GENE EXPRESSION OF BETA-DEFENSINS IN CHICKEN WHITE BLOOD CELLS

A Thesis

by

TIFFANY MARIE SUPAK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2006

Major Subject: Poultry Science

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Approved by:

Co-Chairs of Committee,	James Zhu
	Luc R. Berghman
Committee Member,	David Adelson
Head of Department,	Alan R. Sams

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ABSTRACT

Gene Expression of Beta-Defensins in Chicken

White Blood Cells. (August 2006)

Tiffany Marie Supak, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. James Zhu
Dr. Luc R. Berghman

Infectious agents such as bacteria or viruses can grow rapidly. If a microorganism invades a host, it must be recognized rapidly and destroyed before it overwhelms the immune system. Limiting infection to a minimum in the early stage is critical for the outcome and the recovery from infection. The innate immune system has evolved to recognize a few highly conserved, constitutive structures present only in microorganisms, such as bacterial lipopolysaccharide (LPS), called pathogen-associated molecular patterns (PAMP). Toll-like receptors are the host receptors that recognize PAMP, ultimately activating a variety of transcription factors to induce expression of a wide spectrum of immune related genes, e.g. defensins. Defensins are antimicrobial peptides that play an important role in innate defense against microorganisms in plants and animals. Beta-defensins are the largest family of antimicrobial peptides, which can directly kill microorganisms and have regulatory effects on the immune system. Thirteen beta-defensins have been identified; however, the regulation of these genes has not been well-investigated in the chicken. The objective of this research was to understand constitutive and inducible gene expression of beta-defensins in chicken white blood cells. Real-time RT-PCR was used to quantify gene expression level before and

after LPS stimulation. Transcription factor binding sites in the genes were identified to understand the gene expression regulation. From the expression profile results, most chicken beta-defensins had induced gene expression by LPS stimulation in the early phase (0- to 3-hour) and reduced gene expression in the late phase (3- to 8-hour). As for the level of gene expression, the results show that the induced gene expression in the early phase corresponded to the higher levels of expression at 3-hours after LPS stimulation, and the reduced gene expression in the late phase corresponded to the lower levels of gene expression at 8-hours after LPS stimulation.

To my Mother and Father

and

In memory of my Grannie Riley
(until we meet again, I know you are beside me and I will continue to make you proud)

Thank you God, for the strength you have given me to find my way in the dark.

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CHAPTER I

INTRODUCTION

Animals readily respond to various stimulations in their surrounding environment. The immune response is the main mechanism of defense for the animal against foreign invasion and aids in the restoration of homeostasis (Abbas and Litchtman, 2003). Host defense against invading microbial pathogens is elicited by the immune system which consists of two components: innate immunity and adaptive immunity. Both components of immunity recognize invading microorganisms as non-self which triggers immune responses to eliminate them (Takeda, et al., 2005).

Innate immunity is triggered immediately after microbial invasion in response to highly conserved structures present only in microorganisms, e.g. lipopolysaccharide, which rapidly limits the expansion of invading pathogens and provides time for more effective host adaptive immunity to be generated. Defensins are antimicrobial peptides that play an important role in innate defense against microorganisms in plants and animals. They are induced in response to challenge by lipopolysaccharide, by a regulatory pathway similar to that used by the mammalian immune system, involving toll-like receptors and the transcription factor NFκB (Hancock and Scott, 2000).

This thesis follows the style of Immunogenetics.

This research is concerned with constitutive and inducible gene expression of beta-defensins in chicken white blood cells. Thirteen beta-defensins have been identified; however, the regulation of these genes has not been well-investigated in chickens. This research will analyze these defensins and support their role in innate defense against microorganisms in animals and plants, with real-time RT-PCR and bioinformatics.

CHAPTER II

REVIEW OF LITERATURE

Innate Immunity

Innate immunity provides an ever-present or rapidly inducible initial defense against microbial infection. The innate immune system is an evolutionary conserved system of defense that responds very rapidly in the early phase of the immune response. This naturally occurring first line of defense confers non-specific protection without previous exposure or memory against a large number of pathogens (Diamond, et al., 2000; Froy, 2005). To date, the innate system is not independent, in fact, it is functionally interrelated to the adaptive immune system that develops after a few days to improve and enhance the first line of defense (Tizard, 2004).

In addition to its constitutive quality, there is a rapid response where components of the innate immunity are produced after an initial challenge by pathogens (Kaiser and Diamond, 2000). For example, infectious agents such as bacteria or viruses can grow very rapidly. A single bacterium with a doubling time of fifty minutes can produce about five hundred million offspring within twenty four hours. If a microorganism invades the body it must be rapidly recognized and destroyed before it overwhelms the defenses.

Pathogen-Associated Molecular Patterns and Toll-Like Receptors

The first step in innate immunity is for the body to sense that it is being invaded. The innate system detects infection and eliminates microorganisms without affecting its own tissues through the recognition of conserved molecular structures of invading pathogens, called pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2005). The most studied example of PAMP is bacterial lipopolysaccharide (LPS). LPS is a principle component of the outer membrane of gram-negative bacteria, and a potent activator of innate immune responses (Abbas and Lichtman, 2003; Palsson-McDermott and O'Neil, 2004). The essential structural feature that governs interactions with the innate system is the lipid A portion of LPS. It represents the invariant pattern and is responsible for the proinflammatory effects of LPS, while the O-antigen portion is variable in LPS from different species of bacteria (Aderem and Ulevitch, 2000; Fujihara, et al., 2003). LPS has been shown to initiate multiple intracellular signaling events, including the activation of nuclear factor-κB (NF-κB) (Chow, et al., 1999). Activation of these conserved pathways leads to the induction of numerous genes. The best known receptors that recognize LPS are the family of toll-like receptors (TLRs) (Medzhitov, 2003).

The innate immune cells recognize PAMP through toll-like receptors (TLRs), a family of membrane proteins that serve as pattern recognition receptors (PRRs) for a variety of microbe-derived molecules and play an instructive role in innate immune responses against microbial pathogens, as well as the subsequent induction of adaptive immune responses and inflammation (Fujihara, et al., 2003; Kawai and Akira, 2006).

Inflammation is the response of tissues to invading microorganisms or tissue damage. This involves the activation and directed migration of many different cells, especially macrophages, from the bloodstream to sites of invasion. A cell such as a macrophage thus uses its TLRs to identify the presence of an invader and respond appropriately (Tizard, 2004).

All TLRs share the structural homology and signal transduction pathways with the type I IL-1 receptor and recognize distinct ligands through their leucine-rich repeats in the extra cellular domain (Abbas and Litchtman, 2003). The cytoplasmic portions of the receptors include a conserved cytoplasmic motif, Toll-interleukin-1 (IL-1) receptor (TIR) domain, which is required for initiating intracellular signaling (Brentano et al., 2005).

To date, eleven TLRs have been identified and demonstrated to recognize PAMPs. The engagement of TLRs by pathogenic components results in the induction of specific gene expression profiles that are suited to ensuring efficient removal and destruction of the invading pathogen. The ability of TLRs to trigger induction of tailored profiles of genes is supported by their ability to activate a variety of transcription factors, mainly focusing on NF-κB, because it is a transcription factor activated by all TLRs (Moynagh, 2005).

TLR-4 was the first to be discovered and is most predominantly expressed in immune cells, including macrophages and dendritic cells (Fujihara et al., 2003). TLR-4 is a central component required by LPS as a signal transduction receptor (Gangloff and

Gay, 2004). Binding of LPS by the TLR-4 complex activated the signaling pathways that lead to increased gene expression (Froy, 2005).

Antimicrobial Peptides - Defensins

Antimicrobial peptides, AMPs, are a prevalent mechanism of host defense found throughout nature (Kaiser and Diamond, 2000). AMPs are relatively small molecules, less than 100 amino acids, which have a broad spectrum of antimicrobial activity. They are cationic and amphipathic and serve as an ancient defense mechanism against pathogenic microorganisms that easily come into contact with the host through the environment. These molecules are considered part of the innate immune system of all species (Kaiser and Diamond, 2000; Ganz, 2003; Lynn, et al., 2004; Sugiarto and Yu, 2004).

