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Genetic diversity of the chB6 alloantigen

John Peter O'Laughlin

DePaul University, jolaugh1@students.depaul.edu

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Genetic Diversity of the chB6 Alloantigen

A Thesis Presented in
Partial Fulfillment of the
Requirements for the
Degree of Master of Science

By
J. Peter O'Laughlin
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Department of Biological Sciences
College of Liberal Arts and Sciences
DePaul University
Chicago, Illinois

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Abstract

Chickens are one of the classic models of vertebrate immunity. We have been interested in the role of the alloantigen chB6 (formerly called BU1) in the development of B cells within the bursa and have presented evidence that chB6 can trigger apoptosis. chB6 is an alloantigen and in the original report of its cloning three alleles were reported, chB6.1 in RPL line 6 flocks, chB6.2 in RPL line 7, and chB6.3 in Light Sussex birds. However, no further search for other chB6 alleles has been reported. Using the chicken EST database we have identified four potential new alleles of chB6, although some of these EST clones are not full length cDNAs. We have resequenced these EST clones to confirm the base sequence and amino acid translation. These results suggest greater genetic diversity of the chB6 locus than previously appreciated. Furthermore, rt-PCR was used to clone the turkey homologue of chB6. The results presented here are the beginnings of a larger study to examine chB6's role in B cell development that may provide novel insights into the evolution of immune related genes in all avian species.

Introduction

Role of the Immune System

The immune system is an essential component of an organism's ability to survive, and even simple organisms have elements of a defensive immune system. The purpose of the immune system is to defend an organism from infection while minimizing damage to the host. Vertebrate immune systems are composed of layered defenses with increasing specificity (1).

When an infectious agent tries to gain entry into an organism it first must penetrate through the physical barriers that serve as the initial defense for the host. These external barriers include skin, tears, and mucous membranes. However, if the pathogen overcomes these barriers, the body responds via innate immunity mounting an immediate response in a non specific manner. Once inside the organism the pathogen travels through local tissue and may encounter phagocytes which aid in their removal. Additionally, complement proteins present in the body fluids may target infectious agents for attack (1).

If the pathogen is not removed via the innate immune system adaptive immunity mounts a response using highly specific cells known as lymphocytes. There are two classes of lymphocytes: T cells and B cells. The T cells include cytotoxic T cells, sometimes referred to as killer cells, and helper T cells that provide stimulatory signals to the B cells (1). B cells are essential for pathogen detection and elimination, because they produce antibody molecules (Immunoglobulins) that are specific for a single antigen. The diversity of antibody with distinct specificities allows for the recognition of a wide variety of antigens. The term antigen refers to any substance that can be recognized by the cells of the adaptive immune system (1). The antibody molecules are either cell bound, forming a B cell receptor complex, or are secreted by

the cell as a soluble form into the circulation (1). The antibodies released bind to antigens and aid in their removal. B cells that have participated in active immune responses produce daughter cells that are retained in immune tissues, creating a memory bank of previous exposure. This increases the ability of our body to detect specific antigens upon subsequent encounter and decrease elimination time, thus reducing secondary infections (1).

Each individual immunoglobulin (Ig) molecule is composed of four basic structural units: two heavy chains and two light chains (1). Each light chain consists of a variable region and a constant region while the heavy chain contains a variable region and several constant regions (1). The variable regions of the heavy and light chains compose the paratope of the antibody which forms noncovalent bonds with the small region of the antigen called the epitope (1). Recombination of the gene segments encoding the variable region within individual B cells means that each cell makes a unique paratope (1). This diversity of paratopes ensures that virtually any foreign agent can be detected (1). The gene rearrangements of the variable region are conducted in a two step process in which the variable region of the constant chain undergoes gene recombination followed by recombination of the variable region of the light chain (1). Once complete the two regions join forming a complex of six hypervariable loops (3 on each chain) that extend out from the molecule aiding in the detection and docking of the antigen complexes (1). These loops, also known as complementarity determining regions, aid in the specificity of the molecule (1).

B cell Development

As with all natural processes of such complexity there is room for error. The immune system has comprised a series of checks and balances to regulate B cell development, so that

cells producing antibodies that are self reactive, and may mount an immune response to one's own cells (autoimmune disease), can be recognized and removed. The elimination of these cells is an essential key to regulation of the immune response and is crucial to the host's survival.

In order to prevent the onset of an autoimmune disease self reactive cells must be removed from the cell repertoire before leaving the bone marrow and entering into the circulation. To do so the immune system screens cells within the bone marrow for self reactivity. If the B cell shows any form of self reactivity one of two outcomes are used to rectify the problem; receptor editing or clonal deletion (2-11). Receptor editing is a last chance effort utilized to save the immature self reactive B cells from cell death (2-5). Receptor editing results in a secondary rearrangement of the light chain locus that may help deter self reactivity of the B cell's receptor (13-16). Casellas *et al.* showed that receptor editing plays a major part in shaping the B cell repertoire and takes place during a 2 hour delay during pre B cell development. At least 25% of the pre B cell population undergoes editing and express edited versions of autoreactive antibodies (16, 17). In other models transgenic mice were knocked in (genetically inserted) with autoreactive B cell receptors, providing the potential for secondary rearrangements (16, 18, 19). These models showed that autoreactive B cell receptors were edited at a higher rate than their harmless counterparts (16, 18, 19).

Clonal deletion results in B cells being targeted for removal. This process involves a cell signaling cascade induced by cell surface receptors that trigger apoptosis (programmed cell death) (20-25). Apoptosis results in the degradation of organelles, and the denaturation of proteins, and the digestion of DNA within the cell.

Apoptosis

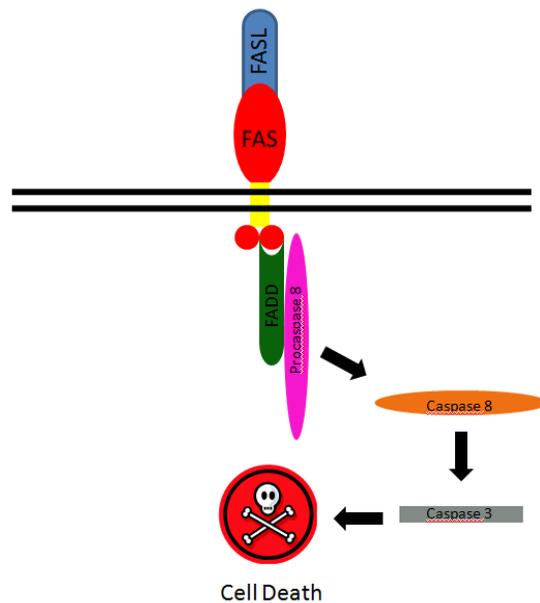
During lymphocyte development deletion of cells by apoptosis is an important process that removes potentially harmful cells produced by improper gene rearrangements. The process of apoptosis involves the detachment of a cell from its surrounding environment. All stimulatory signals are terminated, and the cell undergoes internal suicide. As the DNA and organelles within the cell condense, the mitochondrial induced apoptotic channel becomes more permeable resulting in increased levels of cytochrome c leaving the mitochondria (26). This release of cytochrome c is considered the full commitment step to apoptosis in many cell types (27-30). Cytochrome c has the ability to activate caspase 9, a cysteine protease, which can then activate other caspases within the cell including caspase 3 and 7. Each caspase has a unique function in apoptosis by carrying out the steps involved in cell death, including degradation of chromosomal DNA and proteins. The breakdown of membrane proteins results in increased permeability of the cell membrane exposing the cell's dying state to cells within the surrounding tissue that recruit phagocytes that aid in the removal and digestion of the cell fragments via phagocytosis (31).

