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Identification, Cloning, and Functional Characterization of the IL-1 Receptor Antagonist in the Chicken Reveal Important Differences between the Chicken and Mammals

Mark S. Gibson,* Mark Fife,* Steve Bird,† Nigel Salmon,* and Pete Kaiser* †

The human IL-1 family contains 11 genes encoded at three separate loci. Nine, including IL-1R antagonist (IL-1RN), are present at a single locus on chromosome 2, whereas IL-18 and IL-33 lie on chromosomes 11 and 9, respectively. There are currently only two known orthologs in the chicken, IL-1β and IL-18, which are encoded on chromosomes 22 and 24, respectively. Two novel chicken IL-1 family sequences were identified from expressed sequence tag libraries, representing secretory and intracellular (icIL-1RN) structural variants of the IL-1RN gene, as seen in mammals. Two further putative splice variants (SVs) of both chicken IL-1RN (chIL-1RN) structural variants were also isolated. Alternative splicing of human icIL-1RN gives three different transcripts; there are no known SVs for human secretory IL-1RN. The chicken icIL-1RN SVs differ from those found in human icIL-1RN in terms of the rearrangements involved. In mammals, IL-1RN inhibits IL-1 activity by physically occupying the IL-1 type I receptor. Both full-length structural variants of chIL-1RN exhibited biological activity similar to their mammalian orthologs in a macrophage cell line bioassay. The four SVs, however, were not biologically active. The chicken IL-1 family is more fragmented in the genome than those of mammals, particularly in that the large multigene locus seen in mammals is absent. This suggests differential evolution of the family since the divergence of birds and mammals from a common ancestor, and makes determination of the full repertoire of chicken IL-1 family members more challenging.

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(13). Third, icIL-1RN isoforms may be released from cells and act in a similar way to sIL-1RN by antagonizing membrane-bound IL-1R1 (14–17).

Other, more recently discovered IL-1 family members (IL-1F5–F11) have been less extensively studied. However, functional roles for most of these are beginning to emerge. IL-1F5 (recently renamed IL-36Ra) (18) and IL-1F10 (recently renamed IL-38) (18) suppress inflammation through their common role as receptor antagonists of IL-1R2 (IL-1Rrp2). This prevents the agonists IL-1F6 (IL-36α), IL-1F8 (IL-36β), and IL-1F9 (IL-36y) from binding this receptor to initiate gene transcription via NF-κB and MAPKs (1, 19, 20). IL-1F7 (IL-37) has also been comprehensively characterized as an endogenous inhibitor of the innate immune response (21). The role of IL-1F11 (IL-33) has been described in numerous disease states, central to which is its ability to generate Th2-type immune responses (22).

In humans, nine of the IL-1 genes are clustered in a region of ~370 kb on chromosome 2q13. The two other members, IL-18 and IL-1F11 (IL-33), reside on chromosomes 11 and 9, respectively. The IL-1 cluster is largely conserved across most mammalian species except in the mouse, where IL-1β and IL-1α have become separated from the rest of the cluster (IL-1RN, IL-1F5–F10) following chromosomal rearrangement. The chicken has been extensively studied as a model organism for immune function. Although our understanding of its immunobiology lags behind mammalian species, significant progress has been made over the past decade to elucidate its repertoire of immune function genes (23). In particular, our knowledge of its different cytokine families has grown rapidly, accelerated by the availability of the genome sequence (24).

At present, only two members of the chicken IL-1 family have been cloned and functionally characterized: IL-1β (25) and IL-18 (26). The biological activity of both cytokines resembles that of their mammalian orthologs. Despite the apparent lack of IL-1 ligands, all members of the mammalian IL-1R family have been identified in the chicken (our unpublished observations). This suggests that the chicken may contain further, as yet undiscovered, ligand genes. The current build of the chicken genome sequence does not provide any evidence of additional IL-1 genes, as the nine-member IL-1 cluster found on human chromosome 2 is not present. A limited degree of conserved synteny between these two species at this locus, however, does exist. The region of the chicken genome containing IL-1B includes orthologs of the two genes (SLC20A1 and CKAPL2) that flank the human IL-1 cluster, but all other IL-1 genes are absent.

In this study we report the discovery and characterization of IL-1RN for the first time, to our knowledge, in an avian species.

Materials and Methods

Identification of chicken IL-1 family members

A search of the National Center for Biotechnology Information chicken genome resources expressed sequence tag (EST) database (http://www.ncbi.nlm.nih.gov/genome?term=gallus%20gallus) identified EST sequences that corresponded to putative chicken IL-1 orthologs. For all members of the human IL-1 family yet to be identified in the chicken, the full gene sequence, full amino acid sequence, and the amino acid signature motif were analyzed with the basic local alignment search tool (BLAST) against the chicken genome sequence using the Ensembl genome browser. The chicken IL-1 receptor family was identified by examining conserved synteny between the human IL-1R cluster on chromosome 2 and the chicken genome using the Ensembl genome browser.

Two EST sequences corresponding to novel chicken IL-1 family genes were translated and analyzed by TBLASTN against all eukaryotic animal genomes in Ensembl to confirm putative identities. Sequences (positive hits) from species containing orthologous genes were aligned using ClustalX v1.83 (27).

Following amplification and cloning, two novel chIL-1RN amino acid sequences were analyzed for the presence of a signal peptide using the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP) (28, 29). Novel chIL-1RN protein sequences were analyzed for structural similarity to known protein domains present in the ProDom database (http://prodom.prabi.fr/prodom/current/html/form.php). The chicken sequences were BLASTP queried against all families of protein domains. Phylogenetic analysis was carried out using MEGA v5.0 (30) with bootstrap analysis with 500 bootstrap datasets. The secondary structures of the chIL-1RN proteins were predicted using PSIPRED v3.0 (http://bioinf.cs.ucl.ac.uk/psipred/).

