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Yunfei Chen
Iowa State University

Hyun S. Lillehoj
United States Department of Agriculture

Chung-Hsin Hsu
Iowa State University

Susan L. Carpenter
Iowa State University

Susan J. Lamont
Iowa State University, sjlamont@iastate.edu

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Yunfei Chen · Hyun S. Lillehoj · Chung-Hsin Hsu
Susan L. Carpenter · Susan J. Lamont

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Abstract A 0.7 kilobase (kb) DNA fragment from the 5′ flanking region of a chicken major histocompatibility complex (*MHC*) class II *B* gene was cloned into chloramphenicol acetyltransferase (CAT) reporter vectors and was transfected into a 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 (*NRE*) located further upstream. The conserved *MHC* class II X and Y boxes did not have a significant effect on promoter activity. Sequence analysis of the 0.7 kb class II *B* gene upstream region suggests possible involvement of interferon (IFN), E twenty-six specific (ETS)-related proteins, and other factors in regulating this promoter. A chicken T-cell line culture supernatant increased surface expression of MHC class II antigens, as well as class II promoter activity, in this macrophage cell line. This first functional characterization of a chicken *MHC* class II *B* gene promoter will aid in understanding the regulatory mechanisms that control the expression of these genes.

The nucleotide sequence data reported in this paper have been submitted to the GenBank nucleotide sequence database and have been assigned the accession number L32814

Y. Chen · C. Hsu · S. L. Carpenter · S. J. Lamont (✉)
The Departments of Animal Science and of Microbiology, Immunology, and Preventive Medicine, 201 Kildee Hall, Iowa State University, Ames, IA 50011, USA

H. S. Lillehoj
USDA, Agricultural Research Service, Livestock and Poultry Sciences Institute, Parasite Immunology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

Introduction

The major histocompatibility complex (*MHC*) class II genes encode proteins that present antigens to helper T cells and are thus critical in initiating the cell-mediated immune responses against invading pathogens (Germain et al. 1986). A class II molecules consists of an α chain, which has limited polymorphism, and a β chain, which is highly polymorphic. The class II genes are constitutively expressed in B cells, and are inducible in cells such as macrophages and T cells (reviewed by Benoist and Mathis 1990). IFN-γ is the major factor to induce class II expression in macrophages. Selective expression of these genes is an important step in genetic control of the immune response. 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 tissue-specific transcription of the class II genes (reviewed by Benoist and Mathis 1990; Glimcher and Kara 1992). Reith and coworkers (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 (Boss and Strominger 1986; Tsang et al. 1988; Burkly et al. 1989).

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 genetically unlinked to the *B* complex (Briles et al. 1993; Miller et al. 1994). Significant structural differences between the avian and mammalian *MHC* have evolved. Several chicken *MHC* class II (also named *B-L*) *B* genes have been isolated and sequenced in the B^6 (Xu et al. 1989) and B^{12} (Zoorob et al. 1990) haplotypes. The organization of the DNA elements regulating these genes differs from that in mammalian

