MOLECULAR AND CELLULAR BIOLOGY, Apr. 1992, p. 1535–1545
0270-7306/92/041535-11$02.00/0

Vol. 12, No. 4

A Collection of mRNA Species That Are Inducible in the RAW 264.7 Mouse Macrophage Cell Line by Gamma Interferon and Other Agents

JOSHUA M. FARBER

Division of Infectious Diseases, Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Received 6 November 1991/Accepted 9 January 1992

To identify genes induced during macrophage activation, a cDNA library was prepared from cultures of the RAW 264.7 mouse macrophage cell line that had been treated with conditioned medium from mitogen-stimulated spleen cells, and the cDNA library was screened by differential plaque hybridization. Eleven cDNA clones, designated CRG-1 through CRG-11, corresponding to mRNA species inducible in RAW 264.7 cells by the spleen cell conditioned medium, were isolated. Inductions were not blocked by cycloheximide. All of the mRNAs were inducible by gamma interferon, and some were also inducible by alpha and beta interferons, by lipopolysaccharide, by phorbol 12-myristate 13-acetate, and by the calcium ionophore A23187. Sequencing of the cDNAs revealed that CRG-1, CRG-3, and CRG-5 are cDNAs of recently identified transcription factors IRF-1, zif/268, and LRF-1 respectively. As previously reported, CRG-2 and CRG-10 (MIG) encode new members of the platelet factor 4 family of cytokines. CRG-6 corresponds to a new member of a family of interferon-inducible genes clustered on mouse chromosome 1, CRG-9 corresponds to a prostaglandin synthase homolog, CRG-8 corresponds to β_2-microglobulin, and CRG-4 corresponds to metallothionein II. CRG-11 contains sequences of a truncated L1Md repetitive element as well as nonrepetitive sequences. The nonrepetitive sequence of CRG-11 as well as the sequences of CRG-7 are not closely related to published sequences. The CRG genes and proteins are of interest because of their involvement in macrophage activation, because of their roles as mediators of the effects of gamma interferon and other pleiotropic agents, and because of their usefulness as tools for studying the signal pathways through which gamma interferon and other inducers exert their effects on gene and protein expression.

Monocytes/macrophages play a central role in immune and inflammatory responses and as such are involved in the pathogenesis of disorders as diverse as atherosclerosis (7) and AIDS (24). Macrophages can be activated by endogenous cytokines such as interferons (IFNs) (54, 61, 71), granulocyte/macrophage colony-stimulating factor (26), and tumor necrosis factor (61) as well as by exogenous factors such as lipopolysaccharide (LPS) (15) and muramyldipeptide (84). When activated, macrophages manifest enhanced capabilities in the presentation of antigens, phagocytosis, the killing of intracellular pathogens, and the killing of tumor cells (reviewed in reference 93). Macrophage activation is in large measure the result of the induction of genes by macrophage-activating factors, such as the induction of the genes for the major histocompatibility class II antigens (53, 66) and the Fcγ receptor-α (88) by IFN-γ and induction of the tumor necrosis factor gene by LPS (5). It is likely, given the range and complexity of the responses in which macrophages participate, that many activation-related inducible genes have yet to be identified. Likewise, despite the identification of IFN and LPS receptors (2, 82, 89), the recognition of IFN- and LPS-responsive promoter elements (23, 43, 74, 90), and the characterization of DNA-binding proteins involved in the transcriptional regulation of IFN- and LPS-responsive genes (13, 34, 74), many of the molecular events important for activation-related changes in gene expression, particularly in response to IFN-γ, remain obscure.

This report describes the use of differential screening of a cDNA library prepared from lymphokine-treated mouse macrophage-like RAW 264.7 cells in order to identify genes induced early in the response to macrophage-activating factors. The protein products of such genes are likely to play important regulatory roles, both in the programmed changes in gene expression that accompany macrophage activation and differentiation and in providing signals to other types of cells that react in a coordinated fashion as part of immune and inflammatory reactions. These genes and proteins are of interest not only because of their roles in macrophage activation but also as mediators of the effects of activating factors such as the IFNs and LPS that influence a broad range of biological processes. In addition, the inducible genes can serve as suitable tools for investigations of the signal pathways through which the macrophage-activating factors exert their effects on gene expression.

The current studies describe the isolation of 11 cDNA clones, designated CRG-1 through CRG-11 (for cytokine-responsive genes), corresponding to mRNA species of increased abundance in the early times following cytokine activation of RAW 264.7 cells. Some of the cDNAs correspond to genes that have been identified previously, while others are novel. Full-length clones of two of the novel cDNAs, designated here CRG-2 and CRG-10 (the latter having been named MIG) and encoding members of the platelet factor 4 family of cytokines, have been published previously along with the patterns of expression of their mRNAs (21, 83). Some of these published data on CRG-2 and CRG-10 are included here for purposes of comparison with other members of the CRG collection.

MATERIALS AND METHODS

Cell culture, spleen cell conditioned medium, and cytokines and other activators. RAW 264.7 cells (63) 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 1.5×10^7 to 2.2×10^7 cells per 15-cm dish, and the dishes were used 12 to 24 h later when the cells were 60 to 90% confluent. Lymphokine-rich conditioned medium from concanavalin A (ConA)-stimulated spleen cells was prepared from male C57BL/6 mice according to the procedure of Marcucci et al. (50) as described previously (83), with the harvested medium containing 10 μg of ConA per ml. Control spleen cell conditioned medium was prepared from cells cultured similarly except without ConA, and the medium from control cells was made 10 μg of ConA per ml at the time of harvesting. The spleen cell conditioned medium contained <0.1 endotoxin unit per ml as determined by a chromogenic *Limulus* amebocyte lysate test (Whittaker Bioproducts, Walkersville, Md.). IFN-γ was mouse recombinant protein either prepared from Chinese hamster ovary cells with a specific activity of $\geq 10^7$ U/mg (Amgen Biologicals, Thousand Oaks, Calif.) or purified from *Escherichia coli* to a specific activity of 1.2×10^7 U/mg and generously provided as a gift from Genentech, South San Francisco, Calif. IFN-γ activities were determined by the suppliers by cytopathic reduction assay, using mouse L cells challenged with vesicular stomatitis virus. The IFN-α and IFN-β were murine natural products purified to specific activities of 1.4×10^6 and 1.3×10^8 international reference units per mg, respectively, assayed by the supplier in a cytopathic reduction assay on RtK rat kidney cells challenged with vesicular stomatitis virus (Lee BioMolecular, San Diego, Calif.). Phorbol 12-myristate 13-acetate (PMA) and A23187 were from Sigma. Cells were treated with cytokines and other activators in RPMI 1640 containing 10% fetal bovine serum. Following treatment of the RAW 264.7 cells with each of the IFNs, medium was stored at −70°C, and subsequent assays for endotoxin gave levels of <0.5 endotoxin unit per ml of medium. LPS was from *E. coli* O127:B8 prepared by the Westphal method (Difco Laboratories, Detroit, Mich.).

Construction of the cDNA library. RAW 264.7 cells were plated at 2×10^7 cells per 15-cm dish, and the following day they were exposed for 3 h, in the presence of 10 μg of cycloheximide (CHX) per ml, to 20% conditioned medium from ConA-stimulated spleen cells. Poly(A)$^+$ RNA was prepared from the RAW 264.7 cells, and oligo(dT)-primed cDNA was synthesized. Following treatment with *Eco*RI methylase, addition of *Eco*RI linkers, and cleavage with *Eco*RI, the cDNA was inserted into the *Eco*RI site of λgt10 DNA as described previously (83). Five hundred thousand recombinant phages were amplified in *E. coli* C600 ΔHfl to yield a library that was used for subsequent screening.

Synthesis of complex cDNA probe and screening of the cDNA library. Synthesis of cDNA probe from poly(A)$^+$ RNA from stimulated and control RAW 264.7 cells and differential screening of the cDNA library were done as described by Lau and Nathans (40), with minor modifications. Stimulated RAW 264.7 cells were treated for 3 h with 20% conditioned medium from ConA-stimulated spleen cells in the presence of 10 μg of CHX per ml, while control cells were exposed to medium with identical concentrations of ConA and CHX but without spleen cell conditioned medium. Complex cDNA probe was synthesized as described previously (83). By using 1 μg of poly(A)$^+$ RNA, approximately 7×10^7 cpm was incorporated into the cDNA probe, and the probe was used for hybridization screening at 1×10^6 to 2×10^6 cpm/ml.

Four hundred to eight hundred recombinant phages were plated on agarose dishes (9 by 9 cm), using *E. coli* C600 ΔHfl. Duplicate nitrocellulose lifts were made from each plate, and the filters were processed and hybridized as described previously (40, 60). Purification of plaques showing differential hybridization to the stimulated compared with the control cDNA probe was done as described in Results. The *Eco*RI inserts were isolated, and the purified fragments were used to make probe and/or inserted into the pBlueScript KS phagemid (Stratagene, La Jolla, Calif.).

Northern (RNA) blot analysis. Agarose-formaldehyde gel electrophoresis was done with 1.2% agarose gels according to the procedure of Goldberg (25), and nitrocellulose filters were processed as described previously (83). ^{32}P-labeled cDNA probes were made by nick translation or by using random primers with reagents and according to the protocols provided by Pharmacia or Amersham. The probes had specific activities in the range of 0.5×10^9 to 2×10^9 cpm/μg and were used for hybridizations at 1×10^6 to 5×10^6 cpm/ml. Autoradiography was done with Kodak XAR film, and except as otherwise indicated, an intensifying screen was used at −70°C.

