Cell-specific and interferon-inducible expression of chicken major histocompatibility class II genes

by

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ABSTRACT

The immune system is an adaptive defense system that is capable of producing many kinds of cells and molecules that work together to eliminate foreign invaders of vertebrate animals. In this system, B cells, T cells and macrophages work together through the help of the major histocompatibility complex (MHC) molecules to carry out the task. The MHC class II antigens are mainly restricted in expression to B cells, macrophages, activated T cells and other antigen-presenting cells. They can also be expressed in some other class II-negative cells when induced by Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF - α), Interleukin-4 (IL-4) and other stimulatory factors. In mammals, interferon-gamma (IFN-γ) can induce MHC class I and class II expression on macrophages. This is also true in the chicken. The goal of this research was to characterize cells for differences in constitutive or IFN-inducible regulation of avian MHC class II gene expression. Here we examined chicken cell lines of different cell types with different basal levels of MHC class II surface antigen expression. These cells were evaluated for levels of MHC class II surface antigen expression by flow cytometry analysis, both with and

without interferon exposure. Presence and level of transcription of the gene in a chicken macrophage cell line, before and after induction with IFN, a B cell line and a T cell line was also determined by Northern blot analysis. Here we found that the constitutive expression of chicken MHC class II gene is restricted to the B cells, and is inducible in macrophage to a very high level upon recombinant chicken IFN treatment. The T cell line showed a very weak increase in chicken MHC class II surface antigen level when induced with recombinant IFN but not with the IFN-rich supernatant. The DT-40 pre-B cell was weakly induced by p34(200) IFN-rich supernatant. Northern blot analysis demonstrated the differences in the chicken MHC class II surface antigen level correlated with the transcriptional level of class II gene in B cells and macrophages. However, high abundance of chicken MHC class II RNA transcripts in T cells, comparable to that of the macrophages induced with 1:200 dilution of ChIFN, were detected, even though the class II surface antigen was not detected by flow cytometry. The outcome of this research supported the previous findings of Kasper et al. (1994) that chicken MHC class II antigen expression can be induced in macrophages upon IFN treatment, although a lower level of induction was seen with the IFN-rich supernatant on MQ-NCSU

when compared to the work of Kasper et al. However, the level of chicken MHC class II antigen induction in MQ-NCSU greatly increased when recombinant IFN was used. This result supported Schultz et al (1995) in that this recombinant ChIFN is a chicken homologue of mammalian IFN-γ. The research established a gene regulation system for the study of the chicken MHC class II gene regulation. Further studies of how this cell-specificity is achieved among these different immune cells can contribute to a greater general understanding of cell-specific regulation and the study of constitutive and inducible gene regulation of MHC class II genes in chickens.

INTRODUCTION

The immune system is an adaptive defense system that protects the vertebrate from invading pathogens and cancer (Kuby, 1992). It is capable of producing many kinds of cells and molecules that work together to eliminate foreign invaders of the body. In the immune system (Kuby, 1992), B lymphocytes, T lymphocyte and antigen presenting cells such as macrophages, dendritic cells and B cells that carry antigen in a form that can stimulate lymphocytes (Roitt et al., 1993) work together to achieve this process with the help of major histocompatibility complex (MHC) molecules and many cytokines. The MHC class II antigens are mainly restricted in expression to B cells, macrophages, activated T cells and other antigen-presenting cells. They can also be expressed in some other class II-negative cells when induced by Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF -α), Interleukin-4 (IL-4) and other stimulatory factors. Macrophages can express class II antigens inductively only after stimulation (Benoist and Mathis, 1990). In the chicken, MHC class I and class II are found to have similar function as in mammals, but also have importance in disease resistance and economic traits (Lamont, 1989; Schierman and Collins, 1987). In mammals, interferon-gamma can induce MHC expression in the macrophages, however, IFN induction of chicken MHC

molecules has only been done in the class II genes of the peripheral blood monocytes (Kasper et al, 1994). Although there are similarities between the chicken and mammalian MHC, chicken MHC genes differ from those of mammals in organization and sequence (Guillemot et al., 1989). Especially, the deletion of the S, X, Y boxes in the chicken class II gene caused no significant effect in macrophage expression of chloramphenicol acetyl transferase in CAT assays (Chen et al., 1997). This leads to the hypotheses that either the regulatory mechanism controlling class II gene expression has diverged between avian and mammalian species or that the expression of MHC class II genes is cell-specific in that the X and Y boxes are not required for basal expression of the gene, but are required in the stimulated expression of the gene. To test the hypotheses, immune cells of the chicken must be tested for constitutive levels and inducibility of the MHC class II gene expression. Several chicken cell lines of different cell types were used in this research, and interferon of chicken sources were used for the induction study. In this research, it was found that expression of chicken MHC class II gene was constitutive in the B cell, and that recombinant chicken interferon could induce the class II gene expression in the chicken macrophage cell line, which was due to a change in the level of class II RNA transcription in the cell line. The constitutively MHC class II antigen expression in B cells was not further

induced at any condition tested, and the MHC class II negative T cells were weakly induced ($p<0.05$) by recombinant chicken IFN. MHC class II negative pre-B cells were induced very weakly ($p<0.05$) by partially-purified p34(200) at higher concentration. It was also found that the high abundance MHC class II mRNA transcripts were not translated into MHC class II proteins. The extension of this research such as footprinting and in vitro studies of the transcriptional factors will contribute to a greater general understanding of how cell-specific regulation of the MHC class II gene is achieved in the chicken cell lines through the action of the cis and trans regulatory elements, and also contribute to the the study of inducible transcriptional regulation of the MHC class II gene in chicken.

LITERATURE REVIEW

To generate an effective immune response, two major groups of cells are needed. They are the lymphocytes and the antigen-presenting cells (APCs). Specificity, diversity, memory and self/nonself recognition of the immune response are mediated by the lymphocytes. Lymphocytes recognize antigens by means of membrane receptors specific for the foreign material. The two major population of lymphocytes are the B lymphocytes and the T lymphocytes. B lymphocytes mature within the bone marrow and leave the bone marrow carrying a unique antigen-binding receptor, which is called an antibody molecule. When the B cell encounters an antigen which is specific to the membrane-bound antibody, the B cell begins to divide rapidly and differentiates into memory B cells, which continue to express membrane-bound antibody with the same specificity as the original parent cell, and plasma cells, which produce antibody of the same specificity but in a secreted form. T lymphocytes also arise from hematopoetic stem cells in the bone marrow, but migrate to the thymus gland to mature. T cells express a unique membrane receptor for antigen which recognizes antigen only in association with cell membrane proteins known as major histocompatibility complex (MHC) molecules (Kuby, 1992).

The structure and functions of the major histocompatibility complex

The Major Histocompatibility Complex (MHC) is a chromosomal region containing a family of immune-related genes which are involved in many cellular properties of importance to the immune response (Schierman and Nordskog, 1961; Klein, 1986). In mammals, the MHC is grouped by structure and function into three classes, named class I, class II and class III. The class I genes encode for cell-surface glycoproteins, which are present on almost all types of cells, and are responsible for recognizing foreign tissues and virally infected cells. The class II genes also encode cell-surface glycoproteins which are constitutively present on immunocompetent B cells and dendritic cells (Germain 1986). In addition, a large number of molecules can induce class II antigens on many kinds of cell types. Both class I and class II antigens are involved in the cell-cell interaction in the immune responses in the immune system. Both class I and class II genes participate in non-self recognition (Kuby, 1992). The class III genes encode several complement proteins, as well as a diverse collection of at least 20 other genes (Blair et al., 1995), including 21-hydroxylase and tumor necrosis factor. There are no established functional or structural similarities between class III gene products and the class I or class II molecules.

The structures of class I and class II antigens have been defined in detail. The class I antigen consists of two chains, an glycosylated α chain of molecular weight of about 45 Kd and a non-polymorphic β_2 microglobulin. The α chain anchors in the plasma membrane and has three domains. The β_2 microglobulin is non-covalently associated with the α chain and has a molecular weight of about 12 Kd. This protein is not encoded in the MHC. The class II antigens are heterodimers consisting of a heavy α chain and a light β chain. The molecular weights of the α chain and the β chain are about 34 Kd and 29 Kd respectively (Groenen et al. 1990). Several functional domains can be distinguished: for class I genes, the α_1, α_2, α_3 and β_2 extracellular domains; for class II genes, the α_1, α_2, β_1 and β_2 extracellular domains. Both classes also have a transmembrane segment and cytoplasmic tail.

Class I and class II proteins are responsible for stimulating different populations of T cells. Class I proteins bind endogenous antigens that have been processed within the cell and present them at the surface to T cells. The class I proteins present antigens primarily to CD8 antigen positive, cytotoxic T cells. Class II proteins bind exogenous antigens that have also been processed in the cell but presented by antigen presenting cells (APCs) such as macrophages and dendritic cells. Class II proteins usually stimulate CD4 positive T helper cells. Class I antigens, when

bound by TCR:CD3 complex of the CD8 T cells, initiate CTL killing of intracellular bacteria/virus-infected cells. Class II antigens, when bounded by TCR:CD3 complex of the CD4 T cells, facilitate the degradation of endocytosed extracellular antigens which are presented by APCs (Blair et al., 1995).

The structure and functions of the chicken MHC

The chicken MHC, also designated as the chicken B complex, was first described as a locus controlling blood group antigens in 1948 (Briles et al., 1950). Later, it was identified as the chicken MHC by its polymorphism and its control of alloreactivity (Schierman and Nordskog, 1961). It differs from its mammalian counterpart in size and organization (Guillemot et al., 1989). The biochemical structure, functional attributes and tissue distribution of the B locus antigens show that they are the chicken equivalents of the mammalian MHC molecules. However, the chicken B complex is not only responsible for graft versus host reaction, viral infection, cell to cell interaction and immune responses (Nordskog et al., 1987; Schierman and Collins, 1987; Vainio et al., 1987), but is also associated with resistance to certain diseases and some biological traits such as body weight and egg production (Bacon, 1987).

The chicken MHC genes consist of three classes (Nordskog et al., 1987; Vainio et al., 1987). The class I (B-F) and class II (B-L) antigens are similar to those of mammals in their function and structure. However, the class IV (B-G) molecules are unique, because they are expressed primarily on red cells and their progenitors, and no corresponding polymorphic molecules have been found in other animals (Pink et al., 1977). The *B-G* genes encode differentiation antigens restricted to the erythroid lineage designated as class IV which is not present in the mammals (Salomensen et al., 1991). In addition, six class I, five class II and one class IV genes were found in the B complex of chicken MHC haplotype B^{12} (Guillemot et al., 1989).

As in the other species, the chicken MHC molecules display extensive polymorphism. Both serological tests and restriction fragment length polymorphism (RFLP) analysis have shown that the MHC antigens differ extensively among different haplotypes. In previous studies (Auffray et al., 1986; Hala et al., 1988), five different *B-Lβ* genes from the CB-B^{12} and two from the GB-B^{6} haplotype have been molecularly cloned.

The organization of the class I and class II MHC genes of the chicken is unlike the corresponding mammalian MHC genes. Hala et al.(1988) have searched for recombinations in crosses between the congenic lines CB (B^{12}) and CC (B^{4}) by serological typing, mixed lymphocyte reaction (MLR) and

restriction enzyme fragment length polymorphism (RFLP) analyses and indicated that *B-F* and *B-L* genes are tightly linked together at a distance of below 0.01 centimorgan and therefore is lack of recombination (Hala et al., 1988). Also, the chicken MHC is located on the microchromosome that also contains all of the ribosomal ribonucleic acid (rDNA) genes that are detected as a nucleolar organizer region (Bloom et al., 1987). From the studies of Guillemot and colleagues, it was shown that the *B* complex is much more compact than mouse *H-2* or human *HLA* complexes (Guillemot et al., 1989). The chicken MHC class I and class II genes are interspersed. The *B-L* and *B-F* genes are only separated by a distance of 10 to 20 Kb , which is 2 orders of magnitude closer than the *H-2* and *HLA* genes. In addition, the genes in the *B* complex are about 10 times smaller than the mammalian MHCs because of their shorter introns. The total size of the B complex is not yet known. However, the minimum size of the *B-F/B-L* region is about 250 Kb, and the size of the *B* complex might be a minimum of 850 Kb and a maximum of 2000 Kb (Guillemot et al., 1988). With the studies made by mapping two rDNA genes to one end of a *B* complex cosmid cluster, it was found that the *B* complex is tightly linked to the nucleolus organizing region (NOR) on the chromosome (Guillemot et al., 1988). So, it was suggested that the *B-G* genes which are not directly linked to the *B-F/B-L* loci are located either close to the centromere on the long arm or on the short arm of the

chromosome (Guillemot et al., 1988). Recently, another chromosomal locus, *Rfp-Y*, has been discovered to contain chicken MHC genes but is unlinked to the *B* complex (Briles et al., 1993; Miller et al., 1994). The *Rfp-Y* genes, however, have been mapped to the cosmid cluster II/IV (name of cluster assigned by Guillemont et al., 1988), which is located on the MHC microchromosome 16 (Fig.1) (Miller et al., 1994a; Miller et al., 1994b).

The first study of chicken MHC class I and class II genes was conducted using mammalian probes (Anderson et al., 1987; Auffray et al., 1987; Bourlet et al., 1988; Guillemot et al., 1988; Guillemot et al., 1989). A chicken DNA probe produced by screening a chicken genomic library at low stringency with a human HLA-DQβ cDNA probe was later used as a probe for chicken *B-L* gene analysis (Andersson et al, 1987). This probe was used to isolate three class II MHC gene clones from a highly inbred (99%) chicken line G-B2 (haplotype B6) (Xu et al., 1989).

