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Cloning and Characterization of Chicken IL-10 and Its Role in the Immune Response to *Eimeria maxima*[1]

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We isolated the full-length chicken IL-10 (chIL-10) cDNA from an expressed sequence tag library derived from RNA from cecal tonsils of *Eimeria tenella*-infected chickens. It encodes a 178-aa polypeptide, with a predicted 162-aa mature peptide. Chicken IL-10 has 45 and 42% aa identity with human and murine IL-10, respectively. The structures of the chIL-10 gene and its promoter were determined by direct sequencing of a bacterial artificial chromosome containing chIL-10. The chIL-10 gene structure is similar to (five exons, four introns), but more compact than, that of its mammalian orthologues. The promoter is more similar to that of *Fugu* IL-10 than human IL-10. Chicken IL-10 mRNA expression was identified mainly in the bursa of Fabricius and cecal tonsils, with low levels of expression also seen in thymus, liver, and lung. Expression was also detected in PHA-activated thymocytes and LPS-stimulated monocyte-derived macrophages, with high expression in an LPS-stimulated macrophage cell line. Recombinant chIL-10 was produced and bioactivity demonstrated through IL-10-induced inhibition of IFN-γ synthesis by mitogen-activated lymphocytes. We measured the expression of mRNA for chIL-10 and other signature cytokines in gut and spleen of resistant (line C.B12) and susceptible (line 15I) chickens during the course of an *E. maxima* infection. Susceptible chickens showed higher levels of chIL-10 mRNA expression in the spleen, both constitutively and after infection, and in the small intestine after infection than did resistant chickens. These data indicate a potential role for chIL-10 in changing the Th bias during infection with an intracellular protozoan, thereby contributing to susceptibility of line 15I chickens. ***The Journal of Immunology*****, 2004, 173: 2675–2682.**

In mammals, IL-10 is a 17- to 19-kDa cytokine containing two disulfide bridges that functions as a homodimer. It is produced by activated T cells, B cells, monocytes/macrophages, mast cells, and keratinocytes (1). The genes for human and murine IL-10, both consisting of five exons and four introns, lie on syntenic regions of chromosome 1 in both species. IL-10 is a 4α-helical bundle cytokine (2) and exerts its biological effects through binding to a type II cytokine receptor (3).

Recently, it has emerged that IL-10 is the signature member of a family of cytokines, including both cellular (IL-19 (4), IL-20 (5), IL-22 (6, 7), IL-24 (8), and IL-26 (9)) and virally encoded (BCRF1 of EBV (10, 11) and other IL-10 homologues in equine herpes virus type 2 (12), the Orf (13) and Yaba-like disease (14) poxviruses, and CMV (15, 16)) cytokines, which share limited primary sequence identity and probable structural homology. For most of these IL-10-like cytokines, function has yet to be fully determined.

The predominant effect of IL-10 in mammals is to modify immune responses through direct effects on many cell types, including T cells, B cells, APCs, and NK cells, promoting the development of T2 responses (17, 18). In particular, it inhibits the synthesis of proinflammatory cytokines (including IL-1β, TNF-α, and IL-12) both at the level of transcription and post-transcriptionally, NO production, and MHC class II expression (19–21), thus down-regulating inflammatory (T1) responses. It therefore also promotes T2 responses by inhibiting T1 cytokines (22, 23).

Most of what we know about the role of IL-10 in infectious disease comes from murine models. Manipulating levels of IL-10 in vivo, either with rIL-10 or anti-IL-10 Abs or by using IL-10 knockout mice, alters the T1/T2 bias of the immune response and hence affects protection against infectious agents and/or pathology. In general, low levels of IL-10 increase resistance, and high levels increase susceptibility, to intracellular pathogens, including bacteria, viruses, and protozoa (reviewed in Ref. 24).

The intracellular protozoan *Eimeria* spp. causes a serious enteric disease in a wide range of animal species. Coccidiosis, caused by *Eimeria* spp., is an economically important disease for the poultry industry and is still mainly controlled by medication, but because of ever-present drug resistance and the withdrawal of many drugs, vaccination is increasingly practiced (25). For further progress in vaccination against coccidiosis, a clear understanding of the mechanisms involved in the very effective protective responses normally induced by infection will be required. These mechanisms are now well understood in the mouse model, but to a lesser degree in the chicken. In the former, the response is IFN-γ- and T1-dominated, and with at least some *Eimeria* spp., IFN-γ is produced by $CD4^+$ T cells (26, 27).

Inbred lines of chickens differ in their resistance or susceptibility to infection with *Eimeria maxima* and *Eimeria tenella* (28, 29). In chickens, the level of resistance correlates with the magnitude of the T cell response, with higher proliferative responses in resistant lines of chickens (30). In terms of the role of cytokines, the production of proinflammatory and T1 cytokines, chemokines, and the

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anti-inflammatory cytokine TGF-β has been described after infection of chickens with several species of *Eimeria* (27, 31–40). Differences between inbred lines have been shown for IL-2 and IFN-γ; levels of production of both were higher after either primary infection or secondary challenge in resistant than in susceptible lines (32, 38).

Despite the general lack of cross-reactivity and the low level of sequence identity (both DNA and amino acid) between avian cytokines and their mammalian orthologues, there has been considerable progress recently in cloning avian cytokines (41–43). However, several important cytokines, such as CSFs, TNFs, and the IL-10 family, remain to be cloned in the chicken.

