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VP5 of Infectious Bursal Disease Virus Is Not Essential for Viral Replication in Cell Culture

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Infectious bursal disease virus (IBDV), a member of the *Birnaviridae* family, encodes in its bisegmented double-stranded RNA genome four structural virion proteins, VP1, VP2, VP3, and VP4, as well as a nonstructural protein, VP5. Recently, the establishment of an infectious cRNA system for IBDV has been described (E. Mundt and V. N. Vakharia, Proc. Natl. Acad. Sci. USA 93:11131–11136, 1996). Here, we report the isolation of a VP5$^-$ IBDV mutant constructed by site-directed mutagenesis of the methionine start codon of VP5, followed by cRNA transfection. The resulting virus mutant was replication competent in cell culture, which indicates that VP5 is not required for productive replication of IBDV. Absence of VP5 expression was verified by lack of reactivity with newly established anti-VP5 monoclonal antibodies and polyclonal sera. VP5$^-$ IBDV exhibited a delay in replication in chicken embryo cells compared to the VP5$^+$ parental virus. However, final yields were similar. Our results thus show that VP5 is nonessential for IBDV replication, which makes it a prime candidate for the construction of deleted, marked vaccines.

Infectious bursal disease virus (IBDV) is of major importance to the poultry industry, causing a highly contagious immunosuppressive disease in young chickens. IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae* (12). Two serotypes have been identified by using cross-neutralization assays (7). The genome consists of two segments, A and B, of double-stranded RNA which are localized within a single-shelled icosahedral capsid with a diameter of approximately 60 nm. Recently, the complete genomic sequence of both segments of four strains of IBDV was determined (10). The larger segment, A, encodes a polyprotein of approximately 110 kDa which is autoproteolytically cleaved (4) to form the viral proteins VP2, VP3, and VP4. A second open reading frame (ORF) preceding and partially overlapping the polyprotein gene has been identified (1, 15). A protein encoded by this ORF has been detected in IBDV-infected chicken embryo cells (CEC), as well as in bursal cells of IBDV-infected chickens (9). This protein was named VP5. Segment B encodes a 97-kDa protein, designated VP1, with polymerase (14) and capping enzyme activities (13).

The ORF coding for VP5 of IBDV was found to be conserved in all hitherto published segment A sequences. It encodes 145 amino acids, resulting in a calculated molecular mass of 16.5 kDa for VP5. The apparent molecular mass estimated after expression in *Escherichia coli*, as well as in IBDV-infected CEC, was 21 kDa (9). In the genome of infectious pancreatic necrosis virus (IPNV), another member of the family *Birnaviridae*, a small ORF encoding a 17-kDa protein which partially overlaps the polyprotein gene of segment A, is also present (3). This protein could be detected in IPNV-infected cells (6). Despite the conservation of VP5 in at least two members of the family *Birnaviridae*, the function of this protein is still unknown.

To test for a functional role of VP5 during IBDV replication, a VP5-negative IBDV mutant was isolated by using a recently described infectious cRNA system for IBDV (11). To this end, the *Eco*RI site immediately following the 3′ end of the full-length cDNA of strain D78 segment A (pUC19FLAD78; 11) was deleted. An *Eco*RI-*Kpn*I fragment containing the T7 polymerase binding site followed by the complete segment A sequence was excised and inserted into *Eco*RI-*Kpn*I-cleaved vector pUC18 after inactivation of the unique *Nde*I site within the vector sequence, resulting in plasmid pAD78/EK. Thereafter, the genomic region encompassing the initiation codon for VP5 was amplified in two pieces by using primers A1F5′ and VP5MutR and primers VP5MutF and A2R, respectively (Table 1 shows the sequences and locations of the primers). PCR fragments were cloned separately and subsequently fused via a unique *Afl*II site which had been created by mutations within the respective primers (Fig. 1). An *Eco*RI-*Nde*I fragment containing the T7 polymerase binding site and the 5′ part of segment A including the introduced mutations was excised and used to replace the wild-type *Eco*RI-*Nde*I fragment in pAD78/EK to yield plasmid pAD78/VP5$^-$. Of the three mutations introduced, one turned the initiation methionine codon for VP5 into an arginine codon (Fig. 1).

