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Short sequence-paper

Molecular cloning and characterization of a cDNA encoding the rat interleukin-8 receptor [1]

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Abstract

In this study we present the cloning, characterization and expression analysis of a cDNA encoding a rat interleukin-8 receptor (rIL-8R). A 1324 bp cDNA containing an open reading of 359 amino acids with an 86.1% overall identity with the previously characterized mouse IL-8R was isolated. Genomic DNA analysis using several restriction enzymes revealed a single band suggesting that the *rIL-8R* gene exists as a single-copy, which is in contrast to humans where there are two different *IL-8Rs* genes. Expression of rIL-8R mRNA was found in several tissues including spleen, heart, lung, liver, skeletal muscle and kidney. In brain and testis rIL-8R mRNA was not detectable. Rat IL-8R mRNA expression at the cellular level was studied in the spleen using RNA-RNA in situ hybridization and immunohistochemistry. IL-8R mRNA containing cells were predominately found in the mantle zone of the germinal center. These cells were identified as B lymphocytes using the OX-33 monoclonal antibody.

Keywords: RT-PCR; In situ hybridization; Chemokine; G protein; Gene expression

Interleukin-8 (IL-8) belongs to a family of pro-inflammatory molecules called chemokines which are divided into two groups, the C-C and C-X-C chemokines, based on whether the first two cysteine residues in a conserved region are adjacent to each other, or are separated by an intervening residue [1–3]. IL-8, which can be produced by most cells, belongs to the C-X-C chemokines and is a potent chemoattractant for neutrophils and normal human keratinocytes [2,4,5]. It has been shown that IL-8 is constitutively and commonly produced by various human carcinoma cell lines suggesting a possible role for IL-8 in carcinogenesis [6]. Effects of IL-8 on neutrophils and other cell types are mediated through binding to specific cell-surface receptors [7] belonging to the family of G-protein coupled receptors, characterized as having seven transmembrane domains. Recently, a murine IL-8 receptor (IL-8R) was deleted from the mouse genome using homologous recombination in embryonic stem cells. These mice had lymphadenopathy, resulting from an increase in B cells, and splenomegaly, resulting from an increase in metamyelocytes and mature neutrophils. Thus, the IL-8R could have a role in the expansion and development of neutrophils and B cells [8]. So far, the cDNAs coding for IL-8Rs of several species, includ-

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ing human, rabbit and mouse, have been cloned [9–14]. In the present study we describe the cloning and characterization of a cDNA encoding a rat IL-8R.

In an effort to clone new G-protein coupled receptors in the rat using RT-PCR and degenerate primers with sequences corresponding to conserved amino acids in putative transmembrane regions III and VI of known G-protein coupled receptors [15] we identified a clone with high similarity to previously characterized IL-8Rs. To obtain a full-length cDNA a rat lung cDNA library was screened. Two clones, with inserts of 700 and 800 bp, were isolated and completely sequenced. These clones were partially overlapping and defined a cDNA 1324 bp in length. The cDNA contains an open reading frame of 1077 bp encoding a protein of 359 amino acids (Fig. 1). The predicted translation start site possesses a good consensus sequence for eukaryotic translation initiation (AAGATGG) as described by Kozak [16].

Amino acid sequence analysis of the isolated clone indicated high similarity with previously cloned IL-8Rs in mouse, human and rabbit (Fig. 2) [9–14]. In humans and rabbits two types of specific IL-8R receptors exist (type A and B). The type A receptor binds only IL-8 with high affinity. The B type receptor binds IL-8 but also other chemokines, such as melanoma growth stimulating activity (MGSA) and neutrophil activating protein-2 (NAP-2), with high affinity [3,10]. Our clone showed highest overall similarity to mouse IL-8R (86.1%). Rabbit and human type B IL-8R were 72.4% and 69.9% similar to rIL-8R, respectively. Rabbit and human type A recep-

Fig. 1. The 1324-nt sequence of the rat IL-8R and deduced amino acid sequence. Nucleotide and amino acid sequences are numbered on the right. Underlined (marked I–VII) segments indicate putative transmembrane regions. The * denotes potential N-linked glycosylation sites.

tors showed somewhat lower similarity to rIL-8R (64.3% and 66.9%, respectively). Based on the amino acid sequence similarities we conclude that our clone encodes a rat IL-8R, probably of type B.

