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SYNTHESIS AND CHARACTERIZATION OF RAT INTERLEUKIN-10 (IL-10) cDNA* CLONES FROM THE RNA OF CULTURED OX8$^-$ OX22$^-$ THORACIC DUCT T CELLS

Richard E. Goodman, Jeb Oblak, and Robin G. Bell+

James A. Baker Institute, College of Veterinary Medicine, Cornell University, Ithaca, New York 14853

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SUMMARY A cDNA of the complete coding region of rat IL-10 was cloned and sequenced using RNA isolated from a cultured population of thoracic duct T-lymphocytes obtained from *Trichinella spiralis* infected animals. The OX8$^-$ OX22$^-$T-helper cells were stimulated *in vitro* with Concanavalin A for 24 hours prior to harvest. Reverse transcription of cellular RNA was primed with oligo-dT followed by amplification of IL-10 specific cDNA by polymerase chain reaction with synthetic oligo nucleotide primers chosen from two highly conserved regions of mouse and human IL-10. The sequence of the coding region of the amplified, cloned rat IL-10 cDNA is 90% identical to the mouse and 82% identical to the human IL-10 cDNA coding regions.

Mouse interleukin 10 (mIL-10) was originally named cytokine synthesis inhibitory factor (CSIF) due to the inhibitory activity of this molecule on the synthesis of interferon gamma (IFN-γ) by a mouse T cell clone (1). Analysis of the cDNA and protein sequences of mIL-10 indicated striking homology with a partially characterized product, BCRFI, encoded by the Epstein-Barr virus genome (2). A human cDNA of IL-10 (hIL-10) was characterized and shown to be nearly 73% identical in deduced amino acid sequence with the deduced amino acid sequence of mIL-10. Both hIL-10 and BCRFI have been shown to inhibit IFN-γ production (3). Murine IL-10 has been shown to stimulate proliferation of CD4$^-$ CD8$^-$ thymocytes (4). Human monocytes, in response to LPS stimulation, produce IL-10 which may function in an autoregulatory role by down regulating class II MHC molecules and inhibiting the production of proinflammatory cytokines (5). During parasitic infections the establishment of resistance or

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+To whom correspondence should be addressed at JABIAH, College of Vet. Medicine, Cornell University, Hungerford Hill Rd., Ithaca, NY, 14853. FAX: (607) 277-8399.

Abbreviations: BCRFI, Epstein-Barr virus open reading frame I; CSIF, cytokine synthesis inhibitory factor; Con A, concanavalin A; IL-, interleukin-; RT-PCR, reverse transcriptase-polymerase chain reaction.

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acute disease appears to be at least partly determined by the dominance of IL-10 or IFN-γ, depending on the specific parasite (6,7).

Our preliminary studies examining the cytokine profile of a protective T helper cell population ($OX8^-$ $OX22^-$) isolated from the thoracic duct of rats infected with the nematode *Trichinella spiralis* (8), indicated that these cells exhibit some characteristics of Th2 phenotype (9). Stimulation of the isolated cell population in vitro with Con A or *T. spiralis* specific antigen, led to the secretion of factors that caused isotype switching of B cells from IgM to IgE, an IL-4 response, and the differentiation of eosinophils from bone marrow cells, an IL-5 response (unpublished, K. Ramaswamy and R. Bell). Analysis of RNA from these cells by reverse transcriptase-polymerase chain reaction (RT-PCR) using primers for IL-2, IL-4, IL-5 and IFN-γ indicated that mRNA of all four cytokines were present in the population (unpublished, R.Goodman). Identical analyses of the non-protective T cell population ($OX8^-$ $OX22^+$) which was isolated concurrently from the same animals and stimulated identically, produced a similar mRNA profile but those cells were unable to elicit eosinophil differention even though IL-5 mRNA was present. Both cell populations contain significant levels of IFN-γ mRNA. To determine whether the differences between these cell populations where in part due to differences in IFN-γ or IL-10 production, we have developed a RT-PCR assay for rat IL-10 using an oligonucleotide primer identical to the translation initiation coding region of mIL-10, having three mismatches with human IL-10, and an antisense oligonucleotide for the 3'-untranslated region which is complementary to mIL-10, but differs in the distal half from hIL-10. To verify the identity of the product we have cloned and sequenced the resulting cDNA and report our findings here.