Cloning and sequencing of cDNA

Chicken icIL-1RN cDNA was amplified from RNA from HD11 cells (31) stimulated with LPS for 6 h, by one-step RT-PCR using sequence-specific primers (see Table I) and Ready-to-Go RT-PCR beads (GE Healthcare, Bucks, U.K.). Thermal cycling conditions were 42°C for 30 min, 95°C for 5 min, and 40 cycles of 95°C for 1 min, 60°C for 2 min, and 72°C for 2 min. Sequences specific to different splice variants of icIL-1RN cDNA were synthesized into a PCR product using 1 µl (200 U) SuperScript II (Invitrogen, Paisley, U.K.). Amplification of the cDNA encoding the mature peptide was conducted using sequence-specific primers (see Table I) and 0.625 U GoTaq DNA polymerase (Promega, Southampton, U.K.) in a 25 µl total volume. Thermal cycling conditions were 95°C for 5 min, 5 cycles of 95°C for 30 s, 68°C for 30 s (decrease by 1°C cycle), 72°C for 2 min, and 30 cycles of 95°C for 30 s, 63°C for 30 s, and 72°C for 2 min. Sequence-verified products were directionally cloned into the His-tagged expression vector pHSec (provided by James Birch, Institute for Animal Health; vector details are in Ref. 32) between AgeI and KpnI restriction sites. All splice variant clones were directionally subcloned into pcI-neo (Promega) using EcoRI and MluI restriction sites. The complete amplified cDNA sequences of both wild-type and all isoforms were submitted to the European Molecular Biology Laboratory Nucleotide Sequence Database (accession nos. HE601788–HE601793).

Chicken tissues and cells

Tissues were removed from 6- to 9-wk-old specific pathogen-free (SPF) line 72 chickens, specifically thymus, spleen, bursa of Fabricius, Harderian gland, caecal tonsil, Meckel’s diverticulum, bone marrow, brain, muscle, heart, liver, kidney, lung, and skin. Cell populations were either unstimulated, stimulated (splenocytes, 1 µg/ml Con A [Sigma-Aldrich, Gilgilingham, U.K.]; bursal cells, 500 ng/ml PMA [Sigma-Aldrich]; thymocytes, 25 µg/ml PHA [Sigma-Aldrich], all for 18 h), or separated into specific subsets (splenocytes). Lymphocyte subsets (CD4+, CD8a+, CD8b−, TCR1+, TCR2−, TCR3+, Bu−1, and KUL01+) were isolated from total splenocytes as previously described (33).

Bone marrow-derived dendritic cells, bone marrow-derived macrophages, and blood-derived monocytes were isolated from 4- to 8-wk-old SPF line 72 chickens and stimulated with LPS (200 ng/ml) or CD40L (3 μg/ml) for 1, 2, 4, 8, 12, 24, or 48 h as described (33). RNA from unstimulated and LPS-stimulated (10 μg/ml for 1 h) heterophils was a gift from Dr. Mike Kogut (U.S. Department of Agriculture, College Station, TX).

Six-week-old SPF Rhode Island Red chickens were orally challenged with F. × 108 CFU/IL-1RN/100 g of an avirulent strain of Nalr or Luria-Bertani medium (control). Birds were killed at 3, 7, 14, 21, and 27 d postinfection (dpi), and whole spleens were removed.

Three-week-old resistant (line 61) and susceptible (Brown Leghorn) SPF chickens were challenged intranasally with either 101.3 50% egg infectious dose of avirulent strain F98 Nalr or Luria-Bertani medium (control). Birds were killed at 3, 7, 14, 21, and 27 d postinfection (dpi), and whole spleens were removed.

Total RNA isolation and real-time quantitative RT-PCR analysis of chicken IL-1RN expression

