In Vitro and In Ovo Expression of Chicken Gamma Interferon by a Defective RNA of Avian Coronavirus Infectious Bronchitis Virus

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Coronavirus defective RNAs (D-RNAs) have been used for site-directed mutagenesis of coronavirus genomes and for expression of heterologous genes. D-RNA CD-61 derived from the avian coronavirus infectious bronchitis virus (IBV) was used as an RNA vector for the expression of chicken gamma interferon (chIFN-γ). D-RNAs expressing chIFN-γ were shown to be capable of rescue, replication, and packaging into virions in a helper virus-dependent system following electroporation of in vitro-derived T7 RNA transcripts into IBV-infected cells. Secreted chIFN-γ, under the control of an IBV transcription-associated sequence derived from gene 5 of the Beaudette strain, was expressed from two different positions within CD-61 and shown to be biologically active. In addition, following infection of 10-day-old chicken embryos with IBV containing D-RNAs expressing chIFN-γ, the allantoic fluid was shown to contain biologically active chIFN-γ, demonstrating that IBV D-RNAs can express heterologous genes in vivo.

Infectious bronchitis virus (IBV) is a highly infectious and economically important pathogen of chickens that causes respiratory disease, diminished growth rate, and substantial decline in egg production. Although infectious bronchitis is considered primarily a disease of the respiratory system, strains of IBV have wide and variable tropisms and the clinical manifestations of the disease can be diverse (9). Genetically very similar viruses cause disease in turkeys (6) and pheasants (7). IBV is a group 3 member of the genus Coronavirus of the family Coronaviridae in the order Nidovirales (12), being an enveloped RNA virus with an unsegmented, 5′-end-capped, 3′-end-polyadenylated, single-stranded, positive-sense RNA genome of 27,608 nucleotides (nt) (4). Coronaviruses produce a 3′-coterminal nested set of subgenomic mRNAs (sg mRNAs) that are polycistronic. These are produced by a discontinuous transcription process during synthesis of the negative strand and contain identical 5′ ends due to the addition of a leader sequence derived from the 5′ end of the genomic RNA (gRNA) (35, 36). Preceding the body sequence of each sg mRNA is a consensus sequence, the transcription-associated sequence (TAS) (15), involved in the acquisition of the leader sequence. All coronavirus envelopes contain at least three membrane proteins, the spike glycoprotein, a small membrane protein, and an integral membrane protein. In addition, the coronavirus virion also contains a nucleocapsid protein that interacts with the gRNA.

Coronavirus defective RNAs (D-RNAs), which lack large parts of the genome, are produced following virus passage at a high multiplicity of infection. While all D-RNAs contain cis-acting sequences necessary for replication, only a subset of D-RNAs contain sequences necessary for packaging into virions in the presence of a helper virus. Coronavirus D-RNAs have been used for site-directed mutagenesis of the virus genome (25) and for expression of heterologous genes (1, 2, 13, 17, 20, 22, 39, 42, 43).

Cytokines are regulatory proteins that act as a communication network between cells throughout immunological development. Avian homologues of several mammalian cytokines have been isolated, including chicken gamma interferon (chIFN-γ) (10). Mammalian IFN-γ is a pleiotropic cytokine initially produced by natural killer cells, during immune induction, and then by committed T helper 1 (Th1) cells as a regulator and effector molecule for driving inflammatory responses (for a review, see reference 8). IFN-γ has a major role in activating antiviral immune responses through augmentation of major histocompatibility complex expression on antigen-presenting cells for interaction with T cells, stimulation of antibody (Ab) formation, promotion of Ab isotype class switching, and development of cytotoxic T cells. Several studies have been undertaken to investigate the in vivo potential of IFN-γ as a vaccine adjuvant. Co-administration of bovine IFN-γ with vesicular stomatitis virus G glycoprotein to cattle resulted in an increased formation of protective Ab against vesicular stomatitis virus (41). In a mouse model, fusion of IFN-γ to human immunodeficiency virus gp120 resulted in enhanced primary Ab responses against gp120, enhanced antigen-specific T-cell proliferation, and IFN-γ production (26). The use of recombinant feline IFN-γ as a vaccine adjuvant increased Ab responses against rabies virus and calcivirus antigens (37).

