Identification of CRG-2
AN INTERFERON-INDUCIBLE mRNA PREDICTED TO ENCODE A MURINE MONOKINE*

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In order to identify novel proteins produced by activated macrophages, a cDNA library was made from cultures of the mouse macrophage-like cell line RAW 264.7 that had been treated with conditioned medium from mitogen-stimulated spleen cells, and the library was screened by differential plaque hybridization. A cDNA clone was isolated that detected a 1.4-kilobase mRNA that accumulated dramatically in response to the spleen cell conditioned medium. The 1.4-kilobase mRNA encodes a predicted protein of 98 amino acids, designated CRG-2, molecular weight (Mr) 10,781, with a 21-residue signal peptide. The amino acid sequence indicates that CRG-2 is a member of the platelet factor 4 family (PF4) of cytokines. The CRG-2 mRNA was induced by α-, β-, and γ-interferons (IFNs) and by lipopolysaccharide. In response to IFN-γ, the CRG-2 mRNA level reached a peak between 3 and 6 h. The accumulation of CRG-2 mRNA was not blocked by cycloheximide. Among the known members of the PF4 family, CRG-2 is most closely related to the interferon-inducible human protein IP-10. The 5'-flanking region of the crg-2 gene was isolated, and comparisons between crg-2 and IP-10 genes, mRNAs, and proteins reveal conserved features of possible functional importance. CRG-2 may play a role in host defense, particularly in the response to viral infection.

Activated macrophages have an enhanced ability to perform many functions including phagocytosis, antigen presentation, and pathogen and tumor cell killing. Macrophages can be activated by factors such as interferons (IFN) (1-3), granulo-
of particular functional importance in the crg-2 and IP-10 genes, RNA, and proteins.

MATERIALS AND METHODS
Cell Culture, Macrophage Preparation, Spleen Cell Conditioned Medium, Cytokines, and Other Actiuators—RAW 264.7 cells (26) were obtained from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 supplemented with 10% fetal bovine serum. For preparation of RNA following treatment with lymphokines and other agents, the RAW 264.7 cells were plated at 1 X 10^6 cells/15-cm dish, and the dishes were washed 12-24 h later when the cells were 60-90% confluent. For the experiment with mouse macrophages, peritoneal exudate cells were obtained from male C3H/FeJ and eighteen 2-month-old female BALB/c mice 4 days after intraperitoneal injection of 3 ml of 3% starch. The Balb/c mice were killed immediately with the media as noted under "Results" for 3 h at 37 °C before harvesting for subsequent preparation of RNA. Lymphokine-rich conditioned medium was prepared using spleen cells from male C57BL/6 mice according to the protocol of Gubler and Hoffman (40) using the protocol and reagents supplied by Amersham.

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and bioassays were performed using a cytopathic reduction assay on RtsK rat kidney cells challenged with vesicular stomatitis virus (Lee BioMolecular, San Diego, CA). Recombinant mouse IFN-α was mouse recombinant protein either prepared from Chinese hamster ovary cells with specific activity ≥ 10^6 units/mg (Amgen Biologicals, Thousand Oaks, CA) or purified from Escherichia coli to specific activity of 1.2 X 10^7 units/mg and generously provided as a gift from Genentech, South San Francisco, CA. IFN-γ activities were determined by the suppliers by cytopathic reduction assay using mouse L-cells challenged with vesicular stomatitis virus (Lee BioMolecular, San Diego, CA). Recombinant murine IL-1α, recombinant murine IL-3, recombinant murine IL-4, recombinant murine GM-CSF and recombinant human colony stimulating factor-1 (CSF-1) were obtained from Genzyme, Boston, MA. Recombinant murine IL-13-α and IL-13-β were from Sigma. Unless otherwise indicated, cells were treated with cytokines and other agents, the RAW 264.7 cells were plated at 1.5-2.2 X 10^5 cells/15-cm dish, and the dishes were used 12-24 h later when the cells were 60-90% confluent. For the experiment with mouse macrophages, peritoneal exudate cells were obtained from male C3H/FeJ and eighteen 2-month-old female BALB/c mice 4 days after intraperitoneal injection of 3 ml of 3% starch. The Balb/c mice were killed immediately with the media as noted under "Results" for 3 h at 37 °C before harvesting for subsequent preparation of RNA. Lymphokine-rich conditioned medium was prepared using spleen cells from male C57BL/6 mice according to the protocol of Gubler and Hoffman (40) using the protocol and reagents supplied by Amersham.