Our initial attempts to clone chicken IL-10 (chIL-10)[3] used a genomics approach based on conservation of synteny. As we prepared to shotgun-sequence a candidate bacterial artificial chromosome (BAC) to identify chIL-10, we identified an expressed sequence tag (EST), from a cDNA library derived from RNA from a cecal tonsil of a chicken infected with *E. tenella*, with identity with mammalian IL-10. Because IL-10 can act as a negative regulator of T1 immune responses in mammals, which are important in the control of many intracellular parasites (including the *Eimeria* spp.), we investigated the levels of IL-10 mRNA in various tissues during infection of resistant and susceptible chickens with *E. maxima*.

Materials and Methods

Experimental animals and parasites

Chickens of various inbred White Leghorn lines were produced and maintained at Institute for Animal Health (Compton, U.K.). Inbred lines 7_2 and 15I originate from the Regional Poultry Research Laboratory (East Lansing, MI). Reaseheath C (C line) birds originate from University of Cambridge (Cambridge, U.K.). All three lines have been maintained at Institute for Animal Health for >20 years.

The Houghton strain of *E. maxima* was used (44). The strain is the progeny of a single oocyst. Methods for maintaining the parasites, preparing infective doses, and quantifying infections by detecting or counting the numbers of oocysts in feces between 5 and 10 days postinfection have been described previously (45). *E. maxima* was passaged at frequent intervals and was used within 21 days of sporulation for each experiment. All passage of the parasites in chickens was undertaken in separate isolated rooms, each fitted with two antelobbies and fumigated before and after use with methyl bromide. Furthermore, oocysts were recovered from fecal material in purpose-built rooms, which were disinfected afterward by a minimum of 7 h of exposure to gaseous ammonia. All experiments were conducted in methyl bromide-treated rooms to avoid the ingress of coccidial parasites.

BAC library screening

Partial cDNAs for chicken ligatin (clone 16m15r1, provided by J.-M. Buerstedde, University of Munich, Munich, Germany) and chicken poly-Ig receptor (this laboratory; data not shown) were labeled with [α-^{32}P]dCTP using a random-primed labeling kit (Roche, Lewes, U.K.) and were used to screen the Wageningen chicken BAC library (46). Eight BACs were positive for ligatin, and six were positive for poly-Ig receptor, with four BACs (bW102M1, bW111G11, bW119H6, and bW134F8) containing both genes. After identification of the chIL-10 EST and subsequent cloning of the cDNA, the four BACs identified from the library screening were confirmed as containing chIL-10 by PCR. The positive BACs were subsequently sequenced, initially using primers specific to the cDNA, to generate the full-length chIL-10 gene, including the promoter. The chIL-10 cDNA and gene have been deposited in the databases with accession numbers AJ621254 and AJ621614, respectively.

Phylogenetic analysis

Chicken and selected mammalian IL-10 predicted amino acid sequences were aligned using the CLUSTAL-X program. The alignment generated was manipulated to obtain maximum sequence similarity. The programs in the Phylogeny Inference Package (PHYLIP, version 3.572c, released by J. Felsenstein, University of Washington, Seattle, WA, 1993) were used to analyze the alignment. Distance (d) was calculated for all pairs of sequences according to the formula $d = -\log_e (1 - p - 0.2p^2)$ with p being the proportion of observed aa differences. The tree was constructed by the Fitch-Margoliash method (47) and was drawn unrooted using TreeView (48).

Production of recombinant chIL-10 (rchIL-10) by COS-7 cells

COS-7 cells were routinely grown in DMEM (Life Technologies, Paisley, U.K.) containing 10% FBS (PAA Laboratories, Linz, Austria), 1% nonessential amino acids, 1% L-glutamine, 1 U/ml penicillin, and 1 μg/ml streptomycin at 37°C in 5% CO_2 and passaged using standard conditions (49). Cells were cultured at 5×10^5/ml for 18–24 h at 37°C in 5% CO_2 and washed twice with PBS. Five milliliters of serum-free medium containing 7.5 μg/ml DNA (pCI-neo, pCI-neo-chIL-10, or no plasmid), 258 μg/ml chloroquine, and 600 μg/ml DEAE-dextran was added to the cells, which were then incubated for 3 h at 37°C in 5% CO_2. The transfection medium was then removed and cells washed once with PBS. PBS containing 10% DMSO was added for 2 min, removed, and replaced with 5 ml of growth medium. After 24-h growth at 37°C in 5% CO_2, growth medium was replaced with serum-free DMEM. The cells were then incubated for 72 h, after which supernatant was collected and stored at 4°C before use.

IL-10 bioassay

Spleens were removed from 8-wk-old birds postmortem and immediately placed in DMEM. Using blunt forceps, tissue was then teased apart in 10 ml of DMEM, resulting in a single-cell suspension. Lymphocytes were isolated over Ficoll-Paque (Amersham Pharmacia Biotech, Arlington Heights, IL), washed, and resuspended at 5×10^6 cells/ml in DMEM containing 2 mg/ml BSA, 1% L-glutamine, 1 U/ml penicillin, and 1 μg/ml streptomycin. This cell suspension was added to round-bottom, 96-well plates (100 μl/well) or 24-well plates (1 ml/well) containing serial 2-fold dilutions of rchIL-10 (ex-COS), in a final volume of 200 μl/well (96-well plates) or 2 ml/well (24-well plates) in the presence of 1.2 μg/ml Con A (Sigma-Aldrich, St. Louis, MO), 12.5 μg/ml PHA (Sigma-Aldrich), or no mitogen. Negative controls included serial 2-fold dilutions of supernatant collected from COS-7 cells transfected with pCI-neo alone or medium alone, with or without mitogen. Assays were conducted in triplicate. Cells were incubated at 41°C in 5% CO_2 for 24 h for RNA analysis (isolated from 24-well plates) or for 72 h for analysis of IFN-γ content in the supernatant.

