# Structure and Functional Expression of the Human Macrophage Inflammatory Protein 1α/RANTES Receptor

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## Summary

The chemokine β family is comprised of at least six distinct cytokines that regulate trafficking of phagocytes and lymphocytes in mammalian species; at least one of these, macrophage inflammatory protein 1α (MIP-1α), also regulates the growth of hematopoietic stem cells. We now show that MIP-1α and the related β chemokine, RANTES, induce transient alterations in intracellular $Ca^{2+}$ concentration in polymorphonuclear leukocytes that can be reciprocally and specifically desensitized, suggesting a common receptor. Moreover, we have now cloned both the cDNA and the gene for this receptor, functionally expressed the receptor in *Xenopus* oocytes, and mapped the gene to human chromosome 3p21. Transcripts for the receptor were found in mature and immature myeloid cells as well as B cells. The receptor is a member of the G protein–coupled receptor superfamily. It has ∼33% amino acid identity with receptors for the α chemokine, interleukin 8, and may be the human homologue of the product of US28, an open reading frame of human cytomegalovirus.

Chemokines are structurally and functionally related 8–10-kD peptides that are the products of distinct genes clustered on human chromosomes 4 and 17. Chemokines regulate the trafficking and activation of lymphocytes and phagocytes of the mammalian immune system. In addition, some are able to regulate the proliferative potential of hematopoietic progenitor cells, endothelial cells, and certain types of transformed cells (reviewed in references 1–3). Thus, the chemokines may play an important role in host defense against infection, in the pathogenesis of chronic inflammatory disorders, and in wound healing.

The chemokines can be divided into two groups, α and β, by the arrangement of the first two of four conserved cysteines. Members of the chemokine α group all possess a single amino acid of variable identity interposed between the first two cysteines. IL-8 is the best characterized member of this group. Two human IL-8 receptors have been identified that are 77% identical at the amino acid level (4, 5). IL-8R B can also be activated by the related α chemokines, GROα and neutrophil activating peptide 2 (5, 6).

The first two conserved cysteines of β chemokines are adjacent. The human chemokine β family includes macrophage inflammatory protein 1α (MIP-1α)[1], MIP-1β, regulated on activation, normal T expressed and secreted (RANTES), monocyte chemoattractant protein 1 (MCP-1), MCP-2, MCP-3, and I-309 (3). The biological properties of MIP-1α, RANTES, and MCP-1 have been the most characterized (Table 1). Members of this group attract and activate PMN, eosinophils, monocytes, and lymphocytes with variable selectivity (7–11). In addition, MIP-1α has been shown to regulate the proliferative capacity of myeloid progenitor cells (12–15).

To more precisely delineate the role of chemokines in the inflammatory response and hematopoiesis, we wished to study the structure and signal transduction properties of their receptors. Here we report the cloning of the cDNA and the gene for the first chemokine β receptor, the MIP-1α/RANTES receptor.

[1] *Abbreviations used in this paper:* MCP-1, monocyte chemoattractant protein 1; MIP-1α, macrophage inflammatory protein 1α; RANTES, regulated on activation, normal T expressed and secreted.

## Materials and Methods

The RNA and DNA isolation, Northern and Southern blot analysis, and DNA sequencing were done exactly as described (16).

*Intracellular Calcium Measurements in Blood Cells.* PMN and PBMC were isolated from peripheral blood by Ficoll-Hypaque discontinuous density gradient centrifugation. PMN were further purified by dextran sedimentation and hypotonic lysis. PMN were typically contaminated with ~5% eosinophils. PBMC were ~20% monocytes and 80% lymphocytes. Cells ($10^7$/ml) were washed with HBSS buffered with Hepes (10 mM, pH 7.4) and loaded with 2.5 μM Indo-1 AM (Molecular Probes, Eugene, OR) for 30 min at 37°C in the dark. The cells were subsequently washed twice with buffer and resuspended at $2 \times 10^6$ cells/ml. 3 ml of the cell suspension was placed in a continuously stirred, quartz cuvette maintained at 37°C (DeltaScan; Photon Technology International Inc., S. Brunswick, NJ). Fluorescence was monitored at $\lambda_{ex}$ = 358 nm and $\lambda_{em}$ = 402 nm (bound) and 486 nm (free), and the data are presented as the bound/free ratio. Data were collected every 200 ms. The $R_{max}$ and $R_{min}$ were empirically obtained by adding ionomycin (1 μM) and EGTA (10 mM), respectively. The dissociation constant of Indo-1 ($K_d$ = 250 nM) was used to transform the data to $[Ca^{2+}]_i$ in the relationship $[Ca^{2+}] = K_d \cdot [(R - R_{min}) \div (R_{max} - R) \cdot (S_{f2}/S_{b2})$ (17).