There are many variations of apoptotic induced cell death that depend upon the state of the cell and the form of apoptosis initiation. The two main apoptotic pathways are; one, the result of the loss of signal transduction required for development (Death by neglect) and two, the result of receptor induced signal transduction (Death by instruction) (32). During development, B cells receive multiple stimulatory signals that are responsible for differentiation and proliferation of the cell. If these signals are not present or the cell cannot receive the signals, the cell will die due to neglect. As the B cells undergo gene rearrangements during their development, the cells are screened at multiple checkpoints. Though the cells may be healthy, errors within antigen

receptors may also cause the initiation of apoptosis through a receptor mediated complex (Instruction).

A well studied model that provides a great example of a receptor mediated death domain complex is the Fas (CD95) system, found on cells in the peripheral circulation of mammals, which is illustrated in *Figure 1*. Fas (CD95), is the key component in this apoptotic process (33). The Fas cell receptor (FAS) becomes active when it is bound by Fas ligand (FASL) (33). This activation results in the recruitment of the Fas associated death domain (FADD) protein (33). The interaction between Fas and FADD forms what is known as a death effector domain (DED) which interacts with internal inactive proenzymes known as caspases (34). Procaspase 8 interacts directly with the Fas/FADD complex and becomes active Caspase 8 through substrate cleavage (34). This triggers a cascade of intracellular events eventually leading to the cleavage and condensation of DNA in the nucleus resulting in cell death (33, 35). The Fas receptor is not detected in the bone marrow of mammals, thus indicating that there is another molecule involved in cell apoptosis there (36). Thus suggesting that there is another mechanism by which clonal deletion occurs in both humans and chickens.

Figure 1: Fas (CD95) Death Receptor System



Bursa in Chickens – A Model for B Cell Development

Studying lymphocyte development in the bone marrow of humans poses a difficult task. Multiple blood cell lineages develop in the bone marrow, and there is no clear delineation of a specific region where lymphocytes reside. To isolate individual B cells from the meshwork of cells poses a highly expensive and very difficult task. Within the bone marrow there is no clear delineation of positioning that could provide a timeline for B cell development thus making it difficult to isolate cells at certain stages within their development. Another factor contributing to the difficulty in using bone marrow as a model for study is the identification of individual B cells among millions of other white blood cells and lymphocytes.

The Bursa of Fabricius, a primary lymphoid organ found in avian species, has become a valuable research model to study and understand B cell development. The bursa in chickens is located near the cloaca and originates as an epithelial bud from the gut lining. The bursa is solely devoted to B cell production, thus providing a model to study without interference of other cells or tissues. In experiments conducted where chickens were bursectomized at 60 hours of embryonic development the animals showed an inability to have either functional or specific antibody responses (37-41). DNA analysis of the bursectomized chickens showed Ig light and heavy chains with very minimal differences in V-J and V-D-J joints (37-41). The bursectomized chickens had very few B cell precursors that differentiated into mature Ig producing cells thus the diversity of the B cell receptors was highly restricted (37-41). Collectively this demonstrates that the Bursa is critical for the development of B cells and the immune system within avian species.

During a chicken's embryonic development, starting at day 8 and continuing up to day 14, the bursa becomes seeded with B cell precursors (42). Once in the bursa these precursor cells begin to proliferate, forming lymphoid follicles, each consisting of approximately 10^5 lymphocytes that originated from only 2 to 4 precursor cells (43, 44). As these follicles are generated only a select few, less than 5%, of the mature B cells exit the bursa to travel into the secondary tissues in which they will come into contact with antigens (43). The remaining 95% of the cells undergo apoptosis and are removed from the repertoire of cells.

chB6 & its Role in B Cell Development

The avian chB6 alloantigen, originally called Bu-1, was defined as a marker of B lymphocytes in chickens. Classic experiments by Houssaint showed that chB6 was expressed on the earliest identifiable B cell progenitors and continued to be expressed on B cells until their terminal differentiation into plasma cells (45, 46, 47). Houssaint also reported expression of chB6 on a subset of macrophages in the bursa, liver, and intestine (45, 46, 47). Subsequent work by Funk *et al.* showed that crosslinking chB6 with an anti-chB6 antibody led to apoptosis in both primary B cells and the DT 40 cell line (48). Tregaskes reported the cloning of cDNAs encoding three distinct alleles of chB6; accession numbers X92866 (chB6.1), X92867 (chB6.2), and X92865 (chB6.3) (49). Tregaskes showed that chB6 is a type I transmembrane protein 335 amino acids in length (49). The 184 amino acid extracellular domain contains six sites for potential N-linked glycosylation and a number of conserved cysteine residues (49). The single transmembrane region is followed by a 105 amino acid cytoplasmic domain characterized by a number of acidic residues and high proline content (49). Notably, no similarity with known proteins was reported. Each cDNA sequence retained the unique structure of the chB6 protein with slight variations within amino acid (AA) residues. Using chB6.1 as the reference sequence

chB6.2 has the following amino acids changes: L12 to W12, P47 to L47, N52 to K42, N54 to S54, S66 to L66, K111 to E111, I116 to F116, R120 to W120, D183 to G183 (*Figure 2*). chB6.3 has the following changes in reference to chB6.1: P47 to L47, N52 to K22, N54 to S54, S66 to L66, K111 to E111, K144 to Q144, N147 to Y147, G154 to E154, G172 to V172, E173 to Q173, L-238-I (*Figure 1*). Based on the pattern of amino acids changes Tregaskes *et al.* concluded that the three alleles were equally divergent from one another. Notably all N-linked glycosylation sites and cysteine residues are conserved in all three alleles. They also noted that the cytoplasmic domain was highly conserved with only a single isoleucine to leucine change at position 238.

