<th>ORF D</th>
<th>ORF E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Codons...</td>
<td>377</td>
<td>341</td>
<td>335</td>
<td>375</td>
<td>411</td>
</tr>
<tr>
<td>Expected mRNA</td>
<td>&gt;2·2 kb</td>
<td>&gt;1·1 kb</td>
<td>&gt;1·0 kb</td>
<td>&gt;1·1 kb</td>
<td>&gt;1·5 kb</td>
</tr>
<tr>
<td>Viral transcript(s)</td>
<td>2·5 kb</td>
<td>1·1 kb</td>
<td>1·2 kb</td>
<td>1·2/1·5 kb</td>
<td>1·5 kb</td>
</tr>
<tr>
<td>α (6 h p.i. + CH)</td>
<td>++</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>β (16 h p.i. + PAA)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>γ1 (6 h p.i.)</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>γ2 (16 h p.i.)</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Expected protein</td>
<td>41·3 kDa</td>
<td>38·1 kDa</td>
<td>37·4 kDa</td>
<td>41·5 kDa</td>
<td>45·1 kDa</td>
</tr>
<tr>
<td><em>In vitro</em> translation product</td>
<td>40 kDa</td>
<td>39 kDa</td>
<td>38 kDa</td>
<td>41 kDa</td>
<td>44 kDa</td>
</tr>
<tr>
<td>Viral gene product(s)</td>
<td>40 kDa</td>
<td>34 kDa</td>
<td>38/30 kDa</td>
<td>41 kDa</td>
<td>44 kDa</td>
</tr>
<tr>
<td>3 h p.i.</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6 h p.i.</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>9 h p.i.</td>
<td>–</td>
<td>–</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>12 h p.i.</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>24 h p.i.</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

![Fig. 2. Northern blot analyses. CEK cells were harvested 16 h after infection with ILTV at an m.o.i. of 5. Total RNA of infected (I) and uninfected (N) cells was separated in 1 % formaldehyde/0·8 % agarose gels, transferred to nylon membranes, and hybridized with ³²P-labelled antisense cRNAs of ORF A, B, C, D or E. Sizes (kb) of RNA markers and detected viral transcripts are indicated on the left and right, respectively.](http://vir.sgmjournals.org)
plasmids of the individual ORFs (results not shown). All antisera also detected distinct protein bands in Western blot analyses of ILTV-infected CEK cell lysates (Fig. 3). These proteins were not found in uninfected cells (Fig. 3) nor with the respective preimmune sera, and the specificity of the antisera could be further confirmed by competition experiments with the bacterial fusion proteins which had been used for immunization (results not shown). Detection of the ORF A to E proteins in ILTV-infected cells was in good agreement with the results of RNA analyses since the ORF C, D and E proteins were first recognized 6 h after infection, whereas the ORF A and ORF B proteins were only detectable after 12 h and 24 h (Table 1).

The apparent molecular masses of the viral ORF A (40 kDa), ORF D (41 kDa) and ORF E (44 kDa) proteins (Fig. 3) were similar to those of the respective in vitro translation products and to the masses calculated from deduced amino acid sequences (Table 1), and although two distinct transcripts of ORF D were found (see above), only one protein was detectable. However, the 43 kDa gene product obtained after transient expression of full-length ORF D in plasmid-transfected eukaryotic cells (results not shown) was larger than the 41 kDa protein detected in ILTV-infected cells. Therefore, it cannot be ruled out that, as indicated by the putative TATA-box position (Ziemann et al., 1998a), transcription of the ORF D gene predominantly starts downstream of the first, and in front of the second in-frame start codon at position 24, and that the apparent size of the resulting 38-9 kDa protein is increased by post-translational modifications.

The 34 kDa ORF B gene product of ILTV (Fig. 3) was significantly smaller than calculated and found after in vitro translation (Table 1). In cells transfected with the pcDNA3 expression plasmid of ORF B both the 34 kDa protein and the expected 39 kDa gene product were detectable (results not shown). Since no 39 kDa ORF B protein was found at any time in ILTV-infected cells, translation of ORF B might generally initiate at the second in-frame ATG codon at position 27, which would result in a protein with a predicted molecular mass of 35-2 kDa.

