Identification and characterization of a chicken major histocompatibility complex class II[beta] gene promoter

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Identification and characterization of a chicken major histocompatibility complex class IIB gene promoter

by

Yunfei Chen

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

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1. GENERAL INTRODUCTION

1.1. Organization and functions of the mammalian major histocompatibility complex (MHC)

The MHC was first defined as a locus responsible for acceptance or rejection of transplantation (Gorer 1936a, b, 1937, 1938; Gorer et al. 1948). Later it was demonstrated that the MHC is actually a chromosomal region containing groups of genes that play important roles in the immune system (Klein 1986). Studies on the mammalian MHC have revealed three major classes of genes in this region. Figures 1 and 2 show the organization of the MHC in humans (termed HLA) and in mouse (termed H-2), respectively. Each contains class I, class II, and class III subregions. Within each subregion, multiple genes are located, some of which are pseudogenes (Kappes and Strominger 1988). Class III genes encode complement factors and other proteins such as tumor necrosis factor and 21-hydroxylase (Carroll et al. 1984a, b, 1985, 1987; Spies et al. 1986, 1989; Levi-Strauss et al. 1988; Sargent et al. 1989). Both class I and class II genes are highly polymorphic. They encode cell-surface glycoproteins that present antigens to T cells (Germain 1986, Figure 3). The T cells involved in this process recognize the processed antigens only in association with self MHC molecules, but not with foreign MHC molecules. This phenomenon is known as MHC restriction (Zinkernagel and Doherty 1979; Dausset 1981; Schwartz 1986). Class I proteins are expressed on almost all nucleated cells. They bind to peptides derived from breakdown of proteins inside the cell, which include self proteins as well as viral antigens in an infected cell. The class I protein then is able to present the bound peptide to CD8⁺ T cells, including cytotoxic T cells, which release cytolycic substances upon recognizing particular antigens associated with class I molecules. Class II (also known as Ia) proteins are expressed on limited types of cells, including B cells, activated T cells, and
Figure 1. Genetic organization of the HLA complex. Genetic distances are indicated in centimorgans (cM) and kilobases (kb). R.F. = recombination frequency. (from Xu 1990)
THE HLA COMPLEX

REGIONS

SUBREGIONS

GENES

R.F. (cM)

(1.0)(0.7)(0.3)(0.1)(0.7)
Figure 2. Genetic organization of the H-2 complex. Genetic distances are indicated in centimorgans (cM) and kilobases (kb).
R.F. = recombination frequency. (from Xu 1990)
THE H-2 COMPLEX

REGIONS

I I I I I I I

SUBREGIONS

K IA IE S D Qa Tla

GENES

K2 K Aβ3 Aβ2 Aα Eβ2 21-OHB Slp Bf C2 TNFα D D2,3,4 L Q1,2,4,5,6,7,8/9,10 Tla

R.F.(cM) (0.02)(0.1)(0.11)(0.26)(0.05)(1.5)

SCALE(kb) TOTAL ~2300 kb
Figure 3. Antigen presentation by MHC molecules. Possible pathways of the MHC class I and class II molecules, and the antigens presented by these molecules in an antigen-presenting cell are shown. (from Germain 1986)
CLASS II MHC (Ia)

EXOGENOUS ANTIGEN
- PRESENTED
- DEGRADED

ENDOGENOUS ANTIGEN
- PRESENTED
- DEGRADED

CLASS I MHC (H-2, HLA)

ENDOSOME
LOW pH
LYSOSOME
macrophages. Class II molecules bind to peptides derived from proteolytic cleavage of proteins phagocytosed from the outside environment by the antigen presenting cells. CD4+ T cells then recognize the peptide bound to class II molecules through T cell receptors (TCR) and initiate a cell-mediated immune response against the antigen (Klein et al. 1981; Germain 1986). Because TCR can only recognize MHC-bound antigen peptides, binding of peptides to the MHC molecules becomes a crucial step in immune responses.

1.2. Structure of and antigen binding by the MHC proteins

The MHC class I and class II proteins belong to the immunoglobulin supergene family. A class I molecule consists of a 44 kD α chain associated noncovalently with a 12 kD β2-microglobulin. The gene encoding β2-microglobulin is not linked to the MHC. The α chain is glycosylated and contains five domains, which correspond to the exons of an class Iα gene (Figure 4). Among them, the α1 and α2 domains are responsible for the polymorphic nature of the class I molecules (Klein 1986). A class II molecule consists of a 33 kD α chain and a 28 kD β chain, which are noncovalently associated to each other (Figure 4). Both α and β chains are membrane-bound glycoproteins. The β2 domain is highly polymorphic, while the α chain shows limited polymorphism in the α1 domain (Kaufman et al. 1984). The structure of the genes encoding the class II α or β chains are shown in Figure 4.

The three-dimensional structures of human class I and class II proteins have been determined by X-ray crystallography (Bjorkman et al. 1987a, Brown et al. 1993. See Figure 5). Despite the difference in their subunit organization, the HLA-A2 (class I) and HLA-DR1 (class II) molecules have similar structures, especially at the antigen binding sites. The antigen binding site is supported by two immunoglobulin-like domains. In the class I molecule, the α1 and α2 domains form the antigen-binding groove, while the α3 domain
Figure 4. Structures of the MHC class I and class II antigens and genes. The class I molecule consists of an $\alpha$ chain non-covalently associated with a $\beta_2$-microglobulin. The class II molecule consists of an $\alpha$ chain and a non-covalently linked $\beta$ chain. The domains and their corresponding exons in the genes are shown. Glycosylation sites are represented by small dots attached to the peptide chains. (from Schwartz 1986)
CLASS I MOLECULE

CLASS II MOLECULE

CLASS I α GENE

β2M GENE

CLASS II β GENE

CLASS II α GENE.
Figure 5. Three-dimensional structures of the MHC class I and class II molecules. A. Structure of the HLA-A2 (class I) molecule. The N and C terminus and the domains are shown (from Bjorkman et al. 1987a). B. Structure of the HLA-DR1 (class II) molecule as a dimer. The peptides bound to the MHC class II molecules are shown as dark arrows (from Brown et al. 1993). C. The top view of the antigen-binding site of the HLA-A2 molecule (from Bjorkman et al. 1987a). The bottom of the binding site is formed by β-sheet, and the sides by helices. The antigen-binding site of the HLA-DR1 molecule is very similar to the binding site of the HLA-A2.
and the β2 subunit support from the underside. In the class II molecule, the α1 and β1 domains form the antigen-binding groove, supported by the α2 and β2 domains. The antigen binding groove of both molecules consists of eight strands of antiparallel β-sheet as bottom and two antiparallel helices as sides (Figure 5C). The class II molecule, however, does not have closed ends at the groove as the class I molecule does. This is consistent with the fact that class II molecules bind more extended peptides of 13 to 24 residues (Rudensky et al. 1991; Hunt et al. 1992; Chicz et al. 1992, 1993), whereas class I molecules bind mostly short, nonameric peptides (Falk et al. 1991). Most of the polymorphic residues are located inside the groove (Bjorkman et al. 1987b). This may explain the association between MHC and immune responsiveness to particular antigens, because different alleles of MHC molecules may have different residues in their antigen binding groove, and therefore have different binding affinities to a particular peptide.

The HLA-DR1 molecule was observed as a dimer in crystals. This suggests that T cells are activated through cross-linking when they are bound to dimeric forms of MHC molecules associated with specific peptides.

1.3. Regulation of MHC class II gene expression.

Although the level of expression of class I MHC molecules can be modulated by certain factors, such as interferon (IFN), most cells constitutively express these molecules (Friedman et al. 1984; Rosa et al. 1986). In contrast, expression of class II MHC molecules is strictly regulated. B cells start to express class II molecules after differentiation from the pre-B cell stage and stop expression after becoming plasma cells (McKernan et al. 1984). A variety of inducers increase Ia expression in B cells. These include LPS, phorbol esters, and IL-4 (Monroe and Cambier 1983; Monroe et al. 1984; Rousset et al. 1988). Class II molecules are not expressed in resting T cells, but can be induced upon activation of these cells (Wollman et al. 1980; Robbins et al. 1988). In the macrophage cell lineage, Ia
expression varies depending on the differentiation stage, growth conditions, and the subpopulation (Unanue 1984). γ-interferon (γIFN) plays a very important role in regulation of Ia expression in macrophages. It increases the level of transcription of the class II genes (Basham and Merigan 1983; Koeffler et al. 1984). Some cell-types such as endothelial cells and fibroblasts, which are normally negative for class II antigens, are also induced to express class II genes by γIFN (Collins et al. 1984).

Other agents may down-regulate Ia expression. Sternberg et al. (1986) have found that serotonin suppresses γIFN-induced Ia expression in macrophages, whereas other monoamines tested (dopamine, histamine, tryptamine) have much less effect. Glucocorticoids reduce Ia expressions in B cells and γIFN-induced macrophages, but have no significant effect on the basal level of Ia expression in macrophages (Dennis and Mond 1986; Snyder and Unanue 1982; Warren and Vogel 1985).

1.4. Structure of the chicken MHC

The B locus was first described as a blood group (Briles et al. 1950), but was later identified as linked to the MHC in chickens (Schierman & Nordskog 1961). Extensive serological, biochemical, and genetic studies on this locus have demonstrated that the chicken MHC contains at least three loci: B-F, B-L, and B-G (Ziegler & Pink 1975, 1976; Pink et al. 1977). The B-F and B-L loci encode proteins that function as mammalian MHC class I and class II proteins, respectively. No mammalian counterpart for B-G has been found. The B locus was thus re-named the B complex because it contains multiple genes.

Application of molecular techniques have revealed many more details of the structure of the B complex. As proposed by Pink's three-locus model, the B complex has three major classes of genes (B-F, B-L, and B-G). Each class, however, includes more than one gene. The first chicken MHC genes isolated were B-Lβ genes, by cross-hybridization with a human HLA-DQβ cDNA probe under low stringency (Bourlet et al. 1988). Since
then more B-Lβ genes, as well as B-F genes, which were located on cosmid clones from a B12 chicken, were characterized (Guillemot et al. 1988; Kroemer et al. 1990b; Zoorob et al. 1990). Genomic and cDNA clones were also isolated from a G-B2 (B6) chicken (Xu et al. 1989; Sung et al. 1993).

The B complex is located on one of the microchromosomes which also contains the nucleolar organizer region. The size of the B complex is much smaller than mammalian MHC (Guillemot et al. 1989). Unlike the mammalian MHC, the chicken class I and class IIβ genes are interspersed, rather than being organized into distinct chromosomal regions (Figure 6). No class IIα gene or a class III region have been identified in the B complex, although there is evidence for linkage between class III genes and the B complex (Spike and Lamont 1995). Some non-MHC genes have also been found in the B complex (Guillemot et al. 1988; reviewed by Guillemot 1991; Guillemot et al. 1989). Meanwhile, isolation of B-G cDNA clones from expression libraries (Goto et al. 1988; Kaufman et al. 1989) have made it possible to characterize the B-G genes (Miller et al. 1991). Subsequent analysis of the B-G genes proved that the B-G molecules are highly polymorphic at the DNA level (Miller et al. 1988). The B-G genes are closely linked to the B-F and B-Lβ genes in the B complex.

More recently, Briles et al. (1993) identified a chromosomal location outside the B complex that contains sequences similar to MHC genes. They used a fully pedigreed family of chickens to observe a independently segregating, MHC-like locus designated Rfp-Y. This finding explains in part the inconsistency between serologically defined MHC haplotypes and DNA genotyping using MHC probes and demonstrates the power of DNA technology in genetic studies. One of the cosmid clusters (Guillemot et al. 1988), which contains two class I and two class IIβ genes, has been assigned to Rfp-Y by using a probe specific for that cosmid cluster (Miller et al. 1994).
Figure 6. Molecular organization of the chicken MHC (B complex). This diagram shows the location of the MHC and non-MHC genes on the three non-overlapping cosmid clusters from the $B^{12}$ haplotype. Six $B-F$, five $B-L$, one $B-G$, and eight non-MHC genes (represented by boxes) are located. An arrow above a gene indicates the direction of transcription. (from Kroemer et al. 1990a)
1.5. Association of the chicken MHC with immune and non-immune traits

The chicken MHC influences traits that are important in avian growth, reproduction, and health (reviewed by Bacon 1987; Lamont 1989, 1993). Among them, the association of disease resistance with the MHC is evident. This is not unexpected, because the MHC molecules are responsible for antigen presentation, which is a necessary step in cell-mediated and T cell-dependent humoral immune responses. Also, the MHC region contains or is linked to other genes of the immune system, such as the H-2 gene (Guillemot 1991). The resistance to Marek's disease in birds with MHC haplotypes B\(^{21}\) or B\(^{2}\) and the susceptibility in B\(^{19}\), B\(^{13}\), and B\(^{5}\) birds provides an excellent example of such association (reviewed by Bacon 1987). In the ISU S1 line, the B\(^{1}\) haplotype is more resistant to Marek's disease than B\(^{19}\) (Steadham et al. 1987). Briles et al (1983) have mapped the resistance to Marek's disease to the B-F/B-L subregion of the B complex. Resistance to numerous other diseases have been associated with the B complex. These diseases include Rous sarcoma, fowl cholera, lymphoid leukosis, coccidiosis, and autoimmune thyroiditis (Lamont et al. 1987a; reviewed by Bacon 1987).