A genomic library was constructed and three class II MHC genes were isolated from this phage library using a chicken B-Lβ probe p234 which is a β2 specific probe. The clones were named CCII-2, CCII-4 and CCII-7 (CCII stands for Chicken Class II) (Xu et al.,1989). They were restriction mapped, and the B-L genes in each clone were localized by Southern blotting. The B-Lβ genes in each of the three clones were then subcloned into plasmid vectors and the subclones were

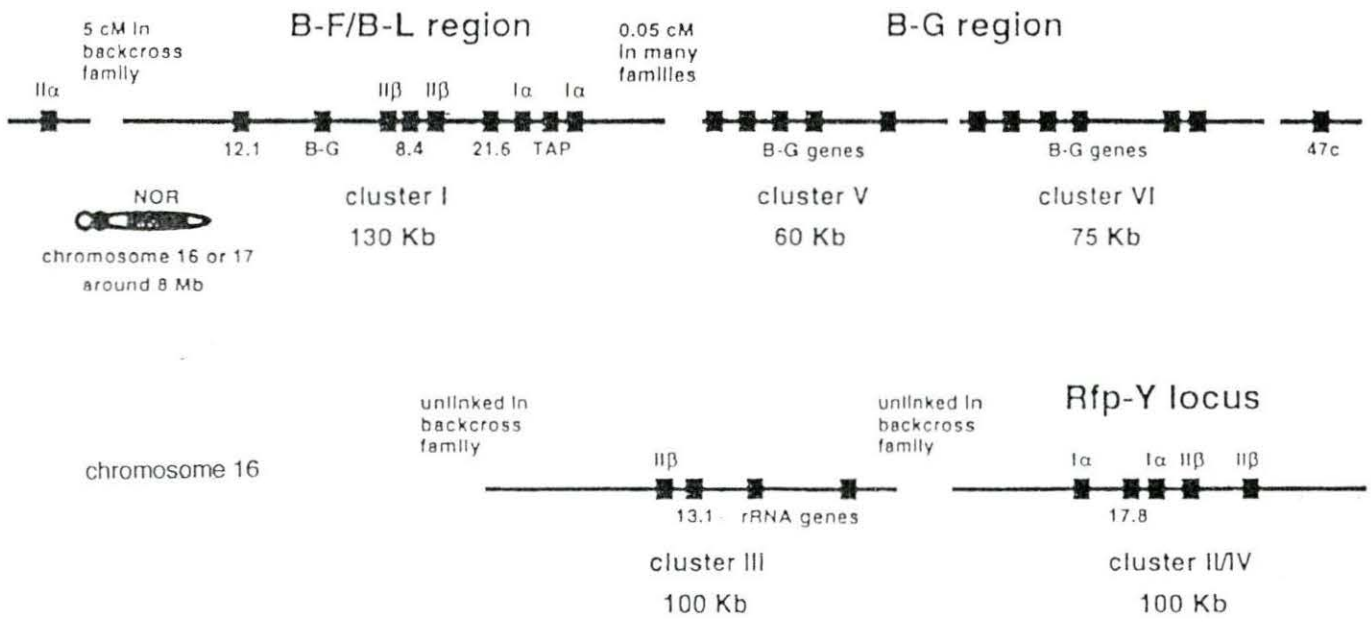


Fig.1 Current view of the chicken Mhc and genomic regions containing related genes. This is a map modified from map produced by Kaufman et. al. (1993)

named CCII-2-1, CCII-4-1 and CCII-7-1. Restriction maps were constructed by Southern hybridizaton with β1 exon oligonucleotide probe [*A2*] and 3'UT oligonucleotide [*39*] probes. It appeared that the CCII-2-1 was a pseudogene because of the absence of β1 exon and 3'UT in the β gene cloned, therefore, only the CCII-4-1 and CCII-7-1 were sequenced. After the sequences of the two genomic subclones were obtained, the results implied that both CCII-4 and CCII-7 were active genes which would be interesting for further study (Xu et al., 1989).

Regulation of MHC expression

Regulation of gene expression of *H-2* and *HLA* has been extensively studied. For the class I MHC gene, a sequence upstream of the *H-2K* gene binds a nuclear factor H2TF1 and stimulates the expression of the class I MHC on all cell types. This sequence was also found to interact with a tissue specific nuclear factor NF-kB (Baldwin and Sharp, 1988), which also binds to the immunoglobulin k chain gene enhancer in B cells.

The class II antigens are mainly restricted in expression to B cells, macrophages, activated T cells and other antigen-presenting cells. They can also be expressed in some other class II-negative cells when induced by

Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF - α), Interleukin-4 (IL-4) and other stimulatory factors. Macrophages can express class II antigens inductively only after stimulation (Benoist and Mathis, 1990). Studies in the human MHC showed that several specific sequences immediately upstream of the class II gene initiation site and the DNA-binding factors are important in the expression of class II antigens on the cell surface (Glimcher and Kara, 1992). These factors can modulate the expression of the class II antigens in a cell-dependent manner by a variety of positive and negative stimuli and the outcome is directly involved in the control of normal and abnormal immune responses.

Little information is available regarding MHC class II gene regulation in the chicken. It was found that sequences in the 5' flanking region of *B-LβII* were similar to S, X and Y boxes in mammals' class II genes, but with some mutations in these elements that rendered the *B-LβII* gene inactive (Zoorob et al., 1990). Also, an ATTGG sequence is located within the Y box and two of the mutations in the *B-LβIII* gene are just within this sequence, implying that CCAAT-binding factor might play a significant role in the expression of B-L genes. The B-L genes lack the typical TATA box or CCAAT box and an adenosine surrounded by pyrimidines may act as the transcription initiation site. Finally, there are SPI-binding sites (Kroemer et al., 1990). The structure and expression of a chicken MHC class I gene has been studied by Kreomer et

al.(1990). Recent studies on the chicken class I genes revealed the sequence of beta 2-microglobulin gene as a small, GC-rich gene with X and Y boxes in the promoter (Riegert et al., 1996). Class I gene expression can be induced by IFN (Zöller et al., 1992).

There is evidence that the expression of class I and class II MHC genes are regulated differently in mouse compared to human (Sartoris et al., 1990). Therefore, although they have similar sequences, one cannot assume that these regulatory elements act in the same manner among different species. Especially, none of the sequences considered to be highly conserved appears exactly like those in *B-LβII* except the transcription initiation site. When examining the chicken MHC in the G-B2 line, the X box is conserved quite well but there are quite a few mismatches in the Y box (Chen et al., 1997).

From our laboratory's past work on the CCII-7-1 clone, it was shown that in the 5' upstream region, the regulatory elements located in the region of about -150 to -500 bp from the translation initiation site have 60% to 100% similarities to the human MHC. Much work has been done to analyze the regulatory elements and their functions in CCII-7-1 (a subclone of CCII-7 which contains the promoter of that gene). The presence of the S, X, Y boxes in the promoter has been identified in the chicken. Comparison between the sequence of the chicken CCII-7 clone to the human MHC class II gene

sequence, indicated that regulatory elements besides S, X, Y boxes were also present. Using the chloramphenicol acetyl transferase (CAT) reporter system, a 0.7 Kb 5' flanking region of the CCII-7-1 subclone was proven to be a functional promoter containing the conserved S, X, Y boxes, negative regulatory elements and other cis-acting elements (Chen et al., 1993), which are crucial for constitutive and interferon-inducible regulation of class II expression in human and murine cells (Dorn et al., 1987). However, surprisingly, deletion analyses indicated that S, X, Y boxes did not have a significant influence on promoter activity (Chen et al., 1993) in macrophages. Glucocorticoids were shown to decrease interferon-induced gene expression in the same transient transfection assay system of chicken class II promoter construct and CAT reporter gene (Chen et al., 1993).

Interferon

Activation of both the humoral and cell-mediated immune response requires lymphokines produced by T_H cells. To ensure careful regulation of the immune response, T_H cells can only be activated by antigen recognition when the antigen is displayed together with MHC on the surface of APCs, which includes macrophages, B cells and dendritic cells.

Interferons (IFNs) are a family of proteins first characterized by antiviral assays, but are also potent immune regulators that modulate cell differentiation and antiproliferation factors. In the humans, they are divided into IFN-α, IFN-β and IFN-γ in accordance to varying degrees in antigenicity, nucleotide and amino acid sequence, structure and biological function (Kuby, 1992). IFN-α is made by leukocytes in response to virus or nucleic acids. It is actually a family of structurally related molecules (Pestka, et al., 1987). Most of them are glycosylated. The apparent molecular weights of the human IFN-α range from 16,000 to 27,000 (Rubinstein et al., 1979; Staehelin et al., 1981). IFN-β is derived from fibroblasts in response to viruses or nucleic acids. The human IFN-β exhibit an apparent molecular weight of approximately 20,000. The amino acid composition is similar to that observed in human IFN-α, and was demonstrated to be a glycoprotein (Knight and Fahley, 1982). Human IFN-γ is produced by T cells in response to antigen or mitogen activation, with an apparent weight of 15,000 to 25,000 (Friedlander et al., 1984; Yip et al., 1984). All three groups of IFN have antiviral activity and induce an increase in surface expression of class I MHC antigens (Heron et al.,1978). The main action of IFN-α and IFN-β is to induce an antiviral state (Kuby, 1992) and are therefore used to inhibit cell growth in certain rare cancers such as renal cell cancer and hairy cell leukemia (Kuby, 1992). Mammalian

IFN-γ has been shown to be an important activator of macrophages, including up-regulation of immunoglobulin Fc receptors, MHC class I and class II antigens (Pestka et al., 1987). Also, when IFN-γ is added together with interleukin-4 to B cells, the class switch to IgE is blocked (Kuby, 1992).

Promoters that respond to IFNs have consensus DNA-binding sites, which were originally called interferon consensus sequences (ICS). They are also known as IFN-responsive elements (IRE). These are the elements to which activators bind and through which gene expression is enhanced (Freidman et al., 1984). It is now known that upon stimulation with interferon, STAT1 and STAT2 are phosphorylated to form a heterodimer with p48, resulting in the complete form of the ISGF3 complex. The ISGF3 complex binds to ICSs in promoters of most IFN-α/β-responsive genes and transactivate the genes through signal transduction pathways (Lee and Benveniste, 1996; Gustafon and Ginder, 1996).

Recombinant DNA technology has led to the availability of large quantities of pure human IFN and thus the studies of the effect of the IFNs and the structural analysis on them. However, relatively little study has been conducted in the avian species due to less readily accessible recombinant cytokines. Kaspers et al. (1994) have reported that expression of MHC class II molecules in chicken macrophages is increased by partially-purfied IFN-rich supernatant

derived from Con A induced spleen cell. Only limited descriptions of avian interferon have been published. Chicken IFN was first discovered as an antiviral activity secreted by virus-infected choriollantoic membranes (Isaac and Lindenmann, 1957). Chicken IFN from virus-infected chicken eggs (Krempien et al., 1985) or embryonic chicken cells (Kohase et al., 1986; Kroemer et al., 1990) was partially purified and found to be a glycoprotein of approximately 25KDa.

Although mammalian IFNs presently are grouped into five families of genes: IFNs-alpha, -beta, -omega, -tau (all are Type I), and -gamma (Type II) (Sekellick et al., 1994), chicken IFN appears to consists of a single antigenic species (Kohase et al., 1986, Beladi et al., 1993) that is largely acid-stable and unusually resistant to heat inactivation. This ChIFN is neutralized with antibody prepared against acid-stable IFN (Yoshida and Marcus, 1990), suggesting a single type of IFN (Sekellick et al., 1994). Recently, a gene encoding chicken IFN was cloned from a cDNA library made from primary chick embryo cells (Sekellick et al., 1994). This cDNA clone showed 80% homology with mammalian type I interferon. This cDNA clone was expressed in E.Coli or COS cells and a recombinant IFN produced (Schultz et al., 1995). Other methods of producing IFN include concanavalin A induction (Kaspers et al., 1994) and virus induction of chicken embryo fibroblast (Sekellick et al., 1994; Yamagata

et al., 1996). The purified recombinant chicken IFN was shown to be a powerful antiviral agent which has a specific antiviral activity of approximately 10^8 IU/mg protein and has high Mx promoter-inducing activity. This recombinant IFN lacks macrophage activating factor (MAF) activity, therefore is not a general activator like LPS, but has specific IFN-γ like action in inducing macrophage to express MHC class II surface antigen. It is a glycoprotein of about 20kDa, and believed to be a chicken homologue of IFN-γ in its action to activate macrophages in releasing NO and induce MHC class II expression. However the activity spectrum resembles mammalian type I IFN (Schultz et al., 1995).

Cell specificity of MHC class II genes

As the embryo develops, cells specialize for function in tissues. In the animal kingdom, the higher eukaryote contains a very wide range of different cell types, each of which expresses specific genes encoding particular products necessary for the specialized function of that cell type. Many of the genes are expressed temporally in specific tissues, at specific development stages or in response to environmental stimuli of many kinds. Examples of the cells and tissues include erythrocytes, islet cells of the

pancreas, liver, skeletal muscle cells, B cells, activated T cells, and many others (Liu et al., 1996).

Mammalian MHC class II genes are also expressed constitutively in B cells, but are inducible in macrophages upon stimulation (Basham and Merigan, 1983). Studies have demonstrated that the conserved DNA elements termed X and Y boxes found in mammalian MHC class II gene (Benoist and Mathis, 1990) are crucial for class II promoter activity in both B cells and IFN-γ-induced cells. Also, many other DNA-binding factors could contribute to the differential expression of the gene in different tissues.

There are many ways to control gene expression in a cell-specific manner. Initiation of messenger RNA synthesis is a primary control point in the regulation of differential expression. Methods include alteration of composition of the transcription complex (Wanatabe et al., 1995), usage of different promoters (Zinn et al., 1983), site-specific methylation of the promoter (Gourdjiet al., 1996) and differential use of the same promoter (Tsai et al., 1996). Additional methods include the effect of the decapping or addition of purines at the 5' cap to regulate the RNA turnover (Hatfeild et al., 1996) and protection of the constitutive promoter from DNase I cleavage although an inducible promoter is present (Young et al., 1996). In conclusion, similar mechanisms involving actions of different transcription factors and DNA elements are utilized to

achieve cell-specific expression in many other cell types. This regulated expression contributes importantly to maintain the dynamic but homeostatic status of the mammalian body.

The specific goal of this research was to analyze cell-specific and IFN-inducible expression of avian MHC class II genes. Cells that differ in constitutive or IFN-inducible regulation of avian MHC class II gene expression will be identified, so as to set up a model in the research for the study of cell-specific gene expression. The extension of this research will contribute to a greater general understanding of cell-specific regulation and the study of transcriptional regulation of MHC class II gene in chickens.

MATERIALS AND METHODS

Cell lines

Several chicken cell lines were selected for this study. RP-9 is a lymphoid leukosis virus transformed B cell line which expresses high levels of chicken MHC class II surface antigen. DT-40 is an avian leukosis virus transformed B cell line having low level of the class II surface antigen expression. MSB-1 is a Marek's disease virus transformed T cell line which does not express class II antigen constitutively. MQ-NCSU is a Marek's disease virus transformed macrophage cell line which does not express class II antigen constitutively. The MQ-NCSU was donated by M.A. Qureshi (North Carolina State University). Both DT-40 and MSB-1 cell lines were donated by C. Thompson (University of Chicago). HD-11 is an MC29 virus transformed chicken macrophage cell line which does not express class II antigen constitutively. RP-9 was donated by Hans Cheng (Avian Disease and Oncology Laboratory, East Lansing, MI) and HD-11 cell line was donated by H.S. Lillehoj (USDA, Agricultural Research Service, Beltsville MD). Characteristics of the chicken cell lines are shown in Table 1.