*cDNA and Genomic Library Screening.* A 2-kb HL-60 neutrophil cDNA library in λ ZAP (Stratagene, La Jolla, CA) was screened with a $^{32}P$-labeled oligonucleotide probe corresponding to nucleotides 238–276 of the cDNA sequence of clone F3R, which encodes the rabbit IL-8R (18), exactly as described (5). DNA sequences were determined on both strands of the two longest hybridizing clones, designated p4 and p7. A human genomic library in λ FIX (Stratagene) was screened exactly as described (19) with a $^{32}P$-labeled probe of p4 cDNA synthesized from random primers. Restriction sites of positive clones were mapped and a 6.5-kb XbaI fragment was sequenced.

*Calcium Efflux Assay.* cRNA was prepared exactly as previously described (5). The materials and methods used for the calcium efflux assay were as described (5) with minor modifications. Defolliculated oocytes from *Xenopus laevis* were microinjected with RNA samples 1–2 d after harvesting and were then incubated at 20–23°C for 3–5 d. Oocytes were then incubated with $^{45}Ca^{2+}$ (100 μCi/ml; Amersham Corp., Arlington Heights, IL) for 4 h. After 10 washes with medium, individual oocytes were stimulated with ligands. Data are presented as the mean ± SEM of the percent of loaded $^{45}Ca^{2+}$ that was released in 20 min by individual oocytes in response to the stimulus. Recombinant human ligands were obtained as follows: MIP-1α (R & D Systems, Minneapolis, MN); IL-8,

**Table 1.** *The Human Chemokine β Family of Proinflammatory Peptides*

| Chemokine* | Percent identity to mature MIP-1α (sequence accession no.) | Source‡ | Targets/actions |
|---|---|---|---|
| MIP-1α | 100 (D90144) | B & T lymphocytes<br>Mast cells<br>Fibroblasts<br>Macrophages<br>Monocytes | Neutrophil and monocyte chemotaxis; §‖ neutrophil§‖ and macrophage§ activation; stem cell suppression; potentiation of GM-CFU stimulation by GMCSF;§ PGE-independent endogenous pyrogen§‖ |
| MIP-1β | 67 (B30574) | B & T lymphocytes<br>Macrophages<br>Monocytes | Inhibits MIP-1α action on stem cells;§ potentiates GM-CFU stimulation by GMCSF;§ inhibits macrophage activation by MIP-1α§ |
| RANTES | 46 (M21121) | T lymphocytes<br>Platelets | Monocyte, granulocyte, and memory T lymphocyte ($CD4^+/CD45Ro^+$) chemotaxis |
| I-309 | 38 (M57506) | T lymphocytes | Monocyte chemotaxis |
| MCP-1 | 39 (M24545) | Monocytes<br>Endothelial cells<br>Fibroblasts<br>Keratinocytes | Monocyte chemotaxis and activation; tumor suppression§ |
| MCP-2 | 40 (P80075) | MG-63 osteosarcoma cells | Monocyte chemotaxis |
| MCP-3 | 34 (P80098) | MG-63 osteosarcoma cells | Monocyte chemotaxis |

* Additional information regarding alternative names and sequence variants of human and murine β chemokines can be found in references 1–3.
‡ The sources listed are based on protein purification and/or chemokine RNA distribution in normal human cell types with the exception of MCP-2 and MCP-3, for which information is currently available only for the transformed cell type from which they were purified.
§ Activities so far reported only for the murine homologue.
‖ Activities so far reported only for native murine MIP-1, which is purified as a heterodimer of MIP-1α and MIP-1β. Human and murine forms of mature MIP-1α and MIP-1β peptides are 74 and 77% identical in amino acid sequence, respectively.

