Comparison of the Genomic Organization and Promoter Function for Human Interleukin-8 Receptors A and B*

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Human neutrophils are highly responsive to the chemokine interleukin-8 (IL-8) owing to high levels of expression of two related receptors encoded by the single copy genes il8ra and il8rb located on chromosome 2q34-q35. To identify nuclear factors that regulate the expression of IL-8 receptors, we have first defined the organization of both genes and characterized their functional promoters. il8ra and il8rb span ~4 and 12 kilobase pairs of genomic DNA, respectively. In both cases, the open reading frame resides on a single exon. In contrast, the 5′-untranslated regions are more complex. For il8ra, it is formed from two exons, whereas for il8rb, seven distinct neutrophil mRNAs are formed by alternative splicing of 11 exons. One of the splice variants, designated IL8RB3, is the predominant form for il8rb. Two equally abundant mRNAs for il8ra, 2.0 and 2.4 kilobases in length, are expressed in neutrophils and arise from usage of two alternative polyadenylation signals. Primer extension analysis identified two major transcription start points for il8ra and 11 for il8rb. Regions extending 300 base pairs (bp) upstream from exon 1 of il8ra and 81 bp upstream from exon 3 of il8rb have limited sequence similarity but had strong constitutive promoter activity when cloned upstream from a chloramphenicol acetyltransferase-encoding reporter gene and transiently transfected into surrogate myeloid (HL-60, and U-937) and lymphoid (Jurkat) cell lines. Neither of these regions has sequences corresponding to classic promoter elements. In contrast, a region 643 base pairs upstream from exon 1 of il8rb had relatively low levels of constitutive promoter activity in all three cell environments, and a conserved TATA element is located 47 bp upstream of the 5′-end of exon 1. Thus, despite marked differences in the complexity of their genomic organization, il8ra and il8rb encode products that are similar in structure, function, and the major cell type of expression.

Activation of neutrophils by the chemokine interleukin-8 (IL-8) is mediated by two structurally and functionally related heptahelical, rhodopsin-like, G protein-coupled receptors designated IL-8 receptor A (IL8RA) and IL8RB (1, 2). The IL-8 receptors have 78% amino acid sequence identity, bind IL-8 with similar affinity, and have a similar mechanism of signal transduction. IL8RA binds only IL-8, whereas IL8RB binds with high affinity at least two other related chemokines, GROα and neutrophil-activating peptide-2 (1–4). Analysis of chimeric receptors has indicated that the ligand profiles of rabbit IL8RA and human IL8RB differ in part because of differences in the sequences of the respective amino-terminal segments (5, 6). Although the relative importance of the two receptors to neutrophil function is not yet known, their respective functional properties suggest that IL8RA could ensure the sensitivity of the neutrophil to IL-8 when IL8RB has been desensitized in the presence of GROα and/or neutrophil-activating peptide-2. IL-8 has little effect on monocytes, but can attract and/or activate basophils, T lymphocytes, and eosinophils in vitro, although the importance of these findings in vivo has not yet been convincingly demonstrated, and the receptors that are involved have not been specifically identified (7–9).

The importance of the IL-8 receptors to the neutrophil is reflected in the high levels of specific transcripts that can be detected by Northern blot hybridization of RNA from resting cells (1–3). In contrast, only low levels of mRNA for IL8RA and IL8RB have been detected by polymerase chain reaction (PCR) in human monocytes (10). mRNA for IL8RA but not for IL8RB has also been detected by PCR in the T cell line Jurkat and in phytohemagglutinin-activated T cells, suggesting that the regulation of gene expression for the two receptors may differ in these cell types (10). Differences in gene regulation in neutrophils for the two receptors have not yet been demonstrated.

The cellular composition of inflammatory infiltrates is regulated in part by the cellular distribution of leukocyte chemoattractant receptors. The precise factors, however, that in turn regulate chemoattractant receptor expression in leukocytes have not yet been identified. We have previously reported that the genes for IL8RA and IL8RB lack introns in the coding block and, with a pseudogene for il8rb (gene il8rp), form a cluster of human genes on chromosome 2q34-q35 (11). To define nuclear factors that regulate IL-8 receptor expression, we have now determined the structural organization of both genes and identified their functional promoters.