Northern Blot Analysis—A agarose/formaldehyde gel electrophoresis was done using 1.2% agarose gels according to the procedure of Goldberg (51). Nitrocellulose filters were prehydrated and hybridized in buffers containing 50% formamide, 5 x SSC (1 X SSC contains 0.15 M NaCl, 0.015 sodium citrate, pH 7.0), 0.08% Ficoll, 0.08% polyvinylpyrrolidone, 0.08% bovine serum albumin, 0.1% SDS, 0.1% sodium pyrophosphate, 100 µg/ml denatured salmon sperm DNA, and 20 mM sodium phosphate, pH 6.5. Hybridization was for >18 h at 42-45 °C. 32P-Labeled cDNA probes were made using random primers (52) with reagents and according to the protocols provided by Pharmacia or Amersham. The probes had specific activities of 0.5-10^7 cpm/µg. 1.5 X 10^6 cpm were incorporated into cDNA probe and the probe was used for hybridization screening at 1-2 X 10^6 cpm/ml. 400-800 recombinant plasmids were plated out on agarose dishes (9 x 9 cm) using E. coli CSH 001 ΔHl. Duplicate nitrocellulose lifts were made from each plate and the filters were processed and hybridized as described (42, 43). Phage DNA was prepared (44) from plates showing differential hybridization to the stimulated as compared to the control cDNA probe. The EcoRI inserts were isolated and the purified fragments were used to make probe and/or inserted into the phagemid RV14, Life Technologies, Gaithersburg, MD. Following hybridization, the filters were washed in 2 X SSC, 0.1% SDS at room temperature for 30 min, followed by four washings in 0.1 X SSC, 0.1% SDS at 50 °C for 30 min each. Autoradiography was done using Kodak XAR film and an intensifying screen at -70 °C.

Cloning and Sequencing of CRG-2 cDNA Clones—CRG-2 cDNA insertst were purified from recombinant phage DNA following incomplete digestion with EcoRI in order to isolate full length clones in which the internal EcoRI sites remained intact. The CRG-2 cDNA library was prepared from the K5 site of the EcoRI cloned into the KS site of the pBlueScript KS phagemid (Stratagene, La Jolla, CA). The recombinant plasmids were used to transform competent E. coli DH5 cells (45) obtained from Bethesda Research Laboratories (Gaithersburg, MD). Plasmid DNA was prepared according to the procedure of Birnboim and Doly (46) as modified by Ish-Horowicz and Burke (47). Conditions for sequencing were performed using available sequencing sites and by the ebensequence III procedure (48) using reagents from Promega (Madison, WI). Sequencing was done by the dideoxy method (49) on double-stranded substrates (50) using reagents supplied by United States Biochemical Corp. (Cleveland, OH).

Cloning and Sequencing of Genomic Library—A BALB/c mouse genomic library in the EMBL-3 vector was obtained from Clontech Laboratories (Palo Alto, CA). 7 X 10^6 recombinant plasmids were plated using a 120-base pair 5′ EcoRI BamHI fragment from CRG-2 cDNA clone 1.1. Two positive plagues were obtained. Analysis of one of the genomic clones revealed a 1.2-
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kb HindIII fragment, which by Southern analysis (31) hybridized to the 5' EcoRI-BamHI fragment of CRG-2 cDNA clone 1.1-1, and the HindIII genomic fragment was subcloned into pBlueScript.

S1 Nuclease Analysis—CRG-2 mRNA was analyzed by S1 nuclease analysis according to the protocol of Weaver and Weissman (32) as modified by Greene and Schildkraut (33). The CRG-2 HindIII genomic fragment containing the 5' sequence of the CRG-2 cDNA and the 5' flanking region was used as template to obtain single-stranded probe for S1 nuclease analysis. The plasmid containing the genomic fragment was denatured with 0.2 N NaOH, 0.2 mM EDTA, for 5 min at room temperature followed by neutralization and precipitation. The denatured template was hybridized with a 32P-labeled oligonucleotide complementary to nucleotides 34 to 51 of the CRG-2 mRNA at 40 °C for 15 min in 10 mM Tris, pH 8.0, and 10 mM MgCl₂. The primer was extended using the Klenow fragment of DNA polymerase I. The duplex containing the probe was cut with BstXI to give a 200-bp fragment and the single-stranded probe purified on an alkaline low melting temperature agarose gel. Probe containing 10⁶ cpm was hybridized to 50 µg of total RNA from control RAW cells or RAW cells exposed for 3 h to 20% conditioned medium from ConA-stimulated spleen cells. Hybridization was in a buffer containing 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 mM EDTA, at 30 °C overnight. The unprotected RNA was digested with 300 units of S1 nuclease in 0.28 M NaCl, 0.05 M sodium acetate, pH 4.5, and 9 mM ZnSO₄, for 60 min at 30 °C, and the reaction was stopped with 0.5 M ammonium acetate and 4 mM EDTA. Following precipitation, the final product was analyzed on a 6% denaturing polyacrylamide/urea gel.

Primer Extension—The transcription initiation site was also determined by primer extension according to the method of McKnight and Kingsbury (34) and Jones et al. (35) as per the protocol of Kingston (36). An oligonucleotide complementary to nucleotides 34 to 51 of the CRG-2 mRNA was end-labeled with 32P to a specific activity of 1.9 × 10⁶ cpm/µg. Fifty µg of total RNA isolated from RAW cells stimulated with 300 units/ml of IFN-γ or medium alone was hybridized to 9 × 10⁶ cpm of labeled oligonucleotide in 1.0 M NaCl, 0.16 M HEPES, pH 7.5, and 0.33 mM EDTA, at 30 °C overnight. The hybridized RNA and oligonucleotide were precipitated, and the primer was extended in a volume of 0.025 ml using 40 units of avian myeloblastosis virus reverse transcriptase for 90 min at 42 °C in the presence of excess deoxynucleoside triphosphates, 50 units of RNasin, 50 mM Tris, pH 8.2, 50 mM KCl, and 6 mM MgCl₂. After stopping the reactions with 20 mM EDTA, the samples were incubated with 1 µg of pancreatic ribonuclease A for 30 min at 37 °C. Reaction products were analyzed on a denaturing 6% polyacrylamide/urea gel.

