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Allelic Variation in *TLR4* Is Linked to Susceptibility to *Salmonella enterica* Serovar Typhimurium Infection in Chickens

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Toll-like receptor 4 (TLR4) is part of a group of evolutionarily conserved pattern recognition receptors involved in the activation of the immune system in response to various pathogens and in the innate defense against infection. We describe here the cloning and characterization of the avian orthologue of mammalian *TLR4*. Chicken *TLR4* encodes a 843-amino-acid protein that contains a leucine-rich repeat extracellular domain, a short transmembrane domain typical of type I transmembrane proteins, and a Toll–interleukin-1R signaling domain characteristic of all TLR proteins. The chicken TLR4 protein shows 46% identity (64% similarity) to human TLR4 and 41% similarity to other TLR family members. Northern blot analysis reveals that *TLR4* is expressed at approximately the same level in all tissues tested, including brain, thymus, kidney, intestine, muscle, liver, lung, bursa of Fabricius, heart, and spleen. The probe detected only one transcript of ca. 4.4 kb in length for all tissues except muscle where the size of *TLR4* mRNA was ca. 9.6 kb. We have mapped *TLR4* to microchromosome E41W17 in a region harboring the gene for tenascin C and known to be well conserved between the chicken and mammalian genomes. This region of the chicken genome was shown previously to harbor a *Salmonella* susceptibility locus. By using linkage analysis, *TLR4* was shown to be linked to resistance to infection with *Salmonella enterica* serovar Typhimurium in chickens (likelihood ratio test of 10.2, $P = 0.00138$), suggesting a role of TLR4 in the host response of chickens to *Salmonella* infection.

Salmonella, a gram-negative bacillus present in the intestinal tracts of humans and other animals, including birds, is capable of producing a wide range of clinical syndromes ranging from asymptomatic carrier state to life-threatening sepsis. Human infection occurs in two major patterns: a systemic disease known as typhoid fever and a gastrointestinal disease termed salmonellosis. Salmonellosis caused by the ingestion of *Salmonella*-contaminated poultry products is one of the most common causes of food poisoning in humans (26). The two serotypes that are the most frequently reported worldwide are serovar Typhimurium and *S. enterica* serovar Enteritidis. Most individuals infected with nontyphoidal *Salmonella* strains develop diarrhea, fever, and abdominal discomfort 12 to 72 h after infection. The disease is self-limiting and usually lasts 4 to 7 days, although 5% of individuals will develop a septic condition.

Incidences of reported nontyphoidal salmonellosis have increased dramatically since 1980 and, at present, up to 3.7 million cases of salmonellosis are estimated to occur annually in the United States (19). In addition to its impact on human health, salmonellosis in young chickens is a major disease (characterized by severe clinical signs of diarrhea and dehydration with a high mortality rate), resulting in economic losses for the poultry industry. Serovar Typhimurium and Enteritidis infections in adult chickens constitute an insidious risk for public health because the two pathogens do not cause significant clinical signs or mortality. In addition to horizontal transfer of the bacteria, localization of *Salmonella* in the ovary or oviduct may result in the contamination of egg contents.

Host genetic factors clearly influence the epidemiology of *Salmonella* infection in chickens. Genetic regulation of chicken resistance to *Salmonella* infection was reported initially by the group of Bumstead and Barrow (6). A survey of inbred and partially inbred lines of chicken showed significant differences in mortality after both oral and intramuscular challenge of newly hatched chicks with serovar Typhimurium (6). Lines W1, 6_1, and N were highly resistant to serovar Typhimurium, with >70% survival during the course of infection, whereas lines C, 7_2, and 15I were highly susceptible, with a 70 to 100% mortality rate. Resistance to infection extended to other *Salmonella* serotypes in these chicken lines: lines resistant to serovar Typhimurium were also resistant to infection with serovars Gallinarum, Pullorum, and Enteritidis, and chicken lines susceptible to serovar Typhimurium were also susceptible to the other *Salmonella* serotypes (5). In susceptible chickens, mortality occurred early during the course of infection (within 7 days postinoculation). Host differences in susceptibility to *Salmonella* infection in chickens have been shown to correlate with the bacterial load in the liver and spleen (2). Significantly higher numbers of salmonellae were isolated from the spleen and liver of susceptible chickens compared to the resistant chickens, suggesting that resistance to salmonellosis in chicken is related to a greater ability of the reticuloendothelial system to control bacterial proliferation during the early stage of infection. Segregation analysis with resistant W1 and susceptible

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C chickens showed that resistance to infection is dominant and inherited as a complex trait and is not associated with the major histocompatibility complex or maternal factors (6).

We used a strategy of comparative genomics, which consists of testing genes known to have a role in resistance to infection in a model organism as candidates for susceptibility to related infections in humans and in economically important farm animals. We have used the knowledge acquired in a well-characterized mouse model of *Salmonella* infection, in particular the identification of the gene underlying the *Ity* (*Nramp1* [natural resistance associated macrophage protein 1]) and *Lps* (*Tlr4* [Toll-like receptor 4]) mutations (3, 23, 24, 30). *Nramp1* and *Tlr4* are known to control the rate of exponential bacterial growth in spleen and liver early during infection (24, 30). *Nramp1* is an integral membrane phosphoglycoprotein located into the late endosome and/or lysosome compartment of resting macrophages and encodes a divalent-cation transporter (11). *Tlr4* belongs to a large family (at least 10 members are known in mammals) of pattern recognition receptors involved in the activation of the immune system in response to different pathogens, including fungi, viruses, and gram-negative and gram-positive bacteria (20). Work in our laboratory has shown that resistance to infection with *Salmonella* in chickens is inherited as a complex trait and that *NRAMP1*, which has been mapped to chicken chromosome 7q13 (13), and the gene for tenascin C, *TNC* (a closely linked marker to *Tlr4* in the mouse genome), are linked to early differential resistance to infection (12).

To directly investigate the role of *TLR4* in the differential resistance and susceptibility of chickens to infection with serovar Typhimurium, we have cloned the chicken *TLR4* gene and performed linkage analysis with a chicken panel composed of 274 progeny issued from *Salmonella*-resistant W1 and *Salmonella*-susceptible C chickens.

