특강 III

MOLECULAR CLONING OF CHICKEN INTERFERON-GAMMA

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닭 인터페론 유전자의 클로닝에 관한 연구

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ABSTARCT

A cDNA encoding chicken interferon-gamma (chIFN-γ) was amplified from P34, a $CD4^+$ T-cell hybridoma by reverse transcription-polymerase chain reaction (RT-PCR) and cloned into pUC18. The sequences of cloned PCR products were determined to confirm the correct cloning. Using this cDNA as probe, chicken genomic library from White Leghorn spleen was screened. Phage clones harboring chicken interferon-gamma (chIFN-γ) were isolated and their genomic structure elucidated. The chIFN-γ contains 4 exons and 3 introns spanning over 14 kb, and follows the GT/AG rule for correct splicing at the exon/intron boundaries. The four exons encode 41, 26, 57, and 40 amino acids, respectively, suggesting that the overall structure of IFN-γ is evolutionarily conserved in mammalian and avian species. The 5′- untranslated region and signal sequences are

may indicate a role in mRNA turnover. The 5′-flanking region contains sequences homologous to the potential binding sites for the mammalian transcription factors, activator protein-1(AP-1), activator protein-2 (AP-2), cAMP-response element binding protein(CREB), activating transcription factor(ATF), GATA-binding factor(GATA), upstream stimulating factor(USF). This suggests that the mechanisms underlying transcriptional regulation of chicken and mammalian IFN-γ genes may be similar.

(**Key words** I; chicken, interferon-gamma, cloning, genomic structure)

I. INTRODUCTION

Interferon-gamma (IFN-γ), or type II IFN, is secreted by subsets of lymphocytes following mitogenic or antigenic stimulation (Pestka et al., 1987). Like interleukin-2 (Farrar et al., 1986) and interleukin-12 (Chan et al., 1991), it plays an important role in both humoral and cellular immunity. IFN-γ stimulates the proliferation of B-cells, enhances the natural immunity against various pathogens, activates the macrophages to produce nitric oxides (Ding et al., 1988), and upregulates the expression of major histocompatibility complex class II antigens (Basham and Merigan, 1983; Song et al., 1997).

Although interferon activity was initially demonstrated in chickens (Isaacs and Lindenmann, 1957), functional and structural characterizations of chicken IFNs has only recently been made possible following gene cloning techniques (Sekellick et al., 1994; Digby and Lowenthal, 1995). Recombinant chicken type I interferon was shown to inhibit virus replication and induces *Mx* gene expression but lacks macrophage activating activity (Schultz et al., 1995a). Chicken recombinant IFN-γ, type II interferon, has macrophage activating activity (Digby and Lowenthal,1995; Weining et al,1996) and induces major histocompatibility complex class I and II gene expression (Weining et al, 1996; Song et al., 1997).

Type I interferons of chicken, turkey and duck are encoded by an 35

intronless family of genes as in mammals (Sick et al, 1996; Surest et al., 1995; Schultz et al, 1995b). In contrast, type II interferons in mammals possess an exon/intron structure (Gray and Goeddel, 1982; Gray and Goeddel, 1983; Dijkema et al., 1985; Dijkmans et al., 1990). Although little is known about the genomic structure of chicken IFN-γ, we recently isolated a cDNA encoding chIFN-γ from chicken $CD4^+$ T cell line (Song et al.,1997). This was used in the present study to isolate the corresponding genomic clone. Sequence analysis of this chIFN-γ gene revealed its exon/intron boundaries and putative transcription factor binding sites in the 5'-untranslated region.

