The non-essential UL50 gene of avian infectious laryngotracheitis virus encodes a functional dUTPase which is not a virulence factor

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The DNA sequence of the infectious laryngotracheitis virus (ILTV) UL50, UL51 and UL52 gene homologues was determined. Although the deduced UL50 protein lacks the first of five conserved domains of the corresponding proteins of mammalian alphaherpesviruses, the ILTV gene product was also shown to possess dUTPase activity. The generation of UL50-negative ILTV mutants was facilitated by recombination plasmids encoding green fluorescent protein (GFP), and expression constructs of predicted transactivator proteins of ILTV (zTIF, ICP4) were successfully used to increase the infectivity of viral genomic DNA. A GFP-expressing UL50-deletion mutant of ILTV showed reduced cell-to-cell spread in vitro, and was attenuated in vivo. A similar deletion mutant without the foreign gene, however, propagated like wild-type ILTV in cell culture and was pathogenic in chickens. We conclude that the viral dUTPase is not required for efficient replication of ILTV in the respiratory tract of infected animals. The replication defect of the GFP-expressing ILTV recombinant is most likely caused by toxic effects of the reporter gene product, since spontaneously occurring inactivation mutants exhibited wild-type-like growth.

Introduction

Infectious laryngotracheitis is a world-wide-occurring, severe respiratory disease of chickens (Bagust & Guy, 1997). The causative agent was designated infectious laryngotracheitis virus (ILTV), or gallid herpesvirus 1, and classified as a member of the Alphaherpesvirinae subfamily of the Herpesviridae (Roizman, 1996). For prevention, chickens are immunized with conventionally attenuated live vaccines. Most of these genetically uncharacterized virus strains are still moderately pathogenic, and they bear the risk of spontaneous reversion to a more virulent phenotype (Bagust & Johnson, 1995).

As a prerequisite for the development of genetically engineered vaccines, most of the ILTV genome has been characterized by DNA sequencing over the last few years. These studies confirmed that ILTV possesses a herpesvirus type D genome (Johnson et al., 1991; Leib et al., 1987; Roizman, 1996) consisting of a long (UL) and a short (US) unique region; the latter is flanked by inverted repeat sequences (IR, TR). In ILTV, as in other alphaherpesvirus genomes, the US region contains a conserved cluster of viral glycoprotein genes (Wild et al., 1996), and the major immediate early (IE) protein ICP4 is encoded within the adjoining IR and TR sequences (Johnson et al., 1995a). Gene content and arrangement of the not yet completely analysed UL region of ILTV DNA (Fuchs & Mettenleiter, 1996, 1999; Griffin & Boursnell, 1990; Griffin, 1991; Johnson et al., 1995b, 1997; Kingsley et al., 1994; Ziemann et al., 1998a, b) also exhibits great similarities to the respective parts of other alphaherpesvirus genomes, and open reading frames (ORFs) were designated according to their herpes simplex virus 1 (HSV-1) homologues (McGeoch et al., 1988). However, recent sequence analyses revealed several unique features of the ILTV genome, including an internal inversion within the UL region, the translocation of UL47 to the US region, the absence of a UL16 homologue, and the presence of several presumably ILTV-specific genes (Fuchs & Mettenleiter, 1999; Wild et al., 1996; Ziemann et al., 1998a, b).

Continuing the sequence analysis of ILTV DNA we have now closed a small gap located between the characterized UL53 (Johnson et al., 1995b) and UL49.5 (Ziemann et al., 1998a).
1998a) genes. Besides UL52 and UL51, the novel DNA sequence contains the entire UL50 ORF of ILTV. The corresponding genes of HSV-1 and -2, varicella-zoster virus (VZV), bovine herpesvirus-1, pseudorabies virus (PrV), Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus were shown to encode functional viral homologues of the ubiquitous enzyme deoxyuridine triphosphatase (dUTPase), which is required during dTTP synthesis and for prevention of uracil-incorporation into DNA (Jöns & Mettenleiter, 1996; Kremmer et al., 1999; Liang et al., 1993; Ross et al., 1997; Williams et al., 1985; Wohlrab et al., 1982). In all herpesviruses investigated in this aspect, the UL50 gene is non-essential for virus replication in cell culture. However, UL50-deletion mutants of both HSV-1 and PrV were found to be significantly attenuated in animals: HSV-1 in mice and PrV in pigs (Jöns et al., 1997; Pyles et al., 1992). Based on these results dUTPase-negative ILTV might be a suitable live vaccine for chickens.