RESULTS

Isolation of CRG-2 cDNA Clones—A cDNA library in the Agt10 vector was prepared from cultures of the mouse macrophage-like cell line RAW 264.7 that had been treated for 3 h with 20% conditioned medium from ConA-stimulated mouse spleen cells. Initial screening of the cDNA library by differential plaque hybridization led to the identification of a 0.6-kb CRG-2 cDNA clone that hybridized to an mRNA species induced in RAW cells by the spleen cell conditioned medium but not detectable in control RAW cells. Multiple additional CRG-2 cDNA clones were isolated, both in the course of further differential screening and by screening the RAW cDNA library with CRG-2 cDNA probes, and two near full-length cDNA clones of approximately 1.1 kb were isolated and designated 1.1-1 and 1.1-2, which were analyzed in greater detail.

Induction of CRG-2 mRNA—On Northern blot analysis, the CRG-2 cDNA probe identified a major 1.4-kb mRNA induced in RAW 264.7 cells following exposure for 3 h to conditioned medium from ConA-stimulated spleen cells (Fig. 1). Minor inducible species of lower mobility were also detected, presumably corresponding to precursors of the 1.4-kb mRNA. The inducing activity was not limited to a single preparation of spleen cell conditioned medium. Induction of the CRG-2 mRNA was not inhibited by 10 µg/ml CHX added simultaneously with the conditioned medium. Treatment of RAW 264.7 cells with 10 µg/ml CHX resulted in a 93% inhibition of protein synthesis at 5 min and a 96% inhibition at 1 h (data not shown), suggesting that new protein synthesis was not required for induction of the CRG-2 mRNA.

To identify the lymphokines in the spleen cell conditioned medium that may have been responsible for inducing the CRG-2 mRNA, as well as to determine which other macrophage-activating factors were capable of enhancing expression of the CRG-2 gene, total RNA was prepared from RAW cells treated with a variety of agents and the RNA analyzed by Northern blot. As shown in Fig. 2, the CRG-2 mRNA was strikingly induced by the three IFNs and, although less so, by LPS. In a separate experiment, poly(I).poly(C) also led to the conspicuous accumulation of the CRG-2 mRNA. While polymyxin B significantly diminished the induction of CRG-2 mRNA by LPS, it had no effect on the induction by the IFNs, suggesting that trace amounts of contaminating LPS were not contributing to the IFN responses. The blot shown in Fig. 2 was also hybridized with an aldolase A cDNA probe, and the aldolase A mRNA signals serve as controls since they remain unchanged in response to all stimuli except poly(I)-poly(C). Poly(I)-poly(C) led to a decrease in the level of aldolase A mRNA.

In order to analyze the time course of accumulation of the CRG-2 mRNA, RAW 264.7 cells were harvested following treatment with IFN-γ for 0–24 h. As shown in Fig. 3, the CRG-2 mRNA was induced rapidly and dramatically, reaching a peak between 3 and 6 h. Treatment of RAW cells with

FIG. 1. CRG-2 mRNA in RAW 264.7 cells treated with concanavalin A-stimulated spleen cell supernatants. Twenty µg of total RNA from RAW 264.7 cells were loaded per lane, fractionated in a 1.2% agarose/formaldehyde gel, transferred to nitrocellulose, and hybridized to CRG-2 cDNA probe. A 0.6-kb cDNA containing the two 5'-EcoRI fragments of CRG-2 (see Fig. 5) was used as probe. The RAW 264.7 cells were treated for 3 h as follows (from left to right): 20% conditioned medium (CM) from ConA-stimulated spleen cells, 20% CM from unstimulated spleen cells plus 10 µg/ml ConA; medium with 10 µg/ml ConA and medium alone. Each of the treatments was done in the presence or absence of 10 µg/ml CHX as noted. Also shown is RNA from RAW 264.7 cells treated in the presence of cycloheximide with CM from a second preparation of ConA-stimulated spleen cells. RNA markers (Bethesda Research Laboratories, Gaithersburg, MD) were detected by hybridization to bacteriophage λ-DNA.
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**FIG. 2.** CRG-2 mRNA in RAW 264.7 cells treated with LPS, IFN-α, −5, and −γ, and poly(I)·poly(C). Total RNA was prepared from RAW 264.7 cells treated for 3, 6, or 2 h with various stimuli as noted and analyzed by Northern blot as in Fig. 1. The blots were hybridized sequentially to CRG-2 cDNA and to aldolase A cDNA probes. The large EcoRI fragment of CRG-2 cDNA clone 1.1-1 which consists of the entire open reading frame and some 5'- and 3'-untranslated sequence, was used as probe (see Fig. 5). Polymyxin B (PB) was used at a concentration of 25 pg/ml with 600 pg/ml DEAE-dextran and 10 μg/ml CHX. The control (CHX alone) was identical except that it lacked poly(I)·poly(C).