A detailed analysis of the amino acid sequence of our rIL-8R clone revealed a number of features. The deduced amino acid sequence have four putative N-linked glycosylation sites in potential extracellular domains (Fig. 1), and hydropathicity analysis [17] demonstrated seven putative transmembrane domains (marked I–VII in Fig. 1) suggesting that this is a G-protein coupled receptor (GPCR). Sequence align-

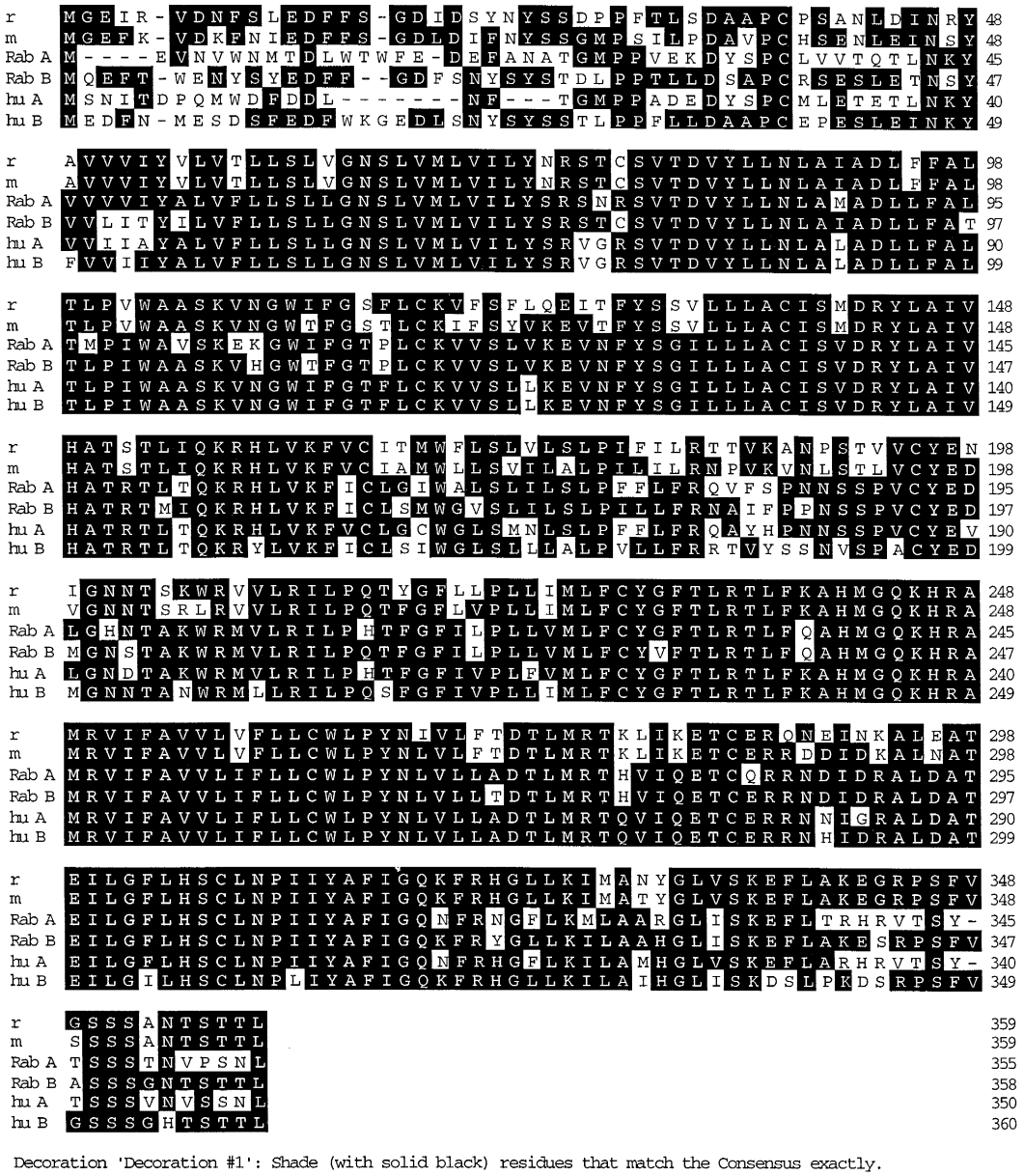

Fig. 2. Alignment of IL-8R amino acid sequences from different species. The sequences of rat (r), mouse (m) rabbit type A and B (RabA and RabB, respectively) and human type A and B (huA and huB, respectively), IL-8Rs are shown. Identical residues are highlighted. Alignments of amino acid sequences were performed using the MegAlign program of the Lasergene software package (DNASTAR, WI, USA).

ments of previously cloned GPCRs have revealed conservation of specific regions within the transmembrane and cytoplasmic regions of the molecules [18]. For example, in the second transmembrane domain of our clone there is an Asp (D) (Fig. 1, aa 93; see also Fig. 2), which is conserved in almost all GPCRs. Replacement of this residue has been shown to abolish ligand binding of the human type A IL-8R [19,20]. Furthermore, our clone contains the highly conserved DRYLAIVHA amino acid motif (Fig. 1 aa 142–150; and also see Fig. 2) which exists in all previously characterized IL-8Rs and is probably involved in signaling by the receptors. Actually, it has been demonstrated in other GPCRs that the DRY motif is necessary for the coupling to G-proteins, and mutation of this motif leads to almost complete loss of biological activity of the receptors [21].