RANTES, and MCP-1 (Genzyme, Cambridge, MA); NAP-2 (Bachem, Philadelphia, PA); and GROα (a gift from M. P. Beckmann, S. Lyman, and D. Cerretti, Immunex Corp., Seattle, WA). The MIP-1α and MIP-1β used in the initial ligand screen (see Fig. 2 *A*) was a gift of U. Siebenlist (National Institute of Allergy and Infectious Diseases) and was used as a diluted supernatant of Sf9 insect cells expressing immunoreactive recombinant human MIP-1α (from clone pAT464) or MIP-1β (from clone pAT744) (20). All proteins were diluted from aqueous stock solutions or culture supernatants into ND96 oocyte media (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, pH 7.45) containing 0.1% BSA.

*Radioligand Binding Assay.* Carrier-free recombinant human MIP-1α (10 μg) was labeled using 5 mCi $Na^{125}I$ (Amersham Corp.) in 100 μl 0.2 M sodium phosphate, pH 7.2, and 50 μl reconstituted Enzymobeads (Bio-Rad Laboratories, Richmond, CA) as previously described (5). The specific activity was 300 Ci/mmol. The labeled protein migrated as a single band on a 14% polyacrylamide gel and was functionally active (not shown). Single oocytes were incubated with $^{125}I$-MIP-1α for 30 min on ice in 10 μl of binding buffer (HBSS with 25 mM Hepes, 1% BSA, pH 7.4). Unbound ligand was removed by centrifugation of the oocyte through 300 μl of F50 silicone fluid (General Electric, Waterford, NY). The tubes were quickly frozen and γ emissions from the amputated tips were counted.

*Chromosomal Localization.* For primary assignment, a panel of 11 Chinese hamster × human hybrid cell lines were used (21). For regional localization, four rodent × human hybrid cell lines were used that contained either the short arm or the long arm of chromosome 3 in the absence of an intact chromosome 3. DNA from somatic cell hybrids and controls were analyzed by PCR using the open reading frame primers. The PCR conditions were 95°C, 5 min; then 30 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 1 min. Fine mapping was performed as described (22) by fluorescence in situ hybridization to peripheral lymphocyte metaphase chromosomes of a 46, XY normal male with a biotin-labeled genomic 6.5-kb XbaI fragment containing the MIP-1α/RANTES receptor gene.

## Results and Discussion

*Activation of PMN by MIP-1α and RANTES.* The α chemokines have previously been shown to mobilize calcium in PMN (1–3). The β chemokines MIP-1α and RANTES were also able to elicit rapid, transient elevations of $[Ca^{2+}]_i$ in human PMN (Fig. 1 *A*) with $EC_{50}$ of 5 and 50 nM, respectively (Fig. 1, *B* and *C*). In contrast, MCP-1 elicited an elevation in $[Ca^{2+}]_i$ in PBMC (Fig. 1 *A*, traces *e* and *f*) but not in PMN (Fig. 1 *A*, trace *d*). These data suggest that PMN possess receptors for MIP-1α and RANTES but not MCP-1. Crossdesensitization experiments performed with MIP-1α, RANTES, and MCP-1 with PMN and PBMC suggested that putative PMN receptors for MIP-1α and RANTES may be identical (Fig. 1 *A*).

*Cloning of cDNA for the MIP-1α/RANTES Receptor.* During the cloning of cDNA for human IL-8R B (5), a related but distinct class of four HL-60 neutrophil cDNAs was identified. The longest cDNA of this group was designated p4. The p4 cDNA is 2,165 bp in length; the longest open reading frame is 1,065 bp, encoding a protein of 355 amino acids. The proposed codon for initiation of translation and its flanking sequence is GGGATGG, which conforms favorably to the consensus rules (23). The 5'- and 3'-untranslated regions are 102 and 998 bp long, respectively. A poly(A) tail is present.

