ANIMAL BIOTECHNOLOGY
Vol. 15, No. 1, pp. 77–88, 2004

The Interferon-α Genes from Three Chicken Lines and Its Effects on H9N2 Influenza Viruses

C. Xia,*,# J. Liu,# Z. G. Wu, C. Y. Lin, and M. Wang

The 211 Key Laboratory of Veterinary Molecular Biology,
College of Animal Medicine, China Agricultural University, Beijing, China

ABSTRACT

The interferon-α genes from three chicken lines were cloned by a direct PCR technique, and the effects of recombinant protein expressed in a prokaryotic system on highly pathogenic H9N2 influenza viruses were investigated. The cloned ChIFN-α gene encoded a protein of 193 amino acids with a signal sequence of 31 amino acids and mature peptides of 162 amino acids. Comparison of ChIFN-α sequences, detected six amino acids substitutions at positions 50, 58, 65, 81, 181, and 183. Homology analysis indicated that ChIFN-α genes could be subdivided into two lineages, SH-ChIFN-α and WJ-ChIFN-α. In addition, both SH-ChIFN-α and WJ-ChIFN-α were expressed with the N-terminal 6 consecutive histidine residues in a high-level prokaryotic expression system. Recombinant chicken interferon-α (rChIFN-α) protein has anti-VSV activity of more than 1×10^8 U/mg. Moreover, High concentration (10,000 U) of rSH-ChIFN-α resulted in over 40% inhibition of the H9N2 virus infection in chicken embryos (*Ovo*), and 100% inhibition from one day-old to five day-old chickens (*Vivo*). The results suggested that rChIFN-α is a potential agent against many Chicken viral strains.

*Correspondence: C. Xia, The 211 Key Laboratory of Veterinary Molecular Biology, College of Animal Medicine, China Agricultural University, Beijing 100094, China; Fax: +86-10-62893154; E-mail: xiachun@cau.edu.cn.
#C. Xia and J. Liu contributed equally to this work.

DOI: 10.1081/ABIO-120037900 1049-5398 (Print); 1532-2378 (Online)

Key Words: Chicken; Interferon-α; rChIFN-α; H9N2 influenza virus.

INTRODUCTION

Interferon (IFN) plays an important role in treating viral diseases by inhibition of virus replication and stimulation of the immune response (1). In mammals, there are two classes of interferons, type-I and type-II (IFN-γ). For type-I it includes five subtypes, such as α-, β-, δ-, ι-, and ω-IFNs, which have related structures and use a common receptor (2). During the last decade, a variety of interferon genes have been cloned from many species, including human and avian (3,4). Especially, successful cloning of human interferon genes have brought a significant benefit to human health care (5).

Chicken interferon-α (ChIFN-α) gene was first cloned in 1994 by Sekellick et al. Since then, a number of IFN genes from birds have been described. The antiviral activity of ChIFN-α have been studied in Marek's disease (MDV) (6), and Rous sarcoma virus (RSV) infections (7), Newcastle disease virus (NDV) (8), Infectious bursal disease virus (IBDV) (9), and infectious bronchitis virus (IBV) (10). In these studies, recombinant ChIFN-α (rChIFN-α) showed to reduce plaque number formation of attenuated MDV, reduce RSV-induced tumor development in susceptible chickens, ameliorate NDV infection, suppress IBDV plaque formation, and ameliorate IBV infection. However, little information exist about rChIFN-α action on AIV in vitro (11).

To investigate rChIFN-α against avian viruses, in this study, three interferon-α genes from two Chinese chicken lines (SanHuangJi, SH; WuJi, WJ) and one American line (Arbor Acres, AA) were cloned. Based on recent nomenclature for ChIFN-α (12), SH-ChIFN-α and WJ-ChIFN-α were subdivided into two subtypes. The SH-ChIFN-α and WJ-ChIFN-α genes were expressed using pQE30 vector in the *E. coli* M15 (pREP4) system. Finally, the effects of rChIFN-α on H9N2 influenza viruses were evaluated by in *Ovo* and in *Vivo* experiment systems.

MATERIALS AND METHODS

Chickens, Cells, and Viruses

Three chicken lines: SH-, WJ-, and AA-chickens were collected in Peking, China. SH-chicken is a yellow broil chicken double egg-type; WJ-chicken is a black broil-type; AA-chicken is a white broil-type chicken used in the Chinese avian industry. Specific pathogen free (SPF) chicken embryos and 1-day-old SH-chickens were obtained from the Institute of Experimental Animals, Beijing. Chicken embryo fibroblasts (CEF) were prepared from 9-day-old SPF chicken embryos by standard methods. Vesicular Stomatitis Virus (VSV) and H9N2 influenza viruses were a present from Dr. Wu Q. M. (13).