RNA from the tissues and cells described above was extracted using an RNaseasy Mini kit (Qiagen, Crawley, U.K.) following the manufacturer’s instructions. TaqMan real-time quantitative RT-PCR (qRT-PCR) was used to quantify the mRNA levels of chicken IL-1RN. Primers and probes specific to different splice variants of chicken IL-1RN (Table I) were designed using Primer Express (Applied Biosystems, Warrington, U.K.). Assays were performed using the TaqMan Fast Universal PCR Master Mix and One-Step RT-PCR Master Mix reagents (Applied Biosystems). Data are expressed in terms of the cycle threshold (Ct) value, normalized for each sample using the Ct value of 28S rRNA product for the same sample, as described previously (34, 35). Final results are shown either as corrected ΔCt, using the normalized value, or as fold difference from levels in un-
Table I. Primers and probes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target for Amplification</th>
<th>Sequence (5’→3’)</th>
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</thead>
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<td>sIL-1RN F</td>
<td>sIL-1RN cDNA</td>
<td>ATGCGGGCTACCAACTGCGCTCCT</td>
</tr>
<tr>
<td>sIL-1RN R</td>
<td>sIL-1RN/cIL-1RN cDNA</td>
<td>GCTGAGCAACAGGCGGAAGTA</td>
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<tr>
<td>sIL-1RNpHL F</td>
<td>Mature sIL-1RN. Includes AgeI site</td>
<td>GAGAGAaaccggTGTGGGTGAGCGGCCG</td>
</tr>
<tr>
<td>sIL-1RNpHL R</td>
<td>Includes KpnI site</td>
<td>GAGAGaggtaccCACAGCTGAAAGTAGA</td>
</tr>
<tr>
<td>icIL-1RN F</td>
<td>icIL-1RN cDNA</td>
<td>GCGATCTGACTGAGGGAGG</td>
</tr>
<tr>
<td>icIL-1RNpHL F</td>
<td>Includes AgeI site</td>
<td>GAGAGAaaccggTGTGGGTGAGCGGCCG</td>
</tr>
<tr>
<td>icIL-1RNpHL R</td>
<td>Includes KpnI site</td>
<td>GAGAGaggtaccCACAGCTGAAAGTAGA</td>
</tr>
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<tr>
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<td>IL-1β</td>
<td>CTTTCATTCTACGTTCAGCAG</td>
</tr>
<tr>
<td>IL-1RN 3β2</td>
<td>IL-1β</td>
<td>GCAGCTTACACGTGGTCGTC</td>
</tr>
<tr>
<td>IL-1RN Int2–3 F</td>
<td>IL-1RN intron 2</td>
<td>GCAGCAACAAAGCTCGTCAC</td>
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<tr>
<td>IL-1RN Int2–3 R</td>
<td>IL-1RN intron 2</td>
<td>GGCAGATCGGCGAGCTGTTAC</td>
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<tr>
<td>IL-1RN Int3–4 F</td>
<td>IL-1RN intron 3</td>
<td>AAACGACGGCTGGCTAC</td>
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<tr>
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<td>IL-1RN intron 3</td>
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<tr>
<td>IL-1RN Int1–2 F</td>
<td>IL-1RN intron 4</td>
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<td>IL-1RN 130bp R</td>
<td>IL-1RN intron 4</td>
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<tr>
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<td>IL-1RN</td>
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<tr>
<td>pHLSec R</td>
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<td>TaqMan F primer</td>
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<td>TaqMan R primer</td>
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<td>TaqMan probe</td>
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<td>TaqMan F primer</td>
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<tr>
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<td>TaqMan R primer</td>
<td>GGCAGATCGGCGAGCTGTTAC</td>
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<tr>
<td>IL-1RN SV1 F</td>
<td>TaqMan probe</td>
<td>CCCTCTCTACGGACGAG</td>
</tr>
<tr>
<td>IL-1RN SV1 F</td>
<td>TaqMan probe</td>
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</tr>
<tr>
<td>IL-1RN SV2 R</td>
<td>TaqMan R primer</td>
<td>GAGAGTGGAGAGGAAGGACCAT</td>
</tr>
<tr>
<td>IL-1RN SV2 R</td>
<td>TaqMan R primer</td>
<td>CCGTCTCGAGAGCTGC</td>
</tr>
<tr>
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<td>TaqMan F primer</td>
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</tr>
<tr>
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<td>TaqMan R primer</td>
<td>TGCTGATGGTCCCGCAAGGA</td>
</tr>
<tr>
<td>IL-1RN 3β2</td>
<td>TaqMan probe</td>
<td>CCACAGCTGACTGAGGGAGGAGGCC</td>
</tr>
<tr>
<td>iNOS F</td>
<td>TaqMan F primer</td>
<td>TTGGAAACCAAAGTCTTCAAGG</td>
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<tr>
<td>iNOS R</td>
<td>TaqMan R primer</td>
<td>CCTCGGCCAATGCTTATG</td>
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<tr>
<td>iNO S</td>
<td>TaqMan probe</td>
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<td>TaqMan F primer</td>
<td>GCCAAGGGCAAGAGAAACT</td>
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<tr>
<td>28S R</td>
<td>TaqMan R primer</td>
<td>GCCAAGGGCAAGAGAAACT</td>
</tr>
<tr>
<td>28S</td>
<td>TaqMan probe</td>
<td>AGGACCAGCTACGCGACCTCCACCACCA</td>
</tr>
</tbody>
</table>

F, Forward; R, reverse.

infected control birds. Bioassay results are expressed as a percentage following conversion from corrected ΔCt values.

Structural analysis of the IL-1RN gene

For the amplification of introns, chicken IL-1 cDNA sequences were aligned with orthologous mammalian cDNA sequences (derived from Ensembl), enabling intron locations for the chicken genes to be predicted. Primers were designed against the known chicken cDNA sequences based on these predictions (Table I). The resulting chicken IL-1RN gene sequence was submitted to GenBank (accession no. HE608245). Putative promoter regions were analyzed using Softberry (http://linux1.softberry.com/berry.phtml). 5′ RACE was carried out using the SMARTer RACE cDNA amplification kit (Clontech) following the manufacturer’s instructions. 5′ RACE-ready cDNA was prepared from HD11 cells stimulated with LPS for 6 h. 5′ RACE PCR used a gene-specific reverse primer (Table I) and the supplied Universal Primer A Mix forward primer. Thermal cycling conditions were 5 cycles of 94°C for 30 s, 72°C for 3 min, 5 cycles of 94°C for 30 s, 70°C for 30 s, 72°C for 3 min, and 25 cycles of 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min.

Expression of recombinant protein in COS-7 and HEK293T cell lines

The different IL-1RN variants and isoforms were expressed in COS-7 cells (ex-COS) using a well-described DEAE-dextran transfection method (36, 37).

HEK293T cells were routinely cultured in DMEM with 10% FCS at 37°C, 5% CO2. For transfection, 50 μg endotoxin-free plasmid DNA (pHLSec-sIL-1RN or pHLSec-iIL-1RN) was added to 5 ml serum-free medium and mixed. Polyethyleneimine (75 μl of 1 mg/ml) was added, briefly mixed, and then incubated at room temperature for 10 min to permit DNA–polyethyleneimine association. During this incubation, HEK293T growth medium was changed for 2% serum-containing DMEM and the DNA-polyethyleneimine mixture was added. After 3 d growth at 37°C, 5% CO2, culture supernatants were harvested and concentrated in a stirred ultrafiltration cell using YM10 ultrafiltration membranes (NMWL 10000) (both from Millipore, Watford, U.K.) followed by a buffer exchange with PBS. Concentrated sIL-1RN and icIL-1RN proteins were purified under native conditions using HIS-Select high-flow cartridges (Sigma-Aldrich). Purified proteins were detected by SDS-PAGE using Mini-PROTEAN TGX gels (Bio-Rad, Hemel Hempstead, U.K.), followed by Western blotting using a PentaHis monoclonal primary Ab (Qiagen) and a polyclonal rabbit anti-mouse IgG HRP-conjugated secondary Ab (Dako, Ely, U.K.).