Transcription of infectious cRNA, purification, and transfection were performed as described in detail previously (11), with the exception that secondary CEC were used for transfection experiments instead of Vero cells. For the transfection experiments, full-length cDNA clones of segment A of strain D78 capable of expressing VP5 (pAD78/EK) or unable to express VP5 (pAD78/VP5$^-$) were transcribed into synthetic RNA and cotransfected with segment B full-length cRNA of IBDV strain P2 (pBP2; 11) into CEC. Three days after transfection, CEC were frozen, thawed and centrifuged and the supernatants were filtered through a 0.45-μm-pore-size filter. The resulting virus progeny was designated IBDV/EK or IBDV/VP5$^-$, respectively.

Generated viruses were passaged three times in CEC to increase virus titers. Thereafter, whole-cell nucleic acid of infected and noninfected CEC was analyzed by reverse transcription (RT)-PCR and PCR, respectively. Reaction products were separated by agarose gel electrophoresis (Fig. 2). A 388-bp fragment amplified by using RNA isolated from IBDV/VP5$^-$ (lane 7)- and IBDV/EK (lane 5)-infected CEC was cloned and

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TABLE 1. Sequences of oligonucleotide primers used to generate mutant constructs[a]

Nucleotide sequence	Orientation	Designation	Nucleotide no.
AGAGAATTC*TAATACGACTCACTATA*GGATACGATCGGTCTGAC	+	A1F5′	1–18
TGGGCCTGTCACTGCTGTCACATGT	−	A2R	716–740
CATTGCTCTGCAGTGTGTAGTGAGC	−	A3R	338–362
CTACAACGCTATCCTTAAGGGTTAGTAGAG	+	VP5MutF	80–109
CTCTACTAACCCTTAAGGATAGCGTTGTAG	−	VP5MutR	80–109

[a] Underlined nucleotides are virus specific T7 promotor sequences are in italics. Mutated nucleotides are boxed, and the sense (+) or antisense (−) orientation of the primer is shown. Primer positions are given according to the published sequence of serotype I strain P2 (13).

sequenced. No PCR product was obtained when the reverse transcriptase step was omitted (lanes 4 and 6) or after RT-PCR using noninfected CEC (lane 3), indicating that the PCR product was derived from RNA of an infected cell culture. PCR with pAD78/EK DNA resulted in the expected 388-bp fragment (lane 2). Sequence analysis of the RT-PCR products showed the presence of the desired alterations in segment A of the generated IBDV/VP5$^-$ (Fig. 3).

For identification of VP5, a monospecific rabbit anti-VP5 serum and 11 hybridoma cell lines producing monoclonal antibodies (MAb) raised against *E. coli*-expressed VP5 of IBDV (9) were established (DIE-1 to DIE-11). To this end, bacterially expressed VP5 was purified by using PrepCell (Bio-Rad, Hercules, Calif.). Fractions were tested by Western blot analysis using a rabbit anti-VP5 serum (9). Appropriate fractions containing *E. coli*-expressed VP5 were combined, precipitated with trichloroacetic acid (7% final concentration), washed in 70% ethanol, and air dried. Fifty to one hundred micrograms of resuspended VP5 mixed with complete Freund's adjuvant was repeatedly injected into specific-pathogen-free rabbits, as well as BALB/c mice (Møllegard), by the intramuscular route. Supernatants of fused spleen cells of the immunized BALB/c mice were first assayed by enzyme-linked immunosorbent assay (ELISA) on IBDV-infected and noninfected Vero cells, respectively. To this end, acetone-methanol-fixed cells were blocked with blocking reagent (Boehringer GmbH, Mannheim, Germany) and 50 μl of hybridoma supernatant was added per well. After incubation in a humidified atmosphere for 1 h at 37°C,