The role of recombinant chIFN-γ as an adjuvant and therapeutic agent has been investigated. Co-administration of chIFN-γ with sheep red blood cells (SRBCs) resulted in an increased secondary Ab response with amounts of SRBCs 10-fold smaller than the dose of SRBCs given alone (23, 24). Administration of recombinant chIFN-γ prior to challenge with avian coccidia resulted in decreased intracellular sporozoite development and oocyst production, with an enhanced level of body weight gain (21). chIFN-γ had an adjuvant effect, reducing parasite replication, when used in a DNA vaccine regimen against Eimeria acervulina (27). Coexpression of New-
castle disease virus antigens with chIFN-γ by using fowlpox virus resulted in an earlier Ab response, with the best protective immune response of the recombinant fowlpox virus vaccines (32).

In this study we describe both the in vitro and in ovo expression of biologically active chIFN-γ from an IBV D-RNA. We demonstrate for the first time the expression of a chicken cytokine from an IBV D-RNA and the in ovo expression of a biologically active heterologous gene from an IBV D-RNA.

MATERIALS AND METHODS

Cells and viruses. IBV Beaudette was grown in 11-day-old embryonated domestic fowl eggs, harvested from allantoic fluid 24 h postinfection, and used as helper virus for the rescue of IBV D-RNAs (31). IBV was passaged and titrated to an MOI of 0.1 for the rescue of IBV D-RNAs by IBV Beaudette. The infected allantoic fluid samples for 2 h at room temperature before storage at −80°C. The cell culture medium was used to infect CK cells, and viruses V2 to V6 were serially passaged every 24 h for six passages (P1 to P6).

Oligonucleotides. The oligonucleotides used in this work were obtained from Invitrogen and are listed in Table 1.

TABLE 1. Oligonucleotides used for generation of TAS-chIFN-γ cassette and hybridization probes

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Position</th>
<th>Polarity</th>
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</thead>
<tbody>
<tr>
<td>IBV51F-γSTART</td>
<td>TCC CCC GGG CAC GTG TTT TAC TTA ACA AAA ACT TAA CAA</td>
<td>NA</td>
<td>+</td>
</tr>
<tr>
<td>SmalF-γ-END</td>
<td>ACC CCC GGG GTA TAG CAA TGG TAT CTI</td>
<td>NA</td>
<td>–</td>
</tr>
<tr>
<td>BG-67</td>
<td>GGC TGG TTT CGA GAG TTA</td>
<td>Beaudette 265–282</td>
<td>+</td>
</tr>
<tr>
<td>BG-2</td>
<td>TCA GGG GTT TAT TGG CAC T</td>
<td>Beaudette 455–473</td>
<td>–</td>
</tr>
<tr>
<td>IFN/3</td>
<td>ATG ACT TGC CAG ACT TAC AA</td>
<td>chIFN-γ 1–20</td>
<td>+</td>
</tr>
<tr>
<td>IFN/7</td>
<td>CAG GTC CAT GAT ATC TTT CAC</td>
<td>chIFN-γ 340–360</td>
<td>–</td>
</tr>
</tbody>
</table>

*Underlined sequences correspond to the IBV sequence. Nucleotides marked in bold correspond to the IBV canonical TAS, and those in italics correspond to restriction endonuclease sites used for cloning.

RESULTS

D-RNAs containing TAS-chIFN-γ. Expression of heterologous genes from coronavirus D-RNAs requires the genes to be under the control of a TAS for synthesis of an sg mRNA. The chIFN-γ sequence was placed under the control of the IBV Beaudette gene 5 TAS (39) by using PCR and a chIFN-γ mRNA-derived cDNA (19), generating a TAS-chIFN-γ cassette for IBV-controlled expression. The gene 5 TAS was originally chosen for expression of heterologous genes because it has the shortest sequence between the 3′ end of the TAS and the AUG of open reading frame (ORF) 5a and also because the Beaudette sg mRNA 5 is one of the most abundantly
expressed sg mRNAs (39). The TAS-chIFN-γ cassette was initially inserted into pBluescript II SK(+) under the control of the T7 promoter. A protein with a size corresponding to that of chIFN-γ was produced in vitro by using the TNT T7 coupled wheat germ extract system (Promega; data not shown), indicating that a product of the expected size could be produced.