FIGURE 1. Amplification of full-length and splice variants of (A) icIL-1RN and (B) sIL-1RN cDNA. For both, two splice variants were cloned from the smaller bands (of ~420 and 450 bp, respectively). Templates for RT-PCR were RNA from Con A-stimulated splenocytes (S) or LPS-stimulated HD11 cells (M).
The signal was detected by ECL (GE Healthcare Life Sciences, Little Chalfont, U.K.).

Characterization of IL-1RN bioactivity

HD11 cells were cultured for 24 h at 41˚C, 5% CO₂, in the presence of either serial dilutions of recombinant chicken (rch) IL-1β (provided by Dr. Benjamin Schusser, University of Munich, Munich, Germany) with or without 30 μl anti–IL-1β polyclonal Ab (also provided by Dr. Schusser) or media alone. Activation of the HD11 cells was measured using a macrophage activation factor assay as previously described (36, 38). This assay measures activation through the induction of inducible NO synthase (iNOS), which produces nitrite in the culture medium, measured by a Griess assay. IL-1β and iNOS mRNA levels were quantified from RNA isolated from the same samples by qRT-PCR.

To determine the antagonistic properties of chIL-1RN, HD11 cells were cultured with either serial dilutions of rchIL-1RN (ex-COS; supernatant or cell lysate), purified rIL-1RN or ricIL-1RN, ex-COS pCI-neo (negative control), or media alone. After 4 h incubation at 41˚C, 5% CO₂, 250 μl 40 ng/ml recombinant chicken IL-1β (synthesized by AMSBio, Abingdon, U.K.) was added with further incubation for 12 h. Activation of the cells and IL-1β and iNOS mRNA expression levels were quantified as previously described.

Statistical analysis

Statistical analyses were carried out using the Mann–Whitney U test within the GraphPad Prism software package. Tests compared the rchIL-1β plus IL-1RN treatment groups to the rchIL-1β only treatment within the HD11 bioassay. For gene expression analyses, tests were performed between groups of different infection status. Statistical significance was determined as *p < 0.05 (significant) or **p < 0.01 (highly significant).

Results

Identification and characterization of chicken IL-1RN, its different variants, and alternatively spliced isoforms

A TBLASTN search of the National Center for Biotechnology Information EST database identified several chicken ESTs representing putative IL-1 family genes (Supplemental Table I). Two of these ESTs (accession nos. CK613932 and BX257557) were combined to create a 554-bp sequence with potential start and stop codons as well as a polyadenylation signal (AATAAA). The pre-determined sequence, TBLASTN analysis against all other genomes in NCBI, resulted in 17 hits with significant homology (data not shown).

A reciprocal BLASTP analysis of the National Center for Biotechnology Information EST database with chIL-1RN identified 10 further ESTs with significant homology (Supplemental Table I). One of these sequences (CK615408.1) was essentially identical to the original sequences. Another EST (BU214831.1) was similar but clearly differed at the 5’ end. This 669-bp sequence located 72 bp from the 5’ end of the exon. The predicted protein from SV2 is in frame but shorter by 24 aa.
Several pairs of primers were designed against the sIL-1RN and icIL-1RN sequences. A full-length 492-bp icIL-1RN coding sequence (CDS) cDNA was amplified by RT-PCR using RNA from LPS-stimulated HD11 cells and splenocytes as template. Gel electrophoresis of the products also revealed an additional smaller band (Fig. 1A). Once both bands had been gel purified, cloned, and sequenced, the smaller band was unexpectedly found to contain two distinct splice variants of the full length, termed SV1 and SV2 (Fig. 2).

Upon closer examination of the splice variant sequences, SV1 lacks exon 2 of the full-length IL-1RN gene and although it is formed through the use of typical splice donor (GT) and acceptor (AG) sites (Fig. 2A), the predicted protein sequence is out of frame and significantly truncated compared with the full-length sequence (Fig. 2B). Analysis of the intron sequences flanking this missing exon identified several conserved sequence motifs associated with exon skipping in mammals (45).

The SV2 transcript sequence was formed through use of an alternative splice acceptor site (AG) within exon 4 located 72 bp from its 5’ end (Fig. 2A). In contrast to SV1, removal of this short stretch of nucleotides did not introduce a frameshift in the predicted protein sequence.

A 522-bp full-length sIL-1RN CDS cDNA was amplified by PCR using cDNA generated from LPS-stimulated HD11 RNA as template (Fig. 1B). As with icIL-1RN, two splice variants of sIL-1RN were identified in addition to the full-length clone. The sIL-1RN splice variant sequences were identical to those of the icIL-1RN variants at all of the splice sites, suggesting that the same mechanism had led to their formation, and they were subsequently termed sIL-1RN SV1 and SV2.

Further characterization of both chIL-1RN sequences was carried out in silico. When aligned with mammalian IL-1RN sequences (Fig. 3), both chIL-1RN variants show relatively high amino acid identity for avian cytokines with their mammalian orthologs. Chicken sIL-1RN is very similar in length to human and mouse sIL-1RN, sharing 38.3 and 37.9% amino acid sequence identity, respectively. Its predicted molecular mass is 19.372 kDa with a theoretical isoelectric point of 8.68. Chicken icIL-1RN has 38.2 and 40.4% amino acid identity with the respective human and mouse sequences, a predicted molecular mass of 18.299 kDa, with an identical isoelectric point to sIL-1RN of 8.68.

