Infectious bursal disease virus (IBDV), an important avian pathogen, exhibits a specific tropism for immature B-lymphocyte populations. We have investigated the ability of IBDV to replicate in chicken B-lymphoid DT40 cells, a tumour cell line derived from the bursa of Fabricius of a chicken infected with avian leukosis virus. Our results show that IBDV persistently infects DT40 cells. Establishment of the persistent infection is associated with an extensive remodelling of the hypervariable region of the VP2 capsid polypeptide, accumulating 14 amino acid changes during the first 60 days of the persistent infection. The amino acid sequence of the non-structural VP5 polypeptide, involved in virus dissemination, is not altered during the persistent infection. Results described in this report constitute the first demonstration of the ability of IBDV to establish a persistent infection in vitro.

Infectious bursal disease virus (IBDV), an avian pathogen responsible for a highly contagious immunosuppressive disease, is the best-characterized member of the family Birnaviridae. Birnaviruses are naked, icosahedral viruses with bipartite, double-stranded RNA genomes (Dobos et al., 1979). Although the last few years have witnessed major progress in the understanding of IBDV molecular and structural biology aspects, key issues concerning the virus replication programme remain poorly characterized. This lack of information is exemplified by the fact that, although the tropism of IBDV for bursal B-lymphocyte populations was first described and characterized extensively over 25 years ago (Becht, 1980; Kibenge et al., 1988), the discovery of the mechanism(s) governing this phenomenon still represents the holy grail of IBDV biology.

Propagation of IBDV field isolates in tissue culture requires previous adaptation involving serial virus passage, a process that invariably leads to the introduction of mutations at specific residues on the VP2 capsid polypeptide, as well as a significant reduction of virus virulence (Cursiefen et al., 1979; Hassan et al., 1996; Lange et al., 1987; Yamaguchi et al., 1996a, b). This phenomenon constitutes a major obstacle to characterizing the interaction of pathogenic IBDV strains with susceptible host cells.

In a recent report, Terasaki et al. (2008) showed that very virulent IBDV isolates can be grown directly in chicken lymphoid DT40 tumour cells infected persistently with avian leukosis virus (ALV) (Baba et al., 1985), without the requirement for a preliminary adaptation process. Most importantly, serial passage in this cell line does not result in the incorporation of mutations at the specific VP2 residues. Additionally, DT40 cells show an extremely high frequency of homologous recombination (Buerstedde & Takeda, 1991). This property has been exploited widely for the generation of derivative cell lines in which selected target genes are inactivated (Winding & Berchtold, 2001). Accordingly, DT40 cells appear to be an excellent candidate system to undertake a systematic analysis aimed at determining the specific roles of virus and cellular proteins during the IBDV replication process.

With the aim of characterizing the ability of IBDV to replicate in DT40 cells grown in suspension, cultures were infected with a plaque-purified isolate derived from the IBDV Soroa strain, a tissue culture-adapted strain (Lombardo et al., 1999), propagated in chicken embryo fibroblasts. For this, cells were sedimented by low-speed centrifugation (1000 g for 5 min at 4 °C), resuspended in 1 ml RPMI 1640 medium supplemented with 50 mM 2-mercaptoethanol at a density of 10^7 cells ml^-1 and incubated with 1, 0.1 or 0.01 p.f.u. IBDV per cell for 30 min at 39.5 °C. After this period, cells were sedimented, washed twice with PBS and resuspended in fresh medium supplemented with 5 % chicken serum and 10 % fetal calf serum at a density of 5 × 10^5 cells ml^-1 and then maintained at 39.5 °C for 10 days. Cultures remained apparently unaffected by the infection, maintaining growth rates and cell-viability counts very similar to those of mock-infected cultures. Interestingly, supernatants harvested from these cultures contained IBDV titres of about 5 × 10^6 p.f.u. ml^-1. This observation led us to hypothesize that IBDV might persistently infect DT40 cells.

