Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 7732–7736, October 1990
Genetics

Identification of three related human *GRO* genes encoding cytokine functions

(inflammatory response/cDNA/tissue-specific expression)

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Contributed by Ruth Sager, June 27, 1990

ABSTRACT **The product of the human *GRO* gene is a cytokine with inflammatory and growth-regulatory properties; *GRO* is also called *MGSA* for melanoma growth-stimulatory activity. We have identified two additional genes, GROβ and GROγ, that share 90% and 86% identity at the deduced amino acid level with the original GROα isolate. One amino acid substitution of proline in GROα by leucine in GROβ and GROγ leads to a large predicted change in protein conformation. Significant differences also exist in the 3′ untranslated region, including different numbers of ATTTA repeats associated with mRNA instability. A 122-base-pair region in the 3′ region is conserved among the three *GRO* genes, and a part of it is also conserved in the Chinese hamster genome, suggesting a role in regulation. DNA hybridization with oligonucleotide probes and partial sequence analysis of the genomic clones confirm that the three forms are derived from related but different genes. Only one chromosomal locus has been identified, at 4q21, by using a GROα cDNA clone that hybridized to all three genes. Expression studies reveal tissue-specific regulation as well as regulation by specific inducing agents, including interleukin 1, tumor necrosis factor, phorbol 12-myristate 13-acetate, and lipopolysaccharide.**

The *GRO* gene is a member of a gene superfamily encoding a set of related cytokines with inflammatory and growth regulatory properties (1–4), including platelet factor 4 (PF4) (5), neutrophil-activating peptide 1/interleukin 8 (6–8), platelet basic protein and its split products connective tissue-activating peptide and β-thromboglobulin (9), γ interferon-inducible protein γIP-10 (10), and macrophage inflammatory protein 2 (11). *GRO*, also called *MGSA* for melanoma growth-stimulatory activity (12), was identified initially by its constitutive overexpression in spontaneously transformed Chinese hamster fibroblasts (1), and a related gene was identified in *v-src*-transformed chicken cells (13, 14). In expression studies with normal fibroblasts, *GRO* showed early response kinetics similar to c-*fos*, leading to the name *GRO* for growth-related (1). Subsequently a gene mapped to chromosome 4 encoding a protein with melanoma growth-stimulating activity (12) was shown to be the same as *GRO* (1), and sequence similarity was reported with the murine early response cDNA called KC (15).

Thus, the *GRO* gene encodes a protein with growth-related properties but whose sequence is related to proteins of the inflammatory response. Recent expression studies have clarified this apparent duality of functions by demonstrating that *GRO* is induced on at least two distinct pathways—one a growth-related serum-response pathway (1) and the other an interleukin 1 (IL-1)- or tumor necrosis factor (TNF)-induced pathway that is equally effective in growing and nongrowing cells (ref. 2; A.A., M. Messineo, S.W.L., and R.S., unpublished data). The IL-1 and TNF response, but not the growth-related serum induction, is mediated through the nuclear factor κB (NF-κB) binding site in the *GRO* promoter region (A.A., M. Messineo, S.W.L., and R.S., unpublished data).

Adherence of monocytes results in the rapid induction of high levels of steady-state mRNAs for a variety of inflammatory mediator genes (16). One of these clones had a predicted amino acid sequence similar to that reported for the original *GRO* gene product, which we call GROα. In the process of isolating a full-length cDNA clone, two different GRO clones, which we call GROβ and GROγ, were identified. In other studies, genomic sequencing of *GRO* led to the independent identification of genes corresponding to GROβ and GROγ.

This paper reports the structures of the GROβ and GROγ cDNAs** and compares them with that of GROα. Sequence data as well as DNA hybridization studies demonstrate the existence of three distinct *GRO* genes. Previous mapping studies of *GRO* (2, 12) which identified a unique *GRO* site at chromosome 4q21, suggest that the three *GRO* genes are closely linked and may have arisen by duplication. In mRNA expression studies using oligonucleotides that distinguish among the three *GRO* genes, we found tissue-specific and induction-specific transcription of these genes, suggesting the evolution of finely tuned transcriptional regulation.

