



Structural characterisation of the distal 5' flanking region of the human interleukin-10 gene

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Interleukin-10 (IL-10) is an important immunoregulatory cytokine. The recent characterisation of the proximal 5' flanking region of IL-10 led to the identification of the promoter region. Two polymorphic dinucleotide repeats and 10 single nucleotide polymorphisms (SNPs) have been identified and suggested to be useful genetic markers in several diseases. We have sequenced a further 5275 bp from –9296 to –4021 of the distal part of the 5' flanking region of the human IL-10 gene from the cosmid clone pWE15-4/11. Our sequence analysis reveals a high density of Alu-repeats within the IL-10 gene locus, including three novel, related structures which we term Alu-IL10 (A-C). Using three overlapping PCR products spanning 5110 bp of this distal part of the IL-10 gene the following single base pair substitutions were identified: at –8571 C/T, –8531 G/A, –6752 A/T, –6208 G/C, –5402 C/G. In addition a heterozygous three base pair deletion at –7400 was observed. The SNPs at –8571 C/T and –8531 G/A are contained within an Alu-repeat. These data should further the understanding of how the IL-10 gene is controlled in man and how its function may vary between individuals. *Genes and Immunity* (2001) 2, 181–190.

Keywords: interleukin-10; distal 5' flanking region; polymorphisms

Introduction

Recently we isolated the proximal 5'-flanking region of interleukin-10 (IL-10), characterized the promoter region and described a 4200-bp fragment.^{1–4} IL-10 is an important multifunctional cytokine and a key regulatory component of many aspects of the immune response.⁵ IL-10 exerts a wide spectrum of biological activities *in vitro* and *in vivo* and is implicated in the regulation of the inflammatory and immune responses.^{5–7} The ability of IL-10 to block activation of cytokine synthesis and several accessory cell functions renders this cytokine a potent suppressor of the effector functions of macrophages, T cells and NK-cells.^{8–10} Among the different cell types affected by IL-10, monocytes/macrophages and lymphocytes appear to be particularly modified with regard to their function, morphology, and phenotype.

The influence of IL-10 in the basic biology of the human immune system is reflected in its involvement with a range of autoimmune and malignant diseases.^{6,7} In particular, IL-10 may contribute to the development and progression of diseases which involve the prolifer-

ation and differentiation of B cells, or the growth of human B-cell lymphomas, where its expression can be profoundly dysregulated^{11,12} IL-10 is able to promote the proliferation and differentiation of B cells and the production of autoantibodies.^{11,13} Indeed, elevated levels of IL-10 are found in the serum of systemic lupus erythematosus (SLE) patients and the number of IL-10 secreting cells is also increased.^{14,15} Recent reports indicate that this cytokine may contribute to Epstein-Barr virus (EBV)-associated transformation.^{16–20} It has been shown that IL-10 is an autocrine growth factor for AIDS-associated lymphoma cells *in vitro* and that IL-10 is a pathogenic factor for lymphoma development in huS-CID-mice.^{18,21} Increased levels of IL-10 were detected in sera of patients with non-Hodgkin's lymphomas as well as in Hodgkin's disease.^{22–25} High IL-10 levels were also associated with poor prognosis in acute infectious diseases, particularly meningococcal meningitis.^{26,27}

The expression of IL-10 is tightly regulated and the levels of constitutive expression in normal leukocytes are extremely low. In contrast, IL-10 is expressed in a range of activated cell-types, including monocytes, T cells and B cells. In addition, IL-10 is apparently constitutively secreted by EBV-immortalised B cells and EBV-positive Burkitt's lymphoma (BL) cells.^{16,17,19,28} However, the mechanism of the IL-10 induction in B cells is still unclear.

Levels of IL-10 secretion are variable between individuals and almost 70% of such intra-individual variation is heritable.²⁹ It is reasonable therefore to examine the human IL-10 gene for polymorphic elements associated with such variation. We isolated the proximal 5'-flanking region of IL-10, characterised the promoter region and

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The sequence data reported in this paper have been submitted to the human genome database and have been assigned the accession number (update to X78437).

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described a 4200-bp fragment as highly polymorphic.¹⁻⁴ Two informative dinucleotide repeats (microsatellites) were described.²⁻⁴ It was demonstrated that these microsatellites combine to form haplotypes which are associated with differential *IL-10* production.²⁹ The haplotype IL10.R2/IL10.G14 was associated with highest *IL-10* secretion overall, whereas the haplotype IL10.R3/IL10.G7 was associated with lowest *IL-10* secretion. Thus, the ability to secrete *IL-10* can vary in man according to the genetic composition of the *IL-10* locus. We and others also demonstrated the presence of multiple single nucleotide polymorphisms (SNPs) in the human *IL-10* 5' flanking region and we recently showed how these combine with the microsatellite alleles to form four major haplotype families (IL10.01, IL10.02, IL10.03 and IL10.04) associated with differential *IL-10* production.^{4,30-32}

However, the variation in *IL-10* secretion attributable to these haplotypes or their component individual loci does not account for the variation in *IL-10* secretion observed between individuals.^{30,33,34} In addition, our original description of the *IL-10* 5' flanking region and recent publications demonstrated the presence of several positive and negative regulatory regions in the 4200 bp described.^{1,35-39} These observations suggest that there may be other, as yet unidentified DNA variations within the *IL-10* gene locus, contributing to variation in *IL-10* secretion.