Cloning and sequencing of CRG cDNA clones. The approximate sizes of the cDNA clones isolated initially were as follows: CRG-1, 1.5 kb; CRG-2, 0.6 kb; CRG-3, 1.2 kb; CRG-4, 0.7 kb; CRG-5, 1.2 kb; CRG-6, 0.6 kb; CRG-7, 1.4 kb; CRG-8, 0.95 kb; CRG-9, 2.2 kb; CRG-10, 1.2 kb; and CRG-11, 0.45 kb. Additional, longer cDNA clones were subsequently isolated for CRG-1 (2.1 kb), CRG-2 (1.1 kb) (83), and CRG-10 (1.4 kb) (21), and a 3.2-kb cDNA of zif/268 (CRG-3; see below) was obtained from B. Christy and D. Nathans (Johns Hopkins University). Both the initial shorter and the longer cDNA clones were used as probes in the experiments described below. CRG-2, CRG-8, and CRG-9 cDNA clones contained internal *Eco*RI sites, and the *Eco*RI fragments were inserted into pBlueScript KS plasmid separately and used individually or in combination as probes. An aldolase A cDNA probe was prepared from a 1.4-kb cDNA clone kindly provided by A. Levy, L. Sanders, and D. Nathans (Johns Hopkins University). Sequencing was done by the dideoxy method (69) on double-stranded substrates, using T3 and T7 primers (Stratagene) and reagents supplied by United States Biochemical Corp. (Cleveland, Ohio).

RESULTS

Construction and screening of the cDNA library. Differential screening of a cDNA library was used to identify genes and proteins induced during macrophage activation. The cDNA library was prepared in the λgt10 vector from oligo(dT)-primed cDNA made from cultures of the RAW 264.7 mouse macrophage cell line that had been treated for 3 h in the presence of CHX with 20% conditioned medium from ConA-stimulated mouse splenocytes. Complex, single-stranded, radiolabeled cDNA probe was prepared from RAW 264.7 cells that had been treated with conditioned medium as described immediately above (stimulated cells) as well as from RAW 264.7 cells that had been treated for 3 h with identical concentrations of ConA and CHX but without spleen cell conditioned medium (control cells). Duplicate phage lifts were prepared and screened as described in Materials and Methods with a cDNA probe from the stimu-

lated and control RAW 264.7 cells. Filters containing a total of approximately 40,000 plaques were screened, and initially 176 phages were identified that showed a greater signal after hybridization to cDNA probe from the stimulated compared with control cells. After elimination of phages with inserts cross-hybridizing to the two most abundant species, the remaining phages showing differential hybridization were plated individually; from each plate, six phages were picked to grow in grids from which lifts were prepared for a second round of screening with a cDNA probe from stimulated and control RAW 264.7 cells. Only phages showing differential hybridization in the second screening were analyzed further. *Eco*RI inserts from these phages were isolated and used to identify cross-hybridizing (i.e., related) phages within this collection. Inserts from 23 unrelated phages were used to synthesize probes for Northern blots of RNA prepared from RAW 264.7 cells. Eleven cDNAs, CRG-1 through CRG-11, hybridized to RNAs of greater abundance in stimulated cells compared with the control RAW 264.7 cells, and these cDNAs were inserted into the pBlueScript KS phagemid (Stratagene) for use as described below (see Materials and Methods).

Induction of the CRG mRNAs by spleen cell conditioned medium. RAW 264.7 cells were treated for 3 h with 20% conditioned medium from ConA-stimulated spleen cells or with various control media both in the presence and in the absence of CHX, and total RNA was harvested for analysis by Northern blot using the CRG and aldolase A cDNA probes. Figure 1 shows that when equal amounts of total RNA were used for analysis, each of the CRG probes hybridized to one or more mRNA species that accumulated in RAW 264.7 cells treated with conditioned medium from ConA-stimulated spleen cells compared with control RAW 264.7 cells. The approximate sizes of the mRNAs are noted in the legend to Fig. 1. Control cells included RAW 264.7 cells treated with conditioned medium from spleen cells not exposed to ConA, RAW 264.7 cells treated with ConA alone, and RAW 264.7 cells that remained in growth medium without additions. Hybridizing a representative blot with an aldolase A cDNA probe demonstrated that the level of the aldolase mRNA did not differ in stimulated versus control RAW 264.7 cells. The panel showing hybridization to the CRG-2 probe and portions of the panel using the CRG-10 probe have been published previously (21, 83) and are shown here for the purpose of comparison with the patterns of expression of the other members of the CRG collection.

For experiments to evaluate the role of new protein synthesis, CHX (10 μg/ml) was added simultaneously with the stimulating or control media. Treatment of RAW 264.7 cells with this dose of CHX resulted in a 93% inhibition of protein synthesis after 5 min and a 96% inhibition after 1 h, as measured by the incorporation of [^{35}S]methionine into acid-precipitable material (data not shown). CHX alone led to a decrease in the levels of the aldolase A control mRNA as well as in the levels of those other mRNAs with readily detectable signals in untreated cells, i.e., CRG-4 and CRG-8. This overall effect precludes attributing lower mRNA levels seen in some cases in the cells stimulated in the presence of CHX compared with cells stimulated without CHX to an inhibiting effect of CHX on induction. CHX alone led to a significant increase in the levels of the CRG-3, CRG-5, CRG-7, and CRG-9 mRNAs, and CHX resulted in superinduction of CRG-1, CRG-5, CRG-7, and CRG-10. For CRG-3 and CRG-9, significant accumulation of which occurred with CHX alone, the data cannot be interpreted in regard to whether induction by the spleen cell conditioned medium required new protein synthesis. For the other CRG mRNAs, induction by the spleen cell conditioned medium clearly occurred in the presence of CHX, and in no case was there evidence that induction required new protein synthesis.

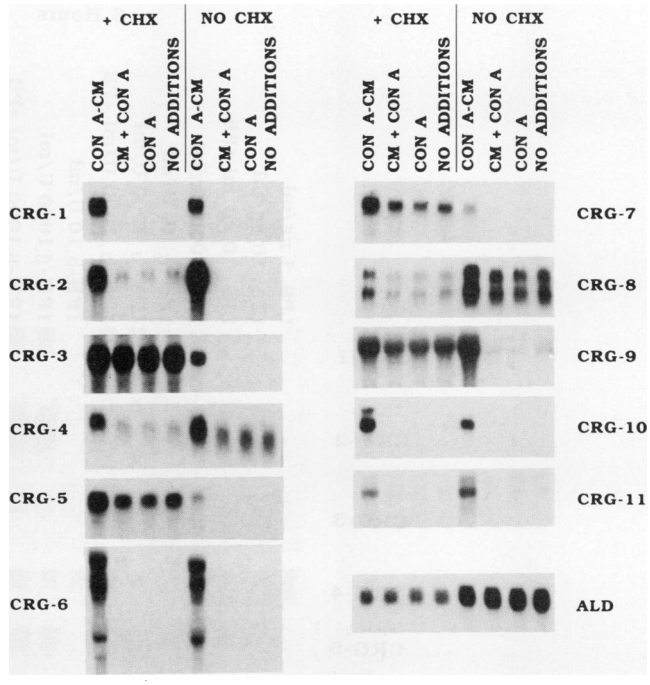

FIG. 1. CRG mRNAs in RAW 264.7 cells treated with spleen cell supernatants. Twenty micrograms of total RNA from RAW 264.7 cells was loaded per lane, fractionated in a 1.2% agarose–formaldehyde gel, transferred to nitrocellulose, and hybridized to CRG cDNA probes (see Materials and Methods). The RAW 264.7 cells were treated for 3 h as follows (from left to right): 20% conditioned medium (CM) from ConA-stimulated spleen cells, 20% conditioned medium from unstimulated spleen cells plus ConA (10 μg/ml), medium with ConA (10 μg/ml), and medium alone. Each of the treatments was done in the presence or absence of CHX at 10 μg/ml, as noted. Exposure for autoradiography ranged from 8 h without an intensifying screen for CRG-2 to 8 days with an intensifying screen for CRG-7 and CRG-11. The sizes of the hybridizing mRNAs were approximated by comparisons with RNA markers (Bethesda Research Laboratories, Gaithersburg, Md.) as follows: CRG-1, 2.3 kb; CRG-2, 1.4 kb; CRG-3, 3.3 kb; CRG-4, 0.6 kb; CRG-5, 2.4 kb; CRG-6, 4.2, 3.4, 2.8 (poorly seen here; see Fig. 2), and 1.7 kb; CRG-7, 2.3 kb; CRG-8, 1.1 and 0.85 kb; CRG-9, 4.4 kb; CRG-10, 1.6 kb; CRG-11, 8.8 kb; and aldolase A (ALD), 1.6 kb. The panel showing hybridization to the CRG-2 probe and portions of the panel using the CRG-10 probe have been published previously (21, 83) and are shown here for the purpose of comparison with the patterns of expression of the other members of the CRG collection.