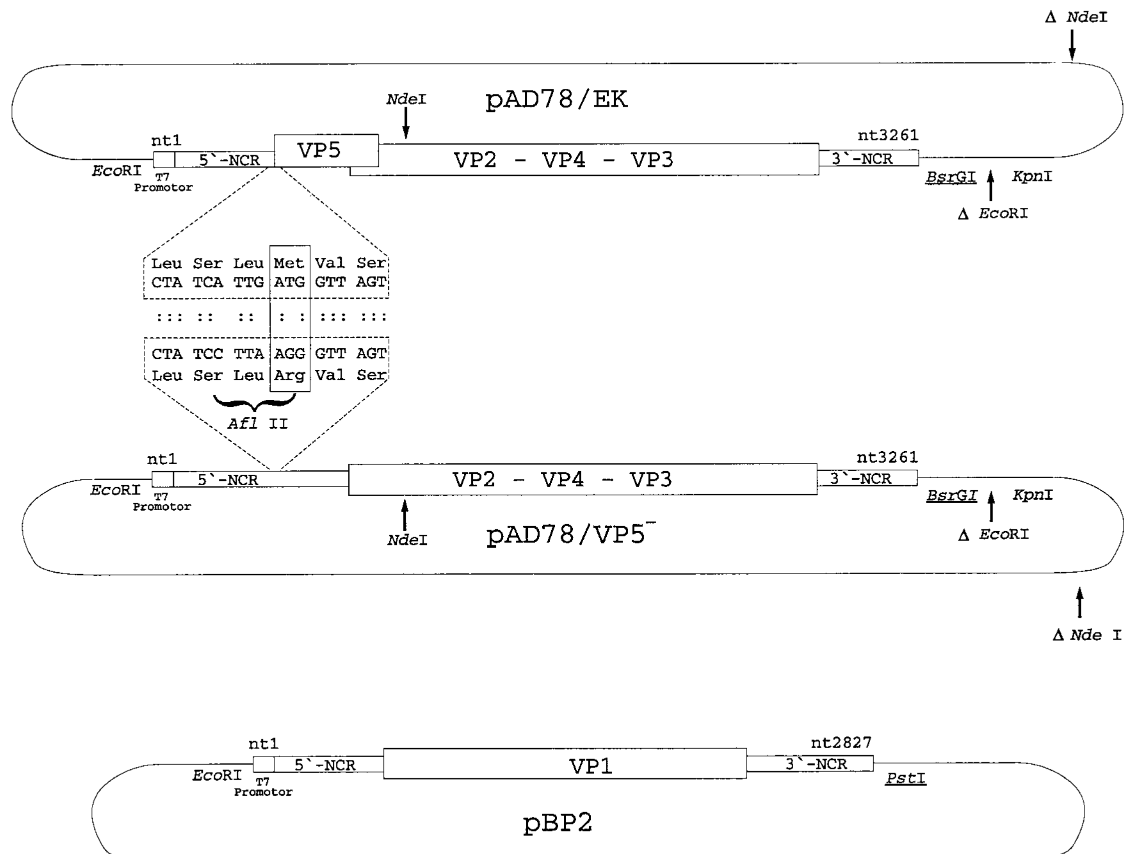

FIG. 1. Construction of genomic cDNA clones. Plasmid pAD78/EK contains the complete strain D78 segment A cDNA encoding the polyprotein (VP2-VP4-VP3) and VP5. Plasmid pBP2 contains the complete strain P2 segment B encoding VP1. Mutations introduced in plasmid pAD78/VP5$^-$ turned the methionine start codon for VP5 into an arginine codon and created an artificial *Afl*II cleavage site, as described in detail in Materials and Methods. Linearization of the recombinant plasmids with the underlined restriction enzymes was followed by T7 polymerase transcription. nt, nucleotides.