In 1997 our lab found that ligation of chB6 by an anti-chB6 antibody induced apoptosis, suggesting that the molecule had a role in apoptosis (50). In addition, we have shown that the chB6 apoptotic pathway can be transferred to mouse cell lines through transfection experiments, providing evidence that it is a regulated form of cell death (48, 51). In more recent studies we have explored the intracellular events that lead up to cell death after crosslinking of the chB6 death receptor induced by ligation via anti-chB6 antibodies. In these studies both caspase 3 and 8 become activated once the receptor is ligated (52). These caspase molecules are part of the intracellular machinery that degrades the cellular components during apoptosis (53). Collectively, these studies indicate that chB6 causes apoptosis via a mechanism similar to that in other death receptor systems, such as Fas (CD95).

EST Database

Expressed sequence tag (EST) databases contain sequences of automated single stranded reads of DNA obtained from cDNA clones (54). The process by which these sequences are

obtained is different from other genomic databases and stems from years of genomic research. EST databases are compiled of very limited information, whereas genomic databases are compiled of entire genomes, and redundant information. For example when looking at a genomic database, the sequences utilized are entire gene sequences (DNA) and include more information than a short sequence read you would find in an EST database. When cells express a particular gene they begin by transcribing the DNA into mRNA which is then later translated into a protein. At any given time within a cell the RNA expressed is a sampling of the genes expressed by that cell. By taking a sampling of the RNA and converting it into complementary copies of DNA (cDNA) researchers can compile profiles of the DNA within the cells under specific conditions or stages. The cDNA can then be ligated into plasmids which are essentially small circular pieces of DNA that can be grown in bacteria. Each specific plasmid contains one of cDNA from the source cell that can be transformed into a bacterium to reproduce pure copies of the cDNA. Collectively these cDNA sequences comprise a library of expressed genes from a particular cell type. The cloned sequences can then be entered into a computer database. However the automated sequencing methods used to derive these cDNAs are known to contain errors. Thus, any cDNA sequences used for genetic information must be obtained and resequenced to ensure the information provided in the database is correct.

Since three alleles of chB6 were identified by Tregaskes (49), we set out to test the hypothesis that there were other, as yet uncharacterized alleles. By searching the EST databases using the known chB6 alleles (chB6.1, chB6.2, and chB6.3) we expected to find other alleles of chB6. The conservation of both the structural and functional components between these new alleles could provide new insight into regions of the molecule that are critical for the function of the molecule as an apoptotic cell receptor. Furthermore, we predicted that we would be able to

clone chB6 homologue(s) in related bird species. Comparison between avian species could also provide information on the degree of conservation of this molecule.

Methods and Materials

Database Searches

Nucleotide sequences of chB6 (Accession numbers X92865, X92866, X92867) were used to search the National Center for Biotechnology Information's (NCBI) Expressed Sequence Tag database nucleotide blast (nblast) search engine. EST retrievals were translated from nucleotide sequence to amino acid sequence using the Expert Protein Analysis System (ExPASy) provided by the Swiss Institute of Bioinformatics. ESTs identified in this search were individually analyzed by alignment to identify both amino acid similarities with chB6.1 and the potential novel alleles. Alignment figures were produced using the multiple sequence alignment program, MAFFT version 6. Alignments with 100% identity to known chB6 were excluded from further analysis. EST clones showing greater than 50% identity to chB6 were analyzed for possible novel allele identification.

EST Clone Preparation

EST clone AM067700 was obtained from pooled chicken lymphoid tissues (Bursa, spleen, and Peyers patch of a Strain Lohman Brown layer/Ross 308 broiler cross) at the ARK-Genomics Center for Functional Genomics in Farm Animals at the Roslin Institute, Midlothian, U.K. Samples were provided as LB agar stabs containing 50 µg/ml of ampicillin. The stab was transferred to liquid culture in LB broth containing 50 µg/ml ampicillin then grown overnight at

37°C. The culture was then streaked out onto LB agar containing ampicillin (25 and 50 µl aliquots). Single colonies were picked and cultured in liquid broth followed by miniprep and sequencing.

EST clones BQ038848, CB016728, CB017782 were obtained from normalized chicken lymphoid tissue (thymus, bursa, spleen, PBL, and bone marrow, strain of bird not indicated) at the Delaware Institute of Biotechnology in Newark, Delaware and provided as bacterial cultures in LB broth with ampicillin and 15% glycerol. Clones were streaked onto LB and Ampicillin plates at 15, 30, and 45 µl aliquots and then incubated over night at 37°C. Single colonies were picked and cultured in liquid broth followed by miniprep and sequencing.

EST clone AJ456867 was obtained from a CB strain inbred chicken from the Jean Buerstedde lab, at the Heinrich-Pette-Institute in Hamburg, Germany and provided as a purified cDNA culture.

Plasmid Isolation

Plasmids were isolated from overnight culture following the QIAprep Spin protocol precisely (www.qiagen.com). An overnight culture (3 mL) was spun in a centrifuge to pellet bacteria. The cells were lysed and DNA was eluted and purified using affinity columns.

Sequencing

Plasmids isolated were sent to Davis Sequencing, L.C. in Davis, California for sequencing analysis. Once sequencing was completed chromatograms were visually analyzed for proper base reads. Each nucleotide sequence was then translated with *Expasy* and aligned with chB6.1, chB6.2, and chB6.3 via MAFFT.

RT-PCR of Turkey cDNA

chB6.1 primers chB6F 5'-ATGGATTTCAACTCTCCTTTTATTC-3' and chB6R 5'-ATGGATTTCAACTCTCCTTTTATTC -3' that were designed by Kent Reed at the University of Minnesota in St. Paul, MN were used to amplify the chB6.1 homologue in turkey cDNA via RT-PCR.

RT-PCR protocol – Synthesis of First- Strand cDNA

The synthesis of the first strand cDNA was conducted according to the Stratagene AccuScript RT-PCR protocol (stratagene.com/manuals/200820.pdf). The cDNA synthesis reaction mixture used for first strand synthesis was composed of 16.5 µl of RNase free H₂O, 2 µl of 10x AccuScript RT buffer , 0.8 µl of dNTP mix, and 0.2 µl of concentrated mRNA (approximately 200ng) from turkey lymphoid tissue (Nicholas line). The reaction mixture was then incubated for five minutes at 65°C. This was then followed by a cool down at room temperature for five minutes. 1 µl of mM DTT was added to the mixture along with 1.0 µl of AccuScript RT. The reaction was then placed in a controlled thermal block at 42°C for thirty minutes and finally chilled on ice for use in later PCR amplifications.