Defensins are a family of antimicrobial peptides abundant in immune cells, white blood cells (specifically neutrophils), intestinal Paneth cells, and barrier epithelial cells, that engage in host defense (Ganz, 2002). Defensins are antimicrobial peptides with a characteristic triple-stranded beta-sheet structure connected with a loop of beta-hairpin turn. The main characteristic of defensins molecules is a framework of six disulphide-linked cysteines (Ganz, 2003; Sugiarto and Yu, 2004). There are many defensins that have been isolated from vertebrates and were classified into three subgroups, alpha-defensins, beta-defensins, and theta-defensins. Two of the subgroups, alpha-defensins and beta-defensins, have been identified in humans, cows, and rodents. Theta-defensins so far have been identified only in leukocytes of rhesus monkeys (Zhao, et al., 2001;

Froy, 2005). There is convincing evidence of conserved characteristics in all vertebrate defensins that indicates alpha-, beta-, and theta-defensins probably originated from a common ancestral defensin gene. Birds only have beta-defensins, and this leads to indicate they are probably the oldest of the three defensin subfamilies (Harwig, et al., 1994; Liu, et al., 1997; Zhao, et al., 2001; Ganz, 2003).

Peptides of the beta-defensin family are involved in and mediate different biological processes based on microbicidal and non-microbicidal activities. Beta-defensins play a more important role in the innate defense system because the avian heterophil lacks oxidative mechanisms (Sugiarto and Yu, 2004). The genes that encode these peptides can either be constitutively expressed or induced by inflammatory mediators and bacterial challenge (Kaiser and Diamond, 2000). Beta-defensins have direct antimicrobial effects through the interaction with the microbial membranes, and have additional functions likely controlled by the interaction with specific receptors (Kluver, et al., 2006). For example, upon challenge with microbes, there is an induction of beta-defensin expression in many tissues. This induction is mediated at the transcription level by TLRs, IκB, and NFκB (Diamond, et al., 2000; Selsted and Ouellette, 2005).

Transcription Regulation of Defensins

One of the greatest challenges facing modern molecular biology is understanding the complex mechanisms regulating gene expression. Two of the most important functional elements in any genome are the transcription factors (TFs) and the genomic

locations within the DNA to which they bind, transcription factor binding sites (TFBS). Transcription factor binding sites, TFBSs, are usually short, around 5 to 15 base pairs in length and they are frequently degenerate sequence motifs. The sequence degeneracy of TFBSs has been selected through evolution and is beneficial because it confers different levels of activity upon different promoters. The function of TFBSs is often independent of their orientation. For example, in yeast, their position within a promoter can vary, and in higher eukaryotes they can occur upstream, downstream, or in the introns of genes that they regulate (Bulyk, 2003).

Various experimental and computational approaches have been used to detect these sites. The publication of an almost complete sequence of the human genome is an enormous achievement; however characterization of the entire set of functional elements encoded in the human and other genomes remains a huge challenge (Bulyk, 2003; Pavesi, et al., 2004). A more complete understanding of transcription factors, their DNA binding sties, and their interactions, will allow for a more comprehensive and quantitative map of the regulatory pathways within cells, as well as a more detailed understanding of the potential functions of individual genes regulated by these binding sites.

Individual TLRs interact with different combinations of adapter proteins and activate various transcription factors such as nuclear factor (NF)-κB, activating protein-1 (AP-1) and interferon regulatory factors (IRF). TFBS are located near the transcription start site (TSS) of the gene; the segment of DNA where the TATAA box is bound on either side (Nelson and Cox, 2005). These activated transcription factors translocate into

the nucleus and bind to TFBSs on target genes to induce the transcription (Kawai and Akira, 2006).

TLR-4 first responds by binding to LPS. LPS recognition *in vivo* is achieved by the cooperation of several molecules including LPB, CD14, MD-2, and TLR-4. The LPS receptor complex is what leads to activation of the intracellular signaling pathways. After recognizing the LPS complex, TLR-4 signals to molecules MyD88, Mal, TRAM, and TRIF inside the cell that then triggers molecular interactions which induce innate immune responses. The TLR-4 pathway activates a key transcription factor, NF-κB, resulting in the production of proinflammatory cytokines, chemokines, and innate immune effectors, and expression of some induced genes lead to the progression of adaptive immune response (Palsson-McDermott and O'Neil, 2004).

The ability to sequence entire genomes has stimulated research directed not only at producing DNA sequence, but also at defining the function of genes on a genome-wide level. Given that genes with related functions are likely to be regulated together, techniques that evaluate global gene expression provide a mechanism for the initial identification and clustering of novel gene sequences with related functions. In the last two decades techniques for the evaluation of gene expression have progressed from methods developed for the analysis of single, specific genes to techniques focused on identifying all genes that differ in expression between or among experimental samples (Moody, 2001).

The observation that relative levels of defensin proteins differ within and across tissues, and that some are inducible while others are constitutive, suggests that defensin

gene regulation is important to the maintenance of a balanced spectrum of antimicrobial activity. Therefore, the identification of the regulatory elements and signaling pathways involved in defensin gene expression is of interest. Studies have shown consistencies in the gene expression of both TAP and the human homolog hBD2. For example, *in vitro* LPS induction of both genes is mediated by the LPS co-receptor CD14 and results in an increase of NF-κB activity (Kaiser and Diamond, 2000).

The first beta-defensin described was isolated from the tracheal epithelium of cattle, in which its expression is inducible by LPS. Bovine beta-defensins have evolved rapidly, as indicated by the 13 beta-defensins isolated from bovine neutrohils and by bioinformatics approaches that have identified about 30 human beta-defensin genes and about 45 beta-defensin genes in mice (Selsted and Ouellette, 2005).

The first human beta-defensin peptide isolated, hBD-1, was purified from hemodialysis fluid; it is also present in plasma, and several N-terminally truncated forms have been found in urine. Two additional beta-defensins, hBD-2 and hBD-3, were subsequently isolated from the scales of psoriatic skin. Expression of a fourth beta-defensin, hBD-4, has been characterized at the mRNA level, and a synthetic form has been produced and functionally characterized, but isolation of natural hBD-4 has not been reported (Selsted and Ouellette, 2005).

These human beta-defensins are widely expressed in epithelium and leukocytes, and their expression is constitutive and / or inducible depending on the site of expression. The mRNA of hBD-1 is constitutively expressed in various epithelia, but expression of all four human beta-defensins is inducible in one or more tissues. hBD-1

expression is up regulated and hBD-2 expression is induced in monocytes exposed to bacterial LPS. hBD-2 is strongly induced by IL-1 produced by LPS stimulated monocytes and this up regulation is dependent on NF-κB (Selsted and Ouellette, 2005).

Objective / Hypothesis

Overall Objective

The overall objective was to determine the gene expression of chicken beta-defensins before and after LPS stimulation in white blood cells. The hypothesis was that the gene expression level of beta-defensins varied between defensin genes and that LPS induced defensin expression at different levels, depending on the beta-defensin.

Specific Aim

The specific aim was to determine the association between transcription factor binding sites in beta-defensin genes and gene expression induction by LPS. The hypothesis was that the presence of transcription factor binding sites recognized by transcription factors of the NFκB pathway was associated with gene induction.

CHAPTER III

LIPOPOLYSACCHARIDE STIMULATED BETA-DEFENSIN EXPRESSION AND THE TRANSCRIPTION FACTOR BINDING SITES ASSOCIATED WITH THEIR INDUCTION

Introduction

Animals are constantly exposed to millions of potential pathogens through contact, ingestion, and inhalation. Hosts' ability to avoid infection depends on their mechanisms of innate immunity (Hancock and Scott, 2000). The innate immune system uses at least two distinct strategies of immune recognition: recognition of microbial non-self and missing self. The first is based on molecular structures that are unique to microorganisms and are not produced by the host. This directly leads to the activation of the immune response. The second is based on molecular structures expressed only on normal, uninfected cells of the host (Medzhitov, 2003). Innate immunity provides an ever-present or rapidly inducible defense against microbial infection. The innate immune system is an evolutionary conserved system of defense that responds very rapidly in the early phase of the immune response. This naturally occurring first line of defense confers non-specific protection without previous exposure or memory against a large number of pathogens (Diamond, et al., 2000; Froy, 2005).