EXPERIMENTAL PROCEDURES

RNA Analysis by Northern Blot Hybridization—Total cellular RNA was prepared from peripheral blood-derived leukocytes from healthy human donors and from the cultured cell lines HL-60, Jurkat, and U-937 using a commercial RNA isolation kit (Stratagene, La Jolla, CA). RNA was also made from HL-60 cells stimulated for 2 days with 750 μM dibutyryl cyclic AMP, a treatment that induces differentiation toward a mature neutrophil-like phenotype (HL-60 neutrophils). Neutrophils were purified from peripheral blood by Hypaque/Ficoll density gradient centrifugation, dextran sedimentation, and hypotonic lysis of residual erythrocytes. Monocytes were separated from lymphocytes by adherence of the mononuclear layer from the Hypaque/Ficoll gradient to tissue culture plastic in RPMI 1640 with 10% fetal bovine serum at 37 °C in 5% CO₂ for 18 h. Pure eosinophils were obtained from the peripheral blood of a healthy individual with an incidental 50% eosinophi...
philia using the methods for neutrophil purification, followed by separation from neutrophils using magnetic beads coated with a monoclonal antibody to CD16. RNA was separated by size by gel electrophoresis and transferred by capillary action to Nytran membranes (Schleicher & Schuell) as described (2). Blots were probed with either the open reading frame (ORF) of il8ra labeled with [α-32P]dCTP using a random primer DNA labeling kit (Boehringer Mannheim) or with the indicated oligonucleotides (oligos) specific for each ILSR exon labeled with [γ-32P]ATP using a 5'-end labeling kit (Boehringer Mannheim).

**RNA Analysis by cDNA Cloning**—Cloning of cDNAs containing the complete ORFs of the IL-8 receptors has been previously described (1, 2). To determine the complete sequences of the 5'-untranslated regions (UTR) of the two PCR-based methods were employed. First, DNA from a human bone marrow 5'-stretch cDNA library in the vector Agt11 (Clontech, Palo Alto, CA) was purified and amplified by PCR using vector-based 5' and 3' primers (Clontech) paired with antisense primers specific for the amino-terminal domain of the ORF of il8ra and il8rb, respectively. Second, 5'-UTR sequences were cloned by 5'-RACE (rapid amplification of cDNA ends) from human neutrophil poly(A) RNA using the 5'-AmplIFINDER RACE kit (Clontech) according to the instructions of the manufacturer. Briefly, antisense il8ra- and il8rb-specific primers were used to synthesize cDNA, and nested gene-specific primers were then used for PCR under stringent conditions (denaturation at 95 °C for 1 min, at 52 °C for 30 s, and extension at 72 °C for 2 min, for 30 cycles). Poly(A) RNA was prepared with the Poly(A) Qiik mRNA purification kit according to instructions of the manufacturer (Stratagene). All PCR products were analyzed on a 1.8% agarose gel, transferred to a nylon membrane, and hybridized with oligos nested within the limits of the PCR product. Hybridizing bands were subcloned and sequenced by the manufacturer's method (12).

**RNA Analysis by Primer Extension**—Human neutrophil total and poly(A)+ RNA was analyzed using the primer extension system according to the instructions of the manufacturer (Promega, Madison, WI). The primers (100–200 ng) were labeled with [γ-32P]ATP (specific activity, 3000 Ci/mmol) and incubated with 1 μg of RNA at 41 °C for 30 min. The reaction products were separated on a 5% acrylamide gel containing 8% urea dried and visualized by autoradiography. A control DNA sequencing reaction was used for precise determination of the product lengths.

**Genomic DNA Analysis**—The isolation of genomic clones containing the ORFs of il8ra and il8rb has been previously reported (11). A fragment from genomic clones (3–5 μg) was cut with restriction enzymes (Boehringer Mannheim) and then fractionated by electrophoresis on an agarose gel (0.8%). After denaturation in alkaline solution, the DNA was transferred to a Nytran filter by capillary action and hybridized with 32P-labeled cDNA or oligo probes specific for il8ra or il8rb. Appropriate fragments were isolated from the probe and isolated by agarose gel electrophoresis, purified with GeneClean (Bio 101, La Jolla, CA), subcloned into pBluescript (Stratagene), mapped, and sequenced on both strands with sequence-based 17-mers by the Sanger method (12). DNA sequences were analyzed with software from the University of Wisconsin Computer Group on a Cray supercomputer maintained by the National Cancer Institute Advanced Scientific Computing Laboratory, Frederick Cancer Research and Development Center, Frederick, MD (13). Potential regulatory sequences in the il8ra and il8rb 5'-flanking regions were identified by computer analysis (MacVector 4.0, IBI and the Eukaryotic Promoter Data Base (EPD), National Center for Biotechnology Information).

