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Recombinant Infectious Bursal Disease Virus Carrying Hepatitis C Virus Epitopes▽

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The delivery of foreign epitopes by a replicating nonpathogenic avian infectious bursal disease virus (IBDV) was explored. The aim of the study was to identify regions in the IBDV genome that are amenable to the introduction of a sequence encoding a foreign peptide. By using a cDNA-based reverse genetics system, insertions or substitutions of sequences encoding epitope tags (FLAG, c-Myc, or hepatitis C virus epitopes) were engineered in the open reading frames of a nonstructural protein (VP5) and the capsid protein (VP2). Attempts were also made to generate recombinant IBDV that displayed foreign epitopes in the exposed loops (P_{BC} and P_{HI}) of the VP2 trimer. We successfully recovered recombinant IBDVs expressing c-Myc and two different virus-neutralizing epitopes of human hepatitis C virus (HCV) envelope glycoprotein E in the VP5 region. Western blot analyses with anti-c-Myc and anti-HCV antibodies provided positive identification of both the c-Myc and HCV epitopes that were fused to the N terminus of VP5. Genetic analysis showed that the recombinants carrying the c-Myc/HCV epitopes maintained the foreign gene sequences and were stable after several passages in Vero and 293T cells. This is the first report describing efficient expression of foreign peptides from a replication-competent IBDV and demonstrates the potential of this virus as a vector.

Infectious bursal disease virus (IBDV), a pathogen causing an immunosuppressive disease in chickens (26), has been used as a therapeutic agent without any toxicity in clinical trials with patients suffering from acute and chronic hepatitis C virus (HCV) infections (2, 8). IBDV belongs to the genus *Avibirnavirus* of the *Birnaviridae* family, and its genome consists of two segments of double-stranded RNA (11). The smaller segment, B, encodes VP1, a 97-kDa multifunctional protein with polymerase and capping enzyme activities (21, 27). The larger segment, A, encodes a 110-kDa precursor polyprotein in a single large open reading frame (ORF) which is cotranslationally processed by the viral protease VP4 (4, 24) into the VP2 precursor (pVP2), VP3, and VP4. pVP2 is further processed by several proteolytic cleavages at its C terminus for conversion into mature VP2 (10). Segment A also encodes VP5, a 17-kDa nonstructural (NS) protein, in a small ORF partly preceding and overlapping the polyprotein ORF. VP5 is dispensable for viral replication *in vitro* and *in vivo* (29), which makes it a prime candidate for the construction of marked vaccines carrying deletions. These marked vectors could be easily distinguished from the wild-type virus and could also trigger a specific cellular immune response in the host species. The available structural data for VP2 (7, 14, 17) reveal that the protein is folded into three different domains (base B, shell S, and projection P). Expression of VP2 by itself leads to dodecahedral subviral particles (SVPs) containing 20 VP2 trimers (6) and exposing the four loops of the P domain (named P_{BC}, P_{DE}, P_{FG}, and P_{HI}). Here, we explore the possibility of displaying foreign epitopes stably in recombinant IBDV by either inserting or replacing sequences in the P_{BC} and P_{HI} loops of VP2.

In this study, we used a 10-amino-acid linear c-Myc epitope (EQKLISEEDL) derived from the C terminus of human c-Myc protein and an 8-amino-acid linear FLAG epitope (DY KDDDDK). c-Myc and FLAG epitope tags were selected as they are well characterized and are recognized by specific monoclonal antibody (MAb) Myc1-9E10 (12) and MAb M2 (5), respectively. These epitope tags allow for systematic determination of sites potentially amenable to insertions or substitutions that are tolerated by the virus during assembly. The specific sites for insertion/substitution of c-Myc or FLAG sequences within segment A were chosen to investigate the following: (i) insertion/substitution of epitopes at sites that are exposed on the surface of the virus (the loops); (ii) substitution, which does not dramatically alter the size or length of segment A (N terminus of the VP5 or VP2 protein), rather than insertion; or (iii) insertion of the tag at the N terminus of VP5 or VP2, which would increase the length of segment A by 30 nucleotides (nt), assuming that it does not interfere in viral packaging. We further explored the vector potential of IBDV by inserting or substituting HCV envelope glycoprotein E2 epitopes, amino acid residues 523 to 535 [$HCV_{(523–535)}$] and 412 to 419 [$HCV_{(412–419)}$], which induce broadly neutralizing anti-HCV antibodies, in VP5 and the external loops of VP2. Consequently, we investigated a series of modifications made in segment A of the IBDV to determine the feasibility of expressing exogenous epitopes.

