High Level Expression of C-terminal Truncated Recombinant Chicken Interferon- γ in Baculovirus Vector System

Kazuaki TAKEHARA¹⁾, Makiyo KAMIKAWA¹⁾, Naomi OHNUKI¹⁾, Tomoshi NAGATA¹⁾, Ayako NAKANO¹⁾, Daisuke YAMAGUCHI¹⁾, Yuichi YOKOMIZO²⁾ and Masayuki NAKAMURA¹⁾

¹⁾Laboratory of Poultry Diseases, School of Veterinary Medicine and Animal Sciences, Kitasato University, Towada, Aomori 034-8628 and ²⁾National Institute of Animal Health, Tsukuba, Ibaraki 305-0856, Japan

(Received 8 August 2001/Accepted 1 October 2001)

ABSTRACT. Chicken interferon- γ (ChIFN- γ) was expressed by baculovirus in a C-terminal truncated form, namely ChIFN- γ T, to accelerate the secretion of the expressed protein. It is also expressed as ChIFN- γ T bearing poly His tag, ChIFN- γ THis, for easy purification. The expressed proteins were detected by SDS-PAGE analysis with Coomassie brilliant blue staining. The purified ChIFN- γ THis with nickel chelated column showed anti-viral activity *in vitro* and stimulation of the secretion of nitrogen intermediates such as nitric oxide in chicken peripheral blood mononuclear cells. Antiserum against ChIFN- γ THis recognized the 15 kDa, 16 kDa, and 32 kDa bands that seemed to be an unglycosylated monomer, a glycosylated monomer, and a homodimer of ChIFN- γ THis in the culture supernatant, respectively. The anti-serum also recognized around 14 kDa and 28 kDa bands in the sera of chickens or concanavalin A stimulated spleen cell culture supernatants that seemed to be monomeric and dimeric forms of a natural ChIFN- γ , respectively.

KEY WORDS: baculovirus, chicken interferon- γ , expression, purification.

J. Vet. Med. Sci. 64(2): 95–100, 2002

Since the gene coding a chicken interferon- γ (ChIFN- γ) was isolated and sequenced by Digby and Lowenthal [2], many reports has been published to produce recombinant ChIFN- γ s [2, 5, 12, 13]. Lowenthal *et al.* [7] demonstrated the poly-His tagged recombinant ChIFN- γ expressed in *Escherichia coli* had anti-viral activity and activation of macrophages. In their expression 2 bands were appeared: one major band seemed to be a full length 18 kDa ChIFN- γ and the other minor band seemed to be a form that has the 12 C-terminal amino acids cleaved [7]. Recently Michalski *et al.* [9] expressed the biologically active recombinant ChIFN- γ constructs bearing an N-terminal poly-His tag in *E. coli*. They demonstrated that a full-length and a truncated protein species (18 kDa and 16 kDa, respectively) had similar biologic activity and that the 16 kDa band had the 12 C-terminal amino acid cleaved. Lillehoj and Choi got the recombinant ChIFN- γ using a baculovirus expression system in cell lysates, but the antiviral activity of their recombinant seemed to be low (2^5 units/ml) [6]. Weining *et al.* expressed ChIFN- γ in *E. coli* with poly-His at N-terminal and they reported that the antiviral activity of the purified product was 500 units/mg [13].

In order to obtain the bioactive recombinant ChIFN- γ in large amount for *in vivo* use, we selected a baculovirus expression system because of its high expression level. Here, we describe cloning ChIFN- γ gene, modification of the gene, expression, and purification of ChIFN- γ using a baculovirus expression system.

MATERIALS AND METHODS

Cloning of ChIFN- γ gene: Spleen cells dispersed from a normal healthy chicken about 2-months old were incubated

in 5×10^6 cells/ml for 6 hr in RPMI-1640 tissue culture medium (Nissui Seiyaku, Tokyo) containing 10% fetal calf serum (FCS) and 10 μ g/ml of concanavalin A (Con A). Then the cells were harvested and total RNA were isolated from 1×10^7 cells using phenol/guanidine thiocyanate solution (ISOGEN; Nippon Gene, Tokyo, Japan), and resuspended in 30 μ l of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Primers ChIFNg-F (5'-gtagatctgagccatcaccaagaaga-3') and ChIFNg-R (5'-ggagatcttaagatgccattagca-3') to amplify the cDNA of the ChIFN- γ gene were designed from the sequence data by Digby and Lowenthal (GenBank U27465). These primers contained *Bgl*III site at both 5 prime ends (underlined). RT-PCR was performed using GeneAmp XL RNA PCR Kit (Perkin Elmer Applied Biosystems Japan, Tokyo, Japan) with the primers and 1 μ l of the total RNA as templates. After RT-PCR, the amplified products were ligated into a plasmid pCR2.1 using TA cloning kit (Invitrogen, San Diego, CA, U.S.A.). The cloned cDNA was sequenced by dideoxy methods using Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer Applied Biosystems Japan).