In order to establish the molecular basis for further studies to determine genetic variations in *IL-10* secretion and predispositions to infectious, autoimmune and malignant diseases, we set out to characterise the distal 5' flanking region of the *IL-10* gene.

Results

Sequence of the distal human *IL-10* 5' flanking region

Using oligonucleotides which allowed direct sequencing 5' of the IL10.R microsatellite the defined sequence upstream of the human *IL-10* gene was extended on the recently described cosmid pWE15-4/11.¹ Because of the presence of a 26 residue mononucleotide T repeat which was difficult to sequence directly, a genomic walking procedure was performed. Cosmid and genomic DNA were digested with *Rsa*I or *Hind*II followed by a subsequent adaptor ligation, which allowed us to generate a 1386-bp fragment (*Rsa*I) and a 1006-bp fragment (*Hind*II). The corresponding amplimers were sequenced in both directions using the corresponding adaptor (T3Ad) and *IL-10* specific primers (IL10/40R). Further primer walking on the cosmid led to the description of an additional 5110 bp giving new information about the distal part of the 5' flanking region of the *IL-10* gene. The sequence of the human *IL-10* gene from -3901 to -9296 is shown in Figure 1. This sequence has been added to our original deposition and can be accessed from the GenEMBL database under the original accession number, X78437. Figure 1 shows the sites of the IL10.R microsatellite and a mononucleotide repeat comprising a stretch of 26 T residues, the polymorphic nature of which remains to be determined.