**Promoter Assay**—A 5.5-kb HindIII fragment of il8ra and 2.3-kb HindIII and 3.3-kb PstI fragments of il8rb were subcloned into pBlueScript SK II to create three template plasmids for PCR. The insert fragments were completely sequenced on both strands, and the sequence was used to design a series of PCR amplifiers. An AccI site was added to the 5'-end of one member of each primer pair, and an XbaI site was added to the 5'-end of the other to facilitate subcloning. Stringent PCR conditions were used (denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s, for 20 cycles). The PCR products were initially subcloned into a Bluescript T-vector (14). They were then subcloned from the resulting plasmids into the gel digestion sites of XhoI and XbaI and were then subcloned into the corresponding sites of the pBASIC plasmid (pCAT-Basic, Promega, Madison, WI) to produce IL8RA- and IL8RB-CAT gene chimeras. pBasic contains the chloramphenicol acetyltransferase-encoding ORF (CAT) without a promoter and enhancer and was used as a negative control. pSV40 (pCAT-Promoter, Promega) has the promoter of simian virus 40 cloned in the orientation upstream of CAT and was used as a positive control. The human cell lines HL-60, Jurkat, and U-937 (American Type Culture Collection, Rockville, MD) were all maintained in RPMI 1640 containing 10% fetal bovine serum and 10 mM HEPES (buffer) at 37°C in a humidified atmosphere containing 5% CO2, 10% unaccompanied DNA (20 μg, prepared by Qiagen columns (Qiagen, Chatsworth, CA)) was used to electroporate 1.5×106 cells in 500 μl of buffer using a Bio-Rad Gene Pulser cuvette at 900 farads and 200 V (Bio-Rad). No carrier DNA was used. The transfected cells were isolated for an additional 2–3 days. Cell lysates were prepared by rapid freeze-thawing 3 times. A portion of each lysate containing 50 μg of protein was incubated with 0.1 μCi of [35S]Chloramphenicol (50 mCi/mmol, DuPont NEN), 9 mM acetyl-coenzyme A (Pharmacia Biotech Inc.), and 0.25 s-Tris-HCl (pH 8.0) in a final volume of 180 μl at 37°C for 12 h. The incubation mixture was extracted with ethyl acetate and then analyzed by thin-layer chromatography. Autoradiography was carried out at room temperature with an intensifying screen. The radioactivity of each spot was quantitated by a System 200 Imaging Scanner (Bioscan, Washington, DC). Cotransfection with the plasmid pSV-β-gal (Promega) demonstrated that the transfection efficiency varied by less than 10% for all constructs. All CAT assays were done using the same stocks of plasmid DNAs, and all three cell lines were transfected and analyzed on the same day for each independent experiment. The relative CAT activity in each lysate was quantitated by Equation 1.

\[
\left(\frac{A_U}{A_U + U}\right)A_U \times 100
\]  

\[\text{(Eq. 1)}\]

Where A and U refer to the counts/min in the acetylated and nonacetylated forms of chloramphenicol, respectively, and and refer to IL8RA- or IL8RB-chimeric CAT constructs and the pBasic control plasmid, respectively.

**RESULTS**

**Expression of IL-8 Receptor mRNA in Blood Phagocytes**—Neutrophils are highly responsive to IL-8, whereas the other types of blood phagocytes, eosinophils and monocytes, respond poorly (7–9). Figure 1 demonstrates that the relative responsiveness of blood phagocytes to IL-8 correlates with the levels of expression of il8ra and il8rb. An il8ra ORF probe that cross-hybridizes with il8rb detected large amounts of the major species of mRNA known to be for il8ra and il8rb in freshly isolated, unstimulated, peripheral blood-derived neutrophils from healthy humans (1–3), whereas peripheral blood-derived eosinophils and mononuclear adherent cells (monocytes/macrophages) from healthy humans had only trace amounts. Prolonged exposures of the neutrophil hybridization revealed a previously unreported minor species of IL8R-specific mRNA at 1.9 kb. Since we had earlier shown that the il8ra ORF probe cross-hybridizes only to il8ra and to a pseudogene for il8rb (il8 rp) and that all three genes lack introns in the coding region (11), the complexity of mRNA transcript size must arise from differences in the composition of the 5'- and/or 3'-UTRs.

**Characterization of the 5'-UTR of IL8RA mRNA**—To characterize the 5'-end of the IL8RA mRNA, we screened HL-60 neutrophil and human bone marrow cDNA libraries but failed to find a clone for IL8RA. Instead, the 5'-UTR was successfully

**FIG. 1. Expression of IL-8 receptor mRNA in normal phagocytes.** Total RNA from neutrophils (N, 20 μg), monocytes (M, 10 μg), and eosinophils (Eo, 10 μg) was hybridized with a radiolabeled full-length il8ra ORF probe that cross-hybridizes with il8rb mRNA. The blot was washed in 0.5× SSPE (1× SSPE = 150 mM sodium chloride, 10 mM sodium phosphate, 1 mM sodium EDTA) at 68°C for 45 min and was then exposed to XAR-2 film in a Quanta III cassette at −80°C for the indicated times. The positions of the ribosomal RNA bands are indicated by hash marks. The positions of bands corresponding to the indicated IL8R genes (A, il8ra; B, il8rb) are indicated at the left. Faint bands in eosinophil and monocyte samples detected after prolonged exposure of the corresponding lanes are indicated by arrowheads at the right.
amplified by PCR from human neutrophil mRNA (by a 5'-RACE procedure) and from a λgt11 library of human bone marrow cDNA. When the neutrophil amplification products were analyzed by gel electrophoresis, a band of ~200 bp was detected, which corresponds to a 5'-UTR of ~100 nucleotides (nt) when the lengths of the anchor primer and the amplified portion of the ORF are subtracted (Fig. 2A, left panel). The amplified products were subcloned, and 10 subclones were picked at random and sequenced. The longest of these had a 5'-UTR of 100 nt, and the other nine were less than or equal to 14 nt shorter (Fig. 2B). All three independent subclones examined from the bone marrow library contained an identical 99-nt 5'-UTR, whose sequence matched those of the neutrophil mRNAs (Fig. 2B). No evidence of alternative splicing in the 5'-UTR of IL8RA was found.