The B complex is also associated with growth and reproduction. There have been reports that MHC diversity affects mortality, egg production, fertilization rate, body weight, hatchability, and feed efficiency (Bacon 1987). Selection for egg production has resulted in alteration of MHC allelic frequencies (Simonsen et al. 1982; Lamont et al. 1987b). It is not yet clear if the MHC genes themselves exert certain functions to influence these traits or if these effects are carried out by genes that are linked to the MHC region.

1.6. Initiation of transcription

The ultimate understanding of genes requires knowledge on their expression. Expression of a gene is regulated at different levels, including activation of the chromosomal region, transcription, post-transcription, translation, and post-translation.
Among them, regulation at the transcription level is a commonly employed mechanism for controlling gene expression. Transcription of a gene starts with binding of the RNA polymerase to the promoter of the gene (reviewed by Mermelstein et al. 1989). In eukaryotes, RNA polymerase II transcribes most of the genes for protein synthesis. However, unlike the prokaryotic RNA polymerase, the eukaryotic RNA polymerase II alone does not recognize specific promoter sequences. It requires a group of proteins called the general transcription factors to form the transcription initiation complex. One of the general transcription factors, TFIID, binds to the TATA box of the promoter and directs accurate initiation of transcription. Other general transcription factors are also involved in transcription initiation complex formation. The binding of TFIID to the promoter is stabilized by TFIIA. TFIIE and TFIIF interact with RNA polymerase II, while TFIIB may interact with the other factors (Mermelstein et al. 1989). Although the TATA box is essential for binding of TFIID in most of the gene promoters, some promoters lack a TATA box. In such TATA-less promoters, it is typical that the sequence immediately upstream of the transcription initiation site has a high content of nucleotides G and C, and contains multiple GC boxes, which are binding sites for the transcription activator, SP1. It has been shown that in promoters lacking a TATA box, the transcription factor TFIID is still required for assembly of the transcription initiation complex (Smale et al. 1990, Carcamo et al. 1991). These TATA-less promoters, however, often have multiple transcription start sites.

1.7. Promoters and enhancers

Accurate initiation of gene transcription requires a functional promoter. Promoters consist of cis-acting DNA elements that are close to the transcription start site. Some of the common DNA elements located in eukaryotic promoter regions include the TATA box, the GC box, and the CAAT box (reviewed by Maniatis et al. 1987; Mitchell and Tjian 1989).
Most of these elements can function independently of their orientation, but the space between them may be important (Takahashi et al. 1986; McKnight 1982), suggesting possible interactions between these elements.

Enhancers increase the level of transcription in an orientation-independent manner. They can activate transcription from a location either adjacent to or separated from the promoter (reviewed by Maniatis et al. 1987; Dynan 1989). Some of DNA elements in enhancers are shared by the promoters. Because promoters and enhancers have the same function of activating transcription initiation, and they may have the same components, they are homologous entities. In fact, when the 5' flanking region of a gene contains a continuous array of DNA elements, it may be impossible to determine a boundary between a promoter and an enhancer.

The DNA elements in promoters or enhancers normally bind to transcription factors. Some of the transcription factors pre-exist in the cells, but are activated by modulation, such as phosphorylation (Imbra and Karin 1986; Wasylyk et al. 1987). These transcription factors interact with each other and with the transcription initiation complex to activate transcription. Because enhancer elements can be quite a distance away from the transcription start site, a widely accepted model for explaining their function to activate transcription is that the transcription factors that bind to the enhancer elements interact with other transcription factors in the proximal promoter region, looping out the length of DNA between the enhancer and the promoter (Ptashne 1986).

1.8. Regulation of tissue-specific and inducible gene expression

Many genes are expressed temporarily, in specific tissues, at specific developmental stages, or in response to environmental changes and stimulations. Tissue-specific and inducible gene expression determines extremely important physiological processes, including development, differentiation, growth, immunity, and metabolism.
Eukaryotic genes usually have a complex pattern of regulatory DNA elements surrounding the transcription start site, although some of the enhancer elements may be several kilobases apart (Grewal et al. 1992). These DNA elements bind specific transcription factors, including activators and repressors, and the interplay of the transcription factors determines whether RNA polymerase will effectively bind to the promoter and therefore initiate transcription. The existence of specific transcription factors that are required for or inhibit expression of a particular gene, or the modification of these transcription factors, varies among different cells. For example, in mature B cells which express immunoglobulin genes, a B cell-specific transcription factor, Oct-2, has been identified to bind to the octamer element in the B cell-specific enhancer, while Oct-1, which binds to the same octamer sequence, is ubiquitously expressed in all cell types (Fletcher et al. 1987; Scheidereit et al. 1987; Müller et al. 1988; Sturm et al. 1988). Another factor, NF-κB, is activated in B cells, but exists in many other cell types in an inactive form (Sen and Baltimore 1986a, b). Transcriptional activation of genes induced by exogenous factors is also mediated by DNA elements in promoters and enhancers. Steroid receptors are activated by binding their ligands, and the activated receptor interacts with a steroid-response DNA element to activate transcription of steroid-regulated genes (reviewed by Yamamoto 1985; Beato 1989).

1.9. The cis-acting elements in control of MHC class II gene transcription

Because cell surface expression of MHC class II molecules correlates with the amount of their corresponding mRNA inside the cell, transcriptional control appears to be the general strategy for MHC class II gene regulation, although there is evidence that post-transcriptional regulation may also be important (reviewed by Glimcher and Kara 1992). During the past decade, numerous studies on transcriptional regulation of mammalian MHC class II genes have revealed a picture of DNA elements and their binding factors that are
involved in this complex process. To date, however, the question of how these elements interact to determine the transcription of these genes in specific cell types still remain to be clarified (reviewed by Benoist and Mathis 1990; Glimcher and Kara 1992).

Sequence comparison of promoter regions of different class II genes has detected conserved DNA sequences termed X and Y boxes (Figure 7; Benoist and Mathis 1990). Studies using DNA transfection (Boss and Strominger 1986; Sherman et al. 1987; Tsang et al. 1988; Thanos et al. 1993) and transgenic mice (Dorn et al. 1987b; Burkly et al. 1989) showed that the promoter region containing the X and Y boxes is crucial for class II promoter activity in both B cells (which constitutively express class II genes) and γIFN-induced cells. The space, but not the exact sequence, between the X and Y boxes is conserved, suggesting interactions between these two elements. Other DNA elements in the proximal promoter region participate in tissue-specific and γIFN-induced expression of class II genes (Figure 8). These include an X2 motif which overlaps with the 3' end of the X box (the classic X box has been named X1 box), a W or Z region which contains an S box, a pyrimidine tract, and an octamer sequence (Benoist and Mathis 1990; Glimcher and Kara 1992). DNA elements that control B cell-specific expression also exist outside of this proximal promoter region (Figure 8). An inverted set of X and Y boxes have been found between -1392 to -1346 upstream of the mouse Eα gene, acting as a B cell-specific enhancer (Dorn et al. 1988; Koch et al. 1989). A “B” motif, which is a binding site for transcription factors NF-κB and H2-TFI, is also located about 1.7 kb upstream of the Eα gene (Dorn et al. 1988). Other enhancer activities have been located in the upstream region of the mouse Eα gene (Gillies et al. 1984), and in the intronic sequences of the human DQ and DR genes (Sullivan and Peterlin 1987; Wang et al. 1987; Peterlin 1991). A negative element, V, is mapped to the -193 to -179 region of the HLA-DRA gene (Cogswell et al. 1990).

Recently, Albert et al. (1994) characterized a silencer at -543 to -534 of the class II-A β gene.
Figure 7. Promoter sequence comparison of MHC class II genes from human, mouse, chicken, and mole-rat. The sequences are arranged to align the S, X, and Y boxes. The numbers on the right of the sequences denote the positions relative to the transcription initiation site, if such a site is experimentally determined. The X2 motif immediately downstream of the X box is underlined. The NF-κB binding sites in Aα, DQα, and the inverted X box in Eα are also underlined. Numbers under the consensus sequences at the bottom represent the frequencies of each nucleotide in that particular position of the 12 gene promoters listed. (from Benoist and Mathis 1990)
Figure 8. Important cis-acting DNA elements that regulate mammalian MHC class II gene expression. Top: two kb of 5' flanking sequence of class II genes. Identified DNA elements are represented by small boxes. The transcription start site and direction are indicated by an arrow. Bottom: a closer look at the proximal regulatory region. PY = pyrimidine tract. (from Benoist and Mathis 1990)
1.10. The trans-acting elements in control of MHC class II gene transcription

Identification of the DNA elements has led to the discovery of the transcription factors that regulate class II gene expression. Some of the X and Y box-binding proteins have been well characterized. Two Y box-binding proteins have been identified, both appear to be ubiquitously expressed. One of them, NF-Y, is required for class II transcription in in vitro transcription assays (Glimcher and Kara 1992). Except for binding to the Y box, which contains an inverted CAAT box, NF-Y can also bind to CAAT boxes in other gene promoters (Dorn et al. 1987a). Another Y box-binding factor, YB1, is suspected to be a repressor, because its mRNA level is negatively correlated with class II expression (Didier et al. 1988).

Multiple XI box-binding activities have also been observed (Liou et al. 1988; Hasegawa et al. 1993). Reith et al. (1989) cloned an XI-binding protein, RF-X. RF-X is absent in cells from severe combined immunodeficiency patients whose MHC class II expression is deficient, thus establishing its significance in regulation of class II gene expression (Reith et al. 1988). The correlation between the existence of RF-X and transcription of class II genes was further demonstrated by Hasegawa et al. (1993). Structural analysis of the RF-X protein revealed a DNA binding domain and a dimerization domain (Reith et al. 1990). The dimerization domain may mediate interaction between the proximal X box with the distal inverted X box in the Ea gene.

The X2 box sequence contains the cyclic AMP response element (CRE) or TPA response element (TRE). It is thus not surprising that XBP1, an X2 box-binding protein, shares homology with Fos, which is a member of a family of transcription factors that bind to CRE. Another distinct X2-binding protein, XBP2, has also been identified (Benoist and Mathis 1990).

Although it is still not clear how these transcription factors work together to accurately regulate MHC class II gene expression in different cell types, cell-free
transcription systems are establishing functional roles for the transcription factors that are already cloned (Glimcher and Kara 1992).

**1.11. Expression of the chicken MHC class II (B-L) β genes**

As in mammals, chicken MHC class II (B-L) molecules are expressed mainly in the B lymphocyte and myeloid lineages (Bourlet et al. 1988) and are induced by chicken IFN (Kaspers et al. 1994). It has been shown by two-dimensional gel electrophoresis that one B-Lα chain can form different heterodimers with two species of B-Lβ chains (Guillemot et al. 1986), suggesting that at least two B-Lβ genes are expressed. Using exon-specific probes, the five B-Lβ genes identified in the B12 haplotype are grouped into two families (Guillemot et al. 1988). B-LβI and B-LβII hybridize to a probe derived from the 3' untranslated sequence of a cDNA clone, but the other three B-Lβ genes represent a family of untranscribed genes. In the B4 haplotype, the sequence of the genomic clone CCII-7-1 resembles that of the cDNA clones (Xu et al. 1989; Sung et al. 1993). CCII-7-1 shares a high degree of sequence homology with the B-LβII gene (Xu et al. 1989; Zoorob et al. 1990) and thus is likely the counterpart of the B-LβII gene in the B4 haplotype. Sequence analysis of two B-Lβ clones from cDNA libraries derived from a B cell line and embryonic liver tissue, respectively, has further confirmed that the CCII-7-1 and the B-LβII genes are expressed. It also showed evidence for expression of the B-LβI gene and its related CCII-4-1 gene of the B4 haplotype (Pharr et al. 1993).