Amplifying the cDNA template

To amplify the desired chB6.1 cDNA the following components were mixed together using a sterile thin walled PCR tube: 40 µl of RNase free H₂O, 5 µl of 10x PCR buffer, 1 µl of dNTP mix (2 mM), 1 µl of chB6F upstream primer (100 ng/µl), 1 µl of chB6R downstream primer (100 ng/µl), 1 µl of first strand cDNA reaction, and 1 µl of PfuUltra HF DNA polymerase. The amplification profile was: first cycle at 95°C for one minute; followed by forty

cycles at 95°C for thirty seconds, 57°C for thirty seconds, and 68°C for three minutes, and one finish cycle at 68°C for ten minutes.

PCR Amplification and Transformation- Strata Clone Blunt PCR Cloning Kit

Once a positive cDNA amplification was verified using an agarose gel the PCR product was cloned using the Strata Clone Blunt PCR Cloning Kit (www.genomics.agilent.com/files/Manual/240207.pdf). Agarose plates treated with 40 µl of 2% X-gal and IPTG were used to culture the bacterial colonies. Aliquots (5 and 100 µl) of the transformation mixture were incubated overnight at 37°C. White colonies showing successful transformations were utilized for further plasmid DNA analysis (55).

Results

Novel Allele Analysis

Based on the genetic diversity seen in Tregaskes cloning work, we hypothesized that there might be other alleles of chB6 in chicken populations. From a preliminary EST database search using each of the three known alleles as the search criteria, a pool of 17 EST hits were retrieved. After an alignment analysis of each individual hit to the known alleles, candidates having an identical match to chB6.1, chB6.2, and chB6.3 were discarded. From the pool of 17 hits, a total of 4 putative novel alleles were identified. Given the errors that might be included in EST reports, candidates with possible novel amino acid changes were acquired and resequenced to confirm base pair changes. For all of the alleles an alignment diagram was composed illustrating the various amino acids that are either similar or different in each of the alleles (*Figure 2*). *Table 1* was composed to show the location of each of the amino acids, and whether

or not the changes seen were conservative or non conservative. Use both *Figure 2 and Table I* as an illustration for the results below.

EST clone BQ038848 (Delaware Institute of Biotechnology) was found to be a truncated cDNA sequence until residue 54 where it had a change from N54 to S54. The overall sequence of BQ038848 is similar to chB6.2 with the following key exceptions; R120, Q144, Y147, E154, A169, R186 and I238 are unlike chB6.2. In fact the arginine at position 186 and the alanine at position 169 are unique among the three previously reported alleles of chB6. With the exception of these two unique substitutions, the pattern of changes seen in BQ038848 appears to be intermediate between the previously described alleles. For instance, R120, Q144, Y147, E154 and I238 do not appear in chB6.2 but are found in chB6.3. In all of these instances amino acids encoded are found in at least one of the other alleles, making it unlikely that the change is due to an error introduced by reverse transcription, PCR, or any subsequent manipulation of the cDNA. Rather, it suggests that the alleles are related with a preferred pattern of substitutions allowed at a particular place in the amino acid chain.

AJ456867 (Heinrich-Pette-Institute) is a full length cDNA with striking sequence similarity to chB6.2. The only difference between AJ456867 and chB6.2 is a single substitution of L12 for W12 in the signal sequence, making this cDNA unique.

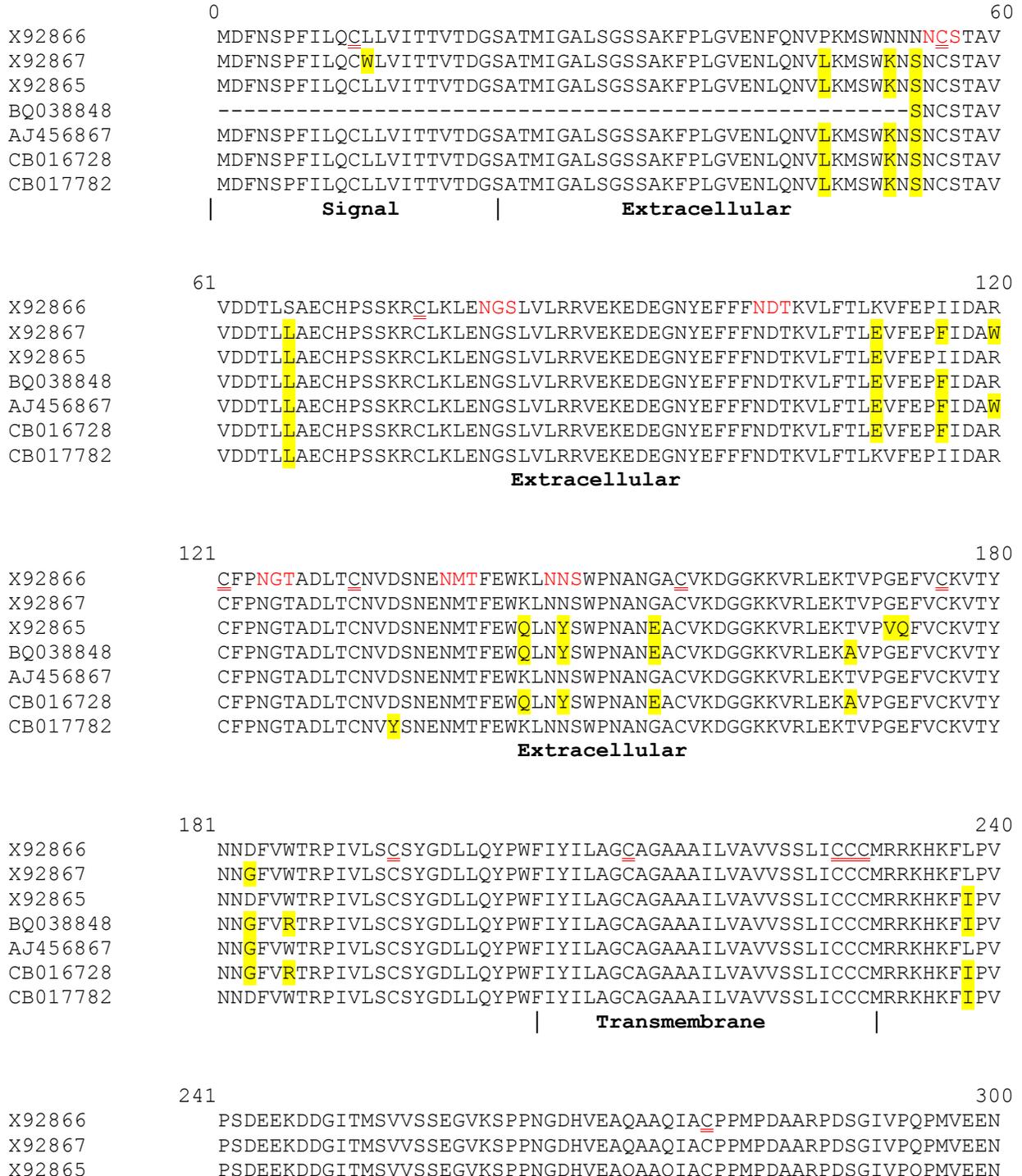
EST clone CB016728 (Delaware Institute of Biotechnology) is very similar to BQ038848 however the cDNA sequence is not truncated and is full length. The full length sequence has similar substitutions to that of both chB6.2 and chB6.3. Within the first 115 amino acids CB016728 has the exact same substitution as chB6.2 and chB6.3, minus the substitution in chB6.2 at position 12. At amino acid 116 it is similar to chB6.2 yet loses its similarity to chB6.2 at amino acid 120 and retains its resemblance to chB6.3. The sequence then retains this similarity