Induction of the CRG mRNAs by IFNs and LPS. It was of interest to determine the responses of the CRG mRNAs to specific macrophage-activating factors. Among those factors tested were LPS and IFN-α, -β, and -γ; the effects of these agents on the CRG mRNA levels in RAW 264.7 cells are shown in Fig. 2. The factors were present throughout the 3- and 6-h incubations. The LPS inhibitor polymyxin B was included as a control in some incubations to rule out the possibility that contaminating LPS was contributing to the responses to the IFNs. The panels showing hybridizations with the CRG-2 and CRG-10 probes have been published previously (21, 83) and are shown here for the purpose of comparison with the patterns of expression of other mem-

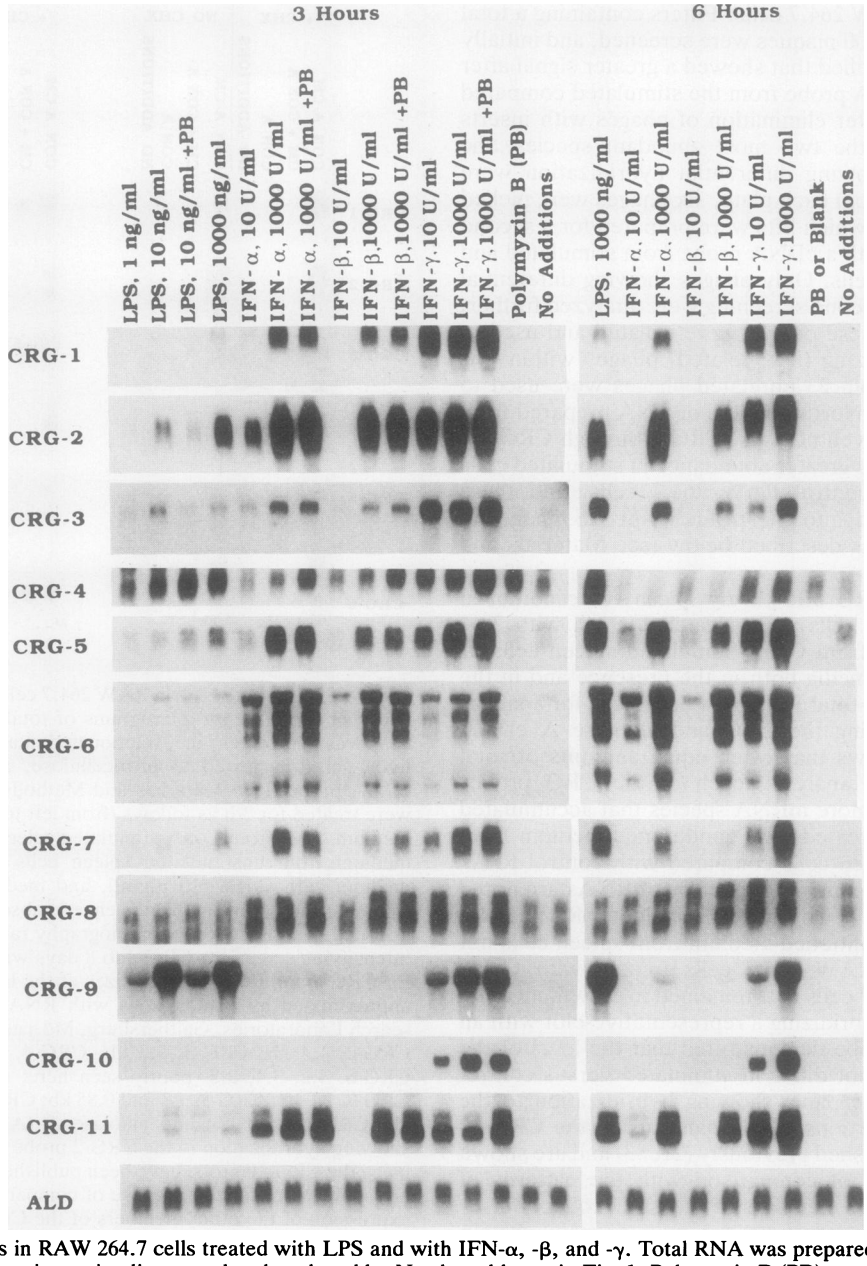

FIG. 2. CRG mRNAs in RAW 264.7 cells treated with LPS and with IFN-α, -β, and -γ. Total RNA was prepared from RAW 264.7 cells treated for 3 or 6 h with various stimuli as noted and analyzed by Northern blot as in Fig. 1. Polymyxin B (PB) was used at a concentration of 5 μg/ml. For the 6-h blots, those probed with CRG-1 through CRG-4 and CRG-8 through aldolase (ALD) included RNA from RAW 264.7 cells treated with polymyxin B, while the corresponding lane was left blank on the blots probed with CRG-5, CRG-6, and CRG-7. Exposures for autoradiography ranged from 12 and 33 h without an intensifying screen for aldolase and CRG-2, respectively, to 12 days with an intensifying screen for CRG-9. The panels showing hybridizations with the CRG-2 and CRG-10 probes have been published previously (21, 83) and are shown here for the purpose of comparison with the patterns of expression of other members of the CRG collection.

bers of the CRG collection. Eight of the eleven CRG mRNAs were induced by LPS, with CRG-1, CRG-8, and CRG-10 being the exceptions. As expected, polymyxin B inhibited the responses to LPS, although incompletely. (For unknown reasons, polymyxin B alone induced accumulation of the CRG-4 mRNA.) In general, the responses to LPS were delayed compared with the responses to the IFNs, with the LPS responses significantly less impressive at 3 h but comparable to the IFN responses at 6 h. An exception was CRG-9, the response of which to LPS was uniformly greater than the responses to the IFNs.

Eight of the eleven mRNAs showed significant responses to IFN-α and -β, with CRG-4, CRG-9, and CRG-10 being the exceptions. In most cases, the levels of induction were higher with IFN-α than with IFN-β when identical antiviral units were used, particularly at the 10-U/ml dose. At 1,000 U/ml for 3 h, some mRNAs such as CRG-6 and CRG-11, showed little differences in their responsiveness to IFN-α versus IFN-β, while CRG-5 and CRG-7, for example, reached significantly higher levels with IFN-α than with IFN-β even at these high doses of the IFNs.

All the mRNAs accumulated in response to IFN-γ, and

induction was evident with both 10 and 1,000 U/ml at both 3 and 6 h. In general, the mRNAs were induced to higher levels and/or in response to lower antiviral doses with IFN-γ than with IFN-α and -β. An exception was CRG-6, which in general appeared more responsive to IFN-α than to IFN-γ. CRG-6 was notable for the multiple species of RNA induced and for the variations in the relative abundance of these species depending on the type of inducer, the dose of inducer, and the duration of exposure. At 3 h of treatment with IFN-α and -β, the third-largest CRG-6 species (2.8 kb) is prominent, while after 3 h of treatment with IFN-γ, this 2.8 kb species is difficult to appreciate. After 6 h, the 2.8-kb species is more prominent than the 3.4-kb species immediately above it in cells treated with either IFN-α, -β, or -γ, although in other experiments, the 2.8-kb band remained the least prominent species in cells treated with IFN-γ, even after 24 h. The differential effect of a given inducer depending on the dose is seen at 6 h with IFN-α and IFN-β, for which the relative levels of the species differ with 10 U/ml compared with 1,000 U/ml.

The dose responses of the CRG genes to the various inducers differed. In response to 3 h of LPS treatment, for example, CRG-2 responses reached a higher level with 1,000 ng/ml than with 10 ng/ml, while CRG-9 responded identically to the two doses. In the case of IFN-γ, CRG-1, CRG-3, and CRG-8, for example, showed little differences in mRNA levels at 3 h whether 10 or 1,000 U/ml was use, while CRG-5, -9 and -10 showed marked differences in the responses at the two doses. Similarly, in response to 3 h of exposure to IFN-α, CRG-6 showed a modest difference at 1,000 versus 10 U/ml, while the differences in the responses of CRG-1, CRG-5, and CRG-7 at the two doses were quite dramatic.

The time course of accumulation of a given CRG mRNA differed depending on the type as well as the dose of inducer. For CRG-1, for example, from 3 to 6 h, levels with IFN-α and -β fell, while the levels with IFN-γ showed little change. The dose effect of a given agent can be seen with CRG-3 and CRG-11. For CRG-3, while there was little difference in the 10- and 1,000-U/ml IFN-γ signals at 3 h, the signals were significantly different at 6 h. For CRG-11, the signals with IFN-α at 10 U/ml went down from 3 to 6 h, while the level with 1,000 U/ml continued to rise.