The 5' upstream DNA sequence of the B-LβII gene (Zoorob et al. 1990) contains the class II regulatory boxes S, X, and Y. An octamer-like sequence, with one nucleotide difference from the conserved sequence ATTTGCAT, is located at -753 to -745. This location is different from that in the mammalian class II gene promoters, where the octamer sequence is immediately downstream of the Y box. Unlike most mammalian class II genes, the B-LβII promoter region lacks a TATA box, but contains a length of sequence that is
rich in nucleotides G and C. This GC-rich region contains three SP1-binding sites. An
adenosine 20 basepairs upstream of the ATG initiation codon has been determined to be the
transcription start site by primer extension study (Zoorob et al. 1990). This adenosine
residue is located within a typical transcription initiation sequence. A mutation in the Y box
has been observed in a putative pseudogene, B-LβIII, suggesting that the class II conserved
boxes may also play a significant role in regulating class II gene expression in the chicken.

Despite the recent progress in understanding the expression of the B-Lβ genes and
the B-F genes (Kroemer et al. 1990b; Zöller et al. 1992), functional analysis of the B-Lβ
gene promoters is still lacking. The objective of this study is to characterize a putative
promoter and the upstream regulatory region of the B-Lβ gene, CCII-7-1.

1.12. Dissertation organization

Each of the Sections 2, 3, and Appendix A derives from a manuscript that is
submitted (Section 2), in press (Section 3), or already published (Appendix A). The Ph.D.
candidate is the first author of all three manuscripts. The identification and functional
characterization of the chicken class IIβ CCII-7-1 gene promoter region is included in
Section 2. The manuscript in Section 3 focuses on the initial establishment of a transfection
system for evaluating chicken class IIβ regulatory elements in the chicken macrophage cell
line, MQ-NCSU, which was needed to conduct the study described in Section 2. Appendix
to Section 3 includes statistical analysis of the data that leads to the conclusions made in
Section 3. Section 4 concludes and discusses the results from Sections 2 and 3. The work
on B-F restriction fragment length polymorphism of the Iowa State University chicken lines,
which is relevant to the major topic of studying chicken MHC genes, is included as
Appendix A. Other appendices include relevant experiments that are not presented in these
three manuscripts or experimental procedures not described in detail in the manuscripts
because of space constraints imposed by journal publication. The bibliography section
includes the references cited in Sections 1 and 4. References cited in other sections and appendices are included within these sections or appendices.
2. IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF A CHICKEN MAJOR HISTOCOMPATIBILITY COMPLEX (MHC) CLASS IIB GENE PROMOTER IN A MACROPHAGE CELL LINE

A manuscript submitted to the journal Gene

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SUMMARY

A 0.7 kb DNA fragment from the 5' flanking region of a class IIB gene was cloned into the chloramphenicol acetyltransferase (CAT) reporter vectors and was transfected into the MQ-NCSU chicken macrophage cell line that expresses a low level of MHC class II antigens. Positive orientation-dependent promoter activity of the chicken DNA was evident in a reporter construct containing an SV40 enhancer. Deletion analysis of this 0.7 kb DNA fragment revealed a short fragment in the 3' end that was crucial for the promoter function and negative regulatory elements located further upstream. The conserved MHC class II X

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and Y boxes did not have a significant influence on promoter activity. Sequence analysis of the 0.7 kb class II\(\beta\) gene upstream region suggested possible involvement of interferon (IFN), ETS-related proteins, and other factors in regulating this promoter. An IFN-rich chicken T cell line culture supernatant increased surface expression of MHC class II antigens as well as class II\(\beta\) promoter activity in this macrophage cell line. This first functional characterization of a chicken MHC class II\(\beta\) gene promoter will aid in understanding the regulatory mechanisms that control the expression of these genes.

(Key words: MHC class II conserved boxes; negative regulatory elements; interferon; glucocorticoids; transfection)

Abbreviations: bp, base pair(s); CAT, chloramphenicol acetyltransferase; GRE, glucocorticoid response element; ICS, interferon consensus sequence; IFN, interferon; kb, kilobase(s); MHC, major histocompatibility complex; NRE, negative regulatory element; PCR, polymerase chain reaction; RCE, retinoblastoma control element.

INTRODUCTION

The MHC class II genes encode proteins that present antigens to helper T cells and thus are critical in initiating the cell-mediated immune responses against invading pathogens (Germain et al. 1986). The class II genes are expressed in limited cell-types including B cells and macrophages, and are inducible by a variety of factors, especially by IFN (reviewed by Benoist and Mathis 1990). In mammals, conserved DNA sequences in the proximal promoter region, namely the S (or Z or W), X, and Y boxes, are crucial in initiating the tissue-specific transcription of the class II genes (reviewed by Benoist and Mathis 1990;
Glimcher and Kara 1992). Reith et al. (1988) have demonstrated that the absence of an X box-binding protein, RF-X, diminishes the expression of MHC class II genes and causes congenital severe combined immunodeficiency. The immediate upstream DNA is also necessary for MHC class II gene expression in vivo (Burkly et al. 1989). In addition, negative and positive elements are located upstream of the conserved class II boxes (Tsang et al. 1988; Burkly et al. 1989; Boss and Strominger 1986).

The chicken MHC, also known as the B complex (Briles et al. 1950; Schierman and Nordskog 1961; Kroemer et al. 1990; Lamont 1993), differs from its mammalian counterpart in size and organization (Guillemot et al. 1989). Another chromosomal locus, Rfp-Y, has been recently discovered to contain chicken MHC genes but is unlinked to the B complex (Briles et al. 1993; Miller et al. 1994). It is apparent that significant structural differences between the avian and mammalian MHC have evolved. Several chicken MHC class II (named B-L) β genes have been isolated and sequenced in the B6 (Xu et al. 1989) and B12 (Zoorob et al. 1990) haplotypes. The organization of the DNA elements regulating these genes differs from that in mammalian species, but the S, X, and Y boxes are well conserved in some of these chicken B-Lβ genes, especially in the B-LβII gene of the B12 haplotype and the CCII-7-1 gene of the B6 haplotype. Analysis of B-Lβ cDNA clones (Zoorob et al. 1990; Sung et al. 1993; Pharr et al. 1993) suggests that the B-LβII gene and its counterparts in other B haplotypes (e.g., the CCII-7-1 gene) are expressed, although there is also evidence for expression of other B-Lβ gene(s) (Pharr et al. 1993; Guillemot et al. 1986). Our objective was to identify and analyze a potential B-Lβ gene promoter by functional characterization of DNA sequences in a 0.7 kb, 5' upstream region of a chicken MHC class IIβ genomic clone.
EXPERIMENTAL AND DISCUSSION

(a) Sequence analysis of the chicken MHC class IIβ CCII-7-1 5' flanking region

The sequence of the CCII-7-1 5' flanking region and the putative transcription factor-binding sites are shown in Fig. 1. The sequence of a 445 bp fragment immediately upstream of the CCII-7-1 translation start site was nearly identical to that of the functional B-LβII gene of the B12 haplotype (Zoorob et al. 1990). No TATA box sequence was observed in this region, but a transcription initiation sequence was present around the transcription start site (Fig. 1) described in the B-LβII promoter by Zoorob et al. (1990). The high GC content in the avian genome, particularly in the immediate 150 bp CCII-7-1 upstream region (81.3% G + C), may explain the lack of TATA box in this gene. Putative S, X, and Y boxes were located in the -120 to -50 region, relative to the transcription initiation site. The Y box includes one inverted CCAAT sequence, and two more inverted CCAAT boxes were found at -195 to -191 and -256 to -252. In addition to the conserved class II boxes, many other elements may regulate this potential B-Lβ promoter. Three potential SP1 binding sites and four retinoblastoma control elements (RCE, Kim et al. 1991; Robbins et al. 1990) were located in the -200 to -1 region, with more RCE-like sequences further upstream. Consistent with the fact that the expression of class II genes is induced by IFN and downregulated by glucocorticoids, three interferon consensus sequence (ICS, Williams 1991)-like motifs and a putative glucocorticoid response element (GRE, Beato 1989) were located. A putative octamer sequence, a half binding site for NF1, multiple binding sites for DNA-binding proteins belonging to the ETS family, including PEA3 sites and a PU box, were also present. Some of the putative regulatory elements overlapped or were adjacent, suggesting that competitive binding and possible interactions between transcription factors may be important in regulation of chicken class II gene expression.
Fig. 1. Nucleotide sequence of the CCII-7-1 5' flanking region (GenBank accession number L32814). The CCII-7-1 subclone is derived from the genomic clone CCII-7 (Xu et al. 1989). It contains a 2.3 kb insert, which includes a chicken B-L\(\beta\) structural gene and 0.7 kb of its 5' flanking sequence. The 5' region upstream of the translation start site was sequenced by the dideoxynucleotide chain termination method. The sequence was revised from Xu et al. (1989) and was confirmed by sequencing PCR products derived from this region of DNA. The previously described transcription initiation site (Zoorob et al. 1990) is shown by a vertical arrow. Putative transcription factor-binding sites are underlined or overlined. The repeat sequence GGTGACC is shown by overlines, and the repeat sequence CCCAGG is shown by underlines. Both of these sequences are repeated three times in this 5' flanking region. Sequences similar to RCE or GGTGACC are not shown in this figure.
PEA3

Oct

PEA3  PEA3  ICS

PU Box

ICS  ICS

GRE

CCAAT Box

RCE  RCE  RCE

S Box  X Box

Y Box  RCE

SPI

↓

+1
(b) Detection of promoter activity in the CCII-7-1 5' flanking region

Because it is known that the MHC contains genes that are not expressed (Guillemot et al. 1989), it is important to confirm that the 5' flanking region of CCII-7-1 contains a functional promoter that activates transcription. A 679 bp fragment (-660 to +19) from this region was tested for its promoter activity in transient CAT expression assays by using a chicken macrophage cell line, MQ-NCSU (Qureshi et al. 1990). When comparing DNA constructs containing an SV40 enhancer, the construct containing the chicken DNA in its natural direction had significantly more CAT activity than the one with the chicken DNA in reversed direction or the vector alone (Fig. 2). This strongly suggests that the -660 to +19 region of CCII-7-1 contains an orientation-dependent promoter that is functional in the chicken macrophage cell line. The relatively low amount of CAT activity with the pCAT-Enhancer vector may be the result of non-specific binding of RNA polymerase to the promoterless DNA. When the SV40 enhancer was absent, all the constructs showed much less CAT activity. Results from other experiments, in which the same or different transfection methods were used (data not shown), as well as deletion studies described in section (d), also confirmed the presence of promoter activity in the CCII-7-1 5' flanking region. The low promoter activity of the CCII-7-1 5' flanking DNA, as indicated by the requirement of additional enhancer for efficiently initiating transcription, may be the result of the low expression of MHC class II molecules of this macrophage cell line. Flow cytometry data indicated that surface MHC class II antigen expression of the MQ-NCSU cell line was much lower than that of the RP9 chicken B cell line, which is known to be class II-positive (Fig. 3). Northern blot analysis of RNA from the MQ-NCSU cells detected an extremely weak signal for MHC class II expression, whereas chick bursal cells and RP9 cells showed strong signals (data not shown). It is possible that additional enhancers exist outside this 0.7 kb region and are required for efficient gene expression. The 3'-untranslated region of the CCII-7-1 gene contains an NF-κB binding site (GGGGACTTTCC), and is therefore a
Fig. 2. Promoter activity of a 679 bp (-660 to +19 from the transcription start site) CCII-7-1 5' flanking DNA fragment. The DNA sequence from the CCII-7-1 upstream region was amplified by polymerase chain reaction (PCR). PCR primers were synthesized in an Applied Biosystems Oligonucleotide Synthesizer (Applied Biosystems, Foster City, CA, USA) and purified by HPLC. The specific PCR product was cloned into the *HindIII* site of the pUC19 vector and subsequently into vector pCAT-Basic or pCAT-Enhancer (Promega, Madison, WI, USA). Both vectors contain a multiple cloning site immediately upstream of a CAT structural gene, but pCAT-Enhancer has an additional SV40 enhancer. The 679 bp fragment was cloned into pCAT-Basic or pCAT-Enhancer in its forward direction to yield the constructs termed pC7-FCAT or pC7-FECAT, respectively, and was cloned in reverse direction to yield pC7-RCAT or pC7-RECAT, respectively. Ten to twenty µg of each plasmid constructs pC7-FCAT, pC7-RCAT, pC7-FECAT, and pC7-RECAT were transfected into the MQ-NCSU chicken macrophage cell line (Qureshi et al. 1990). Plasmids pCAT-Basic, pCAT-Enhancer, and pCAT-Control (which contains an SV40 enhancer and an SV40 promoter upstream of the CAT gene, Promega) were used as controls. The calcium phosphate transfection method was modified from Sambrook et al. (1989). Cells were glycerol-shocked 3 to 5 hours after transfection and were cultured for 36 to 48 hours. They were then harvested and lysed by 3 cycles of freezing and thawing. The volume of cell lysate added to each CAT assay reaction was normalized according to the total protein concentration, which was determined by the BCA protein assay kit (Pierce, Rockford, IL, USA). CAT assays were performed as described by Gorman et al. (1982). After thin-layer chromatography, the amount of radioactivity in acetylated or non-acetylated forms of 14C-chloramphenicol was determined by a Molecular Dynamics PhosphorImager 400E machine (Molecular Dynamics, Sunnyvale, CA, USA). The level of CAT activity was calculated by percentage of conversion into acetylated 14C-chloramphenicol, and was expressed as relative activity normalized to the activity of plasmid pCAT-control (value set at 1). Each construct was transfected at least four times, and the mean and standard error of the observations are shown.
potential location for such enhancers.