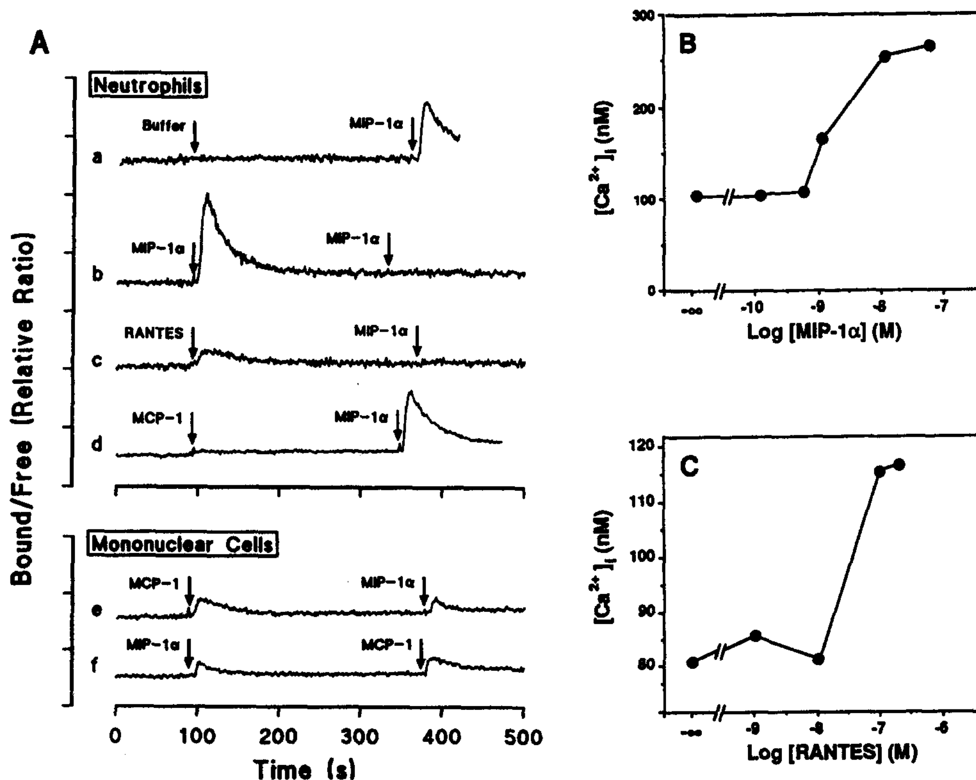


**Figure 1.** MIP-1α and RANTES elicit transient elevation of $[Ca^{2+}]_i$ in human leukocytes. (*A*) PMN (traces *a–d*) or PBMC (traces *e–f*) were loaded with Indo-1 and then stimulated at the indicated times with buffer, 10 nM MIP-1α, 100 nM RANTES, or 50 nM MCP-1. Alterations in the bound/free ratio reflect changes in the $[Ca^{2+}]_i$ as described in Materials and Methods. (*B* and *C*) Ligand concentration dependence of calcium mobilization in PMN by MIP-1α and RANTES. Data are representative of four separate experiments with MIP-1α, two with RANTES, and one with MCP-1.

