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Structure of the Chicken Myelomonocytic Growth Factor Gene and Specific Activation of Its Promoter in Avian Myelomonocytic Cells by Protein Kinases

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In chicken myeloid cells but not in erythroid cells, kinase-type oncogenes activate expression of the chicken myelomonocytic growth factor (cMGF). The autocrine loop established this way plays a key role in lineage-specific cooperation of nuclear and kinase-type oncogenes in retrovirally induced myeloid leukemia. In this report, we describe the cloning of the cMGF gene, including its promoter. The structure of the cMGF gene is homologous to those of the granulocyte colony-stimulating factor and interleukin-6 genes. Expression from reporter constructs containing the cMGF promoter is specific to myelomonocytic cells. Kinases activate cMGF at the transcriptional level in macrophages and strongly induce reporter expression in myelomonocytic cells.

The chicken myelomonocytic growth factor (cMGF) is distantly related to both the mammalian granulocyte colony-stimulating factor (G-CSF) and interleukin 6 (IL-6). cMGF is required for survival and growth of normal and transformed avian myelomonocytic cells (30, 32). In macrophages, cMGF expression can be induced by retrovirally transduced kinase-type oncogenes; by the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), probably through activation of protein kinase C; and by bacterial lipopolysaccharide (LPS) (32, 34), which can activate protein kinase C and cellular tyrosine kinases (48). In myeloid cells transformed by the nuclear oncogenes v-*myc* or v-*myb*, cMGF expression can be induced by retrovirally encoded protein kinases such as v-Mil or v-ErbB. As a consequence, these cells become independent of exogenous cMGF and are highly leukemogenic (1, 24). Thus, kinase-type oncogenes collaborate with nuclear oncogenes in chicken myeloid leukemia formation by inducing an autocrine loop. Recent studies have demonstrated that expression of cMGF from a retroviral construct can fully replace kinase-type oncogenes in augmenting leukemia formation by an attenuated v-*myb* oncogene (34). Interestingly, kinase-type oncogenes fail to induce cMGF expression in cells of other hematopoietic lineages such as transformed erythroblasts (30a). Therefore, we anticipate that the induction of cMGF expression by kinase-type oncogenes might serve as a model to investigate the lineage-specific activity and cooperation of oncogenes.

In this report, we describe the molecular cloning of the cMGF gene and the regulation of its promoter. We found that the structure of the cMGF gene, but not of its promoter, is related to those of the mammalian G-CSF and IL-6 genes. In myelomonocytic cells, cMGF expression is regulated at the transcriptional level, and in transient expression assays, kinases activate expression from the cMGF promoter specifically in myeloid cells. Thus, the activity of the cMGF promoter within a reporter construct parallels the activity of the endogenous gene. The results provide the means to investigate how kinases induce cMGF expression in transformed myeloid cells and thereby collaborate with v-Myb or v-Myc in leukemia formation.

MATERIALS AND METHODS

Cells and culture conditions. HD3 is an avian erythroblastosis virus ES4-transformed chicken erythroblast cell line expressing the viral *erbA* and *erbB* oncogenes (7). HD11 is an MC29-transformed macrophage cell line expressing the v-*myc* oncogene (11). MSB-I refers to the T-lymphoid cell line MDCC-MSB-1 (10). RPL-12 refers to the B-lymphoid cell line LVSCC-TLT induced by the RPL-12 strain of leukosis virus (10). CEF38 is a cell line established from chicken embryo fibroblasts.

All cell types were cultured in Dulbecco's modified Eagle's medium (GIBCO) plus 8% fetal calf serum, 2% chicken serum, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.4) supplemented with an optimal concentration of recombinant cMGF (32) for factor-dependent cell types (E26-, MH2OB-, and CRmyb-HER-transformed cells). myc-HER-transformed cells were supplemented with concanavalin A-spleen conditioned medium as a source of cMGF (9). CRmyb-HER-transformed cells were grown in the presence of 20 ng of murine epidermal growth factor (EGF) (Sigma) per ml unless otherwise indicated. Medium for erythroid and lymphoid cells was supplemented with 500 nM β-mercaptoethanol.