Only very few genetically engineered ILTV mutants carrying deletions in the thymidine kinase or UL10 genes have been described up to now (Fuchs & Mettenleiter, 1999; Okamura et al., 1994; Schnitzlein et al., 1995), probably because cotransfection of permissive cells with ILTV DNA and shuttle plasmids is rather inefficient. Since it was shown in other alphaherpesvirus systems that infectivity of naked virus DNA is significantly increased by viral transactivator proteins (Dargan & Subak-Sharpe, 1997; Moriuchi et al., 1993, 1994), expression constructs of the ILTV UL48 and ICP4 genes were generated and used to improve DNA transfection of a chicken hepatoma cell line which permits ILTV replication (Kawaguchi et al., 1987; Schnitzlein et al., 1994). Thus, we were able to generate UL50-deletion mutants of ILTV.

Methods

*Virus and cells.* ILTV recombinants were derived from a pathogenic virus strain (obtained from D. Lütticken, Boxmeer, NL) which is designated as wild-type (WT) ILTV. Transfection experiments and plaque-assays were performed in chicken hepatoma cells (LMH, Kawaguchi et al., 1987), whereas primary chicken kidney cells (CEK) were used for large-scale virus propagation (Fuchs & Mettenleiter, 1996, 1999).

*DNA sequencing.* From plasmids pILT-ED1 and -ED2 (Fig. 1B), which are independently obtained clones of the genomic 9.8 kb EcoRI-fragment D in pBS- (Stratagene), the 4471 bp EcoRI–Sall fragments were first subcloned and digested with EcoRI and Ndel or Sall and BstEII, and then unidirectionally shortened by nested deletion mutagenesis (nested deletion kit, Pharmacia). Sequencing with vector-specific primers was then unidirectionally shortened by nested deletion mutagenesis (nested deletion kit, Pharmacia). Sequencing with vector-specific primers was performed as described (Fuchs & Mettenleiter, 1996), and remaining gaps were closed using custom-made ILTV-specific primers (GibcoBRL). DNA sequences were assembled and analysed with the GCG software package in UNIX version 9.1 (Devereux et al., 1984).

*Plasmids for mutagenesis of UL50.* To remove unwanted restriction sites, the insert of pILT-ED2 was shortened by double-digestion with EcoRI and CdiI, followed by treatment with Klenow polymerase and religation. Similarly, Sfil and Smal were used for a second deletion step (Fig. 1C). From the resulting plasmid, pILT-CS, a 1067 bp BstEII–Ndel fragment spanning UL50 codons 10–366 was removed (pILT-CSD), or replaced by a 1636 bp Asel–AIII fragment from pEGFP-N1 (Clontech) encompassing a green fluorescent protein (GFP) expression cassette (pILT-CSG, Fig. 1C).

*Transactivator protein expression plasmids.* The UL48 and ICP4 genes of ILTV (Fig. 1A, B) were isolated from cosmids-cloned virus DNA (Fuchs & Mettenleiter, 1999) as 2259 bp Ncol–Spel, or 6608 Sall–Spel fragments, respectively. The fragments were inserted into HindIII–Xbal doubly digested pRc-CMV (Invitrogen) after Klenow fill-in of non-compatible 5′-overhangs. In the obtained plasmids pRc-ICP4 and pRc-UL48 the predicted initiation codons of the ILTV proteins are the first ATG motifs downstream from the human cytomegalovirus IE (pCMV IE) and 7 promoters, which permit constitutive expression in eukaryotic cells, as well as in vivo transcription and translation (Fig. 1D).

*Cotransfection experiments.* Virus DNA was prepared from ILTV-infected CEK cells as described (Fuchs & Mettenleiter, 1996), and plasmids were purified with the Qiagen Maxi kit (Qiagen). For calcium phosphate cotransfection (Graham & van der Eb, 1973) 1 μg ILTV DNA and 10 μg of the desired plasmids were dissolved in 438 μl of 10 mM Tris–HCl (pH 7.4). Subsequently, 62 μl of 2 M CaCl2, 500 μl of 50 mM HEPES, 1.5 mM Na2HPO4 and 280 mM NaCl (pH 7.13) were added slowly, and after gentle mixing the suspension was incubated at room temperature for 1 h. Then the DNA was added to subconfluent LMH cell monolayers in 5 cm Petri dishes containing 2 ml minimum essential medium (MEM) supplemented with 5% foetal calf serum. After 24 h at 37 °C the transfection solution was replaced by 5 ml of fresh medium and incubation was continued for 4–6 days until virus plaques became visible.