**FIG. 3.** Time course of CRG-2 mRNA in RAW 264.7 cells in response to IFN-γ. RNA was prepared from RAW 264.7 cells harvested after treatment with 100 units/ml IFN-γ for 0–24 h as noted, or at 3 h after mock manipulation without the addition of IFN-γ or CHX, or after treatment with CHX alone for 2 h, or with 100 units/ml IFN-γ plus 10 μg/ml CHX for 2 h, and analyzed by Northern blot using CRG-2 and aldolase A cDNA probes as in Fig. 2. The order of the lanes from the original blot has been changed for the figure.

recombinant murine IL-1α, recombinant murine IL-3, recombinant murine IL-4, recombinant murine GM-CSF, recombinant human CSF-1, PMA, and the calcium ionophore A23187 all failed to induce the CRG-2 mRNA, nor was the CRG-2 mRNA induced by serum, i.e. mitogen, stimulation of serum-starved Balb/c 3T3 fibroblasts (data not shown).

To demonstrate the induction of CRG-2 mRNA during the activation of normal macrophages, crg-2 gene expression was analyzed in the adherent population from starch-elicited peritoneal exudate cells obtained from both C3HeB/FeJ and BALB/cJ mice. As shown in Fig. 4, when these cells (>80% macrophages as determined by morphology) were exposed to conditioned medium from ConA-stimulated spleen cells, CRG-2 mRNA was induced. In the case of the C3HeB/FeJ, but not the BALB/cJ mice, conditioned medium from unstimulated spleen cells to which ConA had been added also induced the CRG-2 mRNA. In addition, the C3HeB/FeJ cells were treated with IFN-γ, and this too led to the expression of the crg-2 gene. The electrophoretic mobilities of the CRG-2 mRNA species from peritoneal macrophages as compared to RAW cells were indistinguishable.

**Nucleotide and Predicted Amino Acid Sequence of CRG-2**—As noted above, two CRG-2 cDNA clones of approximately 1.1 kb were isolated. Clone 1.1-1 extended 5 nucleotides 5' beyond the 5' end of clone 1.1-2. As shown in Fig. 5, these cDNAs consisted of 3 EcoRI fragments which, in the case of cDNA clone 1.1-1, were 562, 320, and 215 bp. A partial EcoRI digest of the phage containing cDNA clone 1.1-1 extended 5 nucleotides 5' from the end of clone 1.1-2. As shown in Fig. 5, these cDNAs consisted of 3 EcoRI fragments which, in the case of cDNA clone 1.1-1, were 562, 320, and 215 bp. A partial EcoRI digest of the phage containing cDNA clone 1.1-1 yielded a full length 1.1-1 cDNA clone, which was inserted in pBluescript, and both strands were sequenced in their entirety using deletions made on the basis of available restriction sites (Fig. 5) and deletions generated using exonuclease III as noted under "Materials and Methods." In addition, the 557-bp EcoRI fragment of cDNA clone 1.1-2, containing the long open reading frame corresponding to the presumed coding sequence for the CRG-2 protein, was also sequenced and no discrepancies were found between the 1.1-1 and the 1.1-2 sequences.

In order to determine the start site for transcription of the crg-2 gene and determine the 5'-most sequence of the CRG-2 mRNA, crg-2 genomic clones were isolated from a BALB/c mouse library. A 1.2-kb internal HindIII genomic fragment from a genomic clone was found to hybridize on Southern analysis to the 5' EcoRI-BamHI fragment from cDNA clone 1.1-1 (data not shown) and sequence analysis showed that this genomic fragment extended 5' from the end of the cDNA sequence (Figs. 6 and 10). The start site for transcription of
the CRG-2 mRNA was determined using S1 nuclease analysis and primer extension as shown in Fig. 6. An oligonucleotide complementary to nucleotides 34 to 51 of the CRG-2 mRNA was used both for primer extension on RNA from stimulated and control RAW cells and as the primer for synthesizing the 200-bp single-stranded probe used for S1 nuclease analysis. Primer extension and S1 nuclease analysis both identified a cluster of transcription start sites, with the major site 14 nucleotides 5' to the end of cDNA clone 1.1.1, 65 nucleotides 5' to the methionine codon that begins the long open reading frame and 24 nucleotides 3' to a "TATA" sequence (Figs. 6, 7, and 10).

The complete CRG-2 cDNA sequence is shown in Fig. 7 and consists of 1088 nucleotides exclusive of the poly(A) tail, containing a single long open reading frame of 294 nucleotides, preceded by a 65-nucleotide 5'-untranslated region and followed by a 3'-untranslated region of 729 nucleotides. There is a AAUAAA polyadenylation signal (53) 18 nucleotides 5' of the poly(A) tail. Starting from the first methionine in the long open reading frame, the predicted CRG-2 protein contains 98 amino acids, molecular weight \( M_r \) 10,781. The N-terminal sequence of the CRG-2 protein includes a stretch of hydrophobic residues (amino acids 5 to 14) that is characteristic of the signal peptide of a secreted or transmembrane protein because it lacks a second long hydrophobic region. The \((-3,-1)\) rule of von Heijne (55) predicts signal peptide cleavage after Gly-21. Outside of the signal peptide there are no predicted N-linked glycosylation sites.