Generation of recombinant IBDV carrying foreign epitopes. Construction of the full-length cDNA clones of IBDV segments A and B of strain D78 has been described previously

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TABLE 1. Oligonucleotides used for construction of various plasmids

Designation	Sequence
CMVAF	GGAATTCTCAATATTGGCCATTAGCCA
NheF	CTGACAGATGCTAGCTACAATGGG
NheR	GCTAGCATCTGTCAGTTCACTC
SalR	CCAGAATCCCGTAGTCGACG
IBDV-1F	GATGGAACAGAAGTTGATTTCCGAAGAAGACCTCGATGACAAACCTGCAAGATC
IBDV-1R	GTCTTCTTCGGAAATCAACTTCTGTTCCATCAATGATAGCGTTGTAGAAGG
IBDV-2F	GATGCATGAACAGAAGTTGATTTCCGAAGAAGACCTCACAAACCTGCAAGATCAAACCC
IBDV-2R	GGAAATCAACTTCTGTTCATGCATCGCTGCGATCGTTTGTCTGATCTC
IBDV-3F	GATGGAACAGAAGTTGATTTCCGAAGAAGACCTCGTTAGTAGAGATCAGACAAAC
IBDV-3R	CTTCGGAAATCAACTTCTGTTCCATCAATGATAGCGTTGTAGAAGG
IBDV-4F	GATGCATGAACAGAAGTTGATTTCCGAAGAAGACCTCACAAACCTGCAAGATCAAACCC
IBDV-4R	GGAAATCAACTTCTGTTCATGCATCGCTGCGATCGTTTGTCTGAT
IBDV-5F	CCTACAGCTGGGGTGCAAACGATACGGACGTTAGTAGAGATCAGACAAA
IBDV-5R	CGTTTGCACCCCAGCTGTAGGTAGGCGCGCCCATCAATGATAGCGTTGTAGAA
IBDV-6F	CATCAACACAAACGGGAGCGTTAGTAGAGATCAGACAAA
IBDV-6R	GCTCCCGTTTGTGTTGATGAGTTGCATCAATGATAGCGTTGTAGAA
IBDV-7F	ACAGAAGTTGATTTCCGAAGAAGACCTCACTTCAGGGGTAACAATCACACTGTTCTC
IBDV-7R	GAGGTCTTCTTCGGAAATCAACTTCTGTTCTGATGCTTGGTACTGTGATGAGAATTGG
IBDV-8F	GCCTACCTACAGCTGGGGTGCAAATGATACGGATGGGGTAACAATCACACTGTTCTCA
IBDV-8R	TCATTTGCACCCCAGCTGTAGGTAGGCGCGCCTTGGTACTGTGATGAGAATTGGTAAT
IBDV-9F	GATTACAAGGATGACGACGATAAGCAGATGTCATGGTCGGCAAGAGGGAGC
IBDV-9R	CTTATCGTCGTCATCCTTGTAATCGGAGGTCACTATCTCCAGTTTGATGGA
IBDV-10F	GACCTCCCAACTCATCAACACAAACGGGAGCCAGATGTCATGGTCGGCAAGAGGGAG
IBDV-10R	ATCTGGCTCCCGTTTGTGTTGATGAGTTGGGAGGTCACTATCTCCAGTTTGATGG