for the next 59 amino acids until it has a unique change at position 169 from T to A. At amino acid 172 and 173 it shows a similar resemblance to chB6.2 once again until position 186 where it has a unique substitution of W to R. The latter half of the sequence then retains chB6.3 composition with one highly unique change at amino acid 289 from S to L.

CB017782 (Delaware Institute of Biotechnology) has an amino acid sequence that is very similar to each of the other alleles (chB6.2, chB6.3, BQ038848, AJ456867, and CB016728) within the first 115 amino acids. At position 116 it begins to look more like chB6.1 and chB6.3 until position 134 where it has a unique substitution from D to Y. For the next 104 amino acids the sequence is identical to chB6.1 until it switches back to sharing a similar substitution to chB6.3 at amino acid 238. The cytoplasmic portion of the sequence is identical as seen with all the alleles except for one highly distinctive substitution of Q at amino acid 286, making this cDNA quite different from the others.

In each of the novel alleles there are various amino acid changes that characterize differences within the sequence and the changes appear to cluster in particular stretches of the primary sequence. However, some regions of sequence are absolutely conserved, suggesting a functional importance. The signal sequence, with one exception (amino acid 12 in chB6.2), and transmembrane portions of the molecule are 100% conserved. In each of the alleles the 6 putative N-glycosylation sites and all cysteine residues are 100% conserved suggesting that disulfide bonding and glycosylation are critical to the *in vivo* function of chB6. About 50% of the molecular weight of chB6 on the B cell surface is accounted for by glycosylation (48). The majority of the amino acid changes occur within the extracellular domain. The cytoplasmic region of each of the alleles is also highly conserved thus suggesting the functional importance of this portion of the molecule within the cell.

Figure 2. Alignment of Novel chB6 Alleles. CLUSTAL format alignment by MAFFT (v6.708b). X92866 (chB6.1), X92867 (chB6.2), and X92865 (chB6.3) are the three known alleles published by Tregaskes *et al.* 1996. BQ038848, AJ456867, CB016728, CB017782, and AM077700 are the novel alleles gathered and sequenced from the EST database searches. X92866 is the reference sequence for the results. N-linked glycosylation sites are shown in red font and the cysteine residues are double underlined in red. Each domain of the protein is characterized. Each set row is a total of 60 amino acids long. Polymorphic sites are highlighted in yellow.



BQ038848	PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPD	SGIVPQPMVEEN
AJ456867	PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPD	SGIVPQPMVEEN
CB016728	PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPD	SGIVPQPMVEEN
CB017782	PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAA	SGIVPQPMVEEN

Cytoplasmic

	331		335
X92866	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
X92867	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
X92865	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
BQ038848	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
AJ456867	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
CB016728	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	
CB017782	FPVAVEPGEMPDPEMIADVESQENASNC	FPDPIDN	

Cytoplasmic

Turkey Sequence Analysis

Given the apparent allelic diversity within chickens we attempted to clone a chB6 homologue from a related galliform, the turkey. Turkey bursal RNA was subjected to RT-PCR with chB6 specific primers and the PCR product was cloned using the Strataclone Blunt PCR cloning kit. Twelve different clones were sequenced and the consensus AA sequence is presented (Figure 3). The Turkey cDNA aligned with X92866, X92867, and X92865 shows a high level of conservation between each of the sequences. X92866, X92867, and X92865 are each 335 amino acids long however the Turkey sequence is 2 amino acids shorter (333). The CLUSTAL format alignment by MAFFT inserted two spaces at amino acids 24 and 25 to align the Turkey sequence with the chicken sequences.

The turkey sequence retains a number of the key features seen in the chB6 molecule with very minimal changes, including the N-linked glycosylation sites and cysteine residues. The majority of the amino acid changes occur within the extracellular portion of the sequence (*Figure 3 and Table I*). Both the signal and transmembrane sequence are highly conserved. The turkey sequence has 2 unique amino acid changes at the end of the signal sequence of D20 to A20, and

G21 to D21. The transmembrane sequence is 100% conserved. Out of the 15 cysteine residues 14 of them are conserved with one change of C76 to R76. Of the 5 N-linked glycosylation sites, 4 are identical. The fifth site, at positions 138-140 is shifted by two amino acids. A very distinctive change occurs between amino acids 136-140. The N-linked glycosylation site in X92866, X92867, and X92865 is N138/M139/T140. In the turkey sequence amino acid N138 is changed to S138; thus disrupting that bonding site. However, the two upstream amino acids are N136 and E137, thus forming a new N-linked glycosylation site of N136/E137/S138. Though the amino acid makeup and position shifted is by 2 residues, the structural integrity of the molecule is still intact. The shift may be due to the fact that the turkey sequence is 2 amino acids shorter. The cytoplasmic domain is also highly conserved, however the substitution of two residues with acidic residues further heightens that charge normally found on chB6. Another change is a conservative substitution of D243 with E243, preserving the consensus CK2 phosphorylation site.

Figure 3. Alignment of Turkey chB6 homolog. Using CLUSTAL format alignment by MAFFT (v6.713b) X92866 (chB6.1), X92867 (chB6.2), and X92865 (chB6.3) were used as the reference sequences for alignment of the Turkey cDNA. N-linked glycosylation sites are shown in red font and the cysteine residues are double underlined in red. Each domain of the protein is indicated.