(c) Induction of surface B-L expression and B-Lβ promoter activity by an IFN-rich supernatant

Kaspers et al. (1994) have reported that expression of MHC class II molecules in chicken macrophages is increased by IFN. We used a concentrated p34 chicken T cell line culture supernatant, which is IFN-rich, to treat the MQ-NCSU cell line. Surface class II expression of the macrophage cell line increased after 5, 25, or 43 hours of treatment with the supernatant, with treatment for 25 or 43 hours resulting in a higher level of increase than treatment for 5 hours (Fig. 3). Compared with the RP9 cell line, the MQ-NCSU cell line expressed a low level of B-L molecules, even after induction with the supernatant. Transient expression assays (Fig. 4) also showed an increase in promoter activity of the 0.7 kb CCII-7-1 5' flanking region in the macrophage cell line after treatment with the p34 supernatant. The CAT activity, however, increased only after 2 days, but not after 1 day, of treatment with the supernatant (data not shown). This is in contrast with the flow cytometry data which indicated that the expression of surface class II molecules was induced in 5 hours. This difference may be explained by the requirement for accumulation of CAT enzyme inside the cells to detect the increased level of promoter activity. Consistent with the report in mammals that glucocorticoids inhibit the inducing effect of γ-IFN on MHC class II expression (Warren and Vogel 1985), we observed an approximately 4-fold decrease in CAT activity when dexamethasone or hydrocortisone was added to the macrophages with the p34 supernatant (Fig. 4). In the absence of the IFN-rich supernatant, the effect of glucocorticoids on CAT activity was less obvious. Serotonin also inhibited p34 supernatant-induced CAT activity in this experiment, but had no effect in the absence of p34 supernatant in other experiments (data not shown). The increase of CAT activity after p34 supernatant treatment was also observed in other transient expression experiments, although the level of increase
Fig. 3. Surface MHC class II expression of the MQ-NCSU chicken macrophage cell line. The p34 supernatant was concentrated 200 times by ammonium sulfate precipitation, followed by size exclusion filtration (Amicon membrane size 10 kD). The concentrated supernatant was added to the cell culture at a 1:2000 dilution. After 5 to 43 hours, cells were fluorescent-labeled with a monoclonal antibody against chicken MHC class II antigens using the labeling procedure recommended by Southern Biotechnology (Birmingham, AL, USA). The labeled cells were examined by flow cytometry for the presence of fluorescence on cell surface. A mouse IgG1 was used as isotype control. The cell counts and the intensity of fluorescence (in log scale) are shown. Isotype controls are shown as dotted lines (⋯⋯); cells labeled with anti-class II are shown as broken lines (------); cells treated with p34 supernatant and labeled with anti-class II are shown as solid lines (——). (a) Induction of the MQ-NCSU cell line with the p34 supernatant for 5 hours; (b) induction of the MQ-NCSU cell line for 25 hours; (c) induction of the MQ-NCSU cell line for 43 hours; (d) the RP9 B cell line.
Fig. 4. Induction of CAT activity in transient expression assays by the p34 chicken T cell line supernatant. The MQ-NCSU cell line was transfected with plasmid construct pC7-12ECAT (see Fig. 5), and then treated with concentrated p34 supernatant (p34S, diluted 1:2000), dexamethasone (Dex, 10^{-6}M), hydrocortisone (Hdct, 10^{-6}M), serotonin (5-HT, 5x10^{-7}M), or their combinations before being harvested for CAT assay. CAT activity was expressed as relative activity compared with that of the transfected cells without treatment (No trt). The mean and standard error are shown.
(d) **Deletion analysis of the CCII-7-1 promoter region**

To map DNA elements important in regulating the CCII-7-1 promoter, a series of deletion constructs were generated by PCR with primers that amplify the desired regions (Fig. 5A, B). After transfection into the MQ-NCSU cell line and CAT assay (Fig. 5C), the most 3' end of this promoter region, including the transcription initiation sequence, was found to be required for minimal promoter function. Deletion of the -711 to -405 fragment slightly reduced promoter activity, suggesting possible enhancing effects of the ETS-related proteins and/or octamer-binding proteins in that region. The -404 to -131 region likely contained negative regulatory element(s) (NRE), because removal of this region significantly increased promoter activity. This negative region includes multiple RCE and a repetitive sequence GGTGACC, as well as an ICS-like sequence. RCE is a candidate element for repression of the CCII-7-1 promoter activity, because it negatively regulates human c-fos expression (Robbins et al. 1990) and activates or downregulates the human TGF-β gene, depending upon the cell type (Kim et al. 1991). Surprisingly, deletion of the S, X, and Y boxes had no significant effect in most of our experiments. This contrasts to the mammalian MHC class II gene promoters, where these boxes play crucial roles in regulating expression of the class II genes. It is possible that the macrophages used in our experiments lack, or have reduced levels of, the necessary transcription factors that interact with the S, X, and Y boxes. The low level of MHC class II expression in these cells may support this hypothesis. It is also possible that a different regulatory mechanism controlling class II gene expression have been developed in avian species, and the S, X, and Y boxes represent mere remnants of evolution. Evolutionary divergence between aves and mammals in genetic control of immune response is also observed in antibody diversification mechanisms (Reynaud et al. 1985; Reynaud et al. 1987; Thompson & Neiman 1987).
Fig. 5. Deletion analysis of the CCII-7-1 5' flanking sequence. The CCII-7-1 DNA fragments used in the deletion study were amplified by PCR. The PCR primers had a SalI or PstI restriction site added to their 5' ends for directional cloning. Dimethyl sulfoxide was added to 5% when amplifying DNA fragments spanning a region with high GC content. The PCR products were cloned into the PstI/SalI sites of the pCAT-Enhancer vector in their forward directions. All the constructs were restriction mapped and sequenced to confirm that they contained the expected DNA fragments. Construct pC7-12ECAT contained 730 bp (-711 to +19) of the promoter region, 51 bp longer than the chicken DNA insert in pC7-FECAT, and included an extra PEA3 site. pC7-32ECAT (-404 to +19) deleted all the PEA3 sites, the PU box, and the octamer sequence, but retained all the RCE and the GGTGACC sequences. pC7-42ECAT (-130 to +19) contained the S, X, Y boxes, the transcription initiator, two SP1 binding sites, and one RCE. pC7-52ECAT (-49 to +19) deleted all the S, X, Y boxes from pC7-42ECAT. pC7-16ECAT (-711 to -23) was a 3' deletion construct that deleted the transcription initiator and the most downstream RCE from pC7-12ECAT.

(A) Locations of the PCR primers (numbered heavy lines) in the CCII-7-1 5' flanking region. CCII-7-1 is shown as an open bar, and letters denote putative DNA consensus sequences. C = CCAAT box; F = ICS; G = GRE; I = transcription initiation sequence; O = octamer; P = PEA3 site; R = RCE; S = S box; X = X box; Y = Y box. The PU box, the GGTGACC sequences, the SP1 sites, and the translation start codon (ATG) are also shown. Primers that anneal to the antisense strand of CCII-7-1 are shown as numbered heavy lines above the bar, and those that anneal to the sense strand are shown under the bar. (B) Nucleotide sequences of the primers. Exact positions of the primers in the CCII-7-1 5' region are shown. The restriction site sequences are underlined.
A

1
POPF

3
FFG

4
CCRRS

5
XYRI

ATG

PU box

GGTGACC

SP1

B

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<th>Primer #</th>
<th>Position</th>
<th>Sequence</th>
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<tr>
<td>1</td>
<td>-711 to -687</td>
<td>AGCTGCACACAGGGCATGGGACTGGCTGAA</td>
</tr>
<tr>
<td>2</td>
<td>+19 to +1</td>
<td>CTGTCGACGGCTGCGCGCCGGACGAT</td>
</tr>
<tr>
<td>3</td>
<td>-404 to -385</td>
<td>GACTGCAGGGTGACCATGGAGAGCAACG</td>
</tr>
<tr>
<td>4</td>
<td>-130 to -110</td>
<td>GGCTGCAGAGTGTCAGAAGGCCCGGCG</td>
</tr>
<tr>
<td>5</td>
<td>-49 to -33</td>
<td>ATCTGCAAGCTCCCGCCCGGCGGCGG</td>
</tr>
<tr>
<td>6</td>
<td>-23 to -39</td>
<td>CCGTCGACAGCCCGAGGCCCGGCGG</td>
</tr>
</tbody>
</table>
(C) CAT activity of the deletion constructs, after calcium phosphate transfection into the chicken macrophage cell line. The regions of CCII-7-1 that each insert spans are shown at left. The letters in the bars represent the same DNA consensus sequences as in Fig. 5A. The autoradiogram shows a typical result. The relative CAT activities from eight transfections (mean ± standard error) were normalized to the activity of construct pC7-12ECAT.
C

<table>
<thead>
<tr>
<th>Construct</th>
<th>Relative CAT activity</th>
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<tr>
<td>pC7-16ECAT</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>pC7-12ECAT</td>
<td>1</td>
</tr>
<tr>
<td>pC7-32ECAT</td>
<td>0.73 ± 0.18</td>
</tr>
<tr>
<td>pC7-42ECAT</td>
<td>3.63 ± 1.13</td>
</tr>
<tr>
<td>pC7-52ECAT</td>
<td>3.65 ± 1.18</td>
</tr>
</tbody>
</table>
(e) Conclusions

(1) The 5' flanking region of the chicken MHC class IIβ gene, CCII-7-1, contains a functional promoter.

(2) The CCII-7-1 promoter is inducible by a chicken IFN-rich supernatant in the MQ-NCSU cell line, and the induction can be modulated by other exogenous factors such as glucocorticoids.

(3) This promoter contains negative elements between -404 to -131, and the putative S, X, and Y boxes are not required for promoter activity in the MQ-NCSU macrophage cell line. The most 3' sequence of the promoter is necessary for minimal promoter activity.

(4) The MQ-NCSU chicken macrophage cell line expresses a very low level of MHC class II molecules compared with that of the chick bursal cells or the RP9 B cell line.

ACKNOWLEDGMENTS

Drs. Y. Xu and C. M. Warner warrant special thanks for their roles in the initial cloning of CCII-7-1. The MQ-NCSU cell line was a generous gift from Dr. M. A. Qureshi. Anti-chicken MHC class II monoclonal antibody was kindly provided by Dr. C.-I. H. Chen. Yvonne Wannemuhler provided excellent technical assistance. This is Journal Paper Number J-15881 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project 3073.
REFERENCES


3. TRANSFECTION OF A PUTATIVE CHICKEN MHC CLASS II GENE PROMOTER REGION INTO CHICKEN MACROPHAGES

A paper in press in Advances in Avian Immunological Research

Yunfei Chen, Susan L. Carpenter, and Susan J. Lamont

Abstract

Three transfection methods were evaluated to establish a system for DNA transfection into a chicken macrophage cell line. The calcium phosphate method was determined to be the most efficient. Results from transient expression assays using the chicken macrophage cell line demonstrated that a 0.7 kb DNA fragment from a chicken MHC class II gene 5' flanking region contains a functional promoter, but may need additional enhancers outside this region for maximal promoter activity.

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Introduction

The chicken MHC, or B complex (Briles et al., 1950, Schierman and Nordskog, 1961), contains genes important in immune functions (Lamont, 1993). Among these genes, the class II genes (designated B-L) encode proteins that present antigens to helper T cells and are expressed in limited cell types including B cells and macrophages. Several genomic B-Lβ clones have been structurally analyzed (Xu et al., 1989, Zoorob et al., 1990). Sequence analysis of B-Lβ cDNA clones (Zoorob et al., 1990, Sung et al., 1993, Pharr et al., 1993) suggested that the B-LBII gene of the B12 haplotype and its counterparts in other B haplotypes (e.g., the CCII-7-1 gene of B6) are expressed. Direct demonstration of a functional B-Lβ gene promoter, however, has still been lacking. We report here the establishment of a transfection system for a class II-positive chicken macrophage cell line to conduct functional identification of a chicken B-Lβ gene promoter.

