Familial Eosinophilia Maps to the Cytokine Gene Cluster on Human Chromosomal Region 5q31-q33

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Summary

Familial eosinophilia (FE) is an autosomal dominant disorder characterized by peripheral hypereosinophilia of unidentifiable cause with or without other organ involvement. To localize the gene for FE, we performed a genomewide search in a large U.S. kindred, using 312 different polymorphic markers. Seventeen affected subjects, 28 unaffected bloodline relatives, and 8 spouses were genotyped. The initial linkage results from the genome scan provided evidence for linkage on chromosome 5q31-q33. Additional genotyping of genetic markers located in this specific region demonstrated significant evidence that the FE locus is situated between the chromosome 5q markers D5S642 and D5S816 (multipoint LOD score of 6.49). Notably, this region contains the cytokine gene cluster, which includes three genes—namely, those for interleukin (IL)–3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF)—whose products play important roles in the development and proliferation of eosinophils. These three cytokine genes were screened for potential disease-specific mutations by resequencing of a subgroup of individuals from the present kindred. No functional sequence polymorphisms were found within the promoter, the exons, or the introns of any of these genes or within the IL-3/GM-CSF enhancer, suggesting that the primary defect in FE is not caused by a mutation in any one of these genes but, rather, is caused by another gene in the area.

Introduction

Eosinophilia, defined as >500 eosinophils/μl blood, often is associated with infectious (e.g., tissue-invasive helminths), allergic (e.g., asthma), myeloproliferative (e.g., various lymphomas and leukemias), connective-tissue (e.g., rheumatoid arthritis), and gastrointestinal (e.g., gastroenteritis) diseases. The most dramatic increases in eosinophils occur in idiopathic hypereosinophilic syndromes (HESs), which encompass a spectrum of disorders, including Loeffler syndrome, Loeffler endocarditis, and eosinophilic leukemia. Hardy and Anderson (1968) suggested that these disorders, which have marked eosinophilia and organ dysfunction, could be grouped together as HES. HES is characterized by (1) persistent eosinophilia ($> 1.5 \times 10^{-9}$ eosinophils/liter) for at least 6 mo; (2) no evidence of parasitic, allergic, or other recognized causes of eosinophilia, after comprehensive evaluation; and (3) signs and symptoms of organ dysfunction that can be directly related to eosinophilia or that otherwise are unexplained in the clinical setting (Chusid et al. 1975; Parrillo et al. 1978; Fauci et al. 1982).

Familial eosinophilia (FE; MIM 131400), although rare, has been well documented since the early 1900s (Gaugain 1909; Naiman et al. 1964). FE composes a subset of HES and is transmitted in an autosomal dominant pattern. To determine the molecular basis of FE, we undertook a genomewide linkage approach to the study of a five-generation kindred with documented FE (Lin et al. 1998). The genotyping of 17 affected subjects, 28 unaffected bloodline relatives, and 8 spouses, with 312 highly polymorphic genetic markers, revealed a single locus of interest on chromosome 5q31-q33. The human cytokine gene cluster has been localized to this region and contains the genes for interleukin (IL)–3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF). Since these three cytokines have been shown to activate eosinophils in vitro (Owen et al. 1987; Rothenberg et al. 1988, 1989), the genes encoding them were considered as candidates for the FE locus. Resequencing

of these three genes in five affected and three unaffected individuals from this kindred did not reveal any potentially disease-causing mutations.

Subjects and Methods

Family

The family was referred to the National Institutes of Health by Dr. P. K. Raghuprasad for the study of HES in the proband. Further investigation revealed multiple affected family members. Informed consent was obtained prior to participation in the clinical and/or laboratory evaluations, under an institutional review board–approved clinical study protocol. Age at examination varied as follows: 12–70 years for affected individuals, <1–65 years for unaffected family members, and 33–91 years for spouses. Fifty-three individuals from this kindred had a complete blood count documenting their eosinophil counts. Of them, 17 subjects were affected, and 36 individuals were unaffected. For the purpose of this study, FE was defined by the presence of eosinophilia—that is, $> 1.5 \times 10^{-9}$ eosinophils/liter—without detectable causes. Twenty-three family members also underwent a clinical evaluation at the National Institutes of Health, under an approved clinical study protocol. Clinical examination included routine physical evaluation and examination of stool ova and parasites, echocardiogram evaluation, and cytogenetic study. The other 30 family members were evaluated during field trips that included phlebotomy and a complete blood count.

