

CLONING AND CHROMOSOME MAPPING OF THE HUMAN INTERLEUKIN-1 RECEPTOR ANTAGONIST GENE

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By screening a human genomic library with an interleukin-1 receptor antagonist (IL-1ra) cDNA probe, we have isolated a 15 kb clone which contains the entire coding region of the gene as expressed in monocytes, and includes 6 kb of 5'-upstream sequence. The gene contains four exons which code for the secreted form of the IL-1ra, however, our clone does not contain the alternative first exon used to generate an intracellular form of the protein as found in epithelial cells. Analysis of the sequence reveals a consensus TATA box, and three Alu repeats, two of which are in the upstream region and one in intron 3. The sequence also reveals an 86 bp motif tandemly repeated four times within intron 2, and may reflect the polymorphism known to exist in this region of the gene. By in-situ fluorescence hybridization we have shown that the IL-1ra gene is found on the long arm of chromosome 2 and maps to 2q13-14.1. Previous studies have revealed that IL-1 α , IL-1 β and both type I and type II forms of the IL-1 receptor all map close to this region of chromosome 2.

The increasingly complex cytokine network involves both positive and negative regulatory pathways. Natural inhibitors of cytokine function are of great importance both as analytical tools and as potential therapeutic agents. These inhibitors include soluble extracellular domains of cytokine receptors that may arise by proteolytic cleavage, as in the case of tumour necrosis factor (TNF) receptors,¹ or as alternatively spliced transcripts of the functional transmembrane receptor as in the case of IL-4² and IL-7³ receptors. Another class of inhibitor are true cytokine receptor antagonists, the best characterized of which is the interleukin-1 receptor antagonist (IL-

1ra). The IL-1ra protein was first purified and characterized from human monocyte conditioned medium and the corresponding cDNA subsequently cloned.⁴⁻⁶ The IL-1ra produced by monocytes is a secreted protein structurally related to IL-1 α and IL-1 β that forms a non-productive complex with the IL-1 receptor (IL-1R). Although IL-1ra activity was first characterized from monocytes, it is also expressed in neutrophils and epithelial cells.⁷ Interestingly, epithelial cell IL-1ra is expressed as an alternatively spliced transcript of the monocyte IL-1ra which lacks a leader peptide and consequently is not secreted from the cell. Both IL-1 and IL-1ra bind to the type I and type II IL-1 receptors with comparable affinities,⁸⁻¹¹ and therefore the receptor antagonist is an effective inhibitor of IL-1 induced effects. Indeed a large number of cases have now been reported in which recombinant IL-1ra has been used both in vitro and in vivo to block a range of IL-1-induced biological activities.¹² However the precise physiological relationship between IL-1 and IL-1ra production in normal and pathological processes is as yet poorly understood.

Since monocytes synthesize both IL-1 and the IL-1ra, the pro-inflammatory effects of IL-1 may, in

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part, be counterbalanced by the anti-inflammatory effects of the IL-1ra. Initial studies have suggested that in human peripheral blood monocytes expression of IL-1 β and IL-1ra are differentially regulated;¹³ however, at present little is known about IL-1ra gene regulation. The IL-1ra has been isolated from monocytes stimulated in three different ways; peripheral blood monocytes adhered onto IgG-coated plates,^{4-6,14} phorbol myristate acetate (PMA)-differentiated THP-1 cells¹⁵ and PMA differentiated U937 cells treated with granulocyte macrophage-colony stimulating factor (GM-CSF).⁶ The receptor antagonist may also be induced by LPS¹³ and its expression enhanced by monocyte differentiation and prolonged treatment with GM-CSF.¹⁶ There is clearly a complex relationship between IL-1 and IL-1ra production which may involve the state of differentiation of the monocyte, the particular set of external stimuli to which the cell is exposed and a range of transcriptional and post-transcriptional regulatory mechanisms. To begin studying the regulation of human IL-1ra gene expression in monocytes we report on the cloning and sequencing of the complete gene including six kilobase pairs of 5'-upstream sequence.

