The UL47 gene of avian infectious laryngotracheitis virus is not essential for *in vitro* replication but is relevant for virulence in chickens

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The genome of infectious laryngotracheitis virus (ILTV) exhibits several differences from those of other avian and mammalian alphaherpesviruses. One of them is the translocation of the conserved UL47 gene from the unique long (UL) to the unique short (US) genome region, where UL47 is inserted upstream of the US4 gene homologue. As in other alphaherpesviruses, UL47 encodes a major tegument protein of ILTV particles, whereas the US4 gene product is a non-structural glycoprotein, gG, which is secreted from infected cells. For functional characterization, an ILTV recombinant was isolated in which US4 together with the 3′-terminal part of UL47 was replaced by a reporter gene cassette encoding green fluorescent protein. From this virus, UL47 and US4 single-gene deletion mutants without foreign sequences were derived and virus revertants were also generated. *In vitro* studies revealed that both genes were non-essential for ILTV replication in cultured cells. Whereas US4-negative ILTV exhibited no detectable growth defects, maximum virus titres of the double deletion mutant and of UL47-negative ILTV were reduced about 10-fold compared with those of wild-type virus and rescued virus. Experimental infection of chickens demonstrated that UL47-negative ILTV was significantly attenuated *in vivo* and was shed in reduced amounts, whereas wild-type and rescued viruses caused severe disease and high mortality rates. As all immunized animals were protected against subsequent challenge infection with virulent ILTV, the UL47 deletion mutant might be suitable as a live-virus vaccine.

INTRODUCTION

Infectious laryngotracheitis (ILT) is a worldwide contagious respiratory disease of chickens that causes severe economic losses in the poultry industry (reviewed by Guy & Bagust, 2003). An incubation period of 3–12 days is followed by the acute phase of the infection, which lasts between 1 and 2 weeks. Frequent clinical signs are conjunctivitis, gasping, coughing and expectoration of bloody mucus. ILT significantly reduces weight gain and egg production, and mortality rates of up to 70% have been observed. After the acute phase, an asymptomatic latent infection of the sensory neurones is established, which can be reactivated by various stress factors. For prevention of ILT, chickens can be immunized with live-virus vaccines that have been attenuated by serial passage in cell culture or embryonated chicken eggs (Guy & Bagust, 2003). These vaccines are suitable for mass application via eye drops, aerosols or drinking water. However, they have not been characterized genetically and several of them possess a considerable residual virulence, which may further increase after animal passage (Guy *et al.*, 1990, 1991).

ILT is caused by ILT virus (ILTV; *Gallid herpesvirus 1*), which is classified as a member of the genus *Iltovirus* of the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Davison *et al.*, 2005). Recently, a DNA sequence of the complete ILTV genome was assembled from previously published sequences of genome fragments of different virus strains, which had been analysed in different laboratories (Thureen & Keeler, 2006). This study confirmed that ILTV possesses a type D herpesvirus genome (Roizman & Pellet, 2001), which is ~150 kbp and consists of long (UL) and short (US) unique regions, with inverted repeat sequences (IRs, TRs) flanking the latter (Johnson *et al.*, 1991; Leib *et al.*, 1987) (Fig. 1a). As the gene content and arrangement of the ILTV DNA are similar to those found in other alphaherpesviruses, the designations of open reading frames (ORFs) and proteins have been widely adopted from the homologues in herpes simplex virus type 1 (HSV-1) (McGeoch *et al.*, 1988).

