Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens

Victoria J. Philbin,[1] Muhammad Iqbal,[1] Yvonne Boyd,[2,3] Marianne J. Goodchild,[2] Richard K. Beal,[1] Nat Bumstead,[2] John Young[1] and Adrian L. Smith[1]

Divisions of [1]Immunology and Pathology, and [2]Molecular Biology, Compton Laboratory, Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom

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Present address: [3]Veterinary Research Division, Department for Environment, Food and Rural Affairs, Cromwell House, Dean Stanley Street, Westminster, London, SW1P 3JH, United Kingdom.
Correspondence: Dr Adrian L. Smith, Enteric Immunology, Stewart Building G2D, Division of Immunology and Pathology, Institute for Animal Health, Compton Laboratory, Compton, Newbury, Berkshire, RG20 7NN, United Kingdom.
Email: adrian.smith@bbsrc.ac.uk
Senior author: Dr Adrian L. Smith

Summary

Based upon the recognition of antiviral compounds and single stranded viral RNA the Toll-like receptors TLR7 and TLR8 are suggested to play a significant role in initiating antiviral immune responses. Here we report the molecular characterization of the chicken TLR7/8 loci which revealed an intact TLR7 gene and fragments of a TLR8-like gene with a 6-kilobase insertion containing chicken repeat 1 (CR1) retroviral-like insertion elements. The chicken TLR7 gene encodes a 1047-amino-acid protein with 62% identity to human TLR7 and a conserved pattern of predicted leucine-rich repeats. Highest levels of chicken TLR7 mRNA were detected in immune-related tissues and cells, especially the spleen, caecal, tonsil and splenic B cells. Alternative spliced forms of TLR7 mRNA were identified in chicken, mouse and human and expressed in similar tissues and cell types to the major form of chicken TLR7. The chicken TLR7$^+$ HD11 cell line and fresh splenocytes produced elevated levels of interleukin-1β (IL-1β) mRNA after exposure to the agonists R848 and loxoribine. Interestingly, none of the TLR7 agonists stimulated increased type I interferon (IFN) mRNA whereas poly(I:C) (a TLR3 agonist) up-regulated both chicken IFN-α and chicken IFN-β mRNA. In contrast, TLR7 agonists, particularly R848 and poly(U) stimulated up-regulation of chicken IL-1β, and chicken IL-8 mRNAs more effectively than poly(I:C). Stimulation of chicken TLR7 with R848 was chloroquine sensitive, suggesting signalling within an endosomal compartment, as for mammalian TLR7. The deletion of TLR8 in galliforms, accompanied with the differential response after exposure to TLR7 agonists, offers insight into the evolution of vertebrate TLR function.

Keywords: chicken; comparative immunology; R848; TLR7; TLR8

Introduction

A fundamental requirement of any immune system is the ability to recognize the invasion of pathogens into tissues and then to initiate an effective immune response against the pathogen. This detection is sensed by an array of specialized pathogen recognition receptors, which includes the Toll-like receptor (TLR) family. The 10 human TLR members have been found to recognize a diverse array of ligands including; tri-acyl lipopeptides (TLR1), lipoteichoic acid (TLR2), double-stranded RNA (TLR3), lipopolysaccharide (LPS; TLR4), flagellin (TLR5), diacyl lipopeptides (TLR6), imidazoquinolines (TLR7, TLR8) and CpGs motifs (TLR9) (reviewed in ref. 1).

The TLR family members TLR7, TLR8 and TLR9 form a phylogenetically related cluster, based on sequence similarities and genomic structures[2,3] and are activated by ribonucleic/nucleic acid components or analogues of these molecules.[4,5] In mammals, these TLRs are dependent on the endocytic pathway and are located in lysosomal-like

Abbreviations: ESTs, expressed sequence tags; LRRs, leucine-rich repeats; poly(I:C), polyriboinosinic polyribocytidylic acid; SMART, Simple Modular Architectural Research Tool; ssRNA, single stranded RNA; TIR, Toll-interleukin-1 receptor; TLR, Toll-like receptor.

vesicles.[6,7] Following activation, signals are transduced via the MyD88 adaptor,[8] as well as Bruton's tyrosine kinase for TLR8 and TLR9[9] or the TIR domain-containing adaptor inducing interferon-β (IFN-β) (TRIF) for TLR7.[10] TLR7 activation leads to the production of various inflammatory cytokines [including IFN-α, IFN-β, interleukin-6 (IL-6), IL-12, tumour necrosis factor-α (TNF-α)], up-regulation of costimulatory molecules (CD40, CD80, CD86), major histocompatibilty complex molecules and chemokine receptors (CCR7).[8,11,12] In humans, TLR7 is expressed on a restricted range of cell types with the highest abundance found on plasmacytoid dendritic cells and B cells.[13] In addition to the synthetic antiviral imidazoquinoline compounds, TLR7 has been implicated in recognizing guanosine and uracil-rich single-stranded(ss) RNA such as the U5 region of human immunodeficiency virus type 1 RNA and influenza U-rich ssRNA, leading to up-regulation of IFN-α.[4,14]

Although birds and mammals diverged approximately 300 million years ago evolutionarily conserved chromosomal segments are known to exist in both species,[15,16] that include regions encoding many of the TLR family members[17–19] (our unpublished data). Currently, only studies on chicken TLR2 (chTLR2)[17,18] and chicken TLR4 (chTLR4)[19–21] have been reported in any detail. Two forms of TLR2 (type 1 and type 2) have been identified in the chicken, with differential patterns of tissue distribution and responsiveness to macrophage-activating lipopeptide (MALP-2) or an LPS preparation.[18] The chTLR4 gene is polymorphic and located in a genomic region (E41W17) associated with the resistance/susceptibility of young chicks to *Salmonella enterica* serovar Typhimurium.[19] The chTLR4 mRNA is broadly expressed in a range of tissues including liver, lung, bursa of Fabricius, heart and spleen.[19]

Recent *in silico* analysis revealed expressed sequence tags (ESTs) with high amino acid identities to various chicken TLRs and signalling components including; TLR1/TLR6/TLR10, TLR3, TLR5, TLR7/TLR8, MyD88, TIRAP, IRAK-1, IRAK-2, IRAK-4, and NF-κB1.[22,23] Similar molecules were also identified by ourselves and we can extend the range to include RP-105 (coreceptor with TLR4), Dectin-1 (coreceptor with TLR2), CD14 and LPS-binding protein, TANK-1 (TNF-receptor-associated NF-κB activator). More recently two further TLR adaptors, sterile alpha and TIR motif containing 1 (SARM) and TRIF have been identified in the current Ensemble *Gallus gallus* genome database, which both display conserved synteny with flanking genes (unpublished data, V.J.P. and A.L.S.). Hence, the composition of the chicken TLR family and various signalling components are conserved within mammals, although further analysis is required to assess functional conservation between avian and mammalian pattern recognition pathways.

In this study we focused on chTLR7/8 and discovered that chTLR8 is disrupted in the genome of galliform birds. Using a combination of bioinformatic and experimental strategies we identified the full coding sequence of chTLR7 and alternative splice variants in chicken, mouse and human. The expression of chTLR7 mRNA was assessed in a wide range of tissues, cultured cell types and sorted cell subsets. The specificity and functional attributes of chTLR7 were assessed by exposure of chicken cells to TLR7 agonists (R848, loxoribine and various RNA derivatives) and by examination of the induced cytokine mRNA profile.

Materials and methods

Experimental chickens

Specific-pathogen-free inbred Line 6_1 (White Leghorn) chickens were supplied by the Poultry Production Unit of the Institute for Animal Health (IAH), Compton Laboratory. Birds were reared in wire cages and given *ad libitum* access to water and a vegetable-based protein diet (Special Diet Services, Witham, UK). Birds were wing-banded to allow identification of individuals.

Database mining

Publicly available sequences from human TLR7 (huTLR7) deposited in EMBL were used to identify putative chTLR7 EST from public databases[24,25] between January 2001 and April 2002. To support the identification of the putative chTLR7 ESTs the sequences were re-screened against mammalian sequence databases, only those with the highest matches to mammalian TLR7 being retained for further analysis.