II. MATRERIALS AND METHODS

1. Development of T-cell hybridoma secreting interferon-gamma

Peripheral blood lymphocytes(PBL) were obtained from 6-8 week old SC chickens which has been orally infected with 104 *Eimeria acervulina* oocysts. About 5 ml of PBL suspension was mixed with an equal volume of Hank's balanced salt solution(HBSS) and overlaid onto Histopaque 1077 density gradient medium(Sigma Chemical, St Louis, MO) and centrifuged at 1800 rpm for 20 min at room temperature (20-22℃). Lymphocytes at interface were washed three times in HBSS and fused with thymidine kinase-deficient R24H4 lymphoma cells were resuspended in Iscoves's modified Dulbecco's medium(IMDM), supplemented with 10% fetal calf serum(FCS) and hypo xanthine- aminopterin-thymidine(HAT; Sigma) and plated in U-bottom 96-well microculture plates. After 10-14 days, wells showing cell growth were transferred to 24-well plates and expanded in IMDM supplemented with 10% FCS(IMDM-10) containing hypoxanthine-aminopterin-thymidine. When hibridomas
were confluent, supernatants were tested for IFN production using in vitro method as described(Kaspers et al., 1994). Half of the cells from positive cells were stained with a monoclonal antibody detecting the CD4 antigen and analyzed using EPICS profile II flow cytometer(Coulter Cooperation, Hialeah, FL) (Lillehoj et al., 1988). For each hybridoma, 10^4

viable cells were analyzed. $CD4^+$ T cell hybridomas were cloned by limiting dilution using irradiated spleen feeder cells($2X10^6$ per well) as described(Lillehoj et al., 1988). P34, a $CD4^+$ hybridoma secreting high level of IFN-γ spontaneously was grown and used in this study.

2. Reverse transcription-polymerase chain reaction and cloning of chIFN-γ

The IFN-γ gene was amplified from cDNA from P34 $CD4^+$ T-cell hybridoma or Con-A activated PBLs, using primers derived from the published sequences(Digby and Lowenthal, 1995). The sequences of the primers were 5'-GGATTCAGAAGATGACTTGCCAGACTTACAA-3', and 5'-TTTCTAGA-TTAG CAA TTGCATCTTC-3'(Bioserve Biotech., Laurel, MD). The cDNA was made with the Superscript™ preamplication system(GIBCO BRL, Gaithersburg, MD). mRNA from P34 or Con-A activated lymphocytes from peripheral blood or spleen was mixed with 50ng of random primer and incubated at 70℃ for 10 min followed by 1 min on ice. After adding a reaction mixture consisting of 2$\mu\ell$ of 10X synthesis buffer (200mM Tris-HCl pH8.4, 500mM KCl, 25mM $MgCl_2$, 1μg BSA), 1$\mu\ell$ of 10mM each deoxynucleotside triphosphate and 2$\mu\ell$ of 0.1M DTT were added to the RNA-primer mixture, and the superscript II RT™ was added and incubated at room temperature for 10 min, followed by an additional 50 min incubation. The reaction was terminated by heating at 70℃ for 15 min and chilling on ice. RNase H was added to each tube and the reaction mix was incubated at 37℃ for 20 min. PCR was performed in a thermal cycler(Perkin Elmer Cetus, Bachburg, NJ) for 5 min at 95℃ denaturation step followed by 30 cycles at 55℃ for 1 min and at 72℃ for 2 min. A final elongation step was performed at 72℃ for 10 min. The PCR products were digested with *Eco RI* and *Xba* I (GIBCO BRL, Gaithersburg, MD), agarose gel purified and ligated to *Eco RI* and *Xba* I digested pUC18 plasmid. Recombinant plasmid DNA was used to transform DH5α by electroporation using Gene Pulser II system (Bio-Rad Lab., Hercules, CA) (Sambrook et al. 1989).