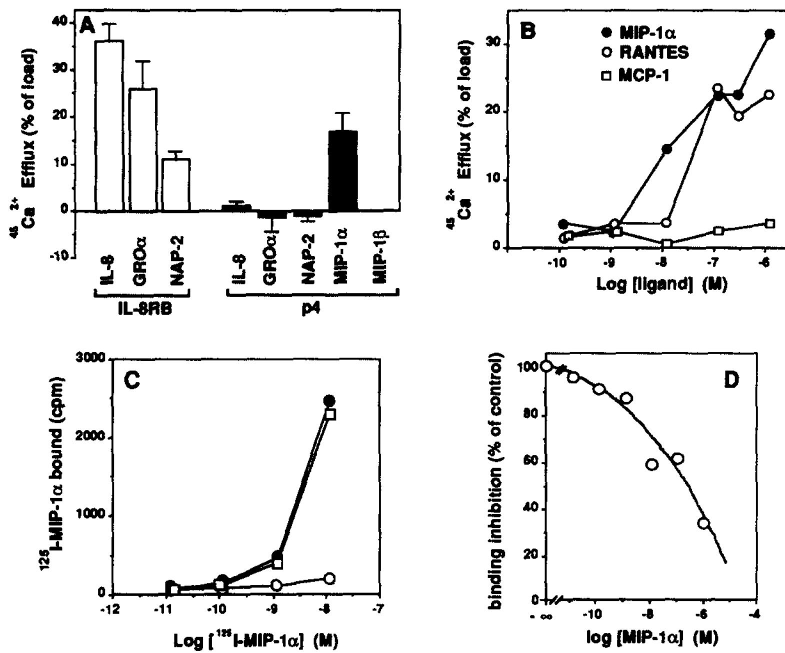


**Figure 2.** Functional expression of the MIP-1α/RANTES receptor in *Xenopus* oocytes. (*A*) The p4 cDNA encodes a receptor selective for a β chemokine. 5 d after injection with 10 ng of either p4 cRNA (*filled bars*) or IL-8R B cRNA (*open bars*), oocytes were stimulated with human rIL-8, GROα, or NAP-2 at 500 nM, or a 1:5 dilution of an Sf9 supernatant containing recombinant human MIP-1α or MIP-1β. Neither MIP-1α nor MIP-1β activated IL-8R B. (*B*) The p4 cDNA encodes a promiscuous receptor for MIP-1α and RANTES. Oocytes were injected with 10 ng p4 cRNA and stimulated 3 d later with the indicated recombinant human β chemokines. Oocytes that had been injected with 50 nl of water did not respond to MIP-1α, RANTES, or MCP-1 over the same concentration range (not shown). The SEM was <10% of the mean value. Data are from a single experiment representative of four separate experiments. In *A* and *B*, the data are derived from five to eight replicate determinations per point. Basal amounts of calcium efflux and calcium uptake were similar for all experimental conditions. (*C* and *D*) Binding of $^{125}$I-MIP-1α to oocytes injected with p4 cRNA. (*C*) Total (*filled circles*) and nonspecific (*open circles*) binding was determined by incubating oocytes injected with p4 cRNA with the indicated concentration of radioligand in the absence or presence of a 100-fold molar excess of unlabeled MIP-1α, respectively. Nonspecific binding was subtracted from total binding to determine specific binding (*open squares*). Specific binding of $^{125}$I-MIP-1α to oocytes expressing IL-8R B was undetectable. (*D*) Oocytes injected with p4 cRNA were incubated with 100,000 cpm $^{125}$I-MIP-1α in the presence or absence of the indicated concentration of unlabeled MIP-1α. 100% represents a mean of 6,401 cpm. Data in *C* and *D* are derived from triplicate determinations per point.

To determine whether IL-8 or a related molecule could activate the p4 product, *Xenopus* oocytes were injected with p4 cRNA and calcium efflux was measured in response to a test panel of ligands. Oocytes injected with p4 cRNA acquired responsiveness to MIP-1α and RANTES but not to MIP-1β, MCP-1, or any of three α chemokines that were tested (Fig. 2, *A* and *B*). The $EC_{50}$ for RANTES was ~50 nM, similar to that observed for activation of a calcium flux response in PMN by this chemokine (Figs. 1 *C* and 2 *B*). The oocyte response to MIP-1α is specific for the entire concentration range tested. However, it has two phases, one that appears to saturate at 100 nM MIP-1α and a second that did not reach a plateau at 5,000 nM MIP-1α (not shown). The first plateau coincides with that demonstrated for PMN over this concentration range (Figs. 1 *B* and 2 *B*).

The threshold for detection of specific binding of $^{125}$I-MIP-1α to oocytes injected with the p4 cRNA was the same as that required for stimulation of calcium efflux. $^{125}$I-MIP-1α did not bind specifically to oocytes expressing human IL-8R B. Specific binding was also detected in COS-7 cells transfected with p4 in the vector pcDNAI, but not in untransfected COS-7 cells (not shown). However, at high concentrations the ligand formed aggregates causing a high background of nonspecific binding that precluded determination of the binding constant.

*Tissue Distribution of the MIP-1α/RANTES Receptor.* A p4 probe hybridized with a single 3-kb band on blots of RNA from the human myeloid precursor cell lines HL-60, U937, and THP-1, from HL-60 cells differentiated with dibutyryl cAMP (HL-60 neutrophils), and from human B lymphocytes. Transcripts were not detectable in total RNA from T lymphocytes activated with PHA, the transformed T cell line Jurkat, EBV-transformed B cells (Fig. 3), and from 10 nonhematopoietic tissues (not shown). This broad expression pattern in mature and immature cells of the immune system is unique among all known chemoattractant receptors and, to