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

Cloning of the ChIFN-α Genes

Genomic DNA was prepared from liver cells of SH-, WJ-, and AA-chickens based on the method described by Sambrook et al. (14). A primer pair corresponding to ChIFN-α1a nucleotide sequences (3) was designed using Oligo 6.0 software. The forward primer was 5′-CCATGGCTGTGCCTGCAAGCC-3′ (54-74nt); The reverse primer was 5′-CTAAGTGCGCGTGTTGCCTGTGAG-3′ (613-636nt). PCR was performed in a Hybaid PCR Machine with a TaKaRa PCR amplification kit (TakaRa Biotechnology (Dalian) Co., Ltd.), and carried out with 1 μg of genomic DNA as template in a total of 50 μL reaction volume containing 50 pmol of each primer and 2.5 U TaKaRa ExTaq polymerase. Reactions were set at 98°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and finally, 72°C for 10 min. PCR products were inserted into pGEM T-Easy Vector (Promega) and the construct was named after pGEM-T/ChIFN-α. Positive clones were screened and the recombinant plasmids were extracted with QIAprep Spin Miniprep kit (QIAGEN) for sequencing. The nucleotide sequence was determined by ABI 377 DNA sequencer using T7 and SP6 sequence primers. Comparison of these ChIFN-α amino acid sequences was performed using GENETYX 9.0 software (Software Development Co., Ltd, Tokyo, Japan).

Expression of ChIFN-α Genes

We used the pQE30 plasmid to construct the expression vector. PCR was performed to generate ChIFN-α genes lacking the signal sequence fragment using pGEM-T/ChIFN-α as a template. The forward primer (5′-TGCCATGCGCCTGC-AACCACCTTCGCG-3′) contains a *Sph*I restriction enzyme site and the reverse primer (5′-TCAAGCTTAGTGCGCGTGTTGCCTGTAAG-3′) a *Hind*III site. The PCR product was digested with *Sph*I and *Hind*III, and then inserted into pQE30 at the *Sph*I and *Hind*III sites. Recombinant expression vectors pQE30/ChIFN-α were sequenced, and transformed into *E. coli* strain M15(pREP4) (QIAGEN), and expressed according to standard procedures (15).

SDS-PAGE, Western Blot, and Purification

A single colony of recombinant M15 (pREP4 plus pQE30/ChIFN-α) was selected and inoculated to 5 mL of LB medium containing 25 μg/mL of kanamycin and 100 μg/mL of ampicillin. Then cultured at 37°C with vigorous shaking until OD_{600} 0.5, isopropylthiogalactose (IPTG) was added to a final concentration of 1 mM to induce expression. After incubation for an additional 5 h, the M15 was harvested by centrifugation. A part of the pellets were resuspended in SDS-PAGE loading buffer, heated at 95°C for 5 min, and loaded on a 15% SDS-PAGE gel. After electrophoresis, the gel was stained with Coomassie Blue-R-25. The density of the expressed protein band was quantitated by thinlayer scanner (Alpha Imager 2000 Documentation and Analysis System). Nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography was used for purification of 6 × His-rChIFN-α according

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

to the manufacture′s instruction (15). Briefly, the M15 was inoculated in a 250 mL LB culture at 1:50 dilution. After overnight culture, IPTG was added to induce expression as described above. The M15 was harvested by centrifugation, resuspended in PBS buffer, and sonicated six times by 10 s with 10 s pauses. The lysate was centrifuged at $10{,}000 \times g$ at 4°C for 10 min. The inclusion bodies were dissolved in 20 mM Tris-HCl buffer with 8M urea, and purified under denaturing condition. Finally, the recombinant protein was refolded using no urea dirasys buffer (20 mM Tris-HCl), containing a redox pair of reduced glutathione (GSH) and oxidized glutathione (GSSG) and phenylmethyl sulfonylfluoride (PMSF). After SDS-PAGE, the M15-expressing proteins was transferred onto a Nitrocellulose membrane (Boeheringer Mannheim). The recombinant protein with $6 \times$ His tag was detected by QIAexpress Detection Kit according to the manual using BIO-RAD Mini-protein II Cell electrophoresis machine system (BioRed).