3. Genomic library screening

Genomic clones of chIFN-γ were isolated by hybridization with a previously described cDNA clone, pUCchIFN-γ (Song et al., 1997). A genomic library constructed from spleen cells of White Leghorn chickens was obtained from Stratagene(La Jolla, CA) and plated at a density of 10,000 plaques per 150-mm petridish with *E.coli* strain *XL-1 Blue MRA*. After incubation for 8 hr, nitrocellulose filters(Amersham, Arlington Heights, IL) were overlaid on each plate, and the filters denatured in 1.5M NaCl, 0.5N NaOH, and neutralized with 1M Tris-Cl pH 7.4, 1.5M NaCl. The filters were baked at 80℃ for 2 hr and prehybridized in 50% deionized formamide, 5X Denhardt's solution, 6X SSC, 0.5% SDS, 100μg /ml denatured calf thymus DNA. The *EcoR*I and *Xba* I insert of pUCchIFN-γ was labeled with [α-^{32}P] dATP by a random labeling kit according to the supplier's instruction(Boehringer Mannheim, Germany) and hybridized to the nitrocellulose filters in 50% deionized formamide, 5X Denhardt's solution, 6X SSC, 0.5% SDS, calf thymus DNA (100 μg/ml) for 18 hr at 42℃. The filters were washed in 2X SSC, 0.2% SDS at room temperature for 5 min, 1X SSC, 0.1% SDS at 68℃ for 1 hr, and 0.2X SSC, 0.2% SDS at 68℃ for 1 hr, and exposed to BioMax MR1 X-ray film(Kodak, Rochester, NY). Ten positive plaques were initially isolated. These were replated and screened until a single homogenous plaque(chIFN-3) was obtained.

4. Subcloning and DNA sequence analysis

Phage DNA was prepared according to the protocol of Sambrook et al. (1989). The DNA was digested with restriction enzymes, transferred to nylon membrane(Amersham) and hybridized with the [α-^{32}P] dATP labeled chIFN-cDNA probe as described above. Positive bands were subcloned into pUC19, the recombinant plasmid used to transform the *E.coli* DH5α and plasmid DNA was prepared from 25-50 ml liquid culture using Qiagen (Hilden, Germany) columns according to the supplier's instruction. Sequence analysis was performed with Sequenase 2.0(USB, Cleveland, OH) or Cy5-Autoread sequencing kits(Pharmacia

Biotech, Uppsala, Sweden) using primers designed on the basis of chIFN-γ cDNA sequence. Sequencing reactions were separated on 0.4mm or 0.2mm 6% polyacrylamide/7-8M urea sequencing gels. Data were analyzed with DNASIS software for IBM PC(Hitachi, Japan) and aligned to the chIFN-γ (GenBank accession number U27465) by BLAST mail server to determine the exon/intron boundaries. To determine putative transcription factor binding sites, the sequence of the 5'-flanking region was analyzed with MatInspector software Release 2.1 program(Quandt et al., 1995).

Ⅲ. RESULTS

1. Chicken IFN-γ cDNA cloning using RT-PCR

Oligonucleotide primers covering the open reading frame of the chicken IFN-γ gene amplified a 517 bp full length DNA fragment from P34 cDNA(Figure 1, lane 2) or Con-A activated PBLs(Figure 1, lane 3). The PCR products were cloned into pUC18 and sequences were verified by dideoxy chain termination DNA sequencing(U.S. Biochemicals, Cleveland, OH).

2. Isolation and sequence analysis of chicken IFN-γ genomic sequences

From a genomic library screened using the chIFN-γ cDNA, 10 independent clones were initially isolated (Figure 2). Restriction analysis revealed that these were overlapping clone representing as one gene, chIFN-3 (Figure 3). The chIFN-3 gene was subcloned and partially sequenced. Exon/intron junctions were identified by comparison with the cDNAs sequences. The chIFN-γ gene is organized into four exons and three introns spanning over 14 kb (Figure 4). As shown in Figure 3, the exon/intron boundaries are demarcated with GT/AG dinucleotides necessary for the correct splicing (Senepathy, et al., 1990).