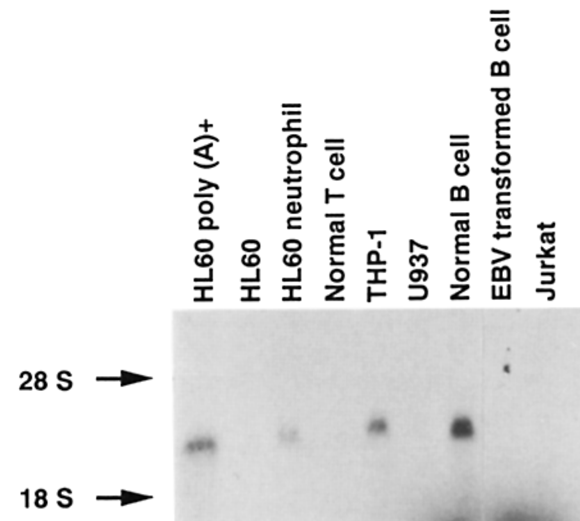


**Figure 3.** Distribution of mRNA encoding the MIP-1α/RANTES receptor. Each lane contains 10 μg of total cellular RNA from the indicated source with the exception of the lane designated *HL60 poly(A)*$^+$, which contains 10 μg of polyadenylated RNA from undifferentiated HL-60 cells. The blot was hybridized with a p4 probe and was washed at 68°C in 0.1× SSPE for 1 h. The blot was exposed to XAR-2 film in a Quanta III cassette at 80°C for 6 d. The position of ribosomal RNA is indicated with arrows.

our knowledge, all other types of receptors, particularly by virtue of the abundant expression in B lymphocytes. We have not tested monocyte RNA by blot hybridization but instead have cloned MIP-1α/RANTES receptor cDNAs from an endotoxin-stimulated human monocyte library (Ahuja, J. K., and P. M. Murphy, unpublished data).

The distribution of MIP-1α/RANTES receptor transcripts differs from the pattern expected based on the functionally defined targets of RANTES action in two regards. First, RANTES is known to attract "memory" T lymphocytes (7), but total RNA from peripheral blood T cells activated with PHA did not contain detectable transcripts. Thus: (*a*) either the receptor gene is expressed at very low constitutive levels in T cells; (*b*) the receptor gene is turned off by PHA treatment of the cells; or (*c*) the RANTES receptor of T cells is distinct from the receptor we have described. Second, MIP-1α/RANTES receptor transcripts are expressed at high levels in B cells, a cell type that reportedly is not a target for RANTES action (7). Perhaps the cellular environment in which the receptor is expressed determines the precise effector function that can be activated.

*Structural Comparison with the IL-8R and with CMV-US28.* The MIP-1α/RANTES receptor contains seven hydrophobic segments predicted to span the plasma membrane, a signature of receptors that are known to be coupled to heterotrimeric G proteins (24). As expected, its closest known structural homologues are human IL-8R A (31% amino acid identity) and B (33% identity); the lengths of all three receptors are ~355 amino acids (Fig. 4). Interestingly, the open reading frame US28 of human cytomegalovirus (25) encodes a protein that is similar in sequence to the MIP-1α/RANTES receptor (33% amino acid identity).

The $NH_2$-terminal segment is poorly conserved for even closely related G protein–coupled receptors, including IL-8R A and B (4–6). Not surprisingly the MIP-1α/RANTES receptor has limited sequence identity with the IL-8Rs in this region. Nevertheless, the three known chemokine receptors and US28 all have acidic $NH_2$-terminal domain; the net charge for IL-8R A and B, the MIP-1α/RANTES receptor, and US28 is −9, −11, −6, and −6, respectively. This is very uncommon in other G protein–coupled receptors (P. M. Murphy, unpublished analysis). It is noteworthy that MIP-1α and RANTES, like other chemokines, are basic polypeptides and could bind via charge interactions with the extracellular $NH_2$-terminal domain of the receptor.

In contrast to the IL-8Rs the $NH_2$-terminal sequences of the MIP-1α/RANTES receptor and US28 are surprisingly similar (56% identity), further highlighting the special evolutionary relationship of these two products. Cytomegalovirus is known to infect myeloid and lymphoid cells in vivo (26), cell types that express the MIP-1α/RANTES receptor. Others have speculated that US28 was acquired by viral hijack of a human gene (25). Thus, the hijacked human gene may be for a β chemokine receptor. We speculate that expression of US28 may alter the responsiveness of infected lymphoid and/or myeloid cells to MIP-1α, RANTES, or another β chemokine. In this way the intracellular environment may be rendered more favorable for viral replication and/or establishment of the latent state. Viral hijack of immunoregulatory ligand and receptor genes has been reported for pox and herpes viruses. These and other viruses may use the viral homologues to elude the immune system by molecular mimicry (reviewed in reference 27). We have cloned US28 by PCR from fibroblasts acutely infected with human cytomegalovirus. No cross-hybridizing human genes were detected by blot hybridization of human genomic DNA with a full-length US28 DNA probe under low stringency conditions (final wash at 45°C in 5× SSPE; data not shown).