Genotyping

Genomic DNA from peripheral blood lymphocytes obtained from 53 family members—including 17 affected individuals, 28 unaffected bloodline relatives, and 8 spouses—was used for genetic analysis. Genotyping was performed by use of a modified version of the Cooperative Human Linkage Center (CHLC) Human Screening Set/version 6.0 panel of polymorphic markers (Dubovsky et al. 1995). Specifically, the screening set comprised 312 fluorescently labeled genetic markers (Research Genetics) with average heterozygosity of .75 and average spacing between markers of 12 cM. Subsequent to the linkage analysis of the results obtained by means of the screening set, additional markers were typed in order to increase the marker density in the region of chromosome 5q.

PCR reactions were set up with a robotic pipetting station (Rosys Robotic Systems) in thin-walled 192-well polycarbonate plates (Corning Costar). Reactions were overlaid with light mineral oil (Sigma Chemical) and were amplified on custom-built thermocyclers (Intelligent Automation Systems), each accommodating 16 192-well plates. PCR products then were multiplexed into panels by pooling (average of eight markers/panel) on the basis of allele-size range and fluorescent label. Aliquots of the multiplexed samples were mixed with either Tamra-labeled GENESCAN 500 and GENESCAN 2500 (Perkin Elmer Applied Biosystems) or rhodamine-labeled MapMarkers (Bioventures) and then were run on ABI377 sequencers (Perkin Elmer Applied Biosystems).

Fluorescent genotyping gels were analyzed in an automated system developed at the Whitehead Institute/MIT Center for Genome Research. The gels were tracked automatically and reviewed manually by use of the Bass/Grace gel-analysis system. Alleles were called automatically by use of software (M.J.D., unpublished data) that implements strict guidelines (to prevent miscalls due to leakage, mistracking, weak signal, or detector saturation) and that identifies alleles on the basis of their characteristic response (including +A and stutter bands). Sizes were determined automatically by comparison with the size standards loaded on every lane. Control genotypes were included on every gel, to ensure accuracy and reproducibility of allele calling. Gels, pedigrees, and markers with aberrantly large numbers of Mendelian-inheritance errors were reexamined carefully, to monitor any systematic laboratory or sample mix-ups and to ensure that each fluorescent marker was producing a consistent assay. The automated genotyping system also was monitored frequently by comparison with manual genotype calls, to ensure consistent performance.

Linkage Analysis

An initial genomewide linkage analysis was conducted by use of the nonparametric approach implemented in the computer program GENEHUNTER (Kruglyak et al. 1996). Because of computational constraints, the pedigree was subdivided into two parts that together included all affected individuals. The order of and the sex-averaged distance between the markers in the screening set were based on the published CHLC map (Murray et al. 1994).

In a second phase, two-point linkage analysis of the data from the higher-density mapping of chromosome 5 was performed by use of the MLINK routine of the LINKAGE package (Lathrop et al. 1984). This parametric analysis was performed for the complete pedigree. Because of the clear pattern of inheritance based on the pedigree, a single model of a dominant, highly penetrant trait was used. The disease gene frequency was assigned a value of .0001. The penetrance values used were .0, .9, and 1.0; incomplete penetrance was specified, since individual IV-8 did not maintain hypereosinophilic status at follow-up (Lin et al. 1998). No new mutations were allowed, and a sex difference was not specified.
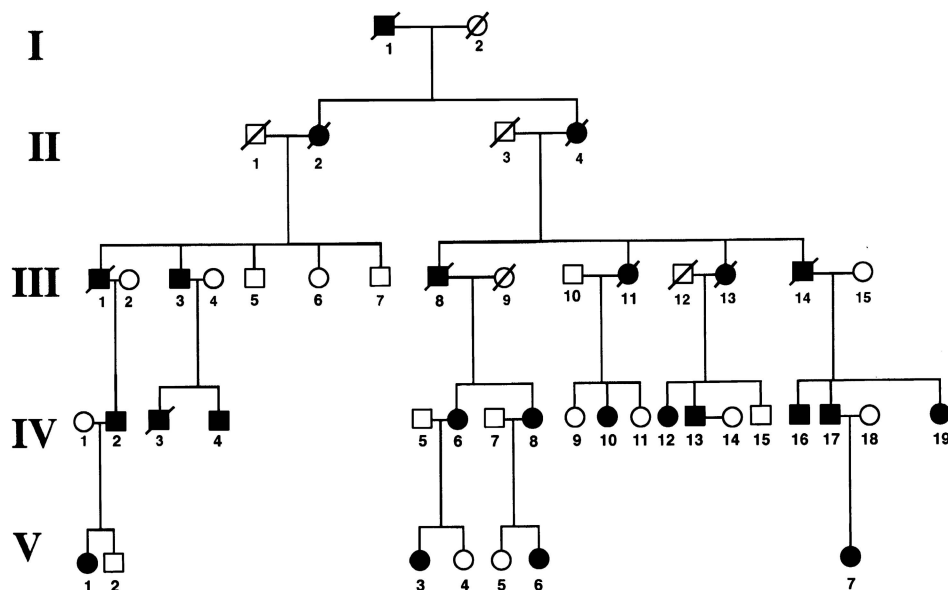
Figure 1 Pedigree used for linkage analysis. Affected individuals are indicated by blackened symbols.