Construction of transfer vectors: The cloned cDNA in pCR2.1 was digested with *Bgl*II and the fragment containing the cDNA was ligated into a *Bam*HI site of a transfer vector pAcYM1 [8] to place the gene under the control of the polyhedrin promoter. The resulted recombinant plasmid was named pAcYM1ChIFNg. For production of C-terminal truncated ChIFN- γ (ChIFN- γ T) and poly-His tagged ChIFN- γ T (ChIFN- γ THis), PCR were performed on pAcYM1ChIFNg as a template using ChIFNg-F as the upstream primer and ChIFNgTR (5'-gagatctcatttgaaactcggagatcc-3') or ChIFNgTHis (5'-gagatctcaatgatgatgatgatgatttgaaactcggagatcc-3') as the down stream primers,

respectively. These primers also contained *Bgl*II site at both 5 prime ends (underlined). The resulting amplified products were digested with *Bgl*II and ligated to a transfer vector pAcYM1, named pAcYM1ChIFN γ T and pAcYM1ChIFN γ THis, respectively. The sequences were verified by dideoxy chain termination sequence analysis as described above.

Isolation of recombinant baculoviruses: To obtain recombinant viruses that would express ChIFN- γ , ChIFN- γ T, and ChIFN- γ THis, the recombinant transfer vectors pAcYM1ChIFN γ , pAcYM1ChIFN γ T, and pAcYM1ChIFN γ THis were transfected into *Spodoptera frugiperda* (Sf) 21 cells with infectious AcRP23-*lacZ* DNA [3] using Lipofectin (Gibco BRL, Gaithersburg, MD, U.S.A.), respectively. The cultures were harvested, plaque-assayed with neutral red and X-gal, and white plaques were selected as recombinant viruses. The resulting viruses were named AcChIFN γ , AcChIFN γ T, and AcChIFN γ THis, respectively. These recombinant baculoviruses were grown and assayed in Sf 21 insect cells in TC-100 medium (Gibco BRL) containing 10% FCS. On occasion the viruses were grown in Sf+ cells (Protein Sciences Co., Meriden, CT, U.S.A.) in ESF921 medium (Expression Systems LLC, Woodland, CA, U.S.A.) without FCS.

Analyses of ChIFN- γ expression: Sf 21 cells were infected with virus at a multiplicity of 5 plaque forming units (PFU)/cell in 35 mm tissue culture dishes and incubated at 28°C for 5 days. The medium and cells were harvested at 1, 2, 3, 4, and 5 days post-inoculation (dpi). After centrifugation at $15,000 \times g$ for 3 min, the resulting supernatants and the cells were applied to SDS-PAGE to determine the presence of the expressed proteins. Wild type baculovirus, *Autographa californica* nuclear polyhedrosis virus (AcNPV), and mock infected cultures were also included. The gel was stained with Coomassie brilliant blue (CBB) or analyzed by Western blotting using antiserum against ChIFN- γ (as described below).

Purification of ChIFN- γ THis: AcChIFN γ THis was inoculated to Sf+ cells in a spinner flask with serum free ESF921 medium. The infected culture supernatants harvested at 4 dpi were ultra-centrifuged with SW28SA (Beckman, Tokyo, Japan) at $80,000 \times g$ for 1 hr and the resulting supernatants were filtered with Diaflo Ultrafilter XM300 (Amicon, Beverly, MA, U.S.A.) to remove debris. The filtrate was concentrated by filtration with Diaflo Ultrafilter YM10 (Amicon), and then the expressed protein, ChIFN- γ THis, was purified using a nickel chelated column of Xpress System Protein Purification kit (Invitrogen) along the manufactured manual under a native condition. The affinity purified protein was gel filtrated with a PD10 column (Amersham Pharmacia Biotech, Tokyo, Japan) in phosphate buffered saline (PBS) to remove imidazole which was used in elution of the protein from the nickel chelated column. The purity was confirmed by SDS-PAGE and silver staining. The protein concentration was determined by Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IN, U.S.A.).