(19), and these clones were used as templates to generate pIBDVA and pIBDVB plasmids. The genome fragments were amplified using the respective primers, as shown in Table 1, and the segments were fused to the cytomegalovirus (CMV) promoter transcription start site of the pCI vector (Promega) at their 5′ ends and a hepatitis delta virus (HDV) ribozyme sequence at their 3′ ends, as described previously (3). The integrity of the plasmid DNA was verified by sequencing, and the proper clones were used for further manipulations. Ten different constructs of IBDV segment A were created by inserting or substituting various foreign sequences in the VP5 or VP2 region, as shown in Fig. 1. First, we replaced the nucleotides encoding the N termini of VP5 (nt 100 to 129) and VP2 (nt 134 to 163) with 30 nucleotides of a c-Myc sequence, thus generating plasmids pIBDV-1 and pIBDV-2, respectively. Second, we inserted the c-Myc sequence between nt 99 and 100 (for pIBDV-3) and nt 133 and 134 (for pIBDV-4) of segment A. We also inserted $HCV_{(523–535)}$ and $HCV_{(412–419)}$ sequences between nt 99 and 100 to make the plasmids pIBDV-5 and pIBDV-6, respectively. Third, we modified the P_{BC} loop of VP2 by mutating the amino acids at positions 222 and 223 to Ala and Ser and inserting the FLAG epitope (for pIBDV-7) and the $HCV_{(523–535)}$ epitope (for pIBDV-8). We also modified the P_{HI} loop of VP2 by replacing P_{HI} loop amino acids 316 to 323 with the FLAG epitope (for pIBDV-9) and the $HCV_{(412–419)}$ epitope (for pIBDV-10). All the manipulations were done by performing overlapping PCR using the respective primers (Table 1). Briefly, two primer pairs (CMVAF/IBDV-1R and IBDV-1F/NheR) were used to construct pIBDV-1. These primer pairs yielded fragments of 800 and 550 bp. These fragments were combined and subsequently amplified using the flanking primers CMVAF and NheR. The amplified fragment was digested by EcoRI and NheI restriction enzymes and inserted into the EcoRI- and NheI-digested fragment of pIBDVA to yield plasmid pIBDV-1. Similarly, plasmid pIBDV-2 was constructed using the CMVAF/IBDV-2R and IBDV-2F/NheR primer pairs. The construction of plasmids pIBDV-3, pIBDV-4, pIBDV-5, and pIBDV-6 was performed similarly using the respective primers (Table 1). Plasmids pIBDV-7 to pIBDV-10 were generated as described above using the flanking primers NheF and SalR along with the respective primers (Table 1). All the plasmids were sequenced to confirm the desired sequence changes in segment A. Using a cDNA-based reverse genetics system developed for IBDV, we generated recombinant viruses by transfecting Vero cells with 1 μg each of a plasmid carrying segment A and a plasmid carrying segment B (pIBDVA and pIBDVB). Successful virus recovery was achieved when cells were cotransfected with the plasmid carrying segment B and with the constructs pIBDVA, pIBDV-3, pIBDV-5, and pIBDV-6 (Table 2), yielding IBDV, IBDV-3, IBDV-5, and IBDV-6, respectively. Generated viruses were passaged further in Vero cells to generate the stock viruses.

Analysis of the recovered viruses. The recovered viruses were characterized by both immunofluorescence and Western blotting analyses. Vero cells infected with IBDV and IBDV-3 were incubated with rabbit anti-IBDV serum and mouse c-Myc MAb (Sigma), respectively, and cells infected with IBDV-5 and IBDV-6 were incubated with HCV polyclonal antibody (My-BioSource, CA); cells were then stained with fluorescein-conjugated secondary antibody. The IBDV polyclonal antibody recognized IBDV antigen in the IBDV-, IBDV-3-, IBDV-5-, and IBDV-6-infected cells. In contrast, the anti-c-Myc MAb readily detected viral antigens in IBDV-3-infected cells but not in mock-infected or IBDV-infected controls (Fig. 2). Similarly, HCV polyclonal antibody reacted with viral antigens in IBDV-5- and IBDV-6-infected cells but not in mock-infected or IBDV-infected controls. Western blot analyses of infected Vero cell lysates with anti-IBDV polyclonal antibody, c-Myc MAb, and HCV polyclonal antibody also confirmed the ex-