Nevertheless, ILTV exhibits a considerable phylogenetic distance from most other avian and mammalian
Fig. 1. Map of the ILTV genome, which consists of long ($U_L$) and short ($U_S$) unique regions with inverted repeat sequences ($IR_S$, $TR_S$) flanking the $U_S$ region (a). The positions of KpnI restriction fragments and selected virus genes (pointed rectangles) are indicated. An enlarged section of the $U_S$ region shows KpnI fragment J cloned in pILT-K16 (b), which contains the translocated UL47 gene and the US4 gene. In plasmid pILT-K16G (c), US4 and the 3' end of UL47 were replaced by an expression cassette encoding EGFP flanked by the immediate-early promoter of human cytomegalovirus ($P_{HCMV-IE}$) and a polyadenylation signal of simian virus 40 ($A^{+}_{SV40}$). Plasmids pILT-K16BA (d) and pILT-K15NB (e) contain deletions of the UL47 or US4 genes, respectively. Retained codon ranges of the analysed genes and relevant restriction sites are indicated (vector site in parentheses). The plasmids were used to generate the indicated ILTV recombinants (in italics).
alphaherpesviruses (Johnson & Tyack, 1995; McGeoch et al., 2000). The virus most closely related to ILTV that has been described is the parrot pathogen *Psittacid herpesvirus 1* (PsHV-1) (Thureen & Keeler, 2006). The genomes of these two viruses contain several common genes that are not found in any other known herpesvirus, and a conserved gene cluster including the homologues of HSV-1 UL22–UL44 is inverted within the U5 region (Thureen & Keeler, 2006; Ziemann et al., 1998a, b). In most alphaherpesviruses, the U1 region contains a gene cluster (UL46, UL47, UL48 and UL49) encoding four major tegument proteins. In ILTV and PsHV-1, however, the UL47 gene is translocated to the US region and is localized between the conserved US3 (protein kinase) and US4 [glycoprotein G (gG)] genes (Thureen & Keeler, 2006; Wild et al., 1996; Ziemann et al., 1998a) (Fig. 1b). As a consequence of this translocation event, UL47 and US4 of ILTV are expressed from 3′-co-terminal mRNAs (Helferich et al., 2007).

Despite its translocation, the UL47 gene encodes an abundant virion protein of ILTV, whereas the N-glycosylated product of the downstream US4 gene is not incorporated into mature virus particles, but is secreted from infected cells (Helferich et al., 2007; Kongsuwan et al., 1993). Secretion of gG has also been found for other alphaherpesviruses such as herpes simplex virus type 2 (HSV-2), pseudorabies virus (PrV), *Bovine herpesvirus 1* (BHV-1) and *Equid herpesvirus 4* (EHV-4) (Crabb et al., 1992; Keil et al., 1996; Rea et al., 1985; Su et al., 1987). The biological relevance of these secreted glycoproteins is largely unknown. It is possible that they modulate the host immune response, as *in vitro* studies have revealed that the gG homologues of EHV-1, BHV-1 and other alphaherpesviruses are able to bind chemokines (Bryant et al., 2003) and that the HSV-2 protein possesses pro-inflammatory properties that affect certain leukocytes (Bellner et al., 2005). In all alphaherpesviruses tested, the gG homologues have been shown to be dispensable for virus replication in cultured cells (Roizman & Knipe, 2001), and for PrV it was also shown that the deletion did not affect virulence in pigs (Kopp et al., 1991). However, the UL47 gene product of HSV-1 appears to be involved in stimulation of viral immediate-early gene transcription in newly infected cells, which is predominantly mediated by the UL48 protein (McKnight et al., 1987; Zhang & McKnight, 1993; Zhang et al., 1991). A role of UL47 during virion morphogenesis has been shown for PrV, where deletion of UL47 impaired secondary envelopment of nucleocapsids in the cytoplasm and led to significantly reduced virus titres (Kopp et al., 2002). Furthermore, UL47-negative PrV is moderately attenuated in model animals, as shown by prolonged survival times of experimentally infected mice (Klopfleisch et al., 2006). However, to our knowledge, the role of the UL47 proteins of alphaherpesviruses during infection of their natural host has not been investigated up to now.

For ILTV, such experiments are of particular interest, as mutants with defined, irreversible gene deletions might be safer vaccines than the attenuated virus strains that are still in use. Therefore, several ILTV recombinants have already been generated, which lack the thymidine kinase gene UL23 (Okamura et al., 1994; Schnitzlein et al., 1995), the dUTPase gene UL50 (Fuchs et al., 2000), the glycoprotein homologues encoded by UL10 (gM, non-glycosylated ILTV protein), UL49.5 (gN), US4 (gG) and US5 (gL) (Devlin et al., 2006; Fuchs & Mettenleiter, 2005; Fuchs et al. 2005), or the iltvirus-specific ORF-A to ORF-E and UL0 genes (Veits et al., 2003b, c). Experimental infection of chickens revealed that UL0-, UL23- and US5-negative ILTV mutants were almost avirulent, whereas deletion of UL50 or US4 led to only moderate attenuation. All tested virus recombinants were able to confer protective immunity against subsequent challenge infection with pathogenic ILTV strains (Fuchs et al., 2000; Schnitzlein et al., 1995; Veits et al., 2003c). Furthermore, ILTV vectors that had been engineered to express H5 or H7 influenza virus haemagglutinin protected chickens against lethal infection with highly pathogenic avian influenza viruses of the corresponding serotypes (Lüscho et al., 2001; Veits et al., 2003c).