Characterization of the 5'-UTR of IL8RB mRNA—When an HL-60 neutrophil cDNA library was screened with a sense oligo corresponding to nt 238-276 of the rabbit IL-8 receptor ORF, 15 independent clones were positive for IL8RB. All contained the same sequence in the ORF, whereas three only extended into the 5'-UTR. However, the sequences of these three longest clones, designated IL8RB1, IL8RB3, and IL8RB10, completely diverged 25 nt upstream from the first ATG initiation codon. This suggested that, unlike for IL8RA, IL8RB mRNA may be differentially spliced in the 5'-UTR. To confirm and perhaps extend this result in normal human sources, the same PCR-based approach for IL8RA was applied to IL8RB.

Unlike the results with IL8RA described above, when the 5'-RACE products for IL8RB were specifically amplified from human neutrophil mRNA and analyzed by gel electrophoresis, three bands ~250, 350, and 550 bp in length were detected (Fig. 2A, right panel). Accounting for the primer lengths and positions, these bands correspond to 5'-UTRs of ~150, 250, and 450 nt in length. These bands were subcloned individually, and DNA from ~100 transformants (approximately equal representation for all three bands) was analyzed. All three of the 5'-UTR sequence patterns previously identified for the HL-60 neutrophil mRNA variants were confirmed and extended in this peripheral blood neutrophil-derived collection (Fig. 2B). Four additional distinct IL8RB mRNA sequence patterns were also found (IL8RB2, IL8RB4, IL8RB7, and IL8RB9). In contrast, all 11 independent PCR products amplified from the bone marrow library that were examined had a 382-nt 5'-UTR identical in sequence to that determined for IL8RB3. Fig. 2B illustrates schematically the length, relative composition, and relative abundance of the seven 5'-UTR variants. The final number in the name of each variant refers to the number of the 5'-most unique sequence segment. IL8RB1 and IL8RB3 mRNAs were the most abundant variants.

Genomic Organization of IL8RA—Using the human IL8RB cDNA as a probe, a human genomic library was screened, and three cross-hybridizing il8ra clones were isolated, mapped, subcloned, and sequenced. An ~5-kb HindIII fragment contained the sequence for the ORF and 5'-UTR arranged on the gene (Fig. 3A). An additional four exons interrupted by a 1.7 kb intron (Figs. 3A and 4A). Exon 1 contains the first 67 nt of the 5'-UTR, and exon 2 contains the remainder of the expressed sequence. The intron has typical splice donor and acceptor boundaries (15). The intron has a complete Alu repeat in reverse orientation (279 bp long, ~83% identity to the consensus sequence) (16).

The analysis of the 5'-UTR suggests a single mRNA species for il8ra. Yet, two IL8RB mRNA species, 2.0 and 2.4 kb in length, are detectable by blot hybridization of neutrophil mRNA with an oligo probe specific for the IL8RB ORF. Fig. 5A indicates that the two mRNAs arise from differential usage of two distinct polyadenylation signals, AATAAA, that are separated by 474 bp on the gene (Fig. 3A). An oligo probe immediately 5' to the proximal AATAAA recognizes both the 2.0 and

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**Fig. 2. Characterization of the 5'-UTR of IL8RA and IL8RB mRNA by analysis of cDNA clones.** A, cDNAs obtained by 5'-RACE. 5'-RACE was performed using specific antisense primers for the amino-terminal segments of the ORFs of the genes indicated at the bottom of each panel in the absence (−) and presence (+) of neutrophil mRNA. Size markers are in bp. B, the boundaries of unique sequence segments (open boxes) composing the indicated 5'-UTRs were defined by correlation of cDNA sequences obtained from neutrophil, HL-60 neutrophil, and bone marrow sources. The numbering of the sequence segments, left-justified above the corresponding box, corresponds to the 5' to 3' arrangement on the corresponding gene (Fig. 3). The name of each type of IL8RB mRNA given at the right is based on the number of the 5' most unique sequence segment. The 5' limit of individual cDNA sequences is indicated along each mRNA form according to the cellular source of the cDNA (tick marks indicate peripheral blood neutrophils; open circles indicate bone marrow; filled triangles indicate HL-60 neutrophils). Additional cDNAs were sequenced whose 5'-end could not be unambiguously assigned to one of the IL8RB mRNAs (data not shown).
DNA sequence of human il8ra (panel A) and il8rb (panel B) genes. The sequence of il8ra is consecutively numbered to nt 1032 bp. The sequence of il8rb is consecutively numbered to nt +1093 bp. The amount of sequence not shown is indicated in the gaps. The sequence of il8ra has not been determined by cDNA sequencing but is believed to be expressed based on the analysis in Fig. 5A. ORF sequence is in boldface, and the derived amino acids are indicated in single-letter code below the first nucleotide of each codon. Exon and intron names are left-justified above their 5' termini. The 5' most expressed nucleotide is chosen arbitrarily to be nt +1 for both genes. The sequence of il8rb is consecutively numbered. The sequence of il8rb is consecutively numbered to nt +1093 bp. The sequence of il8ra is consecutively numbered.
the 2.4 kb mRNA species that were recognized by the ORF probe. However, specific oligo probes 3' to the proximal AATAAA and 5' to the distal AATAAA both recognize the 2.4 but not the 2.0 kb mRNA, whereas an oligo probe located 3' to the distal AATAAA recognizes neither band.