MATERIALS AND METHODS

Cellular RNA was isolated from pulverized frozen rat tissues and from separated Con A stimulated $OX8^-$ $OX22^-$ thoracic duct T lymphocytes (8) of *T. spiralis* infected AO rats, by the guanidine/acidic phenol:chloroform method of Xie and Rothblum (10). RNA concentrations were determined by measuring UV absorbance at 260 nm. The integrity of the RNA was checked by visualizing the ethidium bromide stained rRNA bands in the formaldehyde denaturing gel used for the Northern blot which was performed as described earlier (11).

Oligonucleotides for IL-10 were selected by comparing mouse (1,13) and human (3) sequences to identify highly conserved regions. Specific sequences of the oligos are identical to those of mouse IL-10 at the translation initiation site in exon 1 (oligo#1, 5'-d[catgcctggctcagcactgc], sense strand), in the 3'-untranslated region (oligo#2, 5'-d[gggaactgaggtatcagagg], antisense strand), and in mid-exon 1 (oligo#3, 5'-d[gactgggaagtgggtgcag], antisense strand). Oligo#1 and oligo#2 were used as primers for polymerase chain reaction (PCR) amplification. Oligo#3 was labeled and used to probe Southern blots.

RT-PCR reactions were performed to clone rat IL-10 and to detect small quantities of IL-10 mRNA using cellular RNA, oligo#1 and oligo#2 (above) and a GeneAmp RNA PCR Kit (Perkin-Elmer) following the manufacturers directions. To clone the rat IL-10 cDNA, total RNA was used from $OX8^-$ $OX22^-$ cells (above) which were stimulated in vitro with Con A for 24 hours before collection. First strand synthesis of 0.5 μg of RNA was performed at 42^oC using Oligo-dT as the primer. PCR was accomplished by the addition of AmpliTaq DNA Polymerase and oligonucleotide primers #1 and #2 (to a 250 nM final concentration of each),

followed by 35 cycles of amplification (94°C, 45 s; 50°C, 45 s; and 72°C, 80 s) and a final primer extension at 72°C for 5 minutes. Comparative IL-10 RT-PCR assays using cellular RNA from various tissues were performed in an identical fashion except the PCR was stopped after 25 cycles and identical samples of 0.03 ug of RNA were also analyzed. Southern blotting for detection of the amplified IL-10 cDNA products was accomplished electrophoresis of samples in a 3% NuSieve 3:1 agarose gel (FMC) with 1 X Tris-acetate-EDTA buffer (15), denaturation of the DNA with sodium hydroxide and neutralization with Tris base (pH 8) followed by capillary transfer to GeneScreen Plus membranes (DuPont-New England Nuclear) with 10 X SSC (12). Blots were prehybridized and hybridized in the same solution (20 mM Tris, pH 8; 6 X SSPE ; 1 mM EDTA; 5 X Denhardt's solution; 50 μg/ml sheared, denatured Salmon sperm DNA; and 0.5% SDS) at 50°C. Oligo#3 was labeled with [^{32}P]cordycepin-5'-triphosphate (DuPont-NEN) using Terminal deoxynucleotide transferase (Promega) as described by Tu and Cohen (14) for use as a probe. Hybridization was at 50°C overnight. Unbound probe was washed off with two incubations each in 6 X SSPE, 0.1% SDS at room temperature and at 50°C. Bands were detected by exposing X-OMAT AR film (Kodak) with washed membranes. Band sizes were determined by comparing migration distances with bands from a 123 bp sizing ladder (Gibco-BRL) electrophoresed beside RT-PCR samples and detected by ethidium bromide staining.

To clone the IL-10 cDNA, a sample from RT-PCR was electrophoresed through 1.0% SeaKem GTG agarose (FMC), with 1 X TAE, then excized from the gel after ethidium bromide staining, and purified with Geneclean (BIO 101). The cDNA, approximately 680 bp long, was ligated with pT7Blue(U) plasmid DNA (Novagen, Inc.) using T4 DNA Ligase (New England Biolabs). NovaBlue competent *Escherichia coli* were transformed with ligation products and recombinant colonies were selected on Ampicillin/Tetracycline plates containing color substrates for the Lac Z gene product. White colonies were screened by hybridization with ^{32}P-labeled oligo#3 to select IL-10 bearing plasmids which were amplified and purified from "mini-preps" (15).