Host defense peptides play an important role in the innate immune response, and among these, defensins seem to have a particularly prominent role in antimicrobial defense. Defensins are small, highly cationic peptides, active against gram-positive and

gram-negative bacteria, fungi, parasites, and some enveloped viruses. Defensins kill microorganisms by damaging biological membranes (Boniotto, et al., 2006). There are many defensins that have been isolated from vertebrates and they were classified into three subgroups, alpha-, beta-, and theta-defensins. Beta-defensins are the largest family of antimicrobial peptides, which can directly kill microorganisms and also have regulatory effects on the immune system (Sugiarto, et al., 2004).

The first beta-defensin described was isolated from the tracheal epithelium of cattle, in which its expression is inducible by LPS through a CD14-dependent signaling pathway and the transcription of the bovine tracheal antimicrobial peptide (TAP) gene was found to be regulated by transcription factors such as NF-κB (Tsutsumi-Ishii and Nagaoka, 2002). Bovine 13 beta-defensins have been isolated from neutrophils. By bioinformatic approaches about 30 human beta-defensin genes and about 45 mouse beta-defensin genes were identified (Selsted and Ouellette, 2005). Only beta-defensins were found to be expressed in chickens and all thirteen different genes, Gallinacin 1-13, are clustered densely within an 86-Kb distance on the chromosome 3q3.5-q3.7 (Xiao, et al., 2004). The chicken beta-defensins (cBDs) are predominantly expressed in bone marrow and the respiratory tract for Gallinacin 1-7 and the remaining genes are restricted to liver and the urogenital tract (Xiao, et al., 2004).

One of the greatest challenges facing modern molecular biology is understanding the complex mechanisms regulating gene expression. Two important functional elements in a genome are transcription factor genes (TFs) and genomic sequences; transcription factor binding sites (TFBS) to which TFs bind (Bulyk, 2003). Gene

expression is regulated by binding of transcription factors to the promoter. Over the past few years, a number of beta-defensins have been identified in various animals and found to exhibit constitutive and inducible gene expression. For example, inducible expression of beta-defensins can be detected in blood after stimulation with LPS. NFκB proteins are a family of inducible transcription factors that allow cells to respond to extracellular stimuli, e.g. LPS.

In this experiment, the expression profiles of all thirteen chicken beta-defensins were investigated in white blood cells before and after LPS stimulation. These expression profiles were obtained to illustrate up or down regulation to test the hypothesis that the gene expression level of beta-defensins varies among genes and LPS induces the expression of different defensin genes at different levels. The transcription-factor binding sites (TFBS) were predicted using bioinformatic tools to determine the association of the transcription factor binding sites and gene expression. While researching other studies, similar results were found suggesting that gene expression is induced with LPS stimulation leading to the activation of TFBS such as NF-κB. These studies were the basis of this project in the hope of finding similar results in avian species, proving the hypothesis to be true.

Materials and Methods

Experimental Animals

Eighteen White Leghorns from a local company were used in this study. After wing-banding, these birds were reared in cages at Texas A&M University, Poultry

Science Department. During the experiment, chickens were fed and watered ad libitum according to NRC recommendations. All experimental procedures were approved by the TAMU animal use and care committee.

At the age of five weeks, the layers were randomly divided into three groups (L0, L3, L8) respectively, each including six birds. The two groups (L3 and L8) were injected intravenously with LPS (Sigma, St Louis, MO) solution (5mg/ml, dissolved in PBS) at an appropriate dose of 2.0 mg/kg body weight. At the same time, the first group (L0) was killed with CO_2 to collect the blood at TIME-0 hours. After 3 hours the second group (L3) was killed to collect the blood at TIME-3 hours after stimulation. Finally after 8 hours the third group (L8) was killed to collect the blood at TIME-8 hours after stimulation.

RNA Isolation and cDNA Synthesis

The whole blood samples were used to isolate white blood cells using the HISTOPAQUE®-1177 (Sigma-Aldrich Inc., St. Louis, MO) according to the manufacturer's protocol. Briefly, three milliliters of whole blood was carefully layered onto the 3 ml HISTOPAQUE®-1177. After centrifuging at 400 x g for thirty minutes at room temperature (25°C), the white blood cell was isolated from the plasma and immediately used in RNA isolation.

Total RNA was isolated from the white blood cells with TriZol reagents (Invitrogen, Carlsdad, CA, USA) according to standard protocol. After measuring the concentration and checking the quality of all RNA samples with an Agilent 2100

bioanalyzer (Agilent Technologies, CA), equal amounts of total RNA from each of the three samples in one group were mixed together to pool total RNA. After quantification again for the pooled samples, both the individual and pooled RNA samples were treated with DNase (Invitrogen, Carlsdad, CA, USA) according to the protocol.

cDNA was synthesized from equal amounts (1 mg) of both the individual and pooled total RNA samples with a Thermoscript RT-PCR system kit (Invitrogen, Carlsdad, CA, USA) using random hexamer primers according to the protocol provided by the manufacturer. After incubating for 10 minutes at 25°C followed by a 50°C for fifty minutes and 85°C for five minutes, the prepared cDNA templates were stored at -20°C prior to use in the PCR assay.

Quantitative Real-time PCR

Primers were designed according to fourteen (thirteen chicken beta-defensins and one internal control) chicken mRNA sequences with the Primer Express 2.0 software (Applies Biosystems) for PCR amplification. All the designed amplification fragments were about 50 to 150 base pairs in length and each pair of primers was designed from different exons to further distinguish PCR products amplified from genomic DNA. The specificity of every pair of primer sequences was confirmed at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi. The amplified PCR fragments were checked for their size and specificity in 2.0% to 2.5% agarose gel electrophoresis. These primers and Genbank accession numbers are listed in Table 1.

Real-time PCR was carried out in 20 µl-reactions containing 10 µl of 2X SYBR Green PCR Master Mix (Applied Biosystem Corporation), plus 6 µl cDNA synthesized from 50 ng of total RNA, plus 4 µl of combined primer with the final concentration of 0.3 µM of each forward and reverse oligo-nucleotide primers listed in Table 1. These reagents were loaded on a 384 well plate with an Eppendorf epMotion ™ 5070 Workstation (Eppendorf, Westbury, NY) to decrease the manual variation of each well. A two-step real-time RT-PCR protocol was used that cycled 40 times at 95°C for 15s and at 59°C for 30s in an ABI 7900HT DNA Sequence Analysis System (Applied Biosystems, Foster City, CA). Fluorescence intensity was detected at the end of every cycle that was used to calculate the relative quantification of gene expression. At the end, samples were heated at 95°C for 20 minutes to perform a dissociation curve analysis to further verify single PCR products.

Table 1 Primer sequences used in relative quantitative real-time PCR (qRT-PCR) analysis.

Gene Name	*Access #*	*5' Primer [1] (forward)*	*3' Primer [1] (reverse)*	*Product Size (bps)*
cBD-1	AF033335	5'-CCTTGCTGTACCCTGAGAAACC-3'	5'-AGGTACACGATCCGCATGGT-3'	77
cBD-2	AF033336	5'-CCAGGTTTCTCCAGGGTTGTC-3'	5'-GGCAGGACCCTCCTTTACAGA-3'	65
cBD-3	AF181952	5'-CTGTGGAAGAGCATATGAGGTTGAT-3'	5'-CACGGTCATACCATGGGAGACT-3'	127
cBD-4	AY621306	5'-TTCTCTGCAGTGACAGGATTTCC-3'	5'-AAGCCCACAGCTCCATGAACT-3'	101
cBD-5	AY621307	5'-CATGCAGATCCTGACTCTCCTCTT-3'	5'-GACATGACTTGTGGGAGCAGAA-3'	131
cBD-6	AY621308	5'-CCAGCCCTATTCATGCTTGTAGA-3'	5'-CTGTTCCTCACACAGCAAGATTTTAG-3'	121
cBD-7	AY621309	5'-TGCAGGTCAGCCCTTCATTC-3'	5'-GCCTATTCCATTGTTACATGTTCCA-3'	121
cBD-8	AY621310	5'-TTGGCCGTTCTCCTCACTGT-3'	5'-TGCCCAAAGGCTCTGGTATG-3'	137
cBD-9	AY621311	5'-GCCGTGCTCCTTCAGTTGA-3'	5'-GGTGCCCATTTGCAGCAT-3'	67
cBD-10	AY621312	5'-CAAGATTCCGGCGCAGTAAG-3'	5'-CAAGGCAGTGGAAATGTTGCT-3'	74
cBD-11	AY621313	5'-CTCTTCCTCCTCCAGGCTGTT-3'	5'-CAAGAGCATGTTCCAAATGCA-3'	131
cBD-12	AY621314	5'-CCTTTGTTTCGTGTTCATCTTCATC-3'	5'-CAAAGCAGTACTTAGCCAGGTATTCC-3'	137
cBD-13	AY621315	5'-GGAGGCTCTGCTTCCACATG-3'	5'-AAGGGTCCTGCTCTGCTGTGT-3'	134
β-actin	L08165	5'-CTGATGGTCAGGTCATCACCATT-3'	5'-TACCCAAGAAAGATGGCTGGAA-3'	78