Supernatants were assayed (in triplicate) for IFN-γ content using a quantitative chIFN-γ capture ELISA (BioSource, Nivelles, Belgium) as previously described (50) and by bioassay, using the macrophage activation factor assay, as previously described (51, 52).

Total RNA was isolated from cells in 24-well plates using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer's instructions. Purified RNA was eluted in 50 μl of RNase-free water and stored at −70°C. Real-time quantitative RT-PCR was used to determine IFN-γ mRNA expression levels in splenocytes treated with rchIL-10 or mock-transfected COS cell supernatant (at 1/10, 1/100, and 1/1000 dilutions) with or without mitogenic stimulation (see below).

Experimental design

Three-week-old line 15I and line C.B12 chickens were orally infected with 100 *E. maxima* sporulated oocysts. Tissue samples were collected from four birds of each line at 0, 3, 6, and 9 days postinfection (dpi). Small sections of spleen, cecal tonsil, and small intestine were collected into RNALater (Qiagen) and stored according to the manufacturer's instructions until RNA was purified using the RNeasy mini kit (Qiagen) following the manufacturer's instructions. Purified RNA was then eluted in 50 μl of RNase-free water and stored at −70°C.

Quantitative RT-PCR

Cytokine mRNA levels were quantitated using a method based on that described by Kaiser et al. (51, 53, 54). For both cytokine- and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (Applied Biosystems, Foster City, CA). Details of the probes and primers are given in Table I. All cytokine probes were designed, from the sequence of the relevant genes, to lie across intron:exon boundaries. Cytokine and 28S rRNA probes were labeled with the fluorescent reporter dye FAM at the 5′ end and with the quencher TAMRA at the 3′ end.

RT-PCR was performed using the Reverse Transcriptase Quantitative PCR Master Mix kit (Eurogentec, Seraing, Belgium). Amplification and

[3] Abbreviations used in this paper: ch, chicken; Ct, threshold cycle; dpi, days postinfection; EST, expressed sequence tag; IRE, IFN response element; rch, recombinant chicken; Sp1, specificity protein 1; BAC, bacterial artificial chromosome.

Table I. *Real-time quantitative RT-PCR probes and primers*

RNA Target	Probe/Primer Sequence	Exon Boundary	Accession No.[a]
28S	Probe 5′-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3′ Forward 5′-GGCGAAGCCAGAGGAAACT-3′ Reverse 5′-GACGACCGATTTGCACGTC-3′		X59733
IFN-γ	Probe 5′-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3′ Forward 5′-GTGAAGAAGGTGAAAGATATCATGGA-3′ Reverse 5′-GCTTTGCGCTGGATTCTCA-3′	3/4	Y07922
IL-4	Probe 5′-(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)-3′ Forward 5′-AACATGCGTCAGCTCCTGAAT-3′ Reverse 5′-TCTGCTAGGAACTTCTCCATTGAA-3′	3/4	AJ621735
TGF-β4	Probe 5′-(FAM)-ACCCAAAGGTTATATGGCCAACTTCTGCAT-(TAMRA)-3′ Forward 5′-AGGATCTGCAGTGGAGTGGAT-3′ Reverse 5′-CCCCGGGTTGTGTTGGT-3′	6/7	M31160
IL-10	Probe 5′-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3′ Forward 5′-CATGCTGCTGGGCCTGAA-3′ Reverse 5′-CGTCTCCTTGATCTGCTTGATG-3′	3/4	AJ621614

[a] Refers to the genomic DNA sequence.

detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems) with the following cycle profile: one cycle of 50°C for 2 min, 96°C for 5 min, 60°C for 30 min, and 95°C for 5 min, and 40 cycles of 94°C for 20 s and 59°C for 1 min.

Quantification was based on the increased fluorescence detected by the ABI PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5′ nuclease activity of the *rTth* DNA polymerase during PCR amplification. The passive reference dye 6-carboxy-χ-rhodamine, which is not involved in amplification, was used to correct for fluorescent fluctuations, resulting from changes in the reaction conditions, for normalization of the reporter signal. Results are expressed in terms of the threshold cycle value (C_t), the cycle at which the change in the reporter dye passes a significance threshold.

Standard curves for the cytokine and 28S rRNA-specific reactions were generated as previously described (51). Each RT-PCR experiment contained triplicate no-template controls and test samples and a $\log_{10}$ dilution series of standard RNA. Each experiment was performed in triplicate. Regression analysis of the mean values of five replicate RT-PCRs for the $\log_{10}$ diluted RNA was used to generate standard curves.

Results

Cloning of the cDNA and gene encoding chIL-10

The chIL-10 cDNA was isolated from an EST library derived from RNA from a cecal tonsil of a chicken infected with *E. tenella*. It encodes a polypeptide of 178 aa (Fig. 1), with a predicted mature peptide of 162 aa (Fig. 1) and a calculated molecular mass of 20.56 kDa. The 3′-untranslated region consists of ~1400 nt, with a single potential poly(A) signal. It contains 11 copies of the instability motif AUUUA, commonly found in cytokine and oncogene mRNAs, which mediates rapid mRNA degradation and is a recognition site for an RNase E-like activity that cleaves RNA sequences in man (55). By contrast, the human and murine IL-10 3′-untranslated regions contain seven and six AUUUA motifs, respectively.