In the present study, parts of the adjacent UL47 and US4 genes of ILTV were substituted by an expression cassette encoding enhanced green fluorescent protein (EGFP). The obtained virus recombinant was then used to generate single-gene deletion mutants of UL47 or gG without foreign sequence insertions, as well as corresponding rescued viruses. The growth properties of all virus recombinants in cultured cells were characterized. In addition, an animal trial was performed to investigate the relevance of UL47 for virulence and immunogenicity in chickens.

**METHODS**

**Viruses and cells.** ILTV recombinants were generated by co-transfection of chicken hepatoma (LMH) cells (Kawaguchi et al., 1987; Schnitzlein et al., 1994) with transfer plasmids (see below) and genomic DNA of the virulent ILTV strain A489 (obtained from D. Lütticken, Boxmeer, The Netherlands). Wild-type and mutant viruses were propagated further in primary chicken embryo kidney (CEK) cells, as described previously (Fuchs & Mettenleiter, 1996). Cells were grown in minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen), and after infection were maintained in MEM with 2–5% FCS.

**Plasmid construction.** The 5165 bp *KpnI* fragment J representing nt 125755–130925 of the ILTV genome (GenBank accession no. NC_006623; Thureen & Keeler, 2006), which contains the UL47 and US4 genes, was cloned into the *KpnI*-digested phagemid vector pBSI(−) (Stratagen). From the resulting construct pLIT-K16 (Fig. 1b), three different deletion plasmids were derived. In pLIT-K16G (Fig. 1c), a 2656 bp *AflII*-Sall fragment, which included codons 558–624 of UL47 and codons 1–271 of US4, was replaced by...
an EGFP expression cassette that had been isolated as a 1634 bp AflII–AseI fragment of pEGFP-N1 (Clontech). To generate pILT-K16BA (Fig. 1d), which lacked codons 70–556 of UL47, two separate double digestions of pILT-K16 were performed with AflII and EcoRI, or BssHII and EcoRI, respectively. An isolated 1077 bp BssHII–EcoRI fragment was then used for ligation with the AflII–EcoRI-digested and phosphatase-treated plasmid. In plasmid pILT-K16NB (Fig. 1c), the US4 ORF was removed completely by deletion of a 996 bp Bcl–NheI fragment from pILT-K16. In all cloning experiments, non-compatible fragment ends were blunt-ended by treatment with Klenow polymerase.

Generation of virus recombinants. Virus recombinants were generated by calcium phosphate-mediated transfection (Graham & van der Eb, 1973) of LMH cells with the described transfer plasmids, genomic ILTV DNA and plasmids expressing the ILTV homologues of UL48 and ICP4, which have been shown to increase the infectivity of viral DNA (Fuchs et al., 2000). The EGFP-expressing double mutant ILTV-UL47/US4G was isolated from fluorescent plaques obtained after co-transfection of cells with DNA of wild-type ILTV-A489 and pILT-K16G (Fig. 1c). To facilitate isolation of recombinants with single deletions of UL47 or US4, genomic DNA of ILTV-A47/US4G was used for co-transfection with pILT-K16BA or pILT-K16NB (Fig. 1d, e) and virus progeny was screened for non-fluorescent plaques. The rescued mutants ILTV-UL47R and ILTV-US4R were derived from the corresponding deletion mutants using plasmid pILT-K16 for co-transfection (Fig. 1b). To permit identification of the rescued virus, limiting dilutions of the transfection progeny were propagated in CEK cells grown in 96-well microtitre plates. Aliquots of the cell lysates were spotted onto nitrocellulose membranes (Minifold I; Schleicher & Schuell). For virion disruption and denaturation of viral DNA, the filters were incubated in 0.5 M NaOH and neutralized in 1 M Tris/HCl (pH 7.4)/0.6 M NaCl, followed by 0.6 M Tris/HCl (pH 7.4)/1.5 M NaCl for 5 min each. The membranes were dried in a vacuum oven for 1 h at 80 °C and incubated with 32P-labelled probes (RediPrime II kit; Amersham) of the deleted BssHII–AflII or Bcl–NheI fragments of pILT-K16, as described previously (Fuchs & Mettenleiter, 1999). After three plaque-purification steps, genomic DNA of the ILTV mutants was prepared and analysed by restriction endonuclease digestion and Southern blot hybridization.