MATERIALS AND METHODS

Isolation of cDNA Analogues of *GRO*. Construction and screening of an adherent monocyte cDNA library has been described (16). A 880-base-pair (bp) partial clone (C2m) isolated by subtractive hybridization showed some sequence similarity to the original *GRO* but contained a number of changes in the 3′ coding region. This clone was used to isolate five additional clones from a second library produced from mezerin- and calcium ionophore-stimulated leukocytes (17). One of these, called GROβ, contained a restriction pattern similar to the C2m isolate. Primers were constructed that permitted bidirectional sequencing of the double-stranded templates. In the case of GROβ, a partial clone (C2m) (16), a full-length clones containing two introns (CC2b), and a polymerase chain reaction (PCR) product were used to

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Abbreviations: IL-1, interleukin 1; TNF, tumor necrosis factor; PCR, polymerase chain reaction; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate.

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**The sequences reported in this paper have been deposited in the GenBank data base (accession nos. M36820 and M36821).

confirm the complete cDNA sequence. Another clone (GROγ) was represented three times in this screening. The remaining isolate appeared to have the restriction characteristics of the original *GRO* clone, GROα. All three clones were sequenced (Fig. 1).

Origin and Culture of Cells. Monocytes and lymphocytes were isolated from normal blood by Ficoll/Hypaque and subsequent Percoll density separation as described (18). Neutrophils were purified by sedimentation of the Ficoll/Hypaque pellet in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline containing 3% gelatin for 1 hr at 37°C. Monocytes, lymphocytes, and neutrophils were cultured in serum-free RPMI 1640 medium on plastic dishes that previously had been coated with fibronectin or on plastic with bacterial endotoxin [lipopolysaccharide (LPS)] at 1 μg/ml or were cultured under nonadherent conditions with 5 ng of phorbol 12-myristate 13-acetate (PMA) per ml (18). Fibroblasts and epithelial cells were grown as described (2). Colon carcinoma cells were derived from a fresh surgical specimen. Diagnosis was confirmed by histology.

Isolation of Genomic Clones. Approximately 8×10^5 plaques of a human female leukocyte library in λ phage EMBL3 were screened with ^{32}P-labeled GROα cDNA probe. Positive clones were subcloned into pGEM-3 or pGEM-4Z plasmids, and the whole plasmid or exonuclease III-nested deletion plasmids were sequenced by the dideoxy chain-termination method. The three *GRO* gene clones were distinguished by restriction fragment analysis.

Analysis of RNA and DNA. RNA analysis was carried out by either PCR or RNA blot (Northern) transfer. For PCR, 1 μg of total RNA was converted into first-strand cDNA with random hexamers as described by Kawasaki and Wang (19). Amplification was carried out by using a sense primer that was common to the different *GRO* clones and anti-sense primers specific for each. GROα was detected with GM135 and GM350; GROβ, with ML80 and GM297; and GROγ, with GM135 and GM221 (see Fig. 1). Amplifications were carried out to 25, 30, and 35 cycles to verify that reactions were proceeding exponentially. Data shown are from 30 cycles, at which time all samples had not yet reached maximum intensity. Northern transfers (see Fig. 5) were carried out as described with minor modifications (20). The oligonucleotide probes were labeled by polynucleotide kinase to 10^7 cpm/pM. Southern transfer analysis was carried out after transfer to nitrocellulose from random-primed cDNA probes or by direct oligonucleotide hybridization to dehydrated agarose gels as described (21, 22). Washing was carried out to a final stringency of 2× SSPE (22) at 56°C for oligonucleotides and 0.2× SSC (22) at 65°C for cDNA probes.