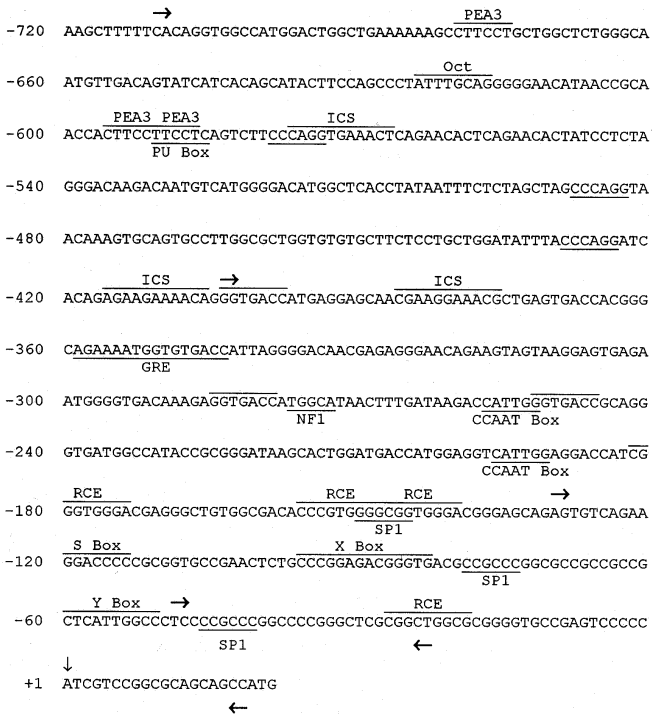

Fig. 1 Nucleotide sequence of the *CCII-7-1* 5′ flanking region. The sequence was revised from Xu and co-workers (1989) and was confirmed by sequencing PCR products derived from this region of DNA. The previously described transcription initiation site (Zoorob et al. 1990) is shown by a *vertical arrow*. 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. PCR primers used in creating the deletion constructs are shown as *horizontal arrows above* (for 5′ primers) or *below* (for 3′ primers) the beginning of the annealing nucleotide sequence, with the arrowheads pointing in the direction of amplification

species, but the S, X, and Y boxes are well conserved in some of these chicken *B-LB* genes, especially in the *B-LBII* gene of the B^{12} haplotype and the *CCII-7-1* gene of the B^{6} haplotype. Comparison of sequence data obtained from the *B-LB* cDNA clones (Zoorob et al. 1990; Sung et al. 1993; Pharr et al. 1993) and the genomic clones suggests that the *B-LBII* gene of the B^{12} haplotype and the *CCII-7-1* gene of the B^{6} haplotype are expressed, although there is evidence for expression of other *B-LB* genes (Guillemot et al. 1986; Pharr et al. 1993; Zoorob et al. 1993). Our objective was to identify and analyze a potential *B-LB* gene promoter by functional characterization of DNA sequences in a 0.7 kb 5′ upstream region of a chicken *MHC* class II *B* genomic clone, *CCII-7-1*. A chicken macrophage cell line was used to study transcriptional activity of the *B-LB* promoter because of its potential inducibility by IFN and because this cell line has been reported as class II-positive (Qureshi et al. 1990).

Materials and methods

The CCII-7-1 subclone

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-LB* structural gene and 0.7 kb of its 5′ flanking sequence. The 5′ region upstream of the translation start site was sequenced by the dideoxy-nucleotide chain termination method.

Cell culture and surface B-L labeling

The MQ-NCSU chicken macrophage cell line was provided by M. A. Qureshi (Poultry Science Department, North Carolina State University, NC, USA). It was maintained in CM (Qureshi et al. 1990) at 40 °C with 5% CO_2. The RP9 chicken B-cell line was obtained from the Avian Disease and Oncology Lab (East Lansing, MI, USA), and was maintained under the same conditions as the MQ-NCSU cells. The p34 chicken T-cell line was developed for IFN production by fusing spleen lymphocytes with thymidine kinase-deficient R24H4 chicken lymphoma cells with polyethylene glycol 4000. The p34 cells were maintained in Iscove's modified Dulbecco's medium, supplemented with 10% fetal calf serum and hypoxanthine-aminopterin-thymidine (Sigma, St. Louis, MO) at 41 °C with 5% CO_2. The p34 culture supernatant was concentrated as described by Kaspers and co-workers (1994). The concentrated supernatant was added to the MQ-NCSU cell culture at a 1 : 2000 dilution. After 5 to 43 h, the macrophage cells were fluorescent-labeled with a monoclonal antibody against chicken MHC class II antigens using the labeling procedure recommended by Southern Biotechnology (Birmingham, AL). A mouse IgG1 was used as isotype control. The labeled cells were examined by flow cytometry for the presence of fluorescence on the cell surface.

Plasmid construction

Polymerase chain reaction (PCR) primers were synthesized in an Applied Biosystems Oligonucleotide Synthesizer (Applied Biosystems, Foster City, CA) and purified by HPLC. A 679 base pair (bp) fragment containing the *CCII-7-1* upstream region (–660 to +19) was generated by PCR amplification of the *CCII-7-1* template using the m13 sequencing primer (GTAAAACGACGGCCAGT; New England Biolabs, Beverly, MA) and a primer CGAAGCTTGGCTGCTGCGCCG-

Table 1 Primers used in cloning the *CCII-7-1* upstream sequences into pCAT vectors

Primer	Position of annealing sequence	Primer sequence[+]
BLp1	−711 to −687	AGCTGCAGCACAGGTGGCCATGGACTGGCTGAA
BLp2*	+ 19 to + 1	CTGTCGACGGCTGCTGCGCCGGACGAT
BLp3	−404 to −385	GACTGCAGGGTGACCATGAGGAGCAACG
NLp4	−130 to −110	GGCTGCAGAGTGTCAGAAGGACCCCCGCG
BLp5	− 49 to − 33	ATCTGCAGTCCCCGCCCGGCCCCGG
BLp6*	− 23 to − 39	CCGTCGACAGCCGCGAGCCCGGGGC