Western blot analyses. CEK cells were infected with wild-type ILTV or virus mutants at an m.o.i. of 2 p.f.u. per cell and incubated for 24 h at 37 °C. Lysates of ~105 infected or uninfected cells were separated by discontinuous SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose filters (Trans-Blot SD Cell; Bio-Rad). Blots were incubated with monospecific rabbit antisera against the UL47 and US4 proteins (Helferich et al., 2007) at dilutions of 1:100 000 or with a mouse monoclonal antibody (mAb) against ILTV glycoprotein gC (Veits et al., 2003a) at a dilution of 1:1000. Binding of peroxidase-conjugated secondary antibodies (Dianova) was detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

Plaque assays and growth kinetics. For determination of plaque sizes, CEK cells were infected with wild-type or mutant ILTV at a low m.o.i. (<0.001). After 2 h, the inoculum was replaced by MEM containing 5% FCS and 6 g methionylcellulose 1–1 and incubation was continued for 2 days at 37 °C. Thereafter, the cells were fixed and plaques were visualized by indirect immunofluorescence (IIF) reactions of a mAb against gI of ILTV (Veits et al., 2003a). Antibody incubation was dispensable for cells infected with the EGFP-expressing mutant ILTV-UL47/US4G. The diameters of 50 plaques each were determined and means ± SD were calculated. One-step growth analyses were performed essentially as described previously (Fuchs et al., 2000). CEK cells were infected at an m.o.i. of 5 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 freeze-thawing and progeny virus titres were determined by plaque assays on CEK cells. The mean results of two independent experiments were plotted.

Animal experiments. White Leghorn chickens were bred from specific-pathogen-free eggs (Lohmann Tierzucht). At the age of 8 weeks, the chickens were divided into three groups of 12 animals each and infected intratracheally with ~2 × 105 p.f.u. of wild-type ILTV-A489, ILTV-UL47 or ILTV-UL47R. The animals were observed for a period of 10 days post-infection (p.i.) and clinical scores were determined as described previously (Fuchs et al., 2005). Briefly, all chickens were classified daily as healthy (0), slightly ill (1: occasional coughing, gasping or sneezing, general condition not affected), ill (2: permanent respiratory disorder, rhinitis, depression), severely ill (3: marked dyspnoea, discharge of bloody mucus, open beaks, exhaustion) or dead (4). The mean values for each group were calculated for the entire monitoring period and dead animals were considered until day 10. One animal from each group was necropsied at days 3 and 4 p.i. and tissue samples of larynx, trachea and lung were fixed for 24 h in 4% phosphate-buffered neutral formaldehyde and then paraffin embedded. Serial sections (3 μm) were dewaxed, mounted on positively charged SuperFrost Plus microscope slides (Menzel) and stained with haematoxylin and eosin (H&E) for light microscopy. For re-isolation of ILTV, tracheal swabs were taken at days 3, 4 and 5 p.i. Virus was released from the swabs by incubation in cell culture medium for 2 h at room temperature, followed by ultrasonic treatment and freeze-thawing. The suspensions were then serially diluted and virus titres were determined by plaque assays on CEK cells. Before infection, as well as at days 17 and 24 p.i., sera were collected and tested for ILTV-specific antibodies by IIF tests on infected CEK cells (Lüscherow et al., 2001). In addition, the sera were tested by IIF on LMH cells that had been fixed 48 h after transfection with pcDNA-UL47 (Helferich et al., 2007) or pRc-IgIc (Fuchs et al., 2005). At day 28 p.i., all survivors as well as five non-immunized control animals were infected by intratracheal administration of 1 × 105 p.f.u. wild-type ILTV-A489. Clinical signs were monitored for 10 days and tracheal swabs were taken at days 3, 4 and 5 post-challenge infection (p.c.). Single animals of each group were necropsied at days 3 and 4 p.c. and investigated for pathological alterations. For antibody detection, sera were prepared at days 11 and 28 p.c. At day 28 p.c., all surviving animals were euthanized and dissected.

RESULTS AND DISCUSSION

Isolation of virus recombinants

For functional analyses of the adjacent UL47 and US4 genes, deletion mutants were generated by homologous recombination between virion DNA and transfer plasmids in transfected LMH cells. In recombinant ILTV-UL47/US4G, major parts of US4 (codons 1–271) and the 3′ end of UL47 (codons 558–624) were deleted and substituted by insertion of an EGFP expression cassette (Fig. 1b). This reporter gene insertion facilitated isolation of the virus mutant. However, as previous studies have demonstrated that overexpression of EGFP might affect virus replication (Fuchs et al., 2000), further recombinants without foreign gene insertions were derived from ILTV-UL47/US4G. These mutants were isolated from non-fluorescent virus plaques for investigation of the effects of single-gene deletions, as they lacked either codons 70–556 of UL47 (ILTV-UL47, Fig. 1d) or the complete US4 gene (ILTV-US4, Fig. 1e). To demonstrate
that replication defects of the mutants were indeed caused by deletion of the investigated genes and not by unexpected second-site mutations, rescuants ILTV-UL47R and ILTV-US4R (Fig. 1b) were derived from both single-gene deletion mutants.

