Cloning and Characterization of cDNAs for Murine Macrophage Inflammatory Protein 2 and its Human Homologues

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Summary

A cDNA clone of murine macrophage inflammatory protein 2 (MIP-2) has been isolated from a library prepared from lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and the nucleotide sequence determined. This cDNA was used to clone cDNAs for human homologues of MIP-2 from a library prepared from phorbol myristate acetate–treated and LPS-stimulated U937 cells. Two homologues were isolated and sequenced. Human MIP-2α and MIP-2β are highly homologous to each other and to a previously isolated gene, human gro/melanoma growth-stimulating activity (MGSA). These three human genes, MIP-2α, MIP-2β, and gro/MGSA, constitute a sub-family within the cytokine family represented by platelet factor 4 and interleukin 8.

Macrophages secrete a wide variety of proteins that mediate many aspects of inflammation (1). We recently described the purification and characterization of a novel monokine, murine macrophage inflammatory protein-2 (MIP-2),[1] which was one of two heparin-binding proteins secreted from RAW 264.7 cells in response to endotoxin stimulation (2). Determination of the NH_2-terminal sequence of purified MIP-2 and comparison with protein and DNA sequence databases indicated that it was a member of a rapidly expanding cytokine family whose members appear to modulate the inflammatory response and to have growth-promoting activities. Members of this family include human and hamster gro (3, 4); human, rat, and bovine platelet factor 4 (5-9); murine KC (10); rat CINC (11); chicken 9E3/CEF4 (12, 13); human platelet basic protein (14), the precursor of connective tissue-activating peptide and β-thromboglobulin; an IFN-γ-inducible protein, human γIP-10 (15); and IL-8, also known as 3-10C, MDNCF, NAF, and MONAP (16-20).

MIP-2 has been shown to elicit a localized inflammatory response when injected subcutaneously into the footpads of C3H/HeJ mice, to have potent chemotactic activity for human PMN, and to induce PMN degranulation of lysozyme but not of β-glucuronidase (2). In addition, MIP-2 has been shown to have CSF-dependent myelopoietic-enhancing activities for granulocyte/macrophage CFU (CFU-GM) (21). To further investigate the role of this cytokine at the molecular level,

we describe here the cloning and sequencing of the cDNA for murine MIP-2, as well as the isolation and nucleotide sequencing of human cDNA homologues.

Materials and Methods

cDNA Library Construction. The isolation of poly(A)$^+$ RNA from Escherichia coli LPS-stimulated murine RAW 264.7 cells and the construction of a cDNA library have been described previously (22).

The stimulation of the human monocytic-like cell line U937 (23), the isolation of total and poly(A)$^+$ RNA, and the construction of a cDNA library were performed as follows. U937 cells (American Type Culture Collection, Rockville, MD) were grown to confluence and stimulated to differentiate by the addition of PMA to a final concentration of 5 × 10^{-8} M. After 24 h in the presence of PMA, LPS (LPS W, E. coli 0127:B8; Difco Laboratories Inc., Detroit, MI) was added to a final concentration of 1 μg/ml, and the cells were incubated for an additional 3 h at 37°C. Total RNA was prepared essentially as described (24). Poly(A)$^+$ RNA was prepared by single passage over oligo-dT-cellulose essentially as described (25). Double-stranded cDNA was prepared using a kit for cDNA synthesis (Pharmacia LKB Biotechnology, Inc., Pleasant Hill, CA) and cloned and packaged into λgt10.

Murine MIP-2 cDNA Isolation. A degenerate oligonucleotide probe pool corresponding to amino acids 9–14 of the NH_2-terminal sequence of MIP-2 (2) was synthesized. This portion of the partial sequence was chosen because it was predicted to be in a highly conserved coding region and because of its lower codon degeneracy when compared with the other parts of the partial sequence. The resulting probe was a 128-fold degenerate pool of oligomers 17 nucleotides in length.

Duplicate nitrocellulose filters lifts of the plated RAW 264.7

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[1] *Abbreviations used in this paper:* GM, granulocyte/macrophage; MGSA, melanoma growth-stimulating activity; MIP-2, macrophage inflammatory protein 2.

cDNA library (5 × 10⁵ plaques) were prehybridized at 42°C in 5× SSC, 2× Denhardt's, 50 mM sodium phosphate buffer, pH 6.5, 50% formamide, 0.2% SDS, and 0.25 mg/ml sonicated salmon sperm DNA, and then were hybridized overnight at 42°C in 5× SSC, 1× Denhardt's, 20 mM sodium phosphate buffer, pH 6.5, 50% formamide, 10% dextran sulfate, 0.1% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 5 × 10⁴ cpm/ml per degeneracy of ³²P-ATP 5' end-labeled synthetic oligonucleotide probe pool. After hybridization, the filters were washed using tetramethylammonium chloride (26). Plaques that were positive on duplicate filters were subjected to a second round of low density plating and screening. Positive phage clones were isolated from which DNA was prepared for further analysis.

Isolation of Human Homologues of Murine MIP-2. Plating of the U937 cDNA library, nitrocellulose filter prehybridization, and hybridization of the filters were performed as described above for the screening of the RAW 264.7 cDNA library. The probe DNA was a 186-bp BalI-BglII fragment isolated from the mu-MIP-2 cDNA. The BglII site was introduced by in vitro mutagenesis using the mutagenic primer 5'-CAAAAGAT<u>CT</u>TGAACAAAG-3'. The BalI-BglII fragment encodes most of the mature mu-MIP-2 amino acid sequence, lacking those base pairs encoding the three NH_2-terminal and eight COOH-terminal amino acids. This fragment was nick-translated, and ~500,000 cpm/ml was included in the hybridization solution.

After hybridization, filters were subjected to three low stringency washes at room temperature for 30 min each in 2× SSC, 0.1% SDS. Plaques positive on duplicate filters were subjected to a second round of low density plating and screening. Positive phage clones were isolated from which DNA was prepared for further analysis.