FIG. 1. Schematic representation of IBDV segment A and B plasmids. The cDNAs of both segments were placed such that transcription from the CMV promoter starts at the first 5′-end nucleotide, whereas the HDV ribozyme sequence was introduced downstream of both segments to ensure the generation of authentic 3′ ends. Segment A was further modified to construct the plasmids with a c-Myc/FLAG/HCV epitope, which is shown in bold letters. Plasmid characteristics are as follows: pIBDV-1, replacement of the 5′ terminus of the VP5 coding sequence with the c-Myc sequence; pIBDV-2, replacement of the 5′ terminus of the VP2 coding sequence with the c-Myc sequence; pIBDV-3, insertion of the c-Myc sequence at the 5′ terminus of the VP5 coding sequence; pIBDV-4, insertion of the c-Myc sequence at the 5′ terminus of the VP2 coding sequence; pIBDV-5, insertion of the $HCV_{(523–535)}$ epitope at the 5′ terminus of the VP5 coding sequence; pIBDV-6, insertion of the $HCV_{(412–419)}$ epitope at the 5′ terminus of the VP5 coding sequence; pIBDV-7, mutation and insertion of a FLAG epitope in the P_{BC} loop of VP2; pIBDV-8, mutation and insertion of the $HCV_{(523–535)}$ epitope in the P_{BC} loop of VP2; pIBDV-9, replacement of the P_{HI} loop of VP2 by the FLAG epitope; and pIBDV-10, replacement of the P_{HI} loop of VP2 by the $HCV_{(412–419)}$ epitope. Vero cells were transfected with various IBDV segment A constructs, plus segment B. The virus infectivity was monitored by immunofluorescence and Western blotting analyses.

pression of c-Myc (fused to VP5) and HCV epitopes by the recovered viruses (Fig. 3). The recovery of the viruses was further confirmed by reverse transcription-PCR (RT-PCR) analysis of viral RNA template. Total cellular RNA from infected and mock-infected cells was extracted and analyzed by RT-PCR. Sequence analysis of the RT-PCR products confirmed the presence of desired alterations in segment A of the generated IBDV-3, IBDV-5, and IBDV-6 viruses. Taken together, these results suggest that IBDV can stably sustain the insertion of small foreign epitopes in VP5 protein without interference in assembly or virus attachment/entry.

Growth kinetics and genetic stability of the recovered viruses. To compare the replication kinetics of the recovered viruses, Vero cells were infected with IBDV, IBDV-3, IBDV-5, and IBDV-6 at a multiplicity of infection (MOI) of 1.0. Infected cell cultures were harvested at different time points, and the titer of infectious virus present in the culture was determined by a focus-forming assay. Briefly, Vero cells were infected with different dilutions of recovered viruses and incubated for 1 h at 37°C. After incubation, the cells were rinsed, overlaid with Dulbecco's modified Eagle medium–5% fetal calf serum, and incubated further at 37°C. After 24 h, the cells were fixed with methanol acetone (1:1), incubated with anti-IBDV polyclonal rabbit antiserum for 1 h, and stained with fluorescein-labeled secondary antibody and foci were counted by using fluorescence microscopy with a Zeiss Axioplan microscope. The results showed that the kinetics and magnitude of replication for IBDV-3 were very similar to those for the wild-type IBDV and that the final virus yields in Vero cells were comparable. However,

TABLE 2. Recovery of infectious viruses using different IBDV segment A constructs[a]

Construct name	Description or change in segment A	Recovery result[b]
pIBDVA	Strain D78 segment A	+
pIBDV-1	Replacement of 5′ terminus of VP5 coding sequence (nt 100–129) with c-Myc sequence	−
pIBDV-2	Replacement of 5′ terminus of VP2 coding sequence (nt 134–163) with c-Myc sequence	−
pIBDV-3	Insertion of c-Myc sequence at 5′ terminus of VP5 coding sequence (between nt 99 and 100)	+
pIBDV-4	Insertion of c-Myc sequence at 5′ terminus of VP2 coding sequence (between nt 133 and 134)	−
pIBDV-5	Insertion of $HCV_{(523–535)}$ epitope at 5′ terminus of VP5 coding sequence (between nt 99 and 100)	+
pIBDV-6	Insertion of $HCV_{(412–419)}$ epitope at 5′ terminus of VP5 coding sequence (between nt 99 and 100)	+
pIBDV-7	Mutation and insertion of FLAG epitope in P_{BC} loop of VP2 (corresponding to nt 794–800)	−
pIBDV-8	Mutation and insertion of $HCV_{(523–535)}$ epitope in P_{BC} loop of VP2 (corresponding to nt 794–800)	−
pIBDV-9	Substitution of FLAG epitope in P_{HI} loop of VP2 (corresponding to nt 1076–1100)	−
pIBDV-10	Substitution of $HCV_{(412–419)}$ epitope in P_{HI} loop of VP2 (corresponding to nt 1076–1100)	−