MATERIALS AND METHODS

Source of chicken lines. Inbred White Leghorn chicken lines W1, C, N, 6_1, 7_2, and 15I were bred and maintained at the Institute for Animal Health (Compton, Newbury, Berkshire, United Kingdom). Lines N, 15I, 6_1, and 7_2 were derived from the Avian Disease and Oncology Laboratory (East Lansing, Mich.). Line C was originally developed at the Northern Poultry Breeding Station (Reaseheath, Cheshire, United Kingdom), and line W1 was obtained from J. Ivanyi (Wellcome Laboratories, Beckenham, United Kingdom). DNA samples from two additional relatively inbred lines (White Leghorn UCD-003 and Red Jungle Fowl UCD-001) (23) lines developed at the University of California at Davis were kindly provided by Hans Cheng (U.S. Department of Agriculture, East Lansing, Mich.). Blood and DNA samples from outbred chickens were kindly provided by Robert McKay (Shaver Poultry Breeding Farms, Cambridge, Ontario, Canada) and Frédéric Lanthier (Institut National de la Recherche Agronomique, Nouzilly, France).

Molecular cloning of chicken *TLR4*. To clone chicken *TLR4*, we used a degenerate PCR approach. Degenerate primers were designed based on the protein alignment of human (GenBank accession no. U93091) and mouse (GenBank accession no. AF110133) TLR4 proteins. A low-stringency PCR amplification was performed in a total volume of 50 μl (50 ng of genomic DNA, 200 μM concentrations of each deoxynucleoside triphosphate, 100 nmol of each degenerate primer [forward, 5′-CA(CT)TA(CT)(AC)G(ACGT)GA(CT)TT(CT)AT(ACT)CC (ACGT)GG(ACGT)GT-3′; reverse, 5′-(AG)TC(CT)TCCCA(CT)TC(ACGT)A(AG)(AG)TA(ACGT)GT(AG)T-3′], 2.0 mM $MgCl_2$, 1× Amersham-Pharmacia *Taq* DNA polymerase buffer, 0.2 U of Amersham-Pharmacia *Taq* DNA polymerase) for 40 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 1 min, with a final extension of 6 min at 72°C. PCR amplification of chicken genomic DNA with the degenerate primers yielded a product of the predicted size (275 bp). The fragment was directly sequenced by using the ThermoSequenase chain terminator radiolabeled cycle sequencing kit (Amersham-Pharmacia), and products were electrophoretically separated in a glycerol-tolerant denaturing 6.5% polyacrylamide gel at a maximum current of 40 mA to verify its identity. The resulting 275-bp PCR product was used to screen an oligo(dT) and random-primed chicken spleen cDNA library, constructed in the λgt10 vector (Clontech catalog no. CL1015a) derived from an adult male Leghorn chicken. Nylon membranes (Hybond N; Amersham-Pharmacia) containing a total of 10^6 lambda clones were hybridized to labeled TLR4 under stringent conditions (6× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.5% sodium dodecyl sulfate [SDS], 5× Denhardt reagent, and 200 μg of fish sperm DNA/ml) for 16 h at 65°C. Seven positive isolates were identified, and further rounds of hybridization-purification followed. Only one 1.6-kb clone appeared to be chicken *TLR4* by sequence analysis and extended the sequence length of the probe. A second chicken *TLR4* probe (300 bp) was derived from the most 5′ sequence of this clone to screen initially an oligo(dT)-primed chicken liver cDNA library (catalog no. 935402; Stratagene, La Jolla, Calif.) cloned in the lambda ZAP vector. The screening resulted in the identification of one *TLR4* clone of 1.2 kb. The 300-bp chicken *TLR4* probe was also used to screen the Compton and the Wageningen BAC libraries. The identification of three positive clones (BAC 51o19, BAC 74 h3, and BAC 128b6) was achieved by hybridization, and the resulting clones were obtained from the UK HGMP Resource Center (http://www.hgmp.mrc.ac.uk/Biology/). BAC DNA was prepared by using the Qiagen Midi Kit (Qiagen, Inc.) for further sequencing.

Sequence analysis. The complete chicken *TLR4* gene was sequenced from BAC DNA with the ThermoSequenase chain terminator radiolabeled cycle sequencing kit (Amersham-Pharmacia). The internal primers used for sequencing are available upon request. Gene structure was determined by comparing chicken *TLR4* cDNA and genomic sequences. Protein alignments were carried out by using CLUSTALW (29), with no adjustment of the default parameters, and were subsequently edited in Genedoc multiple sequence alignment editor and shading utility, version 2.6.002 (22).

Northern blot analysis. Total RNA was isolated from various chicken tissues (brain, thymus, kidney, intestine, muscle, lung, liver, bursa of Fabricius, heart, and spleen) by using Trizol reagent (Gibco-BRL). Total RNA samples (10 μg) were separated on a denaturing 1% agarose formaldehyde-containing gel (27) and then transferred to GeneScreen Plus Hybridization membrane (NEF976; Perkin-Elmer Life Sciences) overnight in 10× SSC. A 2.1-kb probe (forward primer position 393 from initiation methionine in mRNA, 5′-GCTGAAAAAA CTAGTACTGGTGGA-3′; reverse primer position 2499 from initiation methionine in mRNA, 5′-TTTGCCTTCTAGAAGGACTGATGT-3′) consisting of most of exon 3 was [α-^{32}P]dATP labeled and hybridized (820×10^3 counts/ml) in a solution containing 50% formamide, 10% dextran sulfate, 5× SSC, 1% SDS, 5× Denhardt reagent, and 200 μg of herring sperm DNA/ml for 16 h at 42°C. The Northern blot was washed to a final stringency of 0.5× SSC–0.1% SDS for 15 min at 60°C and exposed to Kodak XAR film for 1 week with two intensifying screens at −70°C. The membrane was also hybridized with a chicken β-actin cDNA probe to normalize RNA loading.