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-9300 ... TAACAT ATACATATAT ATGTATATAT AACTATATAC ATATATATAT ATATATAACT
-9240 ATATACATAT ATATGTATAT ATAACTATAT ACATATATAT GTATATATAA CCCACATAGT
-9180 TGAAGCTCT TTTGGGGTTC TTTATAACTT TGAAGATGTA AAGATGCTCT GGATCCACAT
Bam HI
-9120 AGTTGCCCAA CAGCTGACTT ASGGAAACCG GGSCAAGGCT GGACCGAGTT GCTGGCACCC
-9060 ACTCCGCTTC CTTCTCTCTT TAGTCTATGA GCTAAGAGGG GCACATGTAA ATGCAGATCA
-9000 TAGTGGAAAGT AGCATGAATC CAGGTTGGGA GGAAGCAACT GCATTTTCAT GTGTGATGCA
-8940 GCTTGTGCATC CAAATATGAC AAACACACAG GAGTATGGGG AGGATTCGAA GTCAAGTGGCA
-8880 CGTGTGTAGAG GTAAGAGGAC GAATGGGACG GATGATGGGG TCTTGAGCAA AGTGTCTGAC
-8820 TTCTCTCTCT CTCTCTTTTT TTTCTTTTTT TTCTTTTTTT TTTTCTTTTT TTTTCTTTTT
-8760 CACAGAGTCT GCTTGTGGC CACAGCTGGA GGCACATGGC GCGATTTCTG CTCATCGCAG
Alu-Yb8/Sz repeat (composite)
-8700 CCTCAGCTTC CAGAGTTCAA GTGATTCCTC TGCCTCAGCC TCACCACGGC CAGGTAATTT
-8640 TTGATTTTTT AGCAGAGACA GGGTTTTGAC ATGTGTGGCA GGGTGGGTTG AACTACTGAC
-8580 CTAGTATCC CCGCATTTG GCTCCGAAA GTGCTGGATG TAGAGGCGTG AGCCACCGAG
-8520 CTTGGCCCTT CTCTCTCTTT TTATAGAGTT TCTGGAAGGT GTTGTCTCTG TAGGGTACT
-8460 GCAGTGGGAA GGCAGGCCAA ACTATGTCTT ATAACTAGT GACACTCATG GATCAGAGAC
-8400 AAAATGAGAAA CACCATTCTG TCTGAAAAAC CTCCAAGGTA GAAATCTTCC CCTAAGACAA
-8340 AAATATCTAA GCCACCTCA CTATCATPTC CAATTCCTAA AATTAAGGTA CCAAGAGAT
-8280 ACAAAATAGA TTCAATACTC TTATAATGAT GGAAACTTAT TGTCAACAGC TTTCACCTTC
-8220 TTACCAACC ACACCTCAC ACAGAACAA CTCTTAGACT CTTTAGAGAT CCGCTCCCTA
-8160 AGGCCCCAGG AACCCAGAGT GATTTGATAC TTACATGAGT AAAGAATTA TGACTACTGAT
-8100 TGAAAAAGAA TTGCATTGAG ATTTCTACTT GCATATAGAA AGACACGTGA ATTTGATAGG
-8040 GCTCTTCTCT CTAGGCCCTT GTTTGGCCAT TTATAAAGA AGACACGTGA ATTTGATAGG
-7980 ACCCCCTCTT CTTCTGAGCT CCATATAGAA TTTTAAACTT CCAGCAGCCA TGGAGAGAT
-7920 AACATCGCC TGGAAGAGCA CTAATTAGTA ATCAGATCAA TTTCTTTAAA AACTGTGAAA
-7860 GCTATTAATAG GTATAGGCAT ATAGTTTGTG ATCAGACTGG ACATATTTAG TGAAGACCTT
-7800 CTTACTTCTT ACTTTAATAG AGATGGAGA ACAGACATAT AGCCATGTAG CACTACAAA
-7740 AGTTTAGGAA GCACTGTGCT AGCCGCCAG ACAGTCTGAC GGAAGCAACA ACATATTTAG TGAAGACCTT
-7680 GGATGTGCC AGAACCTATG TGASTACTGA GCAAGCAACA CTCTCAAAA ACAGATAGC
-7620 GCGCTGCCCT GGAAGTGTCT ACAGTCTGAC AGAGAGACA GAGGTCATTC AAATGATCAC
-7560 ATAGCCACAT GTCTAATAG AAACCTGAC ATAACTACTA GAGGGTGGG AGCAAGTGTG
-7500 TCTTAGAAGA TACACCCAGG GGGCTGACC TTGACTGGC AATGATGAAC TCTTTATGAG
-7440 AAGAAACATC TGAGCTGAGA GCTGAGAGAT GAGACAGGAG GAGAGCAGG AAGGAGAGAC
-7380 TATGTTGCA AAGGCCCTTT GATAGGAGAT GATAGGGATA AATCACTGGG CATGAGTCTT
Alu-J repeat (half site)
-7320 GGTGCATTTT AAGCCGACC ACTTGGAGC TGAGGCAGA GGAATGACTG AGCTTAGAGC
-7260 TCTCAAAACA GCTTGGGCAA CATAGCAGA CCGTGTCTT TTTAAAABAA AAAAGAGAG
-7200 AGAGAGAGCA TGCGATCAT TTGAAAAAAA AGTGAAGAAA AGTCAATGAG CCGTAGAGTC
-7140 AGAGGGAAGT AGAAATGAAA GAGTGGCCGA ATGTGAAGCA GAAGAGATAG CGAGTGTGAC
-7080 ATGATTAAGG AAGTTATAGA AAACATTAGA AGTATGAGTC TTTATTGATC AAGAGAGGAA
-7020 AAGTGGGAA CCATGGAAGG GTTCAAGTAA CAGCAGAGC ATGATCAGAT TGTGTCTGGG
-6960 AAATAAATAC TCTTGTACA ACCTGGAGA GCTCTAGAGT AATGGATATT TGGTCTCTGG
-6900 TTTGGCAATT ATTTTGGAGT CCAGGAGAGA TGAGCAGCTG TGGGGTGTAT TAGACCCAA
-6840 ACTAGGCCAT GGGGCTACA AAGCTCAGCA GAGTCAAAG AATGAGAAA GACAAGTTAA
-6780 GGGGTACATA AATGGTCCA GTGGGCCAAC ACTGGTATG AGGCTGCAAA GCGCCGTGAG
-6720 TCTGGAGACC CACACTATTT ATTTGGTATC AAACAAGAAA GAGCTGTGTT AGGACGTGGG