Isolation and characterization of BAC clones

Bacterial artificial chromosomes (BAC) clones from the Wageningen chicken BAC library[26] were identified by hybridization to a ^{32}P-labelled (Stratagene, La Jolla, CA) polymerase chain reaction (PCR) amplified genomic DNA probe, based upon the sequence data available from the TLR7 bioinformatic analyses. The MRC HGMP Resource Centre (Hinxton, Cambridge, UK) supplied the library as clones gridded onto nylon filters. Hybridization was performed using standard techniques.[27] Positive clones were selected and the identity of the hybridizing sequence with the cDNA sequence was verified by PCR.

Fluorescent in situ *hybridization (FISH)*

Two BACs were mapped by FISH using previously established protocols.[17] BACs were hybridized independently to metaphases prepared from chick embryo fibroblasts after application of a double thymidine block.[28] BAC DNAs were labelled with biotin-16-dUTP by nick-translation and coprecipitated in 200-ng aliquots with 200 μg of

sonicated chicken genomic DNA before denaturation at 70° for 15 min. After reannealing at 37° for 20 min, 100 ng of BAC DNA was applied per slide in a volume of 10 μl and hybridized for 48 hr at 42° in a humidified chamber. Bound biotinylated probe was detected by incubation with avidin–fluorescein isothiocyanate followed by one round of amplification with biotinylated anti-avidin/avidin–fluorescein isothiocyanate (Vector Laboratories Burlingham, CA).

Preparation of BAC DNA for sequencing

Glycerol stocks of BAC clones were streaked on Luria–Bertani (LB) agar plates containing chloramphenicol (12·5 μg/ml) and incubated overnight at 37°. A single colony was inoculated into 5 ml LB medium containing chloramphenicol (12·5 μg/ml) and agitated for 6–7 hr before transferring into 200 ml LB medium to incubate overnight at 37° on a shaking incubator. The BAC DNA was purified using a Nucleobond AX kit (Macherey-Nagel GmBH, Duren, Germany) according to the manufacturer's protocol.

Bioinformatic analysis

The putative TLR7-containing BAC clones were sequenced by Lark Technologies (Essex, UK) and the DNA sequences were analysed using the web-based software tool NIX (http://www.hgmp.mrc.ac.uk/NIX). Another web-based program (SMART)[29] was used for comparative analysis of structural domains. BLAST searches with chicken TLR sequences were performed using the NCBI blast server (http://www.ncbi.nlm.nih.gov/BLAST) and alignment of cDNA sequences was performed using the CLUSTAL W program. TLR7 and TLR8 sequences were deposited in the EMBL database under the accession numbers: AJ627561, AJ627562, AJ627563, AJ632302, AJ812651, AJ812652, AJ812653 and AJ812654.

Ex-vivo-*sorted cell populations and cultured cells*

Chicken splenic B cells, T-cell receptor type 1 (TCR1; TCR-γδ), TCR2 (TCR-$\alpha\beta_1$) and TCR3 (TCR-$\alpha\beta_2$) were enriched by sorting with magnetic beads using the AutoMACS™ system (Miltenyi Biotech Ltd, Bisley, Surrey, UK) according to the manufacturer's instructions. Briefly, spleens were disrupted by passage through a coarse sieve and lymphocytes were isolated using Ficoll-Paque™ Plus (Amersham Biosciences AB, Uppsala, Sweden). The spleen cell suspension was reacted with primary antibodies (all obtained from Cambridge Biosciences, Cambridge, UK) against B cells or T-cell subsets as follows: anti-Bu-1 phycoerythrin at 0·02 mg/10^8 cells, anti-TCR1 [immunoglobulin G1 (IgG1); TCR-γδ] or anti-TCR3 (IgG1; TCR-$\alpha\beta_2$) at 0·1 mg/10^8 cells or anti-TCR2 phycoerythrin (TCR-$\alpha\beta_1$) at 0·05 mg/10^8 cells in phosphate-buffered saline, 0·5% bovine serum albumin diluent, for 15 min on ice. Following washing by centrifugation and resuspension of the cell pellet in 800 μl phosphate-buffered saline with 0·5% bovine serum albumin, different cell populations were isolated by positive selection (AutoMACS system) using anti-fluorochrome or rat anti-mouse IgG1 conjugated paramagnetic beads, 200 μl (Miltenyi Biotech Ltd) after 15 min incubation on ice. The purity of the sorted cell populations was enriched to greater than 95%, as determined by fluorescence-activated cell sorting (FACS). Peripheral blood monocyte-derived macrophages were obtained from Line 7_2 chickens according to the method of Wigley *et al.*[30] Heterophils were isolated from peripheral blood as described by Kogut *et al.*[31] and were in excess of 90% purity by microscopic analysis of haematoxylin & eosin-stained cytospin preparations.

Primary chicken kidney cells (CKC) were prepared from the kidneys of 2–3 week old Rhode Island Red chicks as previously described.[32] Briefly, kidneys were removed aseptically, teased apart and trypsinized with versene (0·8% NaCl, 0·02% KH_2PO_4, 0·15% Na_2HPO_4, 0·02% KCl and 0·02% EDTA). Cell concentrations were adjusted to 1×10^6/ml in complete minimum essential Eagle's medium supplemented with 12·5% (v/v) heat-inactivated fetal bovine serum, 10% (v/v) tryptose phosphate broth (Difco, Livonia, MI), 100 U/ml penicillin and 1 μg/ml streptomycin (P/S), at pH 7·0 and grown in 1 ml per well in 24-well Nunclon plates (Nunc, Rochester, NY) for 72 hr at 37°, 5% CO_2. Chicken embryonic fibroblasts (CEF) were prepared from 9-day-old embryos and cultured as previously described.[33] HD11 (a chicken macrophage cell line) cells[34] were seeded at 4×10^5/ml and grown at 41°, 5% CO_2 in RPMI 1640 medium containing 20 mM L-glutamine, 2·5% fetal bovine serum, 2·5% chicken serum and 10% tryptose phosphate broth. The bursal lymphoma cell line DT40 (ATCC: CRL2111) were cultured in RPMI-1640 as previously described.[35]

RNA extraction

Chicken tissues were immediately placed in RNAlater™ (Qiagen Ltd. Crawley, UK) and stored at −20°, according to the manufacturer's instructions. Prior to RNA isolation, fragments of chicken tissue were further disrupted by homogenization using a mini-bead beater (Biospec Products Inc., Bartlesville, OK). Isolated cell subsets or cultured cells were disrupted directly in RLT buffer (Qiagen Ltd. Crawley, UK) and frozen at −20° until RNA extraction.

RNA was extracted in the presence of buffer containing β-merceptoethanol and guanidine by using the RNeasy Mini Kit (Qiagen Ltd) according to the manufacturer's instructions. Any contaminating DNA was digested on the column with RNase-Free DNase 1 (Qiagen Ltd) for

90 min at room temperature (20–25°). The column was washed with RW1 and RWP buffers (Qiagen Ltd) and the RNa was eluted with RNase-free water.

Reverse transcription (RT)-PCR

RNA was reverse transcribed using the Promega Reverse Transcription kit (Promega, Southampton, UK) according to the manufacturer's instructions. Briefly, oligo-dT primer (0·5 μg/μl) was used to prime reverse transcription of 1 μg RNA (preincubated at 70° for 10 min then placed on ice) in the presence of dNTP (10 mM), $MgCl_2$ (25 mM), reverse transcriptase buffer (10 mM Tris–HCl, 50 mM KCl, 0·1% Triton^R X-100), AMV Reverse Transcriptase (15 units) and RNasin Ribonuclease inhibitor (1 unit/μl) made up to a final volume of 20 μl with nuclease-free water. Cycle conditions were as follows; 42° for 60 min followed by 99° for 5 min and then a final 4° for 5 min.

All RNA preparations were standardized by RT-PCR for β-actin using primers (see Table 1) and were free from DNA contamination as judged by a lack of signal from samples treated in exactly the same way but with reverse trancriptase omitted (data not shown).

PCR were performed according to standard protocols. Briefly, cDNA (1–2 μg) was incubated with 200 μM dNTP, 1·5 mM $MgCl_2$, 1× reaction buffer [50 mM KCl, 10 mM Tris–HCl (pH 9·0), 0·1% Triton X-100], 0·5 units *Taq* polymerase (Promega Southampton, UK), 5 pmol of each primer (see Table 1), in a 50-μl final reaction volume.