To analyse this possibility, a new set of infections was carried out in triplicate, using an m.o.i. of 0.1. The cell density and viability of these cultures were determined every 72 h by flow-cytometric analysis after incubation
with propidium iodide (Beckman Coulter), and brought to a density of $5 \times 10^5$ live cells ml$^{-1}$ by adding fresh medium. Infected cultures were maintained for 90 days. To analyse virus growth, samples were collected at different times post-infection (p.i.) and subjected to low-speed centrifugation. Supernatants were then used to determine extracellular IBDV titres by plaque assays in QM7 (quail muscle) cells as described previously (Hassan et al., 1996). Cell pellets were used to determine the percentage of infected cells. For this, cells were transferred to glass coverslips precoated with poly-L-lysine by low-speed centrifugation and, after methanol fixation, incubated with rabbit anti-VP3 specific serum (Fernandez-Arias et al., 1998), followed by incubation with goat anti-rabbit Ig coupled to Alexa 594 (Molecular Probes). Cell nuclei were stained with ToPro-3 (Molecular Probes). Fluorescent signals were visualized by epifluorescence. The infected-cell ratio was determined by counting the number of IBDV-positive cells, which show an intense cytoplasmic anti-VP3 red fluorescent signal (Fig. 1c), versus the total cell number.

**Fig. 1.** Characterization of IBDV persistent infection. IBDV-infected DT40 cells were maintained for 90 days. (a) The percentages of live cells (line) and IBDV-infected cells (bars) were determined at the indicated times p.i. Presented data are the means of determinations carried out in three separate infected cultures. SEM is represented by bars. (b) IBDV extracellular titres were determined by plaque assay at the indicated times p.i. Presented data are the means of determinations carried out in three separate infected cultures. SEM is represented by bars. (c) Immunofluorescence analysis used for the detection of infected cells in persistently infected DT40 cultures. Mock-infected and IBDV-infected cells were fixed and incubated with rabbit anti-VP3 specific serum, followed by incubation with goat anti-rabbit Ig coupled to Alexa 594 (red). Nuclei were stained with ToPro-3 (blue). (d) Characterization of the accumulation of the IBDV VP3 protein. The presence of the VP3 protein in persistently infected DT40 cultures was determined by Western blot, using cell extracts collected at the indicated times p.i. A positive control corresponding to purified IBDV (IBDV) and a negative control of mock-infected DT40 cells (M) were also included. (e) Electron microscopy analysis. Ultrathin sections from mock-infected (left panel) and IBDV-infected (central panel) DT40 cells were stained with saturated uranyl acetate and lead citrate. The arrow in the central panel indicates the position of an ALV particle. The image shown in the right panel corresponds to a section from IBDV-infected cells incubated with anti-VP3 serum. The bound antibody was detected by using a 10 nm colloidal gold conjugate. Bars, 200 nm.
as determined by counting nuclei. As shown in Fig. 1(a), cell-viability counts were maintained at values approaching 90 %. Whilst the proportion of infected cells was 3–5 % for the duration of the experiment (Fig. 1a, bars), virus titres showed a small fluctuation, with values ranging from $5 \times 10^6$ to $6 \times 10^6$ p.f.u. ml$^{-1}$ (Fig. 1b). To confirm the specificity of these results, accumulation of the IBDV structural VP3 protein was also analysed by Western blot as described previously (Lombardo et al., 1999). In order to assess total protein loads, Western blots were performed in parallel using an anti-actin monoclonal antibody (AC-15; Sigma). As shown in Fig. 1(d), the presence of a fairly constant amount of VP3 was detected at all time points analysed. Similar results were obtained by using sera specific for the IBDV RNA-dependent RNA polymerase (RdRp) (Lombardo et al., 1999), the VP2 capsid polypeptide and the non-structural VP5 protein (Lombardo et al., 2000) (data not shown).