-6660 GGTAAACAGG TGAGGGCTGT ASGATGTGGG GGTGAAAGG TAGTGTGTGA TGAAGGTATG
-6600 CTTGTGTTGT TTAGCATTTT CTTTGACAGA TATGAAATAT GCTCTGTCTG TCAAGATTAAT
-6540 GGAGGACATG TTTAGGAGC TGGGASAGCA ACCAAAGAT CTTGTGCAT TCCATCCAG
-6540 CCAGAGGAGA TTTTATGCCC TGGGTTTGA TTTTGTGTC TGGAGGCGAG CTTCTTACC
-6420 TTTGGACAG AGCTTGTGTT TCCAAAGGCC AGCAAGGCTT TTGAGCTGTT GACCCGGGAC
-6420 ATCTCCCAAG ACTCTTTTAT ATTGTACAGC AGCAAGGCTT CTTCTGTGAG CTTCTTACC
-6300 AACAGTATG AGCTTGTGTT TCCAAAGGCC AGCAAGGCTT TTTGAGCTGTT GACCAAAATC
-6240 AGTAAATTA AGAATGTTT ATGAGAAA AAATGAGGAG TCTGTGTGAT TCTGTGTGAT GATGATCAT
-6180 AGATTTGCA GAAAGAGGA GGAAGAGGA GGCACCAACA AATCAATTTT AGATGTAAT GATGATCCA
-6120 TTTCCCTTCC TCACTGACAT TGATGAAAGG TGAACCAAGA GAGAGTAT TTTACTGCA
-6060 GATCAGAAAT CAACTACATA AATTCGACAG AAGTCCAGG ACAAAATPAA AATGTGAAA
-6000 CCCCCTCCA ACATTAATG AGAATTTCAA GATGTGGTGA GAGAGTAA GGGAGGAA
-5940 TTTTAGTAT GGGACCTTGT GTACTGAG AGTCACTTAA TCTCAAAACC GACCCCTCT
-5880 TGGATATGTT GGTGTGAGG TCCACGCAAT CCATGTGAGA CTTGAGCAA CTAAAGCCCA
-5820 CCAGAGGAC ACTCTGTGAG TAAAGAAAT CCATTTGTGC CCCACACTCA GAGGCCACT
-5760 AGTATCTCTA GGTAGTATG CAGACAGGC AGAAACACT ATGCTCTCTT GGTGTGCCA
-5700 GGTCAAAACA CCTGTGTGTC TGTCTCTGTA TAAGCCAAAT TTCTCAATG AAGATTTCA
-5640 GAGCAGAGT TCCATCCCTT AAGAAATC CAATTTGCTT GATTTGTGTC CATAGTTGCA
-5580 CAAGGGGAA TTCCACATTT GCTGCCAAA GCTTACCGT TGTCTCTCTC TGTGTTTGTG
-5520 AGTGTGCTC GGCATGATC CTTGGAAGG GCTTAAATCC GPTTGTGATA ACCCTGTGTC
-5460 ATTTGACCT AAGGCCAGC CCGACCCCTA ACTTCTGGC CCACAGGGC
-5400 TTTATCTGA AGTACCAGC ACATATCAA ACCCAATGTC AGGAAAGCC AGAGTAATC
Rsa I
-5340 AGTCTTACA GACTCTGTC TCTTGGACC CCAAAGTGG CACACTTTGG GTGTGTGATA
-5280 GGCATGTGG TCTGTTGAG CACAGCTTCT GAGAGAGGCT CTTCTGTGAG AGCTGAGGAA
-5220 GCTATTTGA GGAATACTA GAAATTTCAA GCTGGCAAG CTATTTTACA CACTAATGAT
-5160 CTGATTAAG AAGAATGAA ACTCCGTTTA TCGCTTTTAA TGAATACACT CTAATGGCAA
-5100 ATGAGATCA ACCCGGAGG GGTCTCTTCA TCAGGACAT TGTCAACCAT CCCAAGATG
-5040 GACAGCTCT CACTTCTCT CCGCTGAGC TGAACCTGCT TGTCTCTGCT CGCTCTGAGC
Hind II
-4980 TPTTCCCAA CAAGCTGTCT AAACCAGAG TCCAGCAGC CCGGATCCCT GAACCTTCCC
Bam HI
-4920 TCTGAGCTC CAGAGCCACC CTCACAAGC TGTAGCCCTG CTTCCCACTC TTGGGTTTGA
-4860 GCTGCGAAT TGCAATGAG CATGGGATA CTACACCCA GAGGCTGCCC ATTTTCTGTA
-4800 CCGATGAGAG TAGCTGGGAG CAGATTTCTT TTTTTTTTTT TTTTTTTTGG AGGAGCTCTC
Alu-Y repeat
-4740 GCTTGTGGC CAGGCTGGA GTGAGTGGC GGTATCTGG CTCACGCAA GCTCCGCTTC
-4680 CAGGCTGAC GCAATTTTC TGCCTGAGC TCCCGATGAG CTGAGCTAC AGGCACTGTC
-4620 CAGCAGGCT AGCTAAATTT TTTTATTTTT TTTTATGAGA GACAGGTTT CAGCGGTTA
-4560 CAGGCTGAG TGTCAATCTC GTCAGCTGCT GATCTGCTCA CTTCCGCTC CAGAGTGT
-4500 GGGATGACG GGTGAGGCA CCGTCCGCA CAAGGGAGGC AGATTCTTTT TTTTTTTTTT
mononucleotide repeat (n=27)
-4440 TTTTTTTT TTTGTAGTG CAAGATTTAA TACAGTAAA ACAGAGCTCC CATCAAAAGG
-4380 GAAGGACCC AAAGAGGTA GCTTATGCG GCTTGAATG CCGGTTTTAT CTCCCGATCA
-4320 TGTCCCTCT CGCTGTGCT TCAGGGGATA GATGATGGC TATTTCTTTA CTTCCGCTTT
-4260 GTTGCATTA AGCATTTTGA TGAACCTCT TTAGACCTG ATTTGGTCGG ATGAGGCTAA
-4200 GTTCAAGCC CCGTGTAAA AGTGGATGC GGTCACTCT CCAGGTAGC TTAGGATTA
-4140 TTTAGTGGT TAGGAAATCC AGCTAGTCT CTTCTTCACT CCGGCTGT AACAGAAA
-4080 CCAAGTGTCT GTTGGGGAG TGGCCGATG ACTGCTTAA TGGAGGAGC ATTTCTTATA
-4020 AGATCTTGA AACTGTAGA TGCACCTCC AAAATCTATT TGCATAAGCA CACACACACA
Bgl II IL10.R (n=13)
-3960 CACACACACA CACACCCAG