PCR conditions were as follows, one cycle, 95° for 2 min, followed by 35 cycles of 95° for 30 s, 58° for 30 s and 72° for 1 min, followed by one cycle at 72° for 3 min using an iCycler (BioRad Laboratories Ltd, Hemel Hempstead, UK). The amplified products were analysed by electrophoresis through 2% agarose (Sigma-Aldrich Company Ltd, Poole, UK) gels in 0·5× Tris-borate-EDTA buffer at 50 mA for 1 hr, and products were visualized by staining with ethidium bromide (BioRad Laboratories Ltd). PCR-amplified products were either directly sequenced or cloned into pCR 2.1 TOPO vector (Invitrogen Ltd. Paisley, UK) prior to sequencing, according to the manufacturer's instructions. Sequencing was carried out using capillary electrophoresis on the CEQ 8000 sequencer according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA).

Quantitative analysis of cytokine mRNA

The mRNA levels of chicken IL-1β, IL-6, IL-8, IFN-α, IFN-β and 28S ribosomal RNA were quantified by real-time RT-PCR using the RT qPCR mastermix kit (Oswell Research Products Ltd Southampton, UK). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE applied Biosystems, Warrington, UK) as previously described.[36,37]

Cells were exposed to R848, loxoribine, Poly(U) RNA, ssRNA 40, ssRNA 33, Poly(I:C) [all ligands purchased from Invivogen (AutogenBioclear, Calne, Wiltshire, UK)], or media alone, for 6 or 24 hr and RNA was extracted using the RNeasy kit (Qiagen Ltd) as indicated above. Primers and probes for ch28S, chIL-1β, chIL-6, chIL-8, chIFN-α and chIFN-β mRNA used in TaqMan have been described previously.[36,38–40]

Table 1. PCR primers

Product	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
avTLR7 (III)	GCCTCAAGGAAGTCCCCAGA	AAGAAACATTGCATGGATTACGG
avTLR7 (III)[1]	GCCTCAAGGAAGTCCCCAGA	CATTATCTTTGGGCCCCAGTC
chTLR7 (II–III)	GCCACTCCTCTCTACCGGATCT	CCCAAGAAACATTGCATGGATTACGG
moTLR7 (II–III)	TCCTCCACCAGACCTCTTGA	AAAGACTCTAGAAACTAAGAGCA
huTLR7 (II–III)	CCAGACCTCTACATTCCATTTTGG	GCAGTCCACGATCACATGGT
avTLR8-chCR1	TCCCATCTGGGAAAAACTTAGC	CCTGAATCTCCTCTGGCACAA
avTLR8-chCR1[1]	TCCCATCTGGGAAAAACTTAGC	TCTGAAAATGGCAGCACAAAA
chβ-actin	TGCTGTGTTCCCATCTATCG	TTGGTGACAATACCGTGTTCA
chMyD88(II–IV)	GCCAATGGGGAAGCGAATA	CAATCGCCCATTTGAACACA
chTRAF6(VI–X)	GTCCCAACAAAGGCTGCTGT	TTCCCTGCGAAGGCTGTTTA
chIRF7 (IV–X)	CCCCAATGACAGAGGTTCTGG	AGGCTGACGTTGCCACTGTT
chTRIF	AAACCCACTGGAGCGGAGTC	CCTCACTGTCCTGTGGACCTG

Accession numbers; moTLR7 NM 133211, huTLR7 NM 016562, β-actin L08165.

[1]Hemi-nested PCR, av, avian.

Exon boundaries are shown in brackets after the product. ChMyD88, chTRAF6, chIRF7 and chTRIF were predicted from the chicken ENSEMBL genome sequence (http://www.ensembl.org/).

Calculation of fold changes in cytokine mRNA

Fold differences (R) in expression of cytokine genes between samples (A and B), each relative to the reference ribosomal RNA, were calculated from the C_T values C (for the cytokine) and C′ (for ribosomal RNA) using the equation $\ln R_{(A/B)} = [(C_A - C_B)/S] - [(C'_A - C'_B)/S']$, where S and S′ are, respectively, the slopes of plots of C_T value against the natural logarithm of concentration for serial 10-fold dilutions of cytokine DNA and ribosomal RNA, assayed on the same plate. This calculation avoids assumptions about the efficiency of the PCR amplifications and reduces to the common $\Delta\Delta C_T$ method in the case that both have perfect efficiency.

5′-RACE

A full-length sequence of TLR7 was obtained using the 5′-RACE kit (Roche Diagnostics GmbH, Roche Applied Science Nonnenwald 2, 82372 Penzberg, Germany). Briefly, 5′ cDNA was generated from 300 ng mRNA extracted from chicken spleen (Line 7_2). 5′-RACE was performed according to the manufacturer's instructions using an antisense gene-specific primer (5′-CATTATC TTTGGGCCCCAGTC-3′ and a second nested antisense gene-specific primer (5′-TGCATTTGACGTCCTTGCAT-3′). The fragments generated by 5′-RACE were gel-purified, subcloned into the pCR2.1-TOPO® vector (Invitrogen Ltd) and sequenced using capillary electrophoresis on the CEQ 8000 sequencer according to the manufacturer's instructions (Beckman Coulter).

Results

The chicken genomic region encompassing TLR7/TLR8 displays conserved synteny with mammals, but lacks a coding TLR8 gene

An EST (AJ393983) was identified with predicted amino acid sequence homology to huTLR7 and used to isolate three BACs, which were mapped onto chicken chromosomal spreads using FISH. ChTLR7 was positioned in the region 1q14-1q31 on chromosome 1, known to contain genes (OTC, ZFX) that lie close to TLR7 on the human and mouse X chromosomes. Analysis of the BAC sequence using the NIX program indicated gene matches in both the protein and DNA databases, in particular to human and rat phosophoribosyl pyrophosphate synthetase II (PRPS2), the hypothetical human protein KIAA0316 (derived from an annotated genomic sequence supported by EST), huTLR7, fragments with homology to huTLR8 and the downstream gene thymosin, beta 4, X-linked (TMB4SX) (Fig. 1a).

All of the coding sequence for mammalian TLR7 except for the initiating methionine lies within a single exon (exon III in man and mouse), which was also the case for the putative chTLR7. The predicted chTLR7 amino acid sequence exhibited 62% identity to huTLR7 (Table 2). In mouse and man, the 5′ untranslated region (UTR) and initiating methionine are located in two upstream exons (I and II) and 5′-RACE was used to identify this sequence in splenic cDNA before mapping onto our genomic sequence. The results indicated a single exon [157 base pairs (bp)], larger than exon II in huTLR7 (101 bp) but did not extend to include any further sequence that might be equivalent to the small exon I in human (37 bp) or mouse (92 bp).

No matches for an intact putative TLR8 open reading frame (ORF) were identified in the BAC-derived chicken genomic DNA but a series of TLR8-like sequences were identified ranging in size from 26 bp to 249 bp, interrupted by a 6·1-kb insertion containing stop codons in the TLR8-like reading frame. The 6·1-kb insertion included a segment with homology to a viral reverse transcriptase gene from the mobile element, chicken repeat 1 (CR1).[41] The entire chTLR7/8 genomic region was condensed compared with mammals, including both intronic and intergenic distances (Fig. 1) with 61 kb and 151 kb between the two flanking genes PRPS2 and TMB4SX of chickens and humans, respectively.

The phylogenetic distribution of the chTLR8-CR1 disruption was analysed using a hemi-nested PCR approach on genomic DNA from a wide range of bird species and 10 inbred lines of White Leghorn chicken. For this, primers were designed to amplify a fragment extending from the chTLR8 match to a CR1 region. The identities of PCR products were confirmed by end sequence. All of the inbred lines of chicken shared the disruption at the TLR8 locus (Fig. 2a). More interestingly, all other galliform bird species tested were also positive for the TLR8-CR1 disruption, including red jungle fowl, guinea fowl, Japanese quail, pheasant and turkey (Fig. 2b). The size of the hemi-nested genomic PCR product was consistent (1·2 kb) with all of the galliform species except Japanese quail. The nucleotide sequence of the PCR products confirmed that these were derived from the expected genomic region. There was no evidence for the CR1-TLR8 disruption in non-galliform bird species although with all DNA samples it was possible to detect a positive signal from TLR7 (Fig. 2b). The identities of the non-galliform TLR7 products were each confirmed by sequence analysis (data not shown).