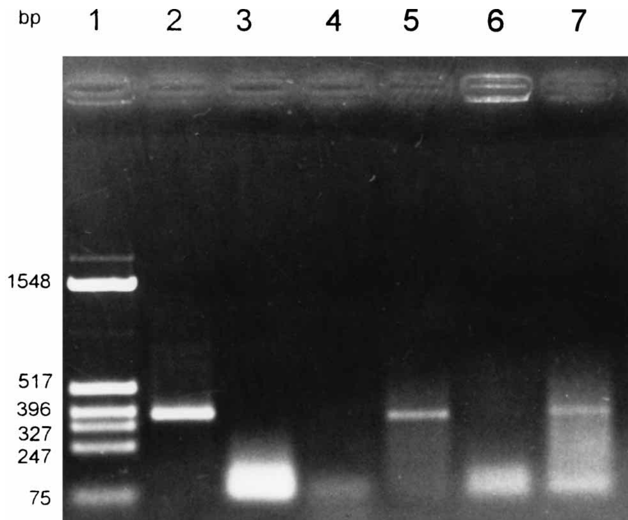

FIG. 2. Amplification of the 5′ region of segment A of IBDV/VP5$^-$ and IBDV/EK. Fragments were amplified by RT-PCR of whole-cell nucleic acids isolated from CEC infected with IBDV/VP5$^-$ (lanes 6 and 7) and IBDV/EK (lanes 4 and 5). Uninfected CEC were used as a negative control (lane 3), and plasmid pUC19FLAD78 (11) was used as a positive control (lane 2). After cotransfection of cRNA transcripts and three blind passages on CEC, RT-PCR resulted in the amplification by primers A1F5′ and A3R of a 388-bp fragment in progeny of IBDV/VP5− and IBDV/EK cRNA transfections. No amplification product was detected when the reverse transcriptase step was omitted, indicating that the resulting signal is derived from reverse-transcribed RNA and not from plasmid DNA inadvertently present in the transfection mixture. Positions of DNA size markers (lane 1) are indicated at the left.

plates were washed three times with PBS-T (0.05% Tween 20 in phosphate-buffered saline). Thereafter, 50 μl of a 1:2,500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin G (Pierce, Rockford, Ill.) was added to each well and the plates were further incubated for 1 h at 37°C. After thorough washing with PBS-T, cells were overlaid with substrate solution (0.01% *o*-phenylendiamine in 0.1 M phosphate-citrate buffer [pH 5.0] with 0.03% H_2O_2). Reactions were stopped by addition of 50 μl of 4 N H_2SO_4. Positive clones were further characterized by immunofluorescence assay (IFA) on IBDV-infected cells, Western blotting on *E. coli*-expressed VP5, and radioimmunoprecipitation assay (RIPA) of IBDV-infected and noninfected CEC and Vero cell lysates. The immunological properties of the MAb produced are summarized in Table 2. One MAb (DIE-7) reacted in all four tests; five MAb (DIE-1, -5, and -9 to -11) showed specific reactivity in ELISA, IFA, and Western blot analysis; and two MAb (DIE-4 and -6) reacted specifically in ELISA, IFA, and RIPA. Three MAb (DIE-2, -3, and -8) showed reactivity only in ELISA and IFA.

IBDV/EK (VP5$^+$) and IBDV/VP5$^-$ were further characterized by IFA. Vero cells infected with IBDV/VP5$^-$ or IBDV/EK and noninfected cells, respectively, were incubated with rabbit anti-IBDV serum, rabbit anti-VP5 serum, and anti-VP5 MAb DIE-7 and stained with fluorescein-conjugated secondary antibodies. Both antisera and the MAb recognized IBDV antigens in the cytoplasm of IBDV/EK-infected cells. In contrast, whereas the anti-IBDV serum readily detected viral antigens in IBDV/VP5$^-$-infected cells, neither the monospecific