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Figure 1 Sequence of the distal 5' flanking region of the human *IL-10* gene from positions -9296 to -3941. The positions of the IL10.R microsatellites, the mononucleotide T repeat and the Alu repeats are shown as well as selected sites for restriction enzymes *Bgl* II, *Bam* HI, *Rsa* I and *Hind* II.

Demonstration of potential transcription factor binding sites

Previously we had found that the known 4200 bp of the *IL-10* promoter could direct the expression of a luciferase reporter gene in different lymphoid cell lines, when inserted in the reverse orientation (own unpublished observation). We therefore searched for potential promoters, transcription factor binding sites related to cytokines as well as for potential open reading frames. Using the NIX software package (available to registered users of the UK Medical Research Council's human genome mapping project website: <http://www.hgmp.mrc.ac.uk>) potential promoters were identified at positions: -8010 and -6340 on the coding strand and positions -8524, -8010, -6340 and -4021 on the noncoding strand of the *IL-10* gene (Figure 2). The potential transcription factor binding sites were defined using TRANSFAC software and our own collection of motifs recently identified as important for the regulation of cytokine promoters, including NFκB, NF-IL6, Oct1, CREB, AP1, GM-CSF; their locations are shown in Figure 2a. As seen on the figure the dense cluster of potential transcription factor binding sites previously described between -3000 and -4000 has to be extended to -4500 because in this part of the new sequence we identified the highest density of potential functional motifs, including a TATA box at -4021, NFκB and AP1 sites.⁴

The *IL-10* 5' flanking region contains multiple novel Alu-repeats

A number of Alu repeats were identified, as illustrated in Figures 2b and 3. The new generated sequence, including also our own published sequence from the proximal 5' flanking region of *IL-10* as well as the *IL-10* sequence deposited under HSJNT4 were analysed. Other SINE and LINE repeats are also shown (Figure 2b). Starting at -4427, immediately upstream of the *IL10.R* microsatellite is the mononucleotide [T]₂₆ repeat, closely followed by an Alu repeat homologous to the Alu-Y family at position -4753 to -4472 (Alu-IL10C). A composite Alu repeat showing homology to Alu-Yb8 in the first half and to Alu-Sc within the second half of the repeat was identified at position -8770/-8514 (Alu-IL10A). A half site Alu repeat is localised at -7332/-7220 (Alu-IL10B) with homology to Alu-J. Overall, four complete Alu sequences (Alu-IL10A, Alu-IL10C, Alu-IL10D and Alu-IL10E) and one Alu half site (Alu-IL10B) within 18 kb of our new 5' flanking sequence, the previously existing 5' flanking sequence and the genomic sequence containing the coding elements and the 3'UTR were identified. It should be noted that the density of repeat elements is higher in the newly-defined distal 5' flanking region compared to the proximal 5' flanking region of the *IL-10* gene. These new *IL-10* Alu sequences are compared against their nearest homologous sequences in Figure 3a while their evolutionary relationships are presented in Figure 3b.⁴⁰

Novel point mutations in the distal 5' flanking region of human *IL-10* gene

Genomic DNAs from European donors and from African donors were sequenced in both directions. A total of 16 individuals were sequenced. These were compared with each other and with the sequence from the pWE15-4/11 cosmid. This led to the discovery of new SNPs within the *IL-10* 5' flanking region, summarised in Table 1. Variant

bases were found at positions: -8571 C/T, -8531 G/A, -6752 A/T, -6208 G/C and -5402 C/G. The pWE15-4/11 cosmid had the following base composition: -8571 C, -8531 G, -6752 A, -6208 G and -5402 C, which we adopted as the reference sequence. Because of the low sample number tested no linkage analysis was performed, however each SNP was observed more than once. Individuals who were -8751 C/C and C/T were identified, as were -8531 G/G and G/A and -5402 C/C and C/G. All possible combinations were observed at -6752 and -6208 and these two SNP loci may be closely linked since homozygotes were always observed to be A/A+G/G or T/T+C/C. The SNPs at positions -8571 and -8531 are part of the Alu-repeat Alu-IL10A. The SNP -8571 is within a potential SP1 binding site and the presence of the 'T' variant would disrupt this.

In addition to the described sequence variations, a trinucleotide insertion/deletion (indel) AGG mutation was noted at -7400 in five individuals. This deletion was only observed as a heterozygous genotype in the samples analysed. This three base pair indel is near to the Alu-J repeat, which is itself a disrupted Alu repeat.

Discussion

We have obtained sequence data for the distal 5' flanking region of the human *IL-10* gene to -9296 bp upstream of the transcription initiation site flanked by a BamHI restriction site and have analysed this new sequence for the existence of potential promoters, transcription factor binding sites and potential new genes. The comparison of our sequence with recently published data (AC068122) is summarised in Figure 4. Some of the deposited unsorted sequences aligned well to the known *IL-10* sequence at positions 17123-20054, 4856-3489, 93517-100618 and 39271-41480 as shown in Figure 4 (base pair numbers are from AC068122 as found in August 2000). This alignment allowed us to localise the *IL-10* sequence within the cosmid pWE15-4/11 near to the T3 site of the cosmid. In addition we used the deposited information to identify neighbouring sequences by PCR and direct sequencing. This revealed that the fragments 7076-9365 and 32892-38895 (AC068122) are immediately 5' from the described 5' distal part of the *IL-10* 5' flanking region.