Haplotype analysis. Sequence variants present between W1 and C *TLR4* alleles were analyzed by using ThermoSequenase chain terminator radiolabeled cycle sequencing in additional *Salmonella*-resistant (lines N and 6_1), *Salmonella*-susceptible (lines 15 and 7_2) and outbred ($n = 18$) chickens. Primer pairs were designed to amplify 7 different cDNA segments of *TLR4* (Table 1). Standard PCR amplification was performed in a total volume of 50 μl (50 ng of genomic DNA, 200 μM concentrations of each deoxynucleoside triphosphate, 100 nmol of each primer, 1.5 mM $MgCl_2$, 1× Amersham-Pharmacia *Taq* DNA polymerase buffer, 0.2 U of Amersham-Pharmacia *Taq* DNA polymerase) for 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min, with a final extension of 6 min at 72°C. The sequenced segments were electrophoretically separated in a glycerol-tolerant denaturing polyacrylamide gel as described previously.

***TLR4* mapping.** A polymorphism within intron 2 of *TLR4*, identified and analyzed by using single-strand conformational polymorphism was used to map the gene in a panel of 425 (W1 × C)F_1 × C chickens previously described (12). This backcross panel has been typed for over 160 markers distributed across the genome at ca. 20-centimorgan intervals (V. Forgetta and D. Malo, unpublished data). One of the PCR primers used to detect the polymorphism (Table 1) was end labeled with [γ-^{33}P]ATP and T4 polynucleotide kinase (New England Biolabs), and the PCR was performed in a total volume of 20 μl. Standard cycling conditions were used as described previously. The PCR products were separated on a 7% nondenaturing polyacrylamide gel containing 6% glycerol at a constant power of 30 W at room temperature. In addition, a coding-region DNA polymorphism predicted to lead to an Arg343Lys substitution in the W1 TLR4 protein was used to map the gene. Primers 5′-GAAACGTTGTCAGAGGTTC CTATG-3′ (position 973 from initiation methionine) and 5′-CTCAGATTAGC

TABLE 1. Primer pairs used to PCR amplify chicken *TLR4* from genomic DNA

Fragment	Direction	cDNA region (5′ to 3′)	Size (bp)
I	Forward	TCCTTTCGTCCGCATCCCTGAGTG	223
	Reverse	AAACCTGAAGCAGAAGAGAAAGTT	
II	Forward	GATTTCCTGACCTTTGGTGCTGAC	243
	Reverse	TGAAGACAAACCAAGAACCACATA	
III	Forward	TGTTTCATCCACATTTACCCTCTT	792
	Reverse	TCTTCCATTCCAGATGTTTCACTT	
IV	Forward	GAAACGTTGTCAGAGGTTCCTATG	596
	Reverse	ACTTTGGTCCACCCATACTAATTT	
V	Forward	GCGGCTCCGCATCTTGGATATTTC	423
	Reverse	GCATGCTACTGTGGTTGGGTTGGG	
VI	Forward	CACAACTTGTTTGAATGCTCTTGC	563
	Reverse	AACAAGCTGCCAGGACCGAGCAAT	
VII	Forward	CCATTGTCACTAATATAATCCAAG	1,551
	Reverse	CTTGAGGCAAAGAACCAAGAGCTG	
VIII (intron 2)	Forward	ACAAAAACTCCCAAGTGGAGCACG	521
	Reverse	CTACAGCTGAACACTTTCATTTCC	

AAATTCTCCAGTC-3′ (position 1292 from initiation methionine) were designed to flank the Arg343Lys codon and used to PCR amplify a 319-bp fragment. The PCR products were sequenced by using an ABI 3700 at the Montreal Genome Center. The location of *TLR4* and the order of all of the loci were determined by minimizing the number of double crossovers with the aid of the MapManager v2.6.5 program (16).

Infection with serovar Typhimurium and statistical genetic analysis. Infection procedures have been described previously (12). Briefly, the effect of *TLR4* on survival to infection with serovar Typhimurium infection was tested by using a Cox proportional hazards model (15, 18) as implemented in R statistical software v1.3.0 (14). The hazard rate is defined as the probability of dying at the time "*t*" given that the individual bird has survived to the time just before *t*. The model assumes that the hazard function is a product of a baseline hazard that is dependent on time but not the covariates, multiplied by a term that is dependent on covariates but not time. The Cox model is semiparametric in the sense that the magnitude of the effect will be estimated for *TLR4*, but there is no explicit estimation of the baseline hazard. Models are tested against the null model, which includes no covariates, by using the likelihood ratio statistical test. We also compared the results found by Hu et al. (12) for *NRAMP1* and *TNC* to the results obtained here.