The ORF C-specific antiserum reacted reproducibly with two proteins not only in plasmid-transfected cells, but also in cells which were harvested at late times after ILTV infection (Fig. 3). In contrast, only the expected 38 kDa ORF C protein was found in cells harvested 6 or 9 h after ILTV-infection (results not shown), indicating that the primary translation product might be partially cleaved by proteases.

The 34 kDa ORF B protein, as well as the 38 and 30 kDa ORF C gene products, were also detectable by immunoprecipitations performed after metabolic labelling of ILTV-infected CEK cells with \[^{35}\text{S}\]methionine (results not shown), whereas the anti-ORF A, anti-ORF D and anti-ORF E sera showed no specific reactions in this assay. Only the ORF C-specific antiserum was also suitable for indirect IF tests of ILTV-infected CEK cells. Confocal microscopy revealed an almost even distribution of the ORF C protein within the virus-induced syncytia (Fig. 4). Most of the punctate green fluorescent signals were localized in the cytoplasm, but the merged yellow fluorescence observed after chromatin counterstaining with propidium iodide indicated that a portion of the ORF C protein also enters the host-cell nucleus (Fig. 4). In contrast, envelope glycoprotein gC of ILTV was completely excluded from the nuclei of infected cells, but accumulated in cytoplasmic vesicles and vacuoles which presumably represent the sites of virion maturation (Granzzow et al., 2001; Guo et al., 1993). In indirect IF analyses of cells transfected with expression plasmids, not only the ORF C, but also ORF B, ORF D and ORF E gene products, exhibited a predominantly cytoplasmic localization (results not shown). However, in ILTV-infected cells expression of the latter proteins was too weak for IF detection, and the ORF A gene product could be localized neither in ILTV-infected nor in plasmid-transfected cells.

---

**Fig. 3.** Identification of the ORF A to E proteins of ILTV. CEK cells were harvested 24 h after infection at an m.o.i. of 2. Lysates of infected (I) and uninfected (N) cells were separated by discontinuous SDS-PAGE, transferred to nitrocellulose membranes, and incubated with monospecific rabbit antisera. Antibody binding was visualized by chemiluminescence reactions of peroxidase-conjugated secondary antibodies. The molecular masses of marker proteins are indicated (kDa). The apparent masses of the detected viral proteins (arrowheads) are listed in Table 1.
Deletion of ORF A to ORF E from the ILTV genome

As a prerequisite for functional investigations of ORFs A to E, a panel of ILTV recombinants was generated by cotransfection of LMH cells with virion DNA of a pathogenic wild-type ILTV strain, and with transfer plasmids in which major parts of the coding sequences of the individual genes were replaced by an EGFP reporter gene cassette (Fig. 1c). In all cases the transfection progeny contained EGFP-expressing virus mutants, which could be plaque-purified in noncomplementing chicken cells. DNA of the virus recombinants ILTV ΔORF A-G, ΔORF B-G,

Fig. 4. Intracellular localization of the ORF C gene product and of glycoprotein C in ILTV-infected and uninfected CEK cells. Cells were grown on coverslips, fixed 48 h after infection with methanol and acetone (1:1) and subsequently incubated with the ORF C-specific rabbit antiserum (α-ORF C) or a glycoprotein C-specific MAb (α-gC) and fluorescein-conjugated secondary antibodies. Prior to confocal fluorescence microscopy, cell nuclei were counterstained with propidium iodide, and the merged green and red fluorescence is shown.
ΔORF C-G, ΔORF D-G and ΔORF E-G, was analysed by digestion with restriction endonucleases and subsequent Southern blot hybridization, as well as by PCR amplification and DNA sequencing of the modified genome fragments (results not shown). These studies demonstrated that all ILTV mutants contained the expected deletions, and were free of parental wild-type virus. However, after repeated passage of ILTV ΔORF A-G and ΔORF C-G, concomitant with increasing numbers of non-fluorescent virus plaques novel submolar DNA fragments became detectable on Southern blots (see below).