The secondary structures of human and mouse IL-1 proteins have been resolved as β-trefoil folds comprised of 12 β-strands. Using PSI-PRED, the secondary structures of both chIL-1RN variants were predicted to have the same three-dimensional configuration, with the 12 β-strands located in almost identical regions to those in the human IL-1RN amino acid sequence (Fig. 3). These regions are the most highly conserved between species, reflecting their likely functional importance. Of the five cysteine residues in the chIL-1RN sequences, three of these are conserved in mammals, are located in β-strands 6 and 10, and two will presumably form a disulfide bond. A single potential N-glycosylation site (NGT) is found in chicken, as in mammals, but the site locations are not conserved.

Both chIL-1RN variants were analyzed for structural similarity to known protein domains in the ProDom database. Sequences were most closely related to domains PDAI6T8 (domain ID, IL-1Ra; closest domain, rat IL-1Ra [to chicken icIL-1RN] and rabbit IL-1Ra [to chicken sIL-1RN]; e values, 2 × 10^{-9} and 4 × 10^{-9} for residues 2–62 and 15–69, with 49 and 52% amino acid identity, respectively) and PD002536 (IL-1; mouse IL-1F10; 2 × 10^{-18}, 32–161/42–171, 37%).

Phylogenetic analysis of the chIL-1RN cDNA and protein sequences showed that the gene is most closely grouped with the mammalian IL-1 receptor antagonist genes (IL-1RN, IL-1F5, and IL-1F10) (40, 41) (Fig. 4). However, the chIL-1RN variants did not group separately with their direct mammalian orthologs, but formed a separate branch, suggesting they share a distant evolutionary relationship.

**Genomic location of IL-1RN**

The chicken genome sequence (v2.1) places chIL-1β on chromosome 22, in a locus with a limited degree of conserved synteny with the IL-1 family gene cluster on human chromosome 2 (Supplemental Fig. 1). The avian orthologs of two genes

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**FIGURE 3.** Alignment of the amino acid sequences of chicken sIL-1RN and icIL-1RN with those of human (secretory and intracellular ic1), mouse, cow, and platypus IL-1RN sequences. Shaded areas represent conservation of amino acid similarity; the darker the shading, the greater the conservation. The secondary conservation of amino acid similarity; the darker the shading, the greater the conservation. The secondary structure of β-strands in humans (5) are indicated by open bars beneath the sequence. Their locations in the chicken, predicted by PSI-PRED, are indicated by closed bars. Conserved cysteines are identified by an asterisk above the alignment and the chicken potential N-glycosylation site by +++ below the alignment.
(SLC20A1 and CKAP2L) that are located adjacent to the human cluster flank chicken IL-1β, although no other genes are shared by the two loci.

A TBLASTN analysis of the chicken genome (v2.1) with both chIL-1RN sequence variants returned no positive hits. Similar analysis of the new chicken genome build (v3.0, unassembled) with chicken icIL-1RN identified two contigs (81757.1 and 113837.1) containing most of the coding sequence of the gene. These contigs, however, were mined from “removed data” sequence reads and are thus unplaced in the assembled genome, so the genomic location of chicken IL-1RN remains unknown.

Closer examination of the locus containing chIL-1β in chicken genome build v2.1 revealed four sequence gaps (estimated at 489, 100, 445, and 100 bp, respectively) immediately adjacent to the 5’ end of the chIL-1β gene. Further chIL-1 family members could be encoded in those gaps. The chicken bacterial artificial chromosome (BAC) map shows that BAC clone TAM32-21N6 covers the entire locus. We attempted to amplify both IL-1β and IL-1RN from BAC clone TAM32-21N6 (and genomic DNA as a positive control) by PCR. Both were readily amplifiable from genomic DNA, but only IL-1β could be amplified from the BAC clone (data not shown), again suggesting that IL-1RN is encoded at a different locus in the chicken genome and not in one of the sequence gaps.

A new, as yet unannotated, assembly (v3.0) of the chicken genome is available and was searched using 8.2 kb sequence from chromosome 22 in v2.1, which included IL-1β and the adjacent sequence gaps. At present, the sequence from this locus in v2.1 is spread across several contigs in v3.0. It does not, therefore, appear that the sequence gaps in v2.1 will be closed in v3.0.

**FIGURE 4.** Phylogenetic analysis of chIL-1RN amino sequences using MEGA v5.0. Analysis was performed using the neighbor-joining method with bootstrap analysis with 500 bootstrap datasets. ch, Chicken; hu, human; IC, intracellular; lz, lizard; m, mouse; pl, platypus; SEC, secretory; zf, zebra finch.
Structural analysis of IL-1RN

A combination of PCR and in silico analyses allowed the genomic structure of chIL-1RN to be determined. cDNA alignments between the human and chicken orthologs of IL-1RN were used to predict the locations of introns in the chicken gene and primers were designed from the flanking sequences to amplify the predicted introns. Sequencing of the resulting PCR-amplified products from chicken genomic DNA showed that the gene structure of chIL-1RN was very similar to its human ortholog. Although readily amplifiable from genomic DNA, the exceptionally high GC content of intron 4 meant it was not completely sequenced. The coding region is comprised of five exons, of similar size to the corresponding human exons. The introns of chIL-1RN, however, are significantly smaller than their human counterparts, resulting in the overall size of the chicken gene being around a 10th of the size of the human ortholog (Fig. 5).