Comparison of the Nucleotide and Predicted Protein Sequence of CRG-2 with Other Sequences—The nucleotide sequence of the CRG-2 cDNA was compared to the sequences in the GenBank (United States Dept. of Health and Human Services) and EMBL Nucleotide Sequence Library data banks using the FASTA program from Genetics Computer Group, University of Wisconsin Biotechnology Center, based on the Lipman and Pearson (56) search for similarity. Greatest homology was found with the human IP-10 cDNA, which contains the sequence TATTTAAT similar to the sequence motifs (62) that have been implicated in the control of the stability of mRNAs of oncogenes and lymphokines (63).

As described above and under "Materials and Methods," a 1.2-kb HindIII erg-2 genomic fragment was isolated, that included the transcription start site and 5' flanking sequence. An oligonucleotide complementary to nucleotides 34 to 51 of the CRG-2 mRNA was used to obtain sequence of the non-coding strand, and an additional oligonucleotide corresponding to positions -296 to -279 of the plus strand was used to obtain the plus strand sequence. This erg-2 genomic sequence along with a comparison of the corresponding sequence from the IP-10 gene is shown in Fig. 9B. This latter region is particularly A:T-rich (69% over 126 positions) and contains the sequence TATTTAAT similar to the sequence motifs (62) that have been implicated in the control of the stability of mRNAs of oncogenes and lymphokines (63).

In addition, previously identified enhancer elements are found in similar positions in the two genes. One of them is the interferon-stimulated response element (ISRE; 5' GGAAAGTGAAACCT 3') found in a series of interferon-inducible genes (64). A second enhancer element closer to the CAAT box is 5' GGGAATTCCT 3', which matches the positive regulatory domain II (PRDII) element containing the virus-inducible NF-κB binding site in the β-interferon gene (65), also shown to mediate gene activation by poly(I)-poly(C) (66). A second NF-κB site is located at -113 to -104 of the erg-2 promoter. An AP-1 site (67) seen at -84 to -78 in erg-2 is not conserved in IP-10.

DISCUSSION

We have identified a novel gene, erg-2, that was induced in mouse macrophages following exposure to conditioned medium from mitogen-stimulated spleen cells. The CRG-2 mRNA accumulated rapidly and dramatically in response to
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A probe was made by extending a \(^*\)P-labeled primer complementary to \(\gamma-, \(\alpha-, \) and \(\beta\)-IFNs, responded less well to LPS, and minimally or not at all to a number of additional cytokines and pharmacologic agents. In response to IFN-\(\gamma\), the CRG-2 mRNA level reached a peak between 3 and 6 h. Induction of CRG-2 mRNA by the spleen cell supernatants and IFN-\(\gamma\) did not require new protein synthesis and therefore CRG-2 is part of the immediate response to IFN-\(\gamma\). The CRG-2 cDNA encodes a predicted protein of 98 amino acids with a 21-amino acid signal peptide that is a member of the PF4 family of cytokines.

**Fig. 6.** Transcription initiation site of CRG-2 mRNA. The start site for initiation of CRG-2 mRNA was determined by \(S1\) nuclease analysis (A) and primer extension (B). Single-stranded DNA probe was made by extending a \(^*\)P-labeled primer complementary to CRG-2 cDNA nucleotides 34 to 51 on a CRG-2 genomic DNA template. The probe was hybridized to total RNA from RAW 264.7 cells stimulated either with conditioned medium from ConA-stimulated spleen cells or from control spleen cells. Following \(S1\) nuclease digestion, the protected DNA fragments were electrophoresed adjacent to the products of dideoxy sequencing reactions done on erg-2 genomic DNA using the 34 to 51 primer. The sequence is written as the reverse complement of the sequence read from the adjacent autoradiograph and the major (+1) and other sites for initiation of transcription are marked by arrows. For primer extension, total RNA from RAW 264.7 cells stimulated with 300 units/ml of IFN-\(\gamma\) or medium alone was hybridized to the \(^*\)P-labeled primer complementary to CRG-2 cDNA nucleotides 34 to 51 and extended using reverse transcriptase. The extended products were electrophoresed adjacent to dideoxy sequencing reactions as size markers (not shown). The length of the major extended product (51 bp) seen in stimulated RNA is indicated. \(P\) indicates unextended primer.

\(\gamma-, \(\alpha-, \) and \(\beta\)-IFNs, responded less well to LPS, and minimally or not at all to a number of additional cytokines and pharmacologic agents. In response to IFN-\(\gamma\), the CRG-2 mRNA level reached a peak between 3 and 6 h. Induction of CRG-2 mRNA by the spleen cell supernatants and IFN-\(\gamma\) did not require new protein synthesis and therefore CRG-2 is part of the immediate response to IFN-\(\gamma\). The CRG-2 cDNA encodes a predicted protein of 98 amino acids with a 21-amino acid signal peptide that is a member of the PF4 family of cytokines. The sequence identities between the predicted CRG-2 and IP-10 proteins suggest that the two proteins may be mouse-human homologues, and the presence of conserved elements of possible functional importance. The presence of conserved sequences in the 3'-untranslated regions is similar to what has been observed in comparisons of the human-mouse homologues of a number of growth factors, oncogenes, and cytokines such as TNF (62), c-myc (68), GM-CSF (62, 63), and gro (24). The region 842 to 967 of the CRG-2 mRNA shows 77% identity with the corresponding region of IP-10 (gaps counted as mismatches), is AU-rich (69% versus 53% for the mRNA as a whole and 56% over the entire 3'-untranslated region), and contains sequence motifs, i.e. UAUUUA and A\(\&\), that have been implicated in the control of mRNA stability (62, 63). These observations suggest that the conserved 3' sequences may be important in regulating the stability of the CRG-2 and IP-10 mRNAs.

