Molecular Organization of the Cytokine Gene Cluster, Involving the Human IL-3, IL-4, IL-5, and GM-CSF Genes, on Human Chromosome 5

By B.H. van Leeuwen, M.E. Martinson, G.C. Webb, and I.G. Young

The human genes for the hematopoietic growth factors interleukin-3 (IL-3), IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor (GM-CSF) belong to a family of glycoprotein growth factors that are believed to play a major role in regulation of hematopoiesis. Human IL-3 and GM-CSF stimulate differentiation and proliferation of myeloid and erythroid progenitors. IL-4 stimulates growth, differentiation, and activation of B cells and is also a growth factor for both T cells and mast cells. Murine IL-3 also stimulates proliferation of mast cells, but whether human IL-3 alone or in combination with human IL-4 promotes growth of mast cells has not been established. Human IL-5 is a more specific cytokine, acting in the later stages of eosinophil differentiation. Murine IL-5 also stimulates B-cell activation, growth, and differentiation, although whether human IL-5 has activity on B cells remains controversial.

The human genes for IL-3, IL-5, and GM-CSF have been cloned and sequenced and localized to 5q23-32 by in situ hybridization in the region deleted in the 5q- syndrome. The human IL-4 gene has also been cloned but only the cDNA sequence has been published. Very recently, the human IL-3 and GM-CSF genes were reported to be only 9 kb apart. Comparison of the three human genes for IL-3, IL-4, IL-5, and GM-CSF with each other and with the murine IL-4 gene indicate similar exon sizes, with the exons being interrupted exactly between codons. There is a region of low homology at the C-terminus of the four proteins and conservation of a potential regulatory element in the promoter region of the GM-CSF and IL-5 genes. However, overall there is little significant sequence homology between the four genes or between the proteins they encode. The murine IL-3, IL-4, IL-5, and GM-CSF genes are expressed in T lymphocytes after stimulation with mitogen or antigen and the corresponding human genes appear to be similarly regulated.

The very close linkage between the IL-3 and GM-CSF genes, together with the similar gene structure, regulation, and biologic function of the four genes, raises the question of whether they had a common evolutionary origin and might be part of a distantly related gene family whose members are clustered on the long arm of chromosome 5.

In the present study, we showed that the human IL-4 gene is also located at 5q31; we also studied the physical organization of the IL-3, IL-4, IL-5, and GM-CSF genes, using pulsed-field gel electrophoresis (PFGE).

MATERIALS AND METHODS

Cloning of the IL-3 gene. Human placental DNA was partially digested with Sau3A, ligated to bacteriophage λ EMBL3A DNA, packaged, and plated out in ED8654 cells. The library was screened with a 45-mer oligodeoxyribonucleotide with sequence corresponding to the 3'-untranslated region of human IL-3. One hybridizing clone was selected for further analysis. A restriction map of this clone was prepared, and the identity of the IL-3 gene was confirmed by partial sequence analysis. Several restriction fragments, encompassing different regions of the gene, were tested as probes. The presence of an Alu element in intron 3 of the gene precluded use of the whole gene, and the probe fragment used in the present work was a 1.3-kb SacI-SacI fragment that contains 800 base pairs (bp) of 3' flanking sequence and gene sequence to the end of exon 2.

Probes for IL-4, GM-CSF, IL-5, and ECGF. The cDNAs encoding human IL-4 and GM-CSF were obtained from S. Gillis, Immunex, Seattle, and G. Wong, Genetics Institute, Cambridge, MA, respectively. The human IL-5 gene has been cloned and sequenced in our laboratory, and the 3.24-kb BamHI-BamHI fragment, encompassing the whole gene, was used as the probe. The ECGF cDNA was obtained from M. Jaye, Rorer Biotechnology, Horsham, Philadelphia.