Retroviruses and oncogenes. MH2 carries the *myc* and gag-*mil* viral oncogenes (16). MH2OB is a deletion mutant of MH2 which does not express v-Mil (6). E26 expresses the gag-*myb-ets* fusion protein (21). CRmyb-HER is a recombinant retrovirus expressing the AMV/E26 recombinant v-myb^{EEA} oncogene and the human EGF receptor (34). The recombinant retrovirus myc-HER, obtained from K. Khazaie, carries an MH2-derived v-*myc* oncogene and the human EGF receptor (50).

Transformation of hematopoietic cells. Primary transformed hematopoietic cells were generated by infecting freshly prepared bone marrow cells of 1-week-old SPAFAS chicks (20) with viruses (filtered medium conditioned by virus-producing fibroblasts) in liquid medium containing 2 μg of polybrene per ml (22). CRmyb-HER-, E26-, myc-HER-,

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and MH2OB-infected bone marrow cells were left in liquid medium plus polybrene until rapidly proliferating transformed cells appeared after 10 to 14 days. Transformed cells were subsequently purified by multiple passages. Alternatively, MH2-infected cells were seeded in semisolid medium (23) and transformed colonies were isolated after 5 to 10 days and propagated in liquid medium. For the experiments described, 10 clones of fast-growing MH2-infected bone marrow cells were combined and grown as a mixed culture.

Screening of the genomic library. A total of 5×10^5 PFU of a chicken genomic library prepared from the DNA of a male SPAFAS chicken in a Lambda-Charon30 vector (46) was screened with [α-^{32}P]dCTP-labelled cMGF cDNA as probe (32) according to standard procedures (4). Briefly, the phages were plated on VCS-257 bacteria and plaques were grown to near confluency and transferred to nitrocellulose filter membranes. Filters were hybridized in 5× Denhardt's solution (33)–100 μg of calf thymus DNA per ml–0.1% sodium pyrophosphate–50 mM Tris-HCl (pH 7.5)–5× SSC (0.75 M NaCl, 75 mM sodium citrate · H_2O, pH 7.0)–1% sodium dodecyl sulfate (SDS)–50% formamide at 42°C for 36 h. Subsequently, the filters were washed in 1× SSC–0.1% SDS at 65°C and exposed to Kodak X-ray film. The initial screen yielded six positive phage plaques, three of which were purified (clones λ1.2, λ4.2, and λ5.3). Phage DNA was prepared and analyzed by restriction analysis and cross-hybridization experiments.

Genomic subclones and sequencing. A 5-kb *Hin*dIII-*Bam*HI restriction fragment of clone λ4.2 was subcloned into Bluescript (Stratagene). In addition, overlapping subfragments of this construct (pλ4.2-HB5) were inserted into M13mp vectors in either orientation. The sequences of both strands were determined by the chain-termination method (40), using the Sequenase kit (United States Biochemical Corp.) with commercial and custom-synthesized primers. Because of the high GC content along the entire cMGF gene, sequencing reactions were performed on single- and double-stranded templates by using both a conventional deoxynucleoside triphosphate mix and a mix in which dITP replaces dGTP.

S1 analysis. S1 analysis was performed with end-labelled single-stranded DNA as protecting probe (4). For preparation of the probe, a fusion of genomic sequence with cDNA sequence at the *Dra*III site enclosing the translation start codon was constructed, since the coding region of the first cMGF exon is only 16 nucleotides long. Briefly, a 1,680-bp genomic *Hin*dIII-*Dra*III restriction fragment (the same fragment as in pM1.6; see "Reporter constructs") and 157 bp of the coding sequence (*Dra*III-*Mae*II restriction fragment) derived from the cMGF cDNA (32) were joined at the *Dra*III site and inserted into Bluescript (pcMGF-F). For the synthesis of the single-stranded DNA probe, pcMGF-F was cut with *Hin*dIII at the 5′ end of the cMGF insert. A custom-synthesized primer matching nucleotides 88 to 117 of the cDNA (32) was labelled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase, annealed to linearized, denatured pcMGF-F, and extended with Klenow. The 1,745-bp polymerization product was purified by alkaline agarose gel electrophoresis. S1 analysis was performed according to the protocol of J. M. Greene in reference 4. Briefly, 50 μg of total RNA extracted from cells by a guanidine thiocyanate method (14) was annealed with 3.5×10^5 cpm of single-stranded probe for 14 h at 30°C. The samples were each digested with 80 U of S1 nuclease for 1 h at 30°C. A fraction corresponding to 8 μg of RNA was loaded on a denaturing 6% polyacrylamide-urea gel.