Amino acid sequence comparisons (Fig. 1) show that sequence identity between chIL-10 and human and murine IL-10 is 45 and 42%, respectively. The mature IL-10 proteins from all species described to date, mammals, *Fugu*, and chicken, all contain four cysteine residues. The chIL-10 lacks potential *N*-linked glycosylation sites compared with two in murine and one in human IL-10. Native human IL-10 is unglycosylated, however. Native murine IL-10 is glycosylated, but this glycosylation is not required for its bioactivity (1, 56). In common with all other IL-10s described to date, chIL-10 contains the IL-10 family signature motif (G-X-X-K-A-X-X-[DE]-X-D-[ILV]-[FLY]-[FILMV]-X-X-[ILMV]-[EKQR]), in this case GIYKAMGEFDIFINYLE.

An unrooted phylogenetic tree was constructed from the amino acid sequences of chicken, *Fugu*, and various mammalian IL-10 (Fig. 2). As expected, the analysis clusters chicken and *Fugu* IL-10 together, apart from the mammalian sequences and separated by a long branch.

The gene structure of chIL-10 is similar to those of the human, mouse, and *Fugu* IL-10 genes, comprising five exons and four introns (Fig. 3), with similar numbers of amino acids encoded by the respective exons across species. Overall, the gene is approximately half the length of the mammalian genes, and 1.5 times longer than the fish gene.

The chIL-10 promoter

The human IL-10 promoter has been well characterized (57, 58) (Fig. 4) and can be divided into regions containing elements that have positive or negative effects on transcription (58). Several transcriptional regulatory elements were also described in the *Fugu* promoter (59) (Fig. 4), and again, these were subdivided into regions with positive and negative effects. Initial investigation revealed little identity (38% nucleotide identity over 1100 nt) between the human and chicken promoters. We then predicted transcriptional regulatory elements in the human, *Fugu*, and chicken promoters using the AliBaba2 program in the TRANSFAC database. Fig. 4 shows the elements previously identified in the human promoter and an NF-κB site and an IFN response element (IRE) identified in the current study; elements (NF-κB, specificity protein 1 (Sp1), IRE, PU box, and YY1) were identified in the *Fugu* promoter (some previously described), and potential NF-κB, IRE, and Sp1 elements were identified in the chicken promoter. All three promoters have TATA boxes and at least one each of NF-κB sites, Sp1 sites, and IREs. Other than these features, there is little similarity between the promoters in terms of numbers or order of the potential transcription factor binding sites. We have yet to determine whether the chicken promoter can be divided into positive and negative regulatory regions.

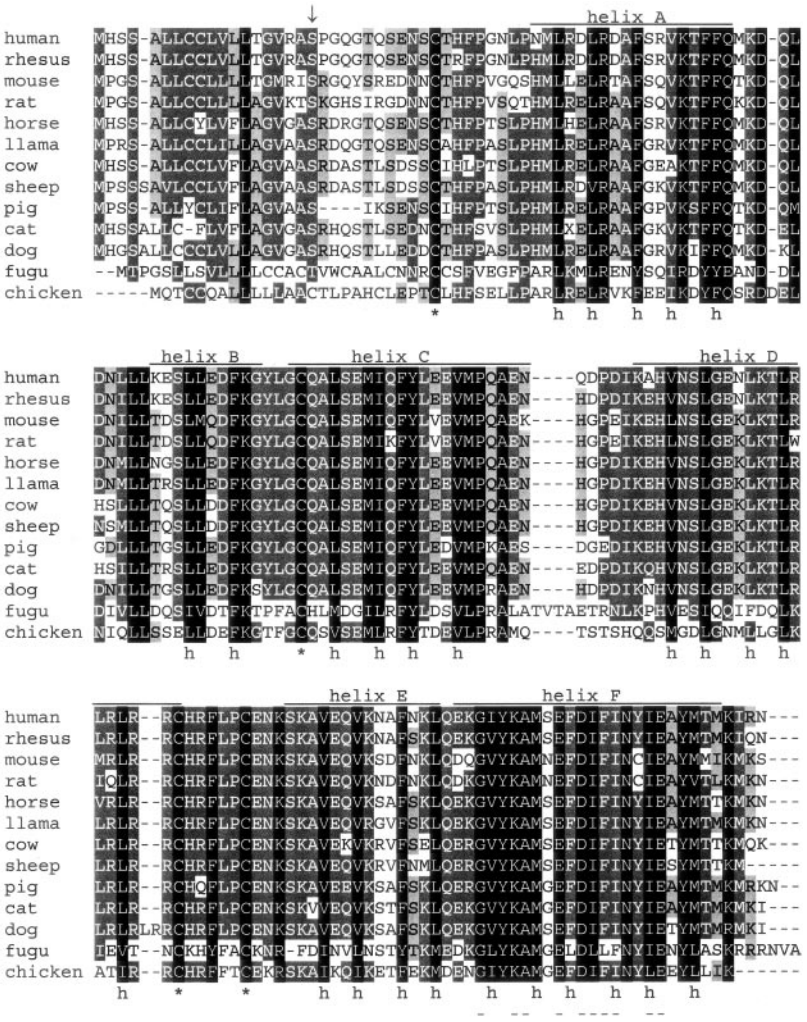

FIGURE 1. Alignment of the predicted chIL-10 amino acid sequence with mammalian IL-10, with reference to secondary structural features and functionally important residues. Shaded areas represent conservation of amino acid similarity; the darker the shading, the more conserved the residue across species. Dashes indicate gaps in the alignment. The predicted N termini of the mature proteins are indicated with an arrow. The cysteines conserved between mammalian and chicken IL-10 are indicated with asterisks. Bases underlined in the chicken sequence represent the IL-10 family signature residues. The α-helical assignments are based on the structure of human IL-10 (68). The hydrophobic amino acids at positions 1 and 4 of the heptad repeats in the amphipathic helices are indicated with an h. The accession numbers of the sequences used are as follows: human, M57627; rhesus macaque, L26029; mouse, M37897; rat, L02926; horse, U38200; llama, AB107649; cow, U00799; sheep, Z29362; pig, L20001; cat, U39569; dog, U33843; fugu, AJ539537; and chicken, AJ621254.