Plasmids were denatured with sodium hydroxide, and sequenced with Sequenase 2.0 (U.S. Biochemicals) using vector specific primers T7 5'-d(taatacgactactataggg) and M13 Forward-40 5'-d(gttttcccagtcacgac), with separation by electrophoresis through a 5% HydroLink Long Ranger gel (J.T.Baker) and as described earlier (11). Sequencing reaction products were detected by autoradiography and were compared with mouse and human IL-10 sequences using the ALIGN program (Scientific and Educational Software, State Line, PA). Protein structure calculations for the deduced mature protein were accomplished using the IBI Pustell program (Kodak, New Haven, CT).

A Northern blot of cellular RNA from rat brain and Con A stimulated T cells was performed as described earlier (11). The cloned rat IL-10 cDNA insert was labeled with [^{32}P]dATP by random primed cDNA synthesis using a Prime-a-Gene kit (Promega) for use as a probe. The IL-10 mRNA size was determined by comparing the migration distance of the detected band with the distances of 28S and 18S rRNA bands detected by ethidium bromide staining of the original gel.

RESULTS AND DISCUSSION

Reverse transcription and PCR amplification of RNA from Con A stimulated OX8$^-$ OX22$^-$ T cells from *T. spiralis* infected rats, using the mouse IL-10 primers, produced a 682 bp cDNA which hybridized specifically to the internal oligo#3 of mouse IL-10. Ligation of this fragment into a pT7Blue plasmid followed by transformation into *E. coli* produced five clones with approximately 680 bp inserts which were partially sequenced to determine the orientation of the clones within the plasmids. Two clones (pIL10-30 and pIL10-39) which had been inserted in the opposite orientation relative to the multiple cloning site were sequenced completely using

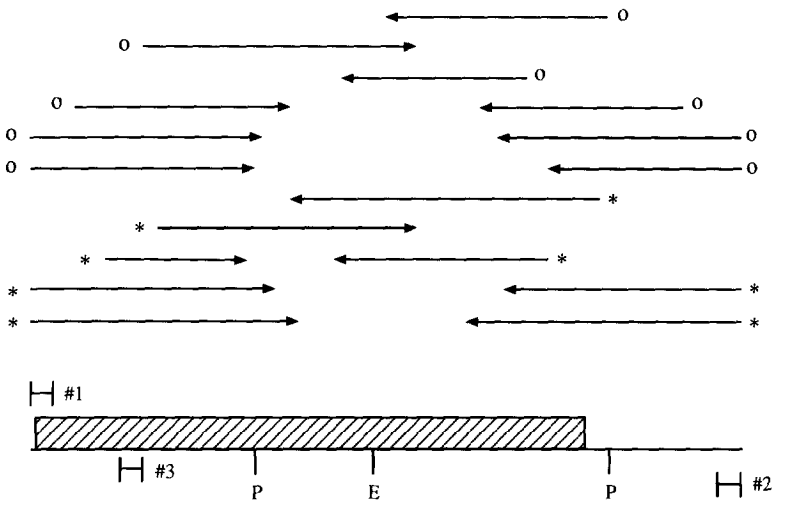

Figure 1. Diagram of the cDNA clone, cDNA sequencing strategy, restriction enzyme sites and deduced protein coding region for rat IL-10. The full sequence of 682 bases includes a single open reading frame (hatched) with a translation initiation codon at base 2 and a termination codon at base 538. The first and last 20 bases of the cDNA represent mouse IL-10. There are two Pst I (P) and one Eco RI (E) restriction sites in the cDNA. The positions of the three oligonucleotides are indicated. Sequence reactions were in the direction indicated by the arrows and represent reactions from pIL10-30 (o), which is oriented with the 5'-end at the T7 end of the multiple cloning site, and pIL10-39 (*), in which the cDNA is oriented oppositely, with the 5'-end near the universal-40 primer site.

replicate sequencing reactions of each strand, with multiple gel loadings to produce a continuous sequence as indicated in Figure 1. The sequence determined for these two clones contained a single open reading frame of 537 bases beginning at base 2, followed by 144 bases of the 3'-untranslated region (rIL-10 in Fig. 2). Other than orientation, the only difference between the two clones is that pIL10-39 is missing the "T" overhang that should exist at the ligation site adjacent to the 5'-end of the cDNA.