Materials and methods

Subcloning of the chicken MHC class II gene putative promoter region

The 5' flanking region of subclone CCII-7-1 (derived from the genomic clone CCII-7, Xu et al., 1989) was examined to identify potential promoter elements. A 679 bp fragment from the CCII-7-1 upstream region was amplified by polymerase chain reaction and cloned into the HindIII site of the pCAT-Basic or pCAT-Enhancer vectors (Promega, Madison, WI) upstream of the CAT structural gene in forward or reverse directions (Fig. 1).
Figure 1. Plasmid constructs used to detect promoter activity of the 679 bp CCII-7-1 5' flanking DNA. The CAT structural gene is shown as an open bar. The closed bar represents an SV40 enhancer. The closed arrow represents an SV40 promoter. The 679 bp chicken sequence is shown as an open arrow, with the arrowhead indicating the forward direction of CCII-7-1.
Chicken macrophage cell culture, DNA transfection, and CAT assay

The MHC class II antigen-positive MQ-NCSU chicken macrophage cell line (kindly provided by Dr. M. A. Qureshi, Poultry Science Department, North Carolina State University, Raleigh, NC) was maintained in Leibovitz-McCoy's complete medium (CM, Qureshi et al., 1990). A highly adhesive subpopulation was used for transfection. Three transfection methods were examined using 10 to 20 µg of supercoiled plasmid DNA in each duplicated transfection. The TransfectACE™ (GIBCO BRL, Grand Island, NY) method was performed according to the recommended protocol. Transfection by the calcium phosphate method was as described by Sambrook et al. (1989), and cells were glycerol-shocked 3 to 5 hours after transfection. Electroporation was carried out by shocking 4 x 10⁶ cells (suspended in phosphate-buffered saline or in CM medium with 20% chicken serum) with appropriate DNA at 280 to 300 V in a 0.4 cm cuvette. After transfection, cells were cultured in CM until harvested 36 to 48 hours later. Cell lysates containing equal amounts of total protein were assayed for CAT activity as described by Gorman et al. (1982).

Results

Establishment of DNA transfection methods for the MQ-NCSU cell line

Transfection efficiency was determined by introducing the pCAT-Control plasmid (Promega, see Fig. 1) into the MQ-NCSU chicken macrophage cell line followed by CAT assay of the transfected cell lysates. Our results showed that all three transfection methods used - calcium phosphate method, electroporation, and TransfectACE™ method - were able to introduce foreign DNA into this cell line. The calcium phosphate method was the most efficient, and electroporation had variable results.
Sequence and functional analysis of the CCII-7-1 5' flanking region

The 5' flanking region of the CCII-7-1 clone contains a transcription initiation sequence previously described (Zoorob et al., 1990), but lacks a TATA box. Putative transcription factor-binding sites were identified based on homology with known consensus sequences. These include the conserved class II boxes, SP1 binding sites, interferon consensus sequences (ICS), a glucocorticoids response element (GRE), and retinoblastoma control elements (RCE).

Plasmid constructs derived from a 679 bp (+19 to -660) CCII-7-1 fragment, pCAT-Basic, pCAT-Enhancer, and pCAT-Control (Fig. 1) were transfected into the MQ-NCSU cell line. Promoter activity of this fragment was demonstrated by the significantly higher promoter activity of construct pC7-FECAT than pC7-RECAT or pCAT-Enhancer. Low CAT activities were observed in all the constructs without an SV40 enhancer, suggesting that this chicken DNA fragment is dependent on additional enhancer elements for optimal promoter activity.

Discussion

We have demonstrated three transfection methods for successful transfection of foreign DNA into a chicken macrophage cell line, and that the calcium phosphate method was the most efficient. We also identified a DNA fragment from the CCII-7-1 5' upstream region as a functional promoter. This DNA fragment requires additional enhancer(s) for efficient promoter activity in the MQ-NCSU chicken macrophage cell line. Because different regulatory mechanisms control mammalian class II gene expression in B cells and macrophages, studies on the regulation of the CCII-7-1 gene in chicken B cells will help
understand the regulatory mechanisms controlling the expression of class II genes in the chicken.

The presence of GRE and ICS in this promoter region is consistent with the glucocorticoid-repressed and interferon-inducible expression of MHC class II genes. RCE is a potential negative regulator, because it represses promoter activity in human c-fos gene (Robbins et al., 1990). It will be interesting to functionally determine the regulatory roles of these elements in this chicken MHC class II promoter.

In conclusion, we have established a transfection system for a chicken macrophage cell line. We have also functionally identified a promoter of the chicken \texttt{B-Lp} gene, CCII-7-1. This information will aid understanding of the evolution of the MHC, and mechanisms of MHC association with disease resistance.

Acknowledgments

The authors thank Yvonne Wannemuhler for technical assistance.

References


Appendix to Section 3

Results and Discussion

1. Comparison of different transfection methods

The three transfection methods, TransfectACE, calcium phosphate, and electroporation, were compared for their transfection efficiencies in separate transient expression experiments. Transfection efficiency was measured by percent conversion of $^{14}$C-chloramphenicol into acetylated forms after the MQ-NCSU cells were transfected with the pCAT-Control plasmid and assayed for CAT activity. In most cases, each method was assayed in 2 to 4 duplicated experiments, and data represent 4 to 7 transfections. The data shown in Table 1 were analyzed by the SAS program for the estimated mean value (LSMEAN) and standard error (SE) for each transfection method. The calcium phosphate method was significantly more efficient than the TransfectACE method (P=0.05). Although the electroporation method was not significantly less efficient than the calcium phosphate method, it caused more cell death in most experiments compared with the other two methods.
Table 1. Transfection efficiency of the different transfection methods

<table>
<thead>
<tr>
<th>Transfection method</th>
<th>LSMEAN</th>
<th>SE</th>
<th>Probability of significant difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TransfectAce (TrAce)</td>
<td>24.5</td>
<td>16.1</td>
<td>.</td>
</tr>
<tr>
<td>Calcium Phosphate (CalPh)</td>
<td>66.8</td>
<td>12.2</td>
<td>.</td>
</tr>
<tr>
<td>Electroporation (Electr)</td>
<td>46.3</td>
<td>12.2</td>
<td>.</td>
</tr>
</tbody>
</table>

* indicates a significant difference.

2. **Comparison of different constructs in transient expression assays.**

The six constructs, pCAT-Basic, pC7-FCAT, pC7-RCAT, pCAT-Enhancer, pC7-FECAT, and pC7-RECAT (see Fig. 1), were compared for their activity to express CAT in the MQ-NCSU cells. The LSMEAN and SE of the relative CAT activities (compared to that of the pCAT-Control plasmid) are shown in Table 2. The data in Table 2 were from five different experiments, with 4 to 8 total transfections for each construct. Statistical analysis showed that construct pC7-FECAT was significantly different from all other constructs. When interactions between transfection methods and constructs were included in the analysis, the relationship between the constructs did not change (data not shown); however, constructs pCAT-Enhancer and pC7-RECAT were significantly different to pCAT-Basic and pC7-FCAT (pC7-RCAT was not estimated).
Table 2. Comparison of relative CAT activity of the pCAT- constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>LSMEAN</th>
<th>SE</th>
<th>B</th>
<th>F</th>
<th>R</th>
<th>E</th>
<th>FE</th>
<th>RE</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>0.017</td>
<td>0.021</td>
<td>0.76</td>
<td>0.99</td>
<td>0.11</td>
<td>0.00*</td>
<td>0.04*</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.026</td>
<td>0.021</td>
<td>0.78</td>
<td>0.20</td>
<td>0.00*</td>
<td>0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td>0.017</td>
<td>0.028</td>
<td>0.17</td>
<td>0.00*</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.064</td>
<td>0.020</td>
<td></td>
<td></td>
<td></td>
<td>0.00*</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>FE</td>
<td>0.176</td>
<td>0.020</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.00*</td>
<td></td>
</tr>
<tr>
<td>RE</td>
<td>0.081</td>
<td>0.021</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Constructs B=pCAT-Basic; F=pC7-FCAT; R=pC7-RCAT; E=pCAT-Enhancer; FE=pC7-FECAT; RE=pC7-RECAT.
* indicates a significant difference.
4. GENERAL DISCUSSION

In summary, a DNA fragment from upstream sequence of the chicken MHC B-Lβ gene, CCII-7-1, has been demonstrated to contain a functional promoter. Further analysis of this upstream region revealed regulatory functions of DNA elements, and inducibility by an IFN-rich supernatant. To investigate the promoter activity of the chicken DNA fragments in the CAT reporter system, a transfection system has been established for the MQ-NCSU chicken macrophage cell line. Among the transfection methods tested, the calcium phosphate method has been most consistently efficient.

4.1. Expression of B-L molecules in the MQ-NCSU cells

The MQ-NCSU chicken macrophage cell line has been previously reported to express a high level of surface B-L molecules (Qureshi et al. 1990). In this study, however, the level of B-L expression by this cell line was very low compared to chick bursal cells or a chicken B cell line, RP9. This low level of B-L expression correlates with the nearly undetectable B-Lβ mRNA in this cell line, even after stimulation with the IFN-rich p34 supernatant (see Appendix B). It is possible that B-L expression of these cells have changed in passage due to heterogeneity of the cell line. This heterogeneity was also demonstrated by the separation of two morphologically different sublines by selection for highly adherent cells. One subline was highly adherent, and had a elongated shape. The other subline was detached from the growing surface after being confluent, and had more rounded cells. These two sublines, however, did not differ significantly in surface B-L expression detected by an anti-B-L monoclonal antibody.
4.2. Additional enhancers that may activate B-LB gene expression

Using the CAT reporter system, a 0.7 kb DNA fragment from the CCII-7-1 5' upstream region has been demonstrated to contain a functional promoter. The relatively low level of promoter activity may be explained by the fact that the chicken macrophage cell line used in this study expresses a low level of MHC class II antigens, and the presence of negative elements in this fragment (as shown by the deletion assays). The results, however, also suggested a possibility that this 0.7 kb DNA fragment requires additional enhancer(s) for optimal promoter activity in this chicken macrophage cell line. In the CCII-7-1 gene, multiple transcription factor-binding sites were located in the extreme 5' end of the 0.7 kb regulatory region, suggesting that there might be more enhancer elements further upstream. It is possible that these upstream enhancer elements include another set of conserved class II boxes, as found in mammalian class II genes (Dorn et al. 1988; Koch et al. 1989). Enhancer elements may also exist downstream. Interestingly, an NF-κB binding site (GGGACTTTCC, Sen and Baltimore 1986a) appears immediately downstream of the CCII-7-1 structural gene. NF-κB is the transcription factor that binds to the B cell-specific enhancer in immunoglobulin genes (Sen and Baltimore 1986a). These observations support the hypothesis that enhancer activities exist outside of the immediate 5' upstream region tested in this study, and may explain the dependence of the 0.7 kb CCII-7-1 5' fragment on additional enhancers for maximal promoter function. Functional assays of the CCII-7-1 promoter region in chicken B cells will provide more information about the requirement of additional enhancers.

4.3. Functions of the class II conserved boxes in the chicken

The 0.7 kb CCII-7-1 promoter region contained a complex pattern of putative cis-acting regulatory elements. Among them, the immediate upstream consensus sequences S, X, and Y boxes are of particular importance in mammalian species. In deletion analysis,
however, these boxes did not significantly influence the activity of the chicken CCII-7-1 promoter. More studies are needed to determine the functions, if any, of these consensus sequences in the chicken \textit{B-LB} promoter. Several hypotheses regarding the seeming lack of regulatory function of these elements can be proposed. First, a different regulatory mechanism controlling class II gene expression may have been developed in avian species, and the S, X, and Y boxes may represent mere remnants of evolution. Evolutionary divergence in genetic control of immune response has been observed in the antibody diversification mechanisms between aves and mammals. In mammals, antibody diversity is achieved by the existence of multiple variable (V), diversity (D), and joining (J) gene segments, the VDJ (or V and J for the immunoglobulin light chains) joining, and the somatic diversification after joining (reviewed by Tonegawa 1983). The chicken employs a quite different mechanism for its antibody diversification (Reynaud et al. 1985; Thompson & Neiman 1987; Reynaud et al. 1987). After a single joining event, multiple V segment pseudogenes contribute part of their sequence to the rearranged gene by the gene conversion mechanism. Second, functional regulation of the chicken \textit{B-LB} gene expression may require interactions between the S, X, and Y boxes and enhancer elements located outside of the immediate upstream region examined in the present study. In mammalian species, the second set of X and Y box sequences in the distal upstream region is proposed to interact with proximal regions through dimerization of the X box-binding protein, RF-X (Reith et al. 1990). A similar interaction may also exist in the chicken \textit{B-LB} genes, and may be required for high activity of the promoter. The proximal S, X, and Y boxes, however, can activate mammalian MHC class II gene expression in the absence of the second set of the identical boxes or other enhancer elements. Third, the macrophages used in our experiments may lack the positive transcription factors that interact with the S, X, and Y boxes. This may explain the weak promoter activity as well as the low level of surface B-L expression of this cell line. It is possible that some of the proteins binding to these boxes in chicken macrophages may in
fact inhibit B-LB gene expression, and these proteins are replaced by positive transcription factors upon induction.