The domain organization of chicken and mammalian TLR7 is broadly conserved

The translated amino acid sequence of chicken TLR7 contains features common to Toll-like receptors, namely a transmembrane domain, an intracellular domain and multiple extracellular leucine-rich repeats (LRRs) (Figs 3

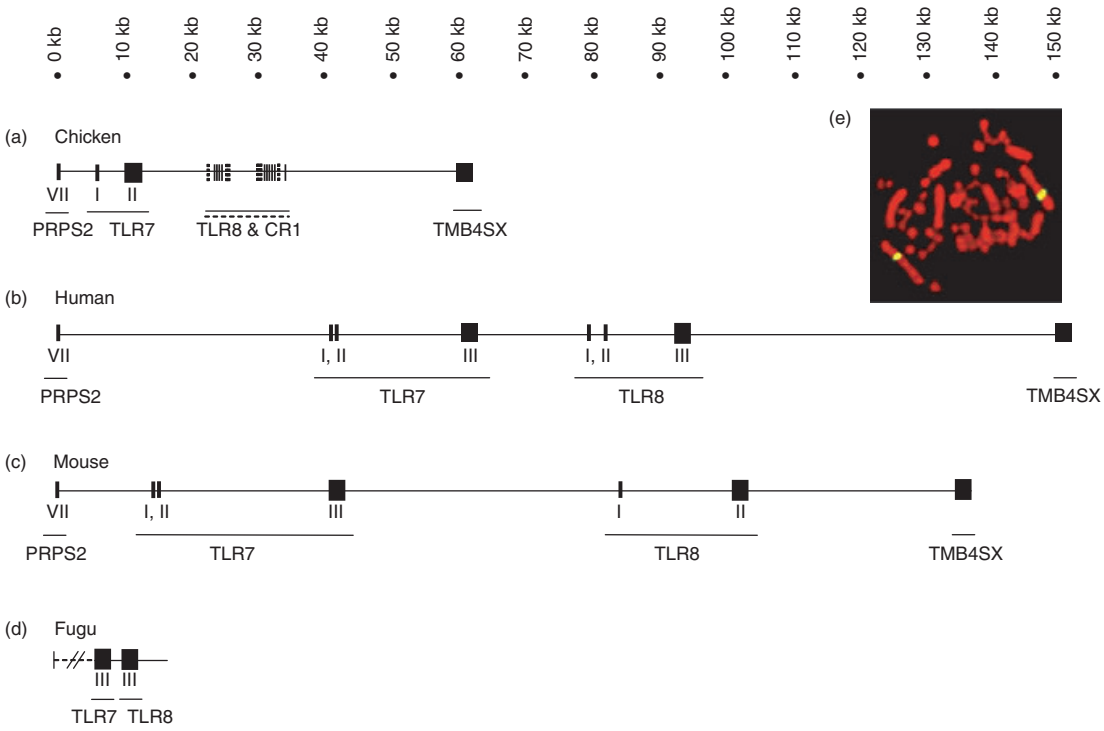

Figure 1. Comparative genomic synteny of TLR7 and TLR8 in chicken (a), human (b), mouse (c) and fugu (d) genomes drawn to scale and FISH mapping of TLR7 (e). Genomic gene distances for human and mouse were taken from the NCBI website (MAPVIEWER) and Fugu from the original citatation.[48] For the Fugu TLR7 and TLR8 region no syntenic flanking genes were evident in the Fugu Ensemble genome database. (e), Representative metaphase illustrating the hybridization of TLR7-containing BAC 404 to chicken chromosome 1. TLR, Toll-like receptor; CR1, chicken repeat one (indicated as a dashed line in the figure above); TMB4SX, thymosin beta 4 X-linked; PRPS2, phosophoribosyl pyrophosphate synthetase II. Exon numbers are indicated by roman numerals.

Table 2. Comparison of amino acid identities and similarities of chicken TLR7 domains with human TLR domains

	Chicken TLR7			
	LRR and TIR		TIR	
Human TLR	% Ident.	% Simil.	% Ident.	% Simil.
TLR 1	23	39	34	53
TLR 2	25	40	39	58
TLR 3	24	42	28	51
TLR 4	20	34	30	52
TLR 5	22	38	25	49
TLR 6	24	42	33	53
TLR 7	**62**	**76**	**72**	**84**
TLR 8	40	56	56	74
TLR 9	35	51	47	66
TLR 10	24	40	34	52

Ident., identity; Simil., similarity.
Highest percentage amino acid identity for chicken TLR7 is highlighted in bold.

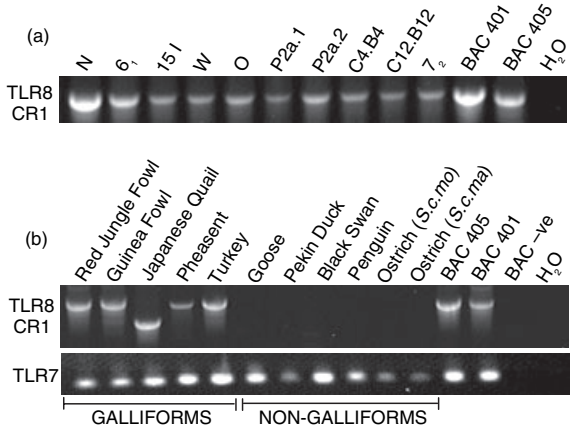

Figure 2. Hemi-nested PCR analysis of the TLR8-CR1 and TLR7 region in various inbred chicken lines (a) and bird species (b). Positive control indicates BACs (405 and 401) which were known to contain chTLR8-CR1 and chTLR7 and negative control shows BAC 404 lacking chTLR8-CR1 and BAC 271 lacking chTLR7. Products were analysed by 1% agarose gel electrophoresis. S.c.mo, *Struthio camelus molybdophane*; S.c.ma, *Struthio camelus massaicus*.

and 4). Chicken TLR7 is highly conserved when compared to mammalian TLR7 and displayed 62% overall amino acid identity with huTLR7 and 72% identity within the TIR domain (Table 2). Alignment of chTLR7 with human and mouse TLR7 illustrated that the conserved proline found at position 712 in the mouse, which