*Isolation and characterization of UL50 mutants.* For generation of UL5UL50G, LMH cells were cotransfected with ILTV WT DNA, transactivator plasmids pRc-UL48 and -ICP4, and transfer plasmid pILT-CSG (Fig. 1C). GFP-expressing virus recombinants were isolated by limiting dilutions of the transfection progeny on CEK cells grown in microtitre plates. The rescue mutant ILTV UL50R and the deletion mutant ILTV AUL50 were obtained after transfections with ILTV AUL50G DNA, transactivator plasmids and pILT-CS or -CSD, respectively (Fig. 1C). In these cases, virus progeny was screened for non-fluorescent plaques. ILTV recombinants were further analysed by Southern blot hybridizations of enzyme-digested virus DNA (Fuchs & Mettenleiter, 1999). Furthermore, the UL50 gene regions of all recombinant ILTV genomes were PCR-amplified with primers UL50-F and UL50-R (reverse of nt 5689–5707) and UL50-R (nt 4169–4188), plasmid-cloned and sequenced as described (Fuchs & Mettenleiter, 1999).

*dUTPase assays.* Activity of the viral dUTPase was determined essentially as described (Jöns & Mettenleiter, 1996; Wohlrab et al., 1982). Confluent monolayers of ca. 106 LMH cells were infected with either ILTV WT, or recombinants ILTV AUL50G, ILTV AUL50 and ILTV UL50R at an m.o.i. of 1. After 16 h at 37 °C infected and non-infected cells were washed once with PBS, scraped into 500 μl hypotonic solution (20 mM HEPES, 1 mM dithiothreitol, 1 mM MgCl2, pH 7.8) and incubated on ice for 30 min after addition of 0.2% IGEPAL CA-630 (ICN). The nuclei were sedimented by centrifugation (500 g for 10 min), washed once with hypotonic solution and finally resuspended in 100 μl of the same buffer supplemented with 80 mM potassium acetate. Five μl of a reaction mixture containing 100 mM MgCl2, 10 mM dithiothreitol, 10 mM EGTA, 20 mM ATP and 30 mM [5′-3H]dUTP (16 Ci/mmol; Amersham) were added to 45 μl of the extracts and incubated for 1 h at 4 °C. The reaction was terminated by subsequent addition of 5 μl of acid.
ILTV dUTPase

Fig. 1. (A) Physical map of the ILTV genome consisting of a unique long (UL) and a unique short (US) region; the latter is bracketed by inverted repeat sequences (IR, TR). Sequenced genome parts are shaded and KpnI restriction fragments as well as selected virus genes are indicated. (B) The genomic EcoRI-fragment D of ILTV was plasmid-cloned (pILT-ED1/2) and sequenced (nt 1–5750; GenBank accession no. AJ249803). Relevant restriction sites are marked, and virus genes are drawn as pointed rectangles. Arrowheads indicate the PCR primers UL50-F and UL50-R, which were used for characterization of UL50 gene mutants. (C) A subcloned Clal-Stul fragment (pILT-CS) was used for manipulation of UL50. In pILT-CSD almost the entire ORF was deleted, and in pBS-CSG it was replaced by an expression cassette (Clontech) containing the enhanced GFP gene (EGFP) flanked by the HCMV IE promoter (PHCMVIE) and a polyadenylation signal (SV40 poly A). As indicated by arrows, the GFP-expressing ILTV mutant ∆UL50G was first isolated, and then rescued (UL50R) or converted into a deletion mutant without foreign sequences (∆UL50). (D) The ORFs encoding the ILTV homologues of αTIF (UL48) and ICP4 were inserted into pRc-CMV (Invitrogen), which permits constitutive gene expression in eukaryotic cells under control of PHCMVIE, as well as in vitro transcription and translation from the T7 promoter (P7). Plasmids pRc-UL48 and pRc-ICP4 were used to enhance the infectivity of ILTV DNA.

of 0.5 M EDTA and 100 µl methanol. For thin-layer chromatography, 5 µl of the probes was mixed with 2.5 µl each of 100 mM solutions of unlabelled dUTP and dUMP, and applied dropwise to polyethyleneimine cellulose sheets containing a fluorescence indicator (Merck). After development in 1 M formic acid, 0.5 M LiCl, the separated spots of dUTP and dUMP were excised under UV light and measured by liquid scintillation counting. Finally, for each sample the ratio between [3H]dUTP and [3H]dUMP was calculated.

Plaque assays and one-step growth kinetics. Two hours after infection of LMH cells with serial dilutions of ILTV in MEM, or 24 h after
transfection with virus DNA the inoculum was removed and replaced by semi-solid MEM containing 5% foetal calf serum and 6 g/l methyl cellulose. After 4–6 days at 37 °C, the cells were fixed for 1 h with 5% formalin, washed and stained for 15 min with 1% crystal violet in 50% ethanol. For quantification of cell-to-cell spread, the average diameters of 30 microscopically measured plaques each of ILTV WT and the different UL50 mutants were calculated. One-step growth of WT and recombinant ILTV was monitored on CEF cells, which were infected at an m.o.i. of 5. After 1 h at 4 °C followed by 1 h at 37 °C, the inoculum was removed and non-penetrated virus was inactivated by a 2 min incubation with 40 mM citric acid, 10 mM KCl, 135 mM NaCl (pH 3). After repeated washing with PBS, fresh medium was added and the cells were further incubated at 37 °C. At indicated time-points the cells were scrapped into the medium, lysed by freeze–thawing and progeny virus was titrated on GDA