In situ hybridization of IL-4. The IL-4 cDNA was nick-translated using tritiated dATP, dCTP, and dTTP (Amersham) and an Amersham nick-translation kit, to a specific activity of 1.3 x 10^8 cpm/μg. The probe was hybridized at 42°C in situ at a concentration
of 200 ng/mL to chromosome slides banded with 5-bromodeoxyuridine as described by Buckle and Craig. The probed slides were stringency rinsed with 50% formamide in 2 x SSC (0.3 mol/L NaCl, 0.03 mol/L sodium citrate, pH 7.0) and then with 2 x SSC, both at 44°C. They were dipped in Ilford L4 emulsion and exposed for 9 to 23 days.

Preparation of genomic DNA for PFGE. RC2A cells were used as the source of human genomic DNA. The cells were grown in RPMI 1640 medium and 10% fetal calf serum (FCS) and embedded in agarose beads by a modification of the method of Jackson and Cooke as outlined in Focus 9:3, Bethesda Research Laboratories, 1987. Cells (1 x 10⁶) in 5 mL PBS (3 mmol/L KCl, 1.5 mmol/L KH₂PO₄, 0.14 mol/L NaCl, 8 mmol/L Na₂HPO₄, pH 6.8) at 45°C, were mixed with 5 mL 1% low MP agarose (Seakem, FMC, Rockland, ME). Mineral oil (20 mL) was added, and the mixture was swirled vigorously and poured into 100 mL ice-cold phosphate-buffered saline (PBS) and stirred to form the beads. The beads were then successively washed with PBS and 1% sodium dodecyl sulfate (SDS) in 25 mmol/L sodium EDTA pH 8, and then resuspended in 1% sodium N-lauroylsarcosine in 25 mmol/L sodium EDTA pH 8. Proteinase K was added to 50 μg/mL, and the beads were incubated overnight at 50°C. The beads were treated with 0.1 mmol/L a large excess of the appropriate restriction enzyme buffer for 30 minutes, with 25 mmol/L sodium EDTA, pH 8, and allowed to sit at room temperature for four hours at the appropriate temperature. The tube was then filled with 25 mmol/L sodium EDTA, pH 8, and allowed to sit at room temperature for five minutes. The beads were centrifuged in a microfuge for 2.5 minutes, 10 μL 10× loading dye added (50% wt/vol sucrose, 25 mmol/L sodium EDTA, pH 8, 0.1% bromophenol blue) and then loaded onto the gel immediately. For double digests, incubation was performed for three hours at the appropriate temperature for the first digest, the beads were resuspended in the buffer for the second digest, and incubation was continued for three hours at the appropriate temperature before 25 mmol/L sodium EDTA, pH 8, and loading dye were added as above. Because each of the probes hybridizes to an EcoRI fragment of known size, an EcoRI digest was included in each PFGE run as a positive control. The EcoRI fragment sizes are 11 kb (IL-4 gene), 17 kb (IL-5 gene), 15 kb (IL-3 gene), 9 kb (GM-CSF gene), and 9.5 kb (EGCF gene), which are all clearly resolved using the PFGE conditions described below.

PFGE. Southern blotting, and hybridization. The method of field-inversion gel electrophoresis (FIGE) was used. The 1% agarose (low electroendosmosis [EEO]) gels used were 20 x 20 cm, and the electrophoresis buffer was 0.5 x TBE (1 x TBE is 90 mmol/L Tris borate, 90 mmol/L borate acid, 2 mmol/L sodium EDTA, pH 8). The gel and electrophoresis buffer were cooled to 10°C, and the electrophoresis buffer was also continuously recirculated to minimize buffer depletion. The FIGE conditions were ramped from 6 to 143 V/cm at 50°C. The beads were treated with 0.1 mmol/L formamide in 2 x SSC (0.3 mol/L sodium phosphate, pH 6.8, 0.5 mol/L sodium pyrophosphate, 1 mmol/L sodium EDTA and 100 μg/mL denatured sonicated salmon sperm DNA) for three to five hours before radiolabeled probe was added. Hybridization was at 65°C for 18 hours for all probes.