Table 1. Characteristics of chicken cell lines

Cell Line	Cell type	Reported MHC class II surface antigen expression	Reference
RP-9	B cell	+++++	Okazaki et al., 1980
DT-40	B cell	-	Baba et al., 1985
MQ-NCSU	macrophage	+	Qureshi et al., 1990
HD-11	macrophage	+	Kaspers et al., 1993
MSB-1	T cell	-	Akiyama et al., 1974

Cell culture conditions

The RP-9 cells were maintained in 2:1 Leibowitz L-15 : McCoy 5A medium supplemented with 20% chicken serum and 10% newborn calf serum (NCS). DT-40 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% NCS. MQ-NCSU cells were maintained in 1:1 Leibowitz L-15 : McCoy 5A medium supplemented with 10% NCS. HD-11 cells were maintained in RPMI supplemented with 10% NCS. MSB-1 cells were maintained in DMEM supplemented with 10% NCS. Chicken embryo fibroblasts were collected from 9-11 day old embryos by decapitation, mincing the tissue into smaller pieces and then trypsinizing at room temperature for 45 min. After elimination of the trypsin by centrifuging the cell

solution at 1500 g for 4 minutes and discarding the fluid, the cells were washed with Hank's balanced salt solution and then resuspended into DMEM supplemented with 10% NCS. All culture media were also supplemented with 1000 U/ml penicillin-streptomycin and 2mM glutamine. All cell lines were incubated at 42°C with 5% CO_2 in 75cm^2 canted-neck flasks (Falcon).

Antibody to chicken MHC class II protein

The monoclonal antibody (mAb) used in this study was CIa-1, which is of IgMκ species and is reactive with a monomorphic determinant of the Ia-like (B-L, or MHC class II) antigens (Ewert et al., 1984). This antibody reacts with determinants on B cells in all avian species tested, but does not detect antigens on lymphocytes of representative mammals, reptiles and amphibians. In addition to labelling the MHC class II antigen on B cells, this antibody defines a subpopulation of the monocyte-macrophage series and reacts with mitogen(Con A)-activated T cells (Ewert et al., 1984). The mAb was kindly donated by Dr. D. L. Ewert (Wistar Institute, Philadelphia, Pensylvania).

Interferon

The p34 IFN-rich supernatant was used in the first stage of this research due to the general unavailability of the chicken recombinant IFN which was used in the later stage of the research. The p34 is a partially-purified IFN-rich supernatant prepared by stimulating spleen lymphocytes obtained from SC chickens with 10 mg concanavalin A for 48 hr. The supernatant was partially purified by first passing the supernatant to a controlled pore glass column equilibrated in PBS, pH 7.2, then collected by washing the column with 0.5 M Tris-HCl, pH 9.5, containing 1.5 M NaCl (Kaspers et al., 1994). This parially purified IFN-rich supernantant was then named p34(200). This IFN was generously donated by Dr. H.S. Lillehoj (USDA, Agricultural Research Service, Beltsville MD). The recombinant chicken IFN (ChIFN) used in the study was prepared by expressing in *E.Coli* a cDNA encoding ChIFN cloned from virus-infected primary chicken embryo cells. The recombinant ChIFN alone has no Macrophage Activity Factor (MAF) activity, which might be caused by an unrelated cytokine (Schultz et al., 1995). It is specific in its mammalian type II IFN action, which is believed to chicken homologue of mammalian IFN-γ. This ChIFN is a gift from Dr. Ursula Schultz (University of Freiburg, Germany).

Flow cytometry

Flow cytometry analysis was used to determine optimum conditions on mAb usage, secondary antibody usage, basal chicken MHC class II surface antigen expression level of different cell types, the amount of IFN and time needed for optimum induction of the chicken MHC class II surface antigen with IFN, and the induced class II antigen expression levels.

CIa-1 mAb was used in all the assays for all the cell types. In all assays, either the RP-9, DT-40, MQ-NCSU, HD-11, MSB-1 cell lines and 9 to 11 days chicken embryo fibroblasts selected for each different experiment were suspended in 1X PBS, supplemented with 4% bovine serum albumin and 0.2% NaN_3 (PBA) at $2x10^8$/ ml; 50µl were transferred to individual 3 ml polypropylene tubes and incubated with 50µl of the appropiate diluted anti-chicken Ia mAb (1:400). Isotype control mAb (Sigma, mouse IgMλ) were used in all assays. The cells were incubated on ice for 30 min, washed two times with PBA , and resuspended in 50 µl PBA buffer. 50µl of fluorescein-conjugated goat antibody to mouse IgG-IgM (Cappel) were added and the cells incubated for 30 min on ice. After a final washing, the cells were resuspended in 1ml PBA and stained with 2µl of 5mg/ml Propidium Iodide, and fluorometric analysis was performed on an EPICS XL flow cytometer (Coulter Corporation, Miami, FL). Live cells gated on the basis of low Propidium Iodide fluorescence and higher

forward scatter, were analyzed for fluorescein fluorescence. The results were displayed as frequency distribution histograms of $\log_{10}$ of the fluorescein fluorescence of at least 5000 cells. In modern flow cytometry, photons produced by the fluorescence emmision are converted into electrons which form voltage pulses. These voltage pulses are digitized to values proportional to the intensity of the fluorescence emitted. The digital values are then converted mathematically for presentation on a log scale. The accuracy of this linear to log conversion permits the comparison of antigen expression level between individual samples by comparing the mean fluorescence values. The flow cytometry data shown in each table and figure were generated by subtracting the mean fluorescence value of the isotype controls from the mean fluorescence value of the CIa-I labeled groups individually and calculated as mean ± SEM. The ranges and means of the isotype control of each cell type is enclosed at the appendix.

Optimization of conditions

To determine optimum conditions for labelling the cells with CIa-I mAb, several tests were conducted. For optimum mAb dilution for the assay, RP-9 cells were subjected to the previous discussed procedure with the exception of using

different dilutions (1:25, 1:50, 1:100, 1:200, 1:400, 1:800 1:1600) of the mAb. Optimum secondary Ab dilution for the assay was also tested by repeating the assay with the exception of using different dilutions (1:25, 1: 50, 1:75) of secondary Ab to RP-9 cells when using 1:400 dilution of the CIa-1 mAb. Flow cytometry analysis was then performed using 1:400 dilution of CIa-I and 1:50 dilution of secondary antibody to measure basal and induced MHC class II expression of each cell type used in this study.

Induction of MHC class II surface antigen by IFN

To determine the best condition of interferon dilution and time that could give the MQ-NCSU and HD-11 macrophages the highest level of induction, 1:1000, 1:2000 and 1:4000 dilutions of p34(200) IFN-rich supernatant were added to the cell cultures for 24, 36, 48 and 60 hour. After the desired incubation time was reached, cells were prepared and subjected to flow cytometry analysis using the previous discussed procedure.

When recombinant IFN became available, flow cytometry analysis was again performed on MQ-NCSU, to determine the equivalent dilution of the recombinant ChIFN to the IFN-rich supernatant. The determined dilution was used as a starting point of reference to determine the optimum concentration of

the recombinant ChIFN to use in the assay. The above determined optimum concentration was then used to determine the optimum chicken class II MHC surface antigen expression level that can be induced in MQ-NCSU. One group of MQ-NCSU was incubated with 1:2000 dilution of the p34(200) IFN-rich supernatant, the others with 1:1000, 1:400, 1:200 and 1:100 dilutions of the recombinant ChIFN.

The MQ-NCSU, RP-9 and MSB-1 cells were cultured in the presence of ChIFN and analysed for the comparison for the level of IFN-induced MHC class II expression using the general flow cytometry analysis protocol described above.

Statistical analysis

Student's t-test was used to analyse the significance of difference in expression level in each paired experimental group. It is designed for the analysis of small sample populations. The null hypothesis of the Student's t-test assumes that there is no difference between two populations tested. Therefore the greater the P value the greater the probability that the two populations are the same and vice versa (Watson, 1992). In this research, the difference between two populations were determined to be significant when p is smaller than 0.05.

p234 probe and ^{32}P-labelling

The p234 is a chicken MHC class II β_2 exon specific probe produced by screening a chicken λL47 genomic library with a human HLA-DQβ cDNA probe at low stringency. The size of this fragment is 234 bp (Bourlet et al., 1988). This probe DNA was a gift from C. Auffray of Institut d' Embryologie, France. 250 µg of denatured p234 DNA was random primed with 500µM dATP, 500µM dGTP, 500µM dTTP and 50µCi [α-^{32}P]dCTP using 5 units of Klenow fragment. The labeled probe was used in the hybridization of the Northern blot.

Chicken β-actin probe

The β-actin probe used in this research was a 590 bp cDNA which encodes for the 3' untranslated region of a chicken β-actin gene. The cDNA was constructed from an mRNA of embryonic chick brain. This cDNA probe specifically for chicken only (Cleveland et al., 1980). This cDNA is a gift from Dr. Donald Beitz (Iowa State University). 120 µg of denatured β-actin DNA was random primed with 500µM dATP, 500µM dGTP, 500µM dTTP and 50µCi [α-^{32}P]dCTP using 5 units of

Klenow fragment. The labeled probe was used in the hybridization of the Northern blot.

Northern blot

To relate the presence of the MHC class II antigen is with the presence of its mRNA transcript, and also, to see if the induced MHC class II antigen expression is due to induction of the messenger RNA (mRNA) transcript, mRNA was isolated for Northern blot analysis. Messenger RNA was isolated from RP-9, DT-40, uninduced MQ-NCSU, and chicken recombinant IFN induced MQ-NCSU, using the Ambion Poly(A)$^+$ RNA isolation kit. RNA quantity was determined by spectrophotometer. Thirty micrograms of mRNA from each of the above mentioned cell types were loaded onto a 1.5% formaldehyde gel, and electrphoresed at 75 volts for 3 hours. At the end of the run, the gel was prepared by immersed shaking in an alkaline solution (1M NaOH,1M Nacl) for 15 min, then in 0.1M Tris pH 7.5 for 15 min, and finally in 20X SSC for 30 min before transferring onto a nylon membrane. Transfer was done by the upward osmosis method overnight. The membrane was fixed by an autolinker (Stratagene). The prepared membranes were first prehybridized and hybridized at 42^{o}C with ^{32}P-labeled p234 and then ^{32}P-labeled chicken β-

actin DNA probe after the p234 probe was striped, for detection of the expression of chicken class II gene and to standardize RNA quantity transfered to the nylon membrane respectively. The prehybridization and hybridization solution consisted of 5X SSPE, 5X Denhart's solution, 0.1% SDS, 100μg/ml denatured salmon sperm DNA, and 50% molecular biology grade formamide. The membranes were washed with 5X SSC, 1% SDS for 20 minutes once and with 0.1 X SSC, 2% SDS for twenty minutes twice, and subjected to autoradiography with Fuji medical X-ray film. The intensity of the detected bands on the two autoradiograms were measured by an imaging densitometer (Bio-Rad, Model GS-670). The abundancy of the chicken MHC class II mRNA detected were normalized by β-actin.

RESULTS

Flow cytometry

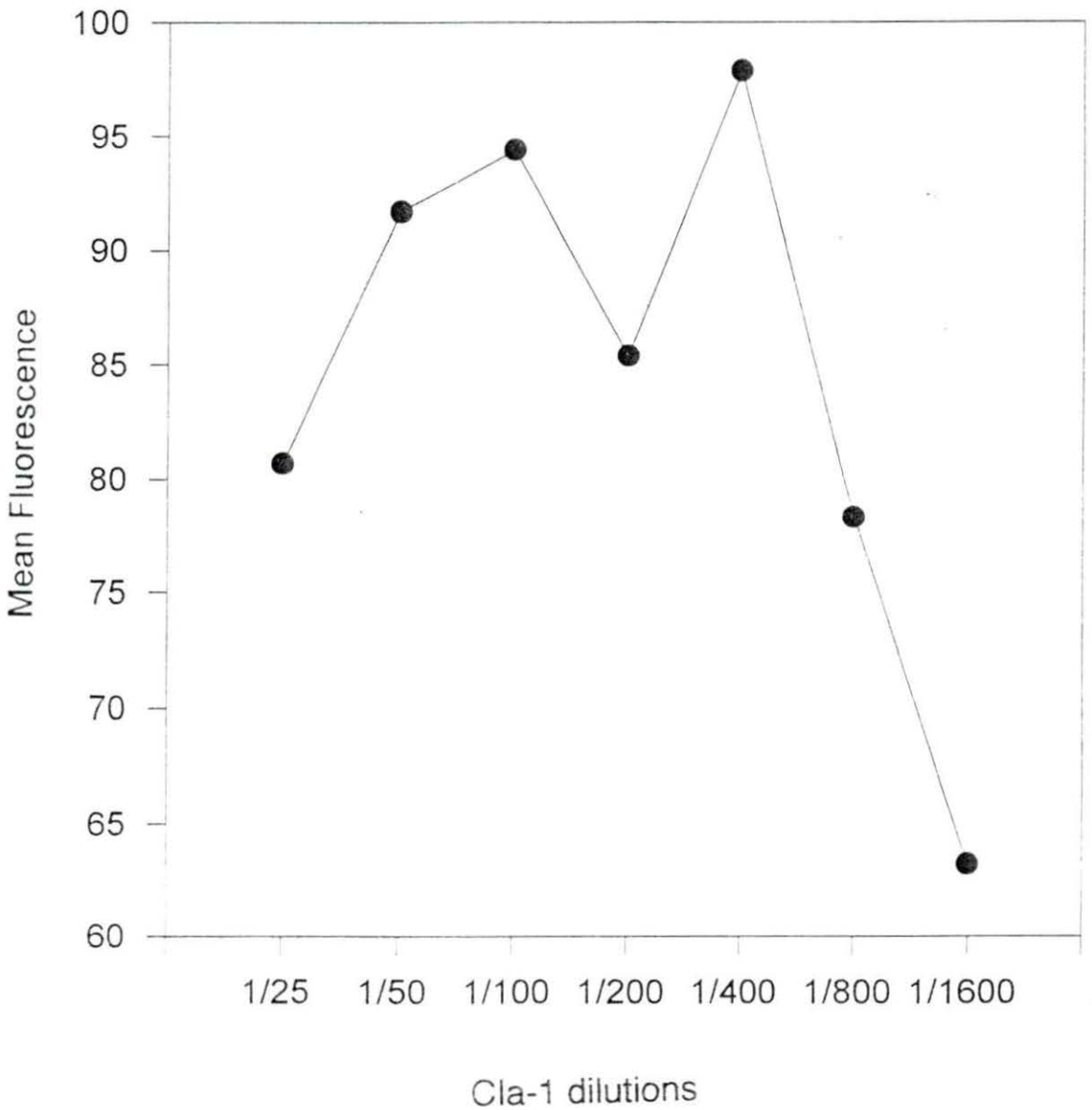
Optimum conditions for detection of chicken MHC class II surface antigen by flow cytometry analysis

A test (n=1) of flow cytometry analysis was conducted using different concentration of CIa-1 monoclonal antibody for labelling chicken MHC class II surface antigens on RP-9 cells. 97.9% of the cells in the suspension were labelled by CIa-1, showing expression of chicken MHC class II surface antigen. The result showed an increase of mean fluorescence value at antibody dilutions of 1:25 to 1:400, which decreased at the 1:800, and 1:1600 dilutions (Fig.2). Therefore, a 1:400 dilution of the monoclonal antibody was used for the rest of the experiments.

Cell-specific expression of chicken MHC class II surface antigen

The basal levels of chicken MHC class II expression were measured in each cell line as a standard point for comparison with the expression levels obtained after IFN was added. RP-9

Fig.2 Mean fluorescence level of chicken MHC class II surface antigen on RP-9 chicken B cell line with varied dilutions of CIa-1 monoclonal antibody. n=1.