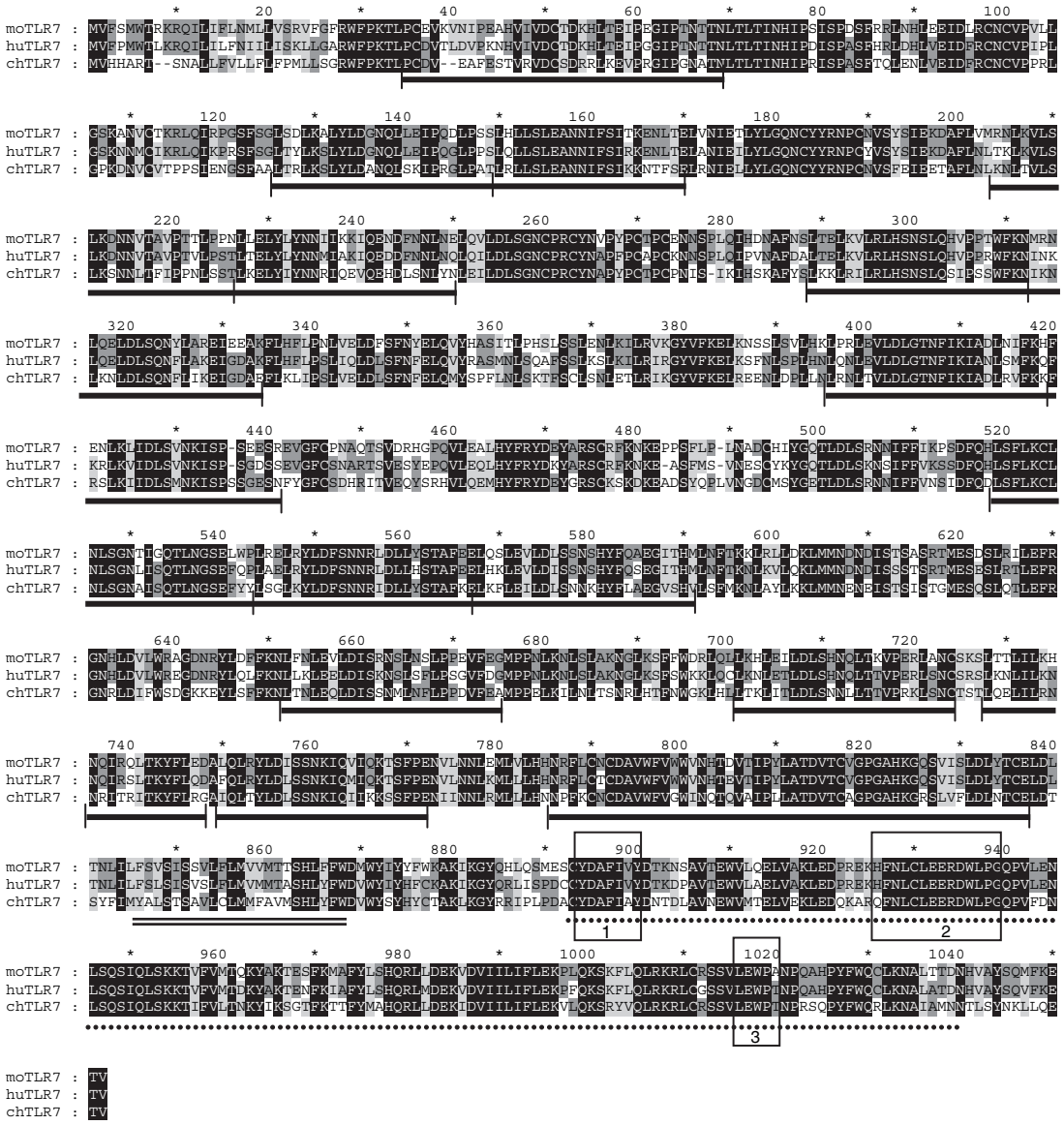

Figure 3. Amino acid sequence alignment of chicken TLR7 protein with human and mouse TLR7 proteins: Accession numbers; chTLR7 (AJ632302), huTLR7 (NM 016562) and moTLR7 (NM133211). Alignment was performed using the CLUSTAL W program and edited in the GENEDOC program, shading was performed using the conserved mode (black, dark grey, light grey shading set to 100%, 80% and 60% amino acid conservation, respectively). The transmembrane domain region is shown by double line, the TIR domain by dotted line and the leucine-rich repeats in chTLR7 by a single line. The three boxes; box 1 (FDAFISY), box 2 (GYKLC-RD-PG) and box 3 (a conserved W surrounded by basic residues) refer to three short consensus motifs defined for the IL-1 receptor family.

is known to be important in signalling, is also present in the chicken (Fig. 3). There was also high conservation of the three subsections of the TIR domain (boxes 1, 2 and 3) known to be important in signalling (boxes 1 and 2) and receptor localization (box 3).[42] The domain structures of chTLR7, huTLR7, murine (mo) TLR7 and fuguTLR7 (fuTLR7) were predicted using the web-based software program, SMART (Simple Modular Architectural Research Tool). This analysis indicated well-conserved groupings of multiple LRRs which are known to be important in ligand recognition (Fig. 4). Although TLRs from different species had a distinct arrangement of LRRs, they were all, including chTLR7, much more similar to each other than to other TLRs. However, with each species TLR7 the precise positioning of some of the LRRs was distinct, for example at the sub-N-terminal position in the chicken. Other domains with high inter-species conservation included the N- and C-terminal cysteine-flanked LRRs, the transmembrane and the TIR domains (Fig. 4).

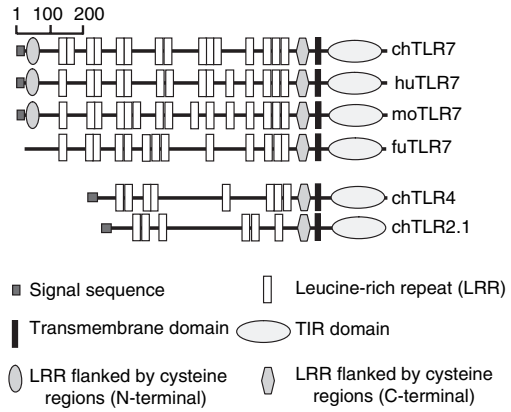

Figure 4. Comparative domain organization of chTLR7 with, human, mouse and fugu TLR7. The domain organization of each TLR was predicted using the SMART software analysis tool. Note that fuTLR7 sequence is missing the initiating methionine and 5′ sequence. The domain organization for chTLR4 and chTLR2·1 is also given to highlight the specificity of the predictions. The scale bar indicates amino acids. TIR, Toll-IL-1-like receptor signalling domain.

TLR7 is alternatively spliced in chicken, mouse and human

TLR7 mRNA expression was analysed using RT-PCR on spleen cDNA using a forward primer designed in exon II and a reverse primer in exon III, which amplified an expected product of 682 bp and two unexpected products of approximately 800 bp and 850 bp. All of the RT-PCR products were confirmed as TLR7 by sequence analysis although the larger products included an additional sequence of either 107 or 149 bp. These sequences were mapped onto the BAC-derived chicken genomic sequence to reveal an extra exon approximately halfway between exon II and exon III, flanked by the appropriate splice acceptor and splice donor sites (AG and GT nucleotides, respectively). Interestingly, the smaller of the additional sequences was identical to the 3′ two-thirds of the larger fragment. These additional exons will be referred to as exon IIA (149 bp) and exon IIA′ (107 bp). Examination of exon IIA and IIA′ sequence revealed that both were largely non-coding except for six amino acids at the 5′ terminus in frame 3. This small coding region included a potential alternative initiating methionine (Fig. 5).

Following the unexpected identification of alternative splice variants in chTLR7, similar experiments were performed with mouse spleen and human B-cell cDNA. This analysis revealed that both mouse and huTLR7 had similar, small alternatively spliced exons located between exon II and exon III. Mouse TLR7 had two alternatively spliced exons, that we have termed exon IIa (81 bp) and exon IIb (68 bp) situated near to exon III, which could be alternatively spliced in all configurations. Neither exon had a large ORF but both did include potential

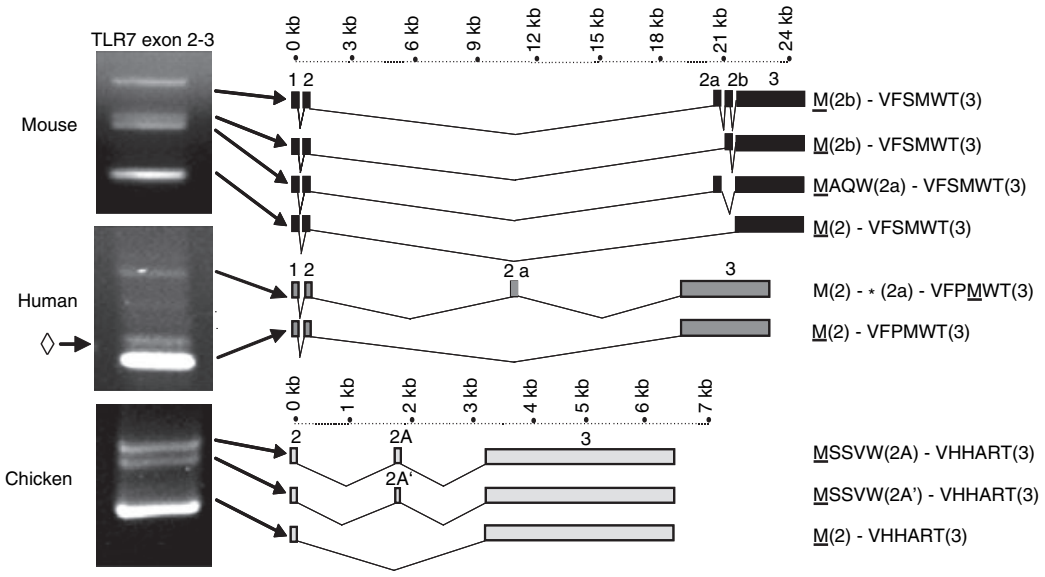

Figure 5. Schematic representation of the TLR7 splice variants identified in mouse, human and chicken. Left side; RT-PCR from exon 2 to exon 3 in mouse, human and chicken. The smallest band on each gel encoded TLR7 without any novel alternatively spliced exon. Products were analysed by 2% agarose gel electrophoresis. Arrows indicate the corresponding band and its exon pattern. The ◇ symbol indicates an uncloned band. Middle centre; splice variants in moTLR7 (top, black), huTLR7 (middle, dark grey) and chTLR7 (bottom, light grey), all drawn to scale. Right side; deduced amino acid sequences for the N terminus of the various alternative splice forms, and the non-spliced forms of TLR7. Known and hypothesized initiating methionines are underlined. Exon numbers are given in brackets. Note huTLR7·2a was noncoding, denoted with an * symbol, alternatively this transcript could start from the next 3′ methionine which is underlined. EMBL accession numbers for the nucleotide sequences of these alternative splice forms; moTLR.2a, AJ812652; moTLR7·2b, AJ812653; moTLR7·2a and 2b, AJ812654; huTLR7·2a, AJ812651; chTLR7·2A, AJ627561; chTLR7·2A′; AJ627562.

alternative initiating methionines situated at the 3′ end of the exon (exon IIa MAQW and exon IIb M). Human TLR7 also had an alternatively spliced exon (123 bp) approximately halfway between exon II and exon III (similar to chTLR7.exon IIA); however, no in-frame ORF was identified and no alternative initiating methionine could be identified.