Filters were washed in 2 x SSC successively for five and 60 minutes at room temperature and then for 60 minutes at 65°C. When used, higher stringency washes of 1 x SSC for GM-CSF probes and 0.5 x SSC for IL-3 probes were used for 60 minutes at 65°C. Autoradiography of filters was at -70°C between two intensifying screens.

Construction of the subchromosomal maps. Sizes of fragments were assigned using the λ multilayer ladders and yeast chromosomes run as size standards on each gel. The middle of the size range covering the hybridizing band was considered the fragment size. Hybridization patterns of bands were generally symmetrical but were subject to variation in gel loading of the bands. When bands were asymmetrical over the gel lane, the center of the most strongly hybridizing region was used.

The subchromosomal maps shown were obtained using standard methods for restriction mapping. Sites were sequenced using the data generated by single digests (Table 1) and double digests (Table 2); fragments generated by partial digestion were used in both assignment and confirmation of sites. The maps satisfy all restriction data obtained. The orientation or exact localization of the genes within the fragments could not be determined because the predicted restriction sites within the genes were not cut under the conditions used. These sites include the ClaI site in the IL-3 gene, the NceI site in the IL-4 gene, the SacI site in the IL-3 gene, and the S/λI site in the GM-CSF gene. Digestion at these sites in genomic DNA was not observed with either PFGE or standard gel electrophoresis. The lack of cleavage at these sites may result from methylation because all enzymes involved are sensitive to methylation.24 Enzymes, such as NaeI, also show marked site preferences.

Table 1. Pulsed-Field Gel Fragments Hybridizing to the IL-4, IL-5, IL-3, GM-CSF, and ECGF Probes

<table>
<thead>
<tr>
<th>Fragment</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-3</th>
<th>GM-CSF</th>
<th>ECGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>BspHI</td>
<td>105</td>
<td>157</td>
<td>300</td>
<td>300</td>
<td>436</td>
</tr>
<tr>
<td>MluI</td>
<td>475</td>
<td>80</td>
<td>&gt;650</td>
<td>&gt;650</td>
<td>&gt;650</td>
</tr>
<tr>
<td>NotI</td>
<td>430</td>
<td>430</td>
<td>420</td>
<td>420</td>
<td>450</td>
</tr>
<tr>
<td>NruI</td>
<td>480</td>
<td>480</td>
<td>&gt;650</td>
<td>&gt;650</td>
<td>580</td>
</tr>
<tr>
<td>SacII</td>
<td>95</td>
<td>160</td>
<td>235</td>
<td>235</td>
<td>&gt;650</td>
</tr>
<tr>
<td>SfiI</td>
<td>85</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ClaI</td>
<td>&gt;650</td>
<td>&gt;650</td>
<td>230</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td>NaeI</td>
<td>240</td>
<td>240</td>
<td>235</td>
<td>235</td>
<td>235</td>
</tr>
<tr>
<td>PvuII</td>
<td>75</td>
<td>165</td>
<td>165</td>
<td>165</td>
<td>165</td>
</tr>
<tr>
<td>Sall</td>
<td>&gt;650</td>
<td>&gt;650</td>
<td>242</td>
<td>242</td>
<td>242</td>
</tr>
<tr>
<td>EagI</td>
<td>465</td>
<td>465</td>
<td>465</td>
<td>465</td>
<td>465</td>
</tr>
<tr>
<td>SmaI</td>
<td>90</td>
<td>140</td>
<td>190</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>455</td>
</tr>
</tbody>
</table>

Fragment sizes are shown in kilobases. Fragments not resolved (>650 kb) were not included unless no other fragments were visible.
represent 68% of the grains over the long arm of chromosome 5.