**Genomic Organization of IL8RB**—Ten il8rb genomic clones were identified during the screening for il8ra (11). The sequence corresponding to the ORF and all of the 5'-UTR variants described in Fig. 2B was found on ~12 kb of one of the genomic clones (Fig. 3B). The 5'-UTR resides on 11 exons that are differentially spliced (Fig. 4B). In three instances, the gene contains sets of exons that are not separated by introns, yet are clearly components of distinct mRNAs (exons 3-6; exons 7 and 8; and exons 10 and 11). Exons 1, 2, 3, 7, 9, and 10 are mutually exclusive in the 7 RNA splice variants. The splice junctions for all of the mRNA variants obey the consensus rules for 5'-intron/exon boundaries. For all mRNA variants except IL8RB10, at least one of the 5'- and/or 3'-boundaries of the functional introns resides within the exon for one of the other mRNA variants (Figs. 3B and 4B). The 5'-boundaries of exons 1, 2, 3, 7, 9, and 10 may be longer than suggested by the longest cDNA clones that were analyzed for them. The 5'-UTR of il8rb lacks significant sequence homology with that of il8ra.

To determine with less bias the relative level of expression of the distinct IL8RB mRNAs, specific oligos were used to probe replicate Northern blots of neutrophil mRNA (Fig. 5B). Exons 3 and 7 were not specifically tested since they are too short for the design of specific probes; exons 4, 6, and 8 were not analyzed since they are common to several mRNA species that could be differentiated by probes for other exons. The probes for exons that were analyzed (1, 2, 5, 9-11) were of similar specific activity. In general the results are consistent with the relative abundance of each mRNA form in the collection of cDNAs from neutrophil, HL-60 neutrophil, and bone marrow sources. The major mRNA species is IL8RB3, which corresponds to an mRNA band ~3.5 kb in length. Surprisingly, IL8RB1 mRNA was not detectable by this analysis even though it was abundantly represented in the collection of neutrophil 5'-RACE by primer extension, shown in Fig. 6. Double underline indicates terminal dinucleotides for the introns defined by each of the mRNA splice variants shown in Fig. 4B. Note that all of these dinucleotides obey the gt/ag rule and that several of them reside within the exons defined by other splice variants.
clones. Also, IL8RB2, which was rare in the collection of cDNAs, clearly corresponds to the 1.9 kb first shown in Fig. 1.

Characterization of the Transcription Start Points (TSP) of IL8RA and IL8RB by Primer Extension of Neutrophil mRNA—To characterize the TSP for il8ra and il8rb without imposing a PCR or cloning step bias and to assess the possibility of additional 5'-exons, human neutrophil RNA was extended using antisense oligo primers corresponding to the ORFs, and the products were analyzed in parallel on the same sequencing gel.

A reaction primed with an oligo specific for the il8ra ORF identified a major and a minor TSP, 90 and 119 nt, respectively, upstream from the initiating ATG (Fig. 6). An additional primer for a different location in the ORF gave identical results. Thus, barring additional exons, the major and minor TSP lie 11 bp downstream and 19 bp upstream, respectively, of the 5' limit of exon 1 determined by PCR and 5'-RACE (Fig. 3A).

In contrast to the simple banding pattern obtained with primers for il8ra, a highly complex banding pattern was obtained with primers for il8rb. Two clusters of major bands were identified (Fig. 6). The same pattern was produced with three different staggered ORF primers of different lengths (data not shown). The bands in the longer cluster are 418, 405, 390, 371, and 354 nt upstream from the initiating ATG. Given that IL8RB3 is the major mRNA species, the longest product in the longer cluster maps to 7 bp upstream of the 5' limit of exon 3, whereas the others map within exons 3-5 (Fig. 3B). The bands in the shorter cluster ranged from 21 to 84 nt in length. Except for the shortest band, these TSP cannot be unequivocally mapped to the gene because of the alternative splicing that occurs for exons 8-10. Additional minor TSP are also apparent.

Molecular Dissection of Functional Promoters for IL8RA and IL8RB—To determine whether portions of the genes immediately upstream from the known expressed sequences of il8ra or il8rb could function in the cell as gene promoters, we constructed IL8RA- and IL8RB-CAT chimeric reporter plasmids and measured CAT activity in transfected HL-60, Jurkat, and U-937 cell lysates. The names, locations, and orientations of these constructs are summarized in Fig. 7A.