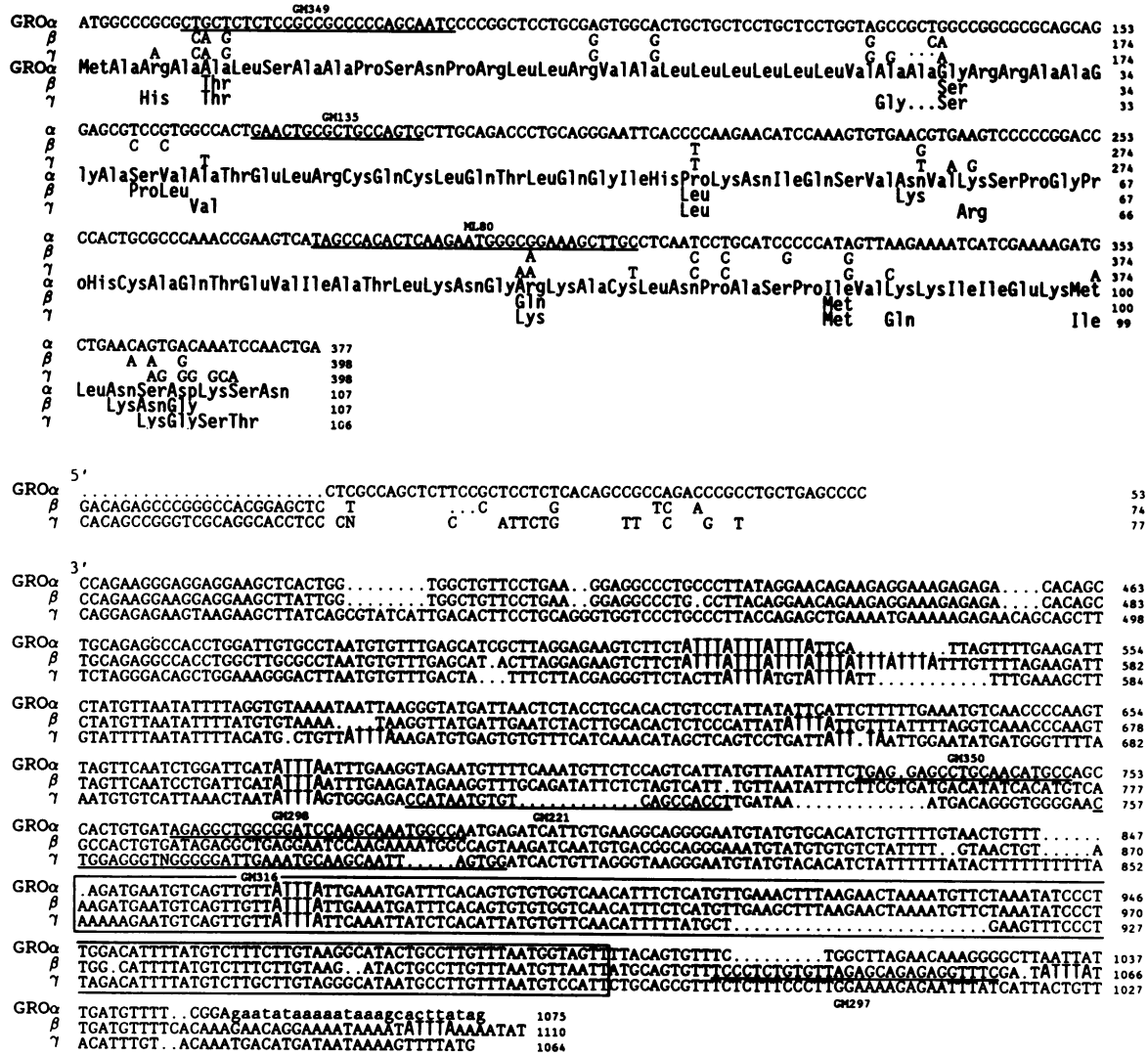

FIG. 1. DNA sequence (*Lower*) and predicted translation sequence of the open reading frame for GROβ and GROγ compared with GROα, noting positions of amino acid differences (*Upper*). Primers used for Southern DNA transfer and for PCR analysis of RNA are underlined. Bold-typed regions indicate the positions of the ATTTA motif, and conserved regions between the three human *GRO* isolates are boxed (beginning with base pair 848 GROα) as well as a region common between human and hamster (beginning base pair 930 GROα).

RESULTS

Characterization of Two Additional *GRO* Gene cDNAs. During isolation of cDNAs by subtractive hybridization from adherent vs. nonadherent monocytes (16) a clone with a *GRO*-related sequence was identified, and, by using this sequence as probe against a leukocyte cDNA library, full-length versions of two sequences we call GROβ and GROγ were recovered.

Sequence comparisons of the α, β, and γ forms are given in Fig. 1. GROβ and GROγ share 93% and 82% identity, respectively, with GROα at the nucleotide level. Compared with GROα, there are 11 amino acid substitutions in GROβ, 8 resulting from first and/or second position changes. Of the 11, 9 are located in the secreted peptide beyond the signal peptide cleavage point. Compared with GROα sequence, there are 15 amino acid changes in GROγ, 11 of which are in the secreted peptide region. In addition there is a codon deletion in GROγ at base pair 157.