Analysis of the 5'-flanking regions of the CRG-2 and IP-10 genes again reveals regions of conserved sequence, some of which contain known regulatory elements. In addition to the TATA and CCAAT boxes (69), both CRG-2 and IP-10 contain the conserved ISRE (64) and binding sites for the transcription factor NF-xB (71). The presence of an ISRE in the CRG-2 and IP-10 promoter is consistent with the induction of CRG-2 by type I and type II interferons as well as by poly(I)-poly(C) (72-74). Induction of IP-10 has been reported to be a relatively specific response to IFN-\(\gamma\), although induction at 18 h by \(\geq 100\) antiviral units of IFN-\(\gamma\)-was in fact demonstrated (23). CRG-2 did appear more sensitive to IFN-\(\gamma\) than to the other IFNs, based on the comparison of the levels of transcription, when analyzed at 6 h (Fig. 2), although the differences at 3 h were less marked.

CRG-2 and IP-10 have 2 conserved NF-xB sites, identical with sequences that have been shown capable of binding NF-xB when used as isolated elements. One sequence, CRG-2 -169 to -160, is identical with that found in the regulatory region of the human IFN-\(\beta\) gene (66, 71, 72) while the other, located at -113 to -104 of the CRG-2 promoter, matches a site in the murine MHC class II E\(\alpha\) gene (75). The NF-xB site in the IFN-\(\beta\) gene has been shown to confer inducibility by virus.
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Fig. 8. Comparison of the amino acid sequences of CRG-2 and other members of the platelet factor 4 family. Amino acid sequences of CRG-2 and other members of the platelet factor 4 family were aligned according to the 4 conserved cysteines. Identical residues between CRG-2 and other members are shaded. Shown below the sequences are the numbers and percents of the identities found in comparisons between CRG-2 and each of the other members of the family, both over their total lengths as far as comparable (TOTAL) and within the highly homologous region between the conserved cysteines (Cys Window). The lower case prefixes refer to the species of origin: m, mouse; h, human; r, rat; and c, chicken. Sequence information was obtained from the following references: hP-10 (23); mMlG' (64); rPF4 (58); hPF4 (59); hPBP (human platelet basic protein) (60); c9E3 (26); mKC/GRO (57); hGRO/MGSA (25); hNAP/ll-8 (22).

and poly(I).poly(C) on a heterologous promoter (66, 71, 72), and the NF-xB sites may play a role in the induction of erg-2 by poly(I).poly(C). Likewise, NF-xB is a mediator of LPS-induced activation of transcription in B cells (76) and may be responsible for the induction of erg-2 gene expression by LPS. Despite the presence of the NF-xB sites in crg-2 and IP-10, IP-10 was found not to be induced by PMA (77). In our experiments, we have not found erg-2 to be induced by PMA or by IL-1 or TNF, all agents that have been reported to activate NF-xB (67, 78). There have been similar findings in the studies of the IFN-g gene, which is inducible by virus through NF-xB, but is not induced by PMA (71).

There are additional conserved sequences in the 5'-flanking regions of erg-2 and IP-10, such as an A:T-rich region between positions -147 and -139 of the erg-2 promoter and the region between the CCAAT and the TATA boxes. At -84 to -78 of the erg-2 promoter, there is a putative AP-1 site (67) that is not conserved in IP-10. Since the AP-1 site, like the NF-xB site, confers inducibility by phorbol esters (67), it is again of interest that in our initial experiments erg-2 was not induced by PMA, nor was it induced by serum in serum-starved Balb/c 3T3 fibroblasts.

The biological activities of the PF4 family of secreted proteins are being actively investigated and defined (reviewed in Ref. 20). While activities of IP-10 have not been reported, IP-10 has been shown to be secreted, in response to IFN-g, and poly(I).poly(C) on a heterologous promoter (66, 71, 72), and the NF-xB sites may play a role in the induction of erg-2 by poly(I).poly(C). Likewise, NF-xB is a mediator of LPS-induced activation of transcription in B cells (76) and may be responsible for the induction of erg-2 gene expression by LPS. Despite the presence of the NF-xB sites in crg-2 and IP-10, IP-10 was found not to be induced by PMA (77). In our experiments, we have not found erg-2 to be induced by PMA or by IL-1 or TNF, all agents that have been reported to activate NF-xB (76, 78). There have been similar findings in the studies of the IFN-g gene, which is inducible by virus through NF-xB, but is not induced by PMA (71).