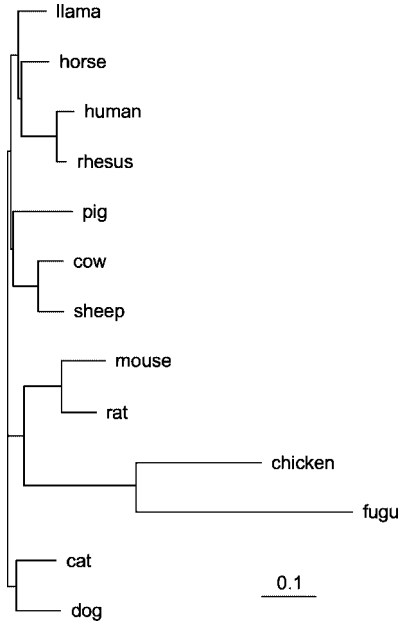

FIGURE 2. Unrooted phylogenetic tree of IL-10. For the branch lengths, the distance given in the scale represents 0.1 amino acid substitution/site. The accession numbers of the sequences used are given in Fig. 1.

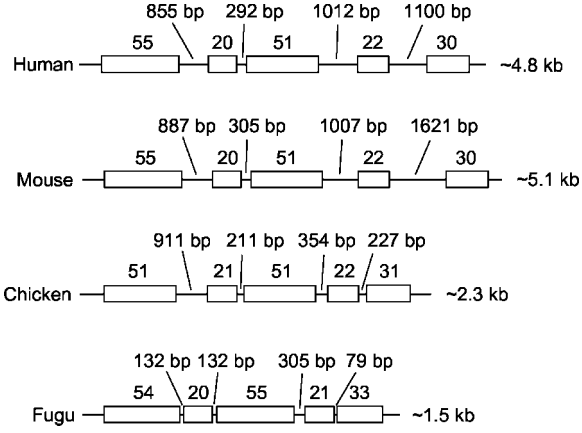

FIGURE 3. Comparison of the gene structures of human, mouse, chicken, and *Fugu* IL-10. All four genes are drawn to the same scale. Exons are shown as boxes; introns are shown as lines joining the boxes. The accession numbers of the sequences used are: human, U16720; mouse, M84340; chicken, AJ621614; and *Fugu*, AJ539537.

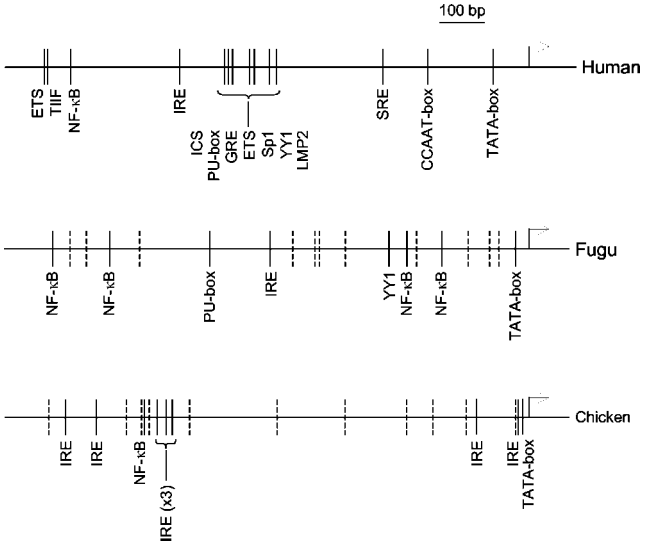

FIGURE 4. Comparison of the human, *Fugu*, and chicken IL-10 promoters. Transcription factor binding sites in the human promoter known to be involved in control of transcription of human IL-10 (57, 58), or predicted (IRE) using AliBaba2 (available online at www.gene-regulation-.com) are shown. For the *Fugu* promoter, sites shown were previously described (59) or predicted (Sp1, NF-κB, IRE) by AliBaba2. All sites in the chicken promoter were predicted by AliBaba2. Predicted Sp1 binding sites are shown as dotted lines. ICS, insulin-like core sequence; GRE, glucocorticoid response element; SRE, serum response element.

Expression of chIL-10 mRNA in tissues and stimulated lymphoid cells

Expression of chIL-10 mRNA was assessed by real-time quantitative RT-PCR (Fig. 5) in lymphoid and nonlymphoid tissues from a line 7_2 chicken and in stimulated lymphoid cells. The chIL-10 message was constitutively expressed in the thymus, bursa of Fabricius, and cecal tonsil, but not in the spleen or bone marrow. It was also expressed in mitogen-activated thymocytes, but not in mitogen-activated splenocytes or bursal cells, and in both primary-cultured, monocyte-derived macrophages and HD11 cells (a chicken macrophage cell line (60)) after LPS stimulation. In nonlymphoid tissues, chIL-10 mRNA expression was detected only in liver and lung.