Western blot analyses of cells infected with the different ILTV recombinants were performed to confirm the absence of the deleted gene products, and to investigate whether expression of adjacent genes was affected (Fig. 5). Whereas similar amounts of glycoprotein C (gC) were found in all ILTV-infected CEK cells, the ORF A, ORF B, ORF C, ORF D or ORF E proteins were not detectable in cells infected with the respective deletion mutants (Fig. 5). These results did not rule out the possibility that the remaining 5′-terminal parts of the deleted ORFs (Fig. 1c) are still expressed, since the resulting proteins could be too small or not sufficiently antigenic for detection. However, it appears unlikely that the truncated gene products, if stable at all, are still functional. The undeleted adjacent ORFs of each of the five ILTV recombinants were still expressed, and the detected proteins exhibited similar sizes like the gene products of wild-type ILTV (Fig. 5). In most cases, the protein expression levels were also comparable, except that the amount of ORF D was reduced in cells infected with ILTV ΔORF E-G, whereas ORF A was overexpressed by ILTV ΔORF B-G. These effects might indicate a functional interdependence of the different ORF proteins, but could also be caused by the deletion or insertion of transcription-regulating DNA sequences. In cells infected with some of the mutants and with wild-type ILTV, smaller than full-length forms of the ORF A and ORF B proteins were visible (Fig. 6). However, these protein bands were not reproducibly detected (see Fig. 3) and presumably represent degradation products.

Successful isolation of deletion mutants already demonstrated that the five unique ILTV genes encoded by ORFs A to E are not required for virus replication in cell culture. For detection of sublethal replication deficiencies one-step growth curves and plaque sizes of the virus recombinants were compared to that of parental wild-type ILTV (Fig. 6). The assays were performed in different cell types, since clear ILTV plaques are only formed in the hepatoma cell line LMH, whereas productive replication is more efficient in primary CEK cells. In repeated experiments all analysed deletion mutants, with the exception of ILTV ΔORF C-G, produced reduced amounts of infectious progeny virus in CEK cells (Fig. 6a). In addition, ILTV ΔORF A-G, ΔORF B-G and ΔORF D-G also exhibited delayed replication kinetics when compared with the parental virus strain (Fig. 6a). Remarkably, the different effects of the individual mutations on one-step growth did not always correlate with that on plaque formation in LMH cells (Fig. 6b). Although productive replication of ILTV ΔORF B-G was significantly affected (Fig. 6a), it produced wild-type-sized plaques (Fig. 6b). In contrast, ILTV ΔORF C-G exhibited the most pronounced plaque-formation defect of all analysed mutants (Fig. 6b), but wild-type-like growth kinetics (Fig. 6a). These findings might indicate that the ORF C protein is only required for direct cell-to-cell spread of ILTV, whereas the ORF B gene product is involved in release of mature virus particles. An analogous role has been described for glycoprotein D of PrV, which is required for formation of infectious virions, but dispensable for cell-to-cell spread (Rauh & Mettenleiter, 1991). For ILTV ΔORF A-G, ΔORF D-G and ΔORF E-G the approximately tenfold titre reductions correlated with plaque diameters of ca. 50% of the wild-type size (Fig. 6).

Previous studies have shown that insertion of an EGFP reporter gene cassette might affect replication of ILTV irrespective of the deleted viral DNA sequences (Fuchs et al., 2000). Therefore, a recombinant LMH cell line carrying the ORF A, B, C, D and E genes of ILTV was generated.