ATTTA repeats have been associated with mRNA instability, typically found in cytokine genes (23, 24). In the 3′ untranslated region, GROβ has 11 ATTTA repeats, 6 of them in a single stretch at base pair 543. In contrast, GROα and GROγ contain 5 and 6 repeats, respectively. In addition a 64-bp region and a 58-bp region 3′ to it are highly conserved among the three GRO cDNA variants (beginning at base pair 847, 870, and 852, in GROα, GROβ, and GROγ, respectively). The intervening 25 bp are conserved in GROα and GROβ. The 3′ 58 bp of that region (930–987) are also conserved in the hamster gene as previously shown (1).

Predicted Secondary Structure of the Encoded Proteins. All but two of the amino acid substitutions are conservative in terms of size and hydrophobicity and in not altering the secondary structural predictions (25). However, there are two proline differences between GROα and GROβ and one between GROα and GROγ. The substitution of proline at position 36 in GROβ for serine in GROα has no apparent effect on structure. The substitution of leucine at amino acid 54 in GROβ and GROγ for proline in GROα has a potentially dramatic effect on α-helix, β-sheet, and β-turn predicted conformation in the secreted protein (25). The possible influence of additional substitutions, particularly in the carboxyl terminus, is difficult to predict.

Genomic Organization of *GRO*. The GROβ gene has been sequenced, as shown diagramatically in Fig. 2. The gene consists of four exons, three introns, and a 3′ untranslated region of about 700 bp terminating at the polyadenylylation site. The full sequence will be published elsewhere. The GROα and GROγ genes have been partially sequenced, including GROα introns 1 and 2 and GROγ intron 1. The intron locations have been conserved, and sequence conservation is about 80%. The upstream regions of the three genes are quite different, but some domains including the NFκB site have been conserved.

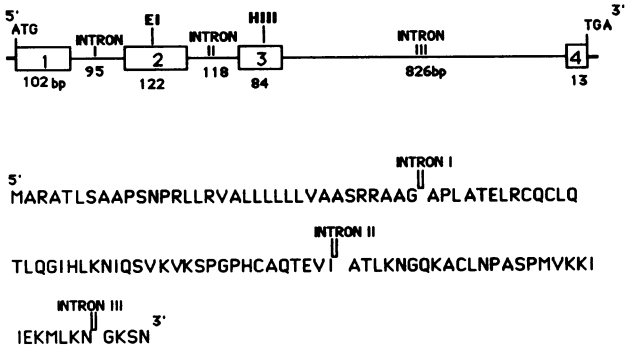

FIG. 2. (*Upper*) Nucleotide lengths in bp and arrangement of exon–intron sequences in the human GROβ gene. (*Lower*) Derived amino acid sequence in single-letter code and intron locations in the human GROβ gene.

DNA Hybridization Analysis Identifies Three *GRO* Genes. Southern blot hybridization with GROα, GROβ, and GROγ cDNA probes gave similar restriction fragment patterns with the enzymes *Xba* I and *Eco*RI as well as extra bands characteristic of each cDNA. In Fig. 3, these patterns (lanes 1, 2, and 3) were compared with Southern hybridizations using oligonucleotide probes common to the three cDNAs (lanes 4 and 5) as well as GROα-specific (lane 6) and GROβ-specific (lane 7) probes. In the *Eco*RI digests, the common probe ML80 identified three bands at 6.6, 4.4, and 3.3 kilobases (kb). With the GROα-specific probe GM350, only the 4.4-kb band was seen (lane 6); and with the GROβ-specific probe GM297, only the 3.3-kb band was seen. ML80 also detected the 6.6-kb GROγ-specific DNA seen only in lane 3. Similarly in the *Xba* I digests, the results are consistent in identifying three genes.

Expression of the Three *GRO* Genes Is Differentially Regulated in a Tissue- and Signal-Specific Manner. Utilizing PCR primer pairs specific for each of the three GRO cDNAs, we have characterized expression of these genes in inflammatory cells from a single donor (neutrophils, lymphocytes, and macrophages) as well as in endothelium and in a freshly isolated colon carcinoma (Fig. 4). Neutrophils adhered to fibronectin produced only the GROα version. Lymphocytes produced low but significant levels of GROα only, while monocytes expressed all three versions. Thus, different cells from the same donor stimulated by adherence to the same extracellular matrix component showed selective *GRO* gene expression.