Analysis of RNA expression. Total RNA was extracted from cells by a guanidine thiocyanate method (4). Selection of polyadenylated RNA by oligo(dT)-cellulose chromatography was performed by standard procedures (33), using SDS instead of *N*-lauryl-sarcosine. RNA was subjected to electrophoresis in 0.8% agarose-formaldehyde gels and transferred to Hybond-N-nylon filters (Amersham) according to standard procedures (33). DNA probes were prepared from purified restriction fragments (see below) with the Multiprime DNA labelling system (Amersham) by using [α-^{32}P]dCTP. Hybridization of filters with DNA probes was performed with 50% formamide–5× SSPE (750 mM NaCl, 50 mM $NaH_2PO_4 \cdot H_2O$, 5 mM EDTA)–5× Denhardt's solution (33)–0.5% SDS–20 μg of salmon sperm DNA per ml at 42°C for 14 to 16 h. Subsequently, the filters were washed with 0.1% SDS–0.2× SSPE (final wash) at 60°C and exposed to Kodak X-ray film.

DNA probes. For the preparation of DNA probes, restriction fragments were isolated from the chicken cDNA clones of GAPDH (1.1-kb *Pst*I fragment of pGAD-28 [19]), cMGF (0.7-kb *Eco*RI-*Ssp*I fragment of pFP1 [32]), c-*jun* (2.4-kb *Hin*dIII-*Eco*RI insert of pGCJ [37]), and c-*fos* (1.1-kb *Nhe*I-*Sma*I fragment [35]).

Nuclear run-on analysis. Preparation of nuclei was performed by the Nonidet P-40 lysis of cells as described previously (25). The nuclei were immediately subjected to transcription assays (2), and the RNA was isolated as described previously (26). Hybridization to immobilized plasmid DNA (1 μg; see "DNA probes") was performed as described above (see "Analysis of RNA expression").

Reporter constructs. To construct pM1.6 and pM1.6-r, a 1,680-bp *Hin*dIII-*Dra*III fragment of pλ4.2-HB5 (see "Genomic subclones and sequencing") which represents the genomic cMGF sequence from positions −1615 to +65 (see Fig. 3) was subcloned into *Hin*dIII-*Hin*cII sites of Bluescript, reisolated after *Hin*dIII-*Kpn*I restriction, and inserted into *Hin*dIII-*Kpn*I sites of pXP2 (pM1.6) or pXP1 (pM1.6-r) luciferase reporter vectors (38). The herpes simplex virus thymidine kinase gene (HSV-tk) promoter constructs pT81 and pT109 are described as pT81luc and pT109luc by Nordeen (38). pT81 contains one GC box and pT109 contains two GC boxes and two CCAAT boxes. pRSV is described in reference 18. The β-galactosidase expression vector carries a Rous sarcoma virus long terminal repeat as promoter/enhancer and is described in reference 12.