Bioassay

The antiviral activity of rChIFN-α was measured by the cytopathic-effect inhibition assay (16). In brief, CEF was seeded in 96-well microplate at a density of 4×10^4 cells per well and cultured for 24 h. Monolayers of CEFs were stimulated with 100 μL of twofold serial dilutions of the various rChIFN-α preparations. After 24 h of culture, the cells were challenged with 100 TCID50 of VSV per well. The rChIFN-α titers were expressed as reciprocals of the dilutions that resulted in 50% virus-induced cell lyses determined 32–48 h after VSV infection.

Effects on H9N2 Viruses Infection

Two experiments were performed to evaluate the effects of rChIFN-α against H9N2 viruses infection in *Ovo* and in *Vivo*.

Experiment 1: Eighty 9-days-old SPF chicken embryos were randomly divided into eight groups as Table 2. Six groups (1–5 and 8) were injected with 0.1 mL solution containing 100 U, 500 U, 5000 U, 10,000 U, and 100,000 U of the purified rChIFN-α in the allantoic liquid, respectively. Two control groups (6 and 7) were injected with 0.1 mL PBS or without injection. After 24 h, chicken embryos were challenged with a 10^5 ELD_{50}/0.1 mL H9N2 HP isolate (A/Beijing/1/96/H9N2) by allantoic injection per embryo. At 24, 48, and 72 h postinfection, the clinical signals, pathological change, and the rate of dead were recorded.

Experiment 2: Just one day-old chickens were grouped as Table 3. First, 1–5 and 8 groups were inoculated in nose/eyes (per 0.05 mL each) with 0.1 mL solution containing 100 U, 500 U, 5000 U, 10,000 U, and 100,000 U rChIFN-α, respectively. Control groups were inoculated with PBS or without. After 24 h, they were challenged with 10^5 ELD_{50}/0.1 mL A/Beijing/1/96/H9N2 by eye-drop plus nasal-drop (per 0.05 mL each). The clinical signals, pathological change, and the rate of dead were recorded until six days of age.

Student's *t*-test was used to evaluate the effects of rChIFN-α on H9N2 viruses.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

RESULTS

Molecular Characteristics of the ChIFN-α Genes

As shown in Fig. 1, the PCR products SH-, WJ-, and AA-ChIFN-α genes were 582 bp long covering an ORF in region 1–582 coding for 193 amino acids. The first 31 amino acid residues constitute the signal peptide. Six sets of cysteines that are likely to form intrachain disulfide bridges within the 32, 61, 68, 129, 155, and 168 sites. The deduced ChIFN-α amino acid sequences displayed four potential *N*-glycosylation sites at amino acid positions 65–67, 71–74, 108–110, and 186–188. The 193 amino acids with a molecular mass are 22.04, 22.20, and 22.11-kDa for three respectively. Both SH- and WJ-ChIFN-α were designated as SH-ChIFN-α and WJ-ChIFN-α and submitted to DDBJ/EMBL/GenBank (AB021153 and AB021154). AA-ChIFN-α is identified with the reported Shiqi-ChIFN-α.

Multiple alignment of the SH-, WJ-, and AA-ChIFN-α amino acid sequences with other IFN-α amino acid sequences from DDBJ/EMBL/GenBank isolated from birds are shown in Fig. 1. Comparison of SH-ChIFN-α, WJ-ChIFN-α, and AA-ChIFN-α with other ChIFN-α showed that SH-ChIFN-α is 98.4% homologous with ChIFN-α1 and ChIFN-α2, is 97.9% homologous with ChIFN-α3 (16), and 99.0% homologous with Shqi-ChIFN-α (9), and ChIFN-α (3); WJ-ChIFN-α is 97.4% homologous with ChIFN-α1 and ChIFN-α2, 99.0% homologous with ChIFN-α3 (16), 97.9% homologous with Shqi-ChIFN-α (9), and ChIFN-α 1994 (3). Among SH-ChIFN-α, WJ-ChIFN-α, turkey interferon-α (TkIFN-α) (17), and duck interferon-α (DuIFN-α) (18), the homology is 50–82%. These results indicated that Avian's interferon could belong to a super family.