Southern Analysis. Genomic DNA from RAW 264.7 cells was isolated as described by DiLella and Woo (27). Human genomic DNA and murine C3H/HeN genomic DNA were purchased from Clontech (Palo Alto, CA).

Genomic DNA was digested with restriction enzymes according to the supplier's specifications. Digested DNA was separated on 1% agarose gels and then transferred to HyBond nylon membranes (Amersham Corp., Arlington Heights, IL). Filters were prehybridized and hybridized in 50 mM sodium phosphate, pH 6.5, 5× SSC, 1 mM sodium pyrophosphate, 40% formamide, 10% dextran sulfate, 5× Denhardt's solution, 0.1% SDS, and 100 µg/ml sonicated salmon sperm DNA. DNAs used for Southern analysis were the 1.1-kb mu-MIP-2 cDNA (clone mMIP-2-20a), the 0.98-kb hu-MIP-2β cDNA (clone hMIP-2-4a), and a 1.05-kb hu-MIP-2α cDNA (clone hMIP-2-5a). All cDNAs were labeled by random priming with ³²P-CTP using a Multiprimer DNA Labeling System (Amersham Corp.). After prehybridization for 2–4 h at 37°C, labeled cDNA was added at 10⁶ cpm/ml. Hybridization was for 16–18 h at 37°C. Filters were rinsed at room temperature for 10 min in 2× SSC, 0.1% SDS, then washed three times at 65°C for 45 min each in 0.1 × SSC, 0.1% SDS. In some cases, hybridized probe was stripped from the blot by treatment for 45 min at 65°C in 0.5× SSC, 0.1% SDS, and 50% formamide to allow rehybridization.

DNA Sequence Analysis. cDNA inserts were subcloned into M13 phage vectors, and DNA sequencing was performed by the dideoxy chain termination method of Sanger et al. (28).

Results

Cloning of Murine and Human MIP-2 cDNAs. Screening of the cDNA library derived from poly(A)⁺ RNA from

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                                                      -27
   .        .        .        .             Met Ala Pro
GCTTCCTCGGGCACTCCAGACTCCAGCCACACTTCAGCCTAGCGCC ATG GCC CCT
        -20                                          -10
Pro Thr Cys Arg Leu Leu Ser Ala Ala Leu Val Leu Leu Leu Leu
CCC ACC TGC CGG CTC CTC AGT GCT GCA CTG GTC CTG CTG CTG CTG
                                  1
Leu Ala Thr Asn His Gln Ala Thr Gly Ala Val Val Ala Ser Glu
CTG GCC ACC AAC CAC CAG GCT ACA GGG GCT GTT GTG GCC AGT GAA
        10                                    20
Leu Arg Cys Gln Cys Leu Lys Thr Leu Pro Arg Val Asp Phe Lys
CTG CGC TGT CAA TGC CTG AAG ACC CTG CCA AGG GTT GAC TTC AAG
                                30
Asn Ile Gln Ser Leu Ser Val Thr Pro Pro Gly Pro His Cys Ala
AAC ATC CAG AGC TTG AGT GTG ACG CCC CCA GGA CCC CAC TGC GCC
        40                                    50
Gln Thr Glu Val Ile Ala Thr Leu Lys Gly Gly Gln Lys Val Cys
CAG ACA GAA GTC ATA GCC ACT CTC AAG GGC GGT CAA AAA GTT TGC
                                60
Leu Asp Pro Glu Ala Pro Leu Val Gln Lys Ile Ile Gln Lys Ile
CTT GAC CCT GAA GCC CCC CTG GTT CAG AAA ATC ATC CAA AAG ATA
        70                73
Leu Asn Lys Gly Lys Ala Asn                .        .
CTG AAC AAA GGC AAG GCT AAC TGA CCTGGAAAGGAGGAGCCTGGGCTGCTGT
 .          .        .        .        .        .        .
CCCTCAACGGAAGAACCAAAGAGAAAGAAAAAAACAAACAGCACCCGGGAAGCCTGGATC
 .          +100                              .        .
GTACCTGATGTGCCTCGCTGTCTGAGAGTTCACTTATTTATTTATCTATGTATTTATTTA
 .        .        .        .        .        .      +200
TTTATTAATTCCATTGCCCAGATGTTGTTATGTTTATTATGATATTTAAAGATATCGATT
 .        .        .        .        .        .        .
CGCTAATTCACTGTAATATCTTAAAAGGTCATTTTAATATGTTAAAGTTTATTTTAATAA
 .        .        .        .        +300             .
TGTTTAATGTGTTCAATTAAAGTTATTTAACTTATATAGTTGGAAGGTGATACATTTTTA
 .        .        .        .        .        .        .
AACCTATTTATTCATTAGTTTCTGGGGAGAGGGTGAGTTGGGAACTAGCTACATCCCACC
 .        .        +400           .        .        .
CACACAGTGAAAGAGACTGGGGATAAGGGGTGGGGGTGGGGACAAATAGATGCAGTCGGA
 .        .        .        .        .        .      +500
TGGCTTTCATGGAAGTAGTGTGCATGTTCACATCATTTTTTGTAAGCACCGAGGAGAGT
 .        .        .        .        .        .        .
AGAACAGCTGTTATTTAGGTTTCAGTGTTTGTAAACTGTATGTACAACATTTTTGATGCT
 .        .        .        +600          .        .
GGATTTCAATGTAATGTTGTGAGTAACCCTTGGACATTTTATGTCTTCCTCGTAAGGCAC
 .        .        .        .        .        .        .
AGTGCCTTGCTTAGCAATTGTTTTGTCATGCCTTTTCGTGTCTTGAAGTGGACACATTTA
 .        +700          .        .        .        .
TTTATTCATGTATTTTTACAAATAACAAAAAATAAAAACGTCTGTTAAAAAAAAAAAAAA
 .
AAAAAAAAAAAA
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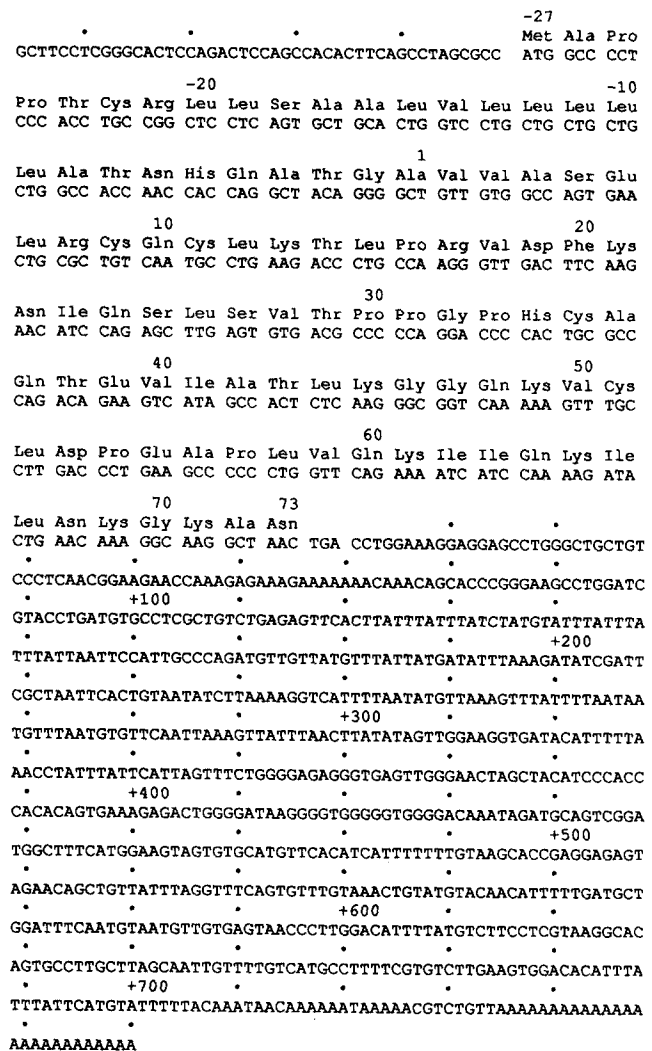**Figure 1.** Nucleotide sequence and predicted protein sequence of murine MIP-2. The molecular weight for the mu-MIP-2 precursor is 10,623. The mature protein sequence, starting at position 1, has a molecular weight of 7,851. These sequence data have been submitted to the EMBL/GenBank Data Library under the accession number X53798.