There are additional conserved sequences in the 5'-flanking regions of erg-2 and IP-10, such as an A:T-rich region between positions -147 and -139 of the erg-2 promoter and the region between the CCAAT and the TATA boxes. At -84 to -78 of the erg-2 promoter, there is a putative AP-1 site (67) that is not conserved in IP-10. Since the AP-1 site, like the NF-xB site, confers inducibility by phorbol esters (67), it is again of interest that in our initial experiments erg-2 was not induced by PMA, nor was it induced by serum in serum-starved Balb/c 3T3 fibroblasts.

The biological activities of the PF4 family of secreted proteins are being actively investigated and defined (reviewed in Ref. 20). While activities of IP-10 have not been reported, IP-10 has been shown to be secreted, in response to IFN-g, and poly(I).poly(C) on a heterologous promoter (66, 71, 72), and the NF-xB sites may play a role in the induction of erg-2 by poly(I).poly(C). Likewise, NF-xB is a mediator of LPS-induced activation of transcription in B cells (76) and may be responsible for the induction of erg-2 gene expression by LPS. Despite the presence of the NF-xB sites in crg-2 and IP-10, IP-10 was found not to be induced by PMA (77). In our experiments, we have not found erg-2 to be induced by PMA or by IL-1 or TNF, all agents that have been reported to activate NF-xB (76, 78). There have been similar findings in the studies of the IFN-g gene, which is inducible by virus through NF-xB, but is not induced by PMA (71).
by keratinocytes, endothelial cells, monocytes, and fibroblasts (79) and has been shown to be expressed in inflammatory lesions in human skin (80, 81). Many members of the PF4 family are chemotaxins. In addition, melanoma growth-stimulatory activity acts as an autocrine growth factor for a human melanoma cell line (25); MIP-2 can act synergistically with GM-CSF or M-CSF in stimulating granulocyte-macrophage colony formation (82); NAP-IL-8 has been shown to inhibit adhesion of neutrophils to endothelial cells (83); and PF4 can act as an angiostatic agent to inhibit the growth of endothelial cells and prevent new blood vessel formation (84). It will be appropriate, therefore, to investigate the role of CRG-2 in the regulation of inflammatory and immune responses, growth, and the repair of tissue injury.

The response of the crg-2 gene to the IFNs and LPS suggests that CRG-2 is a mediator of the functions of the activated macrophage. The conservation of regulatory sequences in the crg-2 and IFN-10 genes suggests that the two proteins may serve analogous biological roles in mouse and human. The response of crg-2 to the IFNs is particularly dramatic and makes the CRG-2 protein a candidate for mediating IFN activities. The dramatic response of CRG-2 mRNA to both type I and type II IFN, the presence in the regulatory region of the crg-2 gene of NF-kB sites that can serve as potent elements for gene induction by virus, and the induction of the crg-2 gene by poly(I):poly(C) suggest that the CRG-2 protein may have a particular role in the host response to viral infection.