Time courses of accumulation of the CRG mRNAs in response to IFN-γ. Because all the CRG mRNAs were induced by IFN-γ, the folds of induction versus time could be determined for all of the mRNAs in response to this agent. RAW 264.7 cells were incubated from 0 to 24 h with 100 U of IFN-γ per ml, and the folds of induction compared with values at time 0 were determined by densitometry following autoradiography/fluorography using preflashed film (Fig. 3). For CRG-2 and CRG-10, the data were derived from experiments published previously (21, 83), and these data are presented here for the purpose of comparison with the time courses for the other members of the CRG collection. The levels of induction varied from slightly more than 2-fold for CRG-4 to more than 800-fold for CRG-2. The patterns of accumulation also varied. Some mRNAs, like CRG-2 and CRG-3, peaked early and declined significantly over the 24 h. Some, like CRG-10 and CRG-1, reached plateaus that were sustained over the 24 h. At 24 h, most mRNAs did not exceed the levels that they had reached at 8 h, with the notable exception of CRG-9, whose accumulation was delayed, with the 24-h signal about four times the signal at 8 h. RAW 264.7 cells that were mock treated and incubated without IFN-γ for 3 h showed no rise in the levels of any of the CRG mRNAs compared with time 0 cells, nor

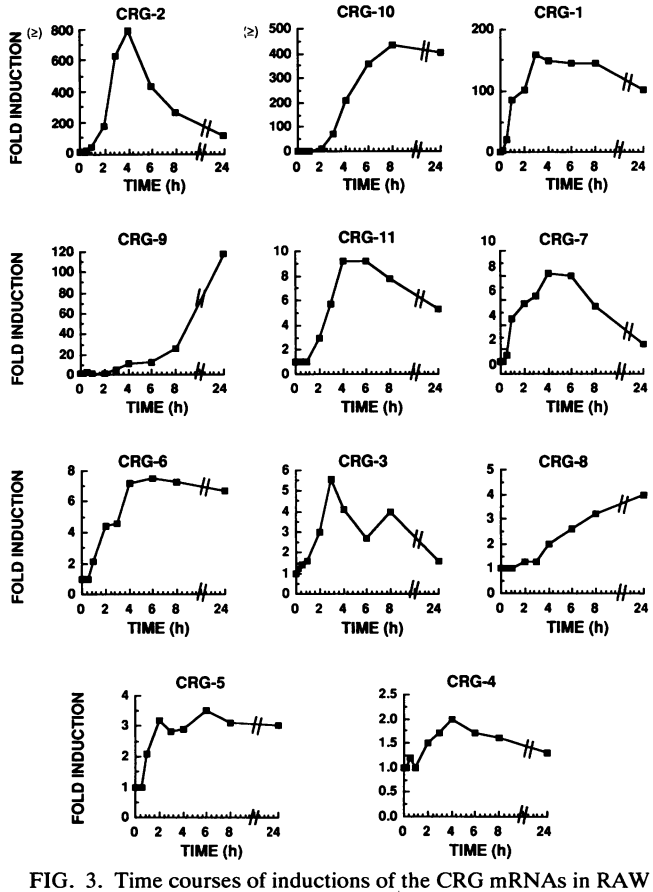

FIG. 3. Time courses of inductions of the CRG mRNAs in RAW 264.7 cells treated with 100 U of IFN-γ per ml. Cells were treated with IFN-γ continuously for the times noted, and total RNA was prepared and analyzed by Northern blot as in Fig. 1. Folds of induction were determined by densitometry of autoradiograms, using film preflashed to an optical density at 540 nm of approximately 0.25, and an intensifying screen, which was found to give a linear relationship between disintegrations per minute and signal intensity, allowing for quantitative comparisons of signals of different intensities and from different exposure times. For CRG-2 and CRG-10, no signals could be detected at 0 h, and folds of induction are expressed as ≥ values calculated versus the minimum signal detectable by the densitometer. For autoradiograms showing more than one major species, e.g., for CRG-6 and CRG-8, the signals from the various species were summed at each time. For CRG-2 and CRG-10, the data were derived from experiments published previously (21, 83), and these data are presented here for the purpose of comparison with the time courses for the other members of the CRG collection.

was there any change with time in the level of the control aldolase A mRNA (data not shown). Similar to the response to the spleen cell conditioned medium, the inductions of the CRG mRNAs by IFN-γ when analyzed at 2 h were not prevented by the simultaneous addition of 10 μg of CHX per ml (data not shown).

Responses of CRG mRNAs to PMA and A23187. Because increases in the intracellular concentration of calcium and activation of protein kinase C have been implicated in the actions of macrophage activators such as IFN-γ and LPS (reviewed in reference 28) and because phorbol ester and calcium ionophore have been shown to provide a portion of the signals required for macrophage activation (8), it was of interest to evaluate the effects of PMA and A23187 on the

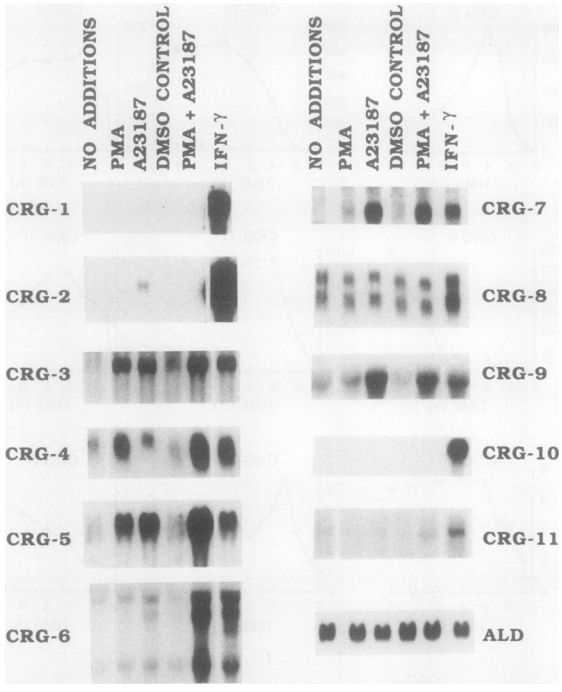

FIG. 4. CRG mRNAs in RAW 264.7 cells treated with PMA and A23187. Total RNA was prepared from RAW 264.7 cells treated for 3 h with various stimuli as noted in medium with 10% fetal bovine serum, and the RNA was analyzed by Northern blot as in Fig. 1. PMA was used at 100 ng/ml, A23187 was used at 10 μM, and IFN-γ was used at 100 U/ml. Because A23187 was added in DMSO, control cells were included that were treated with 0.1% DMSO alone. The results with use of the CRG-2 and CRG-10 probes were reported previously (21, 83), although the data were not shown, and these data are shown here for the purpose of comparison with the patterns of expression of the other members of the CRG collection.

levels of the CRG mRNAs. Figure 4 shows the levels of the CRG mRNAs in RAW 264.7 cells following 3-h exposures to 100 ng of PMA per ml or 10 μM A23187 or the combination of the two. Cells were exposed to 0.1% dimethyl sulfoxide (DMSO) as a control since the A23187 was added in DMSO, and the response, in the same experiment, to 100 U of IFN-γ per ml is included. The results with use of the CRG-2 and CRG-10 probes were reported previously (21, 83), although the data were not shown, and these data are shown here for the purpose of comparison with the patterns of expression of the other members of the CRG collection. Some of the mRNAs, such as CRG-1, CRG-2, CRG-8, CRG-10, and CRG-11, responded minimally or not at all to PMA and/or A23187. CRG-3 responded to PMA and to A23187, with the two together appearing to have an additive but not synergistic effect. CRG-7 and CRG-9 responded only to A23187, while CRG-4 responded minimally to A23187 alone but did respond to PMA alone and responded synergistically to the combination of PMA and A23187. Similarly, CRG-5 and CRG-6 demonstrated the synergistic effects of the two agents. CRG-6 is of particular interest in that PMA caused the induction, albeit to a low level, of the largest (4.2-kb) and smallest (1.7-kb) RNAs, while treatment with A23187 led to the induction of these plus the next-to-largest 3.4-kb species. Neither PMA nor A23187, alone or in combination, led to the appearance of the third-largest 2.8-kb species that was induced minimally in this experiment by IFN-γ and that is prominent after 3 h of treatment with the IFN-α and IFN-β (see above and Fig. 2).

Sequences and identities of CRG cDNAs. For CRG-2 and CRG-10 (MIG), the complete cDNA sequences have been published, demonstrating that the predicted proteins are members of the platelet factor 4 family of cytokines (21, 83). For the other cDNAs, sequences of several hundred nucleotides per cDNA have been determined and compared with entries in the GenBank (Department of Health and Human Services, release 69, September 1991) and EMBL Nucleotide Sequence Library (updated through October 7, 1991) data banks, using the NEWFASTA program from the Genetics Computer Group, University of Wisconsin Biotechnology Center (14). Two of the cDNAs, CRG-8 and CRG-4, represent mRNAs of well-established interferon-inducible genes, β_2-microglobulin (59, 85) and metallothionein II (22, 73), respectively. CRG-7 is related to an unpublished cDNA sequence for human modulator recognition factor I (57), and CRG-7 may represent the mouse homolog of the human protein/gene. CRG-1, CRG-3, and CRG-5 correspond to recently identified DNA-binding proteins with either presumed or demonstrated roles as regulators of transcription. CRG-1 is IRF-1, which binds to the regulatory region of the IFN-β gene (52) and is itself inducible by IFN-β (32). CRG-3 is zif/268, a zinc finger-containing protein induced as part of the immediate-early response to growth factors and other stimuli (10, 51) and for which a DNA recognition sequence has been defined (11). CRG-5 matches almost precisely the sequence of the rat LRF-1, a novel leucine zipper protein that is induced as part of the initial events during liver regeneration (33). The CRG-9 cDNA clone contains two internal *Eco*RI sites with *Eco*RI fragments of approximately 1.6, 0.3, and 0.17 kb, and with one end of the smallest fragment having a poly(A) track preceded by a consensus polyadenylation sequence. Sequence from the termini of the 1.6-kb fragment matches that of the 3′-most *Eco*RI fragment of cDNA clone TIS10, a cDNA clone corresponding to a phorbol ester-inducible mRNA from fibroblasts and encoding a novel prostaglandin synthase homolog (37). The published TIS10 cDNA clone terminates at an internal *Eco*RI site, and the 0.3- and 0.17-kb fragments of CRG-9 presumably correspond to the 3′ end of the mRNA that is missing from the TIS10 sequence. Demonstration that the TIS10 protein is a functional prostaglandin synthase, as suggested from the sequence, has not yet been reported.