Restriction analyses of virion DNA and subsequent Southern blot analyses confirmed the presence of the desired alterations in the genomes of all investigated ILTV mutants (results not shown). Western blot analyses were performed to examine viral protein expression in infected CEK cells (Fig. 2). The blots were incubated with monoclonal antisera against the UL47 protein or against gG encoded by US4 (Helferich et al., 2007). A mAb against gC (Veits et al., 2003a) was used to demonstrate that comparable amounts of this unaffected virion envelope protein were expressed by all ILTV mutants (Fig. 2c). The non-structural gG was not detectable in cells infected with ILTV-ΔUL47/US4G or ILTV-ΔUS4, whereas the UL47 deletion mutant and the rescued virus expressed wild-type-sized US4 gene products of ≥52 kDa (Fig. 2b). No UL47 protein was found in cells infected with ILTV-ΔUL47 (Fig. 2a); however, ILTV-ΔUL47/US4G expressed a UL47 gene product of ~56 kDa, which was smaller than the major 66 kDa proteins of wild-type ILTV and the US4 deletion and rescue mutants (Fig. 2a). It is not clear whether the additional UL47 gene products detected with all viruses resulted from rapid degradation or from functionally relevant processing events. Nevertheless, our studies showed that deletion of US4 did not affect expression of UL47 and vice versa. Apparently, removal of the last 66 codons of UL47 in ILTV-ΔUL47/US4G did not prevent expression of a stable protein. However, it remains to be elucidated whether the truncated UL47 gene product is incorporated into ILTV particles such as the authentic tegument protein of wild-type virions (Helferich et al., 2007).

**In vitro growth properties of UL47 and US4 mutants of ILTV**

Successful isolation and propagation of UL47 and US4 deletion mutants in non-complementing LMH and CEK cells demonstrated that neither of the two genes was essential for productive in vitro replication of ILTV. To examine the effects of the gene deletions more precisely, plaque sizes and growth kinetics of all generated mutants in CEK cell cultures were determined (Fig. 3). At day 2 after infection, fluorescence microscopy revealed that the mean diameters of the virus-induced plaques and syncytia of ILTV-ΔUL47/US4G and ILTV-ΔUL47 were only marginally reduced to ~95% of the wild-type sizes, whereas spread of ILTV-ΔUS4 and the revertants was not affected compared with ILTV-A489 (Fig. 3a). One-step growth kinetics of ILTV-ΔUL47/US4G and ILTV-ΔUL47 appeared to be delayed and maximum titres of both mutants were reduced ~10-fold compared with wild-type ILTV and the rescued mutants (Fig. 3b). In contrast, virus titres were not significantly decreased after deletion of the gG gene in ILTV-ΔUS4 (Fig. 3b). In agreement with this result, the homologous US4 gene products of other alphaherpesviruses such as HSV-1, PrV and BHV-1 have also been shown to be dispensable for virus replication in cell culture, and the respective deletion mutants exhibited no or only moderate replication defects (Balan et al., 1994; Nakamichi et al., 2000; Thomsen et al., 1987; Trapp et al., 2003). The UL47 genes of HSV-1, PrV and MDV-1 are also non-essential for replication in cell culture (Dorange et al., 2002; Kopp et al., 2002; Zhang et al., 1991). However, a UL47-negative PrV mutant was shown to exhibit significant in vitro replication defects with respect to both plaque formation and virus yields (Kopp et al., 2002). The corresponding ILTV mutant showed comparable titre reductions, but plaque formation was barely affected. This discrepancy might be explained by