Results

After 1 h at 4 °C, 30 microscopically measured plaques each of ILTV WT and the different UL50 mutants were calculated. One-step growth of WT and recombinant ILTV was monitored on CEF cells, which were infected at an m.o.i. of 5. After 1 h at 4 °C followed by 1 h at 37 °C, the inoculum was removed and non-penetrated virus was inactivated by a 2 min incubation with 40 mM citric acid, 10 mM KCl, 135 mM NaCl (pH 3). After repeated washing with PBS, fresh medium was added and the cells were further incubated at 37 °C. At indicated time-points the cells were scrapped into the medium, lysed by freeze–thawing and progeny virus was titrated on LMH cells. In vivo studies were performed with 20-week-old, pathogen-free White Leghorn chickens (fertilized eggs purchased from Lohmann, Cuxhaven, Germany). Five animals per group were intratracheally infected with 5 × 10^3 p.f.u. of either ILTV WT, ILTV \( \Delta \)UL50G, ILTV \( \Delta \)UL50 or ILTV UL50R. During the following week, the chickens were examined daily for clinical symptoms (gasping, coughing), and tracheal swabs were taken to titrate shed virus by plaque-assays. After 3 and 4 days, one animal of each group was necropsied and tissues were sampled for histopathological investigations. Before and 14 days after infection, sera were collected and tested for ILTV-specific antibodies by indirect immunofluorescence analyses of infected CEF cells (Ziemann et al., 1998b). Four weeks after primary infection, the remaining animals, as well as four non-infected chickens, were challenged by intratracheal application of 5 × 10^3 p.f.u. of virulent ILTV WT. Again, clinical symptoms and virus shedding were monitored, and 4 days after challenge infection all surviving animals were killed and necropsied.

Histopathology and in situ hybridization. Immediately after necropsy, tissue samples of larynx, trachea and lung were fixed for 24 h in 4% neutral-buffered formalin and paraffin-embedded. Serial sections (3 µm) were dewaxed, mounted on organosilane-coated coverslips and stained with haematoxylin and eosin (HE) for light microscopy. Adjacent sections were investigated by non-radioactive in situ hybridization. As a probe, a 512 bp fragment representing the UL1 gene of ILTV was PCR-amplified from virus DNA in the presence of digoxigenin-labelled dUTP (Boehringer Mannheim) instead of dTTP (Fuchs & Mettenleiter, 1996, 1999). Hybridization and detection reactions were performed as described (Teifke et al., 1998).

DNA sequence of the ILTV UL52–UL50 genes

In previous studies, parts of the ILTV homologues of the HSV-1 genes UL52 (Johnson et al., 1995b) and UL50 (Ziemann et al., 1998a) have been identified, indicating that there should be only a small gap between these two DNA sequences. To close this gap within the genome sequence of ILTV, we analysed two independently generated plasmid clones of the 9.8 kb EcoRI-fragment D of our ILTV WT strain (pILT-ED1/2, Fig. 1B). The newly determined DNA sequence (GenBank accession no. AJ249803) of a 4471 bp EcoRI-Sall subfragment was identical in both clones, and overlapped with published sequences of the ILTV vaccine strain SA-2 (no. ghu80762; Johnson et al., 1997) from position 1–1697. The identity between the different ILTV strains is 99.2%, but one of the few differences (at position 935) causes an additional EcoRI site in the SA-2 genome. To include the entire UL50 gene of ILTV, the new DNA sequence was extended to the right by parts of our previously described data (no. Y14300; Ziemann et al., 1998a). The resulting 5750 bp fragment of ILTV DNA comprises three complete ORFs (Fig. 1B, Table 1), which are flanked by putative TATA-box elements and polyadenylation signals. Since no such signal could be identified behind UL52, this ORF is probably transcribed 3′-coterminally with the downstream UL53 gene. Sequence comparisons of the deduced UL52, UL51 and UL50 proteins of ILTV revealed relatively high degrees of identity to the products of the similarly arranged counterparts in other avian and mammalian alphaherpesvirus genomes (Table 1), including Marek’s disease virus type 2 (MDV-2; Izumiya et al., 1998, 1999), HSV-1 (McGeoch et al., 1988), VZV (Davison & Scott, 1986) and PrV (Baumeister et al., 1995). UL52 encodes a component of the viral helicase–primase complex (Crute et al., 1989), and homologues were also found in genomes of beta- and gammaherpesviruses such as HCMV (Chee et al., 1990) and EBV (Baer et al., 1984). In contrast, UL51 is not significantly conserved in these herpesvirus subfamilies, and a UL50-like ORF was detectable in the genome of EBV, but not in that of HCMV (Table 1). All characterized UL50 proteins of mammalian alphaherpesviruses are dUTPases, which exhibit a specific arrangement of five highly conserved, and presumably functional, motifs (Jöns & Mettenleiter, 1996; McGeoch, 1990). A multiple amino acid sequence alignment of UL50 gene products shows that motifs 1, 2, 4 and 5 are well conserved in the predicted ILTV protein, whereas the most N-terminal, motif 3, is not (Fig. 2). However, further upstream in the alignment, the UL50 gene product of ILTV exhibits a related amino acid sequence containing three of the five conserved residues of motif 3 (underlined in Fig. 2).