[a] Amino acid sequences of epitopes were as follows: c-Myc epitope, EQKLISEEDL; FLAG epitope, DYKDDDDK; $HCV_{(523–535)}$ epitope, GAPTYSWGANDTDV; and $HCV_{(412–419)}$ epitope, QLINTNGS. Vero cells were transfected with various IBDV segment A constructs, plus segment B.
[b] Results for recovery of virus are denoted as + (virus was recovered) and − (no virus was recovered).

IBDV-5 and IBDV-6 showed a slight delay in growth and had titers 1 log lower than IBDV (Fig. 4). To determine the genetic stability of the transfectant viruses *in vitro*, the viruses were propagated in Vero cells (up to 5 passages), total RNA was isolated, and the region corresponding to the modified portion of VP5 was amplified by RT-PCR. Sequence analysis of the RT-PCR product confirmed the expected modifications in the VP5 genes of the recovered viruses. The stability of the recombinant viruses expressing foreign epitopes was further confirmed by immunofluorescence and Western blot analyses of the cell lysates.

The ability of IBDV to propagate in primate cells (16) suggests that IBDV might be capable of replication in humans. Additionally, replication of IBDV in human cells will establish its potential to be used as vectors for prophylactic purposes. With this objective, we infected HEK 293 cells, a specific cell line originally derived from human embryonic kidney cells, with IBDV, IBDV-5, and IBDV-6, and virus replication was confirmed by an immunofluorescence assay (Fig. 2) and Western blot analysis (Fig. 3).

The use of recombinant viral vaccines is a relatively novel and promising approach to combating infectious diseases in humans as well as in veterinary medicine. An efficient viral vector is expected to provide preferentially stable and long-term transgene expression. Several viral systems, including hepatitis B virus, poliovirus, Newcastle disease virus, adenovirus, and influenza virus, have been used as vectors to express foreign epitopes and induce protective immunity against unrelated pathogens (15, 18, 20, 25, 28). Previous studies have shown that vaccination of humans with short synthetic HCV