To investigate the rescue profile of the D-RNAs, Northern blot analyses were carried out on RNA isolated from P0 to P6 CK cells containing D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaiBI by using the IBV 5'UTR, 3'UTR, and chIFN-γ probes (Fig. 3). The 6.8-kb D-RNAs were detected by the two IBV probes in RNA isolated from P1 to P6 CK cells (Fig. 3A, B, D, and E), and analyses using the chIFN-γ probe confirmed that they contained the chIFN-γ sequence (Fig. 3C and F). Both D-RNAs, irrespective of whether the chIFN-γ sequence was inserted into the PmaCI or SnaiBI site, were initially detected at P1 and increased in amount upon serial passage, with the largest amount in P6 CK cells, after which the amount of D-RNA decreased.

RNAs larger than the 6.8-kb chIFN-γ-containing D-RNA and the IBV sg mRNA 2 were detected from P3 to P6 by using both IBV probes. The use of the IBV 5' probe indicated that the RNAs corresponded to new IBV-derived D-RNAs (Fig. 3A, B, D, and E). The new D-RNAs did not contain the chIFN-γ sequence (Fig. 3C and F), indicating that they were derived from IBV gRNA, and were observed in increasing amounts in RNA isolated from P1 to P6 cells, with concomitant gradual loss of the 6.8-kb chIFN-γ-containing D-RNAs (Fig. 3).

Analysis of P5-derived RNA following the rescue of D-RNA
IBV-Vec-IFN-γSnaBI identified a 5-kb D-RNA-derived chIFN-γ mRNA. This mRNA was detected in RNA from P₁ to P₆ CK cells containing IBV-Vec-IFN-γSnaBI, with the amounts detected varying in accordance with the amounts of D-RNA present, the largest amount being detected at P₄ (Fig. 3F). An RNA corresponding to the IBV-Vec-IFN-γPmaCI 1.7-kb chIFN-γ mRNA was not detected following serial passage of the D-RNA (Fig. 3C). However, from the amounts of the RNA detected, the most likely explanation for this result was that the amount of the 1.7-kb chIFN-γ mRNA was below the detection level of the analysis.

The Northern blot analyses showed that the two chIFN-γ-containing D-RNAs with the TAS-chIFN-γ insert in the correct orientation were rescued upon serial passage, but the analyses could not show whether biologically active chIFN-γ was expressed. The biological assay for IFN-γ is based on the fact that macrophages stimulated with IFN-γ produce NO, along with other reactive nitrogen species, as one of several mechanisms to destroy intracellular pathogens. The chIFN-γ bioassay involves stimulation of HD11 cells, a chicken macrophage cell line (3), with chIFN-γ for the induction of NO, which accumulates as stable and quantifiable NO₂⁻ in the HD11 culture medium.