Preparation of antiserum against expressed protein:

Purified ChIFN- γ THis (100 μ g/ml) was mixed with an equal volume of Freund's complete adjuvant and 3 ml of the mixture were administered to a rabbit. After 3 times injection at 2 weeks interval, the serum was collected and used as an antiserum against ChIFN γ THis.

Chicken embryonic fibroblast (CEF) IFN assay: The antiviral activity in the recombinant baculovirus infected culture supernatants was determined by cytopathic inhibition assay in microtiter plates with primary CEF (10^5 cells/100 μ l/well) as indicator cells. Serial five fold dilutions of the supernatant of the recombinant baculovirus infected cultures were made in triplicate in 96 microplate wells in Eagle's minimum essential medium (Nissui Seiyaku, Tokyo, Japan) containing 0.3% of L-glutamine, and after overnight incubation the cells were challenged with the vesicular stomatitis virus (VSV) at 10^2 50% tissue culture infectious dose (TCID $_{50}$). The IFN titer, arbitrary units per ml (AU/ml), was calculated as reciprocal of the highest dilution in the well in which 100% of the monolayer was protected from the cytopathic effect (CPE) of the virus.

Nitrite assay: Production of nitric oxide by chicken peripheral blood mononuclear cell (PBMC) was quantified by accumulation of nitrite in the culture medium and was used as a measure of IFN- γ activity as described previously [2]. In brief, 100 μ l of the serial five fold diluted purified ChIFN- γ with RPMI1640 medium were added to chicken PBMC (5×10^6 cells/ml/well) in 24 microplate wells in triplicate and incubated for 24 hr. Then the concentration of the nitric oxide in the cultured PBMC supernatants was measured. The nitrite assay was performed with Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A.).

Glycosylation assay: To detect glycosylation of the expressed proteins, DIG Glycan Detection Kit (Roche, Tokyo, Japan) was used according to the manufacture's manual. In brief, the purified ChIFN- γ THis sample was applied to SDS-PAGE and blotted onto nitrocellulose membrane. Then the glycosylated polypeptides were detected using the kit.

RESULTS

Cloning and sequencing of the ChIFN- γ gene: Using RT-PCR, about 500 bp band was amplified and the band was cloned into pCR2.1 vector. Using M13 forward and M13 reverse primers, the sequence of the cloned cDNA was determined. The inserted DNA was 538 bp, including the *Bgl*II sites in the primers. Open reading frame was 492 bp and 164 amino acids were encoded. One nucleotide was different from the published sequence at position 292 (the numbering position is according to GenBank U27465), but the deduced amino acid sequence was identical to the published sequence.

Expression of ChIFN- γ by a recombinant baculovirus: The sequenced cDNA was subcloned into the *Bam*HI site of pAcYM1 and transfected into Sf 21 cells with the infectious AcRP23-*lacZ* DNA. After plaque purification twice, the

Table 1. Anti-viral activity of chicken IFN- γ produced in different recombinant baculoviruses^{a)}

Recombinant virus	IFN titer ^{b)}		
	supernatants ^{c)}	after ultra-centrifugation ^{d)}	after heating ^{e)}
AcChIFNg	3.1×10^4	2.5×10^2	nt ^{f)}
AcChIFNgT	7.8×10^5	1.6×10^5	nt
AcChIFNgTHis	7.8×10^5	1.6×10^5	3.1×10^4

a) CEFs were treated overnight with fivefold serial dilutions of ChIFN- γ samples expressed by different recombinant baculoviruses, and were challenged with VSV at 100 TCID₅₀. b) The IFN titer was calculated as reciprocal of the highest dilution in the well in which 100% of the monolayer was protected from the CPE of the virus. c) Culture supernatants harvested and centrifuged at $800 \times g$ for 15 min at 4 dpi. d) Supernatants after ultra-centrifugation at $80,000 \times g$ for 1 hr. e) Heat at 60°C for 30 min. f) Not tested.

Fig. 1. Time course analysis of the expressed proteins by AcChIFNgTHis. Cell culture supernatants infected with AcChIFNgTHis, AcNPV, or mock were harvested at 0, 1, 2, 3, 4, or 5 days post-inoculation (dpi), separated on SDS-PAGE using 15% gel, and stained with Coomassie brilliant blue (CBB). The positions of molecular marker proteins (kDa) are indicated on the left. Positions of 32 kDa, 16 kDa, and 15 kDa bands are indicated with arrow head.

cloned recombinant virus was named AcChIFNg and further passaged in Sf⁺ cells using spinner flasks to get high titered virus stock.