RAW 264.7 cells with a degenerate oligonucleotide pool specific for the NH_2-terminal sequence of murine MIP-2 (2) resulted in the isolation of clone MIP-2-20a. Insert cDNA (~1,100 bp) was isolated, cloned into M13, and the nucleotide sequence determined. The nucleotide sequence and predicted protein sequence are shown in Fig. 1. The predicted mature protein sequence starting at position 1 exactly matches the NH_2-terminal peptide sequence determined previously for purified MIP-2 (2).

To isolate the human homologue(s) of murine MIP-2 cDNA, a fragment encoding most of the mature mu-MIP-2 protein was isolated and used to probe a U937 cDNA library prepared from poly(A)⁺ RNA of PMA-treated and LPS-stimulated cells. DNA from plaques positive on low stringency wash was isolated and subjected to restriction endonuclease analysis, which suggested the presence of two classes

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                                                -34
  •         •         •         •             Met Ala Arg Ala
CTCTCCTCCTCGCACAGCCGCTCGAACCGCCTGCTGAGCCCC    ATG GCC CGC GCC
-30                                      -20
Thr Leu Ser Ala Ala Pro Ser Asn Pro Arg Leu Leu Arg Val Ala
ACG CTC TCC GCC GCC CCC AGC AAT CCC CGG CTC CTG CGG GTG GCG
                    -10
Leu Leu Leu Leu Leu Leu Val Ala Ala Ser Arg Arg Ala Ala Gly
CTG CTG CTC CTG CTC CTG GTG GCC GCC AGC CGG CGC GCA GCA GGA
  1                                  10
Ala Pro Leu Ala Thr Glu Leu Arg Cys Gln Cys Leu Gln Thr Leu
GCG CCC CTG GCC ACT GAA CTG CGC TGC CAG TGC TTG CAG ACC CTG
              20                                      30
Gln Gly Ile His Leu Lys Asn Ile Gln Ser Val Lys Val Lys Ser
CAG GGA ATT CAC CTC AAG AAC ATC CAA AGT GTG AAG GTG AAG TCC
                              40
Pro Gly Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys
CCC GGA CCC CAC TGC GCC CAA ACC GAA GTC ATA GCC ACA CTC AAG
              50                                      60
Asn Gly Gln Lys Ala Cys Leu Asn Pro Ala Ser Pro Met Val Lys
AAT GGG CAG AAA GCT TGT CTC AAC CCC GCA TCG CCC ATG GTT AAG
                              70          73
Lys Ile Ile Glu Lys Met Leu Lys Asn Gly Lys Ser Asn
AAA ATC ATC GAA AAG ATG CTG AAA AAT GGC AAA TCC AAC TGA CCAG
      •         •         •         •         •         •
AAGGAAGGAGGAAGCTTATTGGTGGCTGTTCCTGAAGGAGGCCCTGCCCTTACAGGAACA
      •         •       +100       •         •         •
GAAGAGGAAAGAGAGACACAGCTGCAGAGGCCACCTGGATTGCGCCTAATGTGTTTGAGC
      •         •         •         •         •         •
ATCACTTAGGAGAAGTCTTCTATTTATTTATTTATTTATTTGTTTGTTTTAGAAGA
      •       +200       •         •         •         •
TTCTATGTTAATATTTTATGTGTAAAATAAGGTTATGATTGAATCTACTTGCACACTCTC
      •         •         •         •         •       +300
CCATTATATTTATTGTTTATTTTAGGTCAAACCCAAGTTAGTTCAATCCTGATTCATATT
      •         •         •         •         •         •
TAATTTGAAGATAGAAGGTTTGCAGATATTCTCTAGTCATTTGTTAATATTTCTTCGTGA
      •         •         •       +400       •         •
TGACATATCACATGTCAGCCACTGTGATAGAGGCTGAGGAATCCAAGAAAATGGCCAGTG
      •         •         •         •         •         •
AGATCAATGTGACGGCAGGGAAATGTATGTGTGTCTATTTTGTAACTGTAAAGATGAATG
      •       +500       •         •         •         •
TCAGTTGTTATTTATTGAAATGATTTCACAGTGTGTGGTCAACATTTCTCATGTTGAAGC
      •         •         •         •         •       +600
TTTAAGAACTAAAATGTTCTAAATATCCCTTGGACATTTTATGTCTTTCTTGTAAGGCAT
      •         •         •         •         •         •
ACTGCCTTGTTTAATGTTAATTATGCAGTGTTTCCCTCTGTGTTAGAGCAGAGAGGTTTC
      •         •         •       +700       •         •
GATATTTATTGATGTTTTCACAAAGAACAGGAAAATAAAATATTTAAAAATAAAAAA