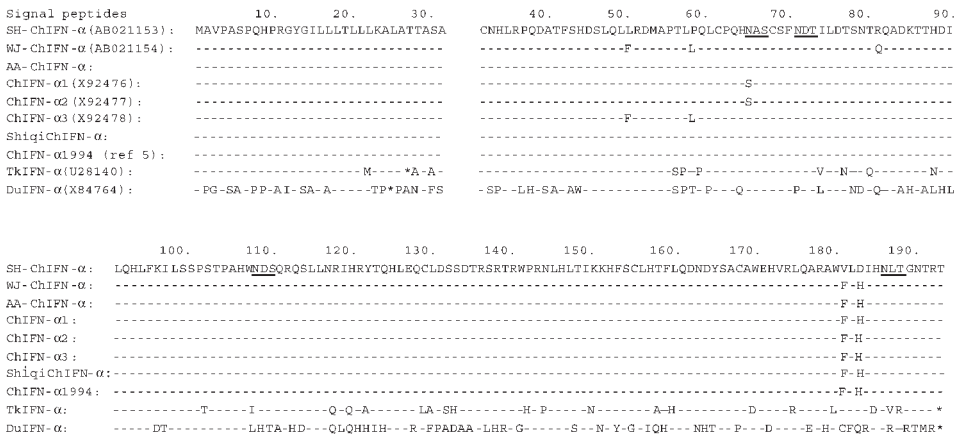

Figure 1. Comparison of amino acid sequences of Avian interferon-α. "–", identity with SH-ChIFN-α. SH-ChIFN-α and WJ-ChIFN-α nucleotide sequence were placed in DDBL/EMBL/GeneBank, accession numbers are AB021153 and AB021154. "*", gaps introduced for alignment purposes. Sequence references are as follows: ChIFN-α1 (X92476), ChIFN-α2 (X92477), and ChIFN-α3 (X92478); Shqi-ChIFN-α (9); ChIFN-α 1994 (3); TkIFN-α (U28140); DuIFN-α (X84764); underline indicate the potential *N*-glycosylation sites.

Table 1. Most variations in the avian interferon proteins.

Species	Protein symbol	AA substitutions in variants	Accession no.	Reference
Chicken	SH-ChIFN-α	L50, D58, N65, R81, V181, D183	AB021153	This article
	WJ-ChIFN-α	F50, L58, Q81, F181, D183	AB021154	This article
	AA-ChIFN-α	F181, H183	—	This article
	ChIFN-α1a	F181, H183	U07868	
	ChIFN-α1b	S65, F181, H183	X92476	(16)
	ChIFN-α1b	S65, F181, H183	X92477	(16)
	ChIFN-α1c	F50, L58, F181, H183	X92478	(16)
Turkey	TuIFN-α	Variations	U28140	(17)
Duck	DuIFN-α	Variations	X84764	(18)

Amino acid sequence variations in all ChIFN-α are shown in Table 1. Six sites of amino acids substitutions were found in ChIFN-α. Three substitutions appeared between SH-ChIFN-α and ChIFN-α1b at positions 65 (S to N), 181 (V to F), and 183 (D to H). Two amino acid substitutions appeared between ChIFN-α1d and ChIFN-α1a at position 181 (V to F) and 183 (D to H). There are five amino acids substitutions between WJ-ChIFN-α and SH-ChIFN-α at positions 50 (L to F), 58 (P to L), 81 (R to Q), 181 (F to V), and 183 (D to H). The amino acid sequences have divaricated positions with ChIFN-α1d only at residue 181 and 183. Interestingly, the molecular characteristics displayed by SH-ChIFN-α (AB021153) and WJ-ChIFN-α (AB021154) could be subdivided into two subtypes of chicken Interferon alpha based on recent nomenclature of avian interferon proteins (12).

SDS-PAGE, Western Blotting and Purification

As shown in Fig. 2, the 6 × His-tagged recombinant SH-ChIFN-α and WJ-ChIFN-α protein were expressed in M15(pREP4) strain using the QIAexpress IV system. The pGEM-T/ChIFN-α was digested with *Sph*I and *Hind*III, and inserted to pQE30 at *Sph*I and *Hind*III sites. After the pQE30/SH- and WJ-ChIFN-α were transformed into M15(pREP4), the M15-expressing system (pREP4, pQE30/SH-, or WJ-ChIFN-α) was constructed. The results of SDS-PAGE and Western blot are shown in Fig. 3. A main band of gel analysis revealed the expressed protein about 21-kDa. Quantitation of the 21-kDa band with thin-layer scanning indicated that the expressed 6 × His-rChIFN-α represented 22.0% of total protein mass of the M15 (Fig. 3A). A purified band appeared with an apparent MW of 21-kDa, corresponding to the monomeric form at position about 21-kDa (Fig. 3B). Western blot with mouse monoclonal antibody against 6 × His confirmed the 21-kDa band to be the recombinant ChIFN-α protein (rSH-ChIFN-α or rWJ- ChIFN-α) (Fig. 3C).