100
95
90
85
80
75
70
65
60
Mean Fluorescence
1/25
1/50
1/100
1/200
1/400
1/800
1/1600
Cla-1 dilutions

B cells showed the highest level of expression compared to the other cell lines (Table 2). DT-40, MSB-1, HD-11 MQ-NCSU and primary chicken embyro fibroblast all showed very little expression (considered as background levels) of the chicken MHC class II surface antigen. Data were collected as the result of four assays.

Table 2. Cell-specific expression of chicken MHC class II surface antigen of chicken cell lines

Cell line	Cell type	Antigen expression level* before induction
DT-40	B cell	1.17 ± 0.03
RP-9	B cell	52.06 ± 2.82
MQ-NCSU	macrophage	1.17 ± 0.31
MSB-1	T cell	2.24 ± 0.29
HD-11	macrophage	1.16 ± 0.12
CEF#	fibroblast	2.27 ± 0.37

*Antigen expression level is expressed as mean fluorescence (mean ± SEM) determined by flow cytometry analysis.

#CEF is primary chicken embryo fibroblast derived from 9 to 11 days old chicks.

Induction of chicken MHC class II surface antigen by IFN-rich supernatant

No increase in chicken MHC class II surface antigen expression was detected on MQ-NCSU when induction was carried out for only 24 hours (Fig.3). The MQ-NCSU cells seemed to have highest induction at 1:2000 dilution of p34(200) for 60 hours, but this was not statistically significant ($p = 0.6316$). Several points on the data were analysed by Student's t-test to see if the observed results were significantly different between IFN-induced groups and un-induced groups. P values of 0.6653 (1:2000, 48 hr), 0.6316 (1:2000, 60 hr), 0.5841 (1:4000, 48hr), and 0.6789 (1:4000, 60hr) determined that the expressions were not significantly different at all those dilution and hours points. An increase in expression level (mean antigen per cell value) was detected by a mean fluorescence shift, and a bimodal peak in the mean fluorescence shift usually occurred after 60 hr of incubation with IFN (Fig.4).

To understand if this time point was a reasonable condition for further induction work, MSB-1 T cell culture supernatant was used as a control of the p34(200) IFN-rich supernatant. Supernatant which is free of Con A stimulation were first passed through a 0.2 mm filter to get rid of dead cells and waste products generated during cell culture and was then incubated with MQ-NCSU. The result showed no change

Fig.3 Time and dose effect of p34 IFN-rich supernatant on MQ-NCSU cell line chicken MHC class II surface antigen expression level. Mean fluorescence (± SEM) determined by flow cytometry analysis. A. No IFN. B. 1:1000 dilution of p34(200). C. 1:2000 dilution of p34(200). D. 1:4000 dilution of p34(200). No significant change in chicken MHC class II gene expression is observed. n=3.

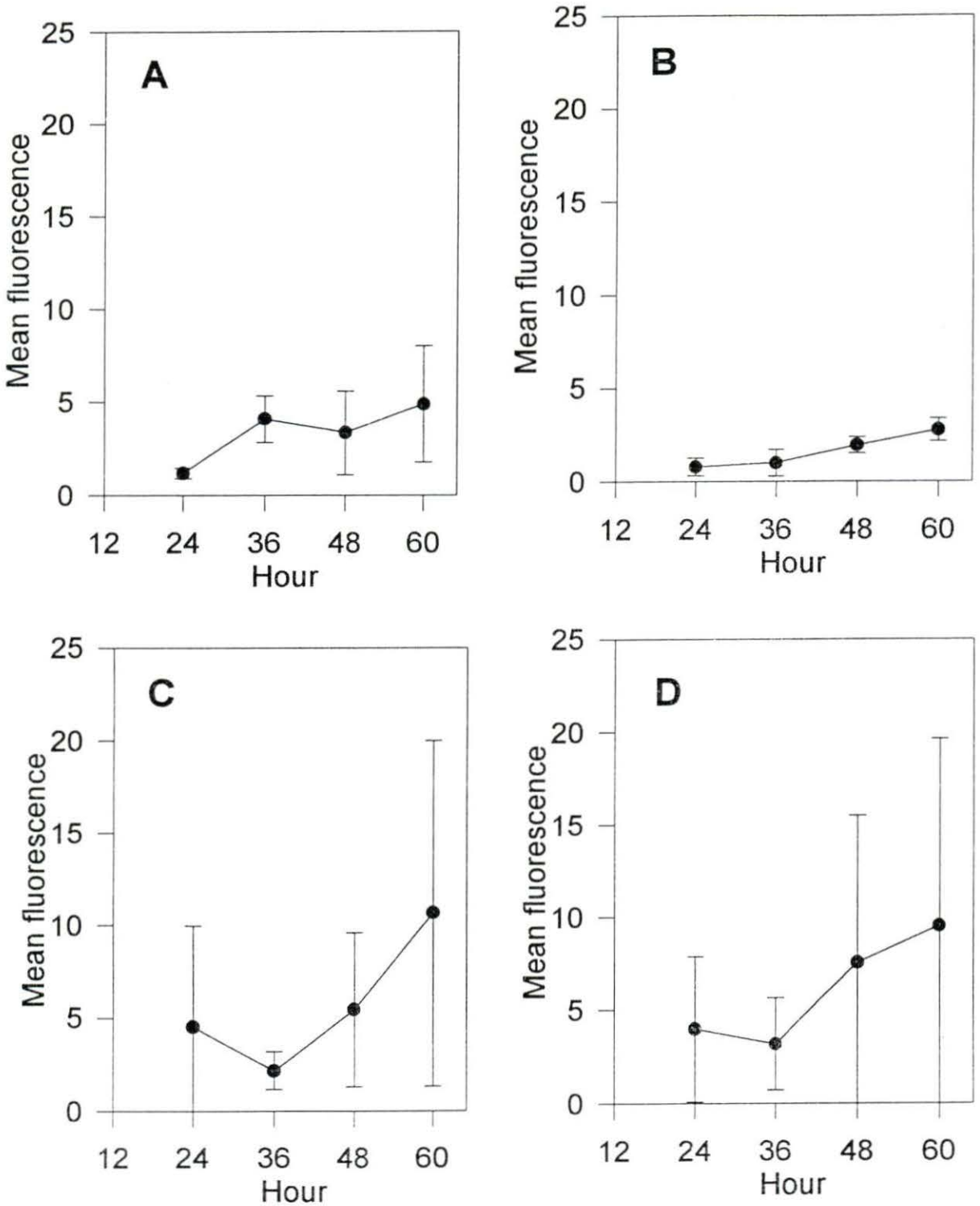
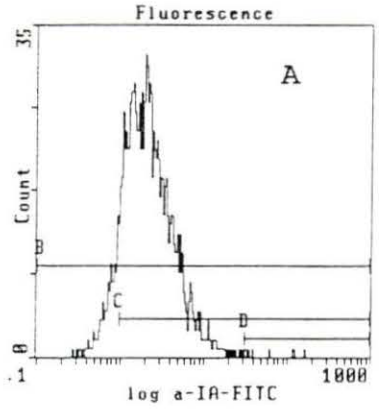
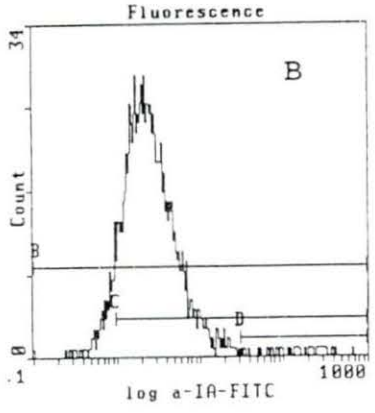

A
B
C
D
Mean fluorescence
Hour
0
5
10
15
20
25
12
24
36
48
60

Fig.4 Expression of labeled chicken MHC class II surface antigen before and after treatment with p34 IFN-rich supernatant on MQ-NCSU. Curve shift towards the right indicates increase in chicken MHC class II expression. A. NO IFN at 60 hr. B. With IFN for 24 hr. C. With IFN for 36 hr. D. With IFN for 48 hr. E. With IFN for 60 hr.


Fluorescence
A
35
Count
0
B
C
D
.1
1000
log a-IA-FITC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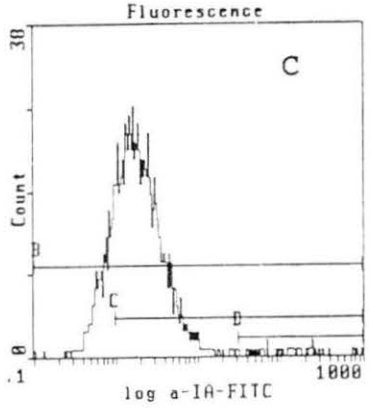
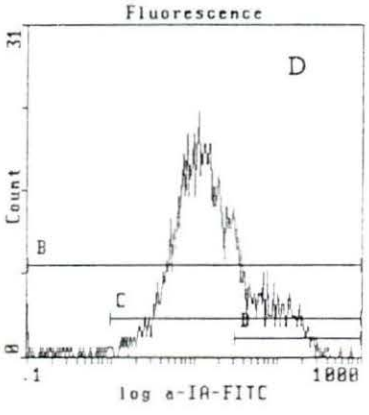
B
34
Count
0
B
C
D
.1
1000
log a-IA-FITC


Fluorescence
C
30
Count
0
B
C
D
.1
1000
log a-IA-FITC


Fluorescence
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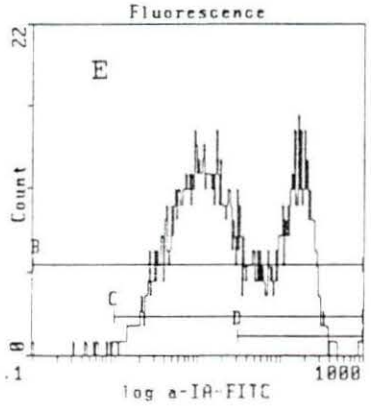
31
Count
0
B
C
D
.1
1000
log a-IA-FITC


Fluorescence
E
22
Count
0
B
C
D
.1
1000
log a-IA-FITC

in the mean fluorescence value (Table 3). Therefore, at this point in the research, 1:2000 dilution of p34(200) IFN-rich supernatant with 60 hr induction was chosen for further experiments.

HD-11 cells died after incubation with p34(200) IFN even at 1:4000 dilution (data not shown). MQ-NCSU cell started to die after 60 hr incubation probably due to accumulation of

Table 3. MSB-1 T cell culture supernatant (TCS) effect on MQ-NCSU

Cell / supernatant	24 hr	36hr	48 hr	60hr
MQ-NCSU/TCS				
test 1	0.508	0.560	0.506	0.283
test 2	0.471	0.957	0.415	0.443
MQ-NCSU only	0.386	0.411	0.495	0.477
MQ-NCSU/p34(200)				
1:2000 dilution	NT	NT	NT	9.399*
RP-9 only	33.426#	NT	NT	NT

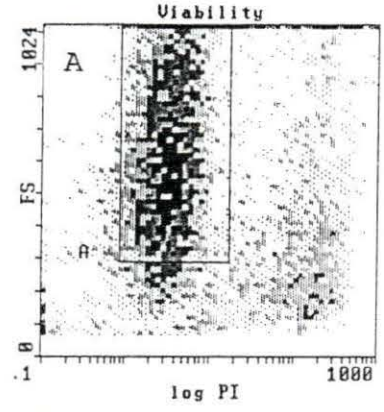
This is a one time test. No statistic analysis is made.
*This served as a positive control for IFN inducibility.
#This served as a positive control for CIa-I labelling.
NT stands for not tested.

waste products produced by the cells. Also, any concentration of IFN higher than the optimum dose led to increased cell death (Fig.5)

MSB-1 cells were not induced by p34 IFN (Fig.6). Points at which mean fluorescence level were slightly higher than that of the average un-induced cells were analysed by the Student's t-test. Expression of the MHC class II proteins was not increased among any of the three groups (1:1000, 48 hr; 1:2000 48hr and 1:4000 24hr) and the uninduced MSB-1 cells, ($p = 0.7892$; $p = 0.9597$; $p = 0.6317$). The same analyses were made on DT-40. Comparison of the effect of 1:1000 dilution of p34(200) on DT-40 for 36 hour and uninduced DT-40 for 36 hr indicated that the expression was weakly induced ($p = 0.0193$) at this time point but fell at 48 hr time point ($p = 0.2243$) (Fig.7). When RP-9 cells were incubated with p34 IFN, the level of MHC class II protein expression was not increased above the already high basal levels of expression at any IFN concentration nor any time point (Fig.8). Points at which mean fluorescence level were slightly higher than that of the average uninduced RP-9 cells were analysed by the Student's t-test. It was determined that MHC class II antigen level was not induced at those points. (P values of 0.8850, 0.6636, 0.5009 and 0.7044 for 1:2000-48 hr, 1:2000-60 hr, 1:4000-48 hr and 1:4000-60 hr respectively).

In general, chicken MHC class II surface antigen level was not increased by p34(200) in the constitutive expressing

Fig.5 Interferon effect on viability of MQ-NCSU. Cells gated at the A region in each panel are live cells. Ungated cells to the right of the panel are dead cells stained with Propidium Iodide. Percentage of cells alive is given below each panel. Larger doses than optimum concentration used for both p34(200) and recombinant ChIFN 5resulted in more cell death. A. 60 hr, no IFN. B. 60 hr, optimum p34(200) concentration. C. 60 hr, 2 fold amount of the optimum concentration. D. 60 hr, optimum recombinant ChIFN concentration. E. 60 hr, 2 fold amount of the optimum concentration.