CRG-6 is closely related to the IFN-activated gene 204 (9), which is part of a cluster of IFN-inducible genes on mouse chromosome 1 (58). Comparisons of segments of 218 and 293 nucleotides from the ends of the CRG-6 cDNA with the gene 204 cDNA sequence, using the BESTFIT program from the Genetics Computer Group (14), revealed percent identities of 86 and 92, respectively (data not shown), which together with the complexity of the pattern of CRG-6 mRNA species compared with the pattern of 204 expression (17) suggests that CRG-6, though a member of the 204 family, is in fact a different IFN-inducible gene. One hundred fifty base pairs at one end of the CRG-11 cDNA, containing a poly(A) RNA track preceded by a consensus polyadenylation sequence, represent the 3′ end of an L1Md (20) repetitive element. In a comparison with the sequences in the GenBank data base, this segment of the CRG-11 sequence most closely matched a portion of a LINE 1 repeat found in the second intron of a major histocompatibility complex class II gene, with which it showed 87.7% identity in a 154-bp overlap (data not shown; 47). The remaining 200 bp of sequence obtained from the CRG-11 cDNA, both adjacent to the L1Md sequence and at

the other end of the cDNA, showed no significant similarity with sequences in the data bases.

DISCUSSION

The genes in the CRG collection encode transcription factors and proteins predicted to be novel cytokines. It is clear that at least some of these proteins, e.g., CRG-3 (zif/268), CRG-5 (LRF-1), and CRG-9 (TIS10), act in a variety of biological contexts (10, 27, 33, 37). All of the CRG mRNAs are inducible by the supernatants of ConA-stimulated spleen cells and by IFN-γ, and in no case was induction prevented by inhibiting new protein synthesis with CHX. The accumulation of the mRNAs in an immediate-early fashion independent of new protein synthesis was to be expected given that the RAW 264.7 cDNA library and probes were prepared from cells treated in the presence of CHX. These results are consistent with those in the literature regarding the induction of the CRG and CRG-related genes that have been identified previously, e.g., the induction of metallothionein II (CRG-4) by IFN-α (22), the induction of the CRG-2-related IP-10 gene by IFN-γ (48), the induction of TIS10 (CRG-9) by phorbol ester (45), and the inductions of zif/268 (CRG-3) (41) and LRF-1 (rat CRG-5) (33) by serum as well as other stimuli. The superinduction of CRG-5 (mouse LRF-1) and of CRG-7 and CRG-10 (MIG) mRNAs by spleen cell supernatants plus CHX (as well as by IFN-γ plus CHX, for which the data are not shown) was also not surprising, since CHX has been shown to augment rates of transcription (22, 39), the duration of transcriptional activation (39, 41), and the stability of mRNAs (41) of inducible genes in a variety of systems. Activation of transcription by IFN-γ and the superinduction of transcription by CHX have been demonstrated directly for the CRG-10 (MIG) gene (90).

The responses of the CRG mRNAs to the IFNs and LPS differed. CRG-1 (IRF-1) and CRG-8 (β_2-microglobulin) responded to IFNs-α, -β, and -γ but minimally or not at all to LPS. CRG-9 (TIS10) responded to IFN-γ and to LPS but not to IFN-α or -β. A similar pattern was found for CRG-4 (metallothionein II). Metallothionein II has been found to be IFN-α inducible in some cell lines (22) but not in others (38). CRG-10 (MIG) was unusual in that it responded only to IFN-γ (21). The patterns of expression of the CRG genes reflect the existence of overlapping yet distinct biological activities for the IFNs and LPS. For example, both LPS (3) and IFN-γ (8) alone are capable of activating macrophages for cytocidal activity, yet at appropriate doses they can be shown to provide complementary and distinguishable signals that are both required to produce a fully activated cell (1, 8). Consistent with these effects, past work has shown that LPS and IFN-γ induce partially overlapping sets of proteins in mouse macrophages (49), and recently a number of LPS-inducible genes have been identified, a subset of which are inducible by IFN-β and/or -γ (30, 79). Likewise, while both type I (α and β, which bind to the same receptor) and type II (γ) IFNs have antiviral, antiproliferative, and immunoregulatory activities, the two types of IFN nevertheless have clearly distinguishable sets of actions in terms of macrophage activation (54, 61) and against viruses (81) and tumor cells (67). Also, the two types of IFN have been shown to induce both similar (12, 65, 87) and unique (19, 65, 87) proteins and genes.

While receptors for IFN-α (82), IFN-γ (2), and LPS (89) have been molecularly cloned, the signal transduction mechanisms whereby the IFNs and LPS induce changes in gene expression are complex and only partly understood. For IFN-α and -β, reports have indicated roles for cyclic AMP (70), protein kinase C (18, 64, 92), and intracellular calcium (91) in producing changes in gene expression and/or biological effects. For IFN-γ, reports have implicated protein kinase C (29), a novel protein kinase(s) (44), increases in intracellular calcium (35), and Na^+/H^+ exchange (62) in signal transduction. For all of the IFNs, however, in cases in which alterations in these signal transduction pathways have been found to be involved, these alterations commonly have been found to be necessary but not sufficient to induce changes in biological behavior and/or gene expression (18, 35, 64, 91). In addition, it is clear that for a given IFN, different pathways are involved in the regulation of different effects (35, 70), and it is difficult to generalize from studies of the induction of a single gene or biological activity. LPS has also been reported to act through protein kinase C (28, 86) and/or through changes in intracellular calcium (28), and PMA or A23187 alone has been found capable of inducing a subset of LPS-inducible genes in macrophages (79). Other LPS-inducible genes do not respond to these agents (79), and it is clear from other evidence that LPS acts through more than one pathway, at least depending in part on the dose of LPS used (72, 89).

As expected, the responses of the CRG collection of genes to the protein kinase C activator PMA and the calcium ionophore A23187 revealed no simple relationships between the IFNs and/or LPS and these two major pathways for signal transduction. It is clear that a number of IFN- and LPS-inducible genes can be induced by PMA or A23187 alone, that some genes fail to respond to either agent, and that some respond additively or synergistically to the action of both agents together. The induction of a subset of IFN-γ-responsive genes by PMA and/or A23187 is consistent with the ability of these pharmacological agents to mimic at least a portion of the effects of IFN-γ as an activator of macrophages for tumor cell cytotoxicity (8, 31), and the products of these genes are therefore candidates as participants in the cytotoxic response. Although the data are limited in terms of the number of genes studied and doses and times of treatment with the activators that were analyzed, mRNAs that were inducible to significant levels only by the IFNs, namely CRG-1 (IRF-1), CRG-8 (β_2-microglobulin), and CRG-10 (MIG), did not respond to PMA and/or A23187. This finding is consistent with the hypothesis of Levy and Darnell (42) that signals following receptor binding by polypeptide ligands, and the IFNs in particular are mediated not by global changes in the intracellular concentrations of small-molecule messengers but rather through direct interactions of specific signal-transducing proteins. The CRG genes that responded to PMA and/or A23187 are all inducible by LPS, a potent and pleiotropic, nonpolypeptide activator.

Of particular note regarding the CRG responses to PMA and A23187 was the pattern of response of the CRG-6 RNAs. As noted above, the sequence of the CRG-6 cDNA clone indicated that CRG-6 is a member of a family of IFN-inducible genes of unknown function on mouse chromosome 1 (9, 58). While the sequence of the CRG-6 cDNA indicates that it is related to the 204 gene as described by Lengyel and coworkers, the complexity of the pattern of expression of the CRG-6 RNAs is reminiscent of the pattern obtained by using a 203 gene probe (17, 58). The 203 gene is one of at least three related genes in the same cluster with the 204 gene, but it does not cross-hybridize with members of the 204 gene family. In addition, the CRG-6 cDNA probe, a partial cDNA of 0.6 kb from the presumed 3′ end of the

mRNA, hybridized to four major RNA species, the largest running just below the 28S RNA, while the 203 probes identified five species, the largest running above the 28S RNA (17). The multiple CRG-6-hybridizing RNAs might arise by any number of possible mechanisms from a single gene or from multiple genes. The observation that the largest 4.2-kb CRG-6 species can be induced by CHX alone, as seen with difficulty in Fig. 1 but more clearly in other experiments not shown, or by low-dose IFN-β (Fig. 2) with no or barely detectable amounts of the smaller species, while the smaller species have not been seen in the absence of the largest 4.2-kb band, is most readily understandable by assuming that the smaller species are derived from the largest one, either by alternative splicing or through an ordered pathway of degradation. The patterns of expression seen in response to the IFNs in Fig. 2 and in response to PMA and A23187 in Fig. 4 suggest that the processing of the CRG-6 RNAs is differentially regulated depending on the species, dose, and duration of the physiologic inducer and that part but not all of the physiologic response can be mimicked by the pharmacologically induced activation of protein kinase C and/or increases in intracellular calcium, again with differential effects depending on which signal transduction pathway is activated. If the regulation of the levels of CRG-6-hybridizing RNA species by IFNs is occurring at the level of mRNA processing, it complements previous observations of posttranscriptional regulation of the levels of inducible mRNAs by both IFN-α (22, 44) and IFN-γ (80). It will be of interest to determine the mechanisms whereby the multiple CRG-6 species are generated and regulated and the structures and functions of the proteins that they encode.