[1] Annealing temperatures for all primers was 59°C

Statistical Analysis

Each individual sample was run in triplicate and the threshold cycle (Ct) values were averaged for data analysis. The expression level of chicken β-actin was used as an endogenous reference and the other, beta-defensins 1-13, were used as target genes. The Ct was defined as the PCR cycle at which the fluorescent signal reached a fixed threshold. A higher Ct value means a lower expression level of that gene. Differences in Ct between reference and the target genes of the same samples, ΔCt, were calculated and used in statistical analysis. The comparative expression (ΔΔCt) between different time intervals with LPS injection was calculated with the following formula:

$$\Delta\Delta Ct = \Delta Ct_{\text{treatment 1}} - \Delta Ct_{\text{treatment 2}}$$

$$= (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 1}} - (Ct_{\text{Target}} - Ct_{\text{Reference}})_{\text{treatment 2}}$$

A positive value of ΔΔCt means treatment 1 expresses a lower level of the target gene than treatment 2.

Statistical analyses (Student's t-test) were performed using Microsoft® Excel 2003 with significance at $P \leq 0{\cdot}05$. The standard deviation, SD, was also calculated for each of the treatment groups. All statistical analyses were based on comparisons between the individual results of LPS stimulation at the early phase, 0- to 3-hour, the later phase, 3- to 8- hour, and the overall phase, 0- to 8-hour, for each target defensin gene.

Sequencing and Assembly of Defensin Genes

Seven of the thirteen chicken beta-defensins to be analyzed in this experiment contained gaps of unknown genomic sequence according to the University of California, Santa Cruz (UCSC) genome browser. These seven gaps had to be filled by sequencing before bioinformatics could be used to locate the TFBS.

All primers were designed with Primer 3, primer designing program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), based on the chicken genome sequences for the seven incomplete beta-defensins on chromosome 3, published by the University of California, Santa Cruz (UCSC) genome website (http://genome.ucsc.edu). Primers were checked with BLAT (UCSC, http://genome.ucsc.edu/cgi-bin/hgBlat?command=start, 2006) and AmplifX 1.37 (Nicolas Jullien, 2004-2005) for specificity, Tm, GC percent, 3' end stability, polyX, self dimer, and self end dimer. The forward and reverse primers were ordered from Integrated DNA Technologies (IDT) at Texas A&M University (http://www.idtdna.com/TAMUBS/Login.aspx). These primers and Genbank accession numbers are listed in Table 2.

Table 2 Primer sequences used in traditional PCR for sequencing gaps in chicken beta-defensins on chromosome 3.

Gene Name	*Accession Number*	*5' Primer [1] (forward strand)*	*3' Primer [1] (reverse strand)*	*Product Size (bps)*
cBD-3[3]	AF181952	5'-AGCACCCCTATAGTGCTGATGG-3'	5'-CCATGAGTGGTAAGGGCTGGAG-3'	783
cBD-4	AY621306	5'-AAAGAAAAAGGGAGACTGTTGC-3'	5'-TCTTCCATGGATAGCTGCTTTA-3'	1198
cBD-6	AY621308	5'-CTGCAGAAATACAGGCTGAGAC-3'	5'-GTCCCTGACTCTTTGAGACCTT-3'	596
cBD-7	AY621309	5'-GGGATTTTTCTCAGACTCCTTG-3'	5'-TCAGGGATAGTTATTGCACTGG-3'	648
cBD-10	AY621312	5'-GGGGTTGAGTTCAGTGATCTTT-3'	5'-TGTACTTTTGCTTTGCCACTTT-3'	600
cBD-11	AY621313	5'-ACTGAATGCCATTTCTGTGCTA-3'	5'-GGTAGGTATCATGCAGGAGCTA-3'	567
cBD-12[3]	AY621314	5'-CCGGGAGTGATTTGTTTCTATC-3'	5'-CCCAGTGTTCTGTTTCCTACAG-3'	955
β-actin[4]	415296	5'-GGGAATCCTCACCCTGAAGTATC-3'	5'-TGGCATACAGGTCCTTCCTGAT-3'	700

[1] Annealing temperatures for all primers was 59°C
[2] cBD-1, -2, -5, -8, -9, and -13 did not need to be sequenced because there was no gap 2 kb up- or down-stream of the transcription starting site.
[3] cBD-3 and -12 were not successfully sequenced due to the inability to design a primer specific enough to these areas. Several programs were used however none were successful.
[4] β-actin was used as a positive control.

Polymerase chain reaction, PCR, was performed according to standard protocol with the oligo-nucleotide primers in Table 2. Briefly, each of the seven reactions were amplified using chicken genomic cDNA reacted with dNTP, reaction buffer, *Taq* polymerase enzyme, with equal parts of forward and reverse primers (Table 2) in a 10 µl final volume. The PCR reaction was performed using a thermocycler (MJ Reasearch, Watertown, MA) under the following conditions beginning with 94 °C for 2 minutes, followed by 35 cycles at 94 °C for 40 seconds, 59 °C for 40 seconds, and 72 °C for 1 minute, ending with 72 °C for 10 minutes. The amplified PCR products were analyzed by 1% agarose gel (containing ethidium bromide, EtBr, for visual analysis of products) electrophoresis in 1 x TBE (Tris-Borate EDTA) buffer at 110 constant volts for 2.75 hours.

After the first PCR analysis, product bands of only 5 of the 7 cBDs were verified to be accurate according to expected product size. The exceptions were cBD-3 and -12. A second PCR reaction following the same procedure was performed, leaving out the reactions for cBD-3 and -12. This time 20 µl of amplified product per reaction was made and loaded into the gel to insure a visible band big enough for easy removal from the gel. After cutting the PCR product from the gel, purification was performed with Ultrafree – DA DNA Extraction from Agarose filters according to the protocol provided by the manufacturers (Millipore Corporation, Bedford, MA). After purification this new product was used as the new template instead of the chicken genomic cDNA.

More PCR product was made with a dilution of 1:10 and 1:100 concentration of the new template beginning with 94 °C for 2 minutes, followed by 19 cycles at 94 °C for

40 seconds, 59 °C for 40 seconds, and 72 °C for 1 minute, ending with 72 °C for 10 minutes. The amplified products were analyzed again by electrophoresis with 1% agarose gels (containing ethidium bromide, EtBr, for visual analysis of products) electrophoresis in 1 x TBE (Tris-Borate EDTA) buffer at 110 constant volts for 2.75 hours. This last electrophoresis was performed to verify the same product band results as the previous PCR and to determine which concentration of template gave the stronger band. It was clear that the 1:100 concentration of template gave the stronger band.

The final step was to amplify 60 µl of PCR product under the exact conditions used in the second PCR at 19 cycles. Next 40 µl of the PCR product was purified with Montage™ PCR centrifugal filter devices according to the protocol given by the manufacturer (Millipore Corporation, Bedford, MA) and the purified product per each reaction along with the matching forward oligo-nucleotide primers were sent to the Gene Technologies Lab, Texas A&M University (http://www.idmb.tamu.edu/gtl/) for sequencing (a total of 5 cBDs were sent for sequencing).