Region ID	%
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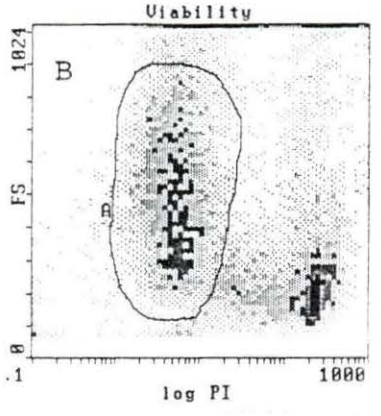
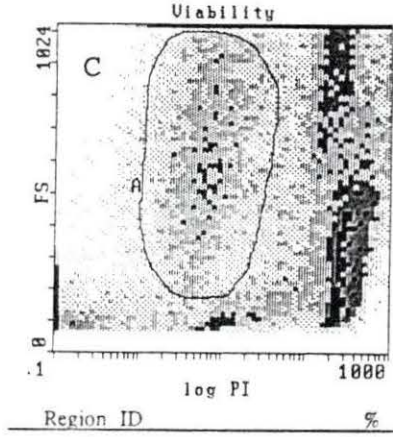


Region ID	%
A	47.9



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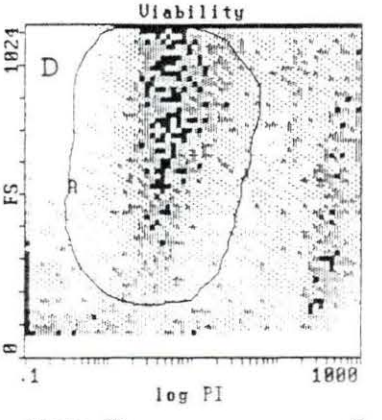
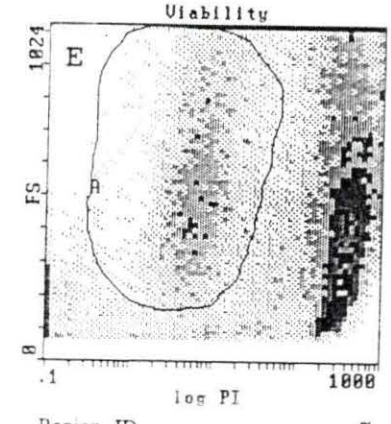
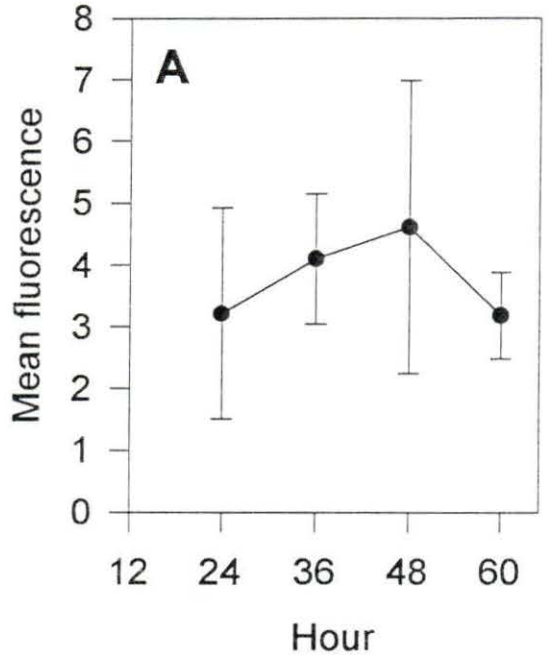


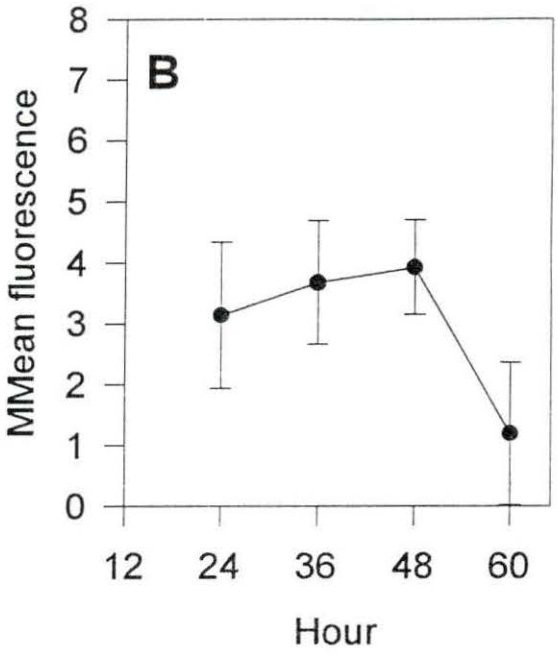
Region ID	%
A	45.9



Region ID	%
A	23.6

Fig.6 Time and dose effect of p34 IFN-rich supernatant on MSB-1 cell line chicken MHC class II surface antigen expression level. Mean fluorescence (± SEM) determined by flow cytometry analysis. A. No IFN. B. 1:1000 dilution of p34(200). C. 1:2000 dilution of p34(200). D. 1:4000 dilution of p34(200). Chicken MHC class II gene expression was not effected by any concentration of p34(200)IFN. n=3.


A
Mean fluorescence
0
1
2
3
4
5
6
7
8
12
24
36
48
60
Hour


B
MMean fluorescence
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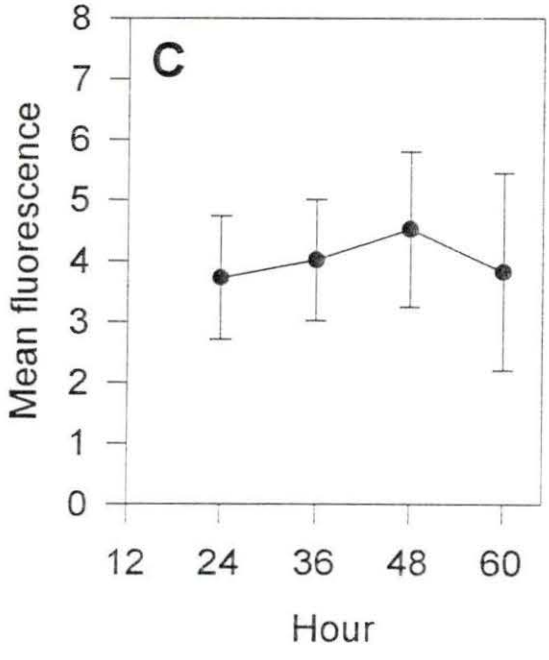
Hour


C
Mean fluorescence
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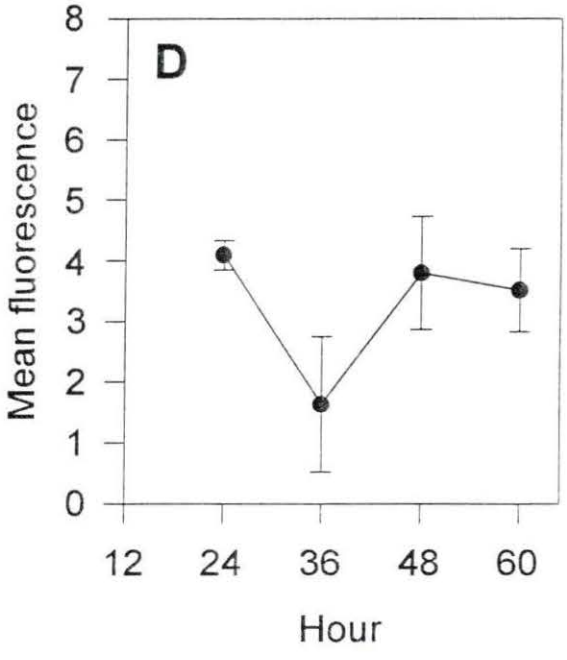
D
Mean fluorescence
0
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12
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48
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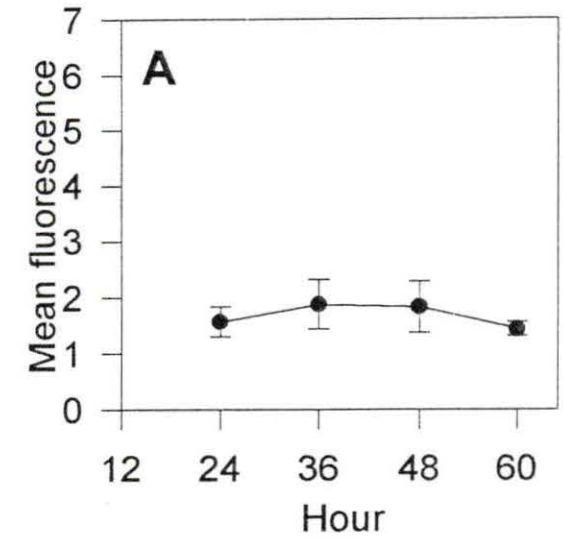
Hour

Fig.7 Time and dose effect of p34 IFN-rich supernatant on DT-40 cell line chicken MHC class II surface antigen expression level. Mean fluorescence (± SEM) determined by flow cytometry analysis. Data without error bar were single experiments. A. No IFN. B. 1:500 dilution of p34(200). C. 1:1000 dilution of p34(200). D. 1:2000 dilution of p34(200). E. 1:4000 dilution of p34(200). Chicken MHC class II gene expression was not effected by any concentration of p34(200)IFN. n=3.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Mean fluorescence
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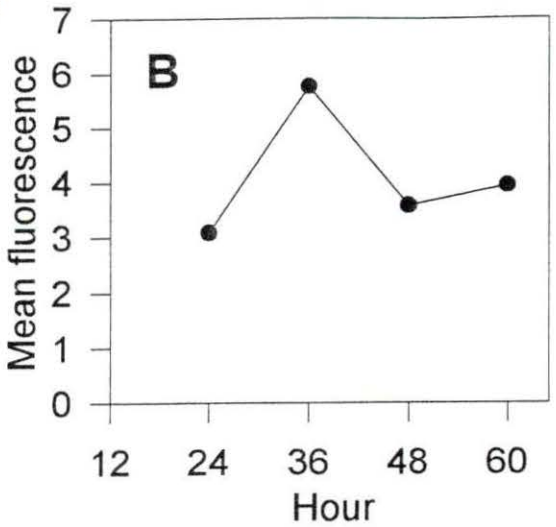
Hour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Mean fluorescence
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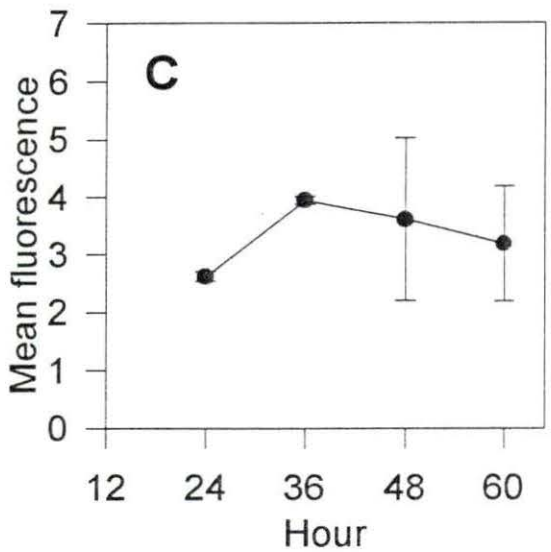
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24
36
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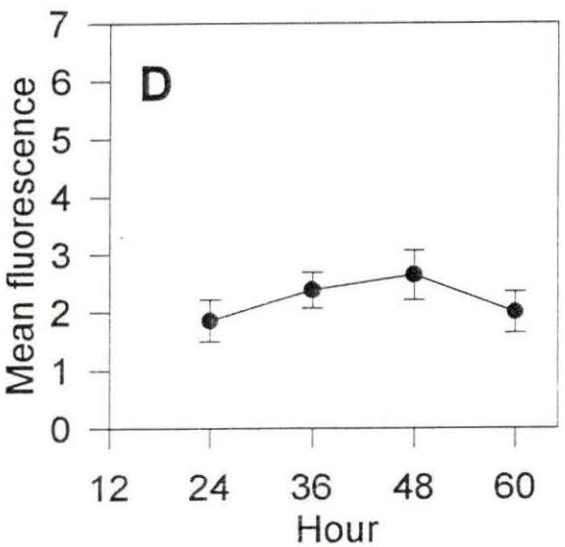
D
Mean fluorescence
0
1
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Hour

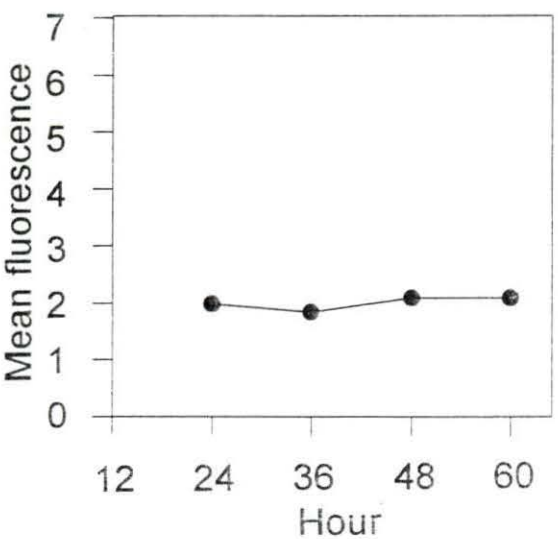
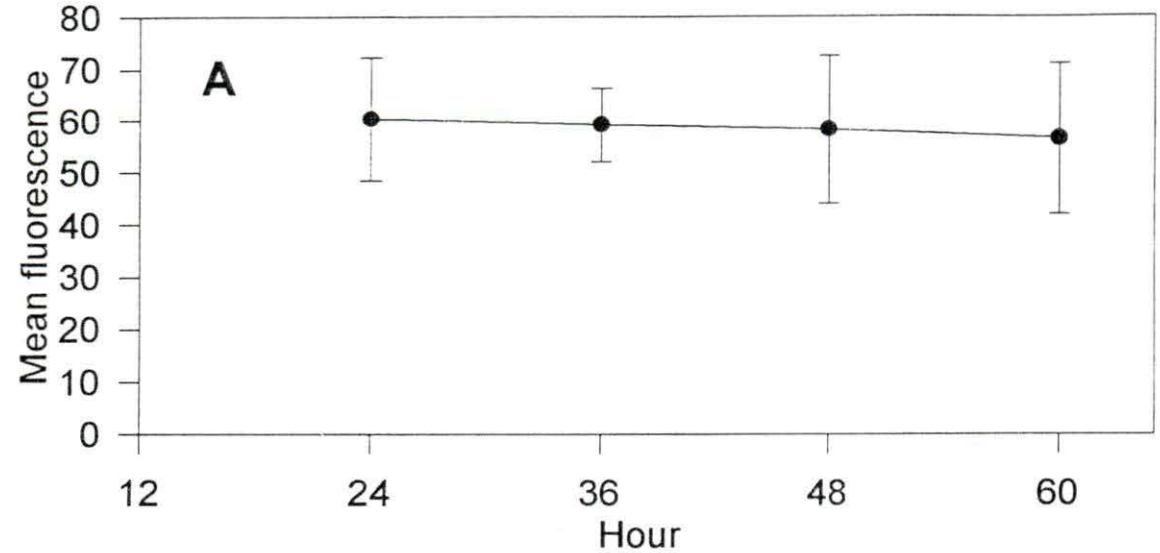