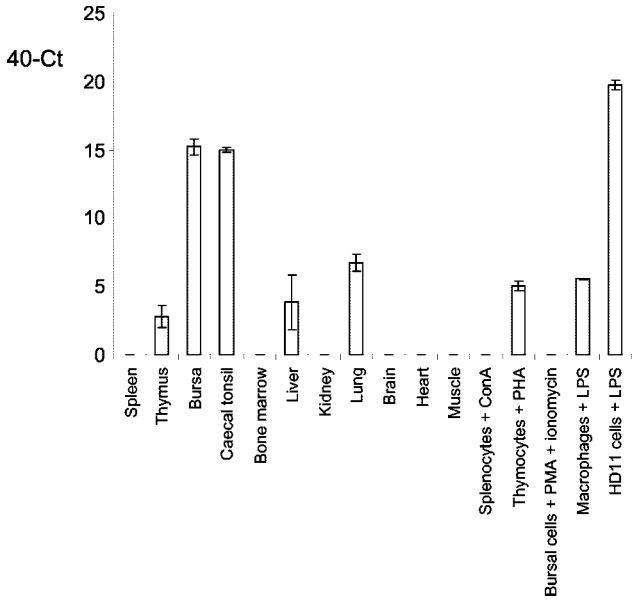

FIGURE 5. Expression patterns of chIL-10 in tissues and stimulated lymphoid cells, as measured by real-time quantitative RT-PCR, with results expressed as 40 − Ct values.

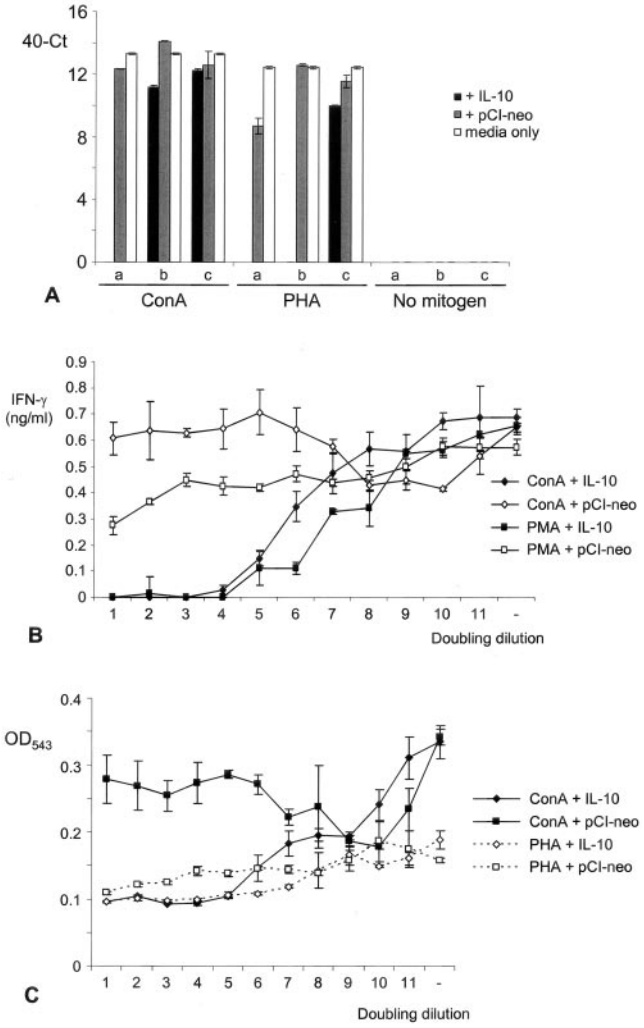

FIGURE 6. Effect of rchIL-10 on the ability of splenocytes to express IFN-γ after mitogen stimulation. Splenocytes were unstimulated (no mitogen) or were stimulated with Con A or PHA, with different concentrations of rchIL-10 ex-COS, equivalent supernatants from COS cells transfected with the expression vector alone (pCI-neo), or COS cell growth medium only. *A*, IFN-γ mRNA expression at 24 h as measured by real-time quantitative RT-PCR, expressed as 40 − Ct values. a, 1/10 dilution; b, 1/100 dilution; c, 1/1000 dilution. *B*, IFN-γ expression (nanograms per milliliter) at 72 h as measured by capture ELISA. The doubling dilutions start from a 1/10 dilution. −, growth medium only. *C*, IFN-γ expression (OD_{543}) at 72 h as measured by the HD11 bioassay. Dilutions are given in *B*. −, growth medium only.

Recombinant chIL-10 inhibits IFN-γ expression from mitogen-stimulated splenocytes at both mRNA and protein levels

We assessed the ability of rchIL-10 (ex-COS) to inhibit IFN-γ expression by splenocytes after mitogen stimulation (Con A and PHA) at both mRNA (TaqMan, Fig. 6*A*) and protein (ELISA (Fig. 6*B*) and bioassay (Fig. 6*C*)) levels. At the mRNA level (24 h poststimulation), a 1/10 dilution of rchIL-10 completely inhibited IFN-γ expression by splenocytes after stimulation with either Con A or PHA. At a dilution of 1/100, rchIL-10 completely inhibited IFN-γ expression after PHA stimulation of splenocytes, but was only partially effective after Con A stimulation. At a 1/1000 dilution, rchIL-10