Different inducers stimulated, however, different patterns of *GRO* expression in the same cell type. Nonadhered monocytes stimulated with PMA expressed only the GROβ and GROγ forms, whereas monocytes treated with LPS expressed all three versions. Expression of the GROγ gene was not restricted to macrophages; freshly isolated and dissected colonic epithelial tumor cells were found to express predominantly the GROγ version by PCR analysis.

Expression of GRO mRNAs was also examined by Northern hybridization with gene-specific oligonucleotide probes (Fig. 5) and a series of total RNAs from IL-1 or TNF-treated fibroblasts, normal mammary epithelial cells, and endothelial cells. The untreated cells did not make detectable levels of GRO message, whereas similar levels of GROα and GROγ and low levels of GROβ were detected in both IL-1- and TNF-treated cells. The LPS-treated monocytes expressed all

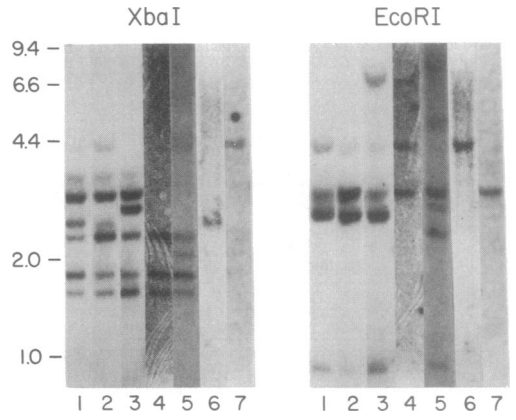

FIG. 3. Southern transfer analysis demonstrating the presence of distinct *GRO* genes. Lanes: 1–3, hybridization with the GROα, GROβ, and GROγ cDNA clones; 4–7, oligonucleotide hybridization to dehydrated agarose gels (24): oligonucleotide ML80 (lane 4), GM349 (lane 5), GROα-specific oligonucleotide GM350 (lane 6), and GROβ-specific oligonucleotide GM297 (lane 7).

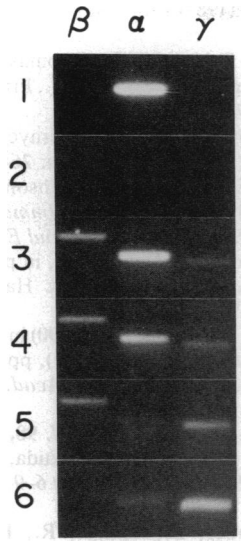

FIG. 4. PCR analysis of *GRO* expression in different cells from the same individual or similar cells stimulated by different signals. Lanes: 1–3, cells adhered for 45 min to fibronectin-coated plastic: neutrophils (lane 1), lymphocytes (lane 2), and monocytes (lane 3); 4, adherent monocytes stimulated with LPS for 4 hr; 5, monocytes stimulated under nonadherent conditions with PMA for 4 hr; 6, fresh biopsy of colonic carcinoma. All data are shown at 30 amplification cycles.

three forms, whereas the bladder carcinoma cell line T24 made only GROα. These results confirm that all three forms of GRO are expressed in the induced cells but do not provide a precise quantitative comparison, since the relative hybridization constants for the three probes may differ.

DISCUSSION

This paper presents evidence for the presence of three different *GRO* genes in the human genome. The genes have been distinguished experimentally by nucleotide differences in the coding region, in the 3′ untranslated region of the mRNA (Fig. 1), and in the 5′ regulatory region (not shown). The differences are also evident in restriction fragment patterns of genomic DNA hybridized with differential oligonucleotide probes as well as with GROα, GROβ, and GROγ cDNAs (Fig. 3). *In situ* chromosome hybridization with GROα cDNA, which cross-hybridizes to all three genes, revealed only a single site, at 4q21 (2, 12). This evidence suggests that the three genes are closely linked and may have arisen by duplication.