* These primers anneal to the sense strand
[+] The underlined sequences are restriction sites added to the 5′ ends of the primers

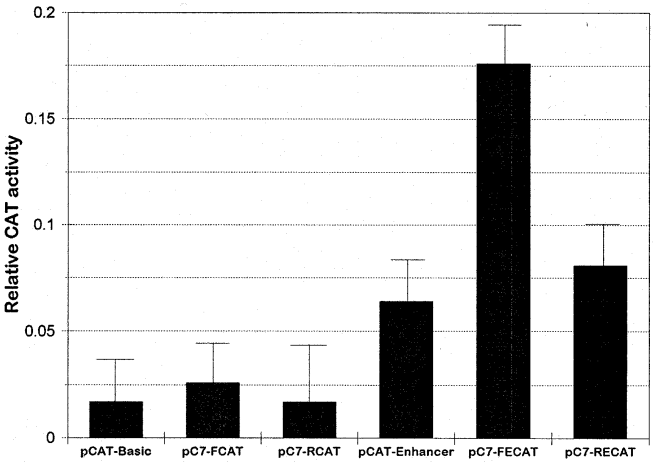

Fig. 2 Promoter activity of a 679 bp (−660 to +19 from the transcription start site) *CCII-7-1* 5′ flanking DNA fragment. This fragment was cloned into pCAT-Basic or pCAT-Enhancer in its forward direction to yield the constructs named pC7-FCAT or pC7-FECAT, respectively, and was cloned in reverse direction to yield pC7-RCAT or pC7-RECAT, respectively. These constructs 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 level of CAT activity was expressed as relative activity compared with the activity of plasmid pCAT-Control (value set at 1). The mean and standard error are shown

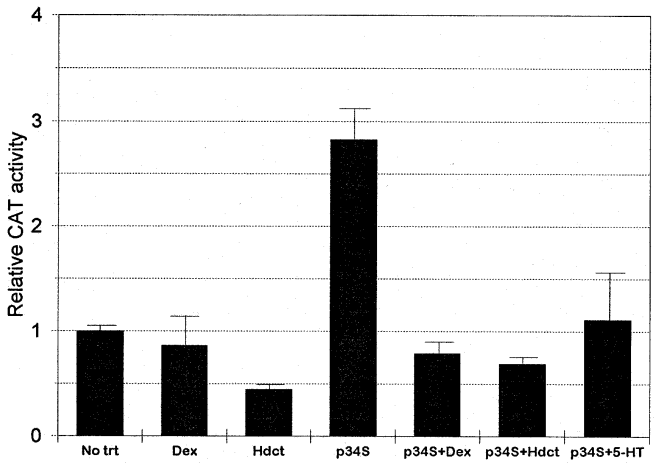

Fig. 3 Induction of CAT activity in transient expression assays by the p34 chicken T-cell line supernatant. The MQ-NCSU cell line was transfected with plasmid construct pC7E-711 (see Figure 4), and then treated with concentrated p34 supernatant (p34S, diluted 1:2000), dexamethasone (Dex, 10^{-6} M), hydrocortisone (Hdct, 10^{-6}M), serotonin (5-HT, 5×10^{-7}M), or their combinations before being harvested for CAT assay. CAT activity was expressed as relative activity compared with the transfected cells without additional treatment (No trt). The mean and standard error are shown

GACGATG, which annealed to the sequence immediately upstream of the translation start site. This fragment was cloned into the *Hin*dIII site of the pUC19 vector, and subsequently cloned into the *Hin*dIII site of vector pCAT-Basic or pCAT-Enhancer (Promega, Madison, WI). Both vectors contain a multiple cloning site immediately upstream of a *cat* structural gene, but pCAT-Enhancer has an additional SV40 enhancer. In the deletion study, the polymerase chain reaction (PCR) primers (see Table 1 and Figure 1) for amplifying the *CCII-7-1* DNA fragments had a *Sal*I or *Pst*I restriction site added to their 5′ ends for directional cloning. To amplify DNA fragments spanning a region with high GC content, dimethyl sulfoxide was added to 5% (Bookstein et al. 1990). The PCR products were cloned into the *Pst*I/*Sal*I 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.