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**Fig. 2.** Protein analyses of CEK cells harvested 24 h after infection with ILTV-A489, ILTV-ΔUL47/US4G, ILTV-ΔUS4, ILTV-ΔUL47, ILTV-US4R or ILTV-UL47R at an m.o.i. of 2. Lysates of infected and uninfected cells (N) were separated by SDS-PAGE, and Western blots were analysed with monoclonal antisera against the UL47 protein (a), the US4 gene product gG (b) or a gC-specific monoclonal antibody (c). The molecular masses of marker proteins (kDa) and detected viral gene products are indicated. Note that ILTV-ΔUL47/US4G expresses a truncated UL47 protein (UL47′).
the observation that ILTV infection rapidly induces cell fusions, leading to the formation of large syncytia (Guy & Bagust, 2003), whereas PrV predominantly moves from cell to cell by the formation of mature virions and subsequent passage through the plasma membranes (Mettenleiter, 2002). Electron microscopy of cells infected with UL47-negative PrV revealed an impairment of secondary envelopment of nucleocapsids in the cytoplasm (Kopp et al., 2002), whereas similar studies of cells infected with ILTV-ΔUL47 indicated significantly reduced amounts of intra- and extracellular virions, but no clear inhibition of any particular maturation step (results not shown). Thus, in addition to its proposed function during virion maturation in the cytoplasm, the UL47 protein of ILTV may play a role during the early steps of the virus replication cycle, such as gene regulation or particle assembly in the cell nucleus. This hypothesis was supported by the detection of considerable amounts of UL47 protein in the nuclei of ILTV-infected cells (Helferich et al., 2007). The UL47 protein of HSV-1 has been shown to stimulate transactivation of herpesviral immediate-early gene promoters mediated by the UL48 gene product (McKnight et al., 1987; Zhang & McKnight, 1993; Zhang et al., 1991) and initial studies indicate that the homologous ILTV proteins might possess similar functions (Helferich et al., 2007).

Interestingly, ILTV-ΔUL47/US4G exhibited in vitro growth defects similar to those of ILTV-ΔUL47, although the double mutant expressed a truncated UL47 protein (see above). This finding indicates that the deleted C-terminal part comprising aa 556–623 of the UL47 protein may contain functionally relevant domains. However, as previous studies have revealed that insertion of foreign genes can affect replication of ILTV (Fuchs et al., 2000; Veits et al., 2003c), it cannot be ruled out that overexpression of the EGFP reporter protein may contribute to the phenotype of ILTV-ΔUL47/US4G.

**Virulence and immunogenicity of ILTV-ΔUL47 in chickens**

Although the precise reasons for the in vitro growth defect of ILTV-ΔUL47 remained unclear, it was conceivable that they would also lead to an attenuated phenotype in chickens. Therefore, an animal trial was performed to test whether ILTV-ΔUL47 might be suitable as a live-virus vaccine. Three groups of 8-week-old chickens were infected by intratracheal administration of 2\(^{6}\) 10\(^4\) p.f.u. per animal of the deletion mutant, rescued ILTV-UL47R or wild-type ILTV-A489. The clinical symptoms observed from days 1 to 10 p.i. were scored (Fig. 4a; Table 1). Between days 3 and 8 p.i., all animals infected with wild-type ILTV or the rescued mutant showed typical signs of ILT, such as dyspnoea and occasional nasal or oral discharge of bloody mucus, which led to mortality rates of 58 and 33 %, respectively (Fig. 4a; Table 1). In contrast, animals infected with the deletion mutant exhibited only moderate respiratory disorders, and all of them survived and convalesced completely after 6–10 days (Fig. 4a). Plaque assays of tracheal swabs revealed that, at day 3 p.i., all animals infected with wild-type ILTV or the rescued mutant showed typical signs of ILT, such as dyspnoea and occasional nasal or oral discharge of bloody mucus, which led to mortality rates of 58 and 33 %, respectively (Fig. 4a; Table 1). In contrast, animals infected with the deletion mutant exhibited only moderate respiratory disorders, and all of them survived and convalesced completely after 6–10 days (Fig. 4a). Plaque assays of tracheal swabs revealed that, at day 3 p.i., all animals infected with ILTV-A489 or ILTV-UL47R shed considerable amounts of virus with mean titres of > 10\(^4\) p.f.u. ml\(^{-1}\) (Fig. 5a). Titres of shed ILTV-UL47 were ~100-fold lower and did not reach their maximum until day 4 p.i. (Fig. 5a). Remarkably, from two
animals infected with the deletion mutant, no virus could be re-isolated at any time (Table 1).