Mean fluorescence
0
1
2
3
4
5
6
7
12
24
36
48
60
Hour

Fig.8 Time and dose effect of p34 IFN-rich supernatant on RP-9 cell line chicken MHC class II surface antigen expression level. Mean fluorescence (± SEM) determined by flow cytometry analysis. A. No IFN. B. 1:2000 dilution of p34(200). C. 1:4000 dilution of p34(200). n=3.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Mean fluorescence
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10
0
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48
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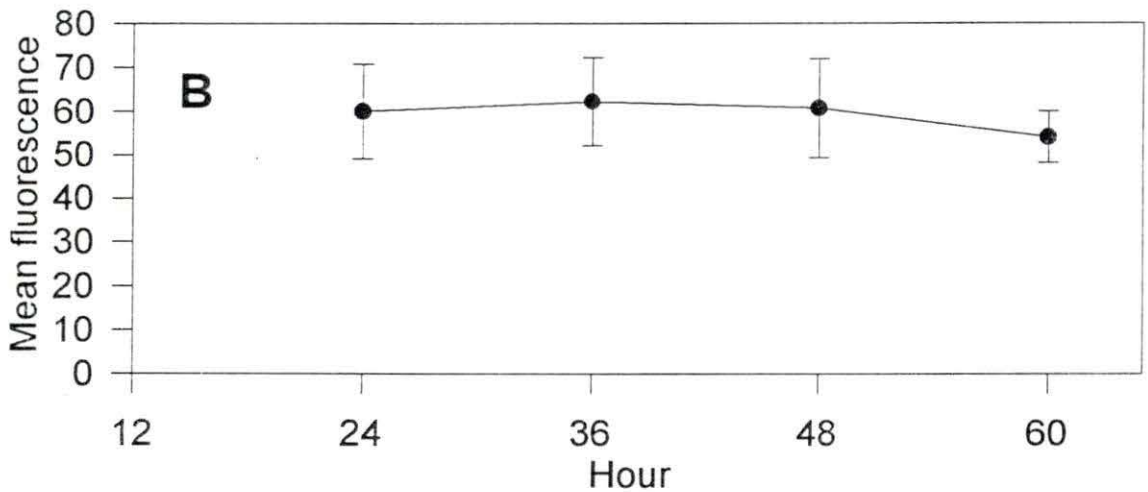
Hour


B
Mean fluorescence
80
70
60
50
40
30
20
10
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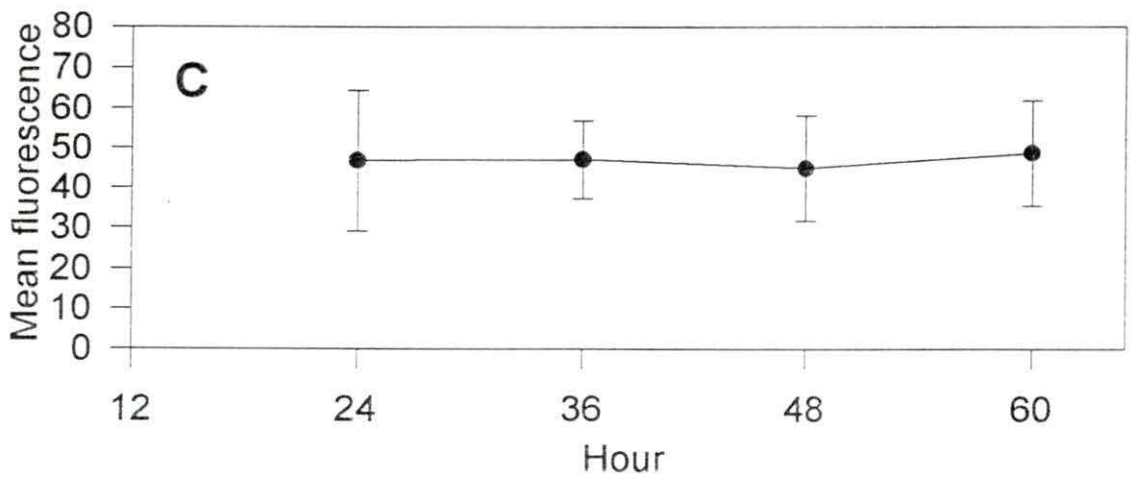
12
24
36
48
60
Hour


C
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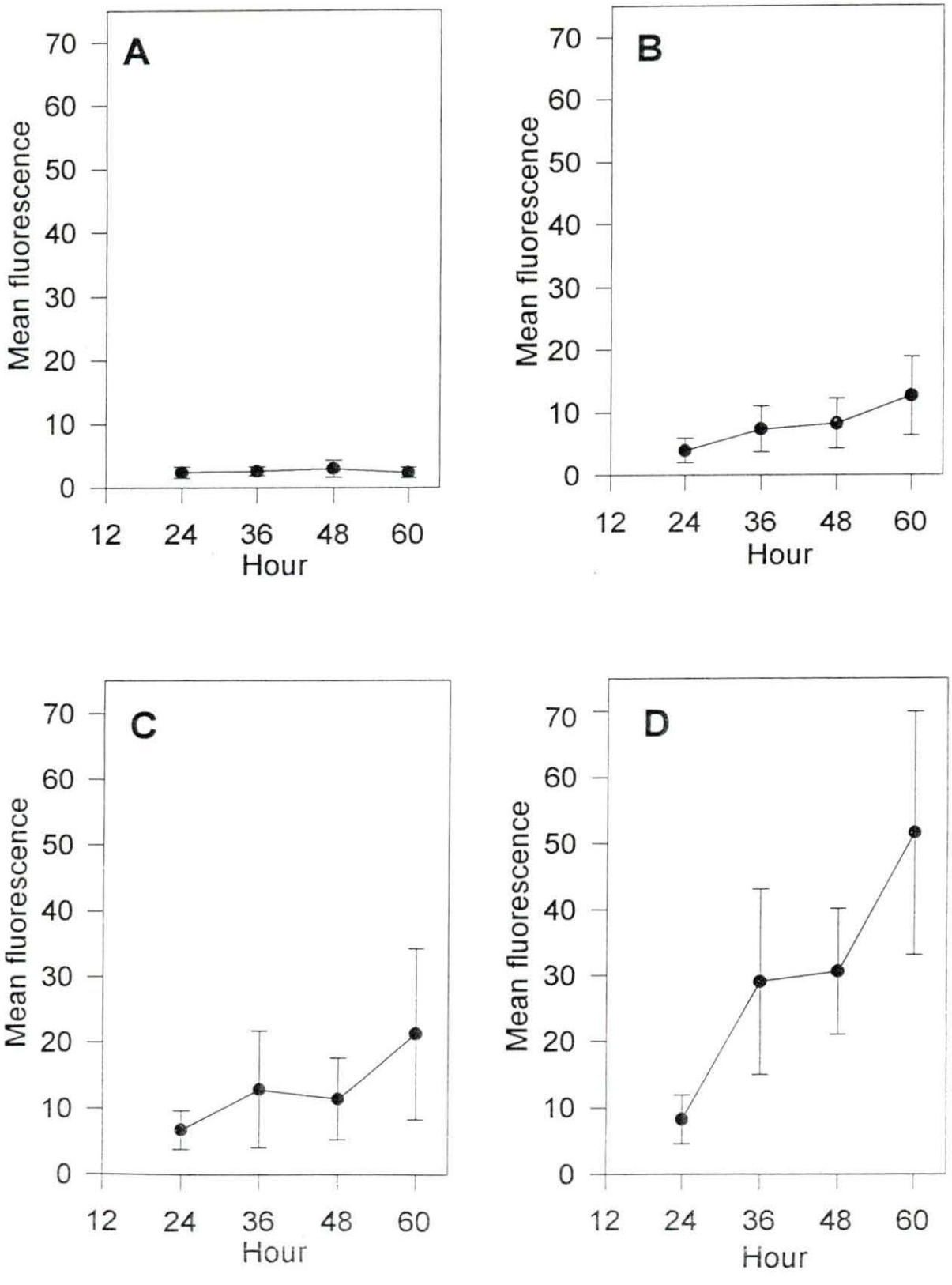
Hour

B cells and non-expressing T cells. The surface antigen level in MQ-NCSU seemed to be increased by this IFN, but was not statistically significant. However, the surface antigen level in DT-40 pre-B cells were weakly but significantly induced.

Induction of chicken MHC class II cell-surface antigen by recombinant chicken IFN

When recombinant chicken IFN (ChIFN) became available, it was used to perform the assays because of its greater purity and specificity. To determine the level of recombinant ChIFN needed to induce MHC class II antigen expression, the concentration of ChIFN that would give an equivalent effect to p34(200) IFN-rich supernatant on MQ-NCSU was first determined. It was found that a 1:2000 dilution of the ChIFN had a similar effect as 1:2000 dilution of p34(200)($p = 0.5095$) (Fig.9). This dilution point was then used as a starting reference point to determine the optimum IFN dilution for induction of the cells. It was found that 1:200 dilution of the CHIFN had a more profound effect of MHC class II antigen induction on MQ-NCSU than 1:2000 p34(200)($p = 0.02617$). The same assay was performed with RP-9 and MSB-1, each chosen as representative of the B cell and T cell categories of the immune cells. The RP-9 cells were not inducible to even higher levels of MHC class II expression with 1:400, 1:200 or even 1:100 dilutions of the ChIFN (p

Fig.9 Comparison of effect of 1:2000 and 1:200 dilution of recombinant chicken IFN to 1:2000 dilution of the p34 IFN-rich supernatant on IFN-induced expression of MQ-NCSU at 24, 36, 48 and 60 hour intervals. A. No IFN. B. 1:2000 dilution of p34(200). C. 1:2000 dilution of the recombinant chicken IFN. D. 1:200 dilution of the recombinant chicken IFN. Results showed 1:200 dilution of the recombinant chicken IFN has a profound inducibility on chicken MHC class II gene expression. n=3.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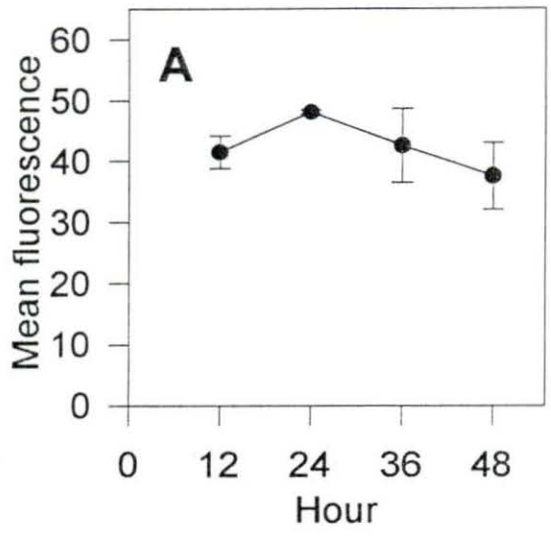
values for each condition tested is in the range of 0.4374 to 0.9787) (Fig.10). The mean fluorescence of MSB-1 cells increased from 2.35±0.29 to 4.97±0.72 after induction with 1:100 dilution of the ChIFN for 48 hr ($p = 0.03409$) (Fig.11). Comparison of levels of MHC class II protein expression between RP-9 cells (which constitutively express chicken MHC class II surface antigen) and MQ-NCSU induced at optimum condition showed that expression of the chicken MHC class II surface antigen in MQ-NCSU cells could be induced by the recombinant ChIFN to an even higher level than the RP-9 cells (Table 4). Fig.12 showed a representative sample of one assay from each cell type.

MQ-NCSU, when induced by a 1:200 dilution of recombinant ChIFN, showed a twenty fold increase in chicken MHC class II surface antigen level. The MSB-1 cell line had a two fold increase in the antigen level when induced with the ChIFN. However, RP-9 B cell line was not induced to a higher surface antigen level from its already high level of MHC class II expression.

Northern blot analysis

MQ-NCSU were induced with 1:400 and 1:200 dilutions of recombinant chicken IFN for 54 hours. This time was chosen to ensure the viability of the cells and was close to the 60 hr

Fig.10 Time and dose effect of recombinant chicken IFN on RP-9 chicken B cell line. Level of chicken MHC class II surface antigen is expressed by mean fluorescence which determined by flow cytometry analysis. A. No IFN. B. 1:2000 dilution of the recombinant chicken IFN. C. 1:400 dilution of the recombinant chicken IFN. D. 1:200 dilution of the recombinant chicken IFN. E. 1:100 dilution of the recombinant chicken IFN. Chicken MHC class II gene expression was not effected by any concentration of IFN. n=3.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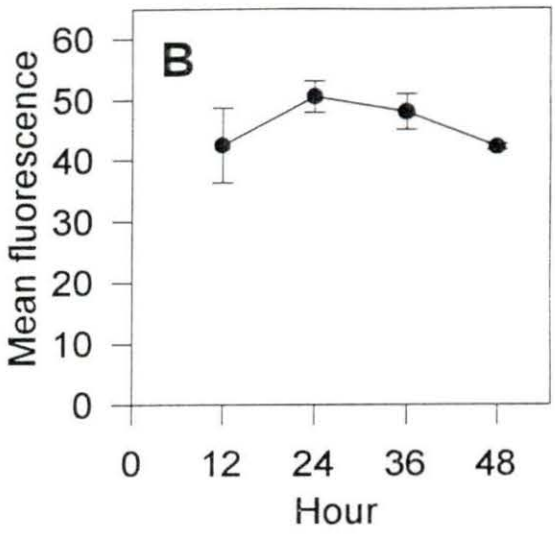
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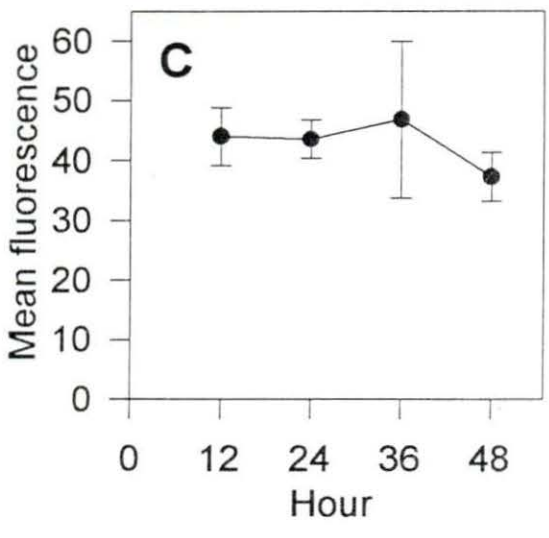
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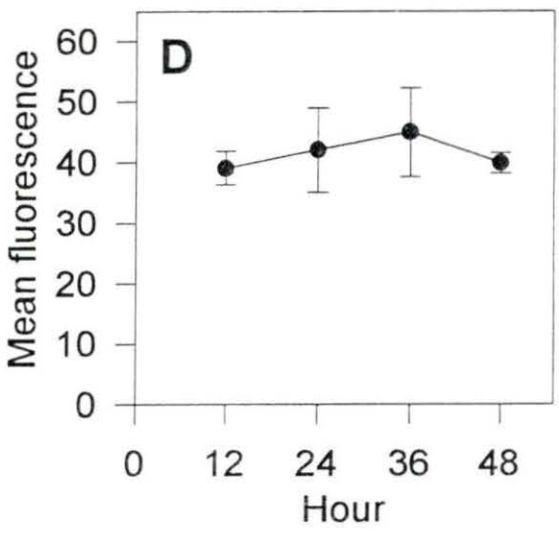
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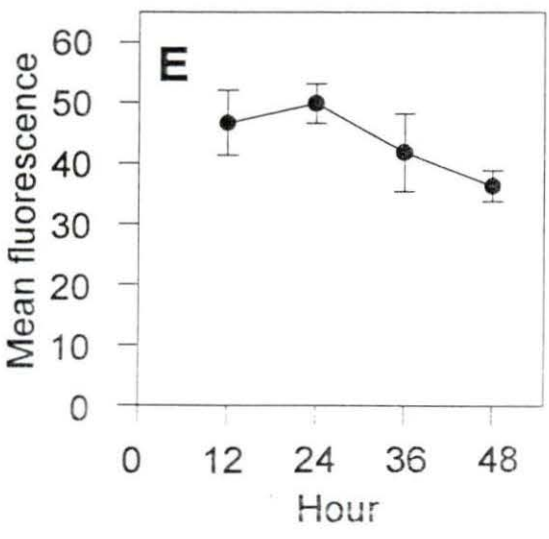
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Mean fluorescence
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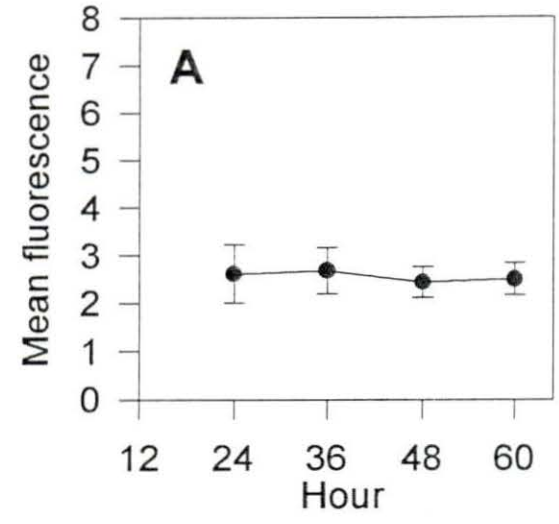
Hour