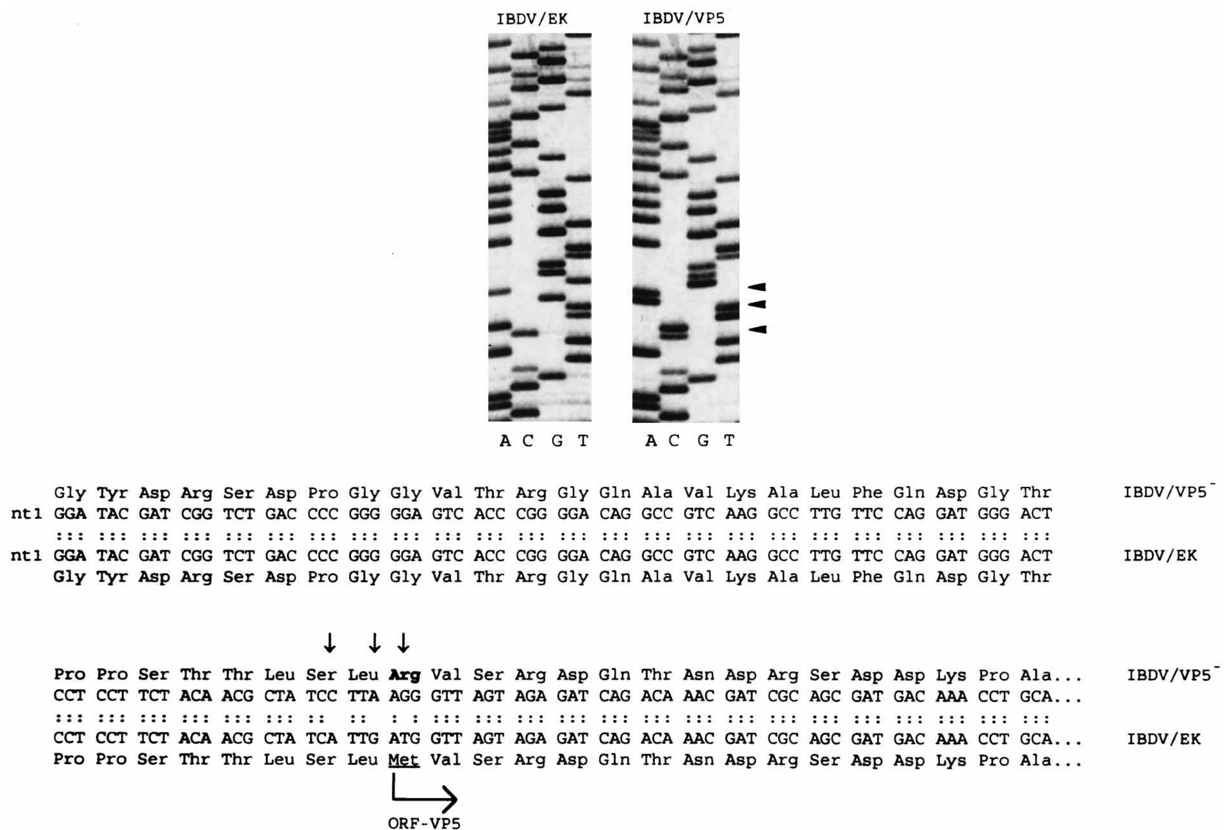

FIG. 3. Sequence of the 5′ end of segment A of IBDV/VP5$^-$. Whole-cell nucleic acids were purified from CEC infected with IBDV/VP5$^-$ or the parental VP5$^+$ strain IBDV/EK, and the corresponding fragment was amplified by RT-PCR, cloned, and sequenced. Modifications in IBDV/VP5$^-$ are marked by arrows. Two silent mutations (nucleotides [nt] 93 [A→C] and 96 [G→A]) and a mutation at nucleotide 98 (T→G) leading to replacement of the VP5 methionine start codon (underlined) with an arginine codon (bold letters) were introduced.

TABLE 2. Reactivities of anti-VP5 MAb DIE-1 to DIE-11 in different immunological tests[a]

MAb	ELISA	IFA	Western blotting	RIPA
DIE-1	+	+	+	−
DIE-2	+	+	−	−
DIE-3	+	+	−	−
DIE-4	+	+	−	+
DIE-5	+	+	+	−
DIE-6	+	+	−	+
DIE-7	+	+	+	+
DIE-8	+	+	−	−
DIE-9	+	+	+	−
DIE-10	+	+	+	−
DIE-11	+	+	+	−

[a] +, positive reaction; −, negative reaction.

anti-VP5 serum nor the anti-VP5 MAb exhibited specific reactivity. None of these immunological reagents reacted with noninfected controls (data not shown).