AcChIFNg was inoculated to Sf⁺ cells and the culture was harvested at 4 dpi. By SDS-PAGE analysis, no specific bands were detected in AcChIFNg infected supernatant (data not shown). After centrifugation at $700 \times g$ for 15 min, the antiviral activity in the resulting supernatant was shown as 3.1×10^4 arbitrary units AU/ml (Table 1). For the further purification, the supernatant was ultra-centrifuged at $80,000 \times g$ for 1 hr. However, the antiviral activity was diminished to 2.5×10^2 (AU)/ml (Table 1). AcNPV or mock infected culture supernatants did not show any antiviral activity (data not shown).

Expression of C-terminal truncated ChIFN- γ . The result-

ing recombinant baculoviruses, AcChIFNgT and AcChIFNgTHis, were inoculated into Sf⁺ cells and the culture supernatants were resolved in SDS-PAGE and analyzed by CBB staining. As shown in Fig. 1 (the data of ChIFN- γ T is not shown), both recombinant viruses expressed specific proteins around 15 kDa, 16 kDa, and 32 kDa, respectively. The antiviral activities were shown as 7.8×10^5 AU/ml and the activity remained in the similar titers (1.6×10^5 AU/ml) even after ultra-centrifugation at $80,000 \times g$ for 1 hr (Table 1). ChIFN- γ is heat labile to compare ChIFN- α [2] and the antiviral activity of ChIFN- γ is a far less than ChIFN- α [13]. To confirm the expressed protein is ChIFN- γ , the supernatant was heated at 60°C for 30 min. After heat treatment, the titer was reduced from 7.8×10^5 AU/ml to 3.1×10^4 AU/ml (Table 1).

Fig. 2. Purified ChIFN- γ THis. After purification with a nickel chelated column, the purified products were separated on SDS-PAGE using 15% gel, then stained with Coomassie brilliant blue (CBB) or blotted onto nitrocellulose membrane and detected by Dig Glycan Detection Kit (Gly). The positions of molecular marker proteins (kDa) are indicated on the left.

Purification and characterization of ChIFN- γ THis: The infected culture supernatants were ultra-centrifuged and concentrated with membrane filters, then ChIFN- γ THis was purified with a nickel chelated column as described in materials and methods. As shown in Fig. 2, 16 kDa and 15 kDa bands were made visible by CBB staining and these bands were detected by anti-polyHistidine mouse monoclonal antibody (SIGMA, St. Louis, MI, U.S.A.) (data not shown). Using glycan detection kit, only the higher band was detected, suggesting that the 16 kDa band was a glycosylated form and the 15 kDa band an unglycosylated form, respectively (Fig. 2). The quantity of the purified ChIFN- γ THis was estimated around 100 μ g/ml using Micro BCA Protein Assay Reagent Kit (PIERCE). The antiviral activity of the purified ChIFN- γ THis was 8×10^5 AU/ml and the efficacy was calculated around 8×10^3 AU/ μ g.

The IFN- γ activity of ChIFN- γ THis for the induction of nitric oxide in chicken PBMC was quantified by accumulation of nitrite in the culture medium. As shown in Fig. 3, around 18 μ M nitrite was induced in PBMC and the titer of purified ChIFN- γ THis was estimated around 1.5×10^5 AU/ml. The minimal concentration of purified ChIFN- γ THis required to activate nitric oxide secretion of PBMC was cal-

Fig. 3. Ability of ChIFN- γ THis to induce nitrite synthesis in PBMC. The serial 5 fold diluted purified ChIFN- γ THis were added to chicken PBMC and incubated for 24 hr. Then the concentration of the nitric oxide in the cultured PBMC supernatants was measured with Nitrate/Nitrite Colorimetric Assay Kit.

culated about 1 ng/ml.

Reactivity of anti-ChIFN- γ THis serum with various ChIFN- γ s: The serum recognized 32 kDa, 16 kDa, and 15 kDa bands in purified ChIFN- γ THis (Fig. 4). The anti-ChIFN- γ THis also recognized the bands around 28 kDa and 14 kDa in Con A stimulated spleen cell culture supernatants and chicken sera (Fig. 4).

When equal volume of 1:100 diluted anti-ChIFN- γ THis serum was mixed with ChIFN- γ , anti-viral activity of ChIFN- γ was reduced around one hundredth (data not shown).