Transient expression assays. DNA was transfected into hematopoietic cells by a DEAE-dextran procedure (4), modified as described previously (41). DEAE was used at concentrations of 0.2 mg/ml for HD3, MSB-1, and RPL-12 and 0.4 mg/ml for CRmyb-HER. The DNA concentration was 2 μg/ml (HD11, MH2OB, MH2, HD3, CEF, and myc-HER) or 5 μg/ml (CRmyb-HER, MSB-1, and RPL-12), one-fifth of the total being the β-galactosidase reporter construct. Following transfection, the cells were seeded in triplicates at 1×10^6 (HD11), 2×10^6 (MH2 and myc-HER), or 4×10^6 (MH2OB) cells in 4 ml of medium (5-cm culture dishes). Myeloblasts, erythroblasts, and lymphoblasts were seeded in 24-well dishes (1 ml per well) at concentrations of 2×10^6 to 4×10^6 cells per ml. If required, TPA (200 nM) or LPS (5 μg/ml) was added after 1 day and 14 to 16 h prior to harvest. CRmyb-HER- and myc-HER-transformed cells were washed twice with serum-free medium 20 h before transfection and replated in growth medium supplemented with recombinant cMGF instead of EGF in order to silence the EGF receptor kinase. Following transfection, cells were grown with cMGF and, where indicated, with murine EGF (40 ng/ml) (Sigma).

Cells were harvested approximately 40 to 48 h after transfection, resuspended in 100 μl of 0.1 M potassium phosphate buffer (pH 7.8), and lysed by three freeze-thaw cycles. After the extract was cleared by centrifugation, 30 μl of each extract was assayed for luciferase activity as described in reference 18. The light units used correspond to relative light units (RLU) as determined with a Lumat lb 9501 luminometer (Berthold) with a 10-s integration time. RLU were obtained after subtraction of background reading and correction for β-galactosidase activity. β-Galactosidase activity was assayed with *o*-nitrophenyl-β-D-galactopyranoside as substrate as described in reference 27. β-Galactosidase activity assayed in each sample of cell extract was used to correct luciferase data for variations in transfection efficiencies within experiments. The protein content of extracts was determined with the Bio-Rad Protein Assay, using chicken lysozyme as a standard.

The constructs pM1.6 and pT109 were transfected in parallel. The transfection protocols (see above) and transfection efficiencies for the different cell types vary. The numerical data (light units per 30-μl cell extract) for the experiments described in Fig. 6 are as follows (pM1.6/pT109): CEF38, 246 ± 53/490 ± 33; HD3, 377 ± 62/2,217 ± 334; MSB-I, 0 ± 78/1,950 ± 226; RPL-12, 3,831 ± 694/60,786 ± 2,273; MH2OB, 349 ± 36/37 ± 14; MH2, 58,594 ± 1,484/427 ± 62; CRmyb-HER + EGF, 5,898 ± 803/513 ± 102; myc-HER + EGF, 10,521 ± 552/30 ± 2.

Computer applications. Compilation and computer-aided analysis of sequence data was performed by use of the University of Wisconsin Genetics Computer Group Package of computer programs (17). "Compare/Dotplot" and "Bestfit/Gap" were applied by using default parameters.

Nucleotide sequence accession number. The sequence reported in this paper has been entered in the GenBank data base and assigned accession number M85034.

RESULTS

The cMGF gene is transcriptionally regulated. To assess at what level cMGF expression can be regulated, we studied the kinetics of cMGF expression following LPS treatment of the v-*myc*-transformed macrophage cell line HD11 (11). We had previously shown that LPS treatment rapidly induces secretion of cMGF activity into the cell culture medium. This activity was not released from intracellular stores but was newly synthesized following LPS stimulation (31). Figure 1 shows that cMGF mRNA expression was induced by LPS treatment, reached a maximum after approximately 90 min, and remained high for at least 6 h. The immediate early gene transcripts of c-*jun* and c-*fos*, used as positive controls, were similarly induced. However, in contrast to what was observed with cMGF, their mRNA levels dropped shortly after stimulation. The level of transcript of the housekeeping glycerin-aldehyde-phosphate-dehydrogenase (GAPDH) gene remained constant throughout the experiment. The tumor-promoting phorbol ester TPA also induced cMGF as well as c-*jun* and c-*fos* mRNAs in HD11 macrophages. In contrast, however, TPA failed to induce cMGF mRNA in v-*rel*-transformed lymphoid cells (REV-NPB4), v-*erbA*/*erbB*-transformed erythroid cells (HD3), and v-*myb*-*ets*-transformed myeloblasts (E26), although it activated c-*jun* and c-*fos* expression in these cells. By using a temperature-sensitive mutant of the E26 virus, it was previously shown that E26 myeloblasts become competent to induction of cMGF expression during differentiation to macrophages (9). Thus,