In humans, the first exons of the intracellular and secretory variants (ic1 and s1, respectively) are separated by 9.6 kb DNA. Expression of the two variants is controlled by large promoters of 4525 and 1680 bp, respectively (42, 43), which precede ic1 and s1, respectively (Fig. 5). In the chicken, however, examination of contig 81757.1 (from genome sequence v3.0, the only genomic sequence information so far available upstream of exon ic1) shows the genomic organization at the corresponding region of the chIL-1RN gene differs markedly, with only 129 bp separating ic1 and s1. Attempts to determine the length of the 5′ untranslated region (UTR) of chicken sIL-1RN by 5′ RACE were unsuccessful. The 5′ UTR of chicken icIL-1RN was determined by 5′ RACE to be 50 nt in length, giving a total length of 57 bp for exon ic1 (Fig. 2A). There is a TATA (TATAAA) box 40 nt upstream of the sIL-1RN start codon. Three potential transcription factor binding sites were identified upstream of the icIL-1RN start codon: a PU.1 site at −41 (relative to the icIL-1RN start codon) in the 5′ UTR in the reverse orientation, an NF-IL-6 site at −69, and an Sp1 site at −88 (both in the forward orientation). Both Sp1 and NF-IL-6 binding sites are present in the human icIL-1RN promoter (42). With limited sequence available upstream of exon ic1, it is unclear whether these elements control only expression of sIL-1RN, or whether those upstream of exon ic1 play a role in controlling expression of icIL-1RN, either uniquely or as well as that of sIL-1RN. Further upstream genomic sequence is required for a thorough analysis of the potential promoter(s).

Expression of IL-1RN in tissues and sorted cell subsets

The expression profile of IL-1RN was examined in a broad range of tissues and cells by qRT-PCR (Fig. 6). Three sets of primers and probes were designed to quantify expression of full-length IL-1RN, SV1, and SV2, but it was not possible to design qRT-PCR assays that could differentiate between the two structural variants of chIL-1RN, regardless of the splice variant. Expression of full-length IL-1RN was ubiquitous, with highest levels in lymphoid tissues in the bone marrow and blood, and highest levels in non-lymphoid tissues in the brain (Fig. 6A).

Constitutive expression of full-length IL-1RN was detected in the entire lymphocyte subset cell panel. Of the 20 different populations investigated, KUL01+ cells (macrophages) and blood-derived monocytes (with or without LPS stimulation) showed the highest expression levels (Fig. 6B). Stimulation of cell subsets with LPS led to an increase in expression levels only in the monocyte population. In both bone marrow-derived dendritic cells and bone marrow-derived macrophages, LPS-stimulation significantly decreased expression of full-length IL-1RN, whereas LPS stimulation of heterophils had no effect on expression levels.

**FIGURE 5.** Exon/intron structure of the IL-1RN gene, and the various splice variants thereof, in humans and chickens. Both genes contain the first exon (s1) for the sIL-1RN transcript. Upstream of this is the first exon (ic1) for the icIL-1RN1 transcript. The ic1 exons are spliced into the middle of exon s1 to form the intracellular structural variants. The human gene also contains a further upstream exon (ic2), which is present in the icIL-1RN2 transcript, located between exons ic1 and s1. PCR amplification of intron 4 indicated it is ~750 bp in length; however, the complete sequence was not determined.
Expression of IL-1RN is increased in response to bacterial and viral challenge

In both a viral (IBDV) and bacterial (S. typhimurium) challenge model, IL-1RN mRNA expression levels were statistically significantly increased, in bursal cells and splenocytes, respectively, in infected birds compared with levels in uninfected, age-matched controls at certain time points after infection (Fig. 7).

At 4 d after IBDV infection, full-length and SV2 IL-1RN mRNA expression levels were statistically significantly increased 4- and 2.5-fold in bursal cells from chickens of a resistant (61) line. Differences in IL-1RN SV1 mRNA expression levels in all groups at all time points were not statistically significant (Fig. 7A).

The expression of full-length, SV1, and SV2 IL-1RN transcripts was also assessed in splenocytes from outbred Rhode Island Red birds following infection with S. typhimurium strain F98. Full-length IL-1RN mRNA expression levels were statistically significantly upregulated 2-fold in comparison with levels in splenocytes from uninfected, age-matched controls, at 3 dpi (Fig. 7B). By 7 dpi, however, mRNA expression levels in both infected and control birds were not significantly different and remained so for the duration of the experiment. IL-1RN SV1 mRNA expression levels were not statistically significantly different between infected and control birds throughout the experiment.

Full-length rdsIL-1RN and icIL-1RN are bioactive but splice variants of both are not

Recombinant chicken sIL-1RN and icIL-1RN were expressed in HEK293T cells and purified (data not shown). The ability of purified rchIL-1RN to inhibit the biological activity of chIL-1β was assessed in an HD11 cell bioassay. The antagonistic activity of both chIL-1RN variants was determined by their ability to inhibit the IL-1β–mediated upregulation of IL-1β and iNOS. The ability of HD11 cells to respond to IL-1β was first tested (Fig. 8A). HD11 cells were either stimulated for 24 h with rchIL-1β with or without anti–IL-1β Ab or cultured in media only. IL-1β and iNOS mRNA expression levels were upregulated in rchIL-1β–stimulated cells compared with levels in unstimulated cells. In cells cultured in the presence of rchIL-1β and anti–IL-1β Ab, the Ab was able to neutralize the biological activity of IL-1β at all but the two highest concentrations, with resulting IL-1β and iNOS mRNA expression levels being similar to those in unstimulated cells.