Marker allele frequencies were calculated on the basis of the alleles observed in the founder individuals. Multipoint analysis was performed by use of the VITESSE algorithm (O'Connell and Weeks 1995). The order of and the sex-averaged distance between the markers in this denser set were based on integrated maps from the Marshfield Medical Research Foundation. Haplotypes were reconstructed from the genotype data, by use of the GENEHUNTER program (Kruglyak et al. 1996).

Mutation Screening

Screening for germ-line mutations in the IL-3, IL-5, and GM-CSF genes was performed by the resequencing of these genes in five affected individuals (IV-6, IV-12, IV-17, V-3, and V-7) and three unaffected individuals (IV-5, IV-14, and IV-18). Regions in which sequence polymorphisms were detected were resequenced in the remaining affected individuals and in an additional 10 unaffected individuals.

The resequencing was performed by PCR amplification of genomic DNA, by use of overlapping primer pairs covering the exons, introns, and 5' UTR of each gene. The PCR products were purified by use of the solid-phase reversible immobilization technique (Hawkins et al. 1994), and both strands were sequenced by use of FS dye primer cycle-sequencing kits (Perkin Elmer Applied Biosystems) and then were run on ABI377 sequencers. The sequences obtained were aligned by use of the Staden (GAP4) package (Staden 1996). The reference genomic sequence for the IL-3 and IL-5 genes was obtained from the Lawrence Berkeley National Lab-

oratory, Human Genome Center. The reference genomic sequence for the GM-CSF gene was taken from the published sequence (Miyatake et al. 1985 [Entrez accession number X03021]). The enhancer region for the IL-3/GM-CSF proximal promoters also was sequenced (nucleotides 42–689 [Entrez accession number L07488]).

Results

Family study

Figure 1 shows the pedigree for the family members used in this genomewide linkage study. The family includes 24 affected individuals (19 were studied), over five generations, and has been described elsewhere (Lin et al. 1998). Cardiac abnormalities were diagnosed in five subjects (III-3, II-11, III-14, IV-6, and IV-12) with sustained eosinophilia, of whom two (III-14 and IV-12) had neurological symptoms (Lin et al. 1998). There was no evidence of parasitic, allergic, or other recognized causes of eosinophilia. A previous study of these family members demonstrated that the serum quantities of three important eosinophilopoietic cytokines were either undetectable (IL-3 and GM-CSF) or not significantly different (IL-5) in our FE patients, as compared with the quantities that were measurable in control individuals (Lin et al. 1998).

Linkage Analysis

Nonparametric analysis of the data from the genomewide screen was performed initially so that the

linkage analysis would be independent of mode-of-disease inheritance and penetrance values. This linkage analysis gave a single peak for chromosome 5, centered at marker D5S1505 (nonparametric linkage score, 2.12; P value, .0097). No other chromosome showed evidence of excess sharing (data not shown).

In a second phase, additional markers on chromosome 5q were genotyped, and the data were combined with those from the initial genomewide screen. Two-point maximum-likelihood calculations based on this combined data set yielded a parametric LOD score of 3.28, between D5S1505 and the disease locus. Table 1 summarizes the two-point LOD scores between chromosome 5q markers and the FE disease locus. To extract more information from this genotype data, four-point linkage analyses were performed by use of the VITESSE algorithm, for markers located between D5S1468 and D5S399. The maximum multipoint LOD score (6.49) was obtained when markers D5S1505, D5S642, and D5S816 were examined, with the disease locus placed between the latter two markers (fig. 2). Analysis of reconstructed haplotypes for the 5q region indicated the presence of obligate recombinants that defined a maximum interval, of 18 cM, between markers D5S642 and D5S1480 (fig. 2).