Comparison of the resulting open reading frame of this sequence with the coding region of mouse and human IL-10 sequences (1,3) indicated that it is 90% identical to the mouse and 82% identical to human coding region sequences (Fig. 2). However, the first and last 20 nucleotides of our cDNA actually represent mouse IL-10 and could differ slightly from the actual rat sequence. Comparison of the three species indicates unusually strict conservation of the 3' untranslated region in the IL-10 gene. As suggested by Moore et al. (1), there are a number of "AT" rich regions in the 3'-end of mIL-10 which may be involved in regulating mRNA stability and the most proximal of these is conserved in both sequence and position in rat IL-10 (Fig. 2, bases 654-658). In addition, significant regions of homology exist between the 3'-untranslated regions of mIL-10 and hIL-10 beyond the extent of the rIL-10 cDNA (not shown), which include the "AUUUA" motif involved in regulated degradation of some mRNA's (16), suggesting that there is post-transcriptional regulation of IL-10 mRNA.

The deduced amino acid sequence (Fig. 3) should be full length at 178 amino acids, based on the mouse and human sequences. The probable signal peptide cleavage site was predicted

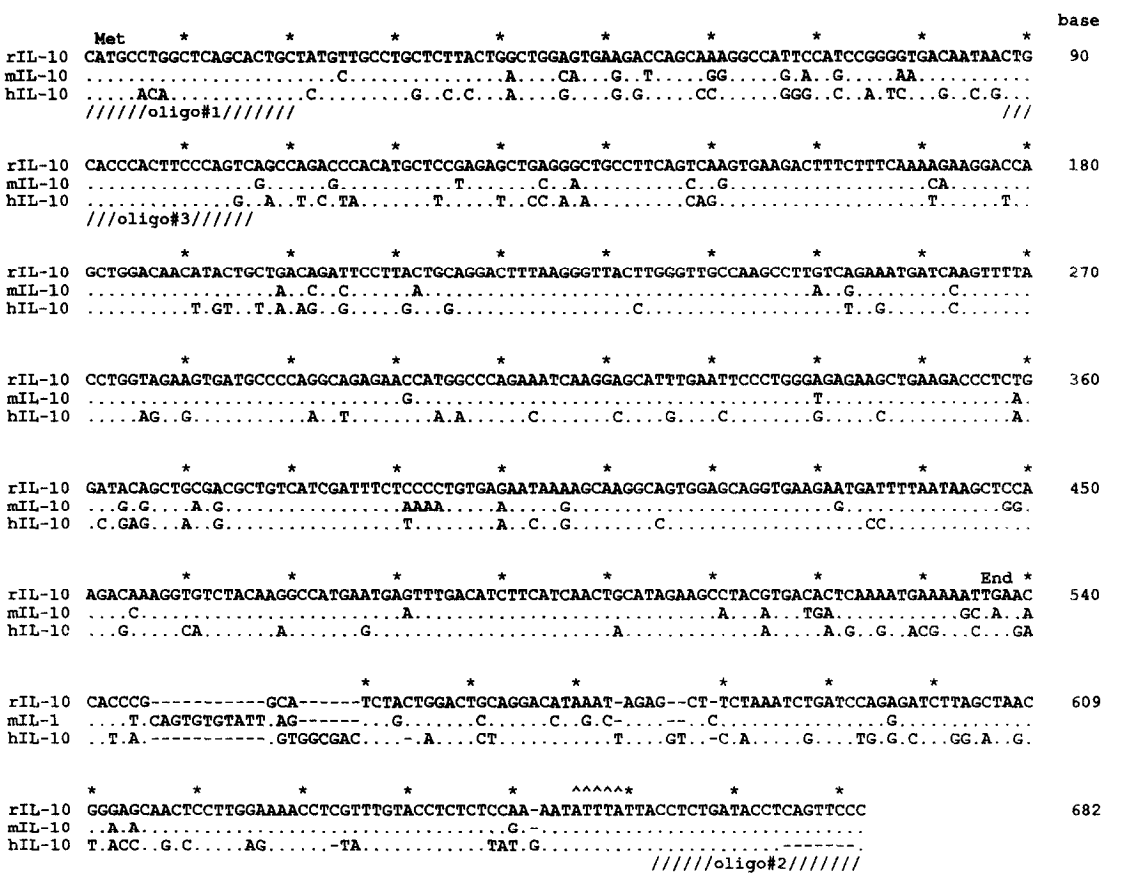

Figure 2. Nucleotide sequence of rat IL-10 cDNA and comparison with mouse and human IL-10 cDNAs. The complete nucleotide sequence of the rat IL-10 cDNA is shown, with the similarities (.) and differences (base code) and gaps (-) in alignment of the mouse and human cDNA sequences are indicated for the corresponding region of the cDNAs. The start codon (Met) and termination (End) are shown. Oligonucleotide positions (hatched) are indicated for the PCR primers (#1 and #2) and for the label used in Southern blots (#3). A putative destabilization signal is marked (^^^^^).

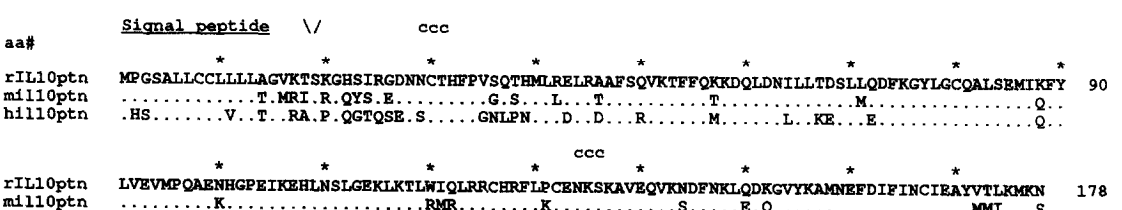

Figure 3. Homology alignment of the deduced amino acid sequences of rat, mouse and human IL-10 peptides. The full-length deduced amino acid sequence of rat IL-10 is shown with the similarities in the mouse and human proteins indicated (.) as well as the differences. The most likely signal sequence clevage site is indicated (V) after amino acid 18, as are the potential N-linked glycosylation sites of mouse and rat IL-10 (ccc).