Cells from cultures maintained for 90 days p.i. were inspected by electron microscopy. For this, cells were washed three times in PBS and then fixed by incubation with 2 % glutaraldehyde and 2 % tannic acid in 0.4 M HEPES buffer (pH 7.5) for 1 h at room temperature. After washing with HEPES buffer, cells were post-fixed with a solution containing 1 % osmium tetroxide and 0.8 % potassium ferricyanide and processed for embedding in EML-812 resin (EML Laboratories). Ultrathin sections were either stained directly with saturated uranyl acetate and lead citrate or immunolabelled with rabbit anti-IBDV VP2 protein (Fernandez-Arias et al., 1998), followed by incubation with goat anti-rabbit Ig conjugated to 10 nm colloidal gold particles (Sigma). As expected, mock-infected cells contained abundant ALV particles with a distinctive retrovirus-like appearance (Fig. 1e, left panel). A fraction of cells (approx. 5 %) from IBDV-infected cultures contained large cytoplasmic inclusions, highly reminiscent of those observed in other IBDV-infected cell lines (Garriga et al., 2006), formed by isometric particles with morphology and size (60–70 nm diameter) akin to that of IBDV particles (Fig. 1e, middle panel). The identity of the inclusions was further confirmed by the specific immunostaining observed following incubation with anti-VP3 antiserum (Fig. 1e, right panel). The presence of ALV particles apparently budding through the cell membrane of IBDV-infected cells (Fig. 1e, middle panel) indicates that IBDV and ALV are able to replicate simultaneously in the same cell. These results demonstrate conclusively that the IBDV strain used in this study establishes a long-term, persistent infection in DT40 cells.

Some cytopathic viruses are capable of persistently infecting a variety of cell lines (Ahmed et al., 1997; Dermody et al., 1993). Although mechanisms underlying these phenomena are not completely understood, it has been shown that, in some cases, virus persistence is associated with the selection of viruses containing mutations that downregulate and/or inactivate a critical virus gene(s) (Borzakian et al., 1992; Hofmann et al., 1993). The IBDV genome harbours three open reading frames (ORFs) encoding: (i) the RdRp; (ii) a large polyprotein containing three domains, corresponding to the capsid protein precursor (pVP2), the viral protease (VP4) and a multitasking structural polypeptide (VP3); and (iii) a 17 kDa polypeptide, VP5, that accumulates at the cell membrane (Lombardo et al., 2000). Although VP5 plays an important role in virus dissemination and pathogenesis (Yao et al., 1998), it is not essential for virus replication in cell culture (Mundt et al., 1997). Mutations affecting VP5 expression and/or functionality might reduce virus spreading and thus contribute to the selection of virus populations with reduced infectivity. The results of Western blot analyses described above indicate that the expression of this protein is not affected significantly during persistent infection. However, the possibility that the VP5 ORF might contain mutations affecting the functionality of the polypeptide remained. To investigate this, samples from persistently infected cultures were collected at 15, 30, 60, 75 and 90 days p.i. and used for RNA purification using TRIzol (Invitrogen), following the manufacturer’s instructions. The resulting RNAs were reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) following the instructions of the manufacturer. The resulting cDNAs were used to PCR-amplify a 570 bp region spanning the VP5 ORF. Reactions were carried out with Vent DNA Polymerase (New England Biolabs) using primers 5'-ATGGTCAAGTAGATCAGAC and 5'-CCCACATCATAATGATCTTGG. Amplified DNA fragments were purified and subjected to nucleotide sequencing using the same primer pair. The results of this analysis showed that the VP5 sequence is strictly conserved in all analysed samples (data not shown), thus ruling out a possible link between the establishment of the persistent infection and defects in VP5 functionality.