Bioassay

The anti-virus titer from five lots of rSH-ChIFN-α and rWJ-ChIFN-α were detected by CPE_{50} method. The results showed that both rSH-ChIFN-α and

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

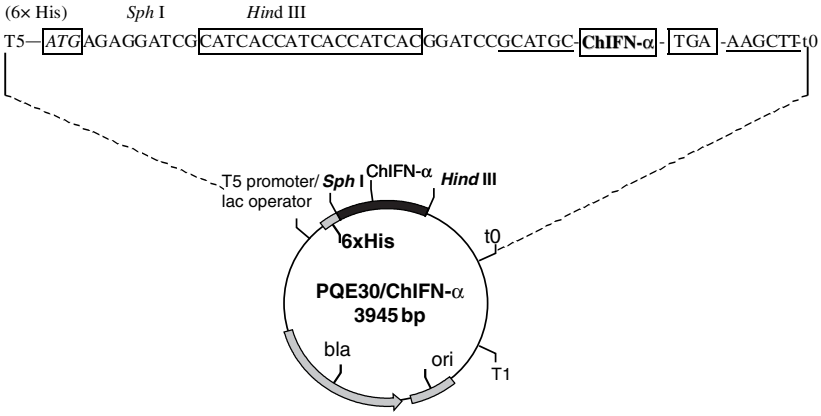

Figure 2. Sequences of the recombinant expression vectors pQE30/SH- or WJ-ChIFN-α gene.

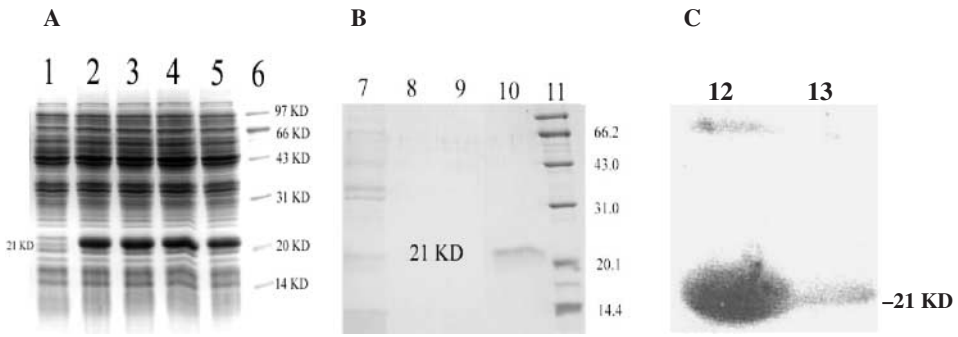

Figure 3. Analysis of recombinant ChIFN-α with SDS-PAGE and Western blot; A: Lane 1, the total cell lysis of M15 strain before IPTG induction; Lane 2–5, the total cell lysis of three M15 strains after IPTG induction; Lane 6, the molecular weight marker; B: Lane 7, the first flow through from the Ni-affinity column; Lane 8, the second flow through from the Ni-affinity column; Lane 9, the third flow through from the Ni-affinity column; Lane 10, the recombinant ChIFN eluted from Ni-affinity column; Lane 11, the molecular weight marker; C: Lane 12, Western blot analyzed the total cell lysis of M15-expressing recombinant ChIFN-α after IPTG induction. Lane 13, Western blot analyzed the purified recombinant ChIFN-α by the Ni-NTA metal-affinity chromatography column.

rWJ-ChIFN-α have antiviral activity of more than 1×10^8 U/mg $\sim 2.2 \times 10^8$ U/mg (Tables 2 and 3).