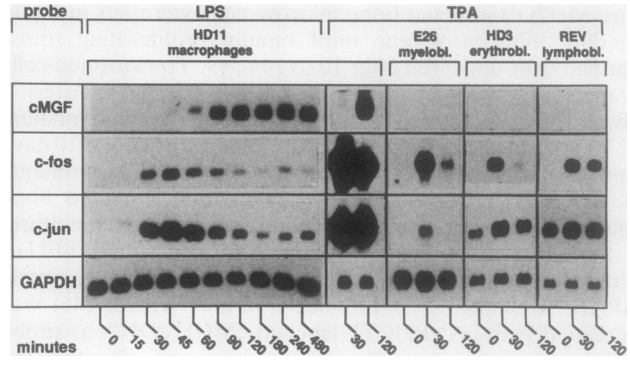


FIG. 1. Expression of cMGF mRNA. The kinetics of induction of cMGF mRNA and comparison of gene expression after TPA treatment in different hematopoietic lineages are shown. Exponentially growing cell lines of macrophages (HD11), erythroblasts (HD3), and *preB preT* lymphoblasts (REV-NPB4) or primary, E26 transformed myeloblasts were plated in aliquots and grown overnight. LPS (100 ng/ml) or TPA (100 nM) was added, and cells were harvested at the indicated times. Polyadenylated RNA was prepared, and 2.5 μg was subjected to Northern blot (RNA) analysis and hybridized to the indicated ^{32}P-labelled DNA probes.

induction of cMGF mRNA expression seems restricted to more mature myelomonocytic cells.

To determine whether the accumulation of cMGF transcripts in LPS-treated HD11 macrophages occurs through transcriptional activation of the cMGF gene, we performed nuclear run-on experiments. As shown in Fig. 2, transcription of the cMGF gene was induced 15 to 30 min after the addition of LPS, and the transcription rate remained constant for at least 2 h. c-*jun* transcription, recorded as a positive control, was also rapidly induced upon LPS stimulation but faded after 30 min. In nuclear run-on experiments using TPA instead of LPS to induce cMGF expression in HD11 macrophages, we obtained similar results (data not shown). Thus, our data show that after stimulation of macrophages by LPS or TPA, cMGF expression is upregulated at the transcriptional level.

Molecular cloning and structure of the cMGF gene. To

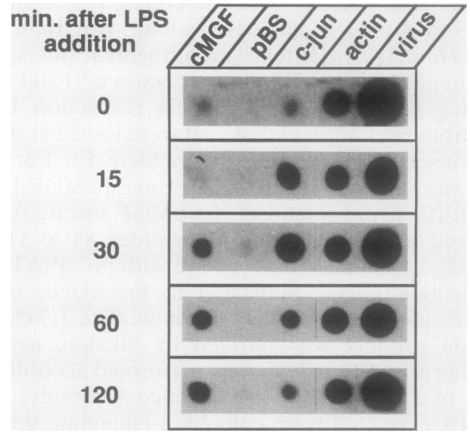


FIG. 2. Nuclear run-on analysis. Nuclei were prepared from unstimulated HD11 cells and HD11 cells treated with LPS (100 ng/ml) for the indicated periods of time. Run-on reactions were performed, and RNA was extracted and hybridized to the indicated probes.

determine the structure of the cMGF gene and to identify *cis*-regulatory sequences responsible for its transcriptional activation, we screened a chicken genomic phage library by using the cMGF cDNA as a probe (see Materials and Methods). The structure of the cMGF gene is schematically shown in Fig. 3A. The sequence from positions −1615 to +2114 is shown in Fig. 3B.