Candidate-Gene Analysis

We screened the IL-3, IL-5, and GM-CSF genes for mutations, because they are three known regulators of eosinophil differentiation and proliferation and because they map to the critical interval. For the initial screen, genomic sequences were obtained for five affected individuals and three spouses from this pedigree. Specifically, the genomic regions from −1860 to 2569, from −925 to 2385, and from −599 to 2043, for the IL-3, IL-5, and GM-CSF genes, respectively, were studied (position 0 refers to the first nucleotide of exon 1). As summarized schematically in figure 3, the entire sequence for all exons and introns was obtained, except for 170 bp of the second intron of IL-3, 70 bp of the second intron of IL-5, and 610 bp of the third intron of GM-CSF. The upstream regions that were sequenced contained all the
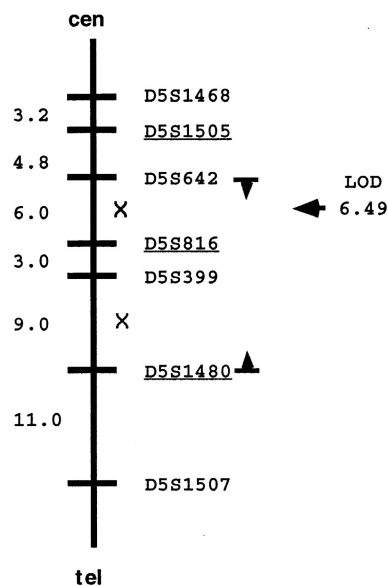

Figure 2   Linkage map of the chromosome 5q region of interest. Markers included in the initial genomewide screen (*underlined*) and the additional markers used to increase marker density are shown, with the estimated distance between loci indicated in centimorgans. "X" indicates the recombinants that define a maximum interval between markers D6S642 and D5S1480 (*vertical arrowheads*). The horizontal arrow indicates the most likely position for the FE gene, based on the four-point linkage analysis using the genotype data from markers D5S1505, D5S642, and D5S816.