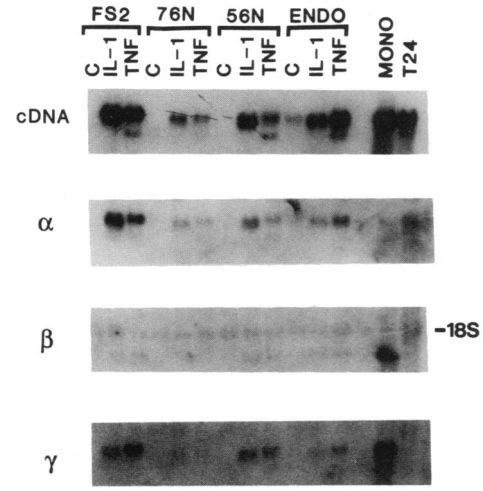

FIG. 5. *GRO* expression in cell lines. Expression in human cells of GROα, GROβ, and GROγ mRNAs by using specific oligonucleotide probes compared with cDNA probes (10 μg of total RNA per lane). Concentrations of IL-1 and TNF giving peak GRO responses were used. Lanes: FS-2, diploid skin fibroblasts; 76N, diploid mammary epithelial cells; 56N, fibroblasts from mammary gland; ENDO, umbilical vein endothelial cells (grown as described in ref. 27); MONO, LPS-treated monocytes; T24, bladder carcinoma cell line; C, control.

Although there are 9 amino acid differences in the secreted protein between GROα and GROβ, and 11 differences between GROα and GROγ, only one of them has a predicted strong effect on protein conformation. Several of the other changes, particularly in the carboxyl terminus, may have an influence on heparin binding because substitutions of lysines in this region markedly alter activity in platelet factor 4 (26). The conformational change resulting from substitution of proline for leu-54 may affect receptor recognition on target cells.

GROβ and GROγ cDNAs initially were detected in activated monocytes and cloned from a leukocyte library. Differential oligonucleotide pairs were constructed primarily on the basis of sequence differences in the 3′ untranslated regions. Differential expression of the three genes in mRNAs has been detected primarily by PCR amplification.

The role of NFκB in IL-1 and TNF-induced GRO transcription was determined with CAT constructs and gel retardation assays using the GROβ regulatory region. The NFκB site has now been identified by genomic sequencing in a similar domain in GROα, GROβ, and GROγ (unpublished data), suggesting that this site is utilized in cytokine stimulation of transcription of all three *GRO* genes.

The inducing effects of IL-1 and TNF on expression of all three GRO mRNAs (Fig. 5) are consistent with the presence of NFκB sites in the GROα, GROβ, and GROγ upstream regions. Fibroblasts, endothelial cells, and mammary epithelial cells have shown similar responses. Serum stimulation of *GRO* expression in fibroblasts, which does not appear to act via NFκB (A.A., M. Messineo, S.W.L., and R.S., unpublished data), elicits a stronger response of GROα mRNA than GROγ mRNA; GROβ mRNA is undetectable (unpublished data).

Recombinant GROα protein (3) was used in studies that demonstrated potent neutrophil chemoattraction by GRO (4). Whether the three GRO proteins are ligands for the same or different receptors is not yet known.

The strong expression in activated monocytes of GROβ and GROγ, which led to their detection, is important evidence of the tissue-specific regulation of *GRO* gene expression. The differential *GRO* gene expression in monocytes depending on the activation of the three genes and the substrate attachment of their products is further evidence that *GRO* gene expression is under finely tuned regulation. T24 (Fig. 5) and melanoma cell line A205 (unpublished data) express predominantly GROα, whereas a colon carcinoma (Fig. 4) expressed primarily GROγ. Both the complex regulatory control and the altered conformation of the GRO proteins encoded by different genes must reflect important functions of the GRO proteins requiring elaborate regulation in the inflammatory response.

Note Added in Proof. The sequences of cDNAs for the human macrophage inflammatory proteins MIP-2α and MIP-2β have just been published (28). They correspond to the cDNA sequences for GROβ and GROγ described in this paper, including not only the coding regions but also the 3′ and partial 5′ untranslated regions. Thus the three *GRO* genes represent the human homologs of the murine *MIP-2* gene.

We thank J. Pober for the endothelial RNAs (Fig. 5); S. Orkin for the leukocyte genomic library; M. Kriegler, C. Perez, K. De Fay, and I. Albert for the SR/α peripheral blood cDNA library; R. Cuenca for the colon carcinoma RNA; and S. Budd for preparation of the manuscript. This work was supported by National Institutes of Health Grants AI26774 (to S.H.) and CA39814 (to R.S.).

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