RESULTS

Identification of the chicken TLR4 gene. It is now widely recognized that Toll-like receptors (TLRs) are part of a family of genes whose proteins are the main sensors used by the innate immune system to detect invading pathogens. *Tlr4* was recently shown to be mutated in the *Salmonella*-susceptible mouse strains C3H/HeJ, C57BL/10ScNCr (23, 24), and B6.mnd (3). In addition, previous studies performed in our laboratory have established linkage between *TNC* (a closely linked marker to *Tlr4* in the mouse genome) and susceptibility to *Salmonella* infection in chicken line C (12). To directly investigate a potential role for *TLR4* in differential resistance or susceptibility of chickens to infection with serovar Typhimurium, we have cloned the chicken *TLR4* gene. We have initially used degenerate PCR to clone a 275-bp genomic fragment corresponding to chicken *TLR4*. The degenerate primers were derived from the highly conserved Toll–interleukin-1R (TIR) domain of the protein (Fig. 1). This region is also known to be encoded by a single exon in all mammalian TLRs, allowing the use of genomic DNA as a template. The 275-bp fragment demonstrated no similarity to any sequence in the NCBI BLAST database at the nucleotide level (BLASTN). However, once the sequence was translated in all frames and compared to all protein sequences (BLASTP), one open reading frame (ORF) showed 61% identity and 77% similarity to the human *TLR4* gene, establishing the proper identity of the PCR product. The chicken partial *TLR4* gene fragment was tested on a Southern blot of chicken spleen genomic DNA samples digested with the restriction enzyme *Bam*HI. The probe recognized a specific band of 4.4 kb (data not shown). To clone the entire chicken *TLR4* gene, several cDNA (spleen and liver) and genomic libraries were screened. The screening of chicken cDNA libraries yielded only two partial *TLR4* clones (1.2 and 1.6 kb) containing the entire 3′ untranslated region (1,017 bp) including the poly(A)$^+$ tail. Three BAC genomic *TLR4* clones averaging 103 kb in size were isolated and partially sequenced (~12 kb of genomic DNA was sequenced in the vicinity of chicken *TLR4*; GenBank accession no. AY064697). Individual *TLR4* exon-intron boundaries were determined by a loss of identity between the genomic and cDNA nucleotide sequence (we have used chicken and mammalian *TLR4* cDNAs as a point of comparison) and also by the presence of consensus donor and acceptor splice site sequences at the point of divergence. To verify that homologous sequences between chicken genomic DNA and mammalian *TLR4* cDNA was present in the chicken *TLR4* mRNA, we performed reverse transcription-PCR by using total chicken spleen RNA as a template and with primers derived from the predicted exons 1, 2, and 3. Sequencing of the resultant PCR products showed that these fragments perfectly matched the exonic part of the genomic clone. A putative initiator methionine was identified by comparing genomic and cDNA sequences. A TGA stop codon was found in the same ORF at 99 bp upstream from the putative initiator ATG. A consensus sequence for eukaryotic translation initiation sites was identified at position −25. The ORF is 2,532 bp in length and encodes for a type 1 transmembrane protein of 843 amino acids. The predicted structure of *TLR4* is identical to that of all other TLR genes with a leucine-rich repeat extracellular domain connected to a TIR domain by a single transmembrane domain. The chicken *TLR4* gene displays remarkable homology at the levels of both protein sequence and structure to other *TLR4* genes, with 46% identity and 64% similarity to its human counterpart (Fig. 1). An analysis of the first kilobase upstream from the start codon revealed several consensus sequences for potential transcription factor binding sites (MatInspector; Genomatix). An octamer factor 1 (Oct-1) motif, a myeloid-specific PU.1 motif, an interferon regulatory factor 2 motif, and an avian CCAAT box were all found in the promoter region (data not shown).

Tissue expression of chicken *TLR4* mRNA. We determined the relative levels of *TLR4* expression in chicken tissues by Northern blot analysis. We used a *TLR4* cDNA subfragment that corresponds to amino acids 131 to 833 of the predicted chicken TLR4 protein as a hybridization probe on blots containing total RNA from different chicken tissues, including brain, thymus, kidney, intestine, muscle, lung, liver, bursa of Fabricius, heart, and spleen (Fig. 2). The *TLR4* probe used in these experiments shows no significant nucleotide identity to