4.4. Negative regulatory elements (NREs) that regulate MHC class II expression

Deletion of a 274 bp fragment (-404 to -131) immediately upstream of the S box caused increase in CAT activity, suggesting the presence of NRE. In recent years, evidence has accumulated to indicate that NREs in eukaryotes play a role just as significant as they do in the prokaryotes, and are as widely used a strategy as positive elements for control of gene expression. Removing the repression by an NRE in a specific environment is an effective means of tissue-specific or developmentally regulated gene activation. Negative control of gene expression is conserved throughout eukaryotic evolution. It has been found from organisms as low as yeast to as high as human (reviewed by Levine and Manley 1989). Several models are proposed for repression of gene expression by NREs. First, NREs may overlap or lie close to positive DNA elements that are needed for activation of gene expression, thus blocking the activating effects of the positive elements to initiate transcription of mRNA. Alternatively, binding of proteins to NREs may directly block binding of the factors of the transcription complex. Second, NRE-binding proteins and transcription activator proteins may bind to the promoter region simultaneously, but the binding of the repressor shields the activator from interacting with the transcription complex, resulting in a failure to function properly. Third, the repressor itself may directly interfere with the transcription complex, either by destabilizing its binding to the promoter, by modifying it, or by other methods such as changing the DNA conformation. Repression may also occur without the presence of NRE. Over-expression of factors that normally interact with a DNA-binding transcription activator can exhaust the supply of the activator, which subsequently diminishes the activation of gene expression. In addition, many repressors are
transcription activators under different conditions, such as the chicken ovalbumin upstream promoter-transcription factor (Kimura et al. 1993).

Negative DNA sequences have also been identified in mammalian MHC class II genes in transiently transfected cells (Tsang et al. 1988; Cogswell et al. 1990; Albert et al. 1994) and in transgenic animals (Burkly et al. 1989). The study by Albert et al. (1994) indicates that active repression by a silencer is partly responsible for cell type-specific regulation of class II gene expression, because an upstream silencer was active only in class II^+ cells, but not in class II^- cells. Cell fusion studies also provide evidence for negative regulation of the class II genes. Fusion of murine (Venkitaraman et al. 1987) or human (Latron et al. 1988; Dellabona et al. 1989) class II^+ B cells with murine class II^- plasmacytoma result in class II^- hybrid cells. Similarly, fusion of murine lymphoma cells with class II^- fibrosarcoma cells (Stuart et al. 1989) or class II^- T lymphoma cells with class II^+ B cell blasts (Aragnal et al. 1986) result in class II^- hybrids. These studies suggest that dominant negative element(s) inhibit class II gene expression in class II^- cell types.

Analysis of the CCII-7-1 5' flanking sequence has revealed a highly (G+C)-rich region immediately upstream of the translation start codon. A TATA box is lacking in the proximal promoter region, but multiple GC boxes (potential binding sites for a transcription activator, SP1) and reversed CAAT boxes, including the one in the conserved MHC class II box Y, are located in the proximal region. These characteristics match the description of a typical housekeeping gene (Reynolds et al. 1984, Kageyama et al. 1989, Zot and Fambrough 1990). Housekeeping genes, however, are constitutively expressed at a low level in all cell types while the MHC class II genes have a very limited range of expression. So how is the expression of the class II genes kept only in specific cell types such as B cells and some macrophages? Whereas the proximal class II boxes S, X and Y and other possible positive elements may provide an activating force for cells that express relatively high level of class II molecules, NREs can be an effective way to prevent class II genes from being expressed in
most cells that normally do not express class II molecules, and to selectively express these molecules by removing the repression in a few cell types.

The MQ-NCSU macrophage cell line is low in class II expression. It is possible that NREs are responsible for the low level expression of the normal class II genes in these cells, as well as the weak activity of the CCII-7-1 promoter in transient expression assays. Candidate NREs in regulating CCII-7-1 gene expression were suggested by sequence analysis. Three retinoblastoma control elements (RCEs), which are potential NREs (Robbins et al. 1990; Kim et al. 1991), were located within the negative region detected by deletion analysis. Functional analysis of RCE in CCII-7-1 promoter is needed to determine if this element has an effect on chicken B-Lβ gene expression. Another sequence, GGTGACC, and its related sequences are abundant in this negative region. It will be interesting to investigate whether this sequence serves as a binding site for transcription factor(s), and if so, whether it is another NRE.

4.5. Interferon induction of B-Lβ expression

In macrophages, MHC class II gene expression is induced by IFN. The induction is slow, and may require synthesis of transcription factors (Benoist and Mathis 1990). Steimle et al. (1994) have identified a transcription activator, CIITA, that mediates γIFN-induced and constitutive expression of MHC class II molecules. In mammalian MHC class II genes, γIFN-inducibility is conferred by DNA sequences within or surrounding the S, X, and Y box region, but no exact sequence motif has been identified (Glimcher and Kara 1992). Unlike the mammalian IFN, which is subdivided into types α, β, ω, τ and γ, the chicken IFN has not yet been found to be divided into subtypes (Sekellick et al. 1994). Induction of chicken MHC class II expression by IFN has been described (Wick et al. 1987; Kaspers et al. 1994). In the chicken macrophage cell line used in this study, B-Lβ promoter activity and surface Ia expression increased after induction by an IFN-rich supernatant, but surface Ia expression
and B-Lβ mRNA level remained low compared with that in bursal cells and a B-L-positive B cell line. Other lymphokines in the inducing supernatant may complicate the induction effect by IFN, although it is possible that this cell line does not respond well to IFN stimulation.

The interferon response element, ICS (also known as interferon-stimulated response element, ISRE), has been identified in many mammalian interferon-stimulated genes (Williams 1991) and in a chicken MHC class I gene (Zöller et al. 1992). As expected, the CCII-7-1 promoter region contained putative ICS sequences. The three putative ICS in the CCII-7-1 promoter, however, had slightly different nucleotide sequences. This may imply more subtle regulation by binding affinities of the ICS to its binding protein, and possible interaction between the multiple ICS. In mammals, ETS proteins may trans-regulate genes that contain ICS (Williams 1991). Interestingly, two of the putative ICS (-377 to -386, and -567 to -578) in the CCII-7-1 promoter contained or were adjacent to a GGAA or TTCC sequence, the core sequence recognized by the ETS family proteins.

4.6. Other regulatory elements

The finding that identical or different transcription factor-binding sites overlapped or were close to each other was not unique to ICS and ETS sites in the CCII-7-1 promoter (see Figure 2.1). This type of organization suggests competitive binding and interaction between transcription factors that bind to these sites. For example, the overlapping of two RCE and an SP1 site around position -147 suggests that possible competition between SP1 (a transcription activator) and proteins that bind to RCE (a potential NRE) might be one of the mechanisms that influence the promoter activity. In addition to the ETS sites mentioned above, putative PEA3-binding sites (CTTCCT) and a PU box (TTCCTC) were identified, consistent with the tissue-specific expression of the class II genes. PEA3 (Xin et al. 1992) is a transcription factor required for activation of the polyomavirus enhancer and functions in the activation of cellular enhancers during development and differentiation (Martin et al.
1988). A PU box-binding protein, PU.1, is expressed in macrophages and B cells, but not in T cells and many other cell types (Klemsz et al. 1990). It is a transcriptional activator, suggesting its role in regulation of the class II promoter, which is active in these cell types.

4.7. Conclusions and future directions

The regulation of MHC class II gene expression is extremely complex, as shown in mammals, and only limited information in this area is available in the avian species. This study functionally identified and analyzed a promoter of the chicken B-Lβ gene, CCII-7-1, and suggested that the mechanism controlling class II expression in the chicken is not identical to that employed by mammals. Understanding the regulation of expression of the chicken class II genes will help elucidate the mechanisms underlying the well-established association of these genes with disease resistance. Further investigation into the regulatory mechanisms developed by the chicken class II genes may also provide significant evolutionary contrasts between the mammals and the birds. Some of the future studies may include:

1. Analyze cell type-specific and IFN-induced B-Lβ promoter activity.

The B-Lβ promoter will be functionally characterized in cells that constitutively express B-L molecules, express B-L only after IFN induction, or are negative for B-L expression. This will be an important step toward understanding the different mechanisms that control class II gene transcription in different cell types.

2. Characterize the NREs in the CCII-7-1 5’ upstream region.

The negative region upstream of the CCII-7-1 gene will be searched for presence of sequence motifs that are responsible for repression of B-Lβ gene expression in B-L-negative cells. It will be interesting to investigate whether these NREs, if present, also decrease the level of expression in cells that constitutively express B-L molecules, and whether they can function as universal silencers. Results from these experiments will test the hypothesis that lack of class II expression in many cell types is achieved by an active repression mechanism.
3. **Identify the functions of the class II conserved boxes.**

Mutagenesis and transcription factor-binding analysis of the S, X, and Y boxes in B-L-positive or negative cells, and in IFN-induced cells may answer the question on how these sequence motifs contribute to regulation of B-Lp gene expression. Alternately, if these sequences seem to be non-functional in chickens, it provides another example of evolutionary divergence between mammals and birds.

4. **Search for regulatory elements outside of the CCII-7-1 upstream region analyzed in this study.**

Because sequence and functional analyses, as well as information obtained from mammalian class II regulation studies suggest the existence of possible enhancers outside the CCII-7-1 0.7 kb upstream region, sequences further upstream, within, or downstream of the CCII-7-1 gene will be screened for enhancer activity. If such enhancers exist, their binding proteins will be characterized, and possible interactions between these enhancers and the proximal regulatory elements will be studied.


Guillemot, F., A. Billault, O. Pourquié, G. Béhar, A.-M. Chausse, R. Zoorob, G. Kreibich, and C. Auffray. 1988. A molecular map of the chicken major histocompatibility complex: the class II β genes are closely linked to the class I genes and the nucleolar organizer. EMBO J. 7:2775-2785.


Reith, W., C. Herrero-Sanchez, M. Kobr, P. Silacci, C. Berte, E. Barras, S. Fey, and B. Mach. 1990. MHC class II regulatory factor RFX has a novel DNA-binding domain and a functionally independent dimerization domain. **Genes Dev.** 4:1528-1540.


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APPENDIX A.
MAJOR HISTOCOMPATIBILITY COMPLEX CLASS RESTRICTION
FRAGMENT LENGTH POLYMORPHISM ANALYSIS IN HIGHLY INBRED
CHICKEN LINES AND LINES SELECTED FOR MAJOR
HISTOCOMPATIBILITY COMPLEX AND IMMUNOGLOBULIN PRODUCTION

A paper published in the journal Poultry Science\textsuperscript{1}

Y. CHEN and S. J. LAMONT

Summary

Selected chicken populations were analyzed by restriction fragment length polymorphism (RFLP) with a chicken MHC Class I (B-F) cDNA probe. The 13 highly inbred chicken lines differed in genetic origin and in MHC (B) haplotype, as distinguished by using hemagglutination with antisera against B-G and B-F antigens. The S1 sublines differed for B haplotype and antibody response to a synthetic polypeptide, GAT. In the highly inbred lines, band-sharing between lines from different origins was less than that between lines from same origin, showing the influence of the genetic background on chicken MHC Class I gene RFLP. In the S1 line, use of three restriction endonucleases (BglII, PvuII, TaqI) produced MHC Class I RFLP patterns that were associated with B haplotype, but not with immune response.

\textsuperscript{1}Reprinted with permission from Poultry Science 1992, 71:999-1006. Copyright © Poultry Science.
to GAT (IrGAT). A previous study (Pitcovsky et al., 1989) also demonstrated an association of MHC class IIβ RFLP patterns with B haplotype, but not IrGAT, in the same line, suggesting that IrGAT is not controlled by MHC Class I or Class IIβ genes.