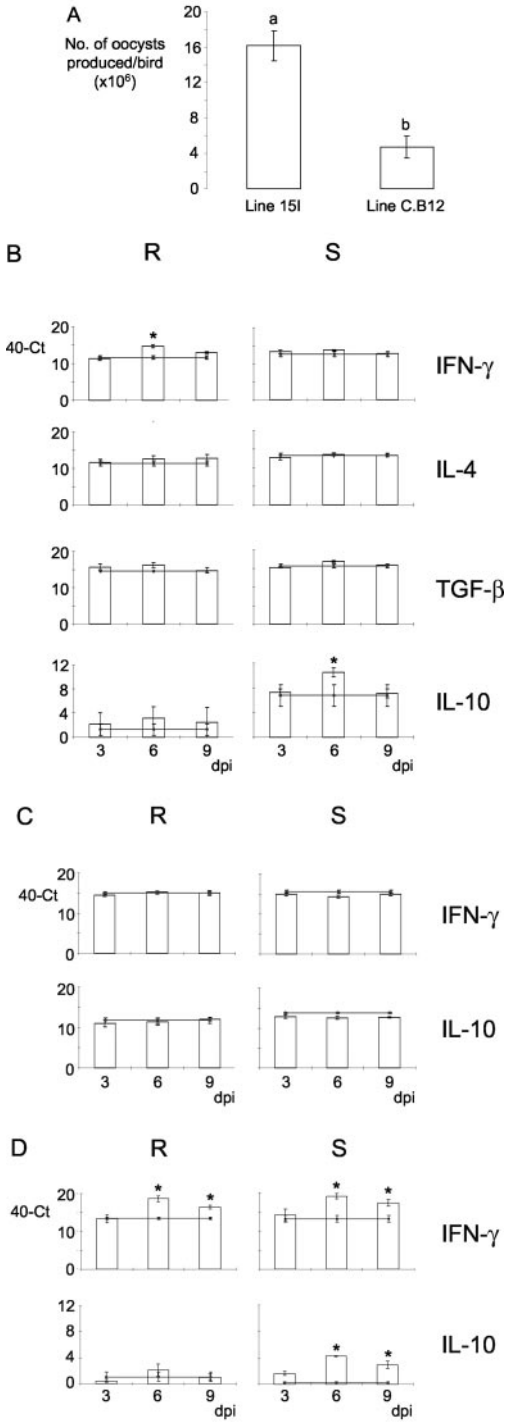

FIGURE 7. Cytokine mRNA expression patterns correlate with susceptibility to infection with *E. maxima*. *A*, Total oocyst output in resistant (line C.B12) and susceptible (line 15I) chickens. Different letters indicate values that differ significantly at $p < 0.05$. Cytokine mRNA expression was determined in the spleen (*B*), cecal tonsil (*C*), and small intestine (*D*) of resistant line C.B12 (R) and susceptible line 15I (S) chickens at 3, 6, and 9 dpi with *E. maxima*. Bars indicate cytokine levels in infected chickens on each of the days. Lines indicate levels in age-matched uninfected chickens at 6 dpi. ∗, Values differ significantly at $p < 0.05$.

was only partially effective in inhibiting IFN-γ expression after PHA stimulation and had no effect on IFN-γ expression after Con A stimulation.

At the protein level (72 h poststimulation), rchIL-10 inhibited the production of IFN-γ, as measured by ELISA, at high concentrations, and this effect titrated out with increasing dilution of rchIL-10. In general, Con A induced more IFN-γ than PHA. No IFN-γ was detected from unstimulated splenocytes (negative control; data not shown).

The bioassay measures the ability of IFNs, including IFN-γ, to stimulate NO production by a chicken macrophage cell line (HD11). Recombinant chIL-10 inhibited the production of NO-inducing agents (probably IFN-γ) after stimulation of splenocytes with either mitogen, although much less IFN-γ was produced after PHA stimulation of splenocytes. For both mitogens, the inhibition titrated out with increasing dilution of rchIL-10. Splenocytes stimulated with mitogen were not inhibited by the presence of supernatant from COS cells transfected with pCI-neo alone (data not shown).

Levels of expression of IL-10 mRNA correlate with susceptibility to infection with the intracellular protozoan E. maxima

E. maxima is a chicken-specific intracellular parasite that resides within enterocytes of the small intestine and induces high levels of IFN-γ in the spleen and gut (35, 37). Resistance to primary infection with *Eimeria* spp. has been shown to be absolutely dependent on the ability to express high levels of IFN-γ (61–64). Bearing in mind the negative influence of chIL-10 on chIFN-γ expression (see above), we chose to assess the expression of IL-10 (and other cytokines) in resistant (line C.B12) and susceptible (line 15I) chickens during the course of infection with *E. maxima*.

The level of resistance/susceptibility of lines C.B12 and 15I to infection with *E. maxima* was assessed by the total numbers of oocysts produced in the feces. The results depicted in Fig. 7*A* confirm the relative susceptibility of line 15I compared with line C.B12 chickens and are consistent with previously published data (28, 29).

We used real-time quantitative RT-PCR to assess the levels of mRNA of T1 (IFN-γ), T2 (IL-4), and regulatory cytokines (TGF-β4 and IL-10) expressed in spleen, cecal tonsils, and sections of small intestine from resistant (line C.B12) and susceptible (line 15I) birds at 3, 6, and 9 dpi with *E. maxima*. In terms of cytokine mRNA expression, in the spleen there were no significant differences between control and infected birds within the same line, between lines, or between time points for IL-4 and TGF-β4 (Fig. 7*B*). For IFN-γ, there was a transient increase in resistant birds at 6 dpi, above levels in age-matched control birds. The major difference between resistant and susceptible birds was for IL-10. Constitutive levels of IL-10 mRNA expression in the spleens of resistant birds were 43-fold lower than in the spleens of susceptible birds. There was also a transient increase in IL-10 mRNA levels in susceptible birds at 6 dpi, above levels in age-matched controls.