Two tallest peaks, which are over the distal half of band 5q23 and all of band 5q31. These distributions indicate a possible single-linkage between all four cytokine genes. The small secondary peak on the long arm of chromosome 17 (Fig 1) is produced in most of our in situ hybridizations and is regarded as technical artefact.25

Physical linkage of the IL-3, IL-4, IL-5, and GM-CSF genes. For convenience, DNA was isolated from the cell line RC2A, which had a normal diploid karyotype. Two normal human chromosome 5's were observed in trypsin G-banded metaphase spreads (results not shown). High-molecular-weight (mol wt) DNA was prepared from cells that had been embedded in agarose beads, and the DNA was then digested with restriction enzymes, which generate large fragments. The fragments were separated by FIGE and, after transfer to nitrocellulose, sequentially hybridized with each gene probe. Figure 3 shows an ethidium bromide-stained gel and the fragments that hybridized to each of the cytokine gene probes. Under the conditions used, only fragments <650 kb were resolved; larger fragments ran together into the gel without separation. Fragments 50 to 650 kb showed a linear relationship between size in kilobases and distance traveled into the gel, whereas this relationship was logarithmic for fragments <50 kb.

Figure 3B, C, D, and E show the bands that hybridized to the IL-4, IL-5, IL-3, and GM-CSF probes, respectively. Table 1 summarizes the sizes of these hybridizing fragments. Many common fragments hybridized to both the IL-3 and GM-CSF probes, indicating very close linkage of these genes, in agreement with the results of Yang et al showing that these genes are only 9 kb apart.13 Three common fragments hybridized to both the IL-4 and IL-5 probes: a 480-kb NaeI fragment, a 430-kb Nol fragment, and a 240-kb Nael fragment, indicating linkage between these two genes as well. The smaller Nael fragments, which hybridized to the IL-4 probe (75 kb) and the IL-5 probe (165 kb) appear to arise from incomplete digestion at an Nael site within the 240-kb Nael band. Nael shows marked site preference, and we obtained variable amounts of cleavage at this site in different digests (Table 2).

Common fragments that hybridized to the IL-3 and GM-CSF probes and the IL-4 and IL-5 probes were also demonstrated. These were the 465-kb Eaug1 (not shown in Fig 3), 460-kb PvuI, and 430-kb Nol fragments. In each case, these digests were only partial and bands were not particularly sharp. However, these results indicate possible linkage between all four cytokine genes.

Table 1 also shows the sizes of fragments that hybridized

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### Table 2. Pulsed-Field Gel Fragments Used in Construction of Restriction Maps Surrounding the IL-4, IL-5, IL-3, and GM-CSF Genes

<table>
<thead>
<tr>
<th>Fragment</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-3</th>
<th>GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaeI - ClaI</td>
<td>240</td>
<td>240</td>
<td>235</td>
<td>235</td>
</tr>
<tr>
<td>NaeI - SacI</td>
<td>95</td>
<td>70</td>
<td>205</td>
<td>205</td>
</tr>
<tr>
<td>SacI - ClaI</td>
<td>95</td>
<td>85</td>
<td>205</td>
<td>205</td>
</tr>
<tr>
<td>SacI - NaeI</td>
<td>270</td>
<td>170</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>ClaI - NaeI</td>
<td>550</td>
<td>550</td>
<td>230</td>
<td>230</td>
</tr>
<tr>
<td>ClaI - Nol</td>
<td>650</td>
<td>650</td>
<td>480</td>
<td>480</td>
</tr>
<tr>
<td>NaeI - SacI</td>
<td>95</td>
<td>155</td>
<td>235</td>
<td>235</td>
</tr>
<tr>
<td>SacI - ClaI</td>
<td>270</td>
<td>170</td>
<td>120</td>
<td>120</td>
</tr>
</tbody>
</table>

Fragment sizes are shown in kilobases. Fragments not resolved (>650 kb) were not included unless no other fragments were visible.