Effects on H9N2 Viruses

Results of experiment 1 are shown in Table 2. In *Ovo*, the effect of rSH-ChIFN-α against H9N2 viruses was remarkable. After challenge, 2–5 groups injected

with various concentrations of rChIFN-α showed 30–90% mortality up to 72 h postinfection. However, the PBS control group (group 6) showed a classical AIV pathogenic changes (Fig. 4), and induced 100% mortality at 60 h postinfection. The results indicated that the treatment with high concentration of rSH-ChIFN-α could be partially (40–70%) protecting against the embryo mortality. Especially, for groups 4 and 5 which were treated with 10,000 U and 100,000 U of rSH-ChIFN-α, the H9N2 virus clinical signs of infection were inhibited for 3 days postinfection. In contrast, the PBS control showed a higher pathogenic change, especially hyperaemia (haemorrhage) from head to foot, and 100% mortality (Fig. 4). Groups 4, 5 groups, and the control group (group 6), showed insignificant difference in mortality rate.

The effects of rSH-ChIFN-α on H9N2 virus infection are shown in Table 3. After challenge, 1–5 groups injected with various concentration of rChIFN-α showed 0–10% mortality by 72 h postinfection. In contrast, the PBS control (group 6) showed 30% mortality by 72 h, and 50% mortality by 120 h postinfection.

Table 2. The effect of rChIFN-α on H9N2 viruses infection in *Ovo*.

Group	Number	Treatment with rSH-ChIFN-α[a]	Challenged	In *Ovo* death		
				48 h	72 h	Rate
1	10	100 U	+	10	0	10/10 (100%)
2	10	500 U	+	7	1	8/10 (80%)
3	10	5,000 U	+	7	2	9/10 (90%)
4	10	10,000 U	+	0	6	6/10 (60%)
5	10	100,000 U	+	0	4	4/10 (40%)
6	10	PBS	+	10	0	10/10 (100%)
7	10	—	−	0	0	0/10 (100%)
8	8	100,000 U	−	0	0	0/8 (0%)

[a]The original concentration of rSH-ChIFN-α is 2.2×10^8 U/mg.

Table 3. The effects of rSH-ChIFN-α on H9N2 viruses infection in *Vivo*.

Groups	Number	Treatment with rSH-ChIFN-α[a]	Challenged	In *Vivo* death			
				48 h	72 h	120 h	Rate
1	10	100 U	+	0	1	0	1/10 (10%)
2	10	500 U	+	0	0	0	0/10 (0%)
3	10	5,000 U	+	0	0	0	0/10 (0%)
4	10	10,000 U	+	0	0	0	0/10 (0%)
5	10	100,000 U	+	0	0	0	0/10 (0%)
6	10	PBS	+	1	3	1	5/10 (50%)
7	10	—	−	0	0	0	0/10 (0%)
8	8	100,000 U	−	0	0	0	0/8 (0%)

[a]The original concentration of rSH-ChIFN-α is 1.6×10^8 U/mg.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

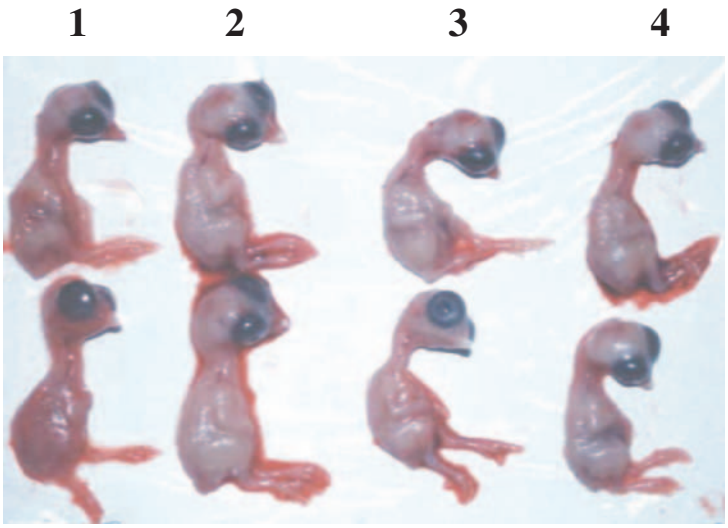

Figure 4. The protected effect against challenge with A/Beijing/1/96/H9N2 before treatment by pur-rChIFN-α in *Ovo*. 1: Treatment without rChIFN-α, 2: Treatment with 5000 U rChIFN-α, 3: Treatment with 10,000 U rChIFN-α, 4: Treatment with 100,000 U rChIFN-α. *(View this art in color at www.dekker.com.)*

Although no death observed in 2-, 3-, 4-, and 5-groups, clinical signs were observed from 24 to 72 h postinfection.