![Fig. 5. Protein expression of ILTV recombinants. Western blot analyses of CEK cells infected with the five single-gene deletion mutants (ΔORF A-G, ΔORF B-G, ΔORF C-G, ΔORF D-G, ΔORF E-G), or wild-type ILTV (WT), and of uninfected cells (N) with the corresponding rabbit antisera and a glycoprotein C-specific MAb, were performed as in Fig. 3. The relevant protein bands are marked by arrowheads.](image-url)
and used for plaque-assays with the EGFP-expressing virus mutants. In these cells the plaque sizes of ILTV ΔORF A-G, ΔORF C-G, ΔORF D-G or ΔORF E-G were not restored to the wild-type level, although cellular expression of all five proteins could be demonstrated by Western blot analyses performed after infection with the respective ILTV deletion mutants (results not shown). At present it is not clear whether the amount of protein provided by the cells was insufficient, whether the ORF deletion mutants possess unexpected second site mutations, or whether the observed plaque formation defects are indeed caused by the reporter gene insertion.

Toxic effects of the strongly expressed reporter protein would also explain the rapid reappearance of non-fluorescent virus plaques during passage of some of the purified EGFP-expressing ILTV recombinants in non-complementing cells. Two such virus mutants derived from ILTV ΔORF A-G and ΔORF C-G, respectively, were plaque-purified, and their genomes were analysed by Southern blot hybridization, PCR amplification and DNA sequencing, which confirmed that in both cases parts of the reporter gene cassette including the HCMV immediate early gene promoter and coding sequences of EGFP were deleted. Interestingly, these spontaneous mutations also affected flanking viral DNA sequences (Fig. 1c). The virus mutant ILTV ΔORF A-C exhibited a deletion including ORF A (codons 99–377), ORF B (complete) and ORF C (codons 315–335), whereas the deletion of ILTV ΔORF C-E contained ORF C (codons 1–278), ORF D (complete) and ORF E (codons 183–411). Thus, not only single, but also triple gene deletion mutants of the ILTV-specific ORFs A to E are viable in cell culture. However, the triple mutants exhibited significant in vitro growth defects. Plaque diameters of both viruses on LMH cells were reduced to ca. 35 % of the wild-type size, and maximum titres on CEK cells were reduced ca. tenfold. These findings indicate that the replication deficiencies of the above described single gene deletion mutants are presumably not solely caused by expression of the EGFP reporter protein. Repeated attempts to generate an ILTV recombinant lacking all five unique ORFs were not successful. Therefore, it remains possible that ORFs A to E possess important but redundant functions for virus replication, like those discussed for several conserved tegument and envelope proteins involved in alphaherpesvirus egress (Mettenleiter, 2002).

In vivo studies have to be performed to assay the influence of the gene deletions on virulence of ILTV. Such studies are of particular interest, since ORFs A to E, like other unique genes of individual herpesviruses, might only be relevant for replication in the natural host organism. For example, several cytomegalovirus-specific gene products were identified as functional homologues of host proteins, and were shown to mediate immune evasion by the virus (Hengel et al., 1998). In a similar manner, the expression of a viral homologue of cellular oncogenes by MDV was discussed as a possible reason for the induction of T-cell tumours in chickens (Jones et al., 1992). ILTV does not induce tumours, and considering its rapid replication in the respiratory tract of chickens during acute infection (Bagust & Guy, 1997), immune evasion strategies might be dispensable. However, like MDV and unlike most other alphaherpesviruses, ILTV exhibits a very narrow host range consisting only of chickens and their closest cognates (Bagust & Guy, 1997). Therefore, it is possible that the species-specific genes of ILTV are adaptations that benefit replication in its avian host. In consequence, some of the generated deletion mutants might be sufficiently attenuated to be suitable as live-virus vaccines against infectious laryngotracheitis of chickens.
ACKNOWLEDGEMENTS

These studies were supported by a grant from the Deutsche Forschungsgemeinschaft (Fu 395/1). The authors thank D. Lüticken for providing the parental ILT virus strain, E. Mundt for help with rabbit immunization, B. G. Klupp for performing confocal microscopy, R. Riebe for preparation of chicken cell cultures, and C. Ehrlich and M. Voß for expert technical assistance.

REFERENCES