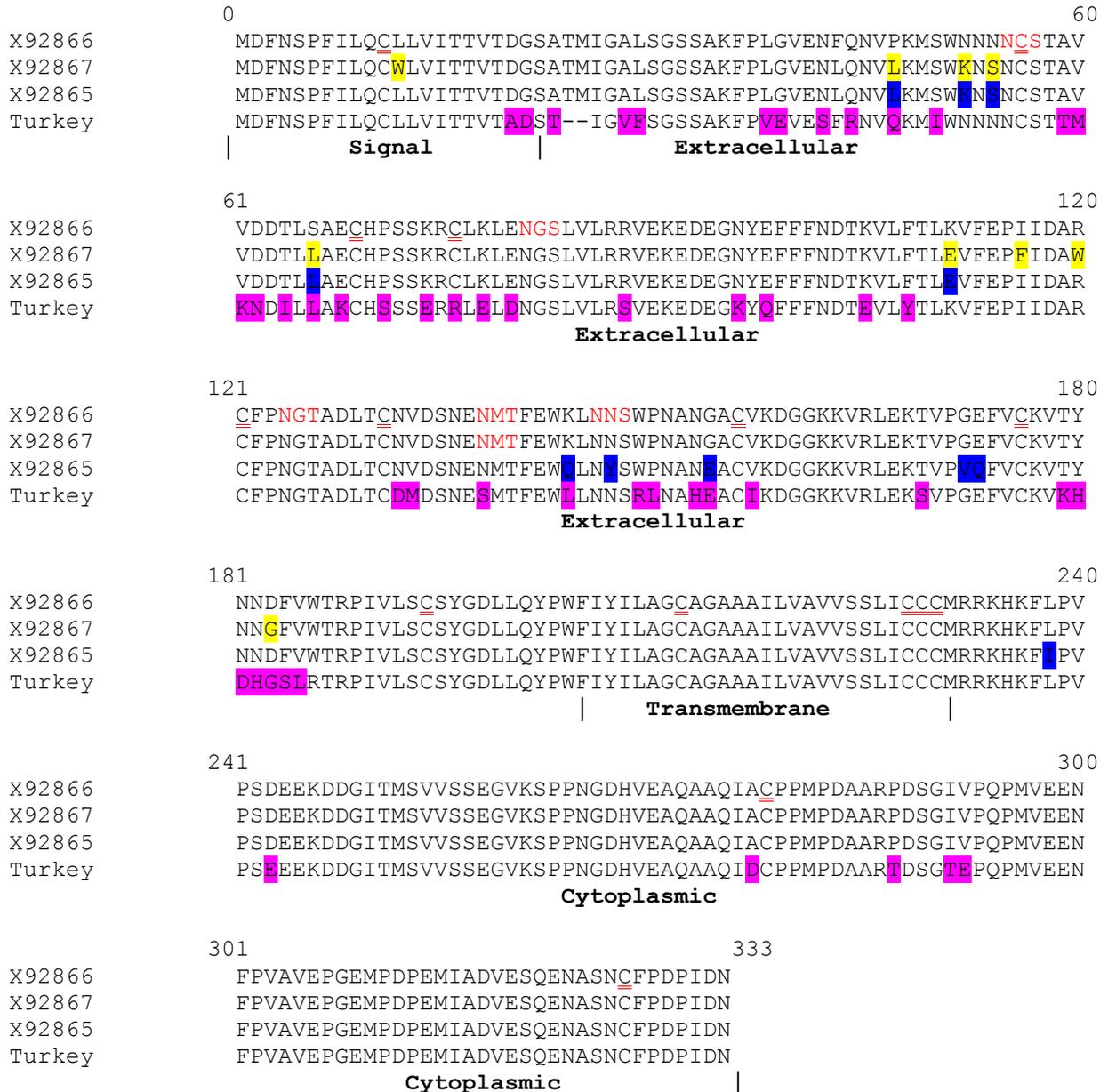


Table I. Summary of Amino Acid Changes among chB6 Molecules. The clones are labeled by their accession numbers. Along the top are the domains of the chB6 sequence. Each of the clones are compared to the chB6.1 sequence and the amino acids that are different are labeled below. For example clone # X92867 has a tryptophan in place of a leucine at amino acid position 12 in chB6.1 (L-12-W). Any changes highlighted are non conservative meaning they are either going from polar to non polar (vice versa), charge changes (+ to -, or - to +), or both depending on the amino acid composition.

Clone Accession #	Signal	Extracellular	Transmembrane	Cytoplasmic
X92867 (chB6.2)	L-12-W	P-47-L, N-52-K, N-54-S, S-66-L, K-111-E, I-116-F, R-120-W, D-183-G	Conserved	Conserved
X92865 (chB6.3)	Conserved	P-47-L, N-52-K, N-54-S, S-66-L, K-111-E, K-144-Q, N-147-Y, G-154-E, G-172-V, E-173-Q	Conserved	L-238-I
BQ038848	Truncated	N-54-S, S-66-L, K-111-E, I-116-F, K-144-Q, N-147-Y, G-154-E, T-169-A, D-183-G, W-186-R	Conserved	L-238-I
AJ456867	Conserved	P-47-L, N-52-K, N-54-S, S-66-L, K-111-E, I-116-F, R-120-W, D-183-G	Conserved	Conserved
CB016728	Conserved	P-47-L, N-52-K, N-54-S, S-66-L, K-111-E, I-116-F, K-144-Q, N-147-Y, G-154-E, T-169-A, D-183-G, W-186-R	Conserved	L-238-I, S-289-L
CB017782	Conserved	P-47-L, N-52-K, N-54-S, S-66-L, K-111-E, D-134-Y	Conserved	L-238-I, R-286-Q
Turkey	D-20-A, G-21-D	A-23-T, A-28-V, L-29-F, L-38-V, G-39-E, N-42-S, Q-44-R, P-47-Q, S-50-Q, S-53-I, A-59-T, V-60-M, V-61-K, D-62-N, T-64-I, S-66-L, E-68-K, P-71-S, K-74-E, C-76-R, K-78-E, E-80-D, R-88-S, N-96-K, E-98-K, K-105-E, F-108-Y, N-132-D, V-	Conserved	D-243-E, A-277-D, P-287-T, I-291-T, and V-292-E

		133-M, N-138-S, K-144-L, W-149-R, P-150-L, N-153-H, G-154-E, V-157-I, T-169-S, T-179-K, Y-180-H, N-181-D, N-182-H, D-183-G, F-184-S, V-184-L		
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Discussion

The chB6 alloantigen has been used as a cell marker throughout various stages of B cell development (45, 46, 47). Prior research has shown that chB6 acts as an apoptotic cell receptor that can also retain this function when transfected into mammalian cell lines (48, 50). However, there has been no success in finding a homologue to the chB6 receptor in mammalian cell lines. Understanding more about the genetic diversity of the chB6 cell receptor in birds may help categorize the functional domains of the molecule and may aid in the search for a mammalian counterpart to the molecule. Utilizing the three published alleles chB6.1, chB6.2, and chB6.3 we were able to identify four putative novel alleles, and an orthologous sequence with high sequence similarity to that of chB6 in turkeys. Our results further characterize the functionally critical domains of the molecule, and regions where substitutions of amino acids are tolerated without presumably interrupting the function of the molecule.