RESULTS AND DISCUSSION

Using an interleukin-1 receptor antagonist cDNA probe we screened 1.7×10^6 plaques of a human genomic DNA library in λ -EMBL 3 and isolated two

positive clones (see Materials and Methods). By Southern blot analysis of restriction enzyme digests, a 15 kb clone containing the complete cDNA sequence and the longest 5'-upstream sequence was identified for further characterization. Figure 1 shows the gene organization and restriction enzyme maps for our human IL-1ra clone which includes 6 kb of 5'-upstream sequence. Based on the restriction enzyme map of our clone, we generated a series of overlapping subclones (Fig. 1B) and sequenced the entire gene and 5'-upstream DNA. The sequencing revealed, in agreement with recent results from Eisenberg et al.,¹⁷ that the gene contains four exons (Fig. 1A). The 5'-untranslated sequence and sequences encoding the leader peptide and the first 14 amino acids of the mature protein are found in the first exon. The fourth exon contains the C-terminal 70 amino acids and the entire 1144 bp of untranslated 3'-sequence. As previously reported this gene architecture bears a striking resemblance to the organization of the IL-1 β and IL-1 α genes. The IL-1ra gene is therefore thought to have arisen from a common ancestral gene to the IL-1 β and IL-1 α genes and is hence a member of the IL-1 gene family.¹⁷

The sequence of 12.5 kb of our clone, including 6 kb of upstream DNA, is shown in Fig. 2. Computer analysis of the upstream sequence revealed a consensus TATA-box 38 bp (-38) from the start of the cDNA sequence (+1). Searches for transcription factor consensus motifs revealed four possible AP1 binding sites (-49, -1821, -3320 and -4665), two

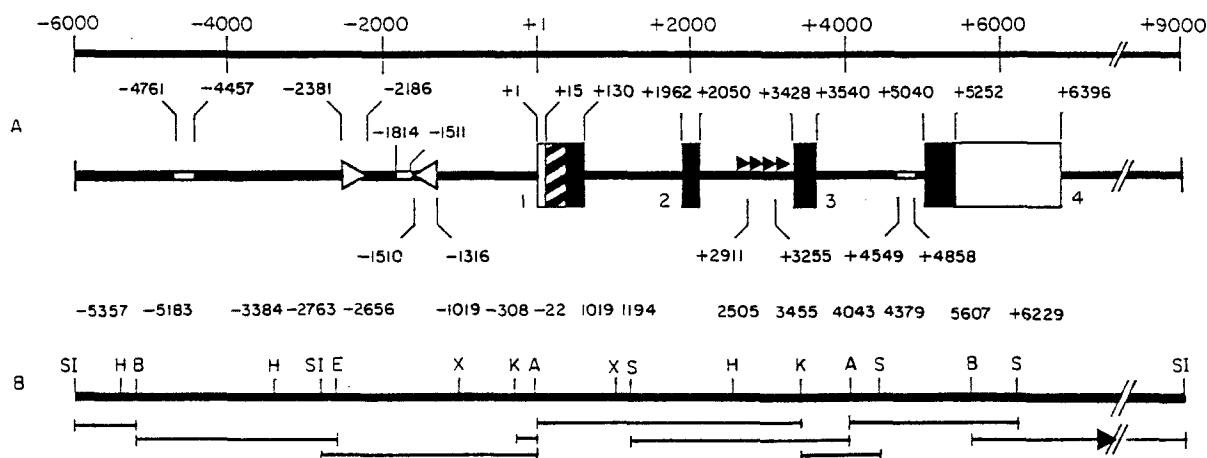


Figure 1. Organization of the interleukin-1 receptor antagonist gene.