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REFERENCES

1. Nathan, C. F., Murray, H. W., Wiebe, M. E., and Rubin, B. Y. (1983) J. Exp. Med. 158, 670–689
2. Schultz, R. M., and Kleinschmidt, W. J. (1983) Nature 305, 239–240
3. Svedersky, L. P., Benton, C. V., Berger, W. H., Rinderknecht, E., Svedersky, L. P., Benton, C. V., Berger, W. H., Rinderknecht, E., Mertz, M. H., and Mertz, M. S. (1987) J. Immunol. 139, 155–161
4. Doe, W. F., and Henson, P. M. (1986) Science 232, 507–508
5. Philip, R., and Epstein, L. B. (1986) Nature 323, 86–89
6. Dinarello, C. A. (1984) Rev. Infect. Dis. 6, 51–95
7. Crawford, R. M., Finkelst, D. S., Ohara, J., Paul, W. E., and Meltzer, M. S. (1987) J. Immunol. 139, 155–161
8. Rich, J. M., and Struhl, K. (1989) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 1, pp. 4.6.1–4.6.13, John Wiley & Sons, New York
9. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 1405–1412
10. Okayama, M., and Berg, P. (1982) Mol. Cell. Biol. 2, 161–170
11. Gubler, U., and Hoffman, B. J. (1983) Gene (Amst.) 25, 263–269
12. Huyten, T. V., Young, R. A., and Davis, R. W. (1986) DNA Cloning Techniques, A Practical Approach (Glover, D., ed) Vol. 1, pp. 49–78, IRL Press, Oxford
13. Latt, L. F., and Nunnan, D. C. (1986) Proc. Natl. Acad. Sci. U. S. A. 84, 1138–1186
14. Peden, K. W. C., Mounts, P., and Hayward, G. S. (1982) Cell 31, 7–10
15. Matzvisis, T., Fritsch, E. F., and Sambrook, J. (1982) in Molecular Cloning: A Laboratory Manual, pp. 317–372, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
16. Hanahan, D. (1983) J. Mol. Biol. 166, 557–580
17. Riehn, H. C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513–1523
18. Ish-Horowicz, D., and Burke, J. F. (1981) Nucleic Acids Res. 9, 2999–2998
19. Heim, S. (1984) Gene (Amst.) 28, 351–359
20. Sanger, F., Nicklen, B., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
21. Chen, E. Y., and Seeburg, P. H. (1985) DNA (NY) 4, 1163–1164
22. Baldwin, R. M., and Opheim, J. (1988) EMBO J. 7, 2025–2033
23. Sugano, S., Stoeckle, M. Y., and Hanafusa, H. (1987) Cell 49, 321–328
24. Raschke, W. C., Baird, S., Raphael, P., and Nakoizn, I. (1978) Cell 15, 261–267
25. Miocori, F., Klein, B., Kirchner, H., and Zawatzky, R. (1982) Eur. J. Immunol. 12, 789–790
26. Meltzer, M. S. (1987) J. Immunol. 139, 135–141
27. Wahl, S. M., Wahl, L. M., McCarthy, J. B., Chedid, L., and Doly, J. (1989) Nucleic Acids Res. 17, 2989–2998
28. Feinberg, A., and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13
29. Fitzgerald, M., and Shute, T. (1981) Cell 24, 201–206
30. Perlman, D., and Halvorsen, H. O. (1983) J. Mol. Biol. 167, 391–409
31. Von Heijne, G. (1986) Nucleic Acids Res. 14, 4693–4690
32. Lipman, D. J., and Pearson, W. R. (1985) Science 227, 1435–1441
33. Oquendo, P., Alberta, J., Wen, D., Graycar, J. L., Derynck, R., and Stiles, C. D. (1989) J. Biochem. 264, 4133–4137
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58. Doi, T., Greenberg, S. M., and Rosenberg, R. D. (1987) Mol. Cell. Biol. 7, 898–904
59. Ponec, M., Surrey, S., LaRocco, P., Weiss, M. J., Rappaport, E. F., Conway, T. M., and Schwartz, E. (1987) Blood 69, 219–223
60. Wenger, R. H., Wicki, A. N., Wals, A., Kieffer, N., and Clemetson, K. J. (1989) Blood 73, 1498–1503
61. Maizel, J. V., and Lenk, R. P. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7665–7669
62. Caput, D., Beutler, B., Hartog, K., Thayer, R., Shimer, S., and Cerami, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1670–1674
63. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
64. Levy, D. E., Kessler, D. S., Pine, R., Reich, N., and Darnell, J. E., Jr. (1988) Genes & Dev. 2, 383–393
65. Goodbourn, S., Burstein, H., and Maniatis, T. (1986) Cell 45, 601–610
66. Visvanathan, K. V., and Goodbourn, S. (1989) EMBO J. 8, 1129–1138
67. Angel, P., Imagawa, M., Chiu, R., Stein, B., Imbra, R. J. H., Rahmsdorf, M. J., Jonat, C., Herrlich, P., and Karin, M. (1987) Cell 49, 729–739
68. Jones, T. R., and Cole, M. D. (1987) Mol. Cell. Biol. 7, 4513–4521
69. Myers, R. M., Tilly, K., and Maniatis, T. (1986) Science 232, 613–618
70. Friedman, R. L., and Stark, G. R. (1985) Nature 314, 637–639
71. Lenardo, M. J., Fan, C.-M., Maniatis, T., and Baltimore, D. (1989) Cell 57, 287–294
72. Fan, C.-M., and Maniatis, T. (1989) EMBO J. 8, 101–110
73. Hug, H., Costas, M., Staeheli, P., Aeby, M., and Weissmann, C. (1988) Mol. Cell. Biol. 8, 3065–3079
74. Wathlet, M. G., Clauss, I. M., Nols, C. B., Content, J., and Huez, G. A. (1987) Eur. J. Biochem. 169, 313–321
75. Blanar, M. A., Burkly, L. C., and Flavell, R. A. (1989) Mol Cell Biol. 9, 844–846
76. Sen, R., and Baltimore, D. (1986) Cell 47, 921–928
77. Fan, C.-D., Goldberg, M., and Bloom, B. R. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5122–5125
78. Osborn, L., Kunkel, S., and Nabel, G. J. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 2336–2340
79. Luster, A. D., and Ravetch, J. V. (1987) J. Exp. Med. 166, 1084–1097
80. Kayden, G., Luster, A. D., Hancock, G., and Colun, Z. A. (1987) J. Exp. Med. 166, 1098–1108
81. Gottlieb, A. B., Luster, A. D., Posnett, D. N., and Carter, D. M. (1988) J. Exp. Med. 168, 941–948
82. Broxmeyer, H. E., Sherry, B., Lu, L., Cooper, S., Carow, C., Wolpe, S. D., and Cerami, A. (1989) J. Exp. Med. 169, 1583–1594
83. Gimbrone, M. A., Obin, M. S., Brock, A. F., Luis, E. A., Hass, P. E., Hebert, C. A., Yip, Y. K., Leung, D. W., Lowe, D. G., Kohr, W. J., Darbonne, W. C., Bechtol, K. B., and Baker, J. B. (1989) Science 246, 1601–1603
84. Maione, T. E., Gray, G. S., Petro, J., Hunt, A. J., Donner, A. L., Bauer, S. I., Carson, H. F., and Sharpe, R. J. (1990) Science 247, 77–79
85. Farber, J. (1990) Proc. Natl. Acad. Sci. U. S. A., in press