### RESULTS

Localization of the human IL-4 gene. Localization of the IL-4 probe to the long arm of chromosome 5 was observed in four individuals, but because of overstaining only one male and one female were scored to show the grains over all chromosomes (Fig 1) and the grains over prophasic number 5 chromosomes (Fig 2). Over the prophasic chromosomes, there was a clear concentration of grains over a "target region" extending from band 5q21.4 to 5q35. Of all the grains over the target region, 58% were in the three major peaks over sub-bands 5q23.3 to 5q31.2. Similarly, in Fig 1, 68% of grains over the long arm of chromosome 5 are in the two tallest peaks over the distal half of band 5q23 and all of band 5q31. These distributions indicate a possible single-band localization of 5q31.1 for the IL-4 gene. The small secondary peak on the long arm of chromosome 17 (Fig 1) is produced in most of our in situ hybridizations and is regarded as technical artefact.

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Supportive evidence for the overlap is that digests of DNA with PvuI, EagI, and NotI, although possibly partial, yield fragments of ~420 to 470 kb that hybridize to probes for all four genes (Table 1). A 340-kb EagI fragment was previously reported to hybridize to probes for both the IL-3 and GM-CSF genes, in addition to a 436-kb fragment.13 The absence of the 340-kb EagI fragment in our digests may result from incomplete digestion under the conditions used or a polymorphism in the DNA used resulting in the loss of an EagI site. Partial digests of DNA with PvuI have been electrophoresed with three different PFGE conditions, and the same fragments in all cases hybridize to probes for both IL-3 and IL-5.

In addition, more support for the overlap comes from partial digestion with NaeI. The proposed overlap of the maps predicts that the 240-kb NaeI fragment containing the IL-4 and IL-5 genes is ~70 kb from the 235-kb NaeI fragment containing the IL-3 and GM-CSF genes. In double digests of NaeI with other enzymes a 310-kb fragment is often observed. The Sall · NaeI and ClaI · NaeI fragments, which hybridize to the IL-3 and GM-CSF probes include a 310-kb fragment, which could be derived from the 235-kb and 70-kb fragments, and the NotI · NaeI and NruI · NaeI fragments, which hybridize to the IL-4 and IL-5 probes also include a 310-kb fragment, which could be derived from the 240-kb and 70-kb fragments. The appearance of this 70-kb band in double digests of NaeI with four different enzymes suggests that it is an NaeI fragment, which can be accounted for by the overlapping map. If the maps in Fig 4 do overlap as described, the distance between IL-3 and IL-4 genes is 200 to 375 kb.

**DISCUSSION**

Our in situ hybridization studies mapped the human IL-4 gene to 5q23.3-q31.2 with a possible single-band localization of 5q31.1. This places the IL-4 gene close to a number of other hematopoietic growth factor and receptor genes that have been assigned to the segment of human chromosome 5 between the bands 5q23 and 5q33. These include the genes for IL-3 and GM-CSF (5q23-q31),8,9,26 IL-5 (5q31),11 macrophage(M)-CSF (CSF-1, 5q33.1),16,27 and the protooncogene FMS, which is believed to be the M-CSF receptor (5q32.2-q33.3).27,28 Other genes that have been mapped to the same general locus include the ECGF gene (5q31.3-q33.2),26 the platelet-derived growth factor receptor gene (5q23-q31),29 and the β1-adrenoceptor gene (5q31-q32).30 Of these genes, the IL-3, IL-4, IL-5, and GM-CSF genes appear to be the most closely related, having similar gene structures, biologic function, and regulation in T lymphocytes. In the present study, we used PFGE to study their molecular organization. We showed that the IL-4 and IL-5 genes are 90 to 240 kb apart and obtained confirmatory evidence for very close linkage of the IL-3 and GM-CSF