The initiating methionine and surrounding sequence identified in these novel chicken and mouse TLR7 splice variants, along with the known mammalian TLR7 variants, were analysed for the presence of a Kozak sequence[43] for translation initiation. However this revealed weak conservation of the Kozak sequence motif, even for the known TLR7 transcripts.

Expression patterns of chTLR7 mRNA

All three splice variants of chicken TLR7 mRNA were expressed at the highest level in lymphoid-associated tissues (spleen, bursa, caecal tonsils), at a lower level in gut-associated tissues (small intestine, caecum, colon) and very weakly in other tissues (thymus, bone marrow, heart, lung, liver, kidney). No products were detected in the brain, muscle, testes, or skin (Fig. 6a).

A range of cell populations were examined *ex vivo* and the TLR7 RT-PCR products (all three isoforms) were most intense with splenic Bu1 + B cells (Fig. 6c).

Low levels of chTLR7 mRNA were detected by RT-PCR of blood-derived monocytes/macrophages, heterophils (avian polymorphonuclear cells) and magnetically sorted splenic TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T-cell populations (Fig. 6b).

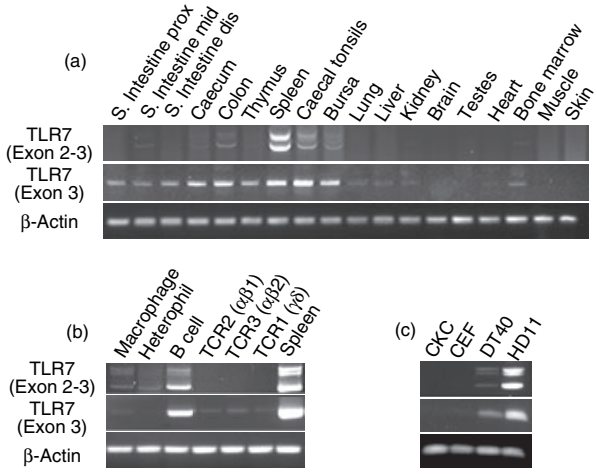

Figure 6. Expression of TLR7 mRNA in chicken tissues, immune cell populations and cultured cells. Each mRNA sample was normalized against β-actin expression levels. All RNA samples were free of genomic contamination because of lack of signal from non-reverse transcribed RNA (data not shown). Spleen in (b) represents positive control. CKC, chicken kidney cells; CEF, chicken embryo fibroblasts.

ChTLR7 mRNA was detected in the HD11 chicken cell line (macrophage-like) and the bursal cell line DT40 (bursal B cell-like) but not in predominately fibroblast CEF or in predominately epithelial CKC cultures.

ChTLR7$^+$ cells respond to agonists of mammalian TLR7 with selective up-regulation of cytokine mRNAs

The response of chTLR7$^+$ (HD-11) and chTLR7$^-$ (CKC) cultured cells to R848 and loxoribine, was assessed by real-time quantitative RT-PCR for chIL-1β or IL-6 mRNA (Fig. 7). IL-1β mRNA in HD11 cells was increased 8·9-fold by treatment with R848 (2·8 μM), and 4·8-fold by treatment with loxoribine (100 μM), compared with

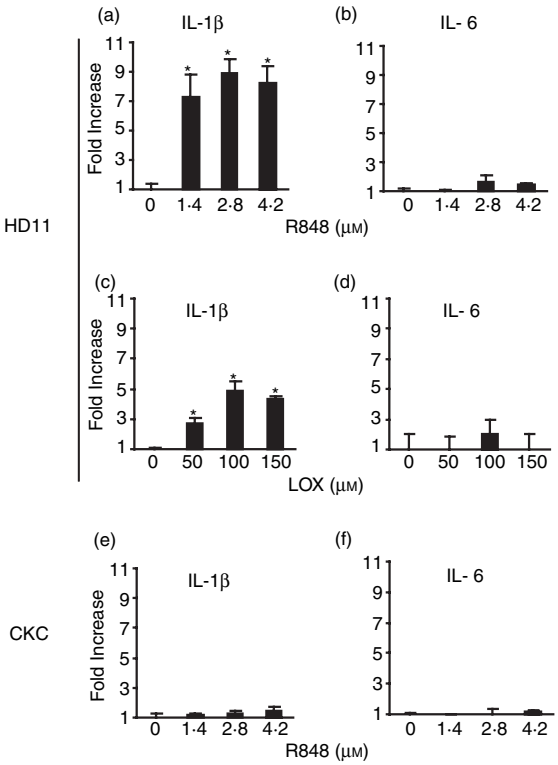

Figure 7. Real-time quantification of IL-6 and IL-1β mRNA extracted from HD-11 cells and CKC, stimulated with R848 or loxoribine. HD11 cells or CKCs were stimulated with R848 or loxoribine for 6 hr over a range of concentrations. RNA was extracted from cells and real-time quantitative RT-PCR was used to determine the levels of cytokine and 28S mRNA in the samples. The 28S mRNA levels were used to normalize the cytokine mRNA levels between the wells and the data are shown as mean relative fold increase, compared with mock-treated wells. * shows statistically significant difference ($P < 0{\cdot}05$) between specific TLR7 ligand treated and mock-treated cultured cells. The error bars show standard error for triplicate samples. (a) IL-1β from HD11 R848-treated cells; (b) IL-6 from HD11 R848-treated cells; (c) IL-1β from HD11 loxoribine-treated cells; (d) IL-6 from HD11 loxoribine-treated cells; (e) IL-1β from CKC R848-treated cells; (f) IL-6 from CKC R848-treated cells.

mock-treated HD11 cells. In contrast, neither R848 nor loxoribine stimulated significant changes in IL-6 mRNA levels in HD11 cells. Exposure of TLR7-negative CKC cells to R848 or loxoribine did not significantly alter levels of IL-1β or IL-6 mRNA (Fig. 7). An examination of the dose–response of HD11 cells to R848 and loxoribine revealed the expected dose dependency of the IL-1β mRNA up-regulation (data not shown).

Similar RT-PCR analyses were performed with freshly isolated chicken splenocytes from 3-month-old, line 6_1 chickens and the range of TLR7 agonists was broadened to include polyuridine [Poly(U)] oligonucleotide complexed with LyoVec™, ssRNA/LyoVec (ssRNA 33 and ssRNA 40) and the TLR3 agonist polyriboinosinic polyribocytidylic acid [poly(I:C)], a known stimulator of chicken type I IFN[44] and a putative chTLR3 agonist. Exposure of splenocytes to each of the TLR7 ligands induced significant increases in the level of IL-1β, IL-6 and IL-8 mRNAs compared with mock-treated splenocytes (Figs 8a,b). Each of the TLR7 agonists were used at concentrations similar to the levels used with mammalian cells.[4,6,14] The synthetic antiviral compound R848 was the most potent of the ligands, inducing 8·1-fold, 4·8-fold and 5-fold increases in IL-1β, IL-6 and IL-8 mRNAs, respectively. The single-stranded poly(U)/LyoVec RNA (1 μg/ml) stimulated 4·1-fold, 3-fold and 3-fold increases in IL-1β, IL-6 and IL-8 mRNAs, respectively. The least effective of the recognized mammalian TLR7 agonists on chicken splenocytes were the ssRNA33 and ssRNA40 derived from virally encoded sequences[4] where small, but significant, increases in both IL-1β and IL-8 mRNAs were detected (Fig. 8). None of the known mammalian TLR7 agonists caused significant up-regulation of IFN-α or IFN-β mRNA levels compared with mock-treated splenocytes. In contrast, treatment of chicken splenocytes with the TLR3 agonist, poly(I:C) led to significant up-regulation of both IFN-α and IFN-β mRNA levels (Figs 8c,d).