The results of histopathology were largely consistent with the clinical observations. At day 4 after infection with either wild-type ILTV or the rescued ILTV-UL47R, acute laryngitis and tracheitis were detected, characterized by oedema of the submucosa, moderate heterophilic infiltration and multifocal degeneration, necrosis and desquamation of the mucosa (Fig. 6b, c, left panels). The lungs were affected by multifocal to coalescent necrosis of the bronchiolar epithelium and heterophilic and lymphohistiocytic infiltration, together with multinucleated syncytial cells (Fig. 6b, c, right panels). These syncyta often contained eosinophilic intranuclear inclusion bodies, which are typical of ILTV infections (Guy & Bagust, 2003). In chickens infected with ILTV-ΔUL47 (Fig. 6a), the tracheal lesions were significantly less pronounced and the lungs retained an alveolar structure similar to that of uninfected animals (not shown).

For detection of ILTV-specific antibodies, sera of all surviving animals were collected at days 17 and 24 p.i. and all showed positive reactions in IIF tests on infected CEK cells (Table 1). As the serum reactions of chickens infected with ILTV-ΔUL47 appeared slightly weaker than those of animals infected with the UL47-positive viruses, we tested whether the UL47 protein itself was relevant for antibody formation. Thus, all sera were subjected to IIF tests on LMH cells that had been transfected with a UL47 expression plasmid (Helferich et al., 2007). Whereas the monospecific rabbit antiserum exhibited strong reactions with these cells, all of the chicken sera were negative.

### Table 1. Summary of animal experiments

Chickens were immunized and challenged intratracheally with the indicated ILTV recombinants or wild-type ILTV (A489). At the indicated time periods after immunization (p.i.) and after challenge infection (p.c.), animals were examined daily and clinical scores as well as morbidity and mortality rates were determined. Shedding of ILTV was detected by plaque assays of tracheal swabs on CEK cells and ILTV-specific serum antibodies were approximately quantified (+, ++) by IIF tests on infected cells. NT, Not tested.

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<th>Characteristic</th>
<th>Days (p.i/p.c.)</th>
<th>Group</th>
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<td></td>
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<td>III(n = 12)</td>
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<td>Virus shedding (p.f.u. ml^{-1})</td>
<td>3, 4 and 5 days (p.i.)</td>
<td>9/9 (4.3 x 10^3)</td>
<td>8/10 (6.5 x 10^1)</td>
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<td>10/10 (+ +)</td>
<td>6/6 (+ +)</td>
<td>NT</td>
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<tr>
<td></td>
<td>24 days (p.i.)</td>
<td>3/3 (+ +)</td>
<td>10/10 (+ +)</td>
<td>6/6 (+ +)</td>
<td>0/5 (−)</td>
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<tr>
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<td>7/10</td>
<td>3/6</td>
<td>5/5</td>
</tr>
<tr>
<td>Mortality</td>
<td>4–7 days (p.c.)</td>
<td>0/3</td>
<td>0/10</td>
<td>0/6</td>
<td>4/5</td>
</tr>
<tr>
<td>Clinical score</td>
<td>1–10 days (p.c.)</td>
<td>0.07</td>
<td>0.17</td>
<td>0.06</td>
<td>2.92</td>
</tr>
<tr>
<td>Virus shedding (p.f.u. ml^{-1})</td>
<td>3, 4 and 5 days (p.c.)</td>
<td>0/3 (0)</td>
<td>0/10 (0)</td>
<td>0/6 (0)</td>
<td>4/4 (1.8 x 10^3)</td>
</tr>
<tr>
<td>ILTV-specific antibodies</td>
<td>11 days (p.c.)</td>
<td>1/1 (+ +)</td>
<td>8/8 (+ +)</td>
<td>4/4 (+ +)</td>
<td>1/1 (+ +)</td>
</tr>
<tr>
<td></td>
<td>28 days (p.c.)</td>
<td>1/1 (+ +)</td>
<td>8/8 (+ +)</td>
<td>4/4 (+ +)</td>
<td>1/1 (+ +)</td>
</tr>
</tbody>
</table>
irrespective of the ILTV used for infection (results not shown). In contrast, almost all sera showed specific IIF reactions with LMH cells expressing the immunodominant envelope glycoprotein gJ of ILTV (Fuchs et al., 2005). Thus, UL47 is not relevant for the humoral immune response to ILTV infections. This finding is not surprising, as the protein is a predicted tegument component (Helferich et al., 2007), which is not present at the surface of virions or infected cells. Consequently, the weaker immune response of chickens infected with UL47-negative ILTV is most likely caused by an impaired in vivo replication of this virus, as also indicated by the reduced virus amounts in tracheal swabs.