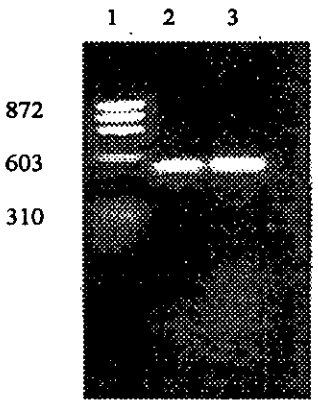

Figure 1. Reverse transcription polymerase chain reaction for chicken IFN-γ gene. Lane 1: φX174-*Hae* III, lane 2: PCR products from P34 T cell hybridoma, lane 3: PCR products from Con-A activated PBL cDNA.

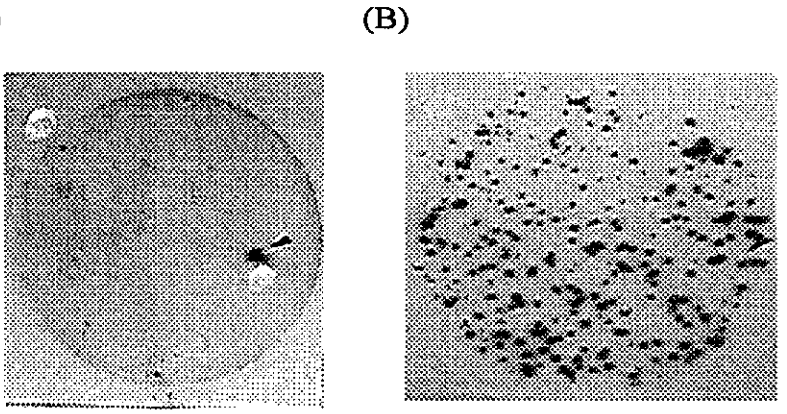

Figure 2. Representative results of chicken genomic library (λFIX II) with radioactive chicken IFN-γ probe. (A) First screening, (B) Third screening. Arrow indicates positive plaque for chicken IFN-γ

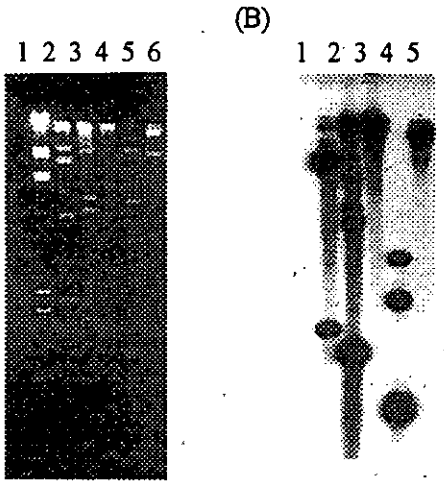

Figure 3. Restriction mapping of λ phages having chicken IFN-γ genomic sequences (λ chIFN-3). (A) Restriction digestions with *Eco RI* (lane 2), *Hind* III (lane 3), *Kpn* I (lane 4), *Pst* I (lane 5) and *Sal* I (Lane 6).(B) Southern hybridization lane 1: *Eco RI*, lane 2: *Hind* III, lane 3: *Kpn* I, lane 4: *Pst* I, lane 5: *Sal* I

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cat gaa agg aca gca cta agg gga ttt aaa att taa aat gat tct gat gtc tgc cac agc
agt atc agt ggt tac ttt aaa aga ata cca gaa atg agt tga ctg ttg tat tga ccc ctt
aac cac atg ata tga ttt ttc aga tag ctt gta aga gta tgc cta tgc cac agg ggc tac
tcc taa atc agt atc ata att tcc ctc ttt ttc ccc acc acg ggt aaa aga aac ttc aga
aag tgc cac gta aat ttt acc ctc tga tga acc gct gaa aaa ttt tcc agg acc atg ctt
taa gaa aga cac ttt cga ggg att ccc tca acc agc cta tag caa aat ctt atc taa cac
tat ctt ggc taa ata tac aca gcc cgt gat gac gaa ata aaa aaa ttt caa gac cag gta
taa ata cca ctc aca ggc aga tga agt tca ttc gtc tac ttt ctt cta at

aga aga cat aac tat tag aag ctg aag ctc act gag ctt ata tct gac atc tcc cag aag  60