DISCUSSION

Poultry industry in China includes the chicken and duck, with populations of about 50–70 billion per year. Several diseases are responsible for consistent serious losses to the chicken industry. Viral diseases caused 8–12% losses in chicken populations in Mainland China. To control these diseases, it is necessary to evaluate the effects of recombinant interferon-α/γ against viral pathogens. In this study, we cloned two subtypes ChIFN-α genes from SH- and WJ-chickens lines. In humans, 13 functional IFN-α genes are clustered on the short arm of chromosome 9, but only eight are known to have allelic variant forms. The antiviral activity of recombinant IFN-α2b was found to be 100-fold higher than other recombinant IFN-α alleles (5,19). In contrast, 10 IFN-α genes were estimated to exist in the chicken genome by Southern blot analysis, but just three ChIFN-α genes were isolated from a genome phage library (16). On the other hand, no information of ChIFN-α allelic forms or loci has been reported.

Most of amino acid residue substitutions are observed between SH- or WJ-ChIFN-α and other reported ChIFN-α (Table 1). For instance, five amino acid residue substitutions appeared between the SH-ChIFN-α and Shiqi-ChIFN-α (9) at positions 50 (L to F), 58 (P to F), 65 (S to N), 81 (R to Q), and 133 (S to R).

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016

Furthermore, two substitutions at positions 181 (L to F), 183 (P to F) are shown among SH-ChIFN-α and ChIFN-αs. The results suggested that the variation of factional ChIFN-α amino acid exists among the chicken lines. Based on recent nomenclature of avian interferon proteins, SH-ChIFN-α and WJ-ChIFN-α could be subdivided into two subtypes (might be designated as ChIFN-α1d and ChIFN-α1e).

For expression of SH- and WJ-ChIFN-α, the genes lacking the signal peptide were inserted into pQE30 vector. The rSH- and rWJ-ChIFN-α with $6 \times$ His tagged were detected by Western blot method. The purified rSH-ChIFN-α has an anti-VSV activity of more than 1.6×10^8 U/mg (Tables 2 and 3). In the start, we assumed that like human INF-α, the antiviral activity of rChIFN-α products might vary in different chicken lines or geographic regions. However, the bioactivity was similar, although we first used the Ni-NTA metal-affinity chromatography to purified rChIFN-α.

The effects of rChIFN-α against avian viral pathogens of MDV, RSV, NDV, IBDV, and IBV have already been reported (6–10). Our results showed that rChIFN-α has strong action on the viruses. Moreover, as an immunologic adjuvant, rChIFN-α could enhance the protective immune responses to IBDV (20). Our results proved that rChIFN-α could protect from H9N2 virus infection in *Ovo* and in *Vivo*. AI is an important disease affecting the avian industry, because highly pathogenic (HP) AIV have caused severe disease with high mortality (13,21). In contract of HP AIV, mildly pathogenic (MP) AIV (a part of H9N2) produce also an outbreak in developing countries, and no vaccine control was available recently. In this study, the H9N2 was isolated in Beijing from a chicken experiencing AI disease. The 10^5 mean embryo lethal dose ELD_{50} was 100% lethal for 9-day old SPF chicken embryos in 72 h postinfection (13). Sekellick et al. (11) have reported ChIFN-α action survival curves for AIV showing that 40–60% of virus were highly sensitive to the inhibitory effects, whereas the rest were up to 100 times less sensitive. Moreover, they suggested that IFN-resistant AIV is generated from a random packaging event that results in virions that contain two or more copies of RNA segment 8, the gene segment that encodes the NS1 protein of AIV. Our data (Tables 2 and 3) address two important issues. First, in *Ovo*, the protection rate rose by high concentrations rSH-ChIFN-α. Second, in *Vivo*, giving rSH-ChIFN-α over 1000 U/per day chicken could be protected from the H9N2 virus challenge.

In conclusion, we cloned and expressed two subtypes of the ChIFN-α genes from Chinese chicken lines, and it's effects on H9N2 influenza viruses were demonstrated in *Ovo* and in *Vivo*.

ACKNOWLEDGMENTS

This work was supported by the grants from the State "863" High Technology R&D Project of China (No. 2001AA249032) and National Laboratory of Agricultural Biotechnology of China (No. 991004-3).