based on frequency calculations (17) to occur after amino acid 18, leaving a mature size of 160 amino acids. The predicted molecular weight for the mature protein is 18.6 kDa however, there are two potential N-linked glycosylation sites (Fig. 3) which, if utilized in the native protein, would alter the molecular weight. The first potential N-glycosylation site is present in the rat and mouse but not human deduced protein sequences, whereas the second site is present in all three. The calculated pI of the mature rat protein is 9.1, and the calculated pI values for mouse is 8.7 and the human is 8.2. There are four conserved cysteine residues in the mature IL-10 peptide from all three species, and a fifth in rat and mouse, however the native proteins will have to be analyzed to determine if there are any internal or external disulfide bridges. Comparison of the deduced amino acid sequences of the three IL-10s indicates that mouse and rat share 83% identity while human and rat have 73% identity (Fig. 3). Mouse and human IL-10 peptide sequences match at 72% of the amino acid residues.

The intact mRNA of rat IL-10 is approximately 1700 bases as determined by the position of a single detectable hybridization band relative to the positions of 28S and 18S RNA bands in a Northern blot of cellular RNA from Con A stimulated $OX8^-$ $OX22^-$ T cells (20 μg RNA). This size is similar to that of hIL-10 mRNA (3). IL-10 mRNA was not detected by Northern blot analysis of 20 mg total RNA samples from brain, Peyer's patch, upper small intestine and freshly isolated thoracic duct T cells from *T. spiralis* infected rats (not shown).

RT-PCR results analyzing RNA from freshly separated, Con A stimulated and *T. spiralis* antigen stimulated populations of thoracic duct $CD8^+$ and $CD8^-$ T cells indicate that IL-10 mRNA is expressed in the Con A stimulated cells in far greater abundance than the other populations (not shown). Whether the protein is expressed in a similar pattern will be tested when a suitable mAb is prepared.

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