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Figure 2. Nucleotide sequence and predicted protein sequence of human MIP-2α. The molecular weight for the hu-MIP-α precursor is 11,391; the molecular weight of the predicted mature protein is 7,894. The NH_2-terminal amino acid of the mature protein was determined based on alignment with mu-MIP-2 and the rules for signal sequence cleavage sites. The nucleotides in the 3' untranslated region from +690 through the poly(A)+ were determined from two cDNA clones isolated from an independently derived cDNA library using a hu-MIP-2α-specific oligonucleotide probe. (M. Fabre, S. van Deventer, personal communication). These sequence data have been submitted to the EMBL/GenBank Data Library under the accession number X53799.

of clones. Insert cDNAs from representative clones of each class were subloned into M13, and the nucleotide sequences were determined. The nucleotide sequence and predicted amino acid sequence of hu-MIP-2α are presented in Fig. 2. This sequence was confirmed on four independent clones and is representative of the more abundant of the two classes of human cDNA homologous to mu-MIP-2. The nucleotide sequence and predicted protein sequence of hu-MIP-2β, representative of a second class of human cDNAs homologous to mu-MIP-2, are shown in Fig. 3. This sequence was confirmed on two independent clones.

Features of Murine and Human MIP-2 cDNAs. The nucleotide sequences of mu-MIP-2, hu-MIP-2α, and hu-MIP-2β each encode a single open reading frame. The nucleotide sequence environment of the initiating ATG codon of mu-MIP-2 conforms to the consensus sequence shared by many mRNAs of higher eukaryotes (29, 30); those of human MIP-2α and

MIP-2β lack the highly conserved purine at position −3 but possess many features of the consensus sequence, including C residues at positions −1, −2, and −4, and a G residue at position +4.

The 3' untranslated region of mu-MIP-2 includes the eukaryotic consensus polyadenylation signal AATAAA (31) at position +719–724 followed by a poly(A) string beginning at nucleotide +735. The consensus polyadenylation signal is present in the hu-MIP-2α cDNA at position +698–703 of the 3' untranslated region followed by a poly(A) beginning at nucleotide +716. No AATAAA polyadenylation signal was found in the 3' untranslated region of clones hMIP-2-4a or hMIP-2-7d of hu-MIP-2β cDNA. This is most likely due to the fact that these clones have a truncated 3' untranslated region since no poly(A) string was present.

The consensus sequence TTATTTAT found in the 3' untranslated region of many cytokine genes (32) and implicated

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                                        -34                -30
    •              •              •      Met Ala His Ala Thr Leu
CTCGCACAGCTTCCCGACGCGTCTGCTGAGCCCC      ATG GCC CAC GCC ACG CTC

                                         -20
Ser Ala Ala Pro Ser Asn Pro Arg Leu Leu Arg Val Ala Leu Leu
TCC GCC GCC CCC AGC AAT CCC CGG CTC CTG CGG GTG GCG CTG CTG

         -10                                              1
Leu Leu Leu Leu Val Ala Ala Ser Arg Arg Ala Ala Gly Ala Ser
CTC CTG CTC CTG GTG GCC GCC AGC CGG CGC GCA GCA GGA GCG TCC

                         10
Val Val Thr Glu Leu Arg Cys Gln Cys Leu Gln Thr Leu Gln Gly
GTG GTC ACT GAA CTG CGC TGC CAG TGC TTG CAG ACA CTG CAG GGA

         20                                      30
Ile His Leu Lys Asn Ile Gln Ser Val Asn Val Arg Ser Pro Gly
ATT CAC CTC AAG AAC ATC CAA AGT GTG AAT GTA AGG TCC CCC GGA

                         40
Pro His Cys Ala Gln Thr Glu Val Ile Ala Thr Leu Lys Asn Gly
CCC CAC TGC GCC CAA ACC GAA GTC ATA GCC ACA CTC AAG AAT GGG

         50                                      60
Lys Lys Ala Cys Leu Asn Pro Ala Ser Pro Met Val Gln Lys Ile
AAG AAA GCT TGT CTC AAC CCC GCA TCC CCC ATG GTT CAG AAA ATC