Bioinformatics program Blast 2 (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) was used to align the chicken genomic sequence from UCSC genome browser with the five chicken beta-defensins' sequencing results received from the Gene Technologies Lab, Texas A&M University (http://www.idmb.tamu.edu/gtl/).

Transcription Starting Site (TSS) Detection

Two different methods, Berkley Drosophila Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and POSSUM (http://zlab.bu.edu/~mfrith/possum/) were used to identify the transcription starting sites, TSSs, of the 13 cBDs except for cBD-3. The promoter regions of the TSSs were detected using the default settings in both programs. These two programs are online and can be used directly via the web.

Transcription Factor Binding Site (TFBS) Prediction

Using the results from the TSS detection programs and the UCSC genome browser, approximately 2000 bps up-stream and down-stream of the promoter regions of the 5' ends of the 13 cBD genes were found except for cBD-3. The TATAA box with in these promoter regions were also found for each cBD. The sequence before the TATAA box was used to identify TFBS up-stream of the TSS and the sequence including the TATAA box and after was used to identify the TFBS down-stream of the TSS. These sequences were identified and analyzed with several TFBS predicting programs:

1. AliBaba (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). All of the parameters were set as the default.
2. MATCH (http://www.gene-regulation.com/pub/programs/alibaba2/index.html). The parameters were set on vertebrates, high quality matrices, and cut to minimize false positives.

3. POSSUM (http://zlab.bu.edu/~mfrith/possum/): The specific matrices for NF-κB, STAT, and IRF were downloaded from TRANSFAC (http://www.gene-regulation.com/pub/databases.html#transfac) website and saved into a text file and uploaded each time per TFBS prediction.

Results

Expression Profile of cBDs in Layer White Blood Cells

Almost all cBDs had inducible gene expression in the early phase, 3-hours, by LPS stimulation of the layer white blood cells. The normalized threshold cycles, ΔCt are shown in Figure 1. Figure 1 reports ten out of the thirteen defensins, cBD-1, 2, 3, 4, 6, 7, 8, 9, 11, and 13, show up-regulation of expression in the early phase, 3- hours after stimulation with LPS. At this 3-hour time interval, the expression of cBD-7 increased the most by 4.84 PCR cycles. cBD-6 also increased expression by more than 3.5 PCR cycles and cBD-8 increased expression by more than 2.5 PCR cycles. There were some cBDs that did not have inducible gene expression in the early phase. Figure 1 shows this down-regulation of expression in cBD-5, -10, and -12. cBD-12 seems to have a constitutive gene expression in the early phase, 3-hours after LPS stimulation, because it is the only cBD that was not statistically different ($P > 0{\cdot}05$); at 0-hours un-stimulated, expression was at 9.73 PCR cycles and at 3-hours after LPS stimulation, expression was at 9.75 PCR cycles.

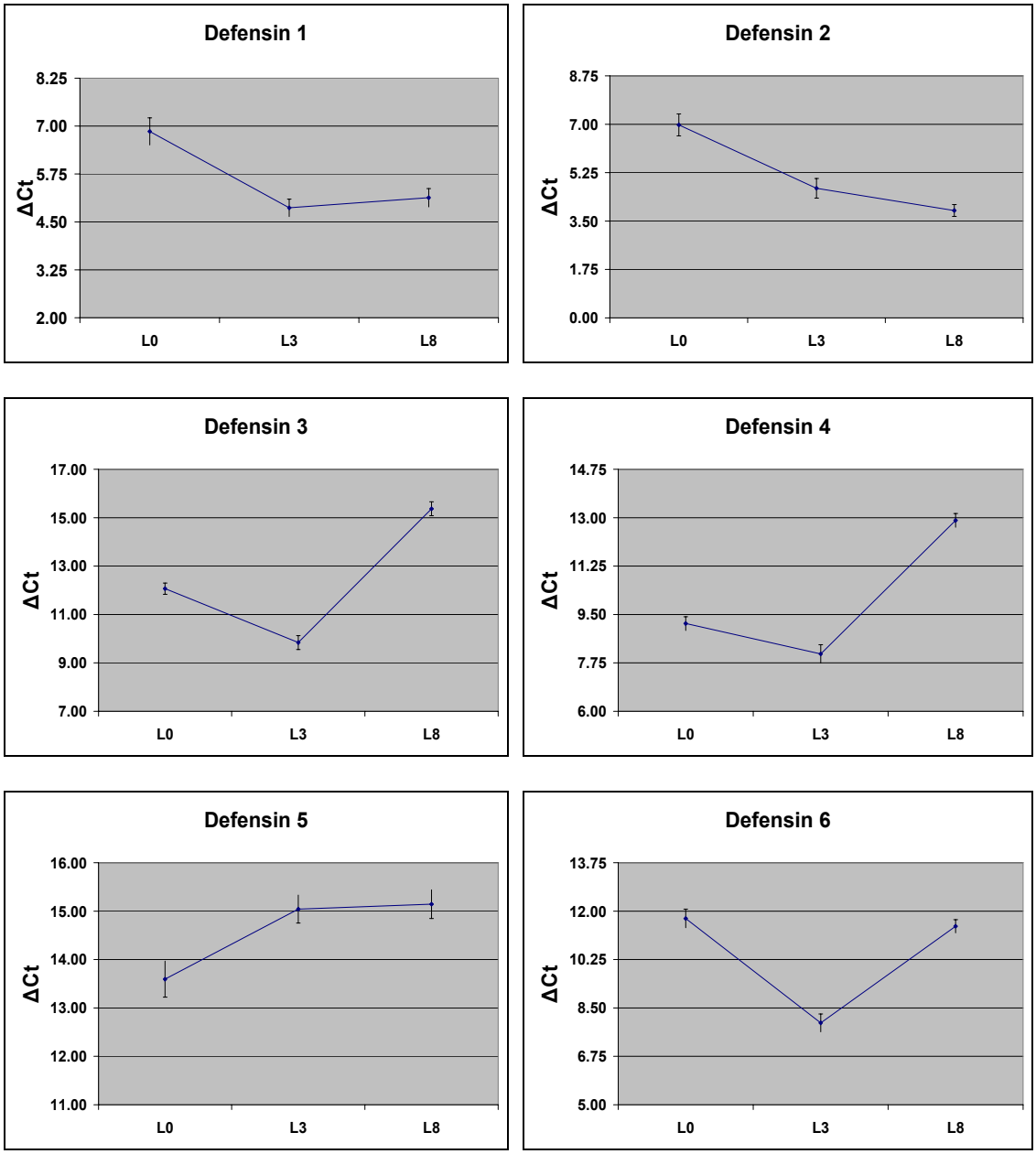

Figure 1 The normalized threshold cycles, ΔCt, for chicken beta-defensins (cBDs) 1-13 before and after lipopolysaccharide, LPS, stimulation of layer white blood cells. A decrease in ΔCt means an increase in induction.

*L0, L3, and L8: layer birds at 0-, 3-, and 8-hour time interval respectively.