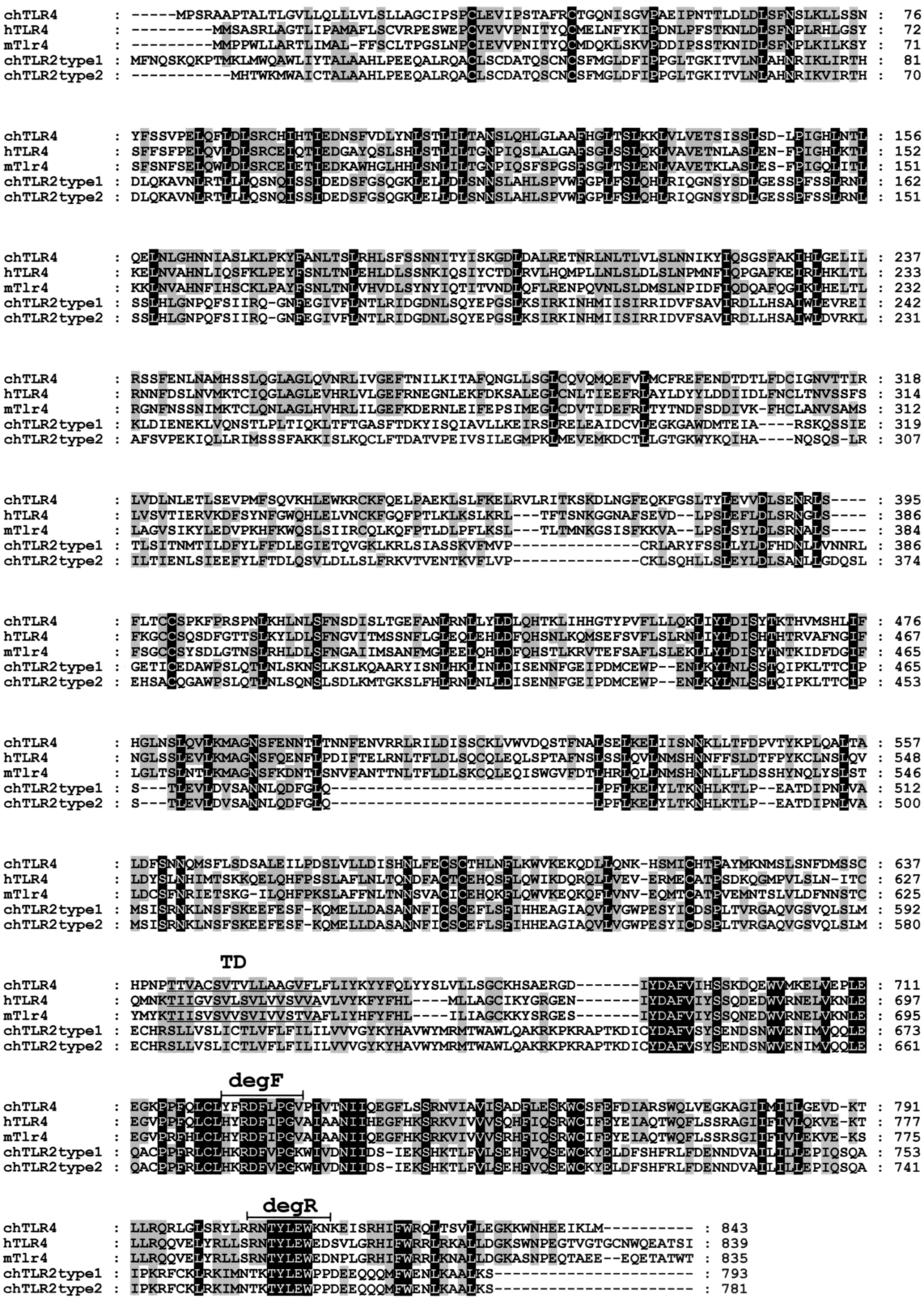

FIG. 1. Predicted protein alignments of chicken TLR4 (chTLR4; GenBank accession no. AY064697), human TLR4 (hTLR4; GenBank accession no. U93091), murine Tlr4 (mTlr4; GenBank accession no. AF110133), chicken TLR2 type 1 (chTLR2type1; GenBank accession no. AB050005), and chicken TLR2 type 2 (chTLR2type2; GenBank accession no. AB046533). Amino acid identity between all five species is indicated in black; the identity or similiarity between three or more species is highlighted in gray. Gaps are represented by a dash. The predicted transmembrane domain (TD) of TLR4 is underlined. The locations of degenerate primers are marked "degF" and "degR."

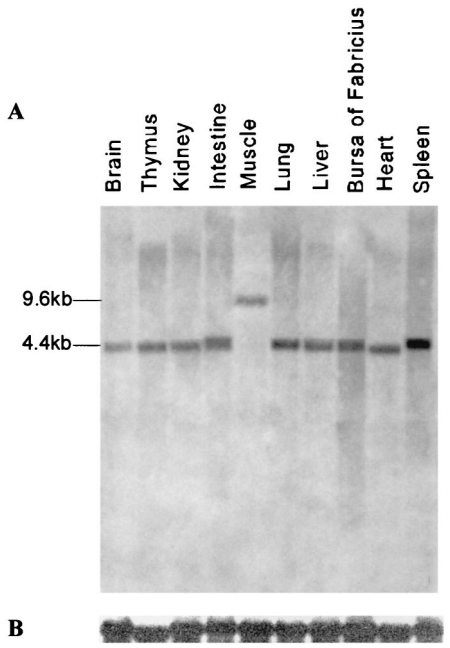

FIG. 2. (A) Northern blot analysis of *TLR4* expression in various chicken tissues. The blot contains 10 μg of total RNA per tissue and was hybridized with a 2.1-kb chicken *TLR4* cDNA subfragment. (B) The Northern blot was probed with a partial chicken β-actin cDNA probe for normalization.

any other known chicken *TLR* cDNAs (10) and was therefore highly specific under high-stringency hybridization conditions. *TLR4* appears to be expressed to the same extent in most tissues studied, although at low levels given the autoradiographic exposure time of one week. The probe recognized a transcript of 4.4 kb in all tissues studied except in muscle, where a single transcript of 9.6 kb was found (Fig. 2). The 9.6-kb transcript could occur as a result of an alternative splicing event or the use of an alternate upstream transcriptional initiation site and/or downstream polyadenylation signals in the *TLR4* gene.

Identification of *TLR4* sequence variants between resistant and susceptible chicken lines. Given the established role of Tlr4 protein in host defense against *Salmonella* infection in mice, we have determined the sequence of *TLR4* from resistant and susceptible chicken lines. We have identified 14 coding nucleotide sequence variants, of which 9 were silent mutations and 5 result in amino acid changes (Table 2). All five amino acid variants (Gly225Glu, Glu301Asp, Arg343Lys, Tyr383His, and Gln611Arg) were located within the leucine-rich region (LRR) of the extracellular domain of TLR4 that is known to be important for ligand recognition (Fig. 3). Two of them were conservative substitutions: a glutamic acid-to-aspartic acid change at position 301 (Glu301Asp) and an arginine-to-lysine change at position 343 (Arg343Lys). These two conservative replacements occur at residues that are not conserved in other TLR proteins, as shown by multiple sequence alignments.

The first nonconservative replacement (Gly225Glu) involves a G-to-A transition at nucleotide 674 and results in a hydrophobic glycine to a hydrophilic glutamic acid change. The second nonconservative replacement (Tyr383His) involves a T-to-C transition at nucleotide position 1147 and results in a nonconservative tyrosine (neutral and hydrophobic amino acid) to histidine (positively charged and hydrophilic amino acid) change. The third nonconservative amino acid change (Gln611Arg) involves an A-to-G transition at position 1832 that results in the replacement of a neutrally charged glutamine with a positively charged arginine (Fig. 3). It is interesting that among the species whose TLR4 protein sequence is known (horse, olive baboon, human, cat, pygmy chimpanzee, Chinese hamster, mouse, rat, cow, dog, gorilla, and orangutan), the tyrosine at position 383 is detected in the mouse, rat, and cow, whereas the neutral and polar amino acid serine is observed at the corresponding positions in horse, baboon, human, cat, chimpanzee, dog, gorilla, and orangutan, suggesting that the presence of a neutral amino acid at this particular position may be important for TLR4 function. The nonconservative change Gln611Arg occurs at a residue that is extremely well conserved in mammals, where a neutrally charged valine residue is present at the equivalent position in all 12 mammalian TLR4 sequenced.

The distribution of conservative and nonconservative amino acid substitutions in TLR4 was studied by determining the haplotype of four additional chicken lines of known susceptibility to infection with *Salmonella* (resistant lines N and 6_1 and susceptible lines 7_2 and 15I). Table 2 illustrates *TLR4* haplotypes identified in the various chicken lines. Five different *TLR4* haplotypes were observed by using 22 sequence variants detected in TLR4; 14 variants were identified in the coding region, and 8 were identified in the 3′ untranslated region. Resistant lines 6_1 and N presented 100% allele identity, and susceptible lines 7_2 and C showed the largest number of sequence variants compared to all other chicken lines. The region of the haplotype covering the coding part of chicken TLR4 is identical in all *Salmonella*-resistant lines and susceptible line 15I shares a similar haplotype (93% allele similarity) for this region. These observations are consistent with data showing that TLR4, and the region surrounding it are not involved in susceptibility of line 15I to *Salmonella* infection (12, 17; unpublished data). Susceptible line C was the most divergent and shared only four sequence variants with the other chicken lines. The two nonconservative changes (Tyr383His and Gln611Arg) initially detected in susceptible line C were also detected in susceptible line 7_2.