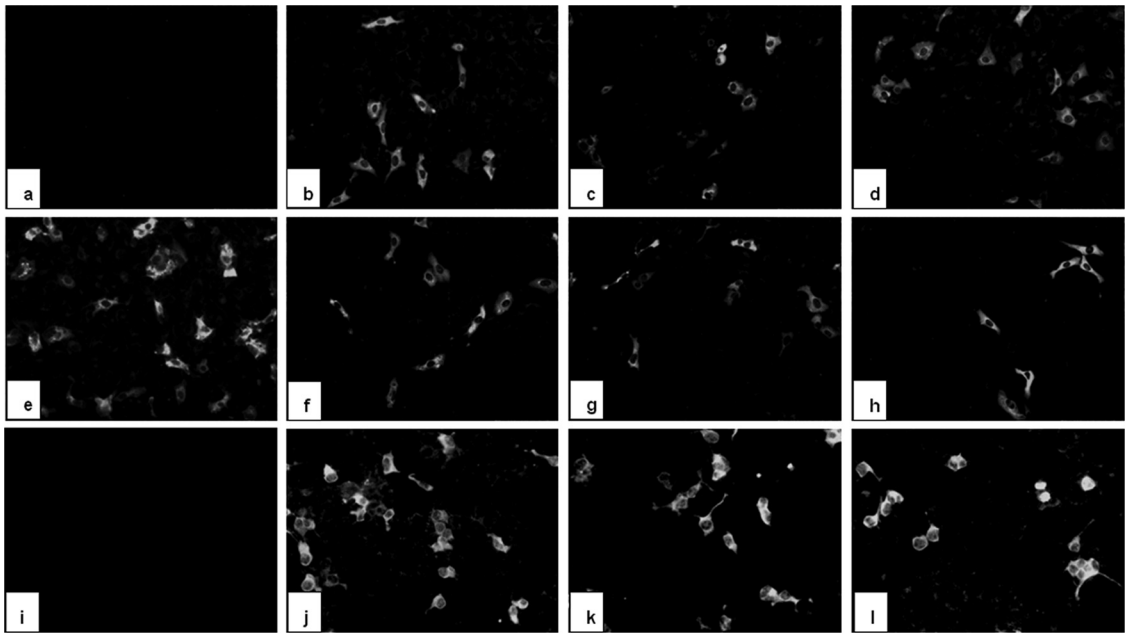

FIG. 2. Immunofluorescence assay results. Vero cells were infected with passage 4 of IBDV (b), IBDV-3 (c and f), IBDV-5 (d and g), and IBDV-6 (e and h) at an MOI of 1.0. Uninfected Vero cells were used as a negative control (a). At 24 h postinfection, the cells were fixed and analyzed by immunofluorescence staining with rabbit anti-IBDV (a, b, c, d, and e), c-Myc monoclonal antibody (f), and HCV polyclonal antibody (g and h). HEK293 cells were infected with IBDV (j), IBDV-5 (k), and IBDV-6 (l), and uninfected HEK293 cells were used as a negative control (i). At 24 h postinfection, the cells were fixed and analyzed by immunofluorescence staining with rabbit anti-IBDV antibody (i, j, k, and l).