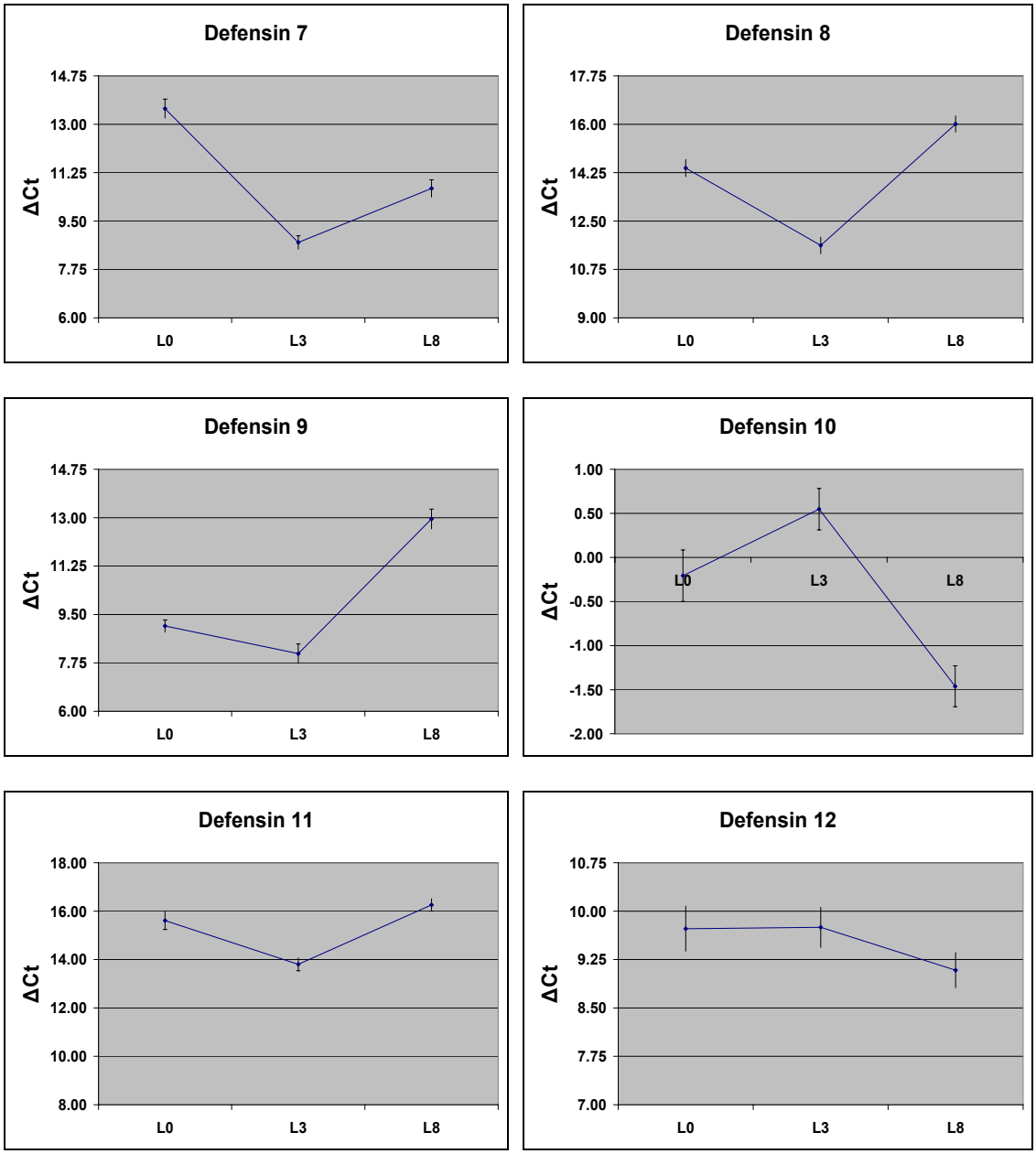

Figure 1 cont.

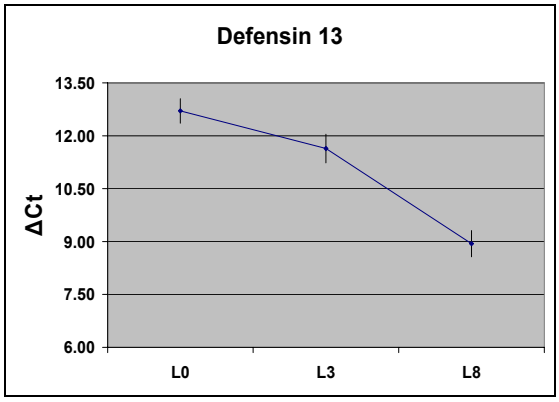

Figure 1 cont.

At 8-hours after LPS stimulation, the expression of most cBDs were down-regulated compared to those at 3-hours post injection. Chicken BD-1 and -5 did not show a significant difference ($P > 0{\cdot}05$) between 3- and 8-hours after stimulation, however all other cBDs did. The overall effect from 0- to 8-hours LPS stimulation was still regarded as inducible gene expression for more than half of the cBDs genes regardless of the expression level at 3-hours post injection. All cBDs except cBD-3, -4, -5, -8, -9, and -11 generally increased expression over the entire time, 0- to 8-hours. Although cBD-6 had induced expression by LPS from 0- to 8-hours, it did not have a significant difference ($P > 0{\cdot}05$).

Almost all cBDs have a lower gene expression level at 0-hours, un-stimulated, verses the level of gene expression at 3-hours after LPS stimulation, except cBD-5, -10, and -12. These exceptions actually had a higher gene expression level at 0-hours, un-stimulated, compared with 3-hours after stimulation, as seen in Table 3 (** a positive value indicates treatment 1 expresses a lower level of the defensin gene than treatment 2). All cBDs show a significant difference in relative expression level ($P \leq 0{\cdot}05$) for the early phase 0- to 3-hour time interval, except cBD-12. This result corresponds to the slight down-regulation of cBD-12 gene expression in Figure 1 in the early phase, further suggesting the possibility of constitutive gene expression; however examination of the later phase still needs to be looked at to confirm this possibility.

Table 3 Relative expression levels (ΔΔCt) of chicken beta-defensins (cBDs) in layer white blood cells before and after LPS stimulation.*

Defensins	0-3 hour	3-8 hour	0-8 hour
cBD-1	1.99	**-0.26**	1.73
cBD-2	2.29	0.81	3.10
cBD-3	2.23	-5.53	-3.30
cBD-4	1.10	-4.83	-3.73
cBD-5	-1.45	**-0.10**	-1.55
cBD-6	3.77	-3.49	**0.28**
cBD-7	4.84	-1.95	2.88
cBD-8	2.80	-4.39	-1.59
cBD-9	1.00	-4.87	-3.87
cBD-10	-0.76	2.01	1.25
cBD-11	1.81	-2.46	-0.65
cBD-12	**-0.02**	0.66	0.64
cBD-13	1.07	2.70	3.77

*Statistical analyses were determined by Student's t-test. Comparisons are based between the individual results of LPS stimulation at the early phase (0- to 3-hour), the later phase (3- to 8-hour), and the overall phase (0- to 8-hour) for each target defensin gene.
**A positive value indicates treatment 1 expresses a lower level of the defensin gene than treatment 2 thus induction expression. A negative value indicates reduction expression.
The differences in bold were not significant (P > 0·05).

More than half of the cBDs had a higher gene expression level at 3-hours after LPS stimulation, verses the level of gene expression at 8-hours after LPS stimulation, except -2, -10, -12, and -13. These exceptions actually had a lower gene expression level at 3-hours verses the 8-hours resulting in continued induced expression by LPS for cBD-2 and -13 and a later phase initial induced expression by LPS of cBD-10 and -12. From this result, the possibility of cBD-12 having constitutive gene expression probably is not true. cBD-1 and -5 did not show a significant difference ($P > 0{\cdot}05$) in the late phase 3- to 8-hours.

The expression levels of seven out of the thirteen cBDs, -1, -2, -6, -7, -10, -12, and -13 had a lower gene expression level at 0-hour unstimulated verses 8-hours after LPS stimulation, however cBD-6 is the only one out of these that is not statistically lower in gene expression in the overall phase 0- to 8-hour. Overall cBD-1, -2, -6, -7, and -13 all show induced gene expression levels by LPS stimulation at both the early and overall phase.

Table 4 Comparing the relative expression levels (ΔΔCt) of selective chicken beta-defensin (cBD) genes between the individual and pooling samples at 0- and 8-hour time intervals.

		Individual Samples		**Pooling Samples**	
Chicken β-defensins		Layer Blood	ΔΔCt	Layer Blood	ΔΔCt
cBD-2	0 hour	6.98 ± 0.39	2.29 [1]	7.0 ± 0.35	2.50 [1]
	8 hour	3.88 ± 0.21	3.10 [1]	3.7 ± 0.20	3.30 [1]
cBD-10	0 hour	-0.21 ± 0.29	-0.76 [1]	-0.3 ± 0.26	-0.70 [1]
	8 hour	-1.46 ± 0.23	1.25 [1]	-1.6 ± 0.21	1.30 [1]

[1]The differences are significant ($P \leq 0{\cdot}05$).