As noted above, 150 bp at one end of the CRG-11 cDNA clone correspond to the 3′ end of a truncated L1Md repetitive sequence, where a full-length repeat contains 6 to 7 kb (20). Previous studies have documented that LINE 1 sequences are transcribed by RNA polymerase II to yield a heterogeneous population of nuclear RNAs as well as discrete poly(A)$^+$ RNA cytoplasmic species (16, 36, 75). One of these discrete species, identified in murine lymphoid cell lines, could be detected only when cytoplasmic RNA was analyzed apart from nuclear RNA, and the discrete species was presumed to represent the transcript of a full-length repeat, since it hybridized to probes that spanned the LINE 1 element (16). In experiments not shown, a 250-bp CRG-11 cDNA fragment lacking L1Md sequences was used to probe a Northern blot of RAW 264.7 RNA, and this probe hybridized to the same inducible CRG-11 band as seen when the complete CRG-11 probe was used. The failure of the complete CRG-11 cDNA probe to identify a heterogeneous population of related but presumably nonidentical L1Md RNAs in the Northern blots shown above may have been due to the limited length of L1Md sequence in the CRG-11 cDNA. The CRG-11 mRNA is not derived from a full-length L1Md repeat but contains a severely truncated repeat, apparently determining the site for polyadenylation of the CRG-11 mRNA and constituting the CRG-11 mRNA's 3′ end. An unpublished cDNA sequence of mouse integrin alpha-4 has also been found to contain a truncated L1Md repeat (55) with partial homology to the repeat sequence in CRG-11, although in the case of the integrin cDNA, the repeat is in the reverse orientation and does not constitute the 3′ terminus of the cDNA.

Of primary interest are the functions of the CRG proteins, i.e., the roles that they play as mediators of the effects of their inducers and as participants in biological processes, including macrophage activation. β_2-Microglobulin (CRG-8) and metallothionein II (CRG-4) have well-defined functions. IRF-1 (CRG-1), zif/268 (CRG-3), and LRF-1 (rat CRG-5) are all recently discovered presumed transcriptional regulators whose physiologic roles are under active investigation. Zif/268 and LRF-1 were identified initially by differential screening, but in systems very different from the one used here. Zif/268 has been found to be induced following a range of stimuli, after growth factor treatment of growth-arrested fibroblasts (41, 78), and in in vitro models of neuronal (51) and cardiac (27, 77) differentiation. IFN-γ treatment of RAW 264.7 cells represents another differentiation pathway whereby zif/268 (CRG-3) is expressed, and with a delayed time course of accumulation compared with serum stimulated fibroblasts (41). LRF-1 (rat CRG-5) was identified on the basis of its accumulation during liver regeneration, peaking at 2 h and almost disappearing at 4 h posthepatectomy (33). This was in contrast to the delayed and more sustained accumulation of CRG-5 following IFN-γ treatment of RAW 264.7 cells (Fig. 2 and 3). It is of interest that the same transcriptional regulators can be induced very rapidly and transiently by growth factors, but with different kinetics and presumably with different effects on gene expression in response to IFN-γ, an antiproliferative and differentiation factor.

CRG-9 has also been identified as TIS10, one of a collection of immediate-early genes in response to phorbol ester stimulation of quiescent fibroblasts, and is predicted to encode a new prostaglandin synthase (37). Previously, surveys had shown expression of the TIS10 mRNA limited to astrocytes (4) and fibroblasts (37). In fibroblasts, the level of the TIS10 (CRG-9) mRNA peaks in the initial hours following treatment with phorbol ester, serum, and other mitogens, in line with the time course of induction of prostaglandin synthesis in these cells but very different from the time course of CRG-9 (TIS10) in RAW 264.7 cells during treatment with IFN-γ (Fig. 3). In contrast to the response to platelet-derived growth factor in quiescent fibroblasts (46), for example, treatment with IFN-γ alone has not been reported to increase the secretion of prostaglandins by macrophages. Several reports have, however, documented that pretreatment of monocytes/macrophages with IFN-γ significantly enhances the production of prostaglandins in response to a subsequent stimulus such as phorbol ester (6) or LPS (56). In contrast, IFN-α and -β have been found to have either no effect or an inhibiting effect on prostaglandin secretion (6). The dramatic induction of the CRG-9 (TIS10) mRNA by IFN-γ but not by the other IFNs (Fig. 2) may represent the basis for the previously noted differential effects of the IFNs on prostaglandin synthesis. The function of the second signal, necessary to demonstrate the augmentation of macrophage prostaglandin secretion by pretreatment with IFN-γ, might be to increase the availability of substrate, i.e., arachidonic acid, and/or to produce an active prostaglandin synthase by inducing translation or posttranslational modification.

CRG-2 and CRG-10 (MIG) are new members of the platelet factor 4 family of cytokines (21, 83). CRG-2 is closely related to the human IP-10 (48), and the two may be homologs (83). The members of the platelet factor 4 family, many of which have been identified only recently, are chemotactic factors, immune cell activators, and positive and negative regulators of cell growth (76). CRG-2 and CRG-10 (MIG) presumably act as mediators of the effects of the IFNs, and the specific induction of CRG-10 (MIG) by IFN-γ raises the possibility that CRG-10 (MIG) mediates a biological activity peculiar to IFN-γ. Preliminary analysis of

the MIG gene promoter has suggested that a novel sequence may mediate the specific response to IFN-γ (90). Studies of the CRG-2 and MIG genes and proteins are ongoing, particularly in regard to the biological functions of the predicted cytokines.

Since the partial sequence available for the CRG-11 cDNA has not been informative, additional cDNA clones and sequence will be obtained to determine whether the CRG-11 gene and encoded protein contain known structural motifs. Investigations of the regulation and functions of the CRG collection of inducible genes and proteins hopefully will shed light on molecular mechanisms underlying a range of biological responses, including growth, differentiation, and immune and inflammatory reactions, and such studies may provide tools for manipulating these complex cellular processes.

ACKNOWLEDGMENTS

This work was begun when I was an Associate in the Howard Hughes Medical Institute, and the work was supported subsequently by a Johns Hopkins University Clinician Scientist Award and by Public Health Service grant CA-48059 from the National Cancer Institute.

I am gratefully indebted to Daniel Nathans and to Hyun Shin for their indispensable advice and support and to Se-Jin Lee, Keith Peden, Michael McLane, Padmavathy Vanguri, Beth Drysdale, Chaim Kahana, Eran Rom, Anthony Lanahan, Barbara Christy, Andrew Levy, Laura Sanders, Sven Willenberger, and Michael Nevins for their expert consultations and assistance.