                                                    M  T  C  Q  T  Y  N  L
  1
cta tct gag cat ttg aac tga gcc atc acc aag aag ATG ACT TGC CAG ACT TAC AAC TTG
120

 F  V  L  S  V  I  M  I  Y  Y  G  H  T  A  S  S  L  N  L  V
28
TTT GTT CTG TCT GTC ATC ATG ATT TAT TAT GGA CAT ACT GCA AGT AGT
CTA             AAT             CTT             GTT             180

 Q  L  Q  D  D  I  D  K  L  K  A  D  F                       41
CAA CTT CAA GAT GAT ATA GAC AAA CTG AAA GCT GAC TTT
       219

                                                  intron1
gt aag gta ttg tct tct act gca ttt ctc ctc ---  < 2 kb  --- att tgt ata aca gat
gct tat gtt ctc tgt tgc ag

 N  S  S  H  S  D  V  A  D  G  G  P  I  I  V  E  K  L  K  N
61
AAC TCA AGT CAT TCA GAT GTA GCT GAC GGT GGA CCT ATT ATT GTA GAG
AAA         CTG         AAG         AAC                       60
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 W  T  E  R  N  E                                                    67
TGG        ACA         GAG         AGA         AAT         GAG
 78
                                    intron2
gt gag tca gaa cca gac acg aca aaa cat ctc  --- > 0.3 kb --- tca cag acc ttt ctt
ttg aat gta ttc ttc aac ag

 K  R  I  I  L  S  Q  I  V  S  M  Y  L  E  M  L  E  N  T  D  87
AAA AGG ATC ATA CTG AGC CAG ATT GTT TCG ATG TAC TTG GAA ATG CTT
GAA         AAC         ACT         GAC                        60

 K  S  K  P  H  I  K  H  I  S  E  E  L  Y  T  L  K  N  N  L
107
AAG TCA AAG CCG CAC ATC AAA CAC ATA TCT GAG GAG CTC TAT ACT CTG
AAA         AAC         AAC         CTT                        120

 P  D  G  V  K  K  V  K  D  I  M  D  L  A  K  L  P
   124
CCT GAT GGC GTG AAG AAG GTG AAA GAT ATC ATG GAC CTG GCC AAG CTC
CCG                                                           141
                                    intron3
gt aag ctc ttt gtt ctg cct cca agg acc ttg  ---  > 0.750 kb  --- cct ttg ttc
tgt ctg ttt ctt gct caa aac ag

 M  N  D  L  R  I  Q  R  K  A  A  N  E  L  F  S  I  L  Q  K
144
ATG AAC GAC TTG AGA ATC CAG CGC AAA GCC GCG AAT GAA CTC TTC AGC
ATC         TTA         CAG         AAG                        60

 L  V  D  P  P  S  F  K  R  K  R  S  Q  S  Q  R  R  C  N  C
164
CTG GTG GAT CCT CCG AGT TTC AAA AGG AAA AGG AGC CAG TCT CAG AGG
AGA         TGC         AAT         TGC                        120