The cMGF gene consists of five exons and four introns, determined by comparing the genomic sequence with the sequence of the cMGF cDNA (32) and the consensus for splice sites (36). Unlike the cap site (see below), the termination site for cMGF transcripts was not determined. Thus, we cannot rule out the possibility of further exons located downstream of the coding region. The upstream sequence and the introns are remarkably GC rich, on average 67 and 73%, respectively, compared with 69% for the coding parts and 44% for the chicken genome in toto (42). Comparison of the cMGF cDNA (32) with the genomic sequence revealed two nucleotide exchanges within the coding region, both of which remain silent. Five and 26 nucleotide exchanges were found in the 5′ leader and the 3′ untranslated region, respectively. These differences could be explained by the different origins of the libraries used, considering that the constraint of sequence conservation is lower in noncoding than in coding regions. Since the cluster of exchanges in the 5′ leader corresponds to the 5′-most nucleotides of the cDNA, they might represent artifacts created by the reverse transcriptase during cDNA preparation (32).

To determine the initiation site(s) for cMGF gene transcription, we performed nuclease S1 (Fig. 4) as well as primer-extension analyses (data not shown). We found that the cMGF mRNA was transcribed from a unique cap site 66 nucleotides upstream of the translation initiation site. Thus, the TATAAA box located 23 nucleotides upstream of the transcription start site of the cMGF gene most likely represents the core promoter (13).

The cMGF promoter drives myeloid-specific, kinase-inducible reporter expression. To investigate whether the DNA sequence upstream of the coding region regulates cMGF gene expression, we inserted a fragment comprising positions −1615 to +65 of the cMGF gene in either orientation into a luciferase reporter vector (pXP). These constructs, termed pM1.6 and pM1.6-r, were transfected into the macrophage cell line HD11. For controls, we transfected luciferase reporter constructs carrying a minimal promoter derived from the HSV-tk gene (pT81) or a promoter from the long terminal repeat of Rous sarcoma virus (pRSV).

Figure 5 shows that pM1.6, harboring the cMGF genomic fragment in its original orientation, gave low basal luciferase expression which was strongly induced by TPA or LPS. In contrast, even in the presence of inducing agents, luciferase expression from pM1.6-r remained low. Expression from the control vectors pXP2 and pT81 was not detectable and close to background levels, respectively. Luciferase activity obtained with pRSV was very high (approximately 3.2×10^6 RLU) and two- to threefold inducible by TPA or LPS (data not shown). These data show that the 1,680-bp sequence of the cMGF gene harbors a directional promoter which contains *cis*-acting elements mediating TPA- or LPS-induced gene expression.

To examine whether the promoter activity of the −1615 to +65 fragment exhibits lineage specificity, we transfected pM1.6 into different cell types. To compare the reporter activities obtained in the different cells, we determined the ratio of luciferase expression from the cMGF promoter (pM1.6) to that from an HSV-tk promoter (pT109). Figure 6A shows that pM1.6 was significantly active in primary transformed macrophages (MH2OB and MH2) and that luciferase activity was strongly enhanced in macrophages expressing the v-Mil serine/threonine kinase (MH2). In fibroblasts (CEF38), B-lymphoblasts (RPL-12), T-lymphoblasts (MSB-1), and erythroblasts (HD3), expression from pM1.6 was low compared with expression from pT109. It is important to note here that HD3 erythroblasts express the v-ErbB kinase which in transformed myeloid cells induces expression of cMGF (34). These data show that the cMGF promoter is active in macrophages and that reporter expression is high in myeloid but not erythroid cells expressing a constitutively active kinase.