In mammals TLR7 induction of IFN-α is dependent on MyD88, TRAF6 and IRF7.[45] Orthologues of these genes in the chicken were identified by sequence homology and conserved synteny and mRNA identified in splenocytes by RT-PCR (Fig. 9).

Mammalian TLR3, TLR7, TLR8 and TLR9 are known to signal from an intraendosomal location within the cell. The addition of chloroquine interferes with endosomal acidification and abrogates signalling via these TLR molecules.[6,46,47] The addition of 100 μM chloroquine to chicken splenocytes for 30 min prior to application of R848 completely abrogated the induction of increased IL-1β message (Fig. 10).

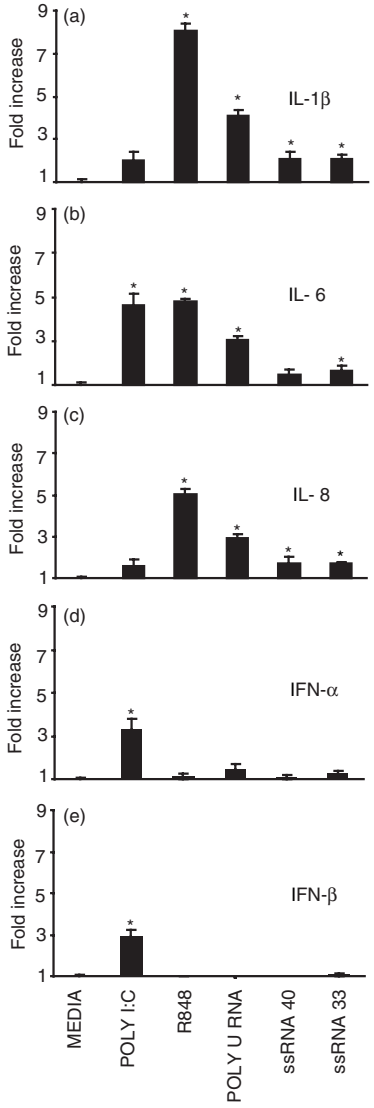

Figure 8. Expression of IL-1β (a), IL-6 (b), IL-8 (c), IFN-α (d) and IFN-β (e) mRNA extracted from chicken splenocytes, stimulated with RNA derivatives by real-time quantitative RT-PCR. Splenocytes from 3-month-old, Line 6_2 chickens were stimulated with either tissue culture medium alone or with poly(I:C) (25 μg/ml), R848 (1 μg/ml), poly(U) RNA (1 μg/ml), ssRNA 40 (1 μg/ml), ssRNA 33 (1 μg/ml) for 24 hr, followed by real-time quantitative RT-PCR. Fold change for mRNA levels was standardized to 28S levels. The error bars show standard error for quadruple samples. The difference between mRNA cytokine production in ligand-treated cells and media-treated cells was tested using the unpaired, two-tailed *t*-test (*, $P < 0{\cdot}05$).

Discussion

The TLR7/8 locus is a highly conserved genomic region in vertebrates with both genes represented in animals as divergent as fish[48,49] and mammals.[1] In humans, application of the synthetic antiviral compound, R848, leads to signalling through both TLR7 and TLR8 which led to the proposal that these TLR represent part of the virus-detecting pattern recognition receptor (PRR) system. We have characterized the chicken TLR7/8 genomic region in detail and identified an intact, transcriptionally active TLR7 gene but only remnants of a TLR8 gene which has

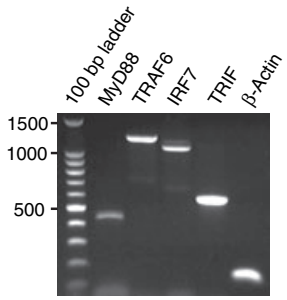

Figure 9. Expression of TLR7 and TLR3 signalling components in chicken splenocytes. RT-PCR was conducted across intron–exon boundaries where possible. All RNA samples were free of genomic contamination (data not shown). MyD88, myeloid differentiation factor; TRAF6, TNF-receptor-associated factor; IRF7, IFN regulatory factor 7; TRIF, TIR-domain-containing adaptor protein inducing IFN-β.

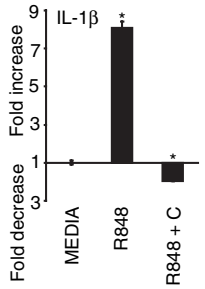

Figure 10. ChTLR7 signalling requires endosomal maturation. Expression of IL-1β mRNA extracted from chicken splenocytes (3-month-old, Line 6_2), stimulated with tissue culture medium alone, R848 (1 μg/ml), or R848 (1 μg/ml) pretreated with chloroquine (100 μM) 30 min before ligand stimulation, for 24 hr, followed by real-time quantitative RT-PCR. Fold change for mRNA levels was standardized to 28S levels. The error bars show standard error for quadruple samples. The difference between mRNA cytokine production in ligand-treated cells and media-treated cells was tested using the unpaired, two-tailed *t*-test (*, $P < 0{\cdot}05$).

stop codons in all three frames and a large 6·1-kb insertion that includes elements with homology to the CR1 retroviral-like insertion element. Nonetheless, the genomic organization of this region in the chicken exhibits conserved synteny of gene order with mammals, that extends beyond the tandemly arranged TLR7 and fragmented TLR8 to the flanking genes. With the chicken TLR7/8 region, both intronic and intergenic distances are smaller than those in this region of the mammalian genomes and are condensed by approximately 2·5-fold (commensurate with other regions of the chicken genome).[50]

The ancestor of the modern-day galliform birds diverged from other avian lineages about 90 million years ago.[51] The TLR8-CR1 insertion event was identified in galliform, but not non-galliform, birds, indicating that a major vertebrate lineage has entirely dispensed with the function of an ancestral TLR8 gene. In humans, TLR7 and TLR8 have been shown to exhibit differential agonist specificity and are expressed differentially in cell subsets[4,11,52] suggesting distinct function. However, in laboratory mice the TLR8 molecule does not respond to R848 or the huTLR8-specific ssRNA agonists and these PRR functions are mediated by moTLR7. Hence, the agonist specificity and function of the TLR7/8 molecules are different in different vertebrate species. The ssRNA33 and ssRNA40 motifs recognized by huTLR8 and moTLR7, respectively, are only weak agonists of chTLR7, which may indicate species-specificity in optimal agonist structure. The optimal nucleotide sequence for CpG motifs (a TLR9 agonist) has also been shown to be different in different vertebrate species.[53]

Several lines of evidence have been presented that the intact gene identified in these studies is the direct chicken orthologue of mammalian TLR7. Namely, genomic localization in a region exhibiting conserved synteny of the genome, the high conservation of amino acid sequence, conservation of predicted structural domains, the patterns of mRNA expression, and agonist reactivity. Nonetheless, it is clear that there are differences between the biology of chTLR7 and mammalian TLR7 molecules that are considered below.