In HD11 cells preincubated for 4 h with either purified sIL-1RN or purified icIL-1RN prior to the addition of rchIL-1β, upregulation of IL-1β and iNOS mRNA expression levels was effectively inhibited (Fig. 8B). In cells stimulated with IL-1β alone, IL-1β and iNOS mRNA expression levels increased significantly compared with levels in unstimulated cells. Differences in expression between the rchIL-1β plus rIL-1RN groups and the rchIL-1β only treatment group were statistically significant up to two and four doubling dilutions of sIL-1RN and icIL-1RN, respectively (Fig. 8B). The antagonistic effect of both chIL-1RN variants gradually declined as they were titrated out in the presence of a fixed concentration of rIL-1β. The induction of iNOS was also measured through the production of nitrite in the culture medium, quantified via a Griess assay. These results correlated with the qRT-PCR data throughout the experiment (Fig. 8C).

The bioactivity of ex-COS icIL-1RN was also tested in the same bioassay (Supplemental Fig. 2). Lysate from COS-7 cells trans-
fected with a vector expressing icIL-1RN inhibited the IL-1β–induced upregulation of IL-1β and iNOS mRNA expression. Supernatant from the same cells, however, exhibited significantly less bioactivity and was only able to antagonize the effect of IL-1β at its highest concentrations. This result is consistent with this variant being the intracellular form and correlates with results from a similar study carried out in mammals (8).

The biological activities of the four identified splice variants of chIL-1RN were tested in the same assay. The bioactivities of cell supernatants and lysates from COS-7 cells transfected with plasmids expressing each splice variant were compared with mock-transfected controls (supernatants and cell lysates from COS-7 cells transfected with pCI-neo lacking a cDNA insert). At their highest concentration, ex-COS lysates containing either icIL-1RN SV1 or SV2 demonstrated greater inhibition of IL-1β–mediated upregulation of IL-1β and iNOS mRNA levels than their respective supernatants, but these were not statistically significantly different from levels with ex-COS lysates of pCI-neo controls (Supplemental Fig. 3). Lysates and supernatants (ex-COS) of both sIL-1RN splice variants, similarly, showed no bioactivity when compared with pCI-neo controls. These results indicate that all four splice variants of chIL-1RN do not act as functional antagonists of IL-1β in this assay.

**Discussion**

Given the potency of IL-1β in immune responses, its regulation is essential to avoid damage to the host through excessive inflammation. In part, this role is fulfilled by the biological activities of IL-1RN. Humans either lacking or possessing mutations in the IL-1RN gene can die prematurely in the absence of treatment with synthetic IL-1RN (44). The identification of IL-1β in the chicken (25) suggested that IL-1RN should also be present, but it is not identifiable in either the existing annotated genome build (v2.1) or the new, yet-to-be-annotated build (v3.0). In this study, we describe the identification and characterization of both secretory and intracellular variants of IL-1RN in the chicken, as are present in mammals. Although obvious similarities between the chicken gene and its mammalian orthologs exist, a number of notable differences were identified.

The chicken has differently spliced isoforms of both the secretory and intracellular variants (differently spliced isoforms have only been described for the intracellular variant in mammals), both structural variants show the same differently spliced isoforms (SV1 and SV2), and the rearrangements generating those isoforms differ between the chicken and mammals. Both SV1 transcripts appear to be formed by exon-skipping, whereas the SV2 transcripts use an alternative splice acceptor site in the final exon of the gene. A number of conserved sequence motifs synonymous with exon skipping (45) were identified in the introns adjacent to the spliced exon in SV1, indicating this gene may be predisposed to splicing at the pre-mRNA stage. This exon-skipping event, however, causes the predicted amino acid sequence to become out of frame, introducing a premature stop codon and considerably truncating any translated protein. Although the predicted SV2 amino acid sequences are in frame, potentially important residues corresponding to two β-sheets of the secondary structure are removed by this splicing event.

Both full-length chicken sIL-1RN and icIL-1RN recombinant proteins antagonized IL-1β–mediated upregulation of IL-1β and iNOS, and as such exhibited biological activity analogous to their
mammalian orthologs. The analysis above of the four splice variants suggested they would be functionally redundant and this proved to be the case. These results suggest that a possible control mechanism exists to regulate chIL-1RN expression, and hence its bioactivity, by generating transcripts that encode functionally redundant proteins.

Apart from IL-1\(\beta\) genes, very little is known regarding the existence of IL-1 family genes in nonmammalian species, with only a single report characterizing a novel IL-1 family member in the rainbow trout (46). Genome sequences are now available for three other avian species: the zebra finch, turkey, and duck. IL-1\(\beta\) is present in all three, but no other IL-1 family members are identifiable, including IL-1RN. Outside of other avian species, the anole lizard is the most closely related species to the chicken for which a genome sequence is available. A search of its genome revealed three IL-1 family genes adjacent to one another in a single locus. None of these genes has been assigned an identity but all contain the IL-1 family signature motif. BLAST analysis (data not shown) indicated they are most likely IL-1F5, IL-1F10, and IL-1RN. However, they form a completely separate clade when analyzed phylogenetically (Fig. 4), and therefore their identities cannot be determined with confidence.