Transfection and CAT assay

Ten to twenty micrograms of supercoiled plasmid DNA was used in each transfection to transfect the chicken macrophage cell line by the calcium phosphate method (modified from Sambrook et al. 1989). Three to 5 h after transfection, medium was removed, and cells were incubated with 15% glycerol in HEPES-buffered saline for 2 min. Cells were cultured for 36 to 48 h after glycerol was removed. They were then harvested and lysed by three cycles of freezing and thawing, and total protein concentration of the cell lysates was determined by using a BCA protein assay kit (Pierce, Rockford, IL). The volume of cell lysate added to each CAT assay reaction was normalized according to the protein concentration. CAT assays were performed as described by Gorman and co-workers (1982). After thin-layer chromatography, the amount of radioactivity in acetylated or non-acetylated forms of ^{14}C-chloramphenicol was determined by a Molecular Dynamics PhosphorImager 400E machine (Molecular Dynamics, Sunnyvale, CA). Level of CAT activity was calculated as percentage of conversion into acetylated ^{14}C-chloramphenicol, and expressed as relative CAT activity compared with the percent conversion of pCAT-Control (Fig. 2) or that of pC7E-711 (Figs. 3, 4).

Results

Sequence analysis of the CCII-7-1 5′ flanking region

The sequence of a 444 bp fragment immediately upstream of the *CCII-7-1* translation start site (Fig. 1) shared a 96% similarity to that of the functional *B-LBII* gene of the B^{12} haplotype (Zoorob et al. 1990). The mismatches included one nucleotide in each of the S and X boxes. Upstream of −444, the sequence of the two clones was quite different (36% similarity), although both had a putative octamer-binding sequence. No TATA box sequence was observed in the *CCII-7-1* 5′ flanking region, but a transcription initiation sequence was present around the transcription start site (Fig. 1), as described in the *B-LBII* promoter by Zoorob and co-workers (1990). The immediate 150 bp *CCII-7-1* upstream region was highly GC-rich (81.3% G+C). Putative S, X, and Y boxes were located in the -120 to -50 region, relative to the transcription initiation site. The Y box included 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-LB* promoter. Three SP1-binding sites and four retinoblastoma control elements (*RCE*; Robbins et al. 1990; Kim et al. 1991) 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-

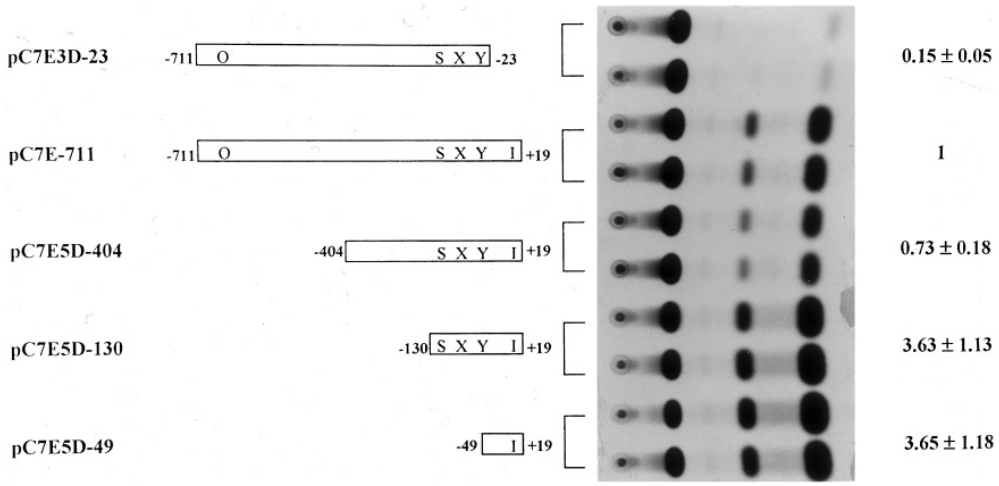

Fig. 4 Deletion analysis of the *CCII-7-1* 5′ flanking sequence. Construct pC7E-711 contained 730 bp (−711 to +19) of the promoter region, 51 bp longer than the chicken DNA insert in pC7-FECAT. pC7E5D-404 (−404 to +19), pC7E5D-130 (−130 to +19), and pC7E5D-49 (−49 to +19) were 5′ deletion constructs. The S, X, Y boxes were deleted in construct pC7E5D-49. pC7E3D-23 (−711 to −23) had a 3′ deletion to remove the transcription initiator. The regions of *CCII-7-1* that each insert spans are shown *left*. I = transcription initiation sequence; O = octamer; S = S box, X = X box, Y = Y box. The autoradiogram shows a typical CAT assay result, after transfecting the deletion constructs into the chicken macrophage cell line by calcium phosphate transfection. The relative CAT activities from eight transfections (mean ± standard error) were normalized to the activity of construct pC7E-711

binding proteins belonging to the ETS family, including polyomavirus enhancer activator 3 (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.