known promoter elements for the IL-3 (Engeland et al. 1995; Nimer et al. 1996; Taylor et al. 1996), IL-5 (Gruart-Gouilleux et al. 1995; Yamagata et al. 1995; Karlen et al. 1996a, 1996b; Stranick et al. 1997), and GM-CSF (Cousins et al. 1994; Wang et al. 1994; Staynov et al. 1995; Cockerill et al. 1996) genes. In addition, the inducible enhancer for the IL-3 and GM-CSF gene promoters (Cockerill et al. 1993), located in the 10-kb region that separates these two genes, also was resequenced. The sequences of all eight individuals were identical at all but three positions in the >10 kb examined, as summarized in table 2. These three differences, all single-nucleotide polymorphisms, were examined in an additional 22 members of this pedigree, for a total of 17 affected and 13 unaffected individuals examined. A common haplotype (CAG) was seen in all 17 affected and in only 2 of the 13 unaffected individuals examined. Finally, none of these polymorphisms appeared to be a functional mutation: the polymorphism found in the IL-5 promoter region is not located in any of the known regulatory elements, the one located in the IL-5 coding region is a silent substitution, and the one located in the IL-3/GM-CSF enhancer is not within the known transcription-factor binding sites. Moreover, all
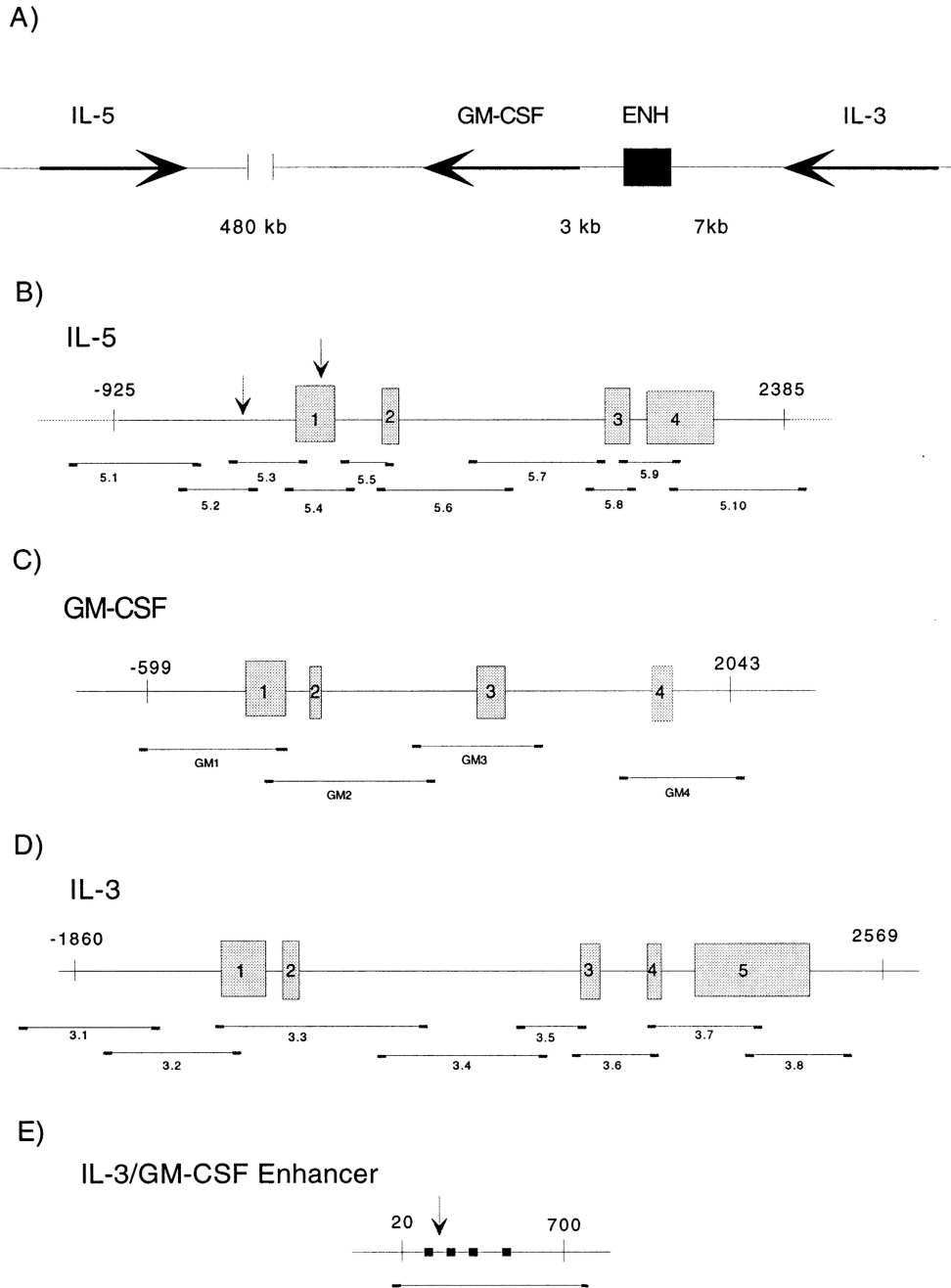
Table 1

Two-Point LOD Scores between Chromosome 5q Markers and the Disease Locus

	LOD Score at Recombination Fraction of						
Locus	.000	.010	.050	.100	.200	.300	.400
D5S1468	−2.15	−1.05	.12	0.46	.54	0.36	.13
D5S1505	−4.00	2.17	3.16	3.28	2.81	1.96	.93
D5S642	1.12	1.74	2.08	2.02	1.58	1.01	.45
D5S816	1.63	1.69	1.76	1.69	1.31	0.82	.32
D5S399	−4.88	−1.75	−.49	−0.03	.16	0.09	.03
D5S1480	−5.05	−1.71	−.48	−0.02	.24	0.22	.11

Figure 3   Schematic diagram of three candidate genes examined for mutations. *A,* Genomic organization of three candidate genes. The orientation of each gene is represented by the direction of the arrows, and the distances between the three cytokine genes and the IL-3/GM-CSF enhancer ("ENH") are indicated below the horizontal line. *B–D,* Genomic structure of and PCR products sequenced for the IL-5 (*B*), GM-CSF (*C*), and IL-3 (*D*) genes. In each panel, the top line includes shaded boxes that represent exons, vertical bars that represent sequence position relative to the first nucleotide of exon 1, and vertical arrows that represent the location of the sequence polymorphisms detected. The PCR products that were sequenced are shown as small horizontal bars and are numbered sequentially. *E,* Genomic structure of the IL-3/GM-CSF inducible enhancer. The blackened squares represent the essential transcription-factor binding sites characterized elsewhere (Cockerill et al. 1993, 1996), and the vertical arrow indicates the location of the sequence polymorphism detected. The nucleotide positions given are taken from reference (Cockerill et al. 1993).