Improvement of viral DNA transfection

In the past it proved to be a problem to generate ILTV recombinants by cotransfection of cells with transfer plasmids and genomic viral DNA. As a consequence of the relatively inefficient replication of ILTV in tissue culture, it is difficult to prepare large amounts of pure viral DNA, and infectivity of the obtained DNA is generally low. Since previous studies showed that transient expression of viral transactivator proteins increased the infectivity of VZV DNA in cultured cells (Moriiuchi et al., 1993, 1994), we tested the effects of expression plasmids encoding the ILTV homologues of aTIF (VP16, UL48 gene product; Ziemann et al., 1998a) or ICP4 (Johnson et al., 1995a) under control of the HCMV IE and T7 promoters (Fig. 1D). In vitro transcription and translation experiments with the generated plasmids pRc-UL48 and pRc-ICP4 revealed expression of properly sized proteins (data not shown). After
Table 1. Properties of the identified ILTV genes

Nucleotide positions of ORFs and transcription signals refer to the DNA sequence (GenBank no. AJ249803), and are marked by (r) if located on the reverse strand. Expected transcript sizes and molecular masses of proteins were calculated from the respective sequence patterns. Other herpesvirus homologues of the deduced ILTV proteins were identified in databases using the GCG program 'blast'; percentage of identical and related (in parentheses) amino acids was determined with the program ‘gap’. In some cases, no significantly conserved homologues were detectable (n.d.).

<table>
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<tr>
<th>ORF</th>
<th>name</th>
<th>UL52 position</th>
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<td>&gt; 1.0</td>
<td>&gt; 1.5</td>
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<tr>
<td>homologues (%)</td>
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calcium phosphate cotransfection of LMH cells with either of these constructs and ILTV DNA the number of progeny virus plaques detectable after overlay with semi-solid medium was reproducibly increased three- to ninefold compared to parallel experiments performed with control plasmids (data not shown). Therefore, pRc-UL48 and pRc-ICP4 were added to all further cotransfections of ILTV DNA.

Generation and genetic characterization of UL50 mutants

To facilitate the isolation of UL50-negative ILTV, recombination plasmid pILT-CSG was constructed in which nearly the entire UL50 ORF (codons 10–366) was replaced by a GFP expression cassette in reverse orientation (Fig. 1C). After cotransfection of LMH cells with ILTV WT DNA, pILT-CSG and transactivator plasmids, plaques of recombinant progeny virus could be easily identified in a fluorescence microscope, and purified to homogeneity by repeated limiting dilution in CEK cells. In a similar manner, the resulting recombinant ILTV AUL50G was used as parental virus of a UL50 rescue mutant (ILTV UL50R), and of a second deletion mutant (ILTV AUL50) which contains no foreign DNA sequences. To this end, non-fluorescent virus plaques were selected from the progenies of cotransfection experiments performed with ILTV AUL50G DNA and plasmids pILT-CS or -CSD, respectively (Fig. 1C).

To verify whether all obtained ILTV recombinants contain the expected mutations, Southern blot analyses of EcoRI-digested virus DNA were performed (Fig. 3). Hybridization with the labelled plasmid pILT-ED (Fig. 3B) revealed that, instead of the 9.8 kbp EcoRI-fragment D of ILTV WT DNA, two novel DNA fragments of ca. 5 kbp each were detected in ILTV AUL50G DNA as a consequence of an additional EcoRI site within the GFP expression cassette (Fig. 1C). As expected, the authentic EcoRI-fragment D is restored in the genome of ILTV UL50R, whereas ILTV AUL50 possesses a ca. 1 kbp shorter fragment (Fig. 3B). Further hybridization experiments confirmed the absence of the deleted UL50 gene fragment from the ILTV AUL50G and AUL50 genomes (Fig. 3C), as well as the presence of the GFP expression cassette in ILTV AUL50G DNA (Fig. 3D). The observed alterations of DNA restriction patterns are in good agreement with the calculated fragment sizes (indicated to the right of Fig. 3). Accuracy of the mutations and purity of the virus stocks were further confirmed by PCR amplification of the UL50 gene region of all ILTV recombinants with primers UL50-F and UL50-R (Fig. 1C), followed by cloning and sequencing of the products (data not shown).