*Analysis of the MIP-1α/RANTES Receptor Gene.* Analysis of human genomic DNA by blot hybridization with a p4 probe under high and low (45°C, 5× SSPE) stringency conditions indicated that the MIP-1α/RANTES receptor must be encoded by a small, single-copy gene that lacks close homologues in human (Fig. 5 *A*). Thus, unshared receptors selec-

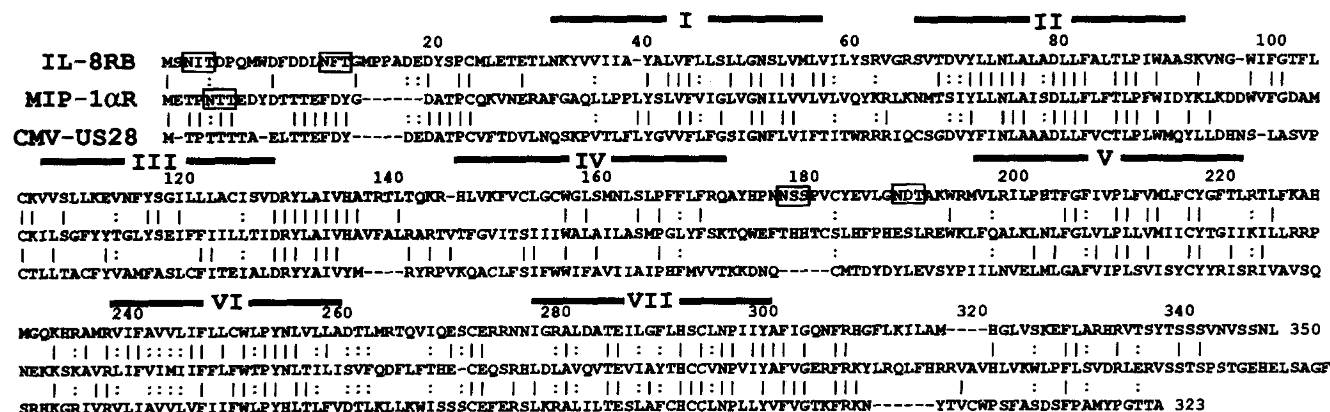

**Figure 4.** Primary structure of the human MIP-1α/RANTES receptor (MIP-1αR) and alignment with that of human IL-8R B (5) and CMV-US28 (25). Vertical bars indicate identical residues for each adjacent sequence position; colons indicate residues that are identical for US28 and IL-8R B but differ from MIP-1αR. The locations of predicted membrane-spanning segments I–VII are noted. Open boxes designate predicted sites for N-linked glycosylation. Arabic numbers above the sequence blocks refer to the MIP-1αR sequence and are left justified. Dashes indicate gaps that were inserted to optimize the alignment. The DNA sequence of p4 has been submitted to GenBank under accession number L10918.