(A) Structural map of the 15 kb *SalI* clone. Diagrammatic representation of the 15 kb *SalI* clone containing the interleukin-1 receptor antagonist gene. The gene is organized into four exons which are represented by large boxes. Solid boxes represent sequences encoding the mature IL-1ra protein, open boxes represent the untranslated sequences in the cDNA, and the dashed box represents the sequences encoding the leader peptide. Tandem repeats (+2911 to +3255) and inverted repeats (-2381 to -2186 and -1510 to -1316) are indicated as solid arrowheads and open arrowheads, respectively. The three Alu sequences are illustrated by small open bars (-4761 to -4457, -1814 to -1511 and +4549 to +4858). Nucleotide positions are marked relative to the start of the published cDNA (+1).⁵ The dimensions of the boxes are an approximation, and not a precise reflection of their actual size. (B) Restriction map of the IL-1ra gene and subclones. The following restriction sites appear in the 15 kb *SalI* clone, and were used to generate a set of overlapping subclones for sequencing, as shown by the bars beneath the restriction map. *Apal* (A), *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Kpn*I (K), *Sac*I (S), *Sal*I (SI), *Xba*I (X).

TCTCCCCACTCTGACCTTTATGTTTGTCTGGGCCGAGGCTGCAAGTCTGGGCTCTGTGGGTGATGAGTGACAAGTCTCTCCCTTCCAGATATGGGGA
GTCTGCTTCCCTAGGTTGCCTCTCCCTGCTCTGATCAGCTAGAAGCTCCAGGAGATCCTCCTGGAGGCCCCAGCAGGTGATGTTTATCCCTCCAGACTGA 1010
GGCTAAATCTAGAAACTAGGATAATCACAAACAGGCCAATGCTGCCATATGCAAGACACTTTGGTTTGCTGGCCAGCCCTCGTCGAGCATGTGGGCTCT
TCAGAGCACCTGTAGGTGGGTACAGTTAGCCACACTTCACAGGTGAAGAGGTGAGGCACAGGTCAGGTCAGGCTGGCCGGAGCTCTGTTTATTACG
TCTCAGAGCTTTGAGTCTGCTCTCAACGAGAGAGGCCCTTTACCAAGAGAAGGATTTGGGACCCGATCAAGGTCAGCTGGCTGAGGTAGAGAGAAGC
CGGGTTGTTCCCAAGGGTAGCTGCTCTCAACGAGGACTCTGAGCAGGTCAACCAGTAATGGAGGAAAGGCTCTAGGGAAGACCCCTTCTGGTCTCAGACTCA
GAGCGAGTTAGCTGCAAGGTGTTCCGTCTCTTGAACACTTCTACCTAGGTGCTATGGTAGCCACTAGTCTCAGGTGGCTATTTAAATTTATACTTAAATGA 1510
ATGAAAAATAGAAGAAATTTAAATTCAGACCCCTTGTCAACATATCCACATTTAAAGAGGTCAATAGGCCACATGTGGTTAGTGGCCACCCTATTGGGCA
GTGACAGCTACAGAACTTTTGTACCCGAAAGTCTCTTTGGATGTTTCTGCTCTACAGCATGCTTTGCTGAAACAGAAGTGCCCTCCCTGGGAATCTC
AGATGGGAAGCAAGTAAAGAGGGGAGTCAAAATGTGGGCTCAGCTGCTGAGGTTGAGGGTTGGGCTGCCTCTTAAACATGTTCAGCCCTCAGTCTTCT
CATCCATCGATGCGCGTGGGTATACTAAATACTATACCCCTGGAAGAGCTGGAATGCAAAATTGACAAGTTCTGGGGGACACAGGAAGGTGCCAAGCACAA

C/EBP sites (−461 and −5083) and single SP1 (−1564), NF- κ B (−2034), AP2 (−5684) and E4TF1 (−3668) sites within the 5′-upstream sequence. Interestingly, phorbol esters, which activate both the AP1 binding Fos-Jun complex and NF- κ B, are one of the few known activators of IL-1ra gene expression. No other full consensus sequences to common transcription factor binding sites were identified. Inspection of the 3′-untranslated sequence revealed a second (+6136) possible consensus polyadenylation site 244 bp upstream to the first polyadenylation site (+6380) though there is no evidence for an alternative 3′-end using the second signal. Similar to the human IL-1 α and IL-1 β genes, our clone for the IL-1ra gene contains three Alu-type sequences, two are present in the 5′-upstream sequence from −4761 to −4457 and another from −1814 to −1510 and one is in the third intron from +4549 to +4858.