The transcriptional control of the human *IL-10* gene is not well-defined, but it is known to be influenced by other cytokines, particularly tumour necrosis factor, *IL-12* and interferon-gamma.^{10,41,42} Several studies have demonstrated that, where gene transcription is influenced by TNF, this influence is exerted through complexes of NFκB and REL proteins.^{43,44} The dense clustering of potential cytokine response elements between -3000 to -4500 suggests that this portion of the human *IL-10* gene may indeed be the area through which proinflammatory cytokines exert influence over *IL-10* transcription, although this hypothesis will be subject to experimental verification (Figure 2a).⁴

Our sequence analysis reveals a high density of Alu repeats within the *IL-10* gene locus with 3 Alu repeats located in the 9296 bp 5' flanking region of the gene (Alu-IL10A/C/D) and an additional one in the 3' flanking region of the *IL-10* gene (Alu-140E). Repetitive elements such as Alu families play a dynamic role in the constant reorganisation of the genome and may be major contributors to evolutionary divergence. Such reorganisation is

Table 1 DNA sequence variations within the distal 5' flanking region of the *IL-10* gene in European (E) and African (A) donors

	-8571C/T ATC[C/T]GCC	-8531G/A CGT[G/A]AGC	-6752A/T CCA[A/T]CAC	-6208G/C AAT[G/C]TGG	-5402G/C AGG[C/G]GTT	-7400del ^a GGA(GGA)GAG
cosmid pWE15-4/11	C	G	A	G	C	+
E01	C/C	G/G	A/A	G/G	C/C	+/+
E02	C/T	G/G	T/T	C/C	G/C	+/-
E03	C/T	G/G	A/T	G/C	C/C	+/+
E04	C/T	G/A	A/T	G/C	G/C	+/-
E05	C/T	G/A	A/T	G/C	G/C	+/-
E06	C/C	G/G	A/A	G/G	C/C	+/+
E07	C/T	G/G	A/T	G/C	C/C	+/+
E09	C/C	G/G	A/A	G/C	C/C	+/+
E10	C/T	G/G	A/T	G/C	C/C	+/+
E11	C/C	G/G	A/T	G/C	G/C	+/-
E12	C/T	G/A	A/T	G/C	C/C	+/-
E61	C/C	G/G	ND	G/C	G/C	ND
A188	C/C	G/G	A/A	G/G	C/C	+/+
A191	ND	G/G	ND	G/C	C/C	+/+
A149	C/C	G/G	A/A	G/G	C/C	+/+
A161	C/C	G/G	ND	G/C	C/C	ND

E, Caucasian; A, African. ^a+ no deletion, - 3 bp deletion, ND not determined.

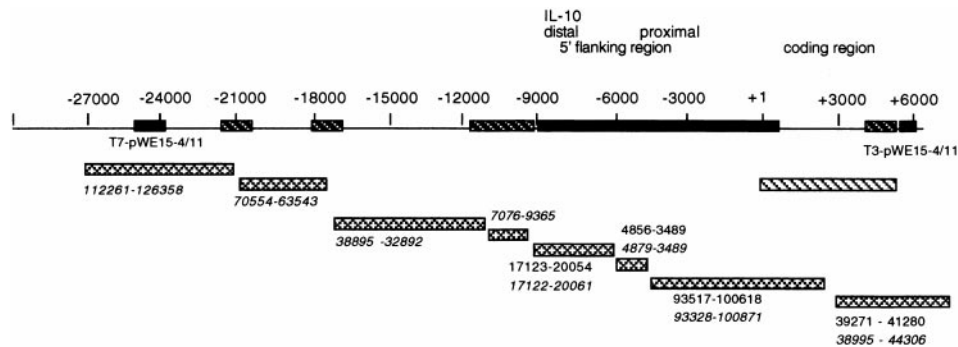


Figure 4 Alignment of sequence data identified in Genbank compared to our own sequence. Used files include sequences from HSJNT4 (coding region) and AC068122. The numbers below indicate the positions within the AC068122 sequence with homology to the presented sequence and the already published *IL-10* sequences. In italic letters are shown the numbers of the corresponding sequence parts as deposited in AC068122. (■) own sequence data, (▨) own sequence data generated knowing published data, (▩) sequence data deposited in HSJNT4, (▧) sequence data deposited in AC068122.

Further analysis of the identified Alu repeats within the *IL-10* gene locus would provide more evidence for this suggestion.