Table 2

Summary of the Sequence Polymorphisms Detected in the IL-3, IL-5, and GM-CSF Genes

Gene	Location	Polymorphism	Nucleotide Position
IL-5	5′ UTR	C or T	−703[a]
IL-5	Exon 1	CG(A or G)	168[a]
IL-3/GM-CSF	Enhancer	G or T	240[b]

[a] Relative to the first nucleotide of exon 1.
[b] Relative to the published sequence (Cockerhill et al. 1993).

three of the alleles found in the shared affected haplotype also were observed in unaffected individuals.

Discussion

We report the results of a genomewide linkage analysis of a large kindred with an autosomal dominant form of FE. The initial results from the genomewide scan provided evidence that the FE locus is situated in the 5q31-q33 region. Subsequent mapping using a higher density of markers further supported the evidence that this region is the location of the FE gene, with a maximum two-point LOD score of 3.28, at recombination fraction .1, for the D5S1505 locus and a maximum multipoint LOD score of 6.49 for the 6-cM region between loci D5S642 and D5S816. This was supported further by analysis of the reconstructed haplotypes, which indicated a critical region delimited by markers D5S642 and D5S1480.

The results of most karyotype analyses for HES have been reported to be normal. However, previous karyotype analysis of affected individuals from this kindred revealed a pericentric-centric inversion of chromosome 10, inv(10)(p11.2q21.2), in two individuals (III-14 and IV-29) (Lin et al. 1998). The observation of genetic linkage to 5q31-q33 suggests that the latter chromosomal variation is unrelated to HES. Linkage to the 5q31-q33 region may be supported further by several reports of chromosomal abnormalities involving 5q31-q33 in patients with eosinophilia associated with malignancies, such as chronic myelomonocytic leukemia, acute lymphocytic leukemia, and myelodysplastic or myeloproliferative syndromes (Hogan et al. 1987; Baumgarten et al. 1989; Berkowicz et al. 1991; Yates and Potter 1991; Baranger et al. 1994; Jani et al. 1994; Matsushima et al. 1994). This also may suggest that the FE gene, or another gene located in 5q31-q33, potentially could play a role in the eosinophilia associated with these neoplastic syndromes. However, the recurrence of translocations in this region may be unrelated to the eosinophilia, per se, since other groups have reported different translocation events in tumor cells related to eosinophilic paraneo-

plastic syndromes (Goh et al. 1985; Matsushima et al. 1994).

The fact that FE maps to 5q31-q33 is particularly interesting in view of the fact that genes encoding IL-3, IL-5, and GM-CSF, three well-described cytokines that play an important role in the differentiation and proliferation of eosinophils (reviewed in Weller 1991), map to this region. A role for these three cytokines in the control of eosinophilic proliferation is supported by the presence of specific cell-surface receptors on eosinophils (Lopez et al. 1989; Chihara et al. 1990) and by studies demonstrating the in vitro activation of normal eosinophils in the presence of any one of these immunoregulatory molecules (Owen et al. 1987; Rothenberg et al. 1987, 1989). Moreover, the serum from some patients with idiopathic eosinophilia has been shown to contain a factor that increases the viability of eosinophils in vitro. This activity was abrogated on the addition of anti–IL-5 but not on the addition of either anti–IL-3 or anti–GM-CSF (Owen et al. 1989). This indicates that IL-5 plays an important role in maintaining the viability of eosinophils in culture.