To analyze the expression of viral proteins in more detail, CEC were infected with IBDV/VP5$^-$ (Fig. 4, lanes 1 to 3) or IBDV/EK (Fig. 4, lanes 4 to 6) or left uninfected (Fig. 4, lanes 7 to 9). Eight hours after infection, cells were labelled for 60 min with Tran^{35}S-Label (ICN, Costa Mesa, Calif.). The label was removed, and cells were further incubated for 2 h in medium prior to lysis. Cells lysates were analyzed by RIPA with rabbit anti-IBDV serum, rabbit anti-VP5 serum, and anti-VP5 MAb DIE-7. The RIPA was performed as previously described (8). IBDV/EK (lane 4)- and IBDV/VP5$^-$ (lane 1)-infected CEC showed VP2, VP3, and VP4 after precipitation with rabbit anti-IBDV serum. The rabbit anti-VP5 serum (lane 5) and MAb DIE-7 (lane 6) precipitated the 21-kDa VP5 (9) only from IBDV/EK-infected cells. No specific reactivity was detectable in IBDV/VP5$^-$-infected CEC after precipitation with rabbit anti-VP5 serum (lane 2) or VP5-specific MAb DIE-7 (lane 3). Uninfected CEC showed no specific reactivity (lanes 7 to 9).

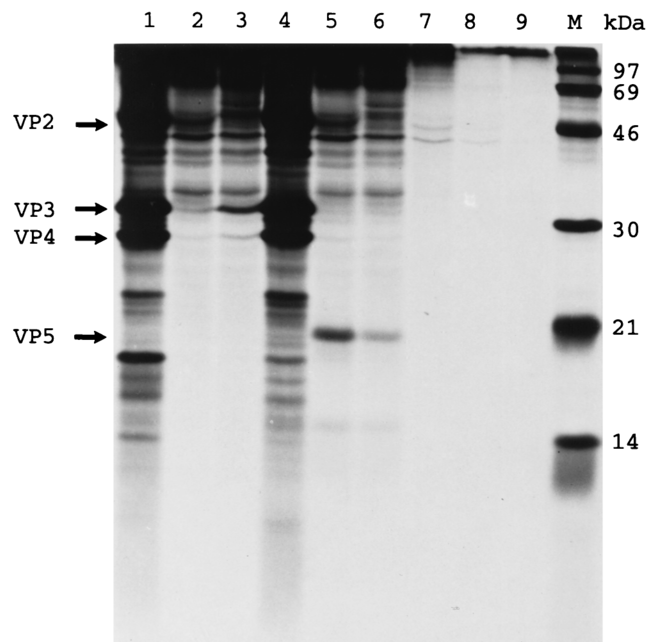

FIG. 4. RIPA of proteins from CEC infected with recombinant IBDV. CEC infected with IBDV/VP5$^-$ (lanes 1 to 3) or IBDV/EK (lanes 4 to 6) and uninfected controls (lanes 7 to 9) were labelled at 8 h p.i. for 60 min with Tran^{35}S-Label, lysed, and immunoprecipitated with rabbit anti-IBDV serum (lanes 1, 4, and 7), rabbit anti-VP5 serum (lanes 2, 5, and 8), and MAb DIE-7 (lanes 3, 6, and 9). Proteins were separated by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. The positions of molecular mass markers (lane M) are indicated, and the locations of viral proteins VP2, VP3, and VP4 are marked, as is that of VP5.

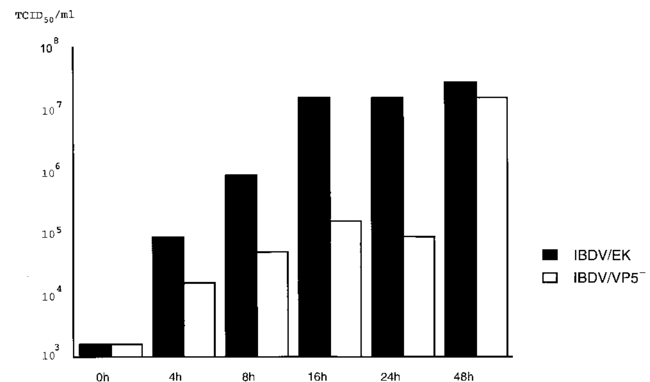

FIG. 5. Replication kinetics of IBDV/EK and IBDV/VP5$^-$. CEC in 60-mm tissue culture dishes were infected with $10^{7.2}$ TCID$_{50}$ of either VP5$^+$ IBDV/EK or IBDV/VP5$^-$. Following incubation at room temperature for 1 h, the inoculum was removed and the cells were overlaid with Dulbecco modified Eagle medium–10% fetal calf serum after being rinsed twice with phosphate-buffered saline. Immediately thereafter and at the times indicated, supernatants were removed and stored at −20°C. After centrifugation, supernatants were titrated on CEC. Infectivity in TCID$_{50}$ per milliliter was calculated as described by Kaerber (5). Values were plotted logarithmically on the vertical axis.