The various polymorphic elements in the *IL-10* promoter and 5' regions have been associated with susceptibility to disease. The best-defined example of this is in SLE where we and others have shown association between IL10.G microsatellite genotype and disease susceptibility.^{45,46} In addition certain SNP genotypes have been associated with disease, or the presence of particular SLE symptoms or disease severity, for example the presence of neuropsychiatric disease.⁴⁷⁻⁴⁹ In less-well replicated studies various markers and alleles in the *IL-10* locus have been associated with rheumatoid arthritis, ulcerative colitis and asthma amongst others.^{47,48,50-55}

It has been shown that patients suffering from chronic hepatitis C and carrying an *IL-10* haplotype associated with high *IL-10* production capacity showed less response to IFN-alpha therapy.⁵⁶

There is also a growing number of reports concerning the potential use of *IL-10* DNA sequence variations in risk prediction in the field of transplantation.^{53,57} For

renal transplantation it was shown that rejection episodes and severe rejections of transplants may correlate with 'high producer' genotypes of the *IL-10* SNP at -1087 and of TNF at position -308 of the recipient.⁵⁸ However in a study by Allen *et al*³³ dysregulated *IL-10* production associated with UVB-induced local immunosuppression did not correlate with any of the known *IL-10* polymorphisms. This suggests that additional, as yet uncharacterised DNA variations within the *IL-10* gene locus may be associated with this and related phenomenon.^{30,33,34}

In addition to the *IL10.G* and *IL10.R* microsatellites, we and others have identified SNPs at positions -3538 T/A, -2774 G/A, -2744 G/A, -2018 G/A, -1354 G/A, -1260 C/T, -1087 A/G, -856 G/A, -824 T/C, -662 C/A, -597 A/C of the human *IL-10* 5' flanking region.^{4,30-32} Two of them -2774 G/A, -2744 G/A are part of an Alu repeat, whereas -2018 G/A is part of a NFκB consensus site, probably important in virus-associated *IL-10* deregulation. In the present report, we found evidence for new SNPs at -8571 C/T, -8531 G/A, -6752 A/T, -6208 G/C, and -5402 C/G, and a heterozygous three base pair deletion at -7400. The SNPs at -8571 C/T and -8531 G/A

are contained within an Alu-repeat. Whether the DNA sequence variations within the Alu repeats at -2769 A/G; -2739 A/G and at -8571 C/T; -8531 G/A are classical SNPs or reflect the existence of polymorphic Alu repeats remains to be analysed. The data presented here now provide new useful information about further DNA variations within the *IL-10* gene. In the future, this information could be used to analyse their frequency and geographical appearance to be useful in their application to epidemiological studies in autoimmune, infectious and malignant diseases. The sequence variations observed so far suggest a geographic specificity between the Caucasian or the African individuals. This however has to be analysed in more detail.

IL-10 is a central component of the immune response. It offers control over inflammatory and cell-mediated immunological mechanisms.⁵⁹ This is reflected in its apparent involvement in many immunological disease states, especially malignant, autoimmune and infectious diseases. Recent evidence has also shown that *IL-10* is a target for many viruses in their attempt to subvert the human immune system.⁶⁰⁻⁶² The area of chromosome 1 where human *IL-10* is encoded is rich with genes encoding proteins important for immune reactions; for example, components of the complement system (RCA = regulator of complement activation). These genes are adjacent to our location for the *IL-10* gene and the genes for many of the Fc-gamma receptors are also nearby as is the cell-surface structure CD34.⁶³ Several lines of evidence indicate that these RCA genes share a common ancestor from which they originated by multiple events of gene duplication.⁶³

Like other cytokines, *IL-10* does not act in isolation but functions within a cytokine network. Recent reports indicate the existence of genes (*mda-7*, *IL-19*) with homology to *IL-10* located on chromosome 1q31/32.^{64,65} On chromosome 12 two other *IL-10* homologues *IL-22* and *Ak155* which are grouped together with *IFN-gamma* were identified.^{66,67} If the RCA gene cluster is the result of multiple gene duplications, it sounds logical to suggest, that this also could happen with an ancestor gene of the *IL-10* gene family and the polymorphic variants we found might affect *mda-7* or *IL-19* regulation.

The DNA sequence variations described in this report appear to be present in Caucasians and the limited number of analysed individuals suggest that they are likely to be common. However further studies will be required to determine their true frequency in Caucasians and other ethnic groups. Thus our structural analysis of the distal 5' UTR may form the basis for further detailed studies about their structure and functional significance in normal physiology and human diseases.

Materials and methods

Sequencing the human *IL-10* 5' flanking region

As a prelude to sequencing the 5' flanking region of the human *IL-10* gene, a cosmid genebank (Stratagene Europe, Amsterdam, The Netherlands) from human placenta was screened for *IL-10* using two different hybridisation probes, as previously described.^{1,4} The nucleotide sequence was determined using the ABI Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequences were analysed on an ABI373

sequencer using ABI software. We have defined the DNA sequence up to position -9296 from the transcription start site of the human *IL-10* gene (with the base immediately preceding the 'A' of the ATG taken as position +30). This sequence has been deposited in the Genebank and EMBL databases as HSINTL10 with accession number X78437 (1996 update, D Kube; 2000 update, D Kube); oligonucleotides used to prime the sequencing reaction are summarised in Table 2.