REFERENCES

1. **Adams, D. O., and T. A. Hamilton.** 1984. The cell biology of macrophage activation. Ann. Rev. Immunol. **2**:283–318.
2. **Aguet, M., Z. Dembic, and G. Merlin.** 1988. Molecular cloning and expression of the human interferon-γ receptor. Cell **55**:273–280.
3. **Alexander, P., and R. Evans.** 1971. Endotoxin and double stranded RNA render macrophages cytotoxic. Nature (London) New Biol. **232**:76–78.
4. **Arenander, A. T., R. W. Lim, B. C. Varnum, R. Cole, J. de Vellis, and H. R. Herschman.** 1989. TIS gene expression in cultured rat astrocytes: induction by mitogens and stellation agents. J. Neurosci. Res. **23**:247–256.
5. **Beutler, B., N. Krochin, I. W. Milsark, C. Luedke, and A. Cerami.** 1986. Control of cachectin (tumor necrosis factor) synthesis: mechanisms of endotoxin resistance. Science **232**: 977–980.
6. **Boraschi, D., S. Censini, M. Bartalini, and A. Tagliabue.** 1985. Regulation of arachidonic acid metabolism in macrophages by immune and nonimmune interferons. J. Immunol. **135**:502–505.
7. **Bowyer, D. E., and M. J. Mitchinson.** 1989. The role of macrophages in atherosclerosis, p. 439–458. *In* M. Zembala and G. L. Asherson (ed.), Human monocytes. Academic Press, San Diego, Calif.
8. **Celada, A., and R. D. Schreiber.** 1986. Role of protein kinase C and intracellular calcium mobilization in the induction of macrophage tumoricidal activity by interferon-γ. J. Immunol. **137**: 2373–2379.
9. **Choubey, D., J. Snoddy, V. Chaturvedi, E. Toniato, G. Opdenakker, A. Thakur, H. Samanta, D. A. Engel, and P. Lengyel.** 1989. Interferons as gene activators: indications for repeated gene duplication during the evolution of a cluster of interferon-activatable genes on murine chromosome 1. J. Biol. Chem. **264**:17182–17189.
10. **Christy, B. A., L. F. Lau, and D. Nathans.** 1988. A gene activated in mouse 3T3 cells by serum growth factors encodes a protein with "zinc finger" sequences. Proc. Natl. Acad. Sci. USA **85**:7857–7861.
11. **Christy, B. A., and D. Nathans.** 1989. DNA binding site of the growth factor-inducible protein zif268. Proc. Natl. Acad. Sci. USA **86**:8737–8741.
12. **Colonno, R. J., and R. H. L. Pang.** 1982. Induction of unique mRNAs by human interferons. J. Biol. Chem. **257**:9234–9237.
13. **Dale, T. C., A. M. A. Imam, I. M. Kerr, and G. R. Stark.** 1989. Rapid activation by interferon-γ of a latent DNA-binding protein present in the cytoplasm of untreated cells. Proc. Natl. Acad. Sci. USA **86**:1203–1207.
14. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
15. **Doe, W. F., and P. M. Henson.** 1978. Macrophage stimulation by bacterial lipopolysaccharides. J. Exp. Med. **148**:544–556.
16. **Dudley, J. P.** 1987. Discrete high molecular weight RNA transcribed from the long interspersed repetitive element L1Md. Nucleic Acids Res. **15**:2581–2592.
17. **Engel, D. A., J. Snoddy, E. Toniato, and P. Lengyel.** 1988. Interferons as gene activators: close linkage of two interferon-activatable murine genes. Virology **166**:24–29.
18. **Faltynek, C. R., G. L. Princler, G. L. Gusella, L. Varesio, and D. Radzioch.** 1989. A functional protein kinase C is required for induction of 2-5A synthetase by recombinant interferon-αA in Daudi cells. J. Biol. Chem. **264**:14305–14311.
19. **Fan, X., G. R. Stark, and B. R. Bloom.** 1989. Molecular cloning of a gene selectively induced by gamma interferon from human macrophage cell line U937. Mol. Cell. Biol. **9**:1922–1928.
20. **Fanning, T. G.** 1983. Size and structure of the highly repetitive BAM H1 element in mice. Nucleic Acids Res. **11**:5073–5091.
21. **Farber, J. M.** 1990. A macrophage mRNA selectively induced by γ-interferon encodes a member of the platelet factor 4 family of cytokines. Proc. Natl. Acad. Sci. USA **87**:5238–5242.
22. **Friedman, R. L., S. P. Manly, M. McMahon, I. M. Kerr, and G. R. Stark.** 1984. Transcriptional and posttranscriptional regulation of interferon-induced gene expression in human cells. Cell **38**:745–755.
23. **Friedman, R. L., and G. R. Stark.** 1985. γ-Interferon-induced transcription of HLA and metallothionein genes containing homologous upstream sequences. Nature (London) **314**:637–639.
24. **Gendelman, H. E., and M. S. Meltzer.** 1989. HIV-infected monocytes in the pathogenesis of AIDS, p. 469–482. *In* M. Zembala and G. L. Asherson (ed.), Human monocytes. Academic Press, San Diego, Calif.
25. **Goldberg, D. A.** 1980. Isolation and partial characterization of the *Drosophila* alcohol dehydrogenase gene. Proc. Natl. Acad. Sci. USA **77**:5794–5798.
26. **Grabstein, K. H., D. L. Urdal, R. J. Tushinski, D. Y. Mochizuki, V. L. Price, M. A. Cantrell, S. Gillis, and P. J. Conlon.** 1986. Induction of macrophage tumoricidal activity by granulocyte-macrophage colony-stimulating factor. Science **232**:506–508.
27. **Gupta, M. P., M. Gupta, R. Zak, and V. P. Sukhatme.** 1991. *Egr-1*, a serum-inducible zinc finger protein, regulates transcription of the rat cardiac α-myosin heavy chain gene. J. Biol. Chem. **266**:12813–12816.
28. **Hamilton, T. A., and D. O. Adams.** 1987. Molecular mechanisms of signal transduction in macrophages. Immunol. Today **8**:151–158.
29. **Hamilton, T. A., D. L. Becton, S. D. Somers, P. W. Gray, and D. O. Adams.** 1985. Interferon-γ modulates protein kinase C activity in murine peritoneal macrophages. J. Biol. Chem. **260**:1378–1381.
30. **Hamilton, T. A., N. Bredon, Y. Ohmori, and C. S. Tannenbaum.** 1989. IFN-γ and IFN-β independently stimulate the expression of lipopolysaccharide-inducible genes in murine peritoneal macrophages. J. Immunol. **142**:2325–2331.
31. **Hamilton, T. A., S. D. Somers, D. L. Becton, A. Celada, R. D. Schreiber, and D. O. Adams.** 1986. Analysis of deficiencies in IFN-γ-mediated priming for tumor cytotoxicity in peritoneal macrophages from A/J mice. J. Immunol. **137**:3367–3371.
32. **Harada, H., T. Fujita, M. Miyamoto, Y. Kimura, M. Maruyama, A. Furia, T. Miyata, and T. Taniguchi.** 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2 bind to the same regulatory elements of IFN and IFN-inducible genes. Cell **58**:729–739.