In order to further analyze the effect of protein kinase activity on cMGF promoter activation in myeloid cells, we transformed bone marrow cells with recombinant retroviruses which carry an inducible tyrosine kinase. We generated v-*myb*-transformed myeloblasts (CRmyb-HER) or v-*myc*-transformed macrophages (myc-HER), both of which express the human EGF receptor kinase. CRmyb-HER transformed cells were previously shown to express cMGF when supplied with EGF (34). Figure 6B shows that in both cell types, the addition of EGF strongly induced reporter expression from the cMGF promoter/enhancer construct whereas the activity of the HSV-tk promoter remained unchanged. In v-*myc*- or v-*myb*-transformed control cells, which do not express the human EGF receptor, EGF treatment had no effect on pM1.6 activity (data not shown). Taken together, our data show that the 1,680-bp cMGF promoter mediates myeloid-specific expression which is inducible by activated serine/threonine as well as by tyrosine kinases.

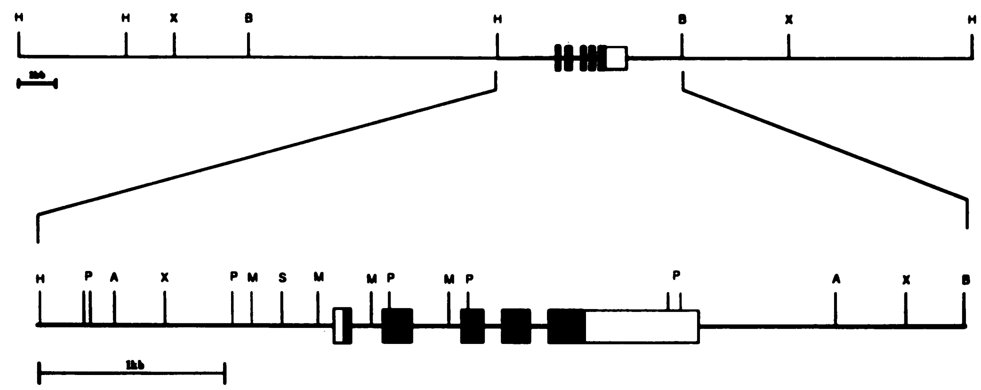
DISCUSSION

The molecular cloning of the cMGF gene revealed that intervening sequences are inserted at codon positions homologous to those of the G-CSF and IL-6 genes (32, 43, 45). This result confirms cMGF as a new member of the family of structurally related cytokines (5). Interestingly, between species the most conserved sequences in either the G-CSF or IL-6 genes are in the promoter regions (43, 45). However, no homology could be identified between the cMGF and G-CSF or IL-6 promoters. Also, in contrast to G-CSF and IL-6 (45, 49), cMGF is transcribed from a single initiation site rather than from multiple initiation sites.

Cytokines and hematopoietic growth factors are usually not constitutively expressed but are activated in response to external stimuli. Different control mechanisms have been found, depending on the cytokine, cell type, and stimulating agent studied (3, 44). Our experiments suggest a predominant role of transcriptional control in the regulation of cMGF expression. The activation of cMGF transcription by LPS or TPA does not require new protein synthesis (data not shown). This suggests that in myelomonocytic cells, activated kinases modify a preformed regulatory factor(s) which then initiates transcription of the cMGF gene.

Expression of cytokines and oncogenes are often regulated at both the transcriptional and the posttranscriptional levels (39). Since the 3′ untranslated region of cMGF transcripts harbors eight copies of the $(ATTTA)_n$ motif (32) which has been implicated in the regulation of mRNA stability (15), we have examined the significance of the cMGF 3′-tail in preliminary experiments. We have, however, failed to detect an important role of posttranscriptional control in cMGF expression. Thus, we conclude that tran-

A.



B.