Comparing Results from Individual to Pooled in Layer Blood

At the end of the real-time RT-PCR statistical analysis, the standard deviations within the pooled groups were also analyzed briefly to compare the individual results to the pooled results, Table 4. The pooled and the individual expression profile results are very consistent and do not show any significant variations in this experiment. The pooled groups were valid and reliable and could have been used instead of the individual results.

Sequencing of Genomic Gaps

The complete genomic sequences for only 5 of the 7 cBDs were obtained using the BLAST 2 program on the NCBI website (http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi) by assembling the sequencing results received from the Gene Technologies Lab, TAMU (http://www.idmb.tamu.edu/gtl/) and the published sequences of the chicken genome from the UCSC Genome Browser (http://genome.ucsc.edu/) (results of assembled sequences not shown). cBD-3 and -12 were not successfully sequenced due to the inability to design a primer specific enough to that area of the genome. This is probably due to the poor quality of the genome in those specific areas.

Predicted TFBS

Both programs Berkley Drosophila Promoter Prediction and POSSUM found TSS of each cBD. When compared together they showed similar detections of the promoter regions and position locations in relation to the cBDs (results of promoters not shown). The results from the three different TFBS programs were examined for similarities and consistencies of TFBS and their position in relation to the TSS of each cBD. The MATCH program did not detect the NFκB in any of the cBDs with default settings and detected only a very few AP-1 and STAT binding sites, this program was found to be very inconsistent with the other two programs and the results were not used in this study. AliBaba and POSSUM were found to be very similar in the TFBS that were predicted with the default settings, however unlike POSSUM, AliBaba did not give the position number of the TFBS so this program was only used to increase the reliability of the predicted TFBS with the POSSUM program. The results were compiled and assembled and shown graphically in Figure 2. Due to the incomplete chicken genome from the UCSC website, the TFBS of cBD-3 were not obtained.

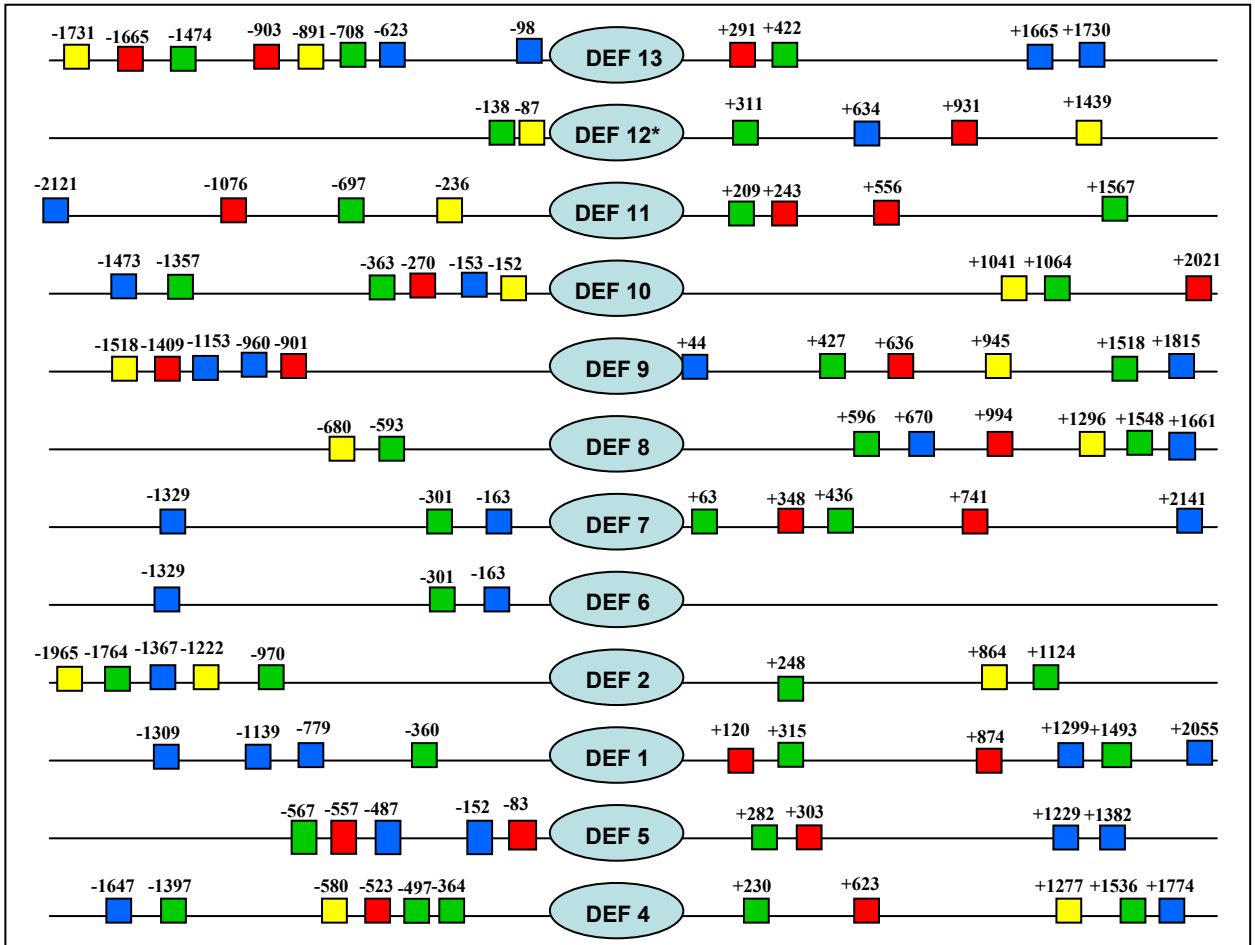

Figure 2 Transcription factor binding sites 2 kb up- and down-stream of the promoters of defensin genes.

* Def 12, position numbers may not be accurate due to the inability to acquire the complete sequence.

Discussion

The expression profiles of thirteen cBDs, from layer white blood cells, before and after LPS stimulation were examined to determine the association between the predicted transcription factor binding sites in the chicken beta-defensin genes and the gene expression induction by LPS stimulation.

The innate immune system is the first line of host defense capable of effectively dealing with the continuous challenge against invading microorganisms (Diamond et al., 2000). Defensins expressed in epithelial and phagocytic cells serve as effector molecules, key components of the innate immune system. All defensins that have been identified to date have the capability to kill or inactivate a variety of bacteria, fungi, and some viruses *in vitro*. In response to microbial infection, production of these defensins is rapidly induced, leading to not only microbicidal activity but also to recruitment of immune cells that play an important role in acquired immunity. The secretion of beta-defensins is controlled primarily at the level of gene transcription. This process is inducible by microbial products through signaling pathways mediated by TLRs which recognize bacterial components such as LPS. LPS stimulation of monocytes and macrophages induces genes that express inflammatory mediators. LPS response elements have been characterized in the 5' region of defensins. The transcription factors that bind to these LPS response elements include, NFκB, AP-1, IRF, and STAT (Guha and Mackman, 2001; Tsutsumi-Ishii and Nagaoka, 2002; Froy, 2005).

In the present research, chicken beta-defensins were evaluated on gene expression level before and after LPS stimulation. These results indicate the induced or reduced expression of the thirteen cBDs before and after LPS stimulation. At 8-hours after LPS stimulation, the expressions of most cBDs were down-regulated to counteract the effects of the increased transcription in the early phase. This counteraction might occur because the host needs to maintain the inner homeostasis via a negative feedback mechanism to counteract the increased transcription level. Without the negative feedback control, eventually there would be some sort of problem for the animal. However, another more likely reason of the reduced gene expression is normalization may not have been necessary because β-actin was also induced by LPS stimulation in the later phase after 8-hours, which could mean that results at 8-hours are not accurate.

As seen in Table 5, before normalization and relative gene expression cBD-11 had the lowest level of gene expression in the blood and cBD-10 had the highest level of gene expression in the blood. When compared together, these 2 cBDs show opposite gene expression induction levels. cBD-10 did not induce expression with LPS stimulation in the early phase, maybe because it was already expressed in high levels in the blood at the start, however cBD-11 did induce expression with LPS stimulation in the early phase, maybe because it was expressed in such low levels in the blood at the start.

Table 5 The threshold cycles (Ct) of cBD target genes in white blood cells before and after LPS stimulation.