                         70          73
Ile Glu Lys Ile Leu Asn Lys Gly Ser Thr Asn     •
ATC GAA AAG ATA CTG AAC AAG GGG AGC ACC AAC TGA CAGGAGAGAAGT
          •         •         •         •         •
AAGAAGCTTATCAGCGTATCATTGACACTTCCTGCAGGGTGGTCCCTGCCCTTACCAGAG
          •         +100      •         •         •
CTGAAAATGAAAAAGAGAACAGCAGCTTTCTAGGGACAGCTGGAAAGGACTTAATGTGTT
          •         •         •         •         •
TGACTATTTCTTACGAGGGTTCTACTTATTTATGTATTTATTTTTGAAAGCTTGTATTTT
          +200      •         •         •         •
AATATTTTACATGCTGTTATTTAAAGATGTGAGTGTGTTTCATCAAACATAGCTCAGTCC
          •         •         •         +300      •
TGATTATTTAATTGGAATATGATGGGTTTTAAATGTGTCATTAAACTAATATTTAGTGGG
          •         •         •         •         •
AGACCATAATGTGTCAGCCACCTTGATAAATGACAGGGTGGGGAACTGGAGGGTGGGGGG
          •         •         •         +400      •
ATTGAAATGCAAGCAATTAGTGGATCACTGTTAGGGTAAGGGAATGTATGTACACATCTA
          •         •         •         •         •
TTTTTTATACTTTTTTTTTAAAAAAAGAATGTCAGTTGTTATTTATTCAAATTATCTCAC
          +500      •         •         •         •
ATTATGTGTTCAACATTTTTATGCTGAAGTTTCCCTTAGACATTTTATGTCTTGCTTGTA
          •         •         •         +600      •
GGGCATAATGCCTTGTTTAATGTCCATTCTGCAGCGTTTCTCTTTCCCTTGGAAAAGAGA
          •
ATTTATCATTACTGTTAC
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Figure 3. Nucleotide sequence and predicted protein sequence of human MIP-2β. The molecular weight of the hu-MIP-2β precursor is 11,345; the molecular weight of the predicted mature protein is 7,867. The NH₂-terminal amino acid of the mature protein was determined based on alignment with mu-MIP-2 and rules for signal sequence cleavage sites. These sequence data have been submitted to the EMBL/GenBank Data Library under the accession number X53800.

in mRNA stability (33) and efficiency of translation (34, 35) is present in multiple copies in all three cDNAs. This sequence is present at four positions, two overlapping, in the 3' untranslated region of mu-MIP-2 (positions +122, +126, +142, +146); and is present two times (positions +158 and +471) and five times, two overlapping (positions +148, +152, +156, +160, and +492) in the 3' untranslated regions of human MIP-2β and MIP-2α, respectively.

Features of the Predicted Amino Acid Sequence. The open reading frames of mu-MIP-2, hu-MIP-2α, and hu-MIP-2β predict polypeptides of 100, 107, and 107 amino acids, respectively. Secondary structural analysis of the predicted protein sequences of the three polypeptides indicates that the initial ∼30 amino acids of each have characteristic features of a signal sequence (36–38). The NH₂-terminal amino acid sequence determined for secreted MIP-2 purified from the conditioned medium of LPS-stimulated RAW 264.7 cells (2) determined

A   cDNA

	hu-MIP-2β	hu-MIP-2α	hu-*gro*	mu-*KC*
mu-MIP-2	54.1	57.2	56.5	46.4
hu-MIP-2β		70.0	69.5	57.0
hu-MIP-2α			87.4	47.6
hu-*gro*				50.8

B   Coding

	hu-MIP-2β	hu-MIP-2α	hu-*gro*	mu-*KC*
mu-MIP-2	71.3	71.3	70.4	75.6
hu-MIP-2β		94.4	90.7	67.9
hu-MIP-2α			93.5	69.4
hu-*gro*				67.3

C   3' Untranslated

	hu-MIP-2β	hu-MIP-2α	hu-*gro*	mu-*KC*
mu-MIP-2	48.1	53.1	53.4	47.3
hu-MIP-2β		59.5	60.9	55.7
hu-MIP-2α			86.2	48.8
hu-*gro*				48.5

Figure 4. Nucleotide sequence homology of MIP-2 homologues. Percentages of nucleotide sequence identity between mu-MIP-2, hu-MIP-2α, hu-MIP-2β, hu-*gro*/MGSA (3, 4), and mu-KC (10) in the cDNA (*A*), coding region (*B*), and 3' untranslated region (*C*) were determined with the ALIGN program (52).

the start of the mature mu-MIP-2 protein in the predicted amino acid sequence. The assignment of the start of mature peptide sequence for hu-MIP-2α and hu-MIP-2β was based on alignment of these sequences with that of mu-MIP-2, as well as consensus rules for signal peptide cleavage sites (36–38). The predicted length of the mature peptide sequence for all three proteins is 73 amino acids. Murine MIP-2 is a basic protein, and the human MIP-2 polypeptides are basic as well, based on predicted isoelectric points of 9.9 and 9.7 for hu-MIP-2α and hu-MIP-2β, respectively. None of the three predicted polypeptides has a consensus signal for N-linked glycosylation.

Murine and Human MIP-2 Homologues. Use of the murine MIP-2 cDNA coding region to isolate a human homologue resulted in the identification of two candidate cDNAs, MIP-2α and MIP-2β. The percentage of nucleotide sequence identity among these three cDNAs, as well as the human *gro*/MGSA cDNA (3, 4) and the murine KC cDNA (10, 39), is presented in Fig. 4 *A*. The human *gro*/MGSA cDNA encodes a protein with MGSA (40); murine KC is a platelet-derived growth factor–inducible gene presumed to be the murine homologue of human *gro*/MGSA. Noteworthy is the high degree of nucleotide homology among the three human cDNAs, particularly between hu-*gro* and hu-MIP-2α. There is an even more striking degree of nucleotide sequence identity among the three human homologues in the coding region, as shown in Fig. 4 *B*. The nucleotide sequence identity in the 3' untranslated regions of the human MIP-2 homologues is considerably less than that observed in the coding regions, with the exception of these regions of hu-MIP-2α and hu-*gro*/MGSA (Fig. 4 *C*). These two cDNAs show a high percentage of sequence homology throughout the 3' untranslated region as well.