Information on the ligand-binding domain of the IL-8Rs comes in large part from domain-swapping experiments and alanine-scanning mutagenesis studies [19,22]. From these studies a ligand binding model was proposed, in which an N-terminal Asp (D) together with the third extracellular loop residues Glu (E) and Arg (R) are involved. These residues are brought into close spatial proximity of one another by a disulfide bridge creating the ligand-binding site [3]. The residues implicated in this model can be found in our rIL-8R and indicates that they are highly conserved through evolution.

Interestingly, the COOH-terminus (Fig. 1; aa 321–354 and also Fig. 2) contains nine serine or threonine residues that may be sites of phosphorylation by cellular kinases. Actually, one group of kinase called G-protein coupled receptor kinases (GRKs) has been implicated in agonist-specific desensitization of GPCRs [23,24]. Furthermore, desensitization was observed in Chinese hamster ovary cells stably transfected with a rabbit IL-8R cDNA after IL-8 addition, and it has been demonstrated that staurosporin (a protein kinase inhibitor) inhibits IL-8 induced desensitization in neutrophils [25,26]. However, the exact role of the different GRKs or other protein kinases (for example protein kinase A or C) in IL-8R desensitization/regulation remains to be elucidated.

Expression of rIL-8R mRNA was studied using a multiple tissue blot (Clontech). We found rIL-8 mRNA expression in several tissues including spleen, heart, lung, liver, skeletal muscle and kidney (lanes 1, 3, 4, 5, 6, and 7, respectively). In brain and testis, expression was not detectable (lanes 2 and 8, respectively). We found two bands 2.4 and 4.4 kb in size (Fig. 3A). The larger band may represent another