Detection of promoter activity in the CCII-7-1 5′ flanking region

Since some of the chicken *MHC* genes may be poorly or not expressed (Guillemot et al. 1988; Zoorob et al. 1993), it is important to determine whether 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 using a chicken macrophage cell line, MQ-NCSU (Qureshi et al. 1990). Figure 2 shows the result of a typical experiment. When DNA constructs containing an SV40 enhancer were compared, the construct containing the chicken DNA in its natural orientation (pC7-FECAT) had significantly more CAT synthesized (2-fold to 4-fold) than the constructs with the chicken DNA in reversed orientation or the vector alone, suggesting the presence of promoter activity in this DNA region. When the SV40 enhancer was absent, all the constructs showed much less CAT expression.

Induction of surface B-L expression and B-LB promoter activity by p34 culture supernatant

Kaspers and co-workers (1994) have reported that expression of MHC class II molecules in chicken macrophages is increased by IFN. We used the concentrated p34 chicken T-cell line culture supernatant to treat the MQ-NCSU cell line. The p34 cell line secretes a high level of chicken IFN (H. S. Lillehoj and co-workers, unpublished data). Surface class II expression of the MQ-NCSU cells increased after 5, 25, or 43 h of treatment with the concentrated supernatant, with treatment for 25 or 43 h resulting in a higher level of increase than treatment for 5 h (Fig. 5). Compared with the RP9 cell line, the MQ-NCSU cell line expressed a low level of B-L molecules, even after treatment with the supernatant. Transient expression assays (Fig. 3) also showed an increase in promoter activity of the 0.7 kb *CCII-7-1* 5′ flanking region in the macrophage cell line 2 days after treatment with the p34 supernatant. Consistent with the report in mammals that gluticorticoids inhibit the inducing effect of IFN-γ 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. 3). In the absence of the p34 supernatant, the depressive 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).

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 was generated by PCR with primers that amplify the desired regions (Fig. 1). After transfecting these constructs into the MQ-NCSU cell line and CAT assay (Fig. 4), the most 3′ end of this promoter region, including the transcription initiation sequence, was found to be required for minimal promoter

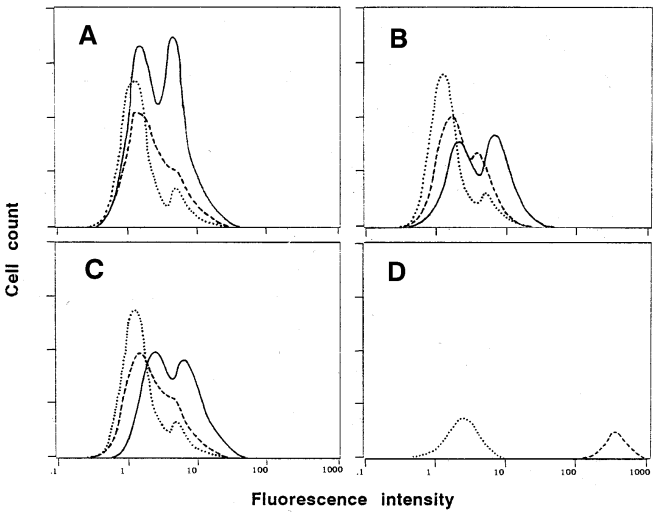

Fig. 5A–D Surface MHC class II expression of the MQ-NCSU chicken macrophage cell line. The cell counts and the intensity of fluorescence (in log scale) are shown. Isotype controls are shown as *dotted lines* (·····); cells labeled with anti-class II are shown as *broken lines* (– – –); cells treated 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 h; **B** induction of the MQ-NCSU cell line for 25 h; **C** Induction of the MQ-NCSU cell line for 43 h; **D** The RP9 B cell line

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 *NRE*, because removal of this region significantly increased promoter activity. Surprisingly, deletion of the S, X, and Y box sequences had no significant effect in most of the experiments. This contrasts with the mammalian *MHC* class II gene promoters, where these boxes play crucial roles in regulating expression of the class II genes (reviewed by Benoist and Mathis 1990; Glimcher and Kara 1992).