Matrix homology analysis within the IL-1ra gene sequence revealed an 86 bp sequence (see Fig. 2) repeated perfectly four times as a tandem repeat within intron 2. Polymorphism is known to exist within the second intron, resulting in three alleles varying in the size of the intron (A1, A2 and A3) as determined by polymerase chain reaction and agarose gel electrophoresis.¹⁸ Our sequence data allow us to determine a precise size for intron 2 of 1377 bp, which lies between the sizes determined for the more predominant A2 and A3 alleles. The functional significance of this polymorphism is as yet unknown. We have also detected an 87% homologous inverted repeat of 195 bp in the 5′-upstream sequence from −2381 to −2186 and from −1510 to −1316.

Recently, it has been shown that epithelial cells express an intracellular form of the IL-1ra which is predicted to arise from the use of an alternative transcriptional start site and a splicing event which replaces the first exon. This rearrangement removes the amino-terminal leader sequence of the protein, consequently blocking secretion via the normal route and resulting in intracellular accumulation.⁷ Although we have sequenced 6 kb of upstream sequence, we have not found the alternative transcription start site and predicted DNA sequence which is specific for this intracellular form of the IL-1ra. Consequently, it appears that the alternative start site used for the epithelial IL-1ra transcript is greater than 6 kb upstream from the start site of the monocyte IL-1ra transcript.

The human interleukin-1 α and IL-1 β genes have been mapped to the same region of chromosome 2, 2q13–21.^{19,20} Furthermore, both type I and II human IL-1 receptors also map to an overlapping region of the same chromosome, 2q12–22 and 2q12, respectively.^{21,22} We therefore mapped the chromosomal

location of human IL-1ra by in-situ fluorescence hybridization (Fig. 3). Simultaneous visualization of banded chromosomes and the biotinylated probe accurately mapped the IL-1ra to chromosome 2q13–14.1, that is, the same region as IL-1 α , IL-1 β and both IL-1 receptors. Chromosomal linkage of ligands and their receptors is very unusual, and the possibility of a functional significance for a cluster of interleukin-1 related genes is intriguing. However, the IL-1 receptor/ligand linkage is not conserved in the mouse since IL-1 α and IL-1 β map to a different chromosome to the receptors. Nevertheless, it remains possible that there is a degree of coordinate expression for human IL-1 related receptor and ligand genes that requires proximity of the genes.

MATERIALS AND METHODS

Screening of the Human Genomic Library

The complete IL-1ra cDNA (British Biotechnology Limited) was removed from the pUC18 vector sequence by *Eco*RI and *Bam*HI cleavage and isolated by low melting point agarose gel electrophoresis. Gel embedded IL-1ra cDNA was labelled with [α -³²P]CTP using standard Multiprime procedures (Amersham International). The probe was used to screen a human genomic DNA library kindly provided by Dr M.F. Ho (Institute of Molecular Medicine, Oxford) by plaque hybridization. The library was constructed from a partial *Mbo*I digest of human genomic DNA ligated into the EMBL 3 lambda vector.²³ Hybridization was carried out at 55°C in 5 × SSC, 0.5%

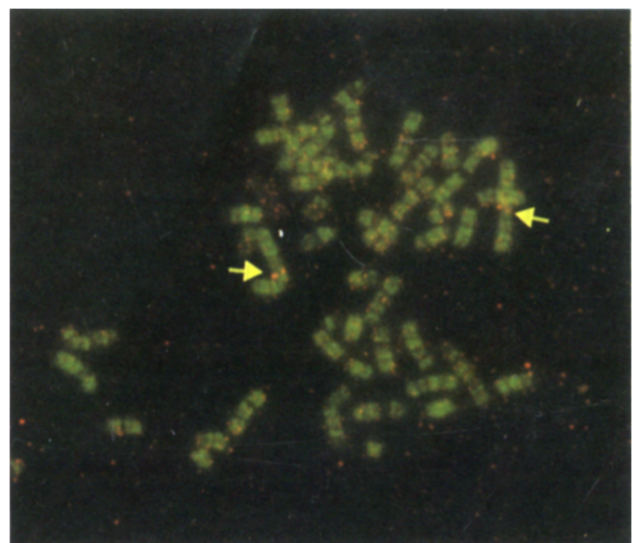


Figure 3. Fluorescence in situ hybridization of the interleukin-1 receptor antagonist to human chromosomes.