However, after 10 rounds of in vitro propagation or after a single in vivo passage of ILTV AUL50G (see below), non-fluorescent virus plaques appeared reproducibly. PCR amplification and sequencing of viral DNA revealed that these ILTV mutants still contain the UL50 deletion, but in addition they exhibit different deletions within the reporter gene. The observed deletions vary in size between 696 and 829 bp, but all of them contain parts of the GFP ORF, and some of them also include the preceding HCMV IE gene promoter or flanking ILTV DNA sequences (data not shown). These results...
demonstrate that the GFP gene insertion within the UL50 locus of ILTV is highly unstable.

The UL50 gene product of ILTV exhibits dUTPase activity

Nuclear extracts of LMH cells, which had been infected at an m.o.i. of 1 for 16 h with either ILTV WT, the UL50-negative virus recombinants ILTV ∆UL50G and ILTV ∆UL50, or the rescue mutant ILTV UL50R, were tested by dUTPase assays (Fig. 4). After incubation of extracts of ILTV WT- or ILTV UL50R-infected cells with $[^2H]$UTP, ca. 45% of the provided radionucleoside-triphosphate was transformed into $[^2H]$dUMP. In contrast, in similarly prepared lysates of cells infected with ILTV ∆UL50G or ∆UL50, only 10–17% dUMP was detectable (Fig. 4). These amounts are apparently produced by cellular dUTPase, since nearly 20% dUMP was also found after incubation of $[^3H]$dUTP with the nuclei of non-infected LMH cells (Fig. 4), whereas no conversion was observed after incubation without any extract. Very similar results were obtained from two independent experiments (Fig. 4).
ILTV dUTPase

ILTV \( \Delta UL50 \) appeared nearly identical to that of WT virus and revertant (Fig. 5A), indicating that the deletion of UL50 has no detectable effect on virus replication in cell culture. However, plaque assays revealed that cell-to-cell spread of ILTV \( \Delta UL50G \) is significantly impaired in both CEK (not shown) and LMH cells (Fig. 5B). Four days after infection of LMH cells, the average plaque diameters of two different ILTV \( \Delta UL50G \) isolates derived from independent transfection experiments were only one-third of the WT size (Fig. 5B). This defect was found to be repaired not only in the UL50 rescue mutant ILTV UL50R, but surprisingly also in the deletion mutant ILTV \( \Delta UL50 \) (Fig. 5B), which differs from \( \Delta UL50G \) only by the absence of the reporter gene (Fig. 1C). Since ILTV \( \Delta UL50 \) was derived from ILTV \( \Delta UL50G \), we conclude that the small-plaque phenotype of the latter mutant is exclusively caused by the GFP gene insertion. This assumption was further confirmed by the fact that the spontaneously occurring GFP inactivation mutants of ILTV \( \Delta UL50G \) (see above) also exhibit WT-sized plaques in cell culture (data not shown).

**Replication of the ILTV mutants in chickens**

An animal trial was performed to investigate whether the UL50 gene mutants of ILTV are attenuated in the natural host, and whether they are able to confer protective immunity. The results of these experiments are summarized in Table 2. From the second day after intratracheal infection of naïve chickens with virulent ILTV WT clinical signs of an acute respiratory disease became obvious, such as gasping, coughing and expectoration of bloody mucus. The symptoms were most severe between 3 and 5 days after infection, and several of the animals died (Table 2). From day 1–5 infectious virus could be reisolated from tracheal swabs, and ILTV-specific antibodies were detectable in sera of convalescent animals by indirect immunofluorescence reactions after 2 weeks. Histopathological investigations of HE-stained sections of ILTV WT-infected chicken tissue revealed lesions in larynx and trachea, including inflammatory infiltration of mucosa and submucosa with lymphocytes and macrophages, and focal epithelial syncytia, which often contained intranuclear inclusion bodies. Later during infection (4 days post-infection), oedema of lamina propria, haemorrhage and desquamation of the mucosa led to formation of a fibrino-sanguino-purulent exudate in the lumen of larynx and trachea (Fig. 6A). In most cases, the lungs were also affected, as shown by the occurrence of necroses and syncytia in the bronchiolar epithelia (Fig. 6E). Parallel in situ hybridization experiments showed that most of these lesions colocalize with ILTV DNA (Fig. 6F). All these observations are consistent with earlier studies (reviewed by Bagust & Guy, 1997).