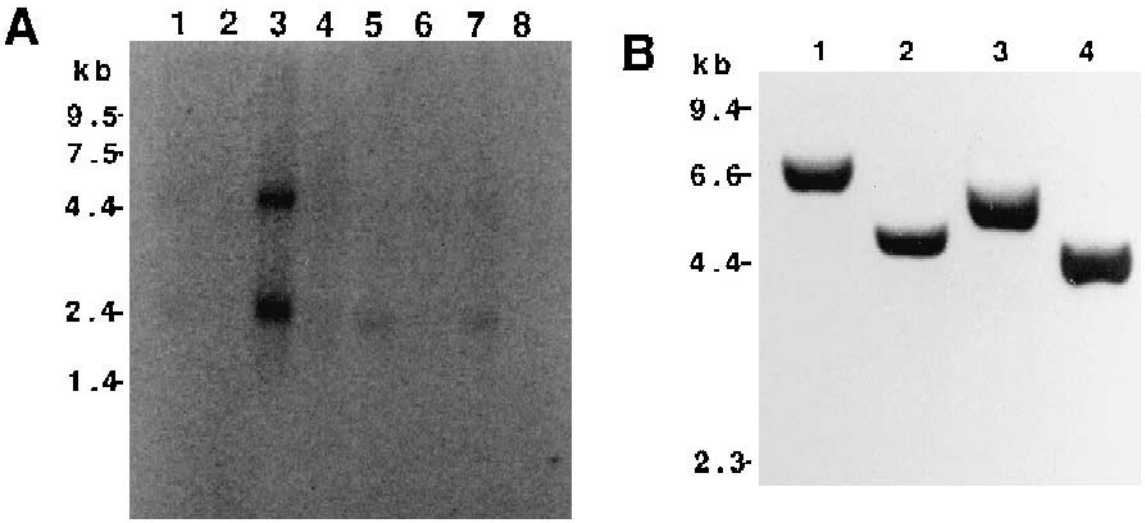

Fig. 3. (A) Tissue distribution of rat IL-8R mRNA. An RNA blot containing 2 μg poly(A)$^+$ RNA from various rat tissues was hybridized with the ^{32}P-labelled rat IL-8R probe. Lanes 1–8 are as follows: heart, brain, spleen, lung, liver, skeletal muscle, kidney, testis. The positions of RNA size markers are shown on the left. The blot was exposed overnight using a PhosphorImager screen (Molecular Dynamics, CA, USA). (B) Southern blot analysis of rat genomic DNA. Genomic DNA (approximately 30 μg) was digested with *Eco*RI (1), *Bgl*II (2), *Xba*I (3), *Sac*I (4) and probed with ^{32}P-labelled rat IL-8R cDNA. The positions of DNA size markers are on the left.