Homology comparisons and alignments of the predicted amino acid sequences of the precursor proteins of MIP-2 homo-

A

	hu-MIP-2β	hu-MIP-2α	hu-*gro*	ha-*gro*	mu-KC	hu-IL-8
mu-MIP-2	59.8	57.9	57.9	68.3	63.0	36.3
hu-MIP-2β		87.9	86.9	61.1	55.1	32.7
hu-MIP-2α			89.7	64.8	58.9	33.6
hu-*gro*				59.3	55.1	34.5
ha-*gro*					80.2	38.2
mu-KC						41.4

B

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hu-gro/MGSA  1 MARAaLS    AAPSNPRLLRVALLLLLLVAAgRRAAGAsvATELRCQCLQT LQGIHpKNIQSVnVKSPGPH
               ||||  ||   |||||||||||||||||||| ||||||  ||||||||||| ||||| |||||| |||||||
hu-MIP-2α    1 MARATLS    AAPSNPRLLRVALLLLLLVAASRRAAGAplATELRCQCLQT LQGIHLKNIQSVkVKSPGPH
               ||  ||||   ||||||||||||||||||||||||||||||  |||||||||| ||||||||||||| | |||||
hu-MIP-2β    1 MAhATLS    AAPSNPRLLRVALLLLLLVAASRRAAGAsVvTELRCQCLQT LQGIHLKNIQSVnVrSPGPH
               ||  || |       |    |  ||    ||||||| | || | ||  ||||||||||  |  ||||||||  |  ||||
ha-gro/MGSA  1 MAPATRS LLrA   P  LL     LLLLLL ATSRLATGAPVANELRCQCLQT MtGvHLKNIQSLKVtPPGPH
               |  |||||| ||  |        ||||  |||||||||||  ||||||||||  |  ||||||||||||  |  |||
mu-KC        1 MiPATRS LLcA         A     LLLL ATSRLATGAPiANELRCQCLQT MaGiHLKNIQSLKVlPsGPH
               | | |    ||  |   |     |||| ||   ||||  |  |||||||| |            ||||||  |  |  |||
mu-MIP-2     1 MaPpT crLLsA         AlvlLLLL ATnhqATGAvvAsELRCQCLKT lprvdfKNIQSLsVtPpGPH
               |          |  |        |     |     |       |      | |||||||  ||     |    |   |||
hu-IL-8      1 M tsklavaLlA         A    fL  isaAlcegAvlprsAkELRCQCiKTyskpfhpKfIkeLrViesGPH
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hu-gro/MGSA 69 CAQTEVIATLKNGrKACLNPASPiVKKIIEKMLnsdKSN
               |||||||||||||| |||||||||| ||||||||||    |||
hu-MIP-2α   69 CAQTEVIATLKNGqKACLNPASPMVKKIIEKMLknGKSN
               ||||||||||||||| ||||||||||| |||||| |   |  .|
hu-MIP-2β   69 CAQTEVIATLKNGkKACLNPASPMVQKIIEKiLnKGstN
               |  |||||||||||  |||||  |||||||  | | |
ha-gro      63 CTQTEVIATLKNGqEACLNPEAPMVQKIVQKML KsGirK
               |||||||||||||| |||| |||| |||||||||| |  |  |
mu-KC       59 CTQTEVIATLKNGrEACLDPEAPLVQKIVQKML K GvpK
               | ||||||||||  |   ||||||||||||||| |  |  |  |
mu-MIP-2    62 CAQTEVIATLKgGqkvCLDPEAPLVQKIiQKiLnK Gkan
               || || |   |   |     ||||  ||   |  |   |
hu-IL-8     61 CAnTEiIvkLsdGrelCLDPkenwVQrvveKfLkraens
```

Figure 5. Amino acid homology and alignment of MIP-2 homologues and human IL-8. (*A*) Percentages of identity between the predicted amino acid sequences of MIP-2 homologues as well as human IL-8 (16) were determined with the ALIGN program (52). (*B*) These amino acid sequences were aligned with GENALIGN, a multiple sequence alignment program based on an algorithm developed by Needleman and Wunsch (53) and Sobel and Martinez (54). An amino acid that is not identical to a corresponding residue in adjacent homologues is designated by a lower case letter. Bold-type amino acid residues indicate the NH₂-terminal amino acid(s) determined by sequencing of the isolated protein (2, 4, 17, 18, 55). The predicted protein sequence of hu-*gro* and ha-*gro* are from Anisowicz et al. (3); hu-IL-8 is from Schmid and Weissman (16). Sequences were verified against GenBank entries (release no. 62) whenever possible.

logues, including hu-MIP-2α, hu-MIP-2β, hu-*gro*/MGSA, ha-*gro*, mu-MIP-2, and mu-KC, are presented in Fig. 5 *A* and *B*. The three human proteins are highly homologous (87–90%), but amino acid differences occur throughout the predicted sequences, particularly at the COOH termini of the mature protein sequences. Based on predicted amino acid homologies alone, it is not possible to assign hu-MIP-2α, hu-MIP-2β, or hu-*gro*/MGSA as the human homologue of mu-MIP-2 or mu-KC.

Southern Analysis. RAW 264.7 DNA was digested with each of three restriction enzymes, BamH1, EcoR1, and EcoRV, separated by agarose gel electrophoresis and probed with ³²P-labeled mu-MIP-2 cDNA. The results, shown in Fig. 6 *A*, are consistent with mu-MIP-2 cDNA defining a single gene. The same results were obtained when mouse C3N/HeJ DNA was similarly analyzed (data not shown).

A Southern analysis of human genomic DNA was performed with hu-MIP-2α and hu-MIP-2β cDNA probes. Hy-
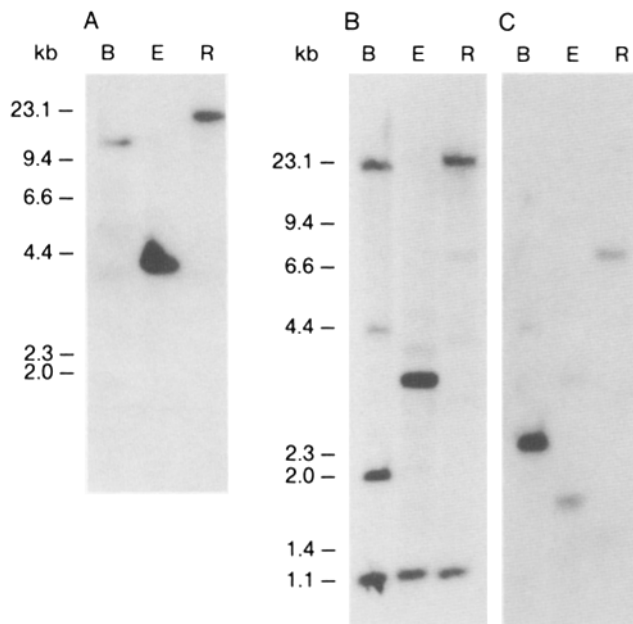
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Figure 6. Southern analysis of genomic DNA with murine and human MIP-2 cDNAs. Genomic DNA, as indicated below, was digested with BamH1, B; EcoR1, E; or EcoRV, R. (*A*) Murine RAW 264.7 DNA hybridized with mu-MIP-2 cDNA. A blot of restricted human DNA was hybridized first with labeled hu-MIP-2β cDNA (*C*), then the filter was stripped and rehybridized with labeled hu-MIP-2α cDNA (*B*).