**FIGURE 8.** Bioassays to determine bioactivity of chIL-1RN. (A) rchIL-1\(\beta\) stimulates HD11 cells to upregulate expression of IL-1\(\beta\) and iNOS mRNA, as measured by qRT-PCR, and this activity is blocked by neutralizing Ab. (B and C) Both secretory and intracellular variants of purified rchIL-1RN antagonize the stimulatory effects of rchIL-1\(\beta\) in the same bioassay, in a dose-dependent manner, as measured by (B) inhibition of mRNA expression levels of IL-1\(\beta\) and iNOS and (C) inhibition of iNOS upregulation, as measured by the induction of nitrite in the culture supernatant, quantified by the Griess assay. Doubling dilutions of recombinant proteins from initial concentrations (x-axis, data point 1) of 480 and 562 \(\mu\)g/ml sIL-1RN and icIL-1RN, respectively. The data are expressed as percentage inhibition of IL-1\(\beta\) or iNOS mRNA expression (A, B) or \(\text{NO}_2^-\) production (C) (from 100% activity in the absence of sIL-1RN or icIL-1RN). Results shown are from three independent experiments. U, Unstimulated cells.
Expression of full-length IL-1RN is ubiquitous and constitutive in the range of cells and tissues investigated, although the TaqMan assay used does not differentiate between sIL-1RN and iCIL-1RN mRNA. Global studies of IL-1RN mRNA expression in mice (47) and rabbits (48) showed it was not constitutive in all tissues. Those analyses, however, used RNase protection assays and Northern blotting, respectively, which lack sensitivity compared with TaqMan. Expression of the full-length chicken IL-1RN was increased in vivo following bacterial or viral infection, as is seen in similar disease models in mammalian species.

Perhaps most interesting from an evolutionary standpoint, in humans the locus encoding IL-1β and IL-1RN on chromosome 2 also encodes seven other IL-1 family members. There is conservation of synteny with the locus encoding chicken IL-1β on chromosome 22, in that two genes that are adjacent to the human IL-1 family locus flank chicken IL-1β. However, we can find no evidence at this locus for chicken IL-1RN. As most multigene families at a single locus are conserved between chickens and mammals, although the precise numbers of members of those families may differ and orthologous relationships may be difficult to ascribe (49), it is surprising that the chicken IL-1 family appears to be more fragmented than the mammalian IL-1 family. Multigene families at a single locus tend to evolve through duplication events from a single ancestral gene. So how can we explain the apparent dissonant genomic location of IL-1RN between the chicken and mammals?

It is possible that human IL-1RN and chIL-1RN represent the results of species-specific convergent evolution. Alternatively, the two molecules evolved from a common ancestor followed by sequence divergence in the two lineages. We think that the latter is the case for IL-1RN, which has multiple members in both lineages: we think that the former is the case for IL-1 family genes in this species.

As we have seen, mammalian IL-1 family genes at a single locus tend to evolve through duplication events from a single ancestral gene. So how can we explain the apparent dissonant genomic location of IL-1RN between the chicken and mammals?

Acknowledgments
We thank Lisa Rothwell, James Birch, and John Hammond for advice and assistance in different aspects of this study.

Disclosures
The authors have no financial conflicts of interest.

References
15. Evans, S., C. A. Dinarello, C. Sick, B. Kaspers, and P. Staeheli. 1998. A chicken homolog of mouse IL-33. In the chicken, one locus could have undergone mutation/deletion in the IL-1RN gene, to leave only IL-1β, while similar events in the other locus might have removed IL-1β and left IL-1RN. Alternatively, a single ancestral locus in the chicken might have become fragmented by an unknown mechanism, although, as stated above, the vast majority of multigene immune families at single loci remain conserved between chickens and mammals. We will continue to try to determine the genomic location of chIL-1RN and to identify the full repertoire of IL-1 family genes in this species.

Acknowledgments
We thank Lisa Rothwell, James Birch, and John Hammond for advice and assistance in different aspects of this study.


Supplementary Figure 1. Limited conserved synteny between the nine-gene human IL-1 family locus on chromosome 2, and chicken chromosome 22. At the chicken locus, only one IL-1 family gene (IL-1β) is currently annotated, although it is flanked by two genes (SLC20A1 and CKAP2L) that lie to one side of the human locus. We found no evidence for the presence of IL-1RN at this locus in the chicken.
Supplementary Figure 2. Bioassays to determine bioactivity of chIL-1RN. Comparison of the ability of unpurified cell lysate (L) and supernatants (S) from COS-7 cells transfected with a vector expressing full-length icIL-1RN to antagonise the stimulatory effects of recombinant chIL-1β on HD11 cells. COS-7 cells were also transfected with empty vector (pCI-neo) to generate negative control supernatants (M). Recombinant chIL-1β stimulates HD11 cells to upregulate expression of IL-1β and iNOS mRNA, as measured by Taqman qRT-PCR. The data are expressed as percent inhibition of mRNA expression (from 100% activity in the absence of icIL-1RN or the control). Doubling dilutions of neat COS-7 cell supernatants or lysates. U = unstimulated cells. Three independent experiments were carried out with similar results and data from one representative experiment is shown.
% inhibition of iNOS expression

% inhibition of IL-1β expression

Doubling dilutions

SV1 Lysate
SV1 Supernatant
SV2 Lysate
SV2 Supernatant
Control Lysate
Control Supernatant
Supplementary Figure 3. Bioassays to determine bioactivity of chIL-1RN. Splice variant (SV1 and SV2) forms of the A. intracellular and B. secretory forms of IL-1RN were expressed in COS-7 cells. COS-7 cells were also transfected with empty vector (pCI-neo) to act as a negative control. The resulting lysates or supernatants from those transfected cells were tested for their ability to antagonise the stimulatory effects of recombinant chIL-1β on HD11 cells. The data are expressed as percent inhibition of IL-1β or iNOS mRNA expression (from 100% activity in the absence of IL-1RN or control lysates or supernatants). Doubling dilutions of neat COS-7 cell supernatants or lysates. U = unstimulated cells. Results shown are from three independent experiments.
Supplementary Table I. Chicken IL-1 family ESTs identified by TBLASTN and reciprocal BLASTP analysis.

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*This EST is a composite of the 5’end of sIL-1RN joined to Tmed4 with NotI linkers, which explains the apparent contradiction between the relatively high % aa identity with a high e-value from the BLAST analysis.