33. **Hsu, J.-C., T. Laz, K. L. Mohn, and R. Taub.** 1991. Identification of LRF-1, a leuine-zipper protein that is rapidly and highly induced in regenerating liver. Proc. Natl. Acad. Sci. USA **88:**3511–3515.
34. **Kessler, D. S., S. A. Veals, X.-Y. Fu, and D. E. Levy.** 1990. Interferon-α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. Genes Dev. **4:**1753–1765.
35. **Klein, J. B., T. M. Schepers, W. L. Dean, G. Sonnenfeld, and K. R. McLeish.** 1990. Role of intracellular calcium concentration and protein kinase C activation in IFN-gamma stimulation of U937 cells. J. Immunol. **144:**4305–4311.
36. **Kole, L. B., S. R. Haynes, and W. R. Jelinek.** 1983. Discrete and heterogeneous high molecular weight RNAs complementary to a long dispersed repeat family (a possible transposon) of human DNA. J. Mol. Biol. **165:**257–286.
37. **Kujubu, D. A., B. S. Fletcher, B. C. Varnum, R. W. Lim, and H. R. Herschman.** 1991. TS10, a phorbol ester tumor promoter-inducible mRNA from Swiss 3T3 cells, encodes a novel prostaglandin synthase/cyclooxygenase homologue. J. Biol. Chem. **266:**12866–12872.
38. **Kusari, J., R. K. Tiwari, R. Kumar, and G. C. Sen.** 1987. Expression of interferon-inducible genes in RD-114 cells. J. Virol. **61:**1524–1531.
39. **Larner, A. C., A. Chaudhuri, and J. E. Darnell.** 1986. Transcriptional induction by interferon. J. Biol. Chem. **261:**453–459.
40. **Lau, L. F., and D. Nathans.** 1985. Identification of a set of genes expressed during the G0/G1 transition of cultured mouse cells. EMBO J. **4:**3145–3151.
41. **Lau, L. F., and D. Nathans.** 1987. Expression of a set of growth-related immediate early genes in BALB/c 3T3 cells: coordinate regulation with c-fos or c-myc. Proc. Natl. Acad. Sci. USA **84:**1182–1186.
42. **Levy, D., and J. E. Darnell, Jr.** 1990. Interferon-dependent transcriptional activation: signal transduction without second messenger involvement? New Biol. **2:**923–928.
43. **Levy, D., D. Kessler, R. Pina, N. Reich, and J. Darnell, Jr.** 1987. Interferon stimulated genes: promoters contain shared response elements that bind interferon-induced nuclear proteins. Genes Dev. **2:**383–393.
44. **Lew, D. J., T. Decker, and J. E. Darnell, Jr.** 1989. Alpha interferon and gamma interferon stimulate transcription of a single gene through different signal transduction pathways. Mol. Cell. Biol. **9:**5404–5411.
45. **Lim, R. W., B. C. Varnum, and H. R. Herschman.** 1987. Cloning of tetradecanoyl phorbol ester-induced 'primary response' sequences and their expression in density-arrested Swiss 3T3 cells and a TPA nonproliferative variant. Oncogene **1:**263–270.
46. **Lin, A. H., M. J. Bienkowski, and R. R. Gorman.** 1989. Regulation of prostaglandin H synthase mRNA levels and prostaglandin biosynthesis by platelet-derived growth factor. J. Biol. Chem. **264:**17379–17383.
47. **Lu, C.-C., and E. K. Wakaland.** Unpublished data. GenBank accession number M60562.
48. **Luster, A. D., J. C. Unkeless, and J. V. Ravetch.** 1985. γ-Interferon transcriptionally regulates an early-response gene containing homology to platelet proteins. Nature (London) **315:**672–676.
49. **MacKay, R. J., and S. W. Russell.** 1986. Protein changes associated with stages of activation of mouse macrophages for tumor cell killing. J. Immunol. **137:**1392–1398.
50. **Marcucci, F., B. Klein, H. Kirchner, and R. Zawatzky.** 1982. Production of high titers of interferon-gamma by prestimulated murine spleen cells. Eur. J. Immunol. **12:**787–790.
51. **Milbrandt, J.** 1987. A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. Science **238:** 797–799.
52. **Miyamoto, M., T. Fujita, Y. Kimura, M. Maruyama, H. Harada, Y. Sudo, T. Miyata, and T. Taniguchi.** 1988. Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-β gene regulatory elements. Cell **54:**903–913.
53. **Nakamura, M., T. Manser, G. D. N. Pearson, M. J. Daley, and M. L. Gefter.** 1984. Effect of IFN-γ on the immune response *in vivo* and on gene expression *in vitro*. Nature (London) **307:**381–382.
54. **Nathan, C. F., H. W. Murray, M. E. Wiebe, and B. Y. Rubin.** 1983. Identification of IFN-γ as the lymphokine that activates human macrophage oxidative metabolism and antimicrobial activity. J. Exp. Med. **158:**670–689.
55. **Neuhaus, H., M. E. Hemler, Y. Takada, and I. L. Weissman.** Unpublished data. EMBL accession number X53176.
56. **Nichols, F. C., and S. W. Garrison.** 1987. Interferon-γ potentiation of lipopolysaccharide-induced eicosanoid release from human monocytes. J. Interferon Res. **7:**121–129.
57. **Oka, T., B. W. Merrills, E. Huang, T. Okada, Y. Tanaka, P. Gertson, and K. Itakura.** Unpublished data. GenBank accession number M62324.
58. **Opdenakker, G., J. Snoddy, D. Choubey, E. Toniato, D. D. Pravtcheva, M. F. Seldin, F. H. Ruddle, and P. Lengyel.** 1989. Interferons as gene activators: a cluster of six interferon-activatable genes is linked to erythroid α-spectrin locus on murine chromosome 1. Virology **171:**568–578.
59. **Parnes, J. R., B. Velan, A. Felsenfeld, L. Ramanathan, U. Ferrini, E. Appella, and J. G. Seidman.** 1981. Mouse β_2-microglobulin cDNA clones: a screening procedure for cDNA clones corresponding to rare mRNAs. Proc. Natl. Acad. Sci. USA **78:**2253–2257.
60. **Peden, K. W. C., P. Mounts, and G. S. Hayward.** 1982. Homology between mammalian cell DNA sequences and human herpes virus genomes detected by a hybridization procedure with high complexity probe. Cell **31:**71–80.
61. **Philip, R., and L. B. Epstein.** 1986. Tumor necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, γ-interferon and interleukin-1. Nature (London) **323:**86–89.
62. **Prpic, V., S. F. Yu, F. Figueiredo, P. W. Hollenbach, G. Gawdi, B. Herman, R. J. Uhing, and D. O. Adams.** 1989. Role of Na^+/H^+ exchange by interferon-gamma in enhanced expression of JE and I-A_β genes. Science **244:**469–471.
63. **Raschke, W. C., S. Baird, P. Ralph, and I. Nakoinz.** 1978. Functional macrophage cell lines transformed by Abelson leukemia virus. Cell **15:**261–267.
64. **Reich, N. C., and L. M. Pfeffer.** 1990. Evidence for involvement of protein kinase C in the cellular response to interferon α. Proc. Natl. Acad. Sci. USA **87:**8761–8765.
65. **Revel, M., and J. Chebath.** 1986. Interferon-activated genes. Trends Biochem. Sci. **11:**166–170.
66. **Rosa, F., D. Hatat, A. Abadie, D. Wallach, M. Revel, and M. Fellous.** 1983. Differential regulation of HLA-DR mRNAs and cell surface antigens by interferon. EMBO J. **2:**1585–1589.
67. **Rubin, B. Y., and S. L. Gupta.** 1980. Differential efficacies of human type I and type II interferons as antiviral and antiproliferative agents. Proc. Natl. Acad. Sci. USA **77:**5928–5932.
68. **Sanders, K. M., and B. H. Littman.** 1986. Lymphokine stimulation of human macrophage C2 production is partially due to interferon-γ. J. Immunol. **137:**876–879.
69. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
70. **Schneck, J., B. Rager-Zisman, O. M. Rosen, and B. R. Bloom.** 1982. Genetic analysis of the role of cAMP in mediating effects of interferon. Proc. Natl. Acad. Sci. USA **79:**1879–1833.
71. **Schultz, R. M., and W. J. Kleinschmidt.** 1983. Functional identity between murine γ interferon and macrophage activating factor. Nature (London) **305:**239–240.
72. **Schumann, R. R., S. R. Leong, G. W. Flaggs, P. W. Gray, S. D. Wright, J. C. Mathison, P. S. Tobias, and R. J. Ulevitch.** 1990. Structure and function of lipopolysaccharide binding protein. Science **249:**1429–1431.
73. **Searle, P. F., B. L. Davison, G. W. Stuart, T. M. Wilkie, G. Norstedt, and R. D. Palmiter.** 1984. Regulation, linkage, and sequence of mouse metallothionein I and II genes. Mol. Cell. Biol. **4:**1221–1230.
74. **Sen, R., and D. Baltimore.** 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a posttranslational mechanism. Cell **47:**921–928.
75. **Skowronski, J., and M. F. Singer.** 1985. Expression of a cyto-

plasmic LINE-1 transcript is regulated in a human teratocarcinoma cell line. Proc. Natl. Acad. Sci. USA **82**:6050–6054.
76. **Stoeckle, M. Y., and K. A. Barker.** 1990. Two burgeoning families of platelet factor 4-related proteins: mediators of the inflammatory response. New Biol. **2**:313–323.
77. **Sukhatme, V. P., X. Cao, L. C. Chang, C.-H. Tsai-Morris, D. Stamenkovich, P. C. P. Ferreira, D. R. Cohen, S. A. Edwards, T. B. Shows, T. Curran, M. M. LeBeau, and E. D. Adamson.** A zinc finger-encoding gene coregulated with c-fos during growth and differentiation, and after cellular depolarization. Cell **53**:37–43.
78. **Sukhatme, V. P., S. Kartha, F. G. Toback, R. Taub, R. G. Hoover, and C.-H. Tsai-Morris.** 1987. A novel early growth response gene rapidly induced by fibroblast, epithelial cell and lymphocyte mitogens. Oncogene Res. **1**:343–355.
79. **Tannenbaum, C. S., T. J. Koerner, M. M. Jansen, and T. A. Hamilton.** 1988. Characterization of lipopolysaccharide-induced macrophage gene expression. J. Immunol. **140**:3640–3645.
80. **Thorens, B., J. J. Mermod, and P. Vassalli.** 1987. Phagocytosis and inflammatory stimuli induce GM-CSF mRNA in macrophages through posttranscriptional regulation. Cell **48**:671–679.
81. **Ulker, N., and C. E. Samuel.** 1985. Mechanism of interferon action: inhibition of vesicular stomatitis virus replication in human amnion U cells by cloned human γ-interferon. J. Biol. Chem. **260**:4324–4330.
82. **Uze, G., G. Lutfalla, and I. Gresser.** 1990. Genetic transfer of a functional human interferon α receptor into mouse cells: cloning and expression of its cDNA. Cell **60**:225–234.
83. **Vanguri, P., and J. M. Farber.** 1990. Identification of CRG-2: an interferon-inducible mRNA predicted to encode a murine monokine. J. Biol. Chem. **265**:15049–15057.
84. **Wahl, S. M., L. M. Wahl, J. B. McCarthy, L. Chedid, and S. E. Mergenhagen.** 1979. Macrophage activation by mycobacterial water soluble compounds and synthetic muramyl dipeptide. J. Immunol. **122**:2226–2231.
85. **Wallach, D., M. Fellous, and M. Revel.** 1982. Preferential effect of γ interferon on the synthesis of HLA antigens and their mRNAs in human cells. Nature (London) **299**:833–836.
86. **Weiel, J. E., T. A. Hamilton, and D. O. Adams.** 1986. LPS induces altered phosphate labeling of proteins in murine peritoneal macrophages. J. Immunol. **136**:3012–3018.
87. **Weil, J., C. J. Epstein, L. B. Epstein, J. J. Sedmak, J. L. Sabran, and S. E. Grossberg.** 1983. A unique set of polypeptides is induced by γ interferon in addition to those induced in common with α and β interferons. Nature (London) **301**:437–439.
88. **Weinshank, R. L., A. D. Luster, and J. V. Ravetch.** 1988. Function and regulation of a murine macrophage-specific IgG Fc receptor, FcγR-α. J. Exp. Med. **167**:1909–1925.
89. **Wright, S. D., R. A. Ramos, P. S. Tobias, R. J. Ulevitch, and J. C. Mathison.** 1990. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science **249**:1431–1433.
90. **Wright, T. M., and J. M. Farber.** 1991. 5′ regulatory region of a novel cytokine gene mediates selective activation by interferon γ. J. Exp. Med. **173**:417–422.
91. **Yan, C., P. B. Sehgal, and I. Tamm.** 1989. Signal transduction pathways in the induction of 2′,5′-oligoadenylate synthetase gene expression by interferon α/β. Proc. Natl. Acad. Sci. USA **86**:2243–2247.
92. **Yap, W. H., T. S. Teo, and Y. H. Tan.** 1986. An early event in the interferon-induced transmembrane signaling process. Science **234**:355–358.
93. **Zembala, M., and G. L. Asherson (ed.).** 1989. Human monocytes. Academic Press, San Diego, Calif.