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-1376  AGCTGCTGTCGGCCTCCGGGGAAATCTGGAATCTCCCGGCACGCCCGTGCTGGGCTGGAGGCCAGGGGCTGTGCCTGCAGCACGGGGCAGCCCCAGGGCATGGATACGGCCCCATCCC -1257
-1256  TGCTCCAGGAACACGGTGGGGCCCCTGGGACAGGACGGGGCTGTGAGCCCGGCCGGCACCTCCAGGGACGCGGGGACACCCCGGAAGCGGTGACTTCCTGGCAGATGCTGGGAGGGGGCT -1137
-1136  GAGGGTCAGCAGAGGAAGAGCGGCCACGAGGCAGAACCGCAGCGCATTGCGCTGACCCCGCACTGCCACCCACGGGGTTTCGGGTCCGGCTGCCACTTCCTTCCTTGGAAGCACAGCAGG -1017
-1016  GGCTTTGGGCTCTGTGGGTGCTGATTCAACCCCACCCTGAACCCAATTCCTGCCGGCACTGACCTGCTGAGGCAGCAGCGCTGCTCGAGGAACGGCCGTCCCCCAATGCCTGCTGTGCTG - 897
- 896  AGGAATGGCCCCGACTGCTCCCTGCAGAGCAGAGACAGAGCTGGAAGGATTTCTCCCAGGAAACCCCACGGATGGGCACACAGCACGAGGTCCCTTCAGGCAGCCGTAGGCAGTGCCTTT - 777
- 776  ATGCTGGTGGGGTCTGTCATCATTAGGCTTCAGGGCGAACACCTGCGTTGTGCTGAGTGAGGCCTCCTGTGCTCACAGGTGGGCAGCGCCACCCGGCTGCTTCCCCTGCTGGGGCTCA - 657
- 656  GGGGGGTAATCCCGTGACCGTACCCCGCTCTGTGCCCACCTGGGCAGCTCAGGGTCCCGGTGCCCGGCAGCGGCCGGCCCAGCTGGCGTCGGCCCATGAGATCCTCTTGGAAGCACTCAT - 537
- 536  TGGAACTGGCCCCAACCCAAAGCCCACGTCACTACGGGGAGGCCGGGATCAGCCAACCCCCCCGGGGCTGCCCCTGGGCGCCGCTTCCCCGAAGGGGCCGGCGGGGAGGGTTCACGTGGG - 417
- 416  GGGGCCGGGCACGGCGGTGCCAAGTGCTGGGTGCTGGCAGCGGCCGCAGGGCTGGTGGTCACCTGGGTCCCATGGCCGTGGTGGTCCCACAGCCACACCGACACCGCGGTGAGGGGCTCC - 297
- 296  GCGAGGGCTGAGAGCCGCCGCCACCGTCCCCACTGCACAGCGGGTTAATGAGTGACCAACCTGACCGGGCACGGGCGCAGCACCGGCACTGCCCACTGCCAGCATTCGGCCGGGCAGCGC - 177
- 176  TGTGCTTCGGTGGCACTGCAGCACCAGGAAAGCCCCATAGCAGCGCAGCACAGGAGCACCCAGCGCCCGGGATTTGCATGCGGGTGATTAAGACAATGAGGCAATCGGCACTGTTGCCAC -  57
                                                              +1           cgg      g  -
-  56  ATCGGTGTGAATCCCCACAGCCCTCTATAAAATGCCCCGATCGATGAGGGCGCGCAGAGCGGGGCAGGGCACTGTGCCGGGCCGGGCTGTGCTGCAGGAGGAGACACAGAGCTGTACACC    63
                                                                           *

   64  ATGTGCTGCCTCACCCGTGAGTACCGCAGACCCATGGGTGGCACCGGGGGGCACGTGGGGCTGCAGCCCTTCCGCCCTCCCCGCATCTCCTCCGTCCCCTCCCCGTGCTCCTTTGGGACC   183
       MetCysCysLeuThrP

  184  CCATCCCCGGGGGGGTCCCCGGAGCACCGTTGGGGTGACCCGGCGTCGGGGCCGTGTGTGACCCGCTGTCCTGCAGCTGTGCTGGCGCTGGCGCTGGTGCTGGGCGCTCCCTGGCAGGCG   303
                                                                                           roValLeuAlaLeuAlaLeuValLeuGlyAlaProTrpGlnAla

  304  CTGCATGGGGCTCCGCTGGCCGAGCTCTCGGGGGACCACGACTTCCAGCTCTTCCTGCACAAGAACCTGGAGTTCACCCGCAAGATCAGGGGGGACGTGGCCGCCCTGCAGCGCGCCGTG   423
       