Discussion

We have cloned the cDNA for murine MIP-2 by using a degenerate oligonucleotide probe pool corresponding to a portion of the NH₂-terminal amino acid sequence determined on the purified protein. Murine MIP-2 purified from the conditioned medium of endotoxin-stimulated RAW 264.7 cells has diverse activities, including CSF-dependent myelopoietic enhancing activity for CFU-GM (21), elicitation of a localized inflammatory response after subcutaneous administration, and a potent chemotactic activity for human PMN (2). The latter activity is characteristic of human IL-8, also known as 3-10C, MDNCF, NAF, and MONAP (16–20). This functional equivalence suggested that mu-MIP-2 could be the murine homologue of hu-IL-8. However, given that the amino acid homology of mu-MIP-2 to hu-IL-8 is low relative to mu-MIP-2 homology to hu-MIP-2α, hu-MIP-2β, or hu-*gro*/MGSA (3, 4) (Fig. 5), it seems unlikely that mu-MIP-2 and hu-IL-8 are murine/human homologues. Redundancy of function among cytokines is not uncommon: cachectin/TNF-α and IL-1 have an overlapping activity profile (41). MIP-2 and IL-8 may be another example of this functional redundancy.

Based on nucleotide and proteins homologies, it is likely that MIP-2 and KC (10) are murine homologues of the human cytokines MIP-2α, MIP-2β, and *gro*/MGSA (3, 4). More specific homologue assignments are difficult to make based only on these data. Further studies of these factors may establish functional homologies that in conjunction with nucleotide and protein identities will allow accurate assignment of interspecies homologues. The identification of two murine factors homologous to three human factors also suggests the existence of an additional murine factor.

We have used the cDNA for murine MIP-2 to clone cDNAs for homologous human genes from the monocytic-like cell line U937, which was stimulated to differentiate by treatment with PMA, and then further stimulated by LPS treatment. We have cloned two cDNAs, hu-MIP-2α and hu-MIP-2β. A noteworthy feature of these cDNAs is the high degree of both nucleotide and predicted amino acid sequence identity among these two cDNAs and the previously cloned cDNA hu-*gro*/MGSA (3, 4). The percentage of nucleotide sequence identity between hu-MIP-2α and hu-*gro*/MGSA is particularly striking, as it extends throughout the entire cDNA. The presence of both MIP-2α and MIP-2β in our U937 cDNA library prepared from poly(A)⁺ RNA from PMA- and LPS-stimulated cells prompted us to screen for *gro*/MGSA as well. Screening of 5 × 10⁵ plaques from the amplified library with oligonucleotides specific for *gro*/MGSA and not MIP-2α/β gave no positive signals; in contrast, 56 MIP-2α-positive signals were detected. This suggests that *gro*/MGSA transcription is not induced in U937 cells stimulated by PMA and LPS.

The results of Southern analysis are consistent with hu-MIP-2β and hu-MIP-2α defining two distinct genes. It is not possible by Southern analysis with a hu-MIP-2α cDNA probe to unequivocally determine if hu-MIP-2α and hu-*gro*/MGSA define separate genes given the high degree of

bridization and wash conditions were determined that made it possible to distinguish between hu-MIP-2α and hu-MIP-2β when probed with their respective cDNAs. These conditions are, however, unlikely to distinguish between hu-MIP-2α- and hu-*gro*/MGSA-specific sequences. The results of Southern analyses of genomic human DNA restricted with BamH1, EcoR1, or EcoRV, and probed with hu-MIP-2α or hu-MIP-2β cDNA, are presented in Fig. 6 *B* and *C*, respectively. The patterns of hybridization obtained with each cDNA clearly differ. MIP-2α cDNA hybridized strongly to EcoRV fragments of ∼23 and ∼1.1 kb, whereas MIP-2β cDNA hybridized to a 7.0-kb EcoRV fragment. Similarly, hu-MIP-2β cDNA hybridized to 1.8- and 3.3-kb (weakly) EcoR1 fragments, whereas hu-MIP-2α hybridized strongly to 3.3- and an ∼1.1-kb EcoR1 DNA fragments and weakly to 4.6- and 3.8-kb EcoR1 fragments. Finally, MIP-2β cDNA hybridizes strongly to a 2.4-kb BamH1 fragment and very weakly to a 4.3-kb BamH1 fragment, whereas human MIP-2α cDNA hybridizes strongly to 20-, 2.0-, and 1.1-kb BamH1 fragments and less strongly to a 4.3-kb BamH1 fragment. The greater complexity of the hybridization pattern obtained with hu-MIP-2α cDNA probe, especially from BamH1-digested DNA, relative to that obtained with hu-MIP-2β cDNA probe, suggests that the former probe is detecting more than one gene, presumably hu-*gro*. Experiments are in progress with specific oligonucleotides to distinguish hu-*gro* and hu-MIP-2α genes. We can conclude from the data shown here that hu-MIP-2β and hu-MIP-2α are two distinct genes.