***
TAA atg acc tcc tgt ctt caa cta ttt taa att tta caa tgc aca att ttt atg ttg tga  180
ttt ttt aac tga gtt tat ata cat tta gct ttt att aat att taa gta ttt taa ata att  240
att tat att aaa aaa aaa cca ggc aaa caa tga aag tat tta tac ctc cta ctg ctg tgt  300
aag aaa cgg att gtg gtc tta aaa tac tgt cta tct gtt gtg tgt ggg ttg act gaa aat  360
acc gaa tga ggt gga tgt tta cca gtt tct gtg tgg gaa ata ctg aat tgg agg tgg atc  420
tgt act caa gaa aac cca ctc atc ccg gtc agt cta gta ttt cta aat cca aat caa gga  480
gtg gct tgt tta aag gga aaa aat gtg agc act ctc tga ctg ggt ctt aga gat ttt act  540
gat ggt ttg gca tga cta aga att tag g                                          568
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Figure 4. Exon/intron boundaries of chIFN-γ. Bold characters indicate the pre-mRNA splicing donor and acceptor sites. The sizes of the introns were approximated by PCR with oligonucletide primers corresponding to the exon sequences.

(A) Chicken

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CATGAAAGGACAGCACTAAGGGGATTTAAAATTTAAAATGATTCTGATGT
50

CTGCCACAGCAGTATCAGTGGTTACTTTAAAAGAATACCAGAAATGAGTT
100
                              STAT
GACTGTTGTATTGACCCCTTACCACATGATATGATTTTTCAGATAGCTT
 150
      ER        AP1       USF
```

GTAAGAGTATGCCTATGCCACAGGGGCTACTCCTAAATCAGTATCATAAT
200

AP2

TTCCCTCTTTTTCCCCACCACGGGTAAAAGAAACTTCAGAAAGTGCCACG
250

USF

TAAATTTTACCCTCTGATGAACCGCTGAAAAATTTTCCAGGACCATGCTT
300

TAAGAAAGACACTTTCGAGGGATTCCCTCAACCAGCCTATAGCAAAATCT
350

STAT

TATCTAACACTATCTTGGCTAAATATACACAGCCCGTGATGACGAAATAA
400

GATA CREB/ATF, AP-1

AAAAATTTCAAGACCAGGTATAAATA 427

TATA box

(B) Human

TGTTCTTTTAACCGCATTCTTTCCTTGCTTTCTGGTCATTTGCAAGAAAA
-370

STAT[1] ER[1]

TTTTTCACTTGTTCCCAACCACAAGCAAATGATCAATGTGCTTTGTGAAT -320

GAAGAGTCAACATTTTACCAGGGCGAAGTGGGGAGGTACAAAAAAATTTC -270

AP-1[1] USF[1]

CAGTCCTTGAATGGTGTGAAGTAAAAGTGCCTCAAAGAATCCCACCAGAA -220

AP2[2]

TGGCACAGGTGGGCATAATGGGTCTGTCTCATCGTCAAAGGACCCAAGGA -170

USF[3]

GTCTAAAGGAAACTCTAACTACAACACCCAAATGCCACAAAACCTTAGTT -120

ATTAATACAAACTATCATCCCTGCCTATCTGTCACCATCTCATCTTAAAA
-70

GATA[4,5] CREB/ATF[4,5]

AACTTGTGAAAATACGTAATCCTCAGGAGACTTCAATTAGGTATAAATAC
-20

AP-1[4,5] TATA box

Figure 5. Comparison of potential transcription binding factor binding sites in the 5′-flanking region of between chicken (A) and human IFN-γ (B) gene. The parameters used to determine the potential chicken regulatory sequences by the MatInspector program are a core similarity is 0.70 and a matrix similarity is 0.80.
[1]Identified in this study, [2]Ye et al., 1994, [3]Cippitelli et al., 1996, [4]Cippitelli et al., 1995, [5]Penix et al., 1993

Table 1. Putative binding motifs of transcription factors in the 5′-flanking region of chIFN-γ gene [a]

Transcription factor	Motif(Consensus sequences)[b]	Nucleotide position[c]	Orientation[d]	Core similarity[e]
AP-1	NTGASTCAG	110, 318	+	1.00,1.00
CREB	TGACGTMA	386	+	1.00
ATF-1	CNSTGACGTNNNYC	387	+	1.00
GATA	NNNNGATANKGNN	345	-	00