It has been shown that, in some instances, the establishment of a persistent infection involves a host–virus co-evolution process resulting in the selection of cell populations with higher resistance to infection, and the generation of virus variants capable of replicating in these cells (Ahmed et al., 1997; Dermody et al., 1993). The analysis of data concerning the effect of IBDV on DT40 infected-cell viability (Fig. 1a) shows that a large proportion, $>90 \%$, of the total cell population remains uninfected during the persistent infection. This takes place in the presence of extracellular virus titres of about $5 \times 10^6$ p.f.u. ml$^{-1}$, exceeding 10 p.f.u. per cell. A simple interpretation of our data suggests that a large proportion of the cell population is naturally resistant to IBDV infection. Low infected-cell ratios were also reported by Terasaki et al. (2008) after infecting fresh DT40 cultures with several pathogenic IBDV field isolates. Data described in that report show that the number of infected DT40 cells increases significantly after three consecutive virus passages in this cell line, indicating that IBDV undergoes an adaptation process that results in the selection of variants with an enhanced ability to infect fresh DT40 cell cultures. The hallmark of this adaptation is the introduction of
mutations in the VP2 hypervariable region. Interestingly, these mutations vary depending upon the virus strain used for infection. This region (residues 206–350), located within the protruding (P) domain of the VP2 capsid polypeptide (Fig. 2a), harbours several neutralizing epitopes and exhibits a much higher degree of genetic variability than other VP2 domains and the rest of the IBDV polypeptides (Schnitzler et al., 1993). To determine whether the establishment of the persistent infection had any effect on the sequence of the hypervariable region, the cDNAs described above were amplified by using primers 5′-CCGTCCTCAGCTTACCCACATC and 5′-CTTCGAGCTGATCCCAAATCC. The resulting DNA fragments, corresponding to nt 65–377 of the polyprotein ORF (GenBank accession no. AF140705), were sequenced in both directions using the same primer pair. The results of this analysis are summarized in Table 1 and Fig. 2. During the first 15 days, 19 nucleotide mutations, 10 of which implied residue substitutions, were detected. As shown, sequencing results showed sequence ambiguities at several positions. This was indicative of the existence of quasi-species within the analysed virus population. The number of nucleotide changes detected at 30 days p.i. was 22; these changes were accompanied by a slight increase in the number of nucleotide ambiguities. At 60 days p.i., 29 nucleotide changes were detected, resulting in 14 amino acid substitutions. The number and position of the amino acid changes was not modified further in samples collected at 90 days p.i. These results suggest that IBDV undergoes an adaptation process, involving a substantial remodelling of the VP2 P domain (Fig. 2), during the early stages of the persistent infection. This process is probably matched by the elimination of cell populations with a higher sensitivity to undergoing a rapid cytolytic infection, resulting in a steady-state situation.

The VP2 protein is responsible for the interaction of IBDV with specific cell receptor(s) that trigger the entry process (Lin et al., 2007; Nieper & Muller, 1996; Ogawa et al., 1998; Setiyono et al., 2001). Additionally, this polypeptide plays a key role in particle assembly (Luque et al., 2007) and is probably also involved directly in the virus egress mechanism. At this point, it is difficult to envisage the effect of the detected VP2 mutations on these different stages of the replication process. Indeed, an exhaustive analysis to determine how establishment of the persistent infection affects the rest of the virus genome is anticipated. Additionally, it would be of interest to determine the contribution of ALV to the establishment of the persistent infection.

The results described here constitute the first evidence for the ability of IBDV to establish a persistent infection in vitro. The availability of a cell system supporting a persistent IBDV infection provides an excellent tool for systematic analysis of the evolution of virus populations, as well as the search for antiviral agents specifically affecting IBDV replication.

### Table 1. Amino acid changes detected in the VP2 hypervariable domain during the persistent infection

Amino acid residues, indicated by using the single-letter code, are numbered as reported by Bayliss et al. (1990). More than one amino acid residue is shown when ambiguities were detected.

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<tr>
<th>Time (days p.i.)</th>
<th>Amino acid residue</th>
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<tr>
<td></td>
<td>P222</td>
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<tr>
<td>0</td>
<td>P/S</td>
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<tr>
<td>15</td>
<td>P/S/L</td>
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