A prominent role for IL-5 in the control of eosinophil differentiation and proliferation in vivo also has been suggested strongly by the study of transgenic mouse models of eosinophilia. These studies demonstrated that eosinophilia resulted when the murine IL-5 gene was uncoupled from its endogenous transcriptional control mechanisms (Dent et al. 1990; Vaux et al. 1990; Schrezenmeier et al. 1993; Lee et al. 1997). In two of these studies the eosinophilia was not accompanied by any detectable histopathology (Dent et al. 1990; Vaux et al. 1990), suggesting that the induction of the IL-5 gene was sufficient for producing eosinophilia but that the expression pattern of the IL-5 gene was likely to be important in the determination of whether any histopathology would result (Lee et al. 1997). The control of eosinophil differentiation and proliferation may not be restricted to IL-5, however, since similar activity has been proposed for the IL-3 and GM-CSF genes, following in vitro studies using cells from patients with either idiopathic eosinophilia or eosinophilia as a paraneoplastic syndrome (Sanderson 1992; Fremand et al. 1993; Schrezenmeier et al. 1993).

Because the eosinophilia in the present kindred segregates as a dominant phenotype, a disease-causing mutation in one of the three cytokines studied might be expected to result in a gain of function or in an increase in gene expression; although less likely, the null mutation could result in haploinsufficiency. Since the only polymorphism found in the coding sequences for the IL-3, IL-5, and GM-CSF genes was silent (table 2) and thus was unable to affect the function of its product, the region upstream of the first exon of each gene was examined for mutations that possibly could affect gene

regulation. The only polymorphism in the proximal promoter regions, found upstream of the IL-5 gene, was not located within any of the known *cis*-acting elements and thus is unlikely to affect gene expression. Likewise, the polymorphism located within the IL-3/GM-CSF inducible enhancer does not colocalize to any of the previously defined transcription-factor binding sites. Although we did not test directly whether the latter two polymorphisms affected gene expression, these sites are unlikely to represent novel regulatory sites, since the promoter regions of these three genes have been studied extensively by a number of groups and have not shown any evidence of regulatory elements in the regions where the polymorphisms were found (Cockerill et al. 1993, 1996; Cousins et al. 1994; Wang et al. 1994; Engeland et al. 1995; Gruart-Gouilleux et al. 1995; Staynov et al. 1995; Yamagata et al. 1995; Karlen et al. 1996*a*, 1996*b*; Nimer et al. 1996; Taylor et al. 1996; Stranick et al. 1997). More importantly, these polymorphisms also were detected both in unaffected relatives and in spouses. It therefore seems unlikely that one of these three cytokines represents the FE gene.

Although abnormally high quantities of IL-5 in the blood of humans and mice have been suggested to account for the histopathology observed for eosinophilia (Owen et al. 1989; Lee et al. 1997), causality is difficult to establish, since activated eosinophils have the ability to produce IL-3, IL-5, and GM-CSF (Kita et al. 1991; Moqbel et al. 1991; Broide et al. 1992; Desreumaux et al. 1992, 1993). Moreover, since the serum quantities of these three cytokines in our FE patients were either undetectable (IL-3 and GM-CSF) or not significantly different from the quantities that were measurable in control individuals (IL-5) (Lin et al. 1998), high systemic levels of these molecules do not appear to be necessary for pathology. However, an intracellular interaction of the cytokines with their receptors within eosinophils or a close intercellular interaction between T lymphocytes and eosinophils possibly could explain the activity of these factors, even under conditions of undetectable serum concentrations.

In summary, we present strong evidence that the FE locus is located in the 5q31-q33 region. Since there are >100 anonymous transcripts in this region of interest (Schuler et al. 1996), the FE gene may be a novel gene. The FE gene may represent a novel eosinophil growth factor, or it could be a regulator of transcription of the known eosinophilopoietins. In any case, the identity of the FE gene will be important to elucidate, in order to gain a better understanding of the control of eosinophil proliferation and differentiation and of the mechanisms responsible for the pathology observed in idiopathic eosinophilia as well as in eosinophilia related to atopy and malignancy.

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Lawrence Berkeley National Laboratory, Human Genome Center, http://www-hgc.lbl.gov/sequence-archive.html (for reference genomic sequences for IL-3 and IL-5)

Marshfield Medical Research Foundation, Center for Medical Genetics, http://www.marshmed.org/genetics/ (for integrated genetic maps)

NCBI Entrez database, http://www.ncbi.nlm.nih.gov/Entrez (reference genomic sequence of the GM-CSF gene [X03021] and enhancer region of the IL-3/GM-CSF proximal promoters [L07488])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for FE [MIM 131400])

Whitehead Institute/MIT Center for Genome Research, http://www.genome.wi.mit.edu/ftp/distribution/software/ (for the Bass/Grace gel-analysis system)

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