DISCUSSION

Since ChIFN- γ gene was isolated by Digby and Lowenthal [2], many reports for the expression of recombinant ChIFN- γ have been published. Most of them were using *E. coli* [7, 9, 12, 13]. Weining *et al.* [13] reported the antiviral activity of purified His-ChIFN- γ was about 500 units/mg and Michalski *et al.* [9] reported the $2\text{--}5 \times 10^6$ units/mg. There are a few reports using baculovirus expression system for ChIFN- γ [5, 6]. Lillehoj and Choi got the recombinant ChIFN- γ using the baculovirus expression system in cell lysates, but the antiviral activity of their recombinant was very low (2^5 units/ml) [6]. Lambrecht *et al.* got antiviral activity around 10,000 units/ml using the recombinant baculovirus but they did not tell whether the recombinant protein was in soluble fraction after $80,000 \times g$ for 1 hr ultra-centrifugation [5]. In the present study the recombinant baculovirus AcChIFN γ that contained full length ChIFN- γ gene expressed a bioactive product in the insect cell culture supernatants (3.1×10^4 AU/ml). However, the anti-viral activity was diminished after ultra-centrifugation at $80,000 \times g$ for 1 hr (Table 1). For the mammalian IFN- γ s, the baculovirus expression system worked well and many IFN- γ s that have bioactivity have been expressed as soluble forms [1, 10]. Then we suspected that two cysteine residues specific in ChIFN- γ C-terminal region were responsible for aggregation. Michalski *et al.* [9] reported that a truncated

Fig. 4. Western blot analysis of various ChIFN- γ s with anti-ChIFN- γ THis serum. Anti-ChIFN- γ THis serum prepared in the present study was used to detect ChIFN- γ s from various sources (spleen cells cultured with or without ConA, chicken serum, recombinant ChIFN- γ THis). Each source and treatment is indicated on the top. Positions of 32, 28, 16, 15, and 14 kDa bands are indicated with arrow head. The molecular weight markers (kDa) are indicated on the left lane.

recombinant construct (His1-Lys133) that had deleted 12 C-terminal region expressed in *E. coli* had biologic activity comparable to that of the full-length construct. We designed the primer to remove 12 amino acids of ChIFN- γ C-terminal region where two of the cysteine residues located. For the purification we also designed the primer to add His-tag to C-terminal of ChIFN- γ T and made ChIFN- γ THis, because an N-terminal signal sequence is important for secretion of a recombinant protein in a baculovirus expression system. As a result, the C-terminal truncation seemed to accelerate the release of the expressed proteins because the truncated forms were expressed in high amounts and the expressed proteins were detected by CBB staining in the culture supernatant (Fig. 1). These proteins were active even after ultracentrifugation (Table 1). Anti-viral activity in culture supernatants infected with AcChIFNgT or AcChIFNgTHis showed 25 times higher than that of AcChIFNg. These data showed that removal of the C-terminal region of the ChIFN- γ made the protein soluble in a baculovirus expression system and accelerate the release of ChIFN- γ . Addition of His-tag to the truncated C-terminal seemed not to affect the bio-

logical activity of the ChIFN- γ . As shown in Table 1, anti-viral titer of ChIFN- γ T and ChIFN- γ THis were similar $1.6\sim7.8 \times 10^5$ AU/ml.

In AcChIFNgT and AcChIFNgTHis infected culture supernatants, 32 kDa, 16 kDa, and 15 kDa specific bands were detected (Fig. 1). After purification, the 16 kDa and 15 kDa bands were purified in high intensity (Fig. 2). There are two potential N-linked glycosylation sites in ChIFN- γ sequence [2]. Using the glycan detection kit, only the 16 kDa band was detected and it appeared that the 16 kDa and the 15 kDa bands are glycosylated and unglycosylated forms of the ChIFN- γ , respectively (Fig. 2). The 32 kDa band was not detected in this assay. Glycosylation, however, is not necessary for induction of IFN- γ activity as described [11], and ChIFN- γ bearing an N-terminal poly-His tag in *E. coli* also showed biological activities [9]. So we think separation of these 2 bands is not necessary for its use.

The purified product of ChIFN- γ THis was also able to induce nitric oxide in chicken PBMC (Fig. 3). The titer 1.5×10^5 AU/ml was comparable to the results of Lambrecht *et al.* who expressed a full length ChIFN- γ using a baculovirus system [5]. This means that the C-terminal truncation did not diminish the activity for induction of nitric oxide that is specific for IFN- γ .