To test whether ILTV-ΔUL47 was nevertheless able to confer sufficient protection against infection with virulent ILTV, all groups were challenged intratracheally with $1 \times 10^5$ p.f.u. wild-type ILTV-A489 per animal at day 28 after immunization. As controls, five naive chickens were infected in the same manner. All control animals developed severe disease and four died between days 3 and 7 p.c. (Fig. 4b).
contrast, all immunized animals survived the infection and none showed any significant clinical symptoms (Fig. 4b). Consistently, no tracheal lesions could be detected in protected animals that were necropsied at day 3 or 4 p.c., irrespective of whether they had been immunized with ILTV-ΔUL47 (Fig. 7a), ILTV-UL47R (Fig. 7b) or wild-type virus (not shown). In control animals, however, the tracheas were affected by marked haemorrhagic necroses (Fig. 7c). No challenge virus could be re-isolated from tracheal swabs of the immunized chickens, whereas the control animals shed amounts of virus similar to those observed for the ILTV-A489 and ILTV-UL47R groups after primary infection (Fig. 5b). However, this did not result in sterile immunity, as analysis by PCR detected ILTV DNA in most of the swabs taken at days 3–5 after challenge of protected animals, but not in swabs from uninfected chickens (results not shown). As expected, antisera prepared at days 11 and 28 p.c. exhibited strong, positive IIF reactions with ILTV-infected CEK cells, and differences between the immunized groups were no longer detectable (Table 1).

Taken together, these results demonstrate that ILTV-ΔUL47, although not completely apathogenic, is significantly attenuated in vivo and is able to protect chickens against subsequent infection with virulent ILTV. The attenuation of ILTV-ΔUL47, as well as its in vitro growth defects, are clearly caused by deletion of UL47, as they could be corrected by restoration of this gene in ILTV-UL47R. Thus, like other genetically engineered gene deletion mutants, ILTV-ΔUL47 would be a candidate for a safer live-virus vaccine than the genetically undefined strains that are currently in use (Guy & Bagust, 2003). Several ILTV recombinants that have been described previously and tested in vivo contain reporter gene insertions (Okamura et al., 1994; Schnitzlein et al., 1995). This facilitates isolation of the mutants, as well as discrimination from field strains. However, spontaneous deletions of the inserted reporter gene cassettes may alter the phenotypes of the mutants not only in vitro but also in vivo. An EGFP-expressing UL50 deletion mutant of ILTV has been shown to become significantly more pathogenic after inactivation of the reporter gene (Fuchs et al., 2000). To exclude this possibility, foreign sequences were removed completely from the genome of ILTV-ΔUL47. Rapid genetic differentiation from wild-type viruses should nevertheless be feasible, e.g. by PCR analysis of the mutated genome region. However, the UL47 gene product is not a suitable marker for serological differentiation of infected and vaccinated animals (van Oirschot, 1999), as, after experimental ILTV infection, antibodies against the UL47 tegument protein were not induced to a detectable level. For the control of other alphaherpesvirus infections of domestic animals such as PrV or BHV-1, marker vaccines lacking the immunogenic gE or gG were or are still utilized (Kaashoek et al., 1995; Marchioli et al., 1987; van Oirschot et al., 1986). For ILTV, a gl-negative virus mutant has been proposed as a putative marker vaccine (Fuchs et al., 2005). As UL47 is translocated to a position immediately upstream of the gG (US4) and gl (US5) genes of ILTV (Fig. 1b) (Wild et al., 1996), and as all three genes are non-essential for in vitro replication, it should be easy to generate double or triple deletion mutants. However, single deletions of UL47 (this study), US4 (Devlin et al., 2006) and US5 (Fuchs et al., 2005) have all been shown to reduce the virulence of ILTV, and therefore multiple deletions may lead to overattenuation, resulting in insufficient protection of vaccinated animals. With respect to immunogenicity, it also remains to be tested whether the available ILTV recombinants, which so far have been administered to chickens individually at relatively high doses, are also suitable for mass application via aerosols or drinking water. Almost avirulent ILTV mutants such as those exhibiting deletions of UL0 or the gl gene (Fuchs et al., 2005; Veits et al., 2003c) might be ineffective at low doses, whereas less-attenuated recombinants such as ILTV-ΔUL47

Fig. 7. Gross pathology of chicken tracheas prepared at day 3 after challenge infection of animals immunized with ILTV-ΔUL47 (a) or ILTV-UL47R (b) and of a non-immunized control animal (c). Note the diffuse tracheal inflammatory haemorrhage in the non-immunized control.
might still be able to confer protection. Therefore, the different ILTV recombinants should be compared in parallel with conventional vaccine strains using equal virus doses and administration routes, as well as chickens of the same breed and age.

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