(Key words: chicken, restriction fragment length polymorphism, major histocompatibility complex class I, GAT, antibody)

Introduction

The B complex, first described as a blood group by Briles et al. (1950), was later identified as the MHC in chickens (Schierman and Nordskog, 1961). There are three classes of highly polymorphic genes encoded in the B complex, designated B-F, B-L, and B-G, or Class I, Class II, and Class IV, respectively. The B-F and B-L genes are interspersed in the B-F/B-L subregion, which is closely linked to the B-G subregion (Guillemot et al., 1988). The B-F and B-L antigens are similar to their mammalian homologues in structure, function, and tissue distribution (Guillemot et al., 1989b). The B-G antigen, however, is unique to avian species and is mainly expressed on erythrocytes (Pink et al., 1977), although other tissues also may express the B-G antigen (Miller et al., 1990).

The B complex has been associated with resistance to many diseases in chickens, including Marek’s disease and fowl cholera, and also with important economic traits (reviewed by Bacon, 1987; Lamont, 1989), illustrating the value in studying it in further detail. One approach that can provide information on the structure of MHC genes is analysis by using RFLP. Previously, RFLP with a chicken MHC Class IIβ probe had been used to analyze highly inbred lines and lines selected for MHC type and Ig production (Warner et al., 1989; Pitcovski et al., 1989), but no data on Class I (B-F) genes were reported. The purpose
of the present study was to examine the B-F genes in these chicken lines by using RFLP analysis.

**Materials and Methods**

**Chicken Lines**

Thirteen highly inbred lines and a partially inbred line selected for MHC and antibody response to GAT were used in this study. The highly inbred lines were produced from a variety of genetic origins by full-sib matings and selection for homozygosity of the B haplotype (Table 1). Birds were serologically characterized for their B haplotype by using antisera against B-F and B-G antigens (Warner et al., 1989). The F2 generation from a cross between the two different B haplotype HN lines also was analyzed. The Iowa State University S1 White Leghorn line, a partially inbred line, was selected for B haplotype (B1 or B19) and for level of secondary antibody response to GAT by ELISA, resulting in four homozygous sublines: B1-IrGATlow, B1-IrGAThigh, B19-IrGATlow, and B19-IrGAThigh (Nordskog and Cheng, 1988). Two birds were sampled from each of these four sublines.

**Deoxyribonucleic Acid Isolation and Preparation of Hybridization Membranes**

Chicken DNA was isolated from whole blood by standard procedures (Maniatis et al., 1989). Samples of DNA were digested completely with restriction endonuclease PvuII, BglII, or TaqI (Promega Corp., Madison, WI 53700). Fragments were electrophoresed and blotted to Hybond N+ nylon membrane (Amersham Corp., Arlington Heights, IL 60005) by capillary transfer. The DNA fragments were then fixed to the membrane by placing the membrane on a Whatman 3MM paper soaked with .4 N NaOH for 20 min and then washing in 5 x standard sodium citrate (SSC, 150 mM NaCl and 15 mM Na3C6H5O7) for 1 min.
TABLE 1. Description of the 13 highly inbred chicken lines

<table>
<thead>
<tr>
<th>Line name</th>
<th>Breed</th>
<th>Year of origin</th>
<th>Inbreeding coefficient (%)</th>
<th>1991</th>
<th>Previous</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Egyptian</td>
<td>1954</td>
<td>98</td>
<td>43</td>
<td>15.2 (or Y)</td>
</tr>
<tr>
<td>M</td>
<td>Egyptian</td>
<td>1954</td>
<td>98</td>
<td>44</td>
<td>5.1 (or Z)</td>
</tr>
<tr>
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<td>Spanish</td>
<td>1954</td>
<td>98</td>
<td>41</td>
<td>21.1 (or X)</td>
</tr>
<tr>
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<td>Leghorn</td>
<td>1954</td>
<td>99</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>HN</td>
<td>Leghorn</td>
<td>1954</td>
<td>99</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>GH</td>
<td>Leghorn</td>
<td>1954</td>
<td>98</td>
<td>47</td>
<td>15.1 (or B)</td>
</tr>
<tr>
<td>GH</td>
<td>Leghorn</td>
<td>1954</td>
<td>98</td>
<td>48</td>
<td>13</td>
</tr>
<tr>
<td>GH</td>
<td>Leghorn</td>
<td>1954</td>
<td>98</td>
<td>49</td>
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</tr>
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<td>G^4</td>
<td>Leghorn</td>
<td>1962</td>
<td>99</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>G^5</td>
<td>Leghorn</td>
<td>1962</td>
<td>99</td>
<td>50</td>
<td>13</td>
</tr>
<tr>
<td>19</td>
<td>Leghorn</td>
<td>pre-1940</td>
<td>98</td>
<td>45</td>
<td>15.1 (or L)</td>
</tr>
<tr>
<td>19</td>
<td>Leghorn</td>
<td>pre-1940</td>
<td>98</td>
<td>46</td>
<td>13</td>
</tr>
<tr>
<td>8</td>
<td>Leghorn</td>
<td>pre-1940</td>
<td>98</td>
<td>42</td>
<td>15.1 (or B)</td>
</tr>
</tbody>
</table>

1 Updated from Warner et al., 1989.
3 Used in previous publications (e.g., Warner et al., 1989; Knudtson and Lamont, 1989).
4 Also known as G-B2.
5 Also known as G-B1.
Probe Preparation and Hybridization

An MHC Class I (B-F) cDNA probe, F10, cloned from a B12 haplotype chicken was used (Guillemot et al., 1988, kindly provided by C. Auffray, Centre National de la Recherche Scientifique, Nogent-sur-Marne, France). The probe was cut from the vector and recovered by using the QIAEX agarose gel extraction protocol (QIAGEN Inc., Chatsworth, CA 91311). The probe DNA was labeled to approximately $5 \times 10^8$ cpm/µg with $^{32}$P-deoxycytidine triphosphate by random priming. Twenty-five to 50 ng of labeled probe was denatured and hybridized with the membrane for 20 to 24 h at 65°C. The membranes were then washed once with $0.263 \ M \ Na_2HP0_4$ and 1% SDS, twice with 2 x SSC and 0.1% SDS, and twice with 0.8 x SSC and 0.1% SDS. All the washes were performed at 65°C for 15 to 20 min. The membranes were exposed to Kodak XAR-5 X-ray films with an intensifying screen at -70°C for 2 to 4 days.

Band-Sharing Calculation

Similarity of the position of the bands was determined by visual inspection and comparison with molecular weight marker. Band-sharing between two lanes of the autoradiogram was calculated by the formula $N_{ab} = (N_a + N_b)$, where $N_a$ = number of bands in Lane a; $N_b$ = number of bands in Lane b; and $N_{ab}$ = number of bands shared in the two lanes. Band-sharing between lines from the same genetic background and band-sharing between lines from different genetic backgrounds were contrasted.

Results

Thirteen Highly Inbred Lines

Among the 13 highly inbred chicken lines, Lines G-B2 (B$^6$), G-B1 (B$^{50}$), and 19-B$^{46}$ (Figure 1, Lanes 1, 2, and 13) shared the same RFLP pattern after DNA digestion with
restriction endonuclease *PvuII* or *BglII* and hybridization with the MHC Class I probe F10. Line 19-B^45 and the three GH lines (Figure 1, Lanes 5, 7, 8, and 9) produced identical MHC Class I RFLP patterns after *BglII* digestion (Figure 1B), but Line 19-B^45 differed from the GH lines in the weakly hybridizing bands after *PvuII* digestion (Figure 1A). All the other lines had unique patterns, confirming considerable polymorphism of the B-F genes. The band-sharing data in these lines are presented in Table 2. The number of strongly hybridizing bands also differed in different lines, ranging from 5 in the Spanish line to 13 in lines M-B^44 and HN-B^15.

To test the association of B-G/B-F serological typing with Class I RFLP, pools were made from blood of 20 individual birds of each serologically identical B haplotype group (B^12^B^12^, B^12^B^15^, or B^15^B^15^) of the F2 generation of a cross between the two HN lines. Analysis with the MHC Class I (B-F) probe showed that the heterozygotes (Figure 2, lane 2) had all the bands present in either of the homozygotes (Figure 2, lanes 1 and 3). Reduction in band intensity of heterozygous bands to approximately half of that in homozygous samples is shown in the three high-molecular-weight bands present in heterozygous form in the B^12^B^15^ samples but in homozygous form in the B^12^B^12^ and B^15^B^15^ samples. The B^12^B^15^ pool had all three bands, whereas the B^12^B^12^ pool had two of them, and the B^15^B^15^ pool had the other band.

**Iowa State University S1 line**

Polymorphism was observed in the S1 line after hybridization with the MHC Class I (B-F) probe. Digestion with *BglII* yielded a pattern in which both B^1^ sublines had a 9.4-kb band, whereas both B^19^ sublines lacked this band but had a 18 kb band instead (Figure 3). No association between IrGAT and RFLP bands was detected. When the restriction enzyme *PvuII* was used, a 9.1-kb band present in all the B^1^ birds was split into two bands (8.6 and .5 kb) in all the B^19^ birds, regardless of the IrGAT trait (Figure 4, bands indicated by arrows).
Figure 1. Autoradiogram of chicken genomic DNA digested with restriction endonuclease A) PvuII or B) BglII, and hybridized with $^{32}$P-labeled MHC Class I (B-F) probe F10. Molecular weight markers are from $\lambda$DNA HindIII digest. Chicken lines are: 1) G-B2 (B6); 2) G-B1 (B13); 3) M-B44; 4) M-B43; 5) 19-B45; 6) Sp-B41; 7) GH-B49; 8) GH-B48; 9) GH-B47; 10) HN-B12; 11) HN-B15; 12) 8-B42; and 13) 19-B46.
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1Generated from Figure 1B.

^2BS1 = average band-sharing with all lines from different genetic backgrounds.

^3BS2 = band-sharing with line(s) from the same genetic background, with different B haplotype(s).

^4N = total number of strongly hybridizing bands.
Figure 2. Autoradiogram of chicken genomic DNA of the F2 generation from a HN-B12 x HN-B15 cross. Each DNA sample was isolated from a blood pool of 20 birds. The DNA was digested with BglII and hybridized with 32P-labeled MHC Class I (B-F) probe F10. Molecular markers are λDNA from HindIII digest. Ea-B types are: 1) B12B12; 2) B12B15; and 3) B15B15.
Figure 3. Autoradiogram of S1 line DNA, digested with BglII and hybridized with $^{32}$P-labeled MHC class I (B-F) probe F10. Molecular weight markers are from λDNA HindIII digest. Sublines are: 1) $\text{B}^1$-IrGAT$_{\text{high}}$; 2) $\text{B}^1$-IrGAT$_{\text{low}}$; 3) $\text{B}^{19}$-IrGAT$_{\text{high}}$; and 4) $\text{B}^{19}$-IrGAT$_{\text{low}}$. 
Ir-GAT:
Blood type: 1

23 -
9.4 -
6.6 -
4.4 -
Figure 4. Autoradiogram of PvuII-digested S1 line DNA, hybridized with $^{32}$P-labeled MHC class I (B-F) probe F10. Molecular weight markers are from λDNA HindIII digest. Sublines are: 1) B$^{1}$-IrGAT$^{\text{low}}$; 2-3) B$^{1}$-IrGAT$^{\text{high}}$; 4-5) B$^{19}$-IrGAT$^{\text{low}}$; and 6-7) B$^{19}$-IrGAT$^{\text{high}}$. 
Use of another enzyme, TaqI, also showed a MHC Class I polymorphism associated with B blood type but not IrGAT (data not shown). Digestion with each of the three enzymes showed association between the MHC Class I (B-F) RFLP patterns and the B blood type (determined by hemagglutination) but not with the antibody response to GAT.