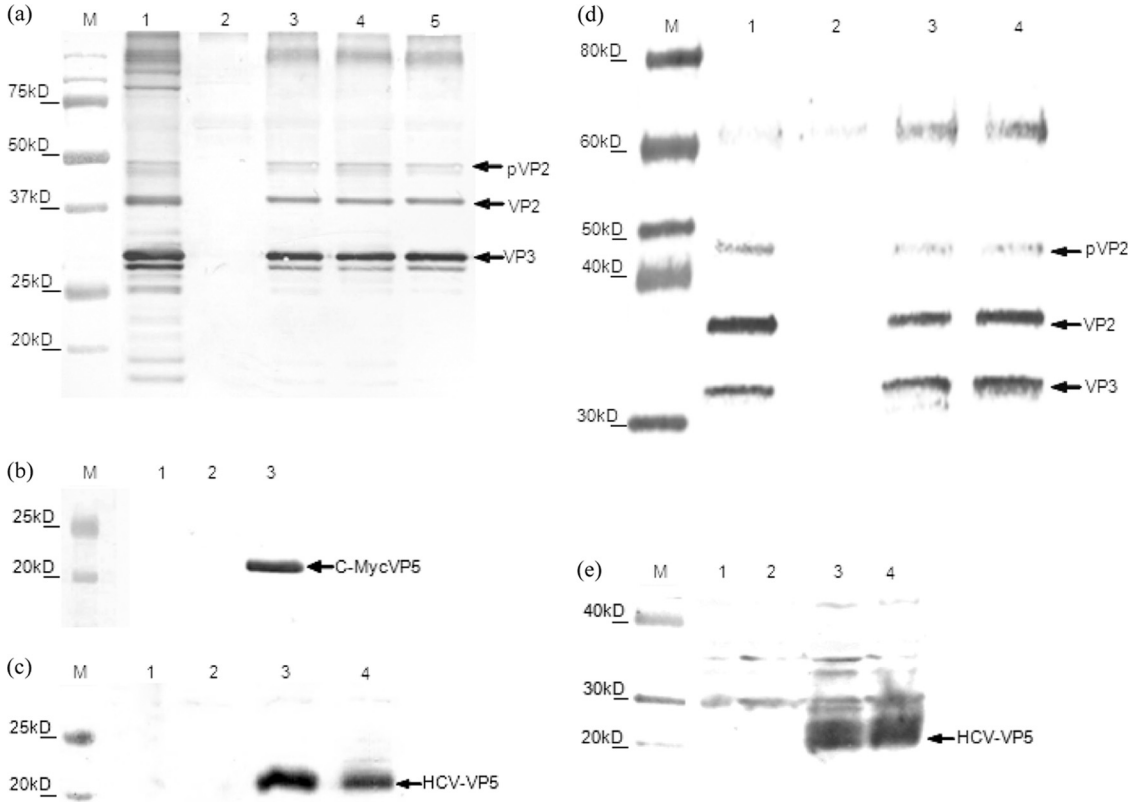

FIG. 3. Immunoblot analysis of IBDV proteins synthesized in virus-infected Vero/HEK293T cells. (a to c) Vero cells were cotransfected with pIBDVB and pIBDVA, pIBDV-3, pIBDV-5, or pIBDV-6. Five days posttransfection, virus was harvested after three cycles of freeze-thawing. Proteins in the cell lysates were separated by SDS–12.5% PAGE; blotted onto nitrocellulose; reacted with polyclonal anti-IBDV serum (a), c-Myc MAb (b), and hepatitis C virus glycoprotein E polyclonal antibody (c); and detected with alkaline-phosphatase and naphthol phosphate fast red color development reagents. (a) Lanes: 1, sample from IBDV-infected cells; 2, sample from mock-infected cells; 3, sample from IBDV-3-infected cells; 4, sample from IBDV-5-infected cells; and 5, sample from IBDV-6-infected cells. (b) Lanes: 1, sample from IBDV-infected cells; 2, sample from mock-infected cells; and 3, sample from IBDV-3-infected cells. (c) Lanes: 1, sample from IBDV-infected cells; 2, sample from mock-infected cells; 3, sample from IBDV-5-infected cells, and 4, sample from IBDV-6-infected cells. (d and e) HEK293T cells were infected with IBDV, IBDV-5, or IBDV-6. Five days postinfection, virus was harvested after three cycles of freeze-thawing. Proteins in the cell lysates were separated by SDS–4 to 20% PAGE, blotted onto nitrocellulose, reacted with polyclonal anti-IBDV serum (d) and hepatitis C virus polyclonal antibody (e), and detected with horseradish peroxidase color development reagents. Lanes: 1, sample from IBDV-infected cells; 2, sample from mock-infected cells; 3, sample from IBDV-5-infected cells; and 4, sample from IBDV-6-infected cells. The positions of pVP2, VP2, VP3, c-Myc–VP5, HCV epitope-VP5, and marker proteins (M) are indicated in kilodaltons.

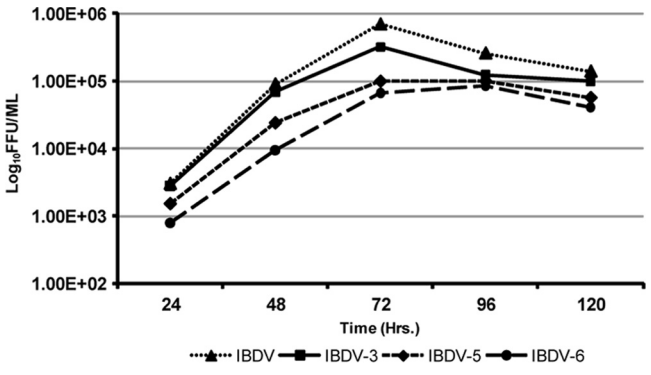

FIG. 4. Replication kinetics of the recovered viruses. Monolayers of Vero cells were infected with the indicated viruses at an MOI of 1.0 and harvested at the indicated time points. Infectious titers were determined by a focus-forming-unit (FFU) assay.

peptides has generated both humoral and cellular immunity (13, 30, 31). In the present study, we explored the potential utility of IBDV to carry foreign epitopes with the aim of developing a safe and efficient viral vector platform. Using a plasmid-based reverse genetics system, we generated a recombinant IBDV (IBDV-3) expressing a c-Myc epitope by introducing the epitope sequence into the VP5 coding region of IBDV, thereby increasing the length of genome segment A by 30 nucleotides. After the recovery of IBDV-3, we further explored the VP5 region by inserting the hepatitis C virus epitopes [$HCV_{(523–535)}$, GAPTYSWGANDTDV, and $HCV_{(412–419)}$, QLINTNGS] and successfully recovered the viruses harboring the HCV epitopes, thus increasing the length of segment A by 42 and 24 nucleotides, respectively. We further explored the VP5 region by inserting the M2e gene (encoding the sequence SLLTEVETPIRNEWGCRCNDSS) from influenza type A but were unsuccessful in recovering this virus (data not shown). We speculate that the reason for the failure