[Image of a diagram]
Fig 3. PFGE analysis of genomic DNA surrounding the IL-4, IL-5, IL-3, and GM-CSF genes. Ethidium bromide-stained gel (A) before transfer of DNA to nitrocellulose filters. Filters were sequentially hybridized to probes encoding sequences from the IL-4 gene (B), IL-5 gene (C), IL-3 gene (D), and GM-CSF gene (E). The size standards used were annealed λ EMBL3A DNA and yeast chromosomes (Y). The restriction enzymes used were BsaHII (1), MluI (2), NorsI (3), NruI (4), SacII (5), SfiI (6), CiaI (7), Nael (8), NariI (9), PvuI (10), SalI (11), and EcoRI (12).
Fig 4. Restriction maps of the subchromosomal regions surrounding the IL-4 and IL-5 genes (A) and the IL-3 and GM-CSF genes (B). Sites used were BssHII (B), CiaI (C), SalI (L), SmaI (M), NcoI (N), NruI (R), and SacII (S). Data used for producing the map are summarized in Tables 1 and 2. Limits of the positions of the probes are indicated by the cross-hatched boxes above the line. Possible linkage of the two loci is depicted by the relative positions of the maps.

genes, which were shown recently by molecular cloning to be 9 kb apart. Our results are consistent with the possibility that the IL-4/IL-5 genes and IL-3/GM-CSF genes may also be closely linked and may be separated by as little as 200 kb, although further work involving separation of larger fragments, as well as isolation of intervening sequences, may be required to verify this possibility. The ECGF gene was also included in this PFGE study because of its close localization to the cytokine genes by cytogenetic methods. However, the restriction mapping data obtained thus far (Table 1 and van Leeuwen BH, Martinson ME, Young IG, unpublished observations) indicates that the ECGF gene is not closely linked to either the IL-4 and IL-5 or the IL-3 and GM-CSF genes.

The proximity of the four related genes, particularly the very close linkage of the IL-3 and GM-CSF genes, supports the possibility that they may have evolved by ancient gene duplication. This model requires relatively rapid divergence of the genes to have occurred because they show little residual sequence homology. There is some homology in the C-terminal region of each of the proteins and conservation of a potential regulatory sequence in the promoter region of the IL-5 and GM-CSF genes. There is evidence for rapid divergence in the IL-3 gene, in which the coding regions show 54% amino-acid homology between mouse and rat and 29% amino-acid homology between mouse and human. Close physical linkage between the IL-3 and GM-CSF genes is also found in the mouse, in which the genes are only 14 kb apart and have the same orientation as the human genome. Similarly, the murine IL-4 and IL-5 genes were shown to be 110 to 180 kb apart. The conservation of the linkage relationships suggests that they may be important in regulation of expression of these genes, but little is known of the mechanisms involved.

If these genes are members of a distantly related gene family, the possibility exists that other as-yet-unidentified members of this family exist within the cluster. It will be of interest to study the DNA both between and surrounding the genes to determine if any other genes with biologic functions related to hematopoiesis are also localized in this region of the chromosome. A common evolutionary origin of the four cytokine genes would also imply that their receptors have the same basic structure and signal transduction mechanism, and there may also be clustering of the receptor genes. Possible verification of these predictions awaits further work.

The molecular organization of the IL-3, IL-4, IL-5, and GM-CSF genes is also of interest with respect to the deletions of Sq commonly found in myeloid disorders. The IL-3, GM-CSF, IL-5, M-CSF, and M-CSF receptor genes were shown to be deleted in the Sq- syndrome and in acute nonlymphocytic leukemia. The present study indicates that the IL-4 gene would also be deleted in the Sq- anomaly. The variability of the proximal and distal breakpoints of the interstitial deletions observed in the Sq- syndrome suggest that juxtaposition of critical elements is not involved. However, the Sq- deletion may affect the expression of genes essential for maintaining normal hematopoiesis. Because the Sq- syndrome involves deletion of a single allele, the altered growth of hematopoietic cells could be caused by haplo-insufficiency or by unmasking of a recessive mutant allele similar to that proposed for retinoblastoma or Wilm's tumor. Because the role of IL-3, IL-4, IL-5, and GM-CSF in maintaining steady-state hematopoiesis is still unclear, further work is required to clarify their involvement, if any, in the Sq- syndrome and related disorders.

ACKNOWLEDGMENT

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Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5

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