Arrows indicate hybridization of IL-1ra probe to chromosome bands 2q13–14.1.

SDS, 5 × Denhardt's solution and 20 µg/ml denatured salmon sperm DNA. Filters were washed at 55°C in 2 × SSC, 0.1% SDS. Following three rounds of hybridization, DNA from individual positive plaques was prepared from plate lysates using polyethylene glycol precipitation of phage particles followed by phenol–chloroform extraction.

Restriction Enzyme Mapping, Subcloning and Sequencing

Phage DNA was digested with a variety of restriction enzymes. Restriction fragments were resolved on 0.8% agarose gels, and subsequently denatured in alkali followed by transfer to Nylon membranes (Hybond N, Amersham International) and fixation under ultraviolet light using a Stratalinker (Stratagene). Southern blots were probed using the IL-1ra cDNA sequence described above. DNA fragments for subcloning were electroeluted and cloned into Bluescript (Stratagene) or pGEM vectors (Promega). Double-stranded, 'mini-prep' DNA from subclones was sequenced by dideoxy chain termination (Sequenase, United States Biochemical) after annealing of commercial or in-house synthesized oligonucleotide primers to alkali-denatured plasmids.²⁴ Oligonucleotides were synthesized on an Applied Biosystems Model 392 oligonucleotide synthesizer using standard phosphoramidite chemistry. Sequences were compiled, aligned and analysed with a DNASTar digitizer and software (DNASTar Inc.), and further computer analyses were performed using Microgenie DNA analysis software (Beckman Inc.). All other standard DNA manipulations were performed as previously described.²⁵

Fluorescence in-situ Hybridization

Peripheral blood lymphocytes were grown for 72 h, 200 µg/ml bromodeoxyuridine (BrdU) was added and the lymphocytes left for a further 16–17 h after which the samples were washed and medium containing thymidine (2.5 µg/ml) added. The cells were harvested 5 hours later and slides prepared and stored at –20°C. Prior to denaturation, the slides were baked at 65°C for 2–3 h. IL-1ra DNA was nick-transcribed and biotinylated (BRL, Bionick), purified through a Sephadex G50 column and then precipitated. Hybridization and detection were modifications of published procedures.²⁶ Labelled probe (120 ng) was mixed with 3 µg Cot 1 DNA (BRL). After denaturation, preincubated probe was placed on a denatured slide and incubated at 37°C for 40 h. Slides were washed, dehydrated and biotin signal detection carried out by incubation for 30 min, at 37°C with 2 µg/ml Texas red-conjugated avidin DCS (Vector Labs) in 4 × SSC, 0.05% Tween 20, 5% low-fat milk (Marvel) pH 7.0 (SSCTM). The signal was then amplified by incubation with biotinylated anti-avidin (Vector Labs; 5 µg/ml in SSCTM), and a second round of Texas red-conjugated avidin DCS (2 µg/ml in SSCTM). Incorporation of BrdU was detected by incubation in 100 µg/ml FITC-conjugated mouse monoclonal anti-BrdU antibody (Boehringer Mannheim) in SSCTM at 37°C for 30 min. Slides were then washed, dehydrated and mounted in Citifluor (Citifluor Ltd, London, UK) containing 0.06 µg/ml 4, 6-diamidino-2-

phenylindole (DAPI). Texas red signals on FITC-banded chromosomes were visualized simultaneously using a dual-band pass filter (Omega). Photographs were taken in Fujicolor HG400 film with a Zeiss Axiophot Photomicroscope.

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Note Added in Proof

The nucleotide sequence data recorded in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X645320

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