Fig.11 Time and dose effect of recombinant chicken IFN on MSB-1 cell line chicken MHC class II surface antigen expression level. Mean fluorescence (± SEM) determined by flow cytometry analysis. A. No IFN. B. 1:2000 dilution of the recombinant chicken IFN. C. 1:400 dilution of the recombinant chicken IFN. D. 1:200 dilution of the recombinant chicken IFN. E. 1:100 dilution of the recombinant chicken IFN. Chicken MHC class II gene expression was not effected by any concentration of IFN. n=3.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Mean fluorescence
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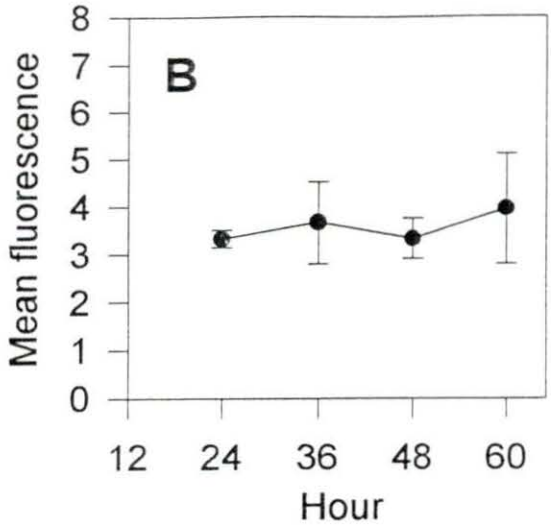
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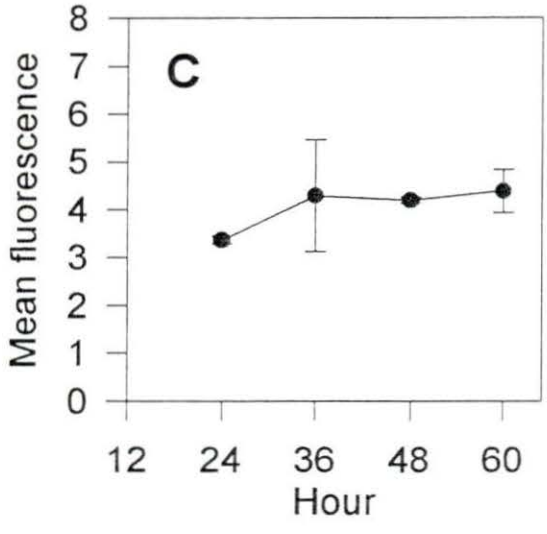
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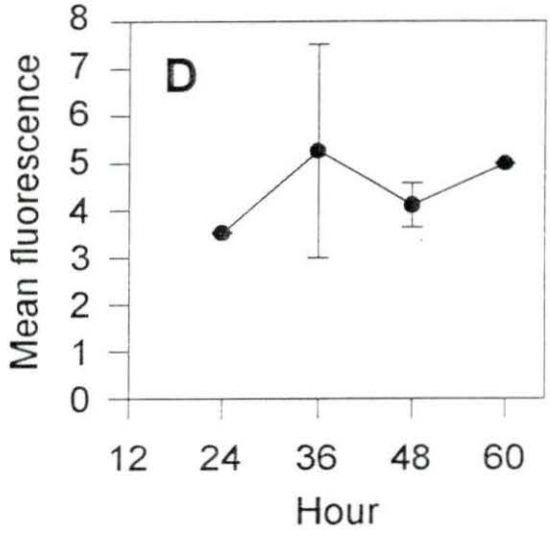
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Mean fluorescence
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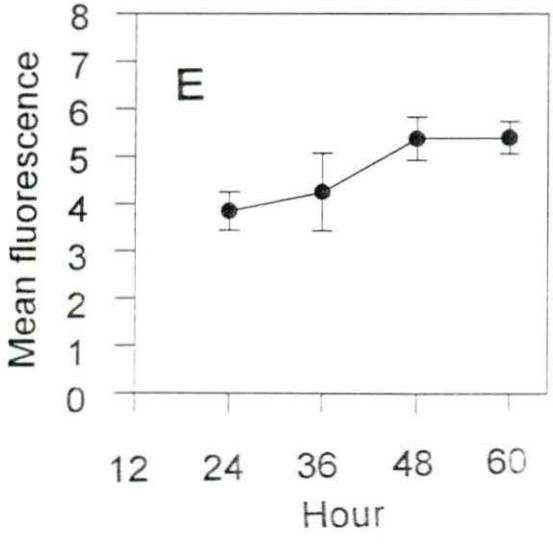
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Mean fluorescence
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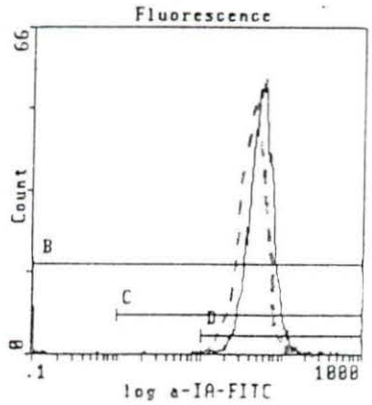
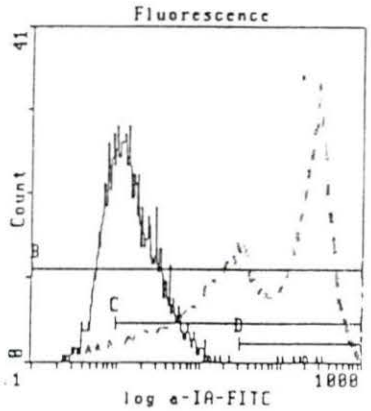
RP-9



MQ-NCSU



MSB-1

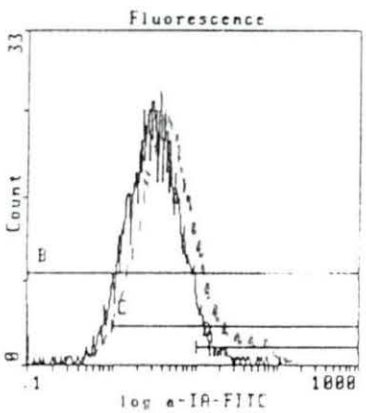


Fig.12 Distribution of mean fluorescence showing chicken MHC class II expression with and without recombinant IFN treatment of RP-9, MSB-1 and MQ-NCSU cell lines. Solid lines indicate distribution of mean fluorescence before IFN treatment. Broken lines indicates curve shift after ChIFN treatment.

Table 4. Recombinant chicken IFN-induced increase in chicken MHC class II surface antigen expression in chicken cell lines

Cell line	Basal level	Level after induction	Fold increase
MQ-NCSU	3.57±2.14	63.97±22.46	20
MSB-1	2.35±0.29	4.97± 0.72	2
RP-9	42.23±5.47	41.61± 5.58	none

*Antigen expression level is expressed as mean fluorescence (mean ± SEM) determined by flow cytometry analysis

point which showed highest induction of MHC class II antigen. Uninduced MQ-NCSU did not have a detectable level of the chicken MHC class II gene transcript, but detectable transcripts were seen with induced MQ-NCSU (Fig.13a). Transcripts shown were of the same size in each sample, around 1.2 Kb, as that of the bursa.

The autoradiogram was probed with β-actin (Fig.13b) and used for normalization of the results of the p234-probed autoradiogram. Comparison among the normalized samples for the level of chicken MHC class II mRNA transcription (Table 5), indicated that bursa had the highest level of chicken MHC class II mRNA transcription. Transcription levels in RP-9 cells were almost as high as the bursa, and were at least 50 fold higher than the transcription in un-induced MQ-NCSU.

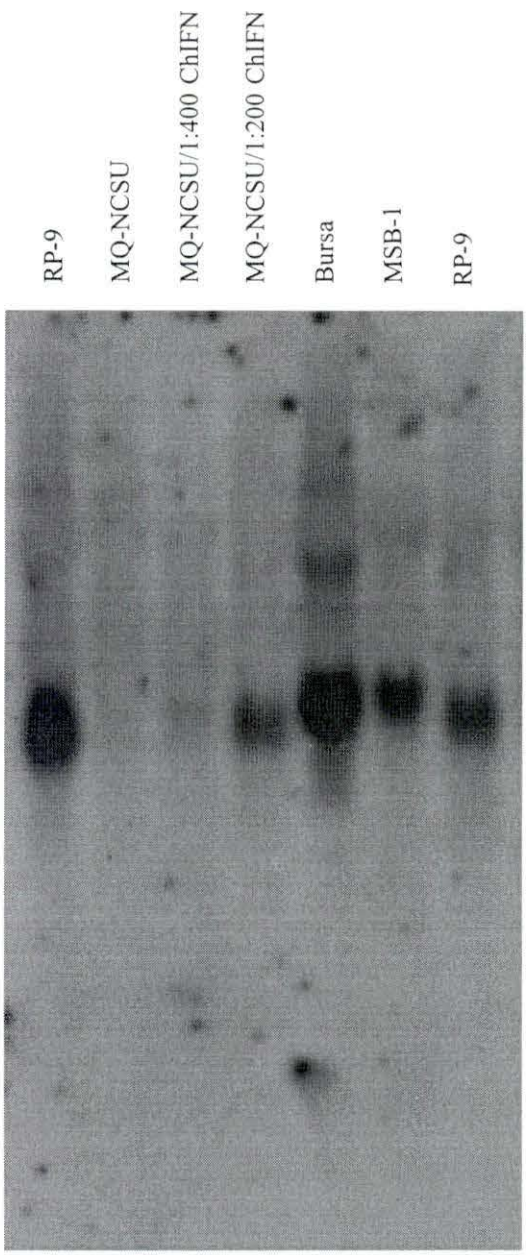


Fig. 13a. Northern blot analysis of mRNA transcription from different uninduced chicken cell lines and bursa cells with MQ-NCSU induced with 1:200 and 1:400 dilutions of CHIFN. The majority of chicken MHC class II gene transcripts are 1.2 Kb in size.

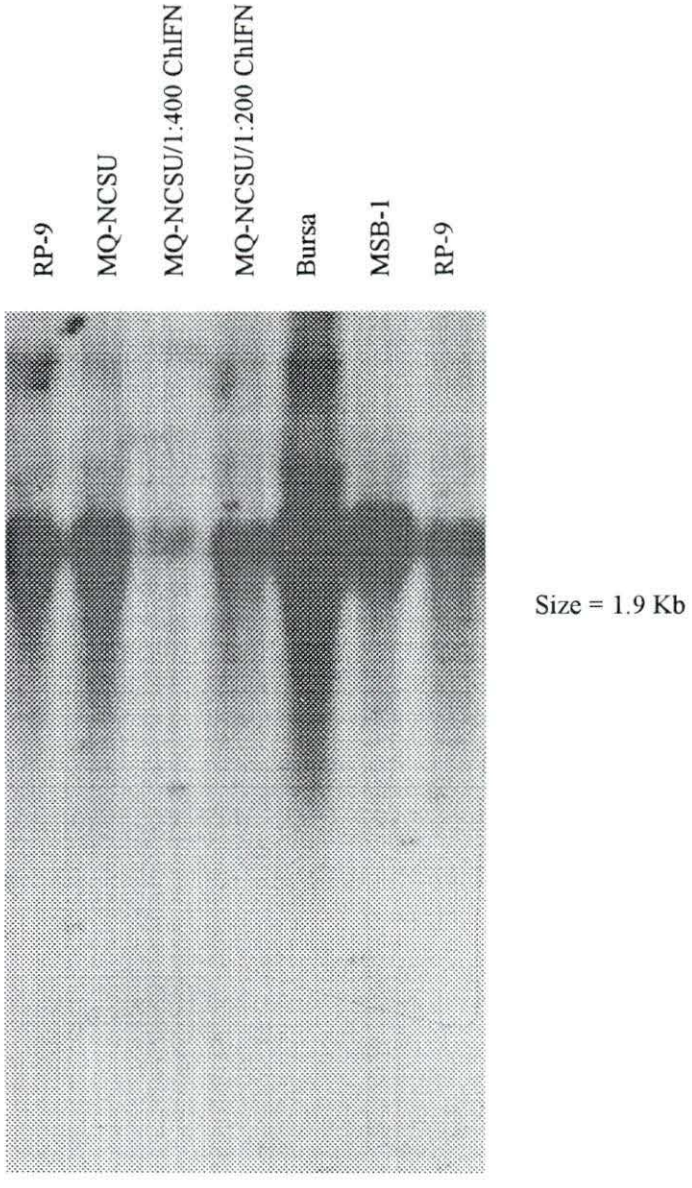


Fig. 13b. Northern blot analysis of chicken B-actin mRNA transcription from different uninduced chicken cell lines and bursa cells with MQ-NCSU induced with 1:200 and 1:400 dilutions of ChIFN.

Table 5. Ratio of chichen MHC class II gene transcription after normalization.

Cell line	intensity p234 /b-actin	ratio of chicken MHC class II gene transcribed
RP-9	27.61 / 32.43	0.8514
MQ-NCSU	0.94 / 32.06	0.0293
MQ-NCSU/ 1:400 ChIFN	2.68 / 11.47	0.2337
MQ-NCSU/ 1:200 ChIFN	11.81 / 26.31	0.4489
Bursa	28.31 / 30.38	0.9319
MSB-1	15.36 / 36.81	0.4172
RP-9	13.29 / 23.72	0.5603

This difference matched the result of the surface antigen level detected by flow cytometry (Table 2). MQ-NCSU stimulated with 1:200 dilution of recombinant ChIFN has about twice the amount of the MHC class II mRNA as MQ-NCSU stimulated with 1:400 dilution of ChIFN. The amount of mRNA transcribed from MQ-NCSU stimulated with 1:200 dilution of

ChIFN was about half that of the RP-9 cells, and about twenty fold greater than that of the uninduced MQ-NCSU.