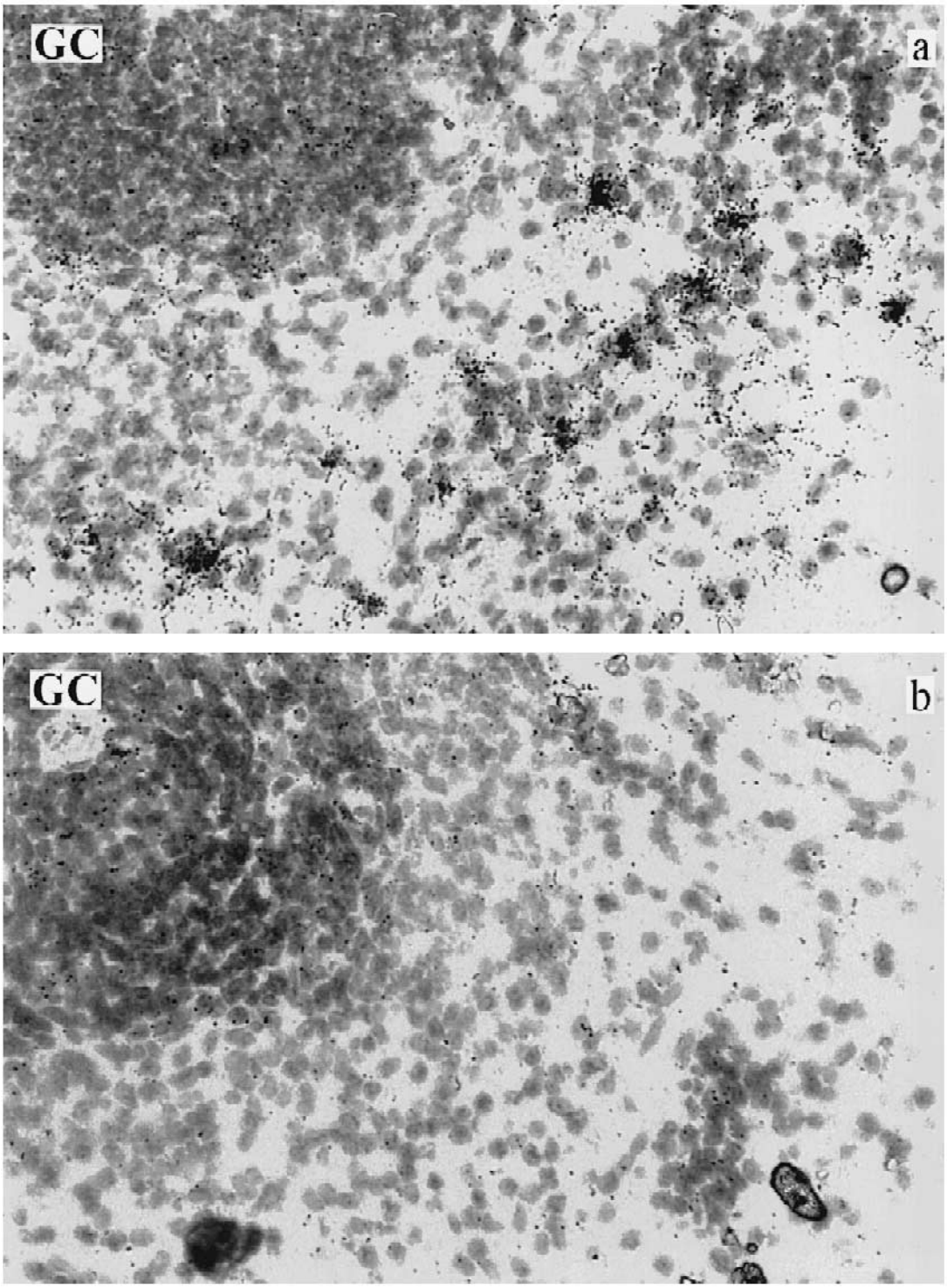

Fig. 4. In situ hybridization of rat spleen sections. Anti-sense (a) and sense (b) ^{35} S-labelled IL-8R cRNA probes were hybridized to slides carrying spleen sections. GC = germinal center. Light micrograph, magnification $\times 40$. Reproduction: 80%.

transcript starting upstream of the IL-8R TATA box having sequences in common with the IL-8mRNA.

Southern blot analysis of rat genomic DNA digested with *Eco*RI, *Bgl*II, *Xba*I and *Sac*I yielded a single band (Fig. 3B lanes 1–4, respectively) suggesting that the rIL-8R is a single copy gene. This result contrasts with that known in human and rabbit where two types of *IL-8R* genes exist. In mice, on the other hand, it has recently been shown that there is only one *IL-8R* gene and this receptor also can bind other chemokines than IL-8 which is characteristic of a type B IL-8R [13,27]. The size of the smallest DNA fragment shown in the Southern blot (Fig. 3B lane 4) was similar to that of the unprocessed rIL-8R mRNA (about 4.4 kb; Fig. 3A) suggesting that, as in the human *IL-8RB* gene, the *rIL-8R* gene lack introns in the coding region [28]. PCR with primers amplifying the rIL-8R coding region also generated a product with the same size (1077bp) using rat genomic DNA or cDNA from spleen as template (data not shown). However, to eliminate the possibility of very small introns in the coding region of the rIL-8R a genomic clone should be isolated and sequenced.