STAT	TTGCCCRKAA	72, 323	+	0.808,1.00
AP-2	MKCCCSCNGGCG	166	-	857
USF	NCACGTGN	123, 217	+	0.876,1.00
ER	NNARGNNANNNTGACCYNN	101	+	1.00

[a, e] : Putative transcription factor binding sites and core similarity were determined with the MatInspector version 2.1 program

[b] Consensus sequences were from TRANSFAC database release 3.2. The single letter codes for ambiguous bases are as follows : K : G or T , M : A or C, N : A, C, G, or T, R : A or G, S : C or G,
Y : C or T

[c] See Figure 3

[d] Oritentation +: sense, -: antisense

IV. DISCUSSION

Ten overlapping genomic clones encoding chIFN-γ were isolated by hybridization to a cDNA clone. Similar to mammalian IFN-γ gene, the chicken IFN-γ gene contains four exons, although there are inter-species variations in the length of each exon. Sequence analysis of the 5′-flanking region and comparison with the 5′-region of human IFN-γ identified several potential transcriptional regulatory elements in the chicken gene (Figure 5). Especially, in the region within 100bp of the TATA box, the chIFN-γ gene has a similar organization of transcription factor binding sites as human IFN-γ. This region contains AP-1/CREB/ATF binding sites which have been shown to play central roles in the selective expression of human IFN-γ in T cells (Penix et al., 1993; Penix et al., 1996; Aune et al., 1997). Within the region between -108 and -40 bps of the human IFN-γ promoter, there are two conserved and essential regulatory elements of IFN-γ which confer

activation-specific expression in T cells. Both elements have sequences, which bind to AP-1/CREB/ATF family of transcription factors(Cippitelli et al., 1995). The proximal element is a target for methylation at its central CpG dinucleotide(Young et al., 1994). In Th1 $CD4^+$ T cells, the methylation status of this site strongly correlates with IFN-γ gene transcription(Young et al., 1994; Penix et al., 1996). Sequences corresponding to USF binding sites were also identified in the chicken IFN-γ gene. Through this site, retinoic acid has an inhibitory effect on the transcription of human IFN-γ promoter(Cippetelli et al., 1996). Putative USF binding sites in the 5'-flanking region of the chicken gene suggests that it is also transcriptionaly modulated by retinoic acid.

V. Summary in Korean

닭 T 임파구 하이브리도마 세포주인 P34로부터 RT-PCR 기법을 이용하여 닭 인터페론 감마 cDNA를 증폭하고 pUC18 벡터에 클로닝하여 염기서열을 결정하였다. 인터페론 감마 cDNA를 프로브로 이용하여 화이트 레그혼으로부터 작성된 박테리오페이지 라이브러리를 스크리닝하였다. 스크리닝 결과, 닭 인터페론 염기서열을 포함하고 있는 10개의 페이지를 분리하여 그중 하나를 이용하여 닭 인터페론 감마 유전자의 구조를 규명하였다. 인터페론 감마는 네 개의 엑손과 세개의 인트론으로 구성되며, 엑손과 인트론 경계는 정확한 스플라이싱에 필요한 GT/AG 규칙을 따르고 있다. 네 개의 엑손은 각각, 41, 26, 57 그리고 40개의 아미노산을 코딩한다. 이들 결과는 인터페론 감마의 유전자 구조가 포유류와 조류에 걸쳐서 진화적으로 보존적임을 암시한다. 5'-UTR과 시그널 서열은 엑손 1번에 존재한다. 엑손 4번에 mRNA 대사에 중요한 역할을 하는AT-rich 염기서열이 존재한다. 5'-flanking 부위에는 사람 인터페론 감마 전사조절에 관여하는 AP-1, AP-2, CREB, ATF, GATA, USF와 같은 전사조절단백질이 결합할 수 있는 염기서열이 존재한다. 이는 닭과 포유류의 인터페론 감마의 전사조절 메커니즘이 유사함을 암시한다.

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