N-linked Glycosylation Sites

N-linked protein glycosylation is most commonly found in eukaryotic cells and has an important role in the modification of secretory and membrane proteins (56). N-linked glycosylations overall course of action is associated with protein translocation and folding (58). In identifying alleles of a gene N-linked glycosylation sites would be presumably conserved in

order to retain folding patterns and binding domains. The typical amino acid sequence of N-linked glycosylation sites occur in three amino acid spans, N-X-S or N-X-T, where X can be any amino acid except for proline. In chB6.1 there are 5 N-linked glycosylation sites that are highlighted in red (*Figure 2*). All of these sites are found in the extracellular domain of the molecule. In each of the other alleles, including the four novel alleles, the location of the sites are entirely conserved with only one amino acid variation at AA 147 from N to Y in alleles chB6.3, CB016728, and BQ03884. One would allow for variation at this amino acid position due to the substitution occurring at the X position where you can have any amino acid except for proline. In the turkey allele the N-linked glycosylation sites are also nearly identical to that of chB6 alleles, except for one site that is shifted two amino acids from NMT and position 138 to NES at 136. Overall the results show that the N-linked glycosylation sites are conserved and thus are important to the structure of the molecule. Since about half of the observed molecular weight of chB6 on the cell surface is accounted for by glycosylation it would seem likely that the carbohydrate moieties are critical to its function (49).

Cysteine Residues

Cysteine residues within protein structures contain thiol groups that are known to be nucleophilic and thus are easily oxidized (57). Cysteine plays an important role in the structural bonding patterns of proteins (57). The reactive thiol groups of the molecule form the disulfide bridges that are critical to the three dimensional folding of the protein (57). In chB6.1 there are 14 cysteine residues that are found in all domains of the molecule. The results show that all 14 of the cysteine residues are 100% conserved in all of the alleles of chicken which helps to further characterize the vital role they have in the structure of the molecule. In the turkey allele, 13 of the 14 cysteine residues are conserved (*Figure 2*). The cysteine residue at position 76 is

substituted by an arginine (R). This substitution is found in the extracellular region where we see the majority of the amino acid substitutions occur, and again may also be due to the fact that the sequence is two amino acids shorter. The loss of these two amino acids may allow for slight variations in the patterns we see in each of the highly conserved alleles.

chB6 and the Novel Alleles

Based on the results presented (Figure 2 and Table I) each of the four putative novel alleles are quite similar to that of the originally published chB6.1, chB6.2, and chB6.3. Overall the majority of the substitutions in all of the alleles occur in the extracellular region of the molecule. In each of the alleles most of the substitutions are similar to that of another allele which suggests that the substitutions seen are tolerated without changing any function of the molecule. The most highly conserved regions between each of the alleles are the regions with defined functions including the signal sequence, transmembrane region, the cytoplasmic tail, and about five stretches of the extracellular domain. In the signal sequence there is only one substitution out of all the alleles seen in chB6.2 at amino acid 12 from L to W. The transmembrane region is 100% conserved even in the turkey homologue.

The cytoplasmic tail is also highly conserved with a common amino acid change seen in chB6.2 and three of the novel alleles BQ038848, CB016728, and CB017782 of L to I at amino acid 238. The other two substitutions are unique to two of the novel alleles. CB016728 has a unique change at residue 289 switching from S to L and CB017782 has a unique change at position 286 of R to Q. The turkey homologue shows a similar strong conservation of this amino acid sequence, thus further providing evidence of the functional importance of the cytoplasmic tail. This would be expected if chB6 had a critical association with cytoplasmic proteins. We

have found that the stretch of 15 amino acids within the cytoplasmic domain is essential for transduction of a death signal (Funk, unpublished observations) and that sequence is conserved in turkey, as is the CK2 phosphorylation site. However, the conservation beyond even this suggests that other parts of the cytoplasmic domain have critical functions necessitating conservation, perhaps regulatory functions. Furthermore, the nearly absolute conservation of the transmembrane and cytoplasmic domain of chB6 provide a target for experiments to clone chB6 homologues from more distantly related aves and perhaps even reptilia.

Within the extracellular domain we find some large blocks of conserved sequence and defined areas where limited changes are allowed. For instance, the first 23 amino acids after the signal sequence are conserved in all chicken alleles. Likewise the sequence from amino acid 66 to 111, from 121 to 144, and 187 to 204 are completely conserved in all chicken alleles with the single exception of a D to Y change at position 134 of CB017782. This would suggest that large portions of sequence here are functionally relevant. As a membrane protein it is reasonable to postulate that chB6 binds an extracellular ligand. If so, this conservation of the potential binding site would be necessary. For instance, in the region from position 66 to 111 there are a number of amino acid changes but the individual changes are most often substituting one charged amino acid for another, or swapping charged and polar amino acids. Specifically, an E to K change at position 66 in the turkey followed by K to E changes at position 72 and 76, an E to D at 78, E to Q at position 97, and another K to E at position 104. If these regions were to be exposed to an aqueous environment on the exterior of the chB6 molecule such changes would be fairly conservative.

Future Research

The results presented here provide evidence for an additional four alleles of chB6 in chickens and a homolog in turkeys. Previous attempts to clone a chB6 homolog in mammals have been unsuccessful. Likewise, database searches for a chB6 homolog have been fruitless. Nevertheless, introduction of cDNA encoding chB6 into mammalian cells can reconstitute death signaling identical to that seen in chicken cells (48, 52), indicating that chB6 engages a death signaling pathway conserved since the divergence of aves and mammals roughly 300 million years ago. This is a fundamental puzzle, if chB6 is a unique avian gene why would it still engage this conserved pathway? Given that genomic studies are uncovering deep similarities among even distantly related species, is it reasonable to conclude that chB6 is in fact unique to Aves?

Death domains and death receptors have been difficult to define. Nevertheless death domain containing proteins have been found in many species and evidence indicates that death receptors are in fact ancient proteins that predate the emergence of vertebrates (58). Furthermore, there is evidence for divergence and functional specialization of death receptors (58). In order to understand the place of chB6 among death receptors, we first need to understand the divergence of chB6 within birds. The work here is a first step towards that understanding.