TLR4 haplotypes were also established in chicken DNA samples of diverse origin, including two inbred lines (White Leghorn and Red Jungle Fowl) and several lines selected for high egg production and meat-type. The ancestor of the domestic chicken is the wild Red Jungle Fowl, which has been domesticated for at least 4,000 years. The breed of greatest commercial significance, the Leghorn, has numerous varieties, but the White Leghorn is the world's leading egg producer (Britannica Student Encyclopedia, Online edition). In addition, these two chicken lines have been used to build the genetic map of the chicken genome because of their genetic divergence (7). Conclusions from these experiments have to take into consideration that some of the animals were related genetically. It was therefore impossible to establish the frequency of a specific allele; however, several interesting observations were made. The first is that all chicken DNA samples tested exhibit either a C or a W allele at a specific TLR4 amino

acid sequence variant, but no new substitutions were identified. The unique coding TLR4 haplotype of line C was also detected in the White Leghorn and Red Jungle Fowl lines. In the outbred population of chickens, half of the DNA samples tested showed heterozygosity for the substitutions at positions 301, 343, and 611 of the TLR4 protein. It appears that the sequence variant at position 383 has been fixed in a homozygous W/W or C/C state for all birds tested.

Chromosomal mapping of chicken *TLR4*. The chromosomal location of the chicken *TLR4* gene was determined by linkage analysis by using a panel of 425 (W1 × C)F_1 × C progeny to map quantitative trait loci associated with resistance to infection with serovar Typhimurium (12). This panel has been recently typed for over 160 microsatellite markers distributed throughout the genome (Forgetta and Malo, unpublished). Two *TLR4* sequence variations were used in the linkage analysis. The first one was a polymorphism in intron 2 of the *TLR4* gene and was detected by single-strand conformational polymorphism analysis; the second sequence variation used for mapping was Arg343Lys (G1028A). These two polymorphisms were typed in a total of 274 (W1 × C)F_1 × C backcross progeny and gave identical results. Chicken *TLR4* was mapped to the microchromosome E41W17 linked to MCW0330 (logarithm of the likelihood ratio score of 20.6) and TNC (LOD score of 37.5) (Fig. 4).

The region surrounding *TLR4* is linked to resistance to infection with serovar Typhimurium in chickens. To test the association of *TLR4* with chicken susceptibility to infection with serovar Typhimurium, we performed linkage analysis in (W1 × C)F_1 × C chicken progeny previously used by us to identify host chromosomal regions associated with disease susceptibility (12). In this model, W1 chickens were highly resistant to infection (5% mortality was observed 15 days postinoculation), whereas C chickens were highly susceptible (89% mortality for the same time period). The overall mortality rate in the backcross progeny was intermediate (35%) between those of resistant W1 and susceptible C lines (12). Mortality rate in line C occurred in two phases: an early phase (day 1 to 7), during which most animals die from infection (ca. 80% of the deaths occurred during this period), and a late phase (days 8 to 15), during which the mortality rate is much lower.

We analyzed the genotype frequencies of *TLR4* in 274 backcross progeny with respect to survival in response to infection. The survival curves for chickens harboring CC or CW1 genotype at *TLR4* are individually presented in Fig. 5A. The major effect of TLR4 is seen early after infection (7 days), and a smaller proportion of chicken progeny that were homozygous CC (64%) survived infection compared with the CW1 (82%) heterozygote. The TLR4 locus yielded a regression coefficient of −0.749 ($P = 0.0017$), and the associated partial likelihood ratio test was 10.2 ($P = 0.00138$) as determined by using the Cox proportional hazards model for the period covering the first 7 days postinfection. The impact of the chromosomal region surrounding *TLR4* on resistance to infection during the first 7 days postinfection is similar to that observed for *NRAMP1* (partial likelihood ratio test of 9.44; $P = 0.00213$), and *TNC* (partial likelihood ratio test of 7.42; $P = 0.00646$). The effect of *TNC* is not independent of *TLR4* since these two genes are loosely linked on the same chromosomal region of E41W17 (Fig. 4). We divided the backcross progeny into

TABLE 2. *TLR4* haplotypes in six chicken lines of known resistance or susceptibility to serovar Typhimurium infection

Chicken line	Phenotype[a]	Nucleotide in sequence variant[b]:																						
		A219G*	A576G*	G674A***	T720A*	G903T**	G912A*	G1028A**	T1147C***	C1327T*	C1416T*	G1455C*	A1644G*	A1832G***	T2487G*	G2818T	A2832G	G2835A	C2847A	A3045G	C3251T	C3282T	T3323C
W	R	A	A	G	T	G	G	T	C	C	C	G	A	A	T	G	A	G	C	A	C	C	C
6_1	R	A	A	G	T	G	G	T	C	C	C	G	A	A	T	T	G	A	C	G	C	C	C
N	R	A	A	G	T	G	G	T	C	C	C	G	A	A	T	T	G	A	C	G	C	C	C
151	S	A	A	G	T	G	G	T	C	C	C	G	A	A	G	T	A	A	C	A	C	C	C
7_2	S	A	A	A	T	G	G	C	T	T	T	C	G	G	G	G	A	G	C	A	T	T	T
C	S	G	G	G	A	T	A	C	C	C	C	C	A	G	G	G	A	G	C	A	C	C	T

[a] "R" indicates the resistant phenotype, and "S" indicates the susceptible phenotype according to 50% lethal dose values after inoculation with serovar Typhimurium (4).

[b] The positions of the sequence variants are given with respect to chicken *TLR4* cDNA (accession number AY064697). *, Silent variant; **, conservative variant; ***, nonconservative variant. Unmarked alleles are found in the 3′ untranslated region.