Cell medium from the P₀ to P₆ CK cells, previously shown to contain the D-RNAs containing chIFN-γ, was assayed for chIFN-γ activity. Medium from cells containing D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI induced significantly larger amounts of NO, following stimulation of HD11 cells with chIFN-γ.
FIG. 3. Northern blot analysis of IBV-specific RNAs following serial passage of D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI. RNAs isolated from P₀ to P₆ CK cells were analyzed for the presence of D-RNAs IBV-Vec-IFN-γPmaCI (A to C) and IBV-Vec-IFN-γSnaBI (D to F). IBV-derived RNAs were detected by using the 309-bp IBV 3'-UTR probe (A and D), the 209-bp IBV 5'-UTR probe (B and E), and the 360-bp chIFN-γ probe (C and F). The RNA samples analyzed were isolated from IBV-infected cells (lane 1), uninfected cells (lane 2), and P₀ to P₆ cells containing D-RNAs IBV-Vec-IFN-γPmaCI (A to C, lanes 3 to 9) and IBV-Vec-IFN-γSnaBI (D to F, lanes 3 to 9). (A to C) Lanes 10 correspond to in vitro T7-derived IBV-Vec-IFN-γPmaCI. Arrows indicate IBV gRNA, sg mRNAs, and TAS-chIFN-γ-containing D-RNAs. The RNAs migrating slower than IBV sg mRNA 2, detected in RNA samples isolated from P₃ to P₆ cells (A, B, D, and E, lanes 6), are new IBV-derived D-RNAs. Analysis of RNA following passage of IBV-Vec-IFN-γSnaBI identified the D-RNA-derived chIFN-γ mRNA (F, lanes 5 to 8). S, spike glycoprotein; E, small membrane protein; M, integral membrane protein; N, nucleocapsid protein.
cells, than any of the controls (Fig. 4A). In contrast, medium from cells containing D-RNAs IBV-Vec-γ-NFIpmaCI and IBV-Vec-γ-NFISnaBI did not induce larger amounts of NO than the controls. Controls involved medium from uninfected CK and IBV-infected CK cells. The amounts of NO induced by using medium from control samples and from cells containing D-RNAs with the TAS-chIFN-γ insert in the incorrect orientation were similar and likely to result from the presence of IFN-α or IFN-β. The IFN-γ bioassay also detects IFN-α and IFN-β, previously shown to be produced by IBV-infected cells (16, 28, 29).

To confirm that the NO detected by the IFN-γ bioassay was due to the induction of HD11 cells by IFN-γ, and not by IFN-α and IFN-β, which also stimulate the production of NO, a neutralizing bioassay was carried out. Samples of cell medium were preincubated with the chIFN-γ-neutralizing MAb Ab 1E-12 and analyzed with the IFN-γ bioassay. Samples were also preincubated with medium and Ab CC305, an isotype control Ab against bovine granulocyte-macrophage colony-stimulating factor. For comparative purposes, recombinant chIFN-γ was included as a positive control sample. NO induction, using medium from cells containing D-RNAs IBV-Vec-IFN-γpmaCI and IBV-Vec-IFN-γSnaBI, with the TAS-chIFN-γ sequence in the correct orientation, was reduced following preincubation of the samples with the neutralizing MAb 1E-12 (Fig. 4B). The amount of neutralizing MAb 1E-12 used was sufficient to neutralize all the chIFN-γ in the solution. To confirm this, the neutralizing activity of the MAb had been titrated before use in this experiment. Levels of remaining activity were assumed to be due to IFN-α and IFN-β. These levels were consistent with those seen in a previous study using this neutralizing MAb (19). A similar reduction in NO induction was observed when the recombinant chIFN-γ was preincubated with the MAb 1E-12. In contrast, the NO induced by using medium from cells containing D-RNAs IBV-Vec-IFN-γ-NFIpmaCI and IBV-Vec-γ-NFISnaBI, with the TAS-chIFN-γ sequence in the incorrect orientation, was not affected by the addition of the neutralizing MAb 1E-12. This indicated that the NO detected resulted from induction with chicken IFN-α or IFN-β rather than chIFN-γ in the cell medium (Fig. 4B).

These results showed that the NO induction observed by using samples taken from cells containing D-RNAs IBV-Vec-IFN-γ-pmaCI and IBV-Vec-γ-SnaBI resulted from the presence of chIFN-γ in the cell medium and confirmed that the D-RNAs expressed biologically active chIFN-γ.

Induction of NO, using medium from cells containing D-RNAs IBV-Vec-IFN-γ-pmaCI and IBV-Vec-IFN-γ-SnaBI, was observed from P3 to P6, and recombinant chIFN-γ at 1:125 and 1:250 dilutions (samples 7 and 8, respectively). Nitrite resulting from the induced NO in the HD11 cell medium samples was quantified by using a variation of the Griess assay, measuring absorbance at 543 nm. The histograms represent the midpoints taken from the linear portions of titration curves calculated from the means of results for triplicates of each sample (A) and the means of results for triplicate samples (± standard errors) (B). OD543, optical density at 543 nm.
The secretion of chIFN-γ into cell medium following expression from the D-RNAs described in this work provided a means of determining whether IBV D-RNAs can be replicated in ovo.