REFERENCES

1. Nnoak R, Ester K, Savic V, Sellilick MJ, Marcus PI, Lowenthal JW, Vainio O, Ragland WL. Immune status assessment by abundance of IFN-alpha and IFN-gamma mRNA in chicken blood. Journal of Interferon & Cytokine Research 2001; 21:643–651.
2. Muller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkerhagel RM, Aguet M. Functional role of type I and type II interferons in antiviral defense. Science 1994; 262:1918–1921.
3. Sekellick MJ, Ferrandino AF, Hopkins DA, Marcus PI. Chicken interferon gene: cloning, expression, and analysis. Journal of Interferon & Cytokine Research 1994; 14:71–79.
4. Schutz U, Rinderle C, Sekellick MJ, Arcus PIM, Staeheli P. Recombinant chicken interferon from *Escherichia coli* and transfected COS cells is biologically active. Eur J Biochem 1995; 229:73–76.
5. Hussain M, Tan T, Ni D, Gill DS, Liao MJ. A new allele of interferon-α17 gene encoding interferon-α17b is the major variant human population. J Interferon Cytokine Res 1998; 18:469–477.
6. Levy AM, Heller ED, Leitner G, Davidson I. Effect of native chicken interferon on MDV replication. Acta Virology 1999; 43:121–127.
7. Plachy J, Weining KC, Kremmer E, Puehler F, Hala K, Kaspers B, Staeheli P. Protective effects of type-I and type-II interferons toward Rous sarcoma virus-induced tumors in chickens. Virology 1999; 56:85–91.
8. Marcus PI, Heide Van D, Selellick MJ. Interferon action on Avian viruses. I. Oral administration of chicken interferon-alpha ameliorates Newcastle disease. J Interferon Cytokine Res 1999; 19:881–885.
9. Mo CW, Cao YC, Lim BL. The in vivo and in vitro effects of chicken interferon α on infectious bursal disease virus and Newcastle disease virus infection. Avian Diseases 2001; 45:389–399.
10. Pei J, Sekellick MJ, Marcus PI, Choi IS, Collisson EW. Chicken interferon type I inhibits infectious bronchitis virus replication and associated respiratory illness. J Interferon Cytokine Res 2001; 21:1071–1077.
11. Sekellick MJ, Carra SA, Bowman A, Hopkins DA, Marcus PI. Transient resistance of influenza virus to interferon action attributed to random multiple packaging and activity of NS genes. J Interferon Cytokine Res 2000; 20:963–970.
12. Lowenthal JW, Staeheli P, Schultz U, Sekellick MJ, Marcus PI. Nomenclature of Avian interferon proteins. J Interferon Cytokine Res 2001; 21:547–549.
13. Liu JH, Okazaki K, Shi WM, Wu QM, Mweene AS, Kida H. Phylogenetic analysis of neuraminidase gene of H9N2 influenza viruses prevalent in chickens in China during 1995–2002. Virus Genes 2003; 279(2):197–2002.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual. 2nd ed. New York: Cold Spring Harbor Laboratory Press, 1989.
15. Qiagen. The QIAexpressionist: A Handbook for High-Level Expression And Purification Of 6 × His-Tagged Proteins. California, USA: Qiagen Inc., 2002.
16. Stick C, Schultz U, Staeheli P. A family of genes coding for two serologically distinct chicken interferons. J Biol Chem 1996; 271:7635–7639.

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

17. Surech M, Karaca K, Foster D, Sharma JM. Molecular and functional characterization of Turkey interferon. J Virol 1995; 69:8159–8163.
18. Schultz U, Kock J, Schlichi HJ, Staeheli P. Recombinant duck interferon: a new reagent for studying the mode of interferon action against hepatitis B virus. Virology 1995; 212:641–649.
19. Dlaz MO, Pomykala HM, Bohlander SK, Maltepe E, Malik K, Bpownstein B, Olopade OI. Structure of the human type-I interferon gene cluster determined from a YAC clone condign. Genomics 1994; 22:540–552.
20. Schijns VCJ, Weining KC, Nuljten P, Rijke EO, Staeheli P. Immunoadjuvant activities of *E. coli*- and plasmid-expressed recombinant chicken IFN-α/β, IFN-γ and IL-1β in 1-day- and 3-week-old chickens. Vaccine 2000; 18:2147–2154.
21. Swayne DE, Beck JR, Kinney N. Failure of a recombinant fowl poxvirus vaccine containing an Avian influenza hemagglutinin gene to provide consistent protection against influenza in chickens preimmunized with a fowl pox. Vaccine 2000; 44:132–137.

Marcel Dekker, Inc.
270 Madison Avenue, New York, New York 10016