An IL8RA-CAT plasmid designated pAls was constructed using the region from 300 bp upstream of the 5'-end of exon 1 to nt 40 of exon 1. This construct was highly active in all three cell environments tested, as compared with transfections with the promoterless parental vector pBasic and the positive control plasmid pSV40 (Fig. 7, A and B).

Given the unusually complex organization of the 5'-UTR of il8rb, we tested a series of chimeric CAT constructs, made from portions of the genomic regions upstream from exons 1, 3, and 10 for independent promoter activity (Fig. 7, A and B). The ~1 kb region of intron 5 immediately upstream of exon 10 (pB9s)

**Fig. 5. Analysis of IL8RA (panel A) and IL8RB (panel B) mRNA variants in human peripheral blood-derived neutrophils.** The gene maps are shown at the top of each panel. Open boxes, exons numbered at the top; dotted lines, gaps; AATAAA, potential polyadenylation signals. Arrows indicate the location and orientation of oligos used for the hybridizations shown below each corresponding gene map. Replicate blots of total neutrophil RNA (10 µg/lane) were hybridized with the indicated oligos and labeled to a similar specific activity. All blots were washed in 2X SSPE at 55 °C for 60 min and exposed to XAR-2 film in a Quanta III cassette at ~80 °C for 2 days in panel A or for the times indicated at the bottom of panel B. Length standards in nucleotides are indicated at the right of each panel.

**Fig. 6. Identification of the major TSP for il8ra and il8rb by primer extension.** Shown are the extended products of total neutrophil RNA primed with primers specific for the divergent amino-terminal segment of the il8ra and il8rb ORFs and separated on the same gel. A companion DNA-sequencing reaction is shown for determination of product lengths, which were precisely determined by longer runs of the same reactions (not shown). The gel was exposed to Kodak XAR-2 film in a Quanta III cassette at ~80 °C for 14 days. Arrows indicate the major extended products, and the lengths are given from the first nt of the corresponding initiating ATG codon. Negative control primer extensions performed in the absence of RNA did not produce any bands (not shown). The results shown are from a single experiment representative of two independent experiments for both il8ra and il8rb using two and three staggered antisense primers, respectively.
FIG. 7. Characterization of functional promoters for *IL8Ra* and *IL8Rb*. A, construct maps and summary data for relative CAT expression. The gene maps are shown by boxed exons numbered at the upper left of each box and connected by lines representing introns with gaps indicated by dotted lines. The portion and orientation of each gene that was used for chimeric CAT constructs are indicated by arrows that project onto the corresponding portion of the gene map. The names of each construct are given on the corresponding position of the *y* axis to the right. Numbers at the ends of the arrows correspond to the limits of the region tested relative to nt +1, the 5′ most expressed nt defined in Fig. 3. pB9s is not consecutively numbered, since regions of upstream introns have not been completely sequenced. The CAT activity of each construct relative to the negative control pBasic is the mean of 3–4 separate experiments with each construct transfected into HL-60 (open bars), Jurkat (hatched bars), and U-937 (filled bars) cells. The mean activity for the least active constructs is indicated to the right of the corresponding bar. The activity of the positive control pSV40 is indicated at the bottom of each graph. B, representative primary data for one experiment in HL-60 cells.
lacked promoter activity relative to pBasic in all three trans- 
certed cell types. In contrast, a CAT construct that contained 
the region from 643 bp immediately upstream from the 5'-end 
of exon 1 through the first 71 bp of exon 1 in the sense orienta-
tion (pB3s) had a mean of 2-8-fold greater CAT activity rela-
tive to pBasic when expressed in the three cell lines. Constructs 
extending pB3s by 420 bp at the 5'-end conferred CAT activity 
of a similar magnitude to Jurkat and U-937 cells when tested in 
both the sense (pB2s) and antisense (pB2a) orientations, but 
they were not reproducibly active in the HL-60 environment.

Next a series of sequential deletions of the region from exon 
1 to 5 was tested (pB4s, pB4a, pB5s, pB6s, pB7s, and pB8s). All 
of these constructs contained exons 3, 4, and the 5'-end of exon 
5, a total of 77 bp of common sequence, and all were highly 
active in all three cell environments tested. The longest region 
tested in this series was ~3-fold more active in the antisense 
orientation (pB4a) than in the sense orientation (pB4s). The 
shortest region tested (pB6s) is 158 bp in length, beginning 81 
bp upstream from exon 3. It retained high activity relative to 
the positive control SV40. The genomic region encompass-
ning the two longest regions with positive CAT activity 
(pB2s +pB4s = pB1s) had ~30-fold increased CAT activity 
relative to pBasic, values that were intermediate to those for 
pB2s and pB4s.

Analysis of the IL8RA and IL8RB Promoter Sequences—No 
long stretches of sequence similarity were found when the il8ra 
and il8rb 5'-flanking regions were compared with each other. 
However, when they were used to search the EMBL and EPD 
data bases, the region of il8rb from -421 to +56 was found to 
have 76% sequence identity in the antisense orientation to a 
region of human genomic DNA located 11 bp from an 81-bp 
sequence that is strongly homologous to the feline leukemia 
virus pol gene (17).