To assay replication of IBDV/VP5$^-$ in more detail, one-step growth was analyzed (Fig. 5). Confluent secondary CEC in 60-mm-diameter petri dishes (approximately 10^6 cells/dish) were infected at room temperature for 60 min with $10^{7.2}$ 50% tissue culture-infective doses (TCID$_{50}$) of IBDV/EK or IBDV/VP5$^-$. After infection, the cells were rinsed, overlaid with 5 ml of Dulbecco modified Eagle medium–10% fetal calf serum, and further incubated at 37°C. Supernatants were removed at different times (0, 4, 8, 16, 24, and 48 h postinfection [p.i.]), stored at −20°C, and titrated in 96-well plates on secondary CEC. Five days later, cells were fixed and stained with crystal violet in 50% ethanol. Titer calculation was done by the method of Kaerber (5). The TCID$_{50}$ at the different time points after infection showed that the VP5-expressing virus (IBDV/EK) replicated faster than the mutant virus lacking VP5 (IBDV/VP5$^-$). At 16 h after infection, IBDV/EK showed a titer approximately 100-fold higher than that of IBDV/VP5$^-$. However, at 48 h p.i., IBDV/VP5$^-$ reached a titer of $10^{7.2}$ TCID$_{50}$/ml, which was similar to that of IBDV/EK ($10^{7.45}$ TCID$_{50}$/ml).

In summary, we describe here the first specific knockout mutant of IBDV. By using the plasmid-based infectious cRNA system, we isolated a replication-competent IBDV mutant unable to express VP5 and demonstrated that VP5 is not essential for replication of IBDV in cell culture. VP5 has recently been identified as the product of an ORF which precedes and partially overlaps the gene for the polyprotein encoded in segment A (9). Both a procaryotically expressed VP5 protein and authentic VP5 present in virus-infected cells have an apparent molecular mass of 21 kDa, which is higher than the 16.5 kDa calculated from the sequence. The reason for this discrepancy is unclear. VP5 does not appear to constitute a component of the IBDV virion but could be detected by IFA in bursal tissue of IBDV-infected chickens (9).

IBDV/VP5$^-$ showed a delay in replication in CEC compared to its parental strain IBDV/EK, recovered after cotransfection of cRNA from full-length P2 segment B with cRNA transcribed from a full-length D78 segment A clone which had been modified outside of the viral insert to facilitate manipulation. Despite the delay in one-step growth, final titers of IBDV/VP5$^-$ were similar to those of IBDV/EK, clearly demonstrating the nonessential character of VP5 for IBDV replication.

The gene for VP5 is conserved in all of the IBDV strains analyzed to date. In addition, in the piscine birnavirus IPNV, a 17-kDa protein is encoded by an ORF located in segment A at a position colinear with the IBDV VP5 gene. This 17-kDa protein seems to be present in virions (3), in contrast to IBDV VP5, which was not detectable in purified virions (9). An infectious cRNA system for IPNV is not available. It is, however, tempting to speculate that the corresponding protein of IPNV might also turn out not to be required for viral replication.

Recombinant techniques for engineering of animal RNA viruses on the basis of cDNA systems are widely used for investigation of viral replication and the function of viral proteins (2). Isolation of IBDV/VP5$^-$ shows that similar techniques can also be used to manipulate birnavirus genomes. This opens the way for a more detailed analysis of requirements for productive birnavirus replication.

In the absence of VP5, in vitro replication of IBDV is delayed. However, final titers are as high as those found after wild-type virus replication. It will be interesting to analyze the growth behavior of IBDV/VP5$^-$ in chickens, the natural host of IBDV. It is conceivable that the delay in replication translates into attenuation in vivo, leading to a virus strain with vaccine potential. Also, lack of a protein which is apparently immunogenic could result in the failure to induce respective antibodies in the animal whereas wild-type virus replication is expected to elicit an appropriate immune response. Whether this approach will eventually lead to a novel marked vaccine against infectious bursal disease remains to be seen.

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