Surprisingly, the MSB-1 cell showed presence of an apparently larger-sized class II mRNA transcript. The size difference may be an artifact of overloading in the lane because the β-actin autoradiogram showed the same phenomena. The abundancy of the MHC class II mRNA in MSB-1 cells was comparable to that of the MQ-NCSU induced with 1:200 dilution of ChIFN. When looked at the protein level, the mean fluorescence value of MSB-1 cells at its optimum inductin condition was only 1/10 of that of the MQ-NCSU stimulated at its optimum induction condition.

It was shown here recombinant ChIFN can induce an increase in mRNA transcription in MQ-NCSU. The macrophages had a twenty fold increase in mRNA level when incubated with 1:200 dilution of ChIFN. This correlated with the increase in MHC class II surface antigen detected by flow cytometry. When the concentration of the ChIFN was lowered by two fold, the mRNA level also decreased by two fold. Therefore, IFN induction of chicken MHC class II expression occurs at the level of RNA transcription in MQ-NCSU. In MSB-1 cells, MHC class II antigen expression did not correlate with the level of mRNA trancription.

DISCUSSION

This research demonstrated that cell-specific expression of MHC class II genes exists in the avian species as well as their mammalian counterparts. The level of the chicken MHC class II surface antigen detected in B cells by flow cytometry was significantly higher than MHC class II antigen levels detected in T cells and macrophages. In macrophages, the chicken MHC class II antigen was inducible by IFN. This induction was time and dose-dependent and occurred at the transcriptional level. These findings support the importance of MHC genes in the immune regulation of chicken and define a model system for the study of inducible gene expression in the chicken using MQ-NCSU and recombinant chicken IFN.

Effect of IFN on cell-specific expression of MHC class II gene expression

Relatively few studies have been reported on the impact of IFN on chicken MHC class II gene expression. Kasper et al. (1994) demonstrated that normal chicken peripheral blood macrophages, when cultured in the presence of the partially purified immune IFN, p34(200), have a dose-dependent increase

in cell surface MHC class II antigen expression. A measurable difference in the RNA transcripts between induced and uninduced cells were observed at 48 hr post-induction with the optimum IFN concentration of 1 to 2.5%. Using recombinant IFN, Schultz et al. (1995) occasionally observed "weak but significant" macrophage activation with replated primary monocytes/macrophages. However, true primary cultured monocytes/macrophages were not induced with ChIFN.

In the present study, the level of the MHC surface antigen on MQ-NCSU started to increase after 36 hours and reached its height of expression after incubation for 60 hours. A 1:2000 dilution of the p34(200) IFN-rich supernatant was the optimum concentration used for the induction of MQ-NCSU. This concentration is equivalent to the 1% level of Kasper et al. However, the level of antigen induction in this research was about one quarter of what Kasper et al.(1994) detected. The optimum concentration for the recombinant IFN was a 1:200 dilution. Schultz et al.(1995) did not report the procedures for the induction of the primary macrophages with IFN. Therefore, we were not able to compare if any factor in the research was different than ours, beside the cells used. The level of the chicken MHC class II surface antigen increased about 20 fold after induction with the recombinant IFN in the present research. However, larger doses of both kinds of IFN above the optimum concentration led to cell death. The ability of recombinant ChIFN to induce chicken

MHC class II expression in macrophages, supports the study of Schultz et al., (1996) that indicates that this recombinant ChIFN could be a chicken homologue of mammalian IFN-γ.

In the RP-9 B cell, added IFN derived from the p34(200) supernatant or recombinant ChIFN, could not further induce a higher level of expression from the already high expression of chicken MHC class II protein. It had been shown in mammals that IFN can be antiproliferative (Pestka et al., 1987), and this property might contribute to the inhibitory effect of IFN on cells with a higher metabolic rate. It is also known that IFN induces an antiviral state by inhibition of viral mRNA transcription, by increasing the rate of decay of viral mRNA, or by production of antiviral proteins. We looked to see if this additional IFN may have had a slight inhibitory effect on the class II surface antigen expression of the cells with extended incubation. However, this difference does not appear to be significant based on Student's t-test.

It is not understood why expression of the MHC class II antigen in B cells is not further inducible, but very weak and statistically significant inducibility for 12 hours was seen in the MHC class II antigen negative DT-40 pre-B cell with higher concentration (1:1000 dilution) of p34(200). Perhaps, when constitutive class II gene expression is functioning in B cells, intracellular mechanisms mediating

the effect of induction by exogenous stimuli of the same genetic system have no ability to function (Sartoris et al., 1990). As for resting B cells, it has been reported that pre-activated T cells were able to induce differentiation and proliferation of class II- deficient small resting B cells (Markowitz et al., 1993). Perhaps, DT-40 pre-B cells could be induced to differentiate to a stage that is starting to express MHC class II antigen. It has been reported that some of the negative regulation of the class II expression in the human DQβ promoter region was relieved after administration of IFN-γ in resting HeLa cells (Tsang et al., 1988). Therefore, how the negative region in the promoter is utilized may determine whether the expression of the MHC class II gene is constitutive or inducible.

During flow cytometry, bimodal peaks in mean fluorescence shift often arose after 60 hr incubation of macrophages with IFN (Fig.4). This occurred when mean values of induced MHC class II expression were very high. This phenomenon may occur because certain cells in the culture respond more promptly to IFN induction and became very highly induced, whereas some others are not so readily induced. This could be due to varying stages of cell differentiation at the time of assay or to heterogeneity of the cell populations in the cell line. The heterogeneity could be studied by sorting the cells that account for the different peaks and incubating the sorted cells with IFN for a longer period to

see if the population with the lower mean fluorescence value will eventually be induced to have higher mean fluorescence value. If the variation in differentiation stages accounts for the bimodal peak, it might suggest that different stages of cells have different sensitivity to IFN induction. If so, which stage of differentiation may contribute to the IFN-inducibility of the macrophages may be interesting to study.

The recombinant IFN resulted in a higher level of induction of the MHC class II antigen expression and a more stable state, with less cell death than p34(200) (Fig.5). This result might be due to the possibility that IFN generated through the induction of T cells with ConA, could at the same time produce elements such as tumor necrosis factors (TNFs). TNFs inhibit the action of the IFN and may be cytolytic to cells (Pestka et al., 1987). Additionally, it has been shown that conditioned medium from ConA stimulated splenocytes is toxic to MDV cell lines (Volpini et al., 1995). This could be the cause of the cell death seen when splenocyte-derived T cell line p34(200) IFN-rich supernatant was incubated with the macrophages.

Recombinant ChIFN induced a higher antigen expression level than p34(200) supernatant. Schultz et al.(1995) determined the specificity and purity of the ChIFN to be very high based on lack of the MAF activity. Macrophage activation can be measured as a function of increased release of NO or H_2O_2 into the culture medium. Bacterial LPS activates

macrophages universally. Schultz et al. (1995) observed increased MHC class II antigen expression on the primary macrophages using partially purified ChIFN and LPS-supplemented ChIFN, but not with pure ChIFN. Therefore, they deduced that activation of macrophages came from a co-induced cytokine present in the partially purified supernatant, and confirmed the purity of the purified ChIFN. The results of the studies with ChIFN, therefore should be considered as representative of the actual effects of isolated IFN, without the complications of other cell-produced factors. Therefore, the higher activity of the recombinant IFN is due to its purity, or its stability, or both. Whether this quality contributes to the stability of the induced RNA transcripts also needs to be explored.

Transcriptional control of chicken MHC class II gene

Transcriptional control is one mechanism for regulating the expression of genes. Transcripts detected in RP-9 and induced MQ-NCSU have the same size as the transcript obtained from the bursa, which is known to be a tissue having high MHC class II expression. Comparison of transcripts from MQ-NCSU cells stimulated with ChIFN showed that transcription increased two fold as the concentration of ChIFN increased two fold. Comparison of transcripts between un-induced MQ-

NCSU, MQ-NCSU induced with 1:200 dilution and 1:400 dilution of ChIFN showed that the increase in the MHC class II mRNA level correlated with the increase in the MHC class II surface antigen level, which were both a twenty fold increase from uninduced MQ-NCSU to MQ-NCSU induced with 1:200 dilution of ChIFN. The increased level was comparable to the level of RP-9 cell line.

A different story was observed with the MSB-1 T cells. The T cells did not demonstrate class II surface antigen during flow cytometry analysis. However, these uninduced T cells have transcripts of about the same size as the MHC class II surface antigen positive cells. The abundancy of the mRNA of uninduced MSB-1 was comparable to that of the MQ-NCSU induced with 1:200 dilution of ChIFN. In MSB-1 cells, the level of MHC class II mRNA transcription did not correlate with the level of the protein expression. Although a two fold increase in mean fluorescence value was observed in MSB-1 induced with 1:100 dilution of ChIFN, the increased mean fluorescence was only 1/10 of that observed in MQ-NCSU induced with 1:200 dilution of ChIFN. Therefore, another mechanism, probably a post-transcriptional modification event, may account for the non-expression of the MHC class II protein in T cells.

It has been suggested that Marek's disease virus (MDV) can influence the regulation of the immune system by altering the expression of MHC class II antigens on immunocompetent

cells (Lessard et al., 1995). It has also been reported that MHC class II expression is down-regulated in antigen presenting cells by an herpesvirus (Epstein-Barr virus) (de Waal Malefyt et al., 1991), and by murine sarcoma virus (Maudsley et al., 1989). This down-regulation by MDV may be a reason for the lower chicken MHC class II expression induced by p34(200) on MQ-NCSU (which were derived from birds that had been infected with MDV) than that of the peripheral monocytes of Kasper et al. (1994). If this cell line is down-regulated, it is possible that MSB-1 T cell is down-regulated too.

At present, the chicken cell lines available are all virally-transformed. We are unable to know at present whether this is the reason why ChIFN did not induce MHC class II expression on truly primary macrophages but worked in the MQ-NCSU. Although a different mAb was used for staining the MHC class II surface antigen, we could postulate the cell difference as one of the factors for the higher expression level induced by Kasper et al.(1994), with p34(200), than what is shown here. It is possible that Marek's disease virus gene has an effect in down-regulating the MHC class II gene expression in this cell line. If the difference truly came from the difference of the cell line used, one should also investigate whether this difference could change the expression pattern and gene regulation of virally-transformed cell types from that of a normal, whole organism. However,

these question can only be addressed when non-virally transformed cell lines become available.

An important and widely used mechanism for regulating gene expression in both prokaryotes and eukaryotes is modulation of the efficiency of transcription initiation, in which cis and trans-acting elements play an important role (Mitchell and Tjian, 1989). In eukaryotes, complex programs of cis-acting elements and trans-acting binding proteins are involved, and IFN could act as one of the regulating factor that either activates or inhibits gene expression through several pathways in different tissues (Pestka et al., 1987).

The previous study (Chen et al., 1997) on the CCII-7-1 promoter showed the presence of a G-C rich region instead of a TATA box. Deletion analysis of S, X, Y boxes of the promoter showed that these boxes are not required for inducing chloramphenicol acetyltransferase activity in CAT expression vector in macrophage (Chen et al., 1997). It is possible that the S, X, Y boxes are not required for basal expresion in unstimulated macrophages, but may be critical in cells expressing high levels of class II antigen. The present research showed that cell specificity does exist in chicken class II gene expression, and it is known that X, Y boxes are essential for the cell-specific and inducible expression of mammalian MHC class II gene (Viville et al., 1991). Thus, there is need to reconsider the role of the X, Y boxes found in the chicken class II promoter. Footprinting and mobility

shift assays may identify transcription factors which act on the class II promoter, and how they contribute to the cell-specificity of the gene. These await further studies, especially, due to the fact that the chicken MHC class II gene promoter has characteristics of a typical housekeeping gene in sequence but, as shown in the present research, has cell-specific expression. Generally, housekeeping genes lack a TATA box and are GC-rich, and are usually constitutively expressed in all cell types.

In the previous study by Xu et al. (1989), three interferon concensus sequences (ICS) were located in the CCII-7-1 promoter. These may account for the IFN-induced expression. Alternately, if the negative regulatory element (NRE) accounts for the inhibitory effect of the expression, the IFN may inhibit the action of the NRE in the macrophage and permit transcription of the gene.

It is not really known yet, how the chickens achieve control of the expression of the MHC class II gene and how IFN changes the expression machinery to achieve the induction action. Therefore, immediate determination of the species of the DNA-binding protein acting on the gene and how the transcriptional factors act on this gene's promoter or interact with each other, with and without IFN, would be a valuable area to research. It is also knwn that different signal transduction pathways (the Stat-1 pathway and the cyclic AMP pathway) are involved in increasing mRNA levels

under the influence of IFN (Pestka et al., 1987; Lee and Benveniste, 1996). Therefore, studies of the cell lines, whether in the kinetical, cellular or molecular aspects of IFN inducibility will yield better understanding of the transcriptional control of the chicken MHC class II gene.

APPENDIX

Representive isotype control mean fluorescence values of each cell type.

cell lines	hr	mean fluoresence value range	mean
RP-9(uninduced)	12	0.263-0.513	0.380±0.100
n=3	24	0.352-0.415	0.384±0.026
	36	0.289-0.419	0.350±0.053
	48	0.278-0.447	0.381±0.074
RP-9(1:2000p34(200))	12	0.281-0.588	0.413±0.065
n=3	24	0.272-0.647	0.426±0.079
	36	0.278-0.517	0.402±0.049
	48	0.276-0.547	0.394±0.057
MSB-1(uninduced)	24	0.248-0.352	0.277±0.025
n=4	36	0.258-0.296	0.274±0.008
	48	0.257-0.404	0.313±0.034
	60	0.296-0.361	0.328±0.015
MSB-1(1:2000p34(200))	24	0.273-0.361	0.300±0.024
n=4	36	0.261-0.333	0.300±0.015
	48	0.260-0.370	0.318±0.023
	60	0.286-0.321	0.309±0.008
DT-40(uninduced)	24	0.362-0.925	0.641±0.162
n=3	36	0.369-0.988	0.685±0.179
	48	0.275-0.953	0.628±0.196
	60	0.279-0.688	0.461±0.120
DT-40(1:2000p34(200))	24	0.248-0.859	0.571±0.177
n=3	36	0.267-0.660	0.513±0.124
	48	0.253-0.946	0.575±0.201
	60	0.269-0.735	0.470±0.138
MQ-NCSU(uninduced)	24	0.320-0.578	0.447±0.053
n=4	36	0.693-1.440	0.960±0.165
	48	0.590-0.920	0.753±0.079
	60	0.609-0.868	0.695±0.062
MQ-NCSU(1:2000p34(200))	24	0.357-1.310	0.833±0.196
n=4	36	0.574-1.960	0.952±0.337
	48	0.223-0.769	1.060±0.393
	60	0.511-1.770	1.002±0.290
CEF(uninduced)	24	1.070-1.290	1.170±0.091
n=3			

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