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Appendix

Figure 1: chB6.1, chB6.2, chB6.3

CLUSTAL format alignment by MAFFT (v6.707b)

```
X92866      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENFQNVPKMSWNNNNNCSTAV
X92867      MDFNSPFILQCWLVITTVTDGSATMIGALSGSSAKFPLGVENLQNVLKMSWKNSNCSTAV
X92865      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENLQNVLKMSWKNSNCSTAV
*****
X92866      VDDTLAECHPSSKRCLKLENGSLVLRVKEKEDEGNYEFFFNNTKVLFTLKVFEPIIDAR
X92867      VDDTLAECHPSSKRCLKLENGSLVLRVKEKEDEGNYEFFFNNTKVLFTLEVFEPFIDAW
X92865      VDDTLAECHPSSKRCLKLENGSLVLRVKEKEDEGNYEFFFNNTKVLFTLEVFEPIDAR
*****
X92866      CFPNGTADLTCNVDSNENMTFEWKLNNWPANANGACVKDGGKKVRLKTVPGFVCKVITY
X92867      CFPNGTADLTCNVDSNENMTFEWKLNNWPANANGACVKDGGKKVRLKTVPGFVCKVITY
X92865      CFPNGTADLTCNVDSNENMTFEWQLNYSWPNANEACVKDGGKKVRLKTVPVQFVCKVITY
*****
X92866      NNDFVWTRPIVLSCSYGDLLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRRKHKFLPV
X92867      NNDFVWTRPIVLSCSYGDLLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRRKHKFLPV
X92865      NNDFVWTRPIVLSCSYGDLLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRRKHKFIPV
**
X92866      PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
X92867      PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
X92865      PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
*****
X92866      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
X92867      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
X92865      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
*****
```

For the following alignments chB6.1 (X92866) was used as the reference for comparison

CLUSTAL format alignment by MAFFT (v6.707b)

```
X92866      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENFQNVPKMSWNNNNNCSTAV
BQ038848    -----NCSTAV
                                           *****

X92866      VDDTLSAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNNTKVLFTLKVFEPIIDAR
BQ038848    VDDTLLAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNNTKVLFTLEVFEPFIDAR
           *****:*****:*****

X92866      CFPNGTADLTCNVDSNENMTFEWKLNNWPANANGACVKDGGKKVRLEKTVPGFVCKVITY
BQ038848    CFPNGTADLTCNVDSNENMTFEWQLNYSWPNANEACVKDGGKKVRLEKAVPGFVCKVITY
           *****:*** ***** *****:*****

X92866      NNDVFWTRPIVLSCSYGDLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
BQ038848    NNGFVRTRPIVLSCSYGDLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRKHKFIPV
           **.* *****:***

X92866      PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
BQ038848    PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
           *****

X92866      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
BQ038848    FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
           *****
```

CLUSTAL format alignment by MAFFT (v6.707b)

```
X92866      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENFQNVPKMSWNNNNNCSTAV
AJ456867    MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENLQNVLKMSWKNSNCSTAV
           *****:*** *****:*.*****

X92866      VDDTLSAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNNTKVLFTLKVFEPIIDAR
AJ456867    VDDTLLAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNNTKVLFTLEVFEPFIDAW
           *****:*****:*****

X92866      CFPNGTADLTCNVDSNENMTFEWKLNNWPANANGACVKDGGKKVRLEKTVPGFVCKVITY
AJ456867    CFPNGTADLTCNVDSNENMTFEWKLNNWPANANGACVKDGGKKVRLEKTVPGFVCKVITY
           *****

X92866      NNDVFWTRPIVLSCSYGDLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
AJ456867    NNGFVWTRPIVLSCSYGDLQYPWFIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
           **.* *****

X92866      PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
AJ456867    PSDEEKDDGITMSVVSSEGVKSPPNGDHVEAQAQIACPPMPDAARPDGIVPQPMVEEN
           *****

X92866      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
AJ456867    FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
           *****
```

CLUSTAL format alignment by MAFFT (v6.707b)

```
X92866      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENFQNVPKMSWNNNNNCSTAV
CB016728    MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENLQNVLKMSWKNSNCSTAV
*****:***  *****:*.*****

X92866      VDDTLSAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNQTKVLFQKVFEPIDAR
CB016728    VDDTLAIECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNQTKVLFQKVFEPIDAR
*****  *****:*****:*****

X92866      CFPNGTADLTCNVDSNENMTFEWKLNSWPNANGACVKDGGKVRLEKQVPGFVCKVQY
CB016728    CFPNGTADLTCNVDSNENMTFEWQLNYSWPNANIEACVKDGGKVRLEKAVPGFVCKVQY
*****:***  *****  *****:*****

X92866      NNDFVWTRPIVLSCSYGDLQYPFYIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
CB016728    NNDFVWTRPIVLSCSYGDLQYPFYIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
**.*  *****:***

X92866      PSDEEKDDGITMSVVSSEGVKSPNGDHVEAQAAQIACPPMPDAARPDGIVPQPMVEEN
CB016728    PSDEEKDDGITMSVVSSEGVKSPNGDHVEAQAAQIACPPMPDAARPDGIVPQPMVEEN
*****  *****

X92866      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
CB016728    FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
*****
```

CLUSTAL format alignment by MAFFT (v6.707b)

```
X92866      MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENFQNVPKMSWNNNNNCSTAV
CB017782    MDFNSPFILQCLLVITTVTDGSATMIGALSGSSAKFPLGVENLQNVLKMSWKNSNCSTAV
*****:***  *****:*.*****

X92866      VDDTLSAECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNQTKVLFQKVFEPIDAR
CB017782    VDDTLAIECHPSSKRCLKLENGSLVLRVVEKEDEGNIEFFFNQTKVLFQKVFEPIDAR
*****  *****:*****:*****

X92866      CFPNGTADLTCNVDSNENMTFEWKLNSWPNANGACVKDGGKVRLEKQVPGFVCKVQY
CB017782    CFPNGTADLTCNVYSNENMTFEWKLNSWPNANGACVKDGGKVRLEKQVPGFVCKVQY
*****  *****:*****

X92866      NNDFVWTRPIVLSCSYGDLQYPFYIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
CB017782    NNDFVWTRPIVLSCSYGDLQYPFYIYILAGCAGAAAILVAVVSSLICCCMRKHKFLPV
*****:***

X92866      PSDEEKDDGITMSVVSSEGVKSPNGDHVEAQAAQIACPPMPDAARPDGIVPQPMVEEN
CB017782    PSDEEKDDGITMSVVSSEGVKSPNGDHVEAQAAQIACPPMPDAARPDGIVPQPMVEEN
*****:*****

X92866      FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
CB017782    FPAVEPGEMPDPPEMIADVESQENASNCFPDPIDN
*****
```