Each promoter region has many sequences similar to the 
consensus sequences for transcription factor DNA binding sites 
(e.g. AP-1, AP-2, and SP-1 sites). The sequence of il8ra up-
stream of the TSP and conserved TATA and CAAT elements 
typical of eukaryotic class I promoters. il8rb contains a con-
served TATA element beginning 47 bp upstream of the known 
5'-end of exon 1. AT-rich regions that could contain TATA-
equivalents are also found 5' to exon 1 of il8ra and exon 3 of 
il8rb. Two potential NFkB binding sites are located 288 
and 331 bp upstream of exon 1 of il8rb. Consensus sequences for a 
transcription factor that regulates the inducible expression of 
several cytokine genes, NF-ATp (nuclear factor of activated T 
cells, Ref. 18), can be found in the promoter regions of both 
receptors (Fig. 3). In addition, il8ra contains a glucocorticoid 
response element 96 bp upstream of the 5'-end of exon 1.

DISCUSSION

The present work clearly establishes the relative expression of 
mRNA for the IL-8 receptors in peripheral blood-derived 
phagocytes from healthy humans, defines the structural or-
ganization of the corresponding genes and mRNAs, and delin-
eates regions of both genes with high constitutive levels of 
 promoter activity in both myeloid and lymphoid cell environ-
ments. While both il8ra and il8rb are expressed at high levels in 
neutrophils and have strong promoters, the complexity of the 
composition of their respective mRNAs differs dramatically.

We found that the levels of IL-8 receptor mRNA in freshly 
isolated, unstimulated, blood-derived neutrophils and eosin-
ophils and in blood-derived adherent mononuclear cells are 
constitutionally skewed, correlating with the rank order of re-
sponsiveness of neutrophils, monocyte/macrophages, and eosin-
ophils to IL-8 (7, 9). Thus, while mechanisms may exist for 
fine tuning the levels of IL-8 receptors on mature phagocytes 
during the evolution of an inflammatory process, the critical 
events regulating receptor gene expression probably occur in 
lineage-committed myeloid precursor cells during differentia-
tion in the bone marrow. Thus, during maturation, neutrophils 
become constitutively sensitive, whereas monocytes and eosi-
nophils remain relatively insensitive to IL-8.

Candidate regions that may be responsible for high levels of 
gene expression in neutrophils were identified by CAT assays 
proximal to exon 1 of il8ra and exon 3 of il8rb. Both regions 
contain AT-rich sequences within 25 bp of the 5'-end of the 
adjacent exon, although neither of these AT-rich sequences con-
tains a consensus TATA sequence. Both regions promoted high 
levels of CAT activity in two different surrogate myeloid cell 
environments, HL-60 and U-937, and in a surrogate lymphoid 
cell environment, Jurkat, whereas an ~1 kb region of il8rb 
proximal to exon 10 did not. A third region of il8rb had lower 
levels of independent promoter activity in all three cell types, 
despite being upstream from exon 1 of il8rb. A sequence motif 
corresponding exactly to the consensus TATA sequence is found 
47 bp upstream of exon 1 of il8rb. Moreover, both functional 
genomic regions of il8rb could promote CAT expression in both 
the sense and antisense orientations in all three cell types. The 
significance of this finding is at present unknown, although it 
have been previously described for several other genes including 
those for type IV collagen a1 and a2 chains and dihydrofolate 
reductase (19, 20). In these examples, however, a small (<1 kb) 
intergenic region regulates the transcription of two adjacent 
 opposes oriented genes from opposite strands. Although il8ra 
and il8rb are both located on human chromosome 2q34-q35 
(11), the relative orientation of and physical distance between 
the two genes is not yet known. Our data indicate clearly that 
the distance is greater than for known genes that use a common 
bidirectional promoter (Fig. 3).

RNA for il8rb was detectable by Northern blot analysis of 
HL-60 and U-937 but not Jurkat cells, whereas RNA for il8ra 
was not detectable in any of these samples (data not shown). 
Moser et al. (10) have found RNA for il8ra but not for il8rb in 
Jurkat samples by PCR analysis. Yet 5'-flanking regions for 
both genes potently promoted CAT activity in all three of these 
surrogate leukocyte environments. Perhaps the factor that is 
limiting for IL-8 receptor gene expression in the genomic con-
text is the methylation state of cis regions of the gene and 
the concentration of specific transcription factors. However, it 
is also possible that tissue-specific control regions are further 
upstream or downstream in the genomic DNA. However, it 
the most important property of the promoter regions of 
il8ra and il8rb is that their sequences have very limited simi-
larity to each other, despite the fact that their products are 
highly related both structurally and functionally and are 
expressed at high levels in the same cell type. Only short 
strategies of sequence identity of unknown significance can be 
identified.