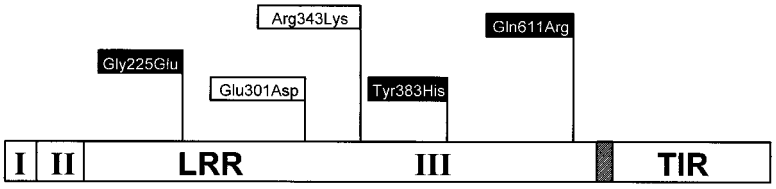

FIG. 3. Graphic representation of the chicken TLR4 coding region and localization of sequence variations detected between W1-resistant and C-susceptible chickens. Roman numerals represent exons. The LRR and TIR homology domain are shown. The hatched area represents the transmembrane domain. Conservative substitutions at the predicted protein level are flagged in white, and nonconservative substitutions are flagged in black.

four two-locus genotypes at *TLR4* and *NRAMP1* (Fig. 5B). All groups were represented equally. The group TLR4^{CW1}-NRAMP1^{CW1} had the highest survival rate at day 7 postinfection (93%) compared to the TLR4^{CC}-NRAMP1^{CC} progeny (58%). The two other groups (TLR4^{CW1}-NRAMP1^{CC} and TLR4^{CC}-NRAMP1^{CW1}) had intermediate survival rates (69 and 73%, respectively), although survival within the TLR4^{CW1}-NRAMP1^{CC} and the TLR4^{CC}-NRAMP1^{CW1} groups dropped significantly from day 6 to day 8 postinfection. These data are consistent with the previous observation in mice that mutation at *Nramp1* affects susceptibility to infection with serovar Typhimurium 1 or 2 days earlier than mutation at *Tlr4* (25, 28). On the basis of these survival analyses, we conclude that the unlinked chromosomal regions harboring *TLR4* and *NRAMP1* have a highly significant impact on resistance to infection during the first 7 days postinfection, although they do not have a significant impact on resistance to infection from day 8 postinfection onward (data not shown).

DISCUSSION

Salmonellosis caused by the ingestion of *Salmonella*-contaminated poultry products is one of the most common causes of food poisoning in humans (26). The economic losses associated with *Salmonella* infections have attracted increasing attention in developed countries in recent years especially in the United States, Canada, and Europe. The financial burden is not only associated with the investigation, treatment, and prevention of human illness but also with the whole chain of food production. Salmonellosis in young chickens is a major disease characterized by severe clinical signs of diarrhea and dehydration with high mortality rate. In adult chickens, serovars Typhimurium and Enteritidis infections do not cause significant disease or mortality, and birds can carry the bacteria for several weeks without presenting any clinical signs, which constitutes an insidious risk for public health.

At the present time, there are active genome projects occurring on agriculturally important species such as cattle, pig, sheep, horse, and chicken (8). This will undoubtedly yield powerful tools for assessing host genes that confer phenotypes of agricultural relevance ("economic trait loci") such as infectious disease resistance (31). Genetic identification and tracking of economic trait loci in animal pedigrees will be of great importance for livestock improvement and production. The strategy of comparative genomics consists of mapping and identifying genes for resistance to infectious disease in model organisms such as the mouse and then testing their orthologues as candidate genes for susceptibility to related infections in other animal species. Candidate genes for a disease phenotype are discovered by scanning the homologous region of the human and mouse gene maps by using comparative anchor loci as landmarks to limit chromosomal regions. Candidate genes within a restricted region may then be tested for association with the phenotype by using linkage analysis. Previous work in our laboratory has shown that this type of approach was successful to test the candidacy of *NRAMP1* in chicken resistance to infection (12). In the present study, we report the candidacy of *TLR4* in innate resistance and/or susceptibility to infection with *Salmonella* in chickens.

In chickens, the TIR domain of TLR4 was the most conserved segment, the cytoplasmic domain alone demonstrates 56% identity and 74% similarity to human TLR4 TIR domain. The extracellular region of TLR4 shows 43% identity and 61% similarity to human TLR4 LRR. As seen for several genes, the gene structure was conserved between avian and mammalian *TLR4*, although we observed major differences in intron sizes. Intron 1 in the chicken was about 6.5 times smaller (0.92 versus 6.0 kb) than murine intron 1 and chicken intron 2 was 5.4-fold smaller (0.97 versus 5.2 kb) than murine intron 2. These observations were expected since the size of the chicken genome is 1,200 Mb, one-third the size of mammalian genomes. Due to

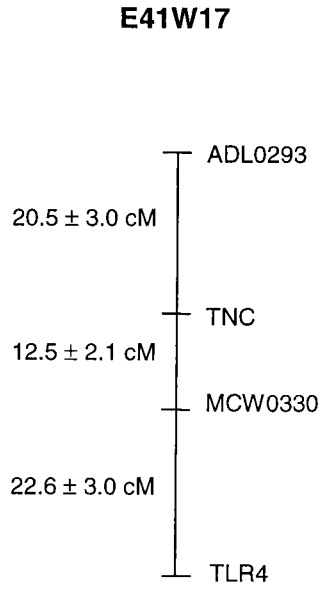

FIG. 4. Chromosomal mapping of chicken TLR4 to microchromosome E41W17 with respect to the microsatellite markers ADL0293, MCW0330, and *TNC*. cM, centimorgans.