The in vivo growth characteristics of the UL50 revertant ILTV UL50R were indistinguishable from those of WT ILTV (Table 2, Fig. 6B). In contrast, the GFP-expressing ILTV mutant \( \Delta UL50G \) appeared to be almost completely apatho-

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**In vitro replication of UL50 mutants**

To analyse the growth properties of the ILTV recombinants in cultured cells, one-step growth kinetics were established and plaque assays were performed (Fig. 5). From CEK cells no progeny virus could be reisolated until 8 h after infection with ILTV at high multiplicity. Then the titres increased rapidly, and reached their maximum of ca. \( 10^8 \) p.f.u./ml at 20 h after infection (Fig. 5A). One-step growth of ILTV \( \Delta UL50G \) and
Fig. 5. *In vitro* replication of WT and recombinant ILTV (ΔUL50G, ΔUL50, UL50R). (A) For one-step growth kinetics CEK cells were scraped into the medium at indicated times after infection at an m.o. i. of 5, and lysed by freezing and thawing. Progeny virus titres were determined by plaque assays on LMH cells. (B) Plaque diameters of WT virus and two isolates (a, b) of each recombinant were determined after a 4-day incubation of infected LMH cells under semi-solid medium. Average diameters of thirty plaques per virus and standard deviations are indicated.

Table 2. Animal experiments

Chickens were intratracheally infected with either WT or recombinant ILTV (ΔUL50G, ΔUL50, UL50R), and challenged with the same dose of WT virus 4 weeks later. Shed virus from tracheal swabs was titrated by plaque assays on LMH cells, and average titres of each virus are indicated. ILTV-specific serum antibodies were detected by indirect immunofluorescence reactions with infected CEK cells. Clinical signs were scored from slightly exerted respiration of some animals (+/−) to heavy gasping with mucus expectoration (+++ ++). Histopathological lesions ranged from mild oedematous thickening (+) to desquamation (++++) of the laryngotracheal mucosa. The noted time periods of investigation refer to the dates of primary infection (d p.i.), or challenge infection (d p.ch.). Some tests were not performed (n.t.) with non-infected (n.i.) control animals.

<table>
<thead>
<tr>
<th>Timescale</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary infection</strong> (5 x 10⁵ pfu/animal)</td>
<td><strong>WT</strong></td>
</tr>
<tr>
<td>clinical signs (gasping, coughing)</td>
<td>2 - 7 d.p.i.</td>
</tr>
<tr>
<td>Mortality</td>
<td>3 - 6 d.p.i.</td>
</tr>
<tr>
<td>virus shedding (pfu/ml in tracheal swabs)</td>
<td>3 - 4 d.p.i.</td>
</tr>
<tr>
<td>pathology (larynx, trachea, lung)</td>
<td>3 - 4 d.p.i.</td>
</tr>
<tr>
<td>ILTV specific antibodies (IFT)</td>
<td>14 d.p.i.</td>
</tr>
<tr>
<td><strong>Challenge infection</strong> (5 x 10⁵ pfu/animal)</td>
<td><strong>WT</strong></td>
</tr>
<tr>
<td>clinical signs</td>
<td>2 - 4 d.p.ch.</td>
</tr>
<tr>
<td>Mortality</td>
<td>3 - 4 d.p.ch.</td>
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<tr>
<td>virus shedding</td>
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</tr>
<tr>
<td>Pathology</td>
<td>4 d.p.ch.</td>
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</tbody>
</table>

In summary, under our experimental conditions both dUTPase-negative ILTV recombinants, as well as WT ILTV, were able to protect chickens from the fatal consequences of a subsequent infection. However, the deletion mutant ILTV ΔUL50 was still pathogenic, and the avirulent substitution mutant ILTV ΔUL50G was genetically unstable *in vitro* and *in vivo*. Besides the GFP-expressing input virus, increasing amounts of non-fluorescent ILTV could be reisolated from all chickens infected with ILTV ΔUL50G after 4 and 5 days. Since the genomes of these viruses exhibit different deletions within the inserted foreign sequence (see above), they probably arose independently in each animal.

Discussion

We determined the DNA sequence of three ILTV genes, which are arranged in a similar way as their homologues UL52, UL50, and GDE.
UL51 and UL50 in the genomes of HSV-1 and other alphaherpesviruses (Baumeister et al., 1995; Davison & Scott, 1986; Izumiya et al., 1998; McGeoch et al., 1988). The UL52, UL8 and UL5 gene products of HSV-1 form a tripartite helicase–primase complex, which is essential for viral DNA replication (Crute et al., 1989). A similar complex is probably formed by ILTV, since homologues of the UL5 and UL8 genes were also detected (Fuchs & Mettenleiter, 1996, 1999). Much
less is known about the UL51 gene, although its product was identified as a virion protein of PrV and HSV-1 (Daikoku et al., 1998; Lenk et al., 1997). The products of the UL50 homologues of many alphaherspesviruses possess dUTPase activity, and a specific arrangement of five conserved sequence motifs was supposed to be required for this function (Jöns & Mettenleiter, 1996; Liang et al., 1993; McGeoch, 1990; Ross et al., 1997; Williams et al., 1985; Wohlrab et al., 1982). Remarkably, the most N-terminal of these motifs (‘motif 3’) is absent from the predicted ILTV protein. However, our results demonstrated that the UL50 gene product of ILTV is an active dUTPase, indicating that ‘motif 3’ might be either dispensable in general, or replaced by a functionally equivalent element which possesses an at least partly different amino acid sequence. One putative substitute, exhibiting three of the five conserved residues of ‘motif 3’, was found at a related position within the ILTV protein.