The antiserum against ChIFN- γ THis detected 32 kDa, 16 kDa, and 15 kDa bands in AcChIFNgT and AcChIFNgTHis culture supernatants, and also detected 28 kDa and 14 kDa bands in Con A stimulated spleen cell culture supernatants and chicken sera (Fig. 4). These bands seem to correspond to homodimer and monomer of ChIFN- γ . This means that the antiserum against ChIFN- γ could be useful for the detection of ChIFN- γ in chicken. The capture ELISA could be developed with this anti-serum and monoclonal antibodies against ChIFN- γ as described [4].

Finally, the bioactive ChIFN- γ THis was highly expressed in baculovirus and purified in the present study. The availability of the bioactive recombinant ChIFN- γ will now enable further characterization of the role of ChIFN- γ in host responses in normal and diseased chickens.

ACNOWLEDGMENT. This work was supported in part by Grant-in-Aid of Recombinant Cytokine's Project provided by the Ministry of Agriculture, Forestry and Fisheries, Japan (RCP-2000-1130).

REFERENCES

- Argyle, D. J., Harris, M., Lawrence, C., McBride, K., Barron, R., McGillivray, C. and Onions, D. E. 1998. Expression of feline recombinant interferon- γ in baculovirus and demonstration of biological activity. *Vet. Immunol. Immunopathol.* **64**: 97-105.
- Digby, M. R. and Lowenthal, J. W. 1995. Cloning and expression of the chicken interferon- γ gene. *J. Interferon and Cytokine Res.* **15**: 939-945.
- Kitts, P., Ayres, M. D. and Possee, R. D. 1990. Linearization of baculovirus DNA enhances the recovery of recombinant

- virus expression vector. *Nucleic Acids Res.* **18**: 5667–5672.
4. Lambrecht, B., Gonze, M., Meulemans, G. and van den Berg, T. P. 2000. Production of antibodies against chicken interferon- γ : demonstration of neutralizing activity and development of a quantitative ELISA. *Vet. Immunol. Immunopathol.* **74**: 137–144.
 5. Lambrecht, B., Gonze, M., Morales, D., Meulemans, G. and van den Berg, T. P. 1999. Comparison of biological activities of natural and recombinant chicken interferon-gamma. *Vet. Immunol. Immunopathol.* **70**: 257–267.
 6. Lillehoj, H. S. and Choi, K. D. 1998. Recombinant chicken interferon-gamma-mediated inhibition of *Eimeria tenella* development *in vitro* and reduction of oocyst production and body weight loss following *Eimeria acervulina* challenge infection. *Avian Dis.* **42**: 307–314.
 7. Lowenthal, J. W., York, J. J., O'Neil, T. E., Rhodes, S., Prowse, S. J., Strom, A. G. and Digby, M. R. 1997. *In vivo* effects of chicken interferon- γ during infection with *Eimeria*. *J. Interferon and Cytokine Res.* **17**: 551–558.
 8. Matsuura, Y., Possee, R. D., Overton, H. A. and Bishop, D. H. L. 1987. Baculovirus expression vectors: the requirements for high level expression of proteins, including glycoproteins. *J. Gen. Virol.* **68**: 1233–1250.
 9. Michalski, W. P., Shiell, B. J., O'Neil, T. E., Beddome, G. and Lowenthal, J. W. 1999. Recombinant chicken IFN- γ expressed in *Escherichia coli*: analysis of C-terminal truncation and effect on biologic activity. *J. Interferon and Cytokine Res.* **19**: 383–392.
 10. Murakami, K., Uchiyama, A., Kokuho, T., Mori, Y., Sentsui, H., Yada, T., Tanigawa, M., Kuwano, A., Nagaya, H., Ishiyama, S., Kaki, H., Yokomizo, Y. and Inumaru, S. 2001. Production of biologically active recombinant bovine interferon-gamma by two different baculovirus gene expression systems using insect cells and silkworm larvae. *Cytokine* **13**: 18–24.
 11. Sareneva, T., Pirhonen, J., Cantell, K., Kalkkinen, N. and Julkunen, I. 1994. Role of N-glycosylation in the synthesis, dimerization and secretion of human interferon- γ . *Biochem. J.* **303**: 831–840.
 12. Song, K. D., Lillehoj, H. S., Choi, K. D., Zarlenga, D. and Han, J. T. 1997. Expression and functional characterization of recombinant chicken interferon-gamma. *Vet. Immunol. Immunopathol.* **58**: 321–333.
 13. Weining, K. C., Schultz, U., Munster, U., Kaspers, B. and Staeheli, P. 1996. Biological properties of recombinant chicken interferon- γ . *Eur. J. Immunol.* **26**: 2440–2447.