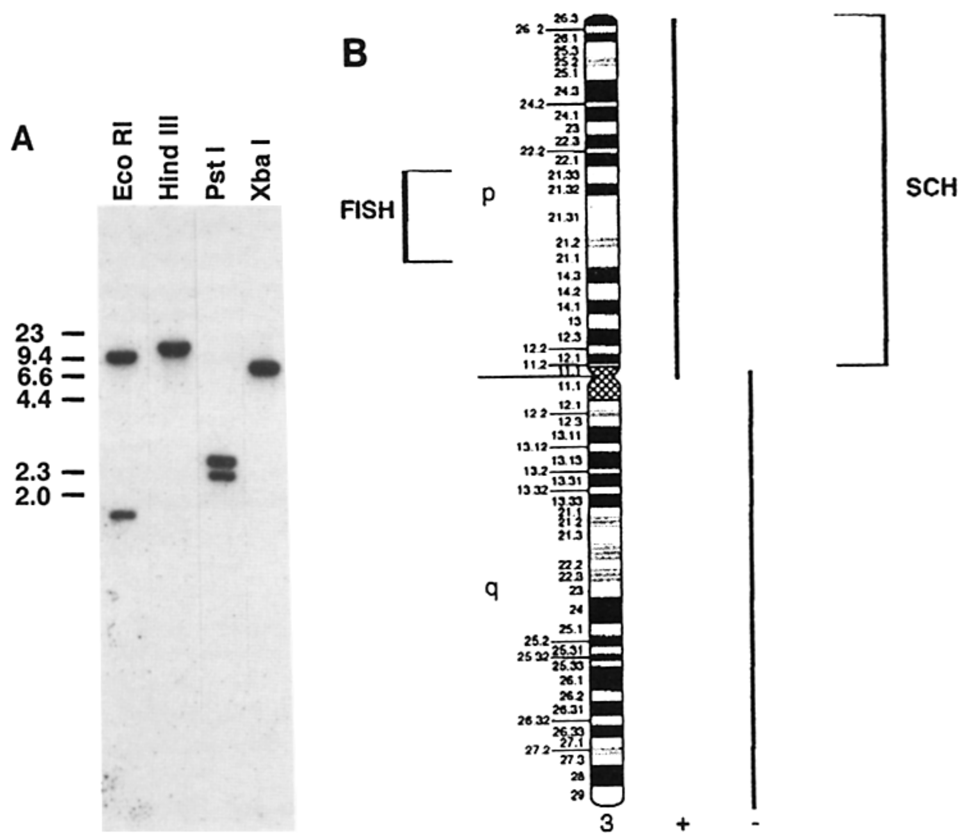


**Figure 5.** Analysis of the human MIP-1α receptor gene. (*A*) A blot of human genomic DNA digested with the indicated restriction endonucleases was hybridized with the p4 cDNA (washed at 68°C in 0.1× SSPE). The blot was exposed to XAR-2 film in a Quanta III cassette at −80°C for 6 d. DNA markers are in kilobases at the left. No crosshybridizing genes were detected under the low stringency conditions (washed at 45°C in 5× SSPE). The open reading frame of the MIP-1α/RANTES receptor gene has a restriction site for EcoRI and for PstI. (*B*) Chromosomal localization. Ideogram of G-banding pattern of human chromosome 3 with vertical bars illustrating the regions present in somatic cell hybrid lines. PCR amplification results are summarized below: +, hybrid positive; and −, hybrid negative for human MIP-1α/RANTES receptor sequence. Bracket labeled SCH shows localization of the gene based on hybrid cell mapping data. Bracket labeled FISH indicates localization of fluorescent in situ hybridization signals at 3p21.

tive for MIP-1α and/or RANTES, if they exist, must be structurally quite divergent from the receptor described here. The 6.5-kb XbaI fragment seen in Fig. 5 *A* was cloned and sequenced. An intron–exon boundary is found at nucleotide −12 relative to the ATG initiation codon, but the open reading frame and 3′ flanking sequences lack introns, a common finding for G protein–coupled receptor genes. By using primers specific for the human MIP-1α/RANTES receptor cDNA for PCR amplification of DNA from rodent × human somatic cell hybrid mapping panels, we have localized the gene to the short arm of chromosome 3. Independent confirmation of this assignment and further sublocalization of this gene to band 3p21 was accompanied by fluorescence in situ hybridization (FISH) of the 6.5-kb genomic XbaI fragment containing the gene (Fig. 5 *B*). The IL-8R genes are clustered on human chromosome 2q34-q35 (19).

MIP-1α has been shown to regulate the growth of stem cells and other more mature myeloid precursors (12–15). The effects of related chemokines, including RANTES, on hematopoiesis have not yet been reported. It will be important to study whether all or only some of the known effects of MIP-1α and RANTES are mediated by the receptor described here. If so, the structure of this receptor suggests that the signal transduction mechanism(s) for these disparate effects will involve G protein(s).

The MIP-1α/RANTES receptor is the first receptor for a hematopoietic growth regulator in the superfamily of receptors with seven transmembrane domains. Identification of the human MIP-1α/RANTES receptor will now facilitate precise delineation of the signal transduction pathways by which MIP-1α and RANTES activate responsive cells of the hematopoietic system and may lead to new insights regarding the pathophysiology of fever and wound healing as well as myelopoiesis and chemotaxis. Moreover, the relationship of the MIP-1α/RANTES receptor and US28 will permit the investigation of new hypotheses regarding the pathogenesis of human cytomegalovirus infection.

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