Following serial passage of D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI, the largest amounts of D-RNA detected with concomitant expression of chIFN-γ occurred in P₃ cells (Fig. 3 and 4). Consequently, we decided to use progeny virus (V₃) containing the D-RNAs from P₃ cells to determine whether the D-RNAs could be rescued in ovo. Virus (V₃) from P₃ cells, representing virus that was one passage away from peak chIFN-γ activity, was used to infect 10-day-old specific-pathogen-free embryonated eggs. At 16 h postincubation, allantoic fluid was clarified and used to infect CK cells to confirm that the D-RNAs could be passed from embryonated eggs. The fluid was also used for chIFN-γ bioassays to analyze for the presence of any chIFN-γ resulting from the replication of the D-RNAs in ovo.

IFN-γ bioassays showed that allantoic fluid and CK cell medium samples, following passage of D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI, contained amounts of chIFN-γ that were up to 64-fold and 10-fold larger, respectively, as measured by NO levels, than those of the controls (Fig. 5A), indicating that the D-RNAs had replicated in ovo. The amounts of NO induced following infection with IBV only and passage of D-RNAs containing the TAS-chIFN-γ gene construct in the antisense orientation were all similar, with NO induction presumably due to the presence of IFN-α or IFN-β. The amounts of chIFN-γ, as measured by NO levels, in allantoic fluid following in ovo passage of D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI were larger than those found in CK cell medium following subsequent in vitro passage of the D-RNAs. This result was anticipated, as the in ovo passage was equivalent to P₃ and CK passage was equivalent to P₃ when equated with in vitro passage of the D-RNAs.

A neutralizing IFN-γ bioassay was again performed to confirm that the NO induced resulted from IFN-γ and not from IFN-α and IFN-β. Allantoic fluid and CK cell medium samples were preincubated with MAb 1E-12, medium, and the isotype control Ab CC305 for 2 h prior to the IFN-γ bioassay (Fig. 5B). The amounts of NO induced by using samples from embryonated eggs (Fig. 5B, samples 1 and 2) and CK cells (Fig. 5B, samples 9 and 10) infected with IBV containing D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI were similar for both D-RNAs both in ovo and in vitro and were similarly reduced following preincubation with MAb 1E-12. A similar reduction in NO induction was observed following preincubation of recombinant chIFN-γ with MAb 1E-12 (Fig. 5B, sample 7), indicating that the IFN activities detected in the allantoic fluid and CK cell medium samples resulted from IFN-γ. In contrast, the amounts of NO induced by using samples from embryonated eggs and CK cells infected with IBV containing D-RNAs IBV-Vec-γ-NFlPmaCI and IBV-Vec-γ-NFlSnaBI and from IBV infection alone were not reduced following preincubation with MAb 1E-12 (Fig. 5B). This indicated that the IFN activities observed following in ovo passage of these D-RNAs and IBV alone resulted from the induction of IFN-α and IFN-β and not from IFN-γ.

These results confirmed that D-RNAs IBV-Vec-IFN-γPmaCI and IBV-Vec-IFN-γSnaBI expressed biologically active chIFN-γ following helper virus-dependent replication in ovo.

**DISCUSSION**

The construction of a series of IBV-based D-RNAs containing the chIFN-γ sequence under the control of an IBV TAS...
has been described. We have shown that IBV D-RNAs containing the chIFN-γ gene were incorporated into virus particles and expressed biologically active chIFN-γ in vitro and in vivo.

Serial passage of the chIFN-γ-containing D-RNAs followed the same pattern of both D-RNA replication and heterologous protein expression that we have previously observed for the expression of the heterologous reporter genes encoding CAT (39) and luciferase (13) from IBV-based D-RNAs. Both detection of the D-RNA and expression of chIFN-γ increased from P0 to P3 and decreased thereafter. No differences in the amounts of D-RNA detected or in chIFN-γ activities were observed whether the TAS-chIFN-γ cassette was inserted in domain I (IBV-Vec-IFN-γSnaBI), interrupting the D-RNA-specific ORF, or domain III (IBV-Vec-IFN-γPmaCI), not interrupting the D-RNA-specific ORF, of D-RNA CD-61.