Discussion

The MHC Class I RFLP analysis of the 13 highly inbred chicken lines demonstrated polymorphism of B-F genes in these lines. Use of a few restriction endonucleases could not distinguish some of the lines with different B haplotypes, especially in lines originated from the same breed (e.g., the two G lines and the three GH lines). A difference in B-F RFLP patterns was noted between line HN-B15 and line 15I5 (Lamont et al., 1990), however, which share the serologically determined B15 haplotype. This observation was in agreement with MHC Class II (B-L)β RFLP studies (Warner et al., 1989), in which lines HN-B12 and HN-B15 also showed different class IIβ RFLP patterns than lines 15.C-B12 and 15I5, respectively. The results of these comparisons illustrate that RFLP is a powerful tool in detecting differences in the B complex that are undetectable by serological typing. Sharing of B-Lβ or B-F RFLP patterns could mean that the B-Lβ or B-F genes are the same, but this conclusion is not definite until those genes are sequenced, because many nucleotide substitutions cannot be detected by any restriction endonucleases. The differences in the RFLP patterns definitely represent subregion diversity, however, and the variation of the number of the strongly hybridizing bands in the B-F RFLP analysis may reflect a difference in the number of B-F genes in different chicken lines; this supports previous observations (Chaussé et al., 1989). Although the B-G and B-F/B-L subregions are closely linked to each other (Guillemot et al., 1989b), the RFLP analysis of the B-L and B-F genes, in addition to
the traditional serological B-G/B-F typing, may help to characterize the B complex. The B-G RFLP also has been successfully applied to genotyping the B complex (Chausse et al., 1989; Miller et al., 1988; Uni et al., in press).

Band-sharing (BS, from Figure 1B) between inbred lines was contrasted for each line between a) the average BS with all the lines that have a different genetic background and b) the BS with the line(s) that have the same genetic background (as indicated by the same line designation). The BS between individual lines ranged from .27 (line HN-B15 with the two G lines and line 19-B46) to 1.0 (line 19-B46 with the two G lines; line 19-B45 with the three GH lines). For all lines (except M-B43), BS with line(s) that have the same genetic origin is greater than the average BS with lines that have different genetic origins. This is especially evident in the G lines, the GH lines, the 19 lines, and line HN-B15. In general, the data in Table 2 suggest that the genetic background of a chicken line influences the MHC Class I (B-F) RFLP pattern. Chicken lines that have the same genetic background tend to have more similar patterns than lines having different genetic backgrounds. But having similar or even identical B-F (or B-L or B-G) RFLP patterns does not always indicate that lines have a close genetic relationship. There are examples (e.g., the two 19 lines) in which a particular line has a B-F RFLP pattern closer to that of line(s) with different genetic background(s) than that of line(s) with the same genetic background. The reason for such instances might be 1) there was mutation in the B complex that happened to be detected by a restriction endonuclease; 2) there was mutation in the flanking region outside of MHC; or 3) recombination occurred between B-G (which is serologically typed) and B-F genes. Previously, a similar approach was taken by Alexander et al. (1987) to show the influence of equine MHC haplotype on the RFLP patterns with an MHC Class I probe, by counting the number of bands shared between lanes and the number of bands different between lanes. The result of their approach showed
that horses sharing the same MHC haplotype had closer MHC Class I RFLP patterns than those with different MHC haplotypes.

Besides the bands strongly hybridizing with the MHC Class I probe, there are many weakly hybridizing bands shown on the autoradiograms, some of which are polymorphic. There are several possible explanations for these weak bands: they could be genes from other members of the immunoglobulin gene superfamily; they could be pseudogenes that contain the polymorphic exon of the B-F gene, and thus hybridize much more weakly to the probe; or, the restriction endonucleases may recognize internal sites in the B-F genes.

Because the immune response to GAT is linked to the B complex (Benedict et al., 1975), and the S1 sublines B^1^-IrGAT^high and B^{19}-IrGAT^low were previously hypothesized to result from recombination between B^1^-IrGAT^low and B^{19}-IrGAT^high (Pevzner et al., 1978), one objective was to determine the association between B-F RFLP pattern and the IrGAT trait. With the three restriction endonucleases used, the B-F RFLP patterns were associated with B blood type, but independent of the IrGAT type. Combining results of this study with those from B-L^B RFLP analysis (Pitcovsky et al., 1989) and other studies using immunological methods (Steadham, 1991), it is proposed that the locus controlling IrGAT is not identical to the B-F or B-L^B genes. Additional genes have been found interspersed among B-F and B-L^B genes (Guillemot et al., 1989b) and these genes may perhaps control or regulate the IrGAT phenotype. One of these genes, 12.3, is hypothesized to be involved in lymphocyte activation (Guillemot et al., 1989a). Nevertheless, both B-F and B-L^B RFLP patterns are associated with the serologically determined B haplotype in the S1 line.

Previously, resistance to two diseases, Marek's disease and fowl cholera, was found to be linked with the B haplotype in the S1 line (Steadham et al., 1987; Lamont et al., 1987), illustrating an association between resistance to these diseases and the MHC Class I and II RFLP patterns.
Acknowledgments

The F10 probe used in this study was provided by Charles Auffray (Centre National de la Recherche Scientifique, France). Betty Young, Yoram Plotsky, and Michael Kaiser provided technical assistance.

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APPENDIX B.

EXPRESSION OF B-Lβ mRNA IN CHICKEN B CELLS AND MACROPHAGES

To compare the level of B-L expression in the MQ-NCSU macrophage cell line to the bursal cells, B cell lines RP9 and DT40, total RNA was isolated from these cell lines or from chick bursa following the protocol described by Chomczynski and Sacchi (1987). The chicken macrophage cell line was treated with the interferon-rich p34 supernatant (concentrated 200 times) at a 1:2000 dilution for 5 or 24 hours before harvested for RNA isolation. The RNA was probed for B-Lβ mRNA by Northern blot analysis (Sambrook et al. 1989). 32P-labeled p234 (kindly provided by Dr. C. Auffray, Centre National de la Recherche Scientifique, Nogent-sur-Marne, France) insert, which contains the conserved β2 exon sequence of the B-Lβ gene (Bourlet et al. 1988), was used as probe.

Results and Discussion

Figure 1 shows the autoradiogram of the Northern blot analysis. Bursal cells were used as positive control, because they consist of mainly B cells, and therefore should synthesize B-Lβ mRNA. The Ia+ B cell line RP9 had a strong signal for B-Lβ mRNA, while the Ia- (as shown by surface labeling experiments, data not shown) DT40 cell line was negative. Although the macrophage cell line was not expected to express high level of B-Lβ mRNA, it was surprising that no significant signal was detected with this cell line in the Northern blot, even after p34 supernatant induction. After two weeks of exposure, however, very weak signals could be observed, indicating that this cell line expresses extremely low level of B-Lβ molecules compared to the RP9 cells or the bursal cells. It is also evident that this cell line is not highly inducible for B-L expression by the interferon-rich supernatant.
Figure 1. Northern blot analysis for B-Lβ expression. Lane 1, bursal cells; lane 2, the RP9 cell line; lane 3, the MQ-NCSU cell line treated with p34 supernatant for 24 hours; lane 4, MQ-NCSU cells treated for 5 hours; lane 5, MQ-NCSU cells without treatment; lane 6, the DT40 cell line. Approximate length of RNA detected by the B-Lβ probe is shown by the arrow at left.
References


APPENDIX C.

ESTABLISHMENT OF STABLE TRANSFECTED CELL LINES

Experimental Procedure

Stable transfected cell lines originating from the MQ-NCSU chicken macrophage cell line were established by using the following procedure:

1. Cells were transfected simultaneously with 10 μl each of the appropriate plasmid construct and the neo-resistant plasmid pSVneo3 (a gift from Dr. Susan L. Carpenter, Department of Microbiology, Immunology, and Preventive Medicine, Iowa State University, Ames, IA 50011) by using the calcium phosphate transfection method.

2. One day after transfection, cells were transferred to a 10 mm culture dish with 15 ml of medium containing 0.3 to 0.5 mg/ml of geneticin (Sigma). Cells started to die after a few days.

3. Drug-resistant cell colonies started to appear after 7 to 10 days. New medium was added to dilute the concentration of the drug when necessary.

4. When the cell colonies grew up to the size that could be visualized without a microscope, the medium in the dishes was discarded. A small piece of filter paper dampened (but not saturated) with trypsin solution was placed on each colony for about half a minute, and transferred into culture medium in a well of a 48-well culture plate. Each piece of the filter
paper should contain a single colony, and the colonies were grown up in the wells until enough cells were obtained.

5. The cells were then screened for the presence of the desired foreign DNA in their genomes. DNA isolated from each colony was subjected to PCR reactions using primers specific for the transfected DNA. Positive cell colonies were frozen down and were further analyzed if necessary.

Results and Discussion

Drug-resistant cell colonies were obtained after co-transfection of plasmid pSVneo3 (which confers resistance to geneticine) and constructs pC7-12ECAT, pC7-42ECAT, or pC7-52ECAT (see Section 2 for description of these constructs), and selection with geneticine. PCR analysis of the genomic DNA isolated from these cell colonies was performed using primers that amplify the chicken DNA cloned into the pCAT-Enhancer vector. Cell colonies containing the desired constructs in their genomes were identified for each of the constructs by the PCR analysis. When cell lysates from these positive colonies were assayed for production of CAT enzyme, however, none showed significant CAT activity. This absence of CAT activity in these stable transfected cell lines may be a result of chromosomal repression for transcription, because the exogenous DNA may be inserted to a location in the chromosome where transcription of genes is not activated. It may also be explained by the fact that the CCII-7-1 promoter is very weak in this macrophage cell line.
Appendix D.

Polymerase Chain Reaction for Amplifying GC-Rich DNA

A specific polymerase chain reaction (PCR) program with a high annealing temperature was designed for amplifying GC-rich DNA fragments:

95°C 2 minutes, 65°C 1 minute, 73°C 2 minutes;
Repeat the cycle once;
95°C 1 minute, 65°C 1 minute, 73°C 2 minutes;
Repeat the cycle 5 times;
94°C 1 minute, 65°C 1 minute, 73°C 2 minutes;
Repeat the cycle 37 times;
75°C 13 minutes.

Ingredients in the PCR reaction mixture (per 50 µl):

- 10 x PCR buffer (Mg²⁺-free) 5 µl
- dNTP (10 mM each) 1 µl
- Primers (2 µM) 5 µl each
- MgCl₂ (25 mM) 2 µl
- Taq DNA polymerase (5 U/µl) 0.5 µl
- DNA template 1 - 200 ng
- Dimethyl sulfoxide 2.5 µl
- H₂O bring the volume to 50 µl
APPENDIX E.

CHLORAMPHENICOL ACETYLTRANSFERASE ASSAY

The following procedure is for chloramphenicol acetyltransferase (CAT) assay with adherent cells. For cells growing in suspension, harvest cells by centrifugation at approximately 1000 g, and wash cells twice with phosphate buffered saline (PBS) before suspending in appropriate amount of 0.25 M Tris (PH 7.8).

I. Harvest cells

1. Discard culture medium, wash with PBS once.
2. Add 1 ml PBS to a 60 mm dish or 75 cm² flask, scrape cells with a rubber policeman.
3. Collect cells by centrifugation, wash cells once with 1 ml PBS.
4. Resuspend cells in 100 µl 0.25 M Tris (PH 7.8). Cells can be kept frozen at this step.

II. Lyse cells and determine protein concentration of the lysate

1. Freeze-thaw cells three cycles using a dry ice-ethanol bath and a 30°C water bath.
2. Centrifuge in a microfuge at full speed for 5 minutes, collect the liquid and discard the pellet.
3. Take appropriate amount of cell lysate (up to 10 µl) for the BCA protein assay (Pierce).

III. CAT assay

1. Heat cell lysate at 65°C for 10 minutes, centrifuge at 4°C for 5 minutes.
2. Take a volume of the liquid which contains desired amount of total protein to a microcentrifuge tube.
3. Add 0.25 M Tris (PH 7.8) to total volume of 87 µl. Add 10 µl of 8 mM acetyl-CoA (water solution) and 3 µl of ¹⁴C-chloramphenicol (50 µCi/ml, NEN). Incubate the
reaction mixture at 37°C for 2 to 3 hours. (Addition of another 5 µl of 8 mM acetyl-CoA  
1.5 hours after the start of incubation may increase acetylation)

4. Add 500 µl of ethyl acetate to the tube. Vortex to mix well, and centrifuge for 2 minutes.

5. Pipette upper organic phase to a new tube. Vacuum dry or air dry in a hood.

6. Add 16 µl of ethyl acetate to dissolve the chloramphenicol (acetylated or non-acetylated).

7. Spot onto a thin-layer chromatography (TLC) sheet (approximately 1 inch from one edge,  
   so that the solvent in the chromatography tank will be just below the spotted samples).

8. Separate the chloramphenicol by chromatography solvent (380 ml of chloroform / 20 ml  
   of methanol).

9. After 50 minutes, dry the TLC sheet, and expose it to an X-ray film at room temperature  
   (usually takes 3 days).

10. Quantify the radioactivity in acetylated and non-acetylated forms of chloramphenicol by  
    a phosphoimager and calculate the acetylation percentage.