Defensins	0 hour	3hour	8 hour
cBD-1 [3]	26.31	25.12	**23.94**
cBD-2 [4]	26.43	24.94	22.69
cBD-3 [3]	31.51	30.09	**34.18**
cBD-4 [3]	28.62	28.32	**31.71**
cBD-5 [1]	33.04	**35.29**	**33.95**
cBD-6 [3]	31.18	28.21	**30.26**
cBD-7 [3]	33.01	28.98	**29.49**
cBD-8 [3]	33.86	31.87	**34.82**
cBD-9 [3]	28.53	28.33	**31.76**
cBD-10 [2]	19.24*	**20.80**	17.35
cBD-11 [3]	35.06*	34.05	**35.06**
cBD-12 [2]	29.18	**30.00**	27.89
cBD-13 [4]	32.15	31.89	27.75
β-actin [2]	19.45	**20.25**	18.81

[1] **cBD-5 in bold** indicates no induction with LPS stimulation.
[2] **cBD-10, -12, and β-actin in bold** indicates reduction with LPS stimulation after 3 hours.
[3] **cBD-1, -3, -4, -6, -7, -8, -9, and -11 in bold** indicates reduction with LPS stimulation after 8 hours.
[4] cBD-2, and -13 indicate induction with LPS stimulation over the entire time interval.
*The smaller Ct value the higher gene expression in blood and vice versa.

The expression of beta-defensins is primarily in epithelial cells, however many other tissues expression them as well. In mammals it is interesting that hBD-1 is constitutively expressed in various epithelial cells and hBD-2 and -3 are induced in response to stimuli such as proinflammatory cytokines or bacterial infection (Ganz, 2003). In contrast, cBD-1 and -2 were both induced in response to LPS stimulation, but cBD-2 showed more induction than cBD-1.

The defensin expression profiles based on gene expression levels were also compared with the predicted TFBS of LPS induced cBD genes. Looking back, Figure 2 shows the assembly of multiple transcription factors (NFκB, AP-1, IRF, and STAT) to their adjacent binding sites around the promoters of the chicken beta-defensin genes. The position number up- or down-stream of the promoter regions for each cBD is also included in Figure 2. TFBS that are clustered together within a short distance, 200 bps, are more powerful than when standing along. In Figure 2, several cBDs have clustered TFBS within several 100 nucleotides; most of these defensins show induction with LPS in the early phase, 3-hours with the exception of cBD-5, -10, and -12.

Chicken BD-3 is the only defensin where TFBS were not found. This is because during the sequencing part of the experiment an oligo-nucleotide primer specific to the area around this defensin was not successfully designed, therefore a complete sequence was not generated. For cBD-12 the TSS and the predicted TFBS were found and assembled around the promoter; however the position numbers may not be accurate because of the inability to obtain 2 kb up- and down-stream of the TSS.

In flies as well as mammals, most inducible host defense genes are critically regulated, at least in part, by the NFκB pathway. In un-stimulated cells, IκB, an inhibitor of κB, masks the nuclear localization signal on NFκB and thus blocks its nuclear translocation. Upon stimulation, IκB is rapidly phosphorylated and NFκB can translocate to the nucleus, where it turns on expression of the target gene. (Medzhitov, 2003).

From the experiment results it was found that ten out of thirteen cBDs induced early phase related TFBS that were not significantly induced which is generally consistent with the predicted result from Figure 2. These results are supportive in combination with similar studies where *in vitro* LPS induction of genes resulted in an increase of NFκB activity. Other studies support the hypothesis that the predicted transcription-factor binding sites of Figure 2 are associated with induced gene expression by LPS stimulation. For example, Diamond et al., 2000 demonstrated that transcription of the bovine beta-defensin TAP gene is cooperatively regulated by NFκB in response to LPS. Therefore, activating NFκB is probably the signal transduction pathway that induced cBDs expression in white blood cells after LPS stimulation. Under-expression of NFκB may be caused by a lower expression level of a particular cBDs in blood

However, there are inconsistencies when related to cBD-5, -10, and -12. According to the gene expression experiment results compared with the predicted TFBS results, cBD-10 and -12 was not induced 3 hours after LPS stimulation but was induced 8 hours after LPS stimulation and the NFκB sites were found in these genes. The transcription of cBD-5 was not induced at all with LPS stimulation even though there

were an abundance of NFκB and AP-1 sites predicted, as seen in Figure 2. This contradicts the hypothesis that LPS induction expression is associated with the NFκB pathway.

It is unclear why cBD-5 was not induced by LPS, since several predicted TFBS were clustered together and found near the promoter region on the gene, Figure 2. However, cBD-5 is one of the lowest expressed defensins in blood, Table 5, and the results show 33.04 PCR cycles at 0-hour, 35.29 PCR cycles at 3-hour and 33.95 PCR cycles at 8-hour. Since there is no inducible response by LPS in the experiment results this could mean that defensin 5 possibly could have had induced response to LPS earlier than the 3 hour time interval. If the experiment checked fluorescent intensity at more time intervals then the induction of cBD-5 might have been detected. This is a very good indication as to why NFkB and AP-1 sites were so frequently predicted by the TFBS programs.

Another possibility why these predicted TFBS were so abundant even though no induction took place before or after LPS stimulation could be speculated that since blood is stimulated *in vivo* the complex regulation mechanism involved within the defensin gene expression may be caused by some other signal pathway or suppressed mechanism involved in expression of cBD-5, -10, and -12, that cannot just be explained through bioinformatics. Also, the transcription of cBD-5 gene could be controlled by other transcription factors. So in the future, a reason would need to be found as to why these three beta-defensins are not induced in this experiment.

Looking ahead, experiments could be done with deletion expression. Simply speaking, delete one specific TFBS, several nucleotides long, of one of the defensin genes and then check to see if the inducible defensin stopped inducing. If induction stopped then that would mean that the TFBS that was deleted is critical for gene expression of that defensin gene.

CHAPTER IV

CONCLUSION

Innate immunity provides immediate and quick response to invading microorganisms. Beta-defensins are cationic peptides with a broad spectrum of antimicrobial activities that contribute to the innate host defense. The observations that gene expression levels vary among defensins and that some have inducible gene expression while others have constitutive gene expression, suggested that defensin gene regulation is important to the maintenance of a balanced spectrum of antimicrobial activity.

Induction was observed *in vitro* by stimulating layer white blood cells with bacterial lipopolysaccharide. Higher levels of gene expression were shown to correspond with induction in most cBDs in the early phase, 3 hours after LPS stimulation and lower levels of gene expression were shown to correspond with reduced induction in the later phase, 8-hours after LPS stimulation. Gene regulation occurs via signal transduction pathways common to other innate immune responses, utilizing transcription factors such as NFκB. Together these observations indicate that gene expression levels do vary among genes and LPS stimulated gene induction is associated with the presence of transcription factor binding sites recognized by transcription factors of NFκB pathway.

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APPENDIX

NOMENCLATURE

AMP	Antimicrobial Peptide
AP-1	Activating Protein-1
Ct	Threshold Cycles
ΔCt	Normalized Threshold Cycle
ΔΔCt	Relative Expression Level
cBDs	Chicken Beta-Defensins
hBD	Human Beta-Defensins
IDT	Integrated DNA Technologies
IκB	Inhibitor κB
IL	Interleukin
IRF	Interferon Regulatory Factor
LBP	Lipopolysaccharide Binding Protein
LPS	Lipopolysaccharide
NFκB	Nuclear Factor Kappa B
NRC	National Research Council (for Poultry)
PAMP	Pathogen-Associated Molecular Patterns
PCR	Polymerase Chain Reaction
PRRs	Pattern Recognition Receptors

qRT-PCR	Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction
SD	Standard Deviation
TAMU	Texas A&M University
TAP	Bovine Tracheal Antimicrobial Peptide
TF	Transcription Factor
TFBS	Transcription Factor Binding Site
TIR	Toll-Interleukin
TLRs	Toll-like Receptors
TSS	Transcription Starting Site

VITA

Tiffany Marie Supak

Permanent Address:

355 Leisure Circle
China Spring, Texas 76633

Email Address:

tiffanysupak02@aggienetwork.com

Education:

B.S. Poultry Science, Texas A&M University, 2003