A D-RNA-derived chIFN-γ mRNA synthesized from IBV-Vec-IFN-γSnaBI was detectable upon serial passage of the D-RNA (Fig. 2B and 3F). Although a D-RNA-derived chIFN-γ mRNA synthesized from IBV-Vec-IFN-γPmaCI was initially detected (Fig. 2B), it was synthesized in smaller amounts, in relation to the D-RNA, than the mRNA from IBV-Vec-IFN-γSnaBI. The observed levels of subgenomic RNAs (sgRNAs) transcribed, under the control of the gene 5' TAS, from CD-61-derived D-RNAs were far less than the amounts observed for the transcription of sg mRNA 5 from genomic RNA. Similar observations have been made following transcription of sgRNAs from other coronavirus-derived D-RNAs. The altered transcription levels may be connected to transcription of sgRNAs from other coronavirus-derived D-RNAs. Similar observations have been made following transcription of sgRNAs from other coronavirus-derived D-RNAs.

Although its main role is in driving a Th1 cell-mediated response against intracellular pathogens, including viruses, IFN-γ can have a direct antiviral effect. Like IFN-α and IFN-β, but to a much lesser extent, it can induce oligoadenylate synthetase and RNA-dependent protein kinase PKR, both important components of IFN-induced antiviral responses (8, 14, 34). It was therefore possible that expression of chIFN-γ from the D-RNAs might interfere with replication of the helper virus. A murine IFN-γ-containing D-RNA of the murine coronavirus mouse hepatitis virus (MHV) expressed murine IFN-γ that resulted in a slight reduction in virus titer (0.5 log10) at low multiplicities of infection, suggesting that the murine IFN-γ had a weak antiviral activity (42). In contrast, we observed no significant differences in virus titers resulting from expression of the chIFN-γ from any of the IBV D-RNAs (data not shown), suggesting that the chIFN-γ did not have an appreciable antiviral effect on the helper IBV.

The murine IFN-γ-containing MHV D-RNA was not detected beyond P2 (42), whereas the IBV-based chIFN-γ-containing D-RNAs were rescued for at least six passages. This observation reflects our previous studies on the expression of heterologous genes from IBV-CD-61-based D-RNAs. Expression of heterologous genes or coronavirus-derived genes from other coronavirus D-RNAs also resulted in loss of the D-RNAs. For example, expression of CAT and hemagglutinin esterase from MHV D-RNAs was not detected beyond P2 (20) and P3 (43), respectively. The most likely explanation for the instability of coronavirus D-RNAs expressing heterologous genes is intolerance of the heterologous sequence within the D-RNA. D-RNAs evolve by the removal of unnecessary sequences. Therefore, the presence of a heterologous sequence may impart some innate instability into the RNA or affect replication and/or packaging of the D-RNA; i.e., D-RNAs containing heterologous or nonrequired sequences are less fitted for replication than D-RNAs with minimal required sequences. We detected additional RNA species, which were larger than the chIFN-γ-containing D-RNAs and IBV sg mRNA 2, from P3 which increased in amount upon serial passage. The new RNAs were not detected by the chIFN-γ-specific probe but by the IBV 5' probe, showing that they contained sequences derived from the 5' end of the IBV genome and were therefore new D-RNAs. As determined from their sizes, the new D-RNAs were not from the loss of the chIFN-γ sequence. An explanation for the loss of the chIFN-γ-containing D-RNAs beyond P2 was the generation of more stable D-RNAs from P2 that eventually resulted, due to competition, in the loss of the chIFN-γ-containing D-RNAs.

Overall, we have demonstrated that in vitro passage of the chIFN-γ-containing D-RNAs resulted in expression of biologically active chIFN-γ secreted into cell culture medium. This observation allowed us to demonstrate that IBV D-RNAs can be replicated in ovo from the presence of biologically active chIFN-γ secreted into the allantoic fluid and subsequent passage on indicator CK cells.

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REFERENCES