Discussion

The increased CAT activity with the pC7FECAT construct demonstrates that the -660 to +19 region of *CCII-7-1* contains an orientation-dependent promoter which is functional in the chicken macrophage cell line used in this study. Results from additional experiments, in which the same or different transfection methods were used (data not shown), as well as the deletion studies, also confirmed the presence of promoter activity in the *CCII-7-1* 5′ flanking region. The *CCII-7-1* 5′ flanking DNA was dependent on an additional enhancer for efficiently initiating transcription. This may be due to low expression of MHC class II molecules of this macrophage cell line. Flow cytometry data (Fig. 5) indicated that surface MHC class II antigen expression of the MQ-NCSU cell line was much lower than in the RP9 chicken B-cell line, which is classified as class II-positive. Northern blot analysis of RNA from the MQ-NCSU cells detected an extremely weak signal for MHC class II expression, while chick bursal cells and RP9 cells showed strong signals (data not shown). Therefore, a class II promoter may have restricted activity in this cell line. It is possible that enhancers located outside the 0.7 kb fragment tested for promoter activity in the present study are required for efficient gene expression. The extreme 5′ end of the *CCII-7-1* 0.7 kb regulatory region gene contains multiple transcription factor-binding sites, suggesting that there may be additional 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 (GGGGACTTTCC) 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 1986). Functional assays of the *CCII-7-1* promoter region in chicken B cells will provide more information about the requirement of additional enhancers.

The immediate upstream consensus sequences S, X, and Y boxes are of particular importance for MHC class II expression in mammalian species. In our 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 *B-LB* promoter. Several hypotheses regarding the seeming lack of regulatory function of these elements can be proposed. First, functional regulation of the chicken *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 *B-LB* genes, and may be required for high activity of the promoter. Second, the macrophages used in our experiments may 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. Expansion of this *B-LB* promoter study to more cell types may reveal other interesting patterns of transcriptional regulation. Such studies are currently being conducted. Third, 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. However, the sequence and location of these boxes are well conserved in the *B-LB* promoters, suggesting that they have a functional significance. Evolutionary divergence in genetic control of immune response has been observed in the antibody diversification mechanisms between aves and mammals. For example, the chicken employs a gene conversion mechanism for its antibody diversification (Rey-

naud et al. 1985; Thompson and Neiman 1987; Reynaud et al. 1987) instead of the *VDJ* joining of mammals. Unique features also exist in the promotor region of the chicken β_2-microglobulin (*B_2m*) gene. Recently, Riegert and coworkers (1996) cloned the chicken *B_2m* gene. The promoter of this gene contains putative S, X, and Y boxes, although expression of the *B_2m* gene differs from that of class II genes, and the S, X, Y box sequences have not been reported in mammalian *B_2m* or class I promoters.

Deletion of a 274 bp fragment (-404 to -131) immediately upstream of the S box caused an increase in CAT activity, suggesting the presence of *NRE*. Negative DNA sequences have 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 and coworkers (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. The lack of TATA box and the presence of a highly (G+C)-rich region immediately upstream of the translation start codon suggests that the *CCII-7-1* promoter resembles a typical housekeeping gene promoter. The *NRE*s may prevent B-L expression in most cells that normally do not express class II molecules, and selectively removing the repression in a few cell types may be the mechanism that leads to expression of the B-L molecules. Candidate *NRE*s in regulating *CCII-7-1* gene expression were suggested by sequence analysis. Three *RCE*s, which are potential *NRE*s (Robbins et al. 1990; Kim et al. 1991), were located within the negative region detected by deletion analysis. 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*.

The interferon response element *ICS* 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 *ICS*-like sequences. In mammals, ETS proteins may trans-regulate genes that contain *ICS* (Williams 1991). Interestingly, two of the *ICS*-like elements (-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. In the chicken macrophage cell line used in this study, *B-LB* promoter activity and surface I a expression both increased after induction by the p34 supernatant, but surface class II expression and B-L mRNA level remained low compared with bursal cells and a B-L-positive B-cell line. Other lymphokines in the p34 supernatant may mask the induction effect by IFN, a question that will be able to be answered when recombinant chicken IFN becomes available.

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