Genome walking

The method is based on the separate digestion of genomic DNA with each of two restriction enzymes creating blunt ends (in this protocol: *RsaI* or *HindII*). After digestion a T3-adaptor is ligated to the DNA. Using a biotinylated primer from a known sequence, a linear PCR is performed making use of the T3 adaptor to allow with a subsequent magnetic purification of the single strand. This purified single strand is amplified with the biotinylated oligonucleotide and the corresponding adaptor primer. For specificity it is recommended to use a nested PCR. One µg of DNA (cosmid, genomic DNA) was digested with *RsaI* or *HindII* (Roche Diagnostics, Mannheim, Germany). The digested DNA was precipitated. The DNA pellet was dissolved in 50 pmole of the corresponding adaptors. The following oligonucleotides (Eurogentec, Seraine, Belgium, for all used oligonucleotides in genome walking, all other oligonucleotides purchased from Interaktiva, Germany) have been used after phosphorylation using polynucleotide kinase according to the manufacturer (Roche Diagnostics):

Table 2 Primers used

Primer ^a	Sequence in 5'-3'-direction
-8953	CATGTGTGATGCAGCTGTGCATCC
-8876	TTAGAGGTAAGGACCAGAATGGGG
-8600	GGCTGGCTTGAACACTACTGACCTAG
-8341R	TTGTCTTAGGGGAAGATTTTC
-8263	CTGTTAATGGATGGAACTTATTGTC
-7766	ACATATAGCCATGTAGCACTAC
-7320R	CAGCCACTATGCCAGTGAT
-7279	GATTACTTGAGCCTAGGAGTTCAA
-6962R	CAAGCACCAATCTGATCATGAC
-6950	TCTTGCTACAACGTGGAGAACG
-6398	CAAAGGCCACAGCGGTT
-6396R	TTGGAACACCAAGCTCTGTGCCAA
-6315R	GGCAGGACTGGCTTGTCTGTCATA
-5951R	CTTACTCTGCTACCACCATTCTGA
-5854	CCATCCAGTGGAGACTGGAGC
-5574R	ACATTGGCTGCCCAAAGA
-5559R	GCCAATGTGGAATTCCTTTGTGCAA
-5524	TTGCAGTGTCTGCCGCCAT
-5317R	AGGAGACAGGAGTCTGTGAGC
-5268	TGTTGCAGCACAGCTTCTGAG
-5217R	CCTCAGCTCCTAGGACAGAGGC
-5015	GCATCGTCAACCTGCCTGCTC
-4762R	AAAAAAAAAAGAATCCCTCC
-4693	GCAAGCTCCGCCTCCAGGTT
-4651R	TGCGGTAAGAAGACGGAGTCG
-4608R	CTGGCGGTGGTGGCAGG
-4459R	CTGCCCTTGTCCGGCACGG
-4258	GCCTAATTAGCATTTTAGTGA
-4235R	AGCTCACTAAAATGCTAATTAGG
-4148	CCTTCCCAGGTAGGCTT

^aThe primer name corresponds to the position within the *IL-10* sequence.

T3Ad: 5'
 GGAGATCTCGAAATTAACCCTCACTAAAGGG;
 T3AdR: 5'
 CCCTTTAGTGAGGGTTAATTTTCGAGATCTCCGCA.

After adaptor ligation using TAKARA ligation kit (Takara Shuzo, Otsu, Japan) the reaction mix was heat inactivated by incubation at 70° for 10 min followed by a purification using the Qiagen PCR purification kit (Qiagen, Hilden, Germany). Afterwards a linear PCR was performed using Pfu polymerase (Stratagene) and a biotinylated *IL-10* specific primer (5' ATCACTTTAGCTGC AGAGGAATCT). The amplimers were heat inactivated by incubation at 70° for 10 min and chilled on ice before performing the magnetic separation. Magnetic-Streptavidin coated beads were used as recommended by the manufacturer (Promega GmbH, Mannheim, Germany). The single strand DNA was eluted using sterile water and processed directly to a nested PCR using Pfu polymerase and T3Ad and the *IL-10* specific primer -3963R (5' TCTGTGTGTGCTTATGCAAATAGATTTTG) as primers. 1/1000 of this PCR reaction was used for an additional seminested PCR using T3Ad/-3981R (5' GG AGATCTAATAGATTTTGGAGGGTGCATTCTACAG). The amplimers were extracted from the agarose gel using the QiaExII kit (Qiagen). The purified amplimers were sequenced directly using the adaptor primer or a corresponding *IL-10* specific primer.

The following software and websites have been used for the sequence analysis:

'Sequence Navigator' (Applied Biosystems Inc),

'DNASIS2.1' (Hitachi Software, Japan),
 sponding *IL-10* specific primer.
<http://genius.embnet.dkfz-heidelberg.de>,

BLAST: <http://www.ncbi.gov/blast>,

Nix UK HGMP Resource Centre:
<http://www.hgmp.mrc.ac.uk/Nix>,

TRANSFAC: <http://www.gsf.de/biodv/matinspector>,

CENSOR: <http://www.girinst.org/RepbasetUpdate>LoginForm.html>.^{68,69}

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