The relevance of the regulatory regions defined by CAT con-
structs in surrogate myeloid and lymphoid cell types to regu-
lation of the IL-8 receptor genes in normal leukocytes and their 
precursors remains to be established. Expression of the IL8R 
promoter constructs that we have described in peripheral blood 
progenitor cells treated with lineage-specific cytokines may 
permits a more physiologic dissection of the elements required 
for IL8R gene expression. Additional transfections in human 
embryonic kidney 293 cells suggested that both regions can be 
quite broadly active (data not shown). Thus, it may be possible 
to use one or the other of these small powerful IL-8 receptor 
promoter regions in a variety of gene therapy applications.
In addition to the promoter sequences, a second dramatic difference between il8ra and il8rb is their organizational and splicing complexity. In human neutrophils, the two mRNAs for il8ra arise from usage of two different polyadenylation signals in the 3'-UTR, whereas at least seven distinct mRNA forms for il8rb arise from alternative splicing of 11 exons in the 5'-UTR. Transcription of il8rb is variably initiated relative to il8ra (Fig. 6). The 5'-UTRs of most of the il8rb mRNAs are much longer than those of most other kinds of G protein-coupled receptors, including those for il8ra. Aside from these differences, the organization of il8ra and il8rb has prominent similarities; both lack introns in the coding block, and both have TSP that are separated by long genomic distances from the corresponding translation start sites. These common features are shared with the genes for the FMet-Leu-Phe, platelet-activating factor (22), and MIP-1α/RANTES (24) chemokreptor receptors, which along with the IL-8 receptor genes form a subfamily of the rhodopsin-like, G protein-coupled receptor gene superfam-

ily (25). Interestingly, the human C5a receptor gene has a single intron that splits the first exon (26).

The splicing pattern of the il8rb mRNA variants has three particularly interesting and unusual features. First, three sets of alternatively spliced exons lack introns between the exons that comprise the set (Fig. 4B). Second, while the splice junctions obey the gt/ag rule for most of the mRNA variants, the gt and/or the ag dinucleotide in most instances reside in exon for at least one of the other mRNA variants. Third, exon 5 is part of IL8RB3, the most abundant mRNA variant, yet it is a classic intron with 5'-gt and 3'-ag terminal dinucleotides from the point of view of IL8RB4 mRNA. Although it is clear that all seven of the variants are expressed in normal neutrophils, the way in which they are each used by the cell is not clear. Mapping of each variant in different types of leukocytes known to express low levels of IL-8 receptor RNA will be necessary to determine whether there are any cell type-specific forms. Moreover, studies of human neutrophils at the single cell level will be needed to address whether il8ra and il8rb and individual variants thereof are truly coexpressed as it appears from analysis of bulk neutrophil mRNA.

Computer analysis indicated that several variant-specific stem-loops could form in the 5'-UTR (analysis not shown). These could potentially affect RNA stability and translatability. Furthermore, given the unusual splicing patterns in the 5'-UTR and the demonstration that several of the spliced exons are adjacent to each other, it is even conceivable that different mRNA forms and even mRNAs bearing the same sequence in the 5'-UTR could encode distinct polypeptides owing to additional splicing in the ORF. However, the sequences of the ORFs for variants IL8RB2, B3, and B10 cloned as HL-60 neutrophil cDNAs were identical (data not shown).

Alternative splicing of the 5'-noncoding region has also been described for the FMet-Leu-Phe chemokreptor receptor gene (22). In contrast, the platelet-activating factor receptor gene may use alternative TSP to generate two distinct mRNA forms that differ in the 5'-UTR (23). The 5'-boundary of the coding exon of leukocyte chemokreptor receptors is typically found a short distance upstream from the ATG initiation codon; 39 bp for il8ra, 25 bp for il8rb, 12 bp for the MIP-1α/RANTES receptor, 11 bp for the FMet-Leu-Phe receptor, and 38 bp for the platelet-activating factor receptor. The organizational similarities among the leukocyte chemokreptor receptor genes are not surprising given the obvious common ancestry that is revealed by alignment of the deduced sequences of their products (25).

The present study provides a foundation for the future delineation of protein factors and DNA sequences that are specifically responsible for transcription of both the IL-8 receptor genes at physiologic sites. Such studies could have immediate relevance to other myeloid genes. They may also contribute important new insights into the molecular basis of normal myeloid differentiation and perhaps spawn new ideas about how it becomes impaired in both congenital and acquired disease states. Finally, the powerful promoter activity that we have demonstrated for small regions of il8ra and il8rb in both myeloid and lymphoid environments may be useful for the construction of improved expression vectors for gene therapy applications.

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Note Added in Proof—Sprenger et al. (27) have recently reported the genomic sequence for il8rb, and identified a single mRNA that corresponds to IL8RB3 in our paper. They also found constitutive CAT-promoter activity for the region upstream of the 5'-most identified exon, which corresponds to exon 3 in our paper, transcribed into HL-60 cells. They further found that the activity could be increased by treatment of the cells with granulocyte colony-stimulating factor.

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