	hu-MIP-2α	hu-gro	hu-IL-8	hu-pbp	hu-pf4	hu-pf4v	hu-γip10
hu-MIP-2β	87.9(107)	86.9(107)	38.9(95)	48.1(77)	43.0(93)	37.6(93)	26.1(69)
hu-MIP-2α		89.7(107)	40.0(95)	48.8(82)	43.6(94)	39.2(102)	30.6(72)
hu-gro			41.1(95)	51.2(82)	46.8(94)	42.2(102)	29.0(69)
hu-IL-8				46.4(60)	30.6(85)	25.5(98)	27.7(83)
hu-pbp					55.4(65)	48.6(70)	35.9(64)
hu-pf4						88.2(93)	35.5(62)
hu-pf4v							36.7(60)

Figure 7. Amino acid homologies between members of the human platelet factor 4 cytokine family. Homologies were determined with the FASTP sequence alignment program (56). The numbers in parenthesis indicate the number of amino acids used in the calculation of the percent identity. Amino acid sequences for the indicated proteins were from the following references: hu-gro (3); hu-IL-8 (16), human platelet basic protein, hu-pbp, (14); human platelet factor 4, hu-pf4 (6); variant of human platelet factor 4, hu-pf4v (48); and IFN-γ-inducible protein, γip10 (15). When possible, sequences were verified against GenBank (release no. 62) or Dayhoff (release no. 22) entries.

nucleotide homology between these cDNAs. The complexity of the hybridization pattern with hu-MIP-2α cDNA, compared with that with hu-MIP-2β cDNA, especially to BamH1-digested genomic DNA, is consistent with the detection of more than one gene. The pattern we observe with hybridization of MIP-2α cDNA to EcoR1-restricted genomic DNA is similar to that reported by Richmond et al. (4) for MGSA cDNA. Differences between the two may reflect differences in the size of the cDNA used as probe and/or the completeness of digestion of genomic DNA.

The high degree of homology among these three cDNAs suggests that their genes may have arisen by duplication and predicts that these genes should be located near each other on the chromosome. Anisowicz et al. (42) have reported in situ hybridization of hu-gro cDNA to a single loci, chromosome band 4q21. Richmond et al. (4) have mapped hu-MGSA to region q13-q21 of chromosome 4 by Southern analysis of human/hamster hybrids using a 700-bp cDNA probe. Given the sequence homology among gro/MGSA and MIP-2α/β genes, these experiments may not have distinguished among them. Thus, the detection of only a single chromosomal loci for gro/MGSA is indirect evidence for close proximity of the three genes. Thus far, four members of the platelet factor 4 cytokine family, including platelet factor 4 (43), gro/MGSA (4, 42), γIP-10 (44), and IL-8 (45), have been localized to this chromosomal region.

Previous studies using hu-gro/MGSA cDNA as a probe for gene expression have shown expression in various transformed cell lines, fibroblasts, epithelial cells, and endothelial cells (3, 4, 42, 46, 47). Expression in some of these different cell types was shown to be markedly induced by stimuli including serum, PMA, IL-1, TNF, LPS, and thrombin (3, 4,

42, 46, 47). IL-1-mediated induction of hu-gro in human fibroblasts has also been confirmed by NH2-terminal sequence analysis of the induced protein (47). The high homology of hu-gro, hu-MIP-2α, and hu-MIP-2β makes problematic the interpretation of the pattern of expression of any one gene from Northern data using cDNA probes. In fact, in several of the above studies, RNA species of two different sizes were noted. Northern analysis with specific oligonucleotide probes will be required to accurately determine the pattern of expression of these genes.

The degree of nucleotide and predicted amino acid homology among human MIP-2α, MIP-2β, and gro/MGSA is particularly high compared with their homology with other human members of this cytokine family (Fig. 7). Interestingly enough, there is another example of highly homologous proteins within the platelet factor 4 family of cytokines. A genomic clone has been isolated that encodes a platelet factor 4 variant that is highly homologous to platelet factor 4; there is 85% amino acid identity in the predicted precursor protein and 96% amino acid identity in the mature protein (48).

The existence of these highly homologous human MIP-2α/β and gro/MGSA peptides raises the question of their functional independence. Recent structural studies indicate that two members of this cytokine family, platelet factor 4 and IL-8, which have 31% amino acid identity (Fig. 7), share many structural features (49–51), including a COOH-terminal helix that has been postulated to be involved in receptor binding. It is interesting to note that one of the main regions of greatest amino acid variability among hu-MIP-2α, hu-MIP-2β, and hu-gro/MGSA is at their COOH termini. Further studies will be required to address the significance of this observation.

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