To study rIL-8R mRNA expression at the cellular level RNA-RNA in situ hybridization was performed as described in [29]. Rat IL-8R mRNA containing cells from at least 20 germinal centers in different spleens were investigated. Positive cells were predominately found in the mantle zone of the germinal centers (Fig. 4a). No positive cells were observed with the sense cRNA probe (Fig. 4b). To further investigate the identity of the rIL-8R positive cells in spleen we performed immunohistochemistry with monoclonal antibodies recognizing monocytes/macrophages (ED1), T-cells (R73), and B-cells (OX-33) [30–33]. As shown in Fig. 5A, the OX-33 antibody stained cells in the germinal celler but also cells in the mantle zone of the germinal center where rIL-8R mRNA containing cells are found (see Fig. 4). The R73 and ED1 antibodies distinctly stained cells in the spleen but not in the area of the mantle zone (Fig. 5B and C). Therefore, most rIL-8R expressing cells in rat spleen are B-cells.

In conclusion, a novel cDNA encoding for rat IL-8R was isolated and sequenced. The receptor showed high homology with previously cloned mouse, rabbit and human IL-8Rs. Northern blot analysis showed that this clone is expressed in several rat tissues. Distinct IL-8R mRNA containing cells outside the germinal centers were identified in the spleen using RNA-RNA in situ hybridization. These cells were identified as B lymphocytes using the OX-33 antibody. Southern blot analysis showed that rIL-8R is probably a single-copy gene. Finally, this cDNA will be a useful tool in further investigations of the IL-8R in rat.

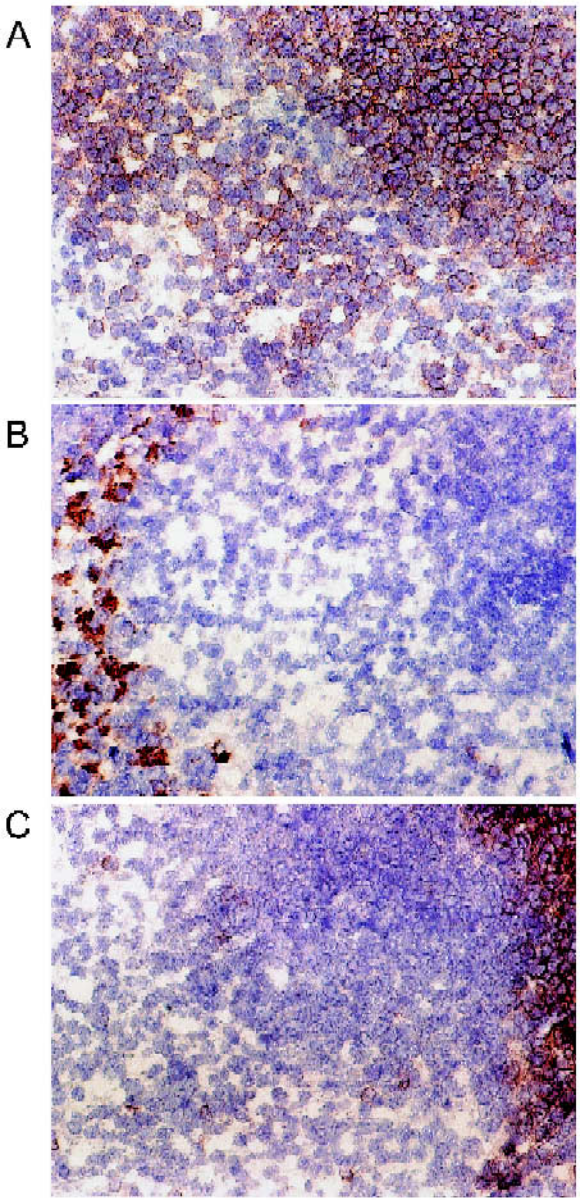

Fig. 5. Immunohistochemistry of rat spleen sections with OXy-33 (a), ED1 (b) and R73 monoclonal antibodies. Dilutions were 1/200, 1/800 and 1/1600, respectively. Antibody binding was detected using the PAP method.

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