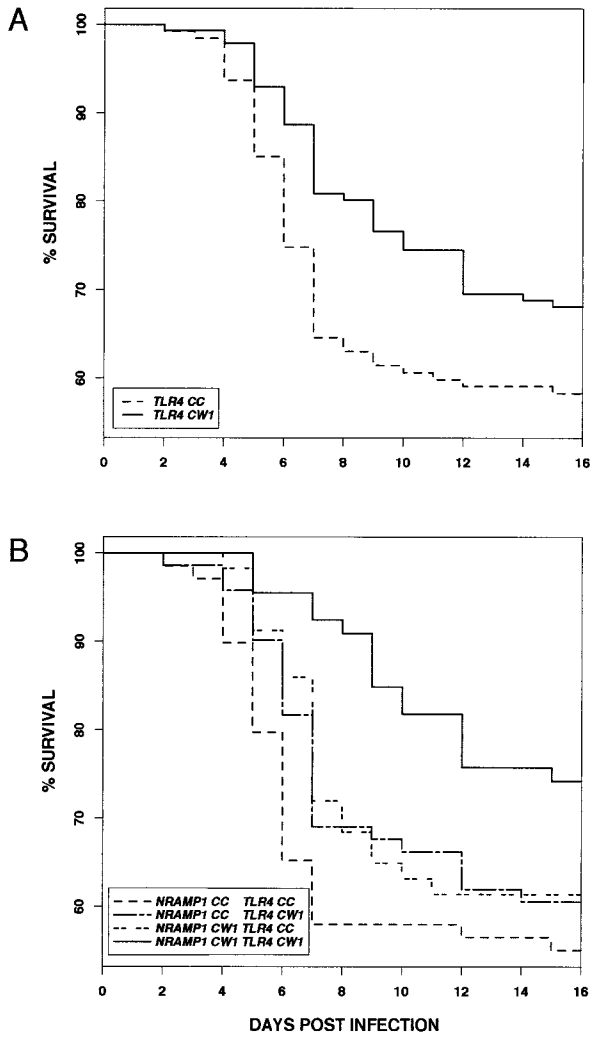

FIG. 5. Effects of *TLR4* and *NRAMP1* genotypes on survival of chickens to infection with serovar Typhimurium. (A) Survival curves for (W1 × C)F_1 × C progeny harboring CC or CW1 genotype at *TLR4*. (B) Survival curves for backcross progeny grouped according to their genotypes at *NRAMP1* and *TLR4* jointly.

the presence of small introns, more coding genetic information is stored within an equivalent length of DNA in chickens compared to mammals, which results in a more compact genome.

Two additional chicken TLRs (chTLR2 types 1 and 2) were recently cloned (4, 10). These two TLRs correspond to mammalian TLR2 since they show the greatest homology to human TLR2 with 52% identity and 70% similarity. Both genes were mapped to chromosome 4q1.1, another well-conserved region between the chicken and human genomes (4). Similarly to TLR4, chTLR2 type 2 was detected in all chicken organs tested and was reported to respond to whole inactivated *Escherichia coli*, *Staphylococcus aureus*, and *Mycobacterium bovis* (10). Chicken *TLR4* shows only 25% identity and 41% similarity to chTLR2 types 1 and 2 at the protein level. Alignment of chicken TLR4, human TLR4, mouse Tlr4, and chicken TLR2 type 1 and type 2 proteins showed that there are regions of strong amino acid identity especially in the cytoplasmic domain of TLR proteins. It is interesting that the Pro^{712} in the TIR domain of murine *Tlr4*, which is critical for the responsiveness of C3H/HeJ mice to gram-negative lipopolysaccharide (LPS), is also conserved in chicken and human TLR4, as well as in chTLR2 types 1 and 2. TLR4 in chickens is expressed in all tissues analyzed at levels comparable with those in the spleen. In mammals, TLR4 is also expressed broadly in adult tissues; however, the levels of expression vary considerably from one tissue to another. Monocytes and macrophages are major sites of TLR4 expression in both mammals and birds, suggesting a biological role of chicken TLR4 in innate immune defense in vivo (9, 32).

We have mapped *TLR4* to microchromosome E41W17 in a region harboring several genes, including *TNC* and *AMBP* that are conserved between birds and mammals (21, 25). This chromosomal region was also previously shown by us to carry a *Salmonella* susceptibility locus (12). The localization of *TLR4* to microchromosome E41W17, together with its role in the regulation of the immune response to LPS and *Salmonella* infection in mammals (1, 23, 24), makes *TLR4* an attractive candidate as a *Salmonella* susceptibility gene in our chicken model of infection. To gain further insight into the candidacy of *TLR4* as a *Salmonella* resistance gene in the chicken, we compared the genomic sequence of *TLR4* in susceptible and resistant chickens. We have identified 14 coding region sequence variants between resistant and susceptible chicken lines; 5 of them were predicted to result in amino acid changes. Among chicken lines of known susceptibility to infection with *Salmonella*, two nonconservative changes present in the LRR domain (Tyr383His and Gln611Arg) were detected only in susceptible lines C and 7_2. These two amino acid changes were also shown to be present in the current commercial chicken gene pool. In humans, two common missense mutations within the LRR domain of TLR4 (Asp299Gly and Thr399Ile) have been associated with the host response to inhaled LPS (1). It is possible that nonconservative amino acid substitutions at positions 383 and 611 may alter the topology of the chicken TLR4 extracellular binding domain and potentially disrupt dimerization and ligand-receptor interaction and, as a consequence, the initial signaling events, although the actual relationship between these sequence variations and the integrity and function of the chicken TLR4 protein is not known.

To analyze the impact of *TLR4* in resistance of chicken to infection with serovar Typhimurium, we have performed a linkage analysis by using survival time postinfection as the phenotype. Survival analysis was carried out by using a chicken DNA panel created from two birds derived from inbred White Leghorn lines that differed in their resistance to disease. Early genetic regulation of chicken resistance to *Salmonella* infection was shown to be linked to *TLR4*. The magnitude of the *TLR4* effect in the differential resistance or susceptibility of chicken lines C and W1 is similar to that observed with *NRAMP1*. Chickens carrying at least one W1 (resistant) allele at *NRAMP1* and *TLR4* showed the greatest degree of resistance to infection (93% of the progeny survived infection) compared to chickens bearing C alleles at *NRAMP1* and *TLR4* (58% survived the first week of infection). In this case, the odds ratio for survival until day seven is 0.62. Together with *NRAMP1*, *TLR4* explains 35% of the phenotypic variance and suggests that additional *Salmonella* resistance genes involved in innate or acquired immunity have yet to be identified in the chicken. A genome scan per-

formed on the same backcross panel verified the significant linkage of these two loci with resistance to *Salmonella* infection in chickens validating the approach of comparative genomics to identify host resistance genes (Forgetta and Malo, unpublished). The importance of *TLR4* in the host response of birds to infection with serovar Typhimurium needs to be further explored; however, the role of TLRs in the control of innate and adaptive immune responses makes them excellent targets for genetic intervention.

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