Both UL51 and UL50 were shown to be non-essential for replication of HSV-1 in cell culture (Barker & Roizman, 1990). The UL50 homologue of ILTV is apparently also not required for virus growth in vitro, since we isolated viable virus recombinants carrying deletions of nearly the entire gene. Probably because of the almost ubiquitous presence of cellular dUTPases, deletion of the UL50 gene of ILTV has no detectable effects on virus replication in cell culture, as quantified by one-step growth kinetics and plaque sizes. Similarly, UL50-negative HSV-1, PrV and VZV mutants were also phenotypically inconspicuous in vitro (Barker & Roizman, 1990; Jöns & Mettenleiter, 1996; Ross et al., 1997). However, the same recombinants of HSV-1 and PrV were shown to be attenuated in mice or pigs, respectively (Jöns et al., 1997; Pyles et al., 1992). In contrast, virulence of ILTV ∆UL50 in chickens was not significantly reduced. An obvious explanation for this difference might be the different target organs of these viruses. Whereas clinical symptoms and mortality caused by HSV-1 in mice, and by PrV in its natural host are predominantly a consequence of neuroinvasion, lytic ILTV infection is restricted predominantly to the respiratory tract of chickens (Bagust & Guy, 1997). This was confirmed by our studies, since none of the animals infected with either ∆UL50 or WT ILTV exhibited any histopathological lesions in the brain (data not shown). Because expression of cellular dUTPase is regulated in a cell-cycle-dependent manner (Duker & Grant, 1980), it was suggested that the ‘redundant’ herpesvirus enzyme is required for efficient lytic replication in non-dividing cells like neurons (Jöns & Mettenleiter, 1996; Pyles et al., 1992). Although ILTV is not neurovirulent, it is able to establish latent infections in the trigeminal ganglia of chickens (Williams et al., 1992). Therefore, it will be interesting to investigate whether the deletion of the ILTV UL50 gene has any effects on establishment of, or reactivation from, latency.

Although the UL50-deleted ILTV recombinant AUL50 is virulent, a similar recombinant, ILTV AUL50G, which differs from the other mutant only in the insertion of a GFP expression cassette, is not. Moreover, ILTV AUL50G exhibits a small-plaque phenotype in cultured cells. Probably due to this negative effect, expression of GFP from the UL50 locus of ILTV was unstable, and various deletion mutants spontaneously arose in vitro and in vivo, which all result in abolishment of GFP expression. Obviously, there is a strong selective pressure against GFP expression in these viruses. Similar phenomena were also observed with other virus recombinants expressing GFP from the thymidine kinase (UL23) or the unique UL0 gene locus of ILTV (unpublished results). Since one-step growth kinetics of any of these virus recombinants are not affected, we speculate that only after infection at low multiplicity does the overexpressed GFP accumulate to concentrations that impair productive virus replication, or virus spread. Thus, the value of GFP under control of the strong and constitutively active HCMV IE gene promoter as an insertion marker for ILTV is questionable.

Up to now, construction of ILTV recombinants suffered from the low infectivity of viral DNA, which is also difficult to purify in large enough quantities. Since the UL48 gene product of HSV-1, αTIF, and the IE CIP4 protein function in subsequent transactivation of viral genes and, thus, enhance infectivity of viral DNA (Dargan & Subak-Sharpe, 1997), we tested whether plasmids from which expression of ILTV UL48 and ICP4 is driven by the HCMV IE promoter have any effect on virus yield in DNA cotransfection assays. Our results show that both ILTV proteins increased the infectivity of ILTV DNA ca. fivefold. Interestingly, their effects are not additive and inclusion of either plasmid gave similar results. This is in contrast to results with the homologous transactivators of VZV, where the ICP4 homologue showed a much more pronounced effect on infectivity of virion DNA than αTIF (Moriuchi et al., 1994). We therefore speculate that in our assays infectivity might still be limited by the low input amount of replication-competent virus DNA. Nevertheless, inclusion of the UL48 and ICP4 expression plasmids significantly facilitated the generation of ILTV recombinants. Thus, this technique will be used to generate further candidates for an improved ILTV vaccine.

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References


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