As with mammalian TLR7 the coding sequence of chTLR7 lies mostly in a single large exon (exon III in mouse and human). With mammals the exon I and exon II encode the 5′ UTR and the initiating methionine;[2,3] our analysis of chTLR7 revealed an upstream exon that terminated with an initiating methionine. However, no further upstream exons could be defined and the chTLR7 'exon II' was longer (157 bp) than exon II in mouse (99 bp) or human (101 bp) TLR7. With mouse and huTLR7 exon I is 92 bp and 37 bp, respectively, and the genomic structure of chTLR7 'exon II' may represent a fusion of the first two mammalian exons. Our RT-PCR analysis using primers designed across the chTLR7 'exon II–exon III' boundary revealed two alternative splice forms for chTLR7. These variants led to the identification of a novel exon in the chicken that we have termed exon IIA. Three different chTLR7 transcripts can be described, one that does not include exon IIA, a second that includes the full exon IIA sequence and a third that includes a 3′-segment of exon IIA. Appropriate splice acceptor and donor signals were identified with the three chTLR7 sequences. Similar splice variants and alternative exons were identified with both mouse and huTLR7. In huTLR7 a single alternative exon was identified with a similar length to the chTLR7 exon IIA, whereas in moTLR7, two small novel exons were identified that were each approximately one-half the length of the chTLR7 exon IIA. The biological significance of these alternative forms of TLR7 mRNA remains to be determined. Analysis of the sequences of the splice variants

indicates the introduction of a longer 5′ UTR, a requirement for the use of an alternative initiating methionine and in some cases an altered signal sequence. No previous reports have identified alternative splicing of TLR7 but huTLR8 can be alternatively spliced into two transcripts (accession numbers NM016610; NM138636) which affect the N terminus of TLR8 and change the identity of the initiating AUG codon. Similarly, huTLR9, can be alternatively spliced into four isoforms by utilizing three small exons situated 5′ to the major coding exon.[3]

As reported for mammalian TLR7,[54] the expression of chTLR7 mRNA was most prevalent in tissues with significant lymphoid components, in particular the bursa of Fabricius, spleen and caecal tonsil. The bursa of Fabricius is a primary lymphoid organ, unique to birds, which is important in the development, diversification and amplification of antibody-producing cells.[55] Moreover, RT-PCR analysis of various cell subsets indicated that the highest levels of TLR7 mRNA were found within splenic B cells, with much lower levels on TCR-αβ and TCR-γδ T-cell subsets, macrophages and heterophils (the major avian polymorphonuclear cell type). ChTLR7 mRNA was also easily detected in the bursal B-cell-like DT40 cell line and at high levels in the HD11 macrophage-like cell line but was not detected in either CKC or CEF cultures, which are dominated by somatic cell types. Collectively, these results are comparable to huTLR7, which is highly expressed on B cells and plasmacytoid dendritic cells, is expressed at lower levels in monocytes, eosinophils, neutrophils, and at very low levels on natural killer and T cells.[52,56]

The LRR domains of the TLR are predicted to form a horseshoe structure[57] and it is clear that this region of the TLR molecule affords agonist specificity. With the chTLR7 molecule the pattern of LRR closely resembled the predicted mammalian LRR patterns, although there were some subtle differences including an extra sub-N terminal LRR. How these differences might translate into differences in ligand specificity is unclear but, as noted above, the fine specificity of TLR7 and TLR8 differs according to species and chickens respond poorly to the recently described viral-derived GU-rich ssRNA sequences. Nonetheless, the responses of fresh splenocytes and the HD11 cell line show that the chTLR7 agonists are similar to those described for mammals. The chloroquine sensitivity of the R848-induced response of chicken splenocytes supports the view that chTLR7 is dependent upon intraendosomal acidification as seen with mammalian TLR3, TLR7, TLR8 and TLR9.[6,46,47]

The effectiveness of TLR–agonist interactions is dependent upon the activation of various signalling pathways that lead to changes in cellular physiology, including the production of pro-inflammatory cytokines. To determine the effects of TLR7 agonists in the chicken we examined changes in expression of mRNA for various chicken cytokines. In mammals, the exposure of cells to R848, loxoribine, poly(U), ssRNA33 or ssRNA40 leads to increased production of IL-1β, IL-6, TNF-α, IL-12 and type I IFN.[4,11,14] With the HD11 TLR7$^+$ chicken macrophage-like cell line both R848 and loxoribine stimulated increased levels of IL1-β mRNA but not of IL-6 mRNA. Similar treatment of fresh splenocytes induced up-regulation of IL-1β and IL-6 mRNA, as did treatment with poly(U) and to a lesser degree ssRNA33 and ssRNA40. The discrepancy in the IL1-β/IL-6 results with HD11 cells and splenocytes is probably the result of the differential response of different cell types to the TLR7 agonists as has been described for dendritic cell subsets in mammals.[58]

Agonist-induced up-regulation of type I IFN is a well-documented effect of signalling via the TLRs implicated in recognition of viruses, including TLR3, TLR4, TLR7 and TLR8.[4,14,59] Hence, we were surprised at the ineffectiveness of a wide range of TLR7/8 agonists in the up-regulation of chIFN-α or chIFN-β mRNA. This observation does not appear to be related to the assay timing because R848 and loxoribine also failed to stimulate up-regulation of chIFN-α or chIFN-β mRNA at 1, 6, or 24 hr post-application onto chicken splenocytes or the HD11 cell line (data not shown). The poly(I:C)-induced up-regulation of both IFN-α and IFN-β mRNA at 24 hr indicates an intact TLR-dependent pathway of type I IFN induction in chicken splenocytes. Interestingly, poly(I:C) exposure induced up-regulation of chIL-6 mRNA but not chIL-1β or chIL-8 mRNA. Collectively, these data demonstrate that signalling via different chTLR molecules leads to differential induction of chicken cytokine mRNA.

The imidazoquinolinamine, S-28828 has been reported to induce type I IFN in chickens and chicken splenocytes.[60] The structural relatedness of S-28828 to other synthetic TLR7 agonists suggests that this may be mediated via TLR7 but we did not observe any induction of IFN-α or IFN-β mRNA with a broad range of TLR7 agonists. However, the S28828 induced IFN was characterized by bioassay and the activity may be from non-α/β type I IFN. The primers and probe used in our studies would have amplified product from all of the published chIFN-α or chIFN-β sequences[61–63] but we cannot exclude the possibility that other chIFN-α/β RNAs were not detected. The differences may also be related to the chicken Line, although we obtained identical results with two different inbred Lines of chicken (Line C and Line N; unpublished data). It is also possible that different TLR7 agonists may differ in their capacity to induce type I IFN in the chicken.

The failure of a broad range of TLR7 agonists to induce up-regulation of IFN-α or IFN-β mRNA may be peculiar to the cell types tested in this study. However, similar

exposure of human peripheral blood lymphocytes to R848 led to increases in production of IFN-α and IFN-β.[11] TLR7 agonist-induced up-regulation of IL-1β, IL-6 and IL-8 clearly indicates that chTLR7 is signalling proficient, but it is possible that the chTLR7 TIR domain does not recruit the appropriate adaptor molecule for IFN up-regulation. With mammalian TLR7, induction of type I IFN is reported to be dependent upon MyD88, TRAF6 and IRF7, which are all detectable by RT-PCR in chicken splenocytes. Also the TLR7 TIR box amino acids that serve as adaptor contact sites are conserved between mammals and chickens. Only two amino acid substitutions are present in the chTLR7 TIR boxes compared with mammalian TLR7 molecules, valine-alanine (box 1, position 900) and a histidine-glutamine (box 2, position 927). In mammals, the TRIF adaptor molecule is implicated in strong type I IFN up-regulation[10] and the TRIF gene can be identified in the *Gallus gallus* genome (chromosome 28) in a region with conserved synteny to mammalian genomes. Moreover, in mammals, TLR3 signals exclusively via the TRIF adaptor and our poly(I:C) results indicate intact TLR3-mediated up-regulation of chIFN-α and chIFN-β mRNA. Hence, none of these explanations is satisfactory for the differences between chTLR7-mediated and mammalian TLR7-mediated cytokine induction. Nonetheless, the lack of IFN-α/β may significantly impact on the induction of avian antiviral immune mechanisms via TLR7.

The genomic and functional data we have presented with chTLR7/8 illustrate the evolutionarily dynamic nature of the TLR repertoire that remains under constant pressure from different pathogens. Further examples of species-specific variation in the TLR repertoire is evident in the literature; including those between mouse and man (TLR7/8, TLR10, TLR11 and TLR12,13[4,64,65]), between mammals and fish (TLR21 and TLR22 in Zebrafish and Fugu[48,49,66]) and chickens (TLR2[18] and TLR7/8, this paper). The diversity in cytokine response of chickens and mammals after exposure to TLR7 agonists adds a further level of complexity. These differences in TLR repertoire and TLR signalling may influence the patterns of host susceptibility to infections (e.g. influenza) and the immune mechanisms responsible for control of infectious challenge in different host species. Practical implications include the development of suitable adjuvant systems for use in farm animals and indicate the importance of functional characterization of TLR in the target species.

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