For gut tissues (cecal tonsils and small intestine), levels of mRNA expression were only measured for IL-10 and IFN-γ. For cecal tonsils, there were no significant differences between control and infected birds within the same line, between lines, or between dpi (Fig. 7*C*). In contrast, in the small intestine, IL-10 mRNA expression was increased significantly above levels in age-matched control birds after *E. maxima* infection in susceptible birds, but was not increased significantly in resistant birds (Fig. 7*D*). IFN-γ mRNA levels were increased significantly in the small intestine of both susceptible and resistant birds at 6 and 9 dpi (Fig. 7*D*).

Discussion

We isolated a chIL-10 cDNA from a cecal tonsil cDNA library and obtained the full gene sequence by direct sequencing of a BAC positive for chIL-10. Comparisons of the chIL-10 sequence at the

amino acid level with its mammalian counterparts show 45 and 42% aa identity with human and mouse IL-10, respectively, somewhat higher than the levels of identity between chickens and mammals for most cytokines cloned to date in the chicken (e.g., chIFN-γ has 32% amino acid identity with human IFN-γ (65) and chIL-18 has 30% amino acid identity with human IL-18 (66)), but similar to the 46% amino acid identity seen between human and chicken IL-12β (67).

In man, IL-10 has a secondary structure comprising six α-helices (68). The clear heptad repeats with hydrophobic amino acids at positions 1 and 4 in the assigned amphipathic helices (Fig. 1) suggest that overall the structure of chIL-10 is very much like that in the human.

We identified the chIL-10 promoter and some of the potential transcription factor binding sites that lie within it. In general, chicken cytokine gene promoters share high nucleotide identity with their mammalian orthologues (43, 67, 69–71). The chIL-10 promoter has little identity with the human IL-10 promoter or with the *Fugu* promoter. However, there is a prominent role for Sp1 during control of human IL-10 gene expression (72, 73), and there are multiple potential Sp1 binding sites in the promoter of the chIL-10 gene. We have shown that chicken IL-10 is expressed at the mRNA level, and that rchIL-10 has at least some similar biological activities to mammalian IL-10. It will be interesting to try to understand regulation of chIL10 expression because its promoter is very different from those of mammalian IL-10 genes.

RT-PCR analyses of lymphoid tissue samples from a line 7_2 chicken or activated lymphoid cells showed that chIL-10 was not expressed constitutively in the spleen or bone marrow, mitogen-activated splenocytes, or mitogen-activated bursal cells. In non-lymphoid tissues it was only detected in liver and lung. This pattern of expression mirrors that for IL-10 in human and mouse, but it is interesting to note that chIL-10 mRNA expression was not detected in mitogen-activated bursal cells when it was expressed in the bursa of Fabricius. Either IL-10 is being expressed in the bursa by non-B cells (e.g., stromal cells), or mitogen stimulation of B cells switches off IL-10 expression. In mammals, proliferating B cells and B cell lymphomas constitutively express IL-10 (74, 75).

Recombinant chIL-10, like mammalian IL-10, inhibits the expression of IFN-γ by mitogen-activated splenocytes. The results shown in Fig. 6 are from splenocytes coincubated with IL-10 and mitogen. Similar results were found when splenocytes were incubated in the presence of IL-10 for 6 h before addition of mitogen (data not shown).

Our IL-10 cDNA clone was isolated from RNA from a cecal tonsil of a chicken infected with *E. tenella*. As such, and considering the role of IL-10 in controlling the T1-T2 balance and in resistant/susceptible phenotypes during protozoan infections in mammals (76, 77), we examined the expression of IL-10 and other key cytokines (IFN-γ, IL-4, and TGF-β4) during the course of an *Eimeria* infection. Tissue samples included spleens, cecal tonsils, and sections of small intestine from birds sampled at 3, 6, and 9 dpi, from both resistant (line C.B12) and susceptible (line 15I) lines of chickens. Perhaps not surprisingly, because *E. maxima* does not infect the ceca, there were no significant differences in cytokine expression in the cecal tonsils between control and infected birds within the same line, between lines, or between dpi (Fig. 7*C*). For the small intestine, susceptible birds showed significantly increased expression of IL-10 mRNA after infection, whereas resistant birds did not. However, both lines of birds showed similarly increased expression of IFN-γ mRNA at 6 and 9 dpi (Fig. 7*D*), which may be considered surprising bearing in mind the differences in expression levels of IL-10 mRNA. Nonetheless, in susceptible birds the increased levels of IL-10 may counteract the protective effect of the IFN-γ, and this contributes to the inability of these birds to limit the growth of the parasite (Fig. 7*A*).

For the spleen, resistant birds up-regulated IFN-γ at 6 dpi compared with those in uninfected birds, whereas the expression of IL-4 and TGF-β4 mRNA was not increased in response to infection. The most informative result was that IL-10 mRNA levels in the spleens of uninfected, susceptible birds were 43-fold higher than those in the spleens of uninfected, resistant birds, suggesting that levels of constitutive expression of IL-10 mRNA may be dependent on host genetics. Susceptible birds also up-regulated IL-10 mRNA expression at 6 dpi.

Taken together, these data suggest that IL-10 may play a crucial role in changing the Th bias during infection with *E. maxima*, preventing the development of strong, IFN-γ-driven responses, which have been shown to be crucial for control of *Eimeria* infections.

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