to recover the virus with the flu sequence inserted into VP5 may be the size of the epitope. The coding sequence is 66 nucleotides, longer than the c-Myc or HCV epitope sequence, and the longer foreign sequence may have hindered the efficient packaging of the IBD virus. This indicates the limitation of IBDV in its intolerance of longer foreign sequences, as well as the importance of the amino acid sequences selected for insertion, which may interfere with protein folding, assembly, or replication of the IBDV genome. Based on the crystal structure of VP2, we also explored insertion into the P_{BC} and P_{HI} loops but failed to recover any recombinant virus. Recently, it was shown that the foot-and-mouth disease virus (FMDV) immunodominant epitope could be effectively inserted into the P_{BC} loop of IBDV to produce IBDV SVPs with chimeric VP2 in insect cells (23). The inability to recover any of the recombinant viruses with modification in the loops may suggest that though it is possible to generate chimeric SVPs, any insertion of sequences into these loops may limit or interfere with efficient packaging or the function of VP2 in virus attachment/entry. The same may be true for the lack of recovery of virus from cells transfected with pIBDV-2 and pIBDV-4 constructs, suggesting the importance of protein sequences in viral function. Although the tolerable size and sites for the insertion were not fully explored in this study, IBDV certainly could be exploited for the expression of small therapeutic epitopes. One of the fundamental requirements for currently available viral vectors is their safety. IBDV is an avian virus and, despite its worldwide distribution in domestic fowl, is not known to be a hazard for transmission to other species. While remaining cautious, we believe that this may not be a risk to humans. The use of IBDV as a therapeutic agent in patients suffering from chronic hepatitis infections has already been documented (2, 8). The results of a phase II clinical trial of IBDV coinfection therapy in 42 acute hepatitis patients (infected with hepatitis B virus and HCV) showed the safety and efficacy of IBDV therapy and provided encouraging evidence that the progression to chronic infection was marginally reduced in IBDV-treated patients compared to the controls (9). It has also been reported that IBDV therapy improved the condition of an HCV patient that had become resistant to conventional treatment, developed decompensated chronic viral hepatitis, and received disability status (2). Importantly, during the treatment of patients, it emerged that to ensure an "artificial viremia" with IBDV, the viral preparation needs to be given in large doses and continuously over a long period (1). These findings further support the therapeutic application of IBDV in humans without detrimental effects. IBDV is not known to multiply in human cells but is able to propagate in primate cells. To establish the potential of an IBDV vector for prophylactic purposes in HCV infections, we infected HEK293T cells, a specific cell line originally derived from human embryonic kidney cells, and compared the growth of IBDV in Vero cells to that in HEK293T cells. The virus replicated to yield similar titers in the two cell lines, and the replication was confirmed by immunofluorescence and Western blot analyses. The ability of IBDV-HCV vectors to replicate in human cells ascertains the potential of IBDV to be useful as an expression vector in humans. A recent study described mice as a potential carrier for IBDV (22). The study also confirmed that IBDV maintained its viability and pathogenicity in mice and that virus excreted from mice induced clinical disease in chickens (22), suggesting that IBDV might be capable of replication in humans. Additionally, most human and animal populations are not exposed to IBDV, in contrast to vaccinia virus or adenovirus and poliovirus, on which some viral vectors and vaccines are based. Therefore, *in vivo* expression of a heterologous immunogen from an IBDV-based vaccine vector would not be limited by prior immunity. Furthermore, because of its natural heat stability, ease of production, and widespread use, IBDV lends itself as a low-cost recombinant veterinary vaccine, especially for poultry. In this study, for the first time we demonstrated the insertion of foreign epitopes in segment A of IBDV, thus generating a replication-competent recombinant virus. Further exploration of the insertion sites used in the present study and other sites with different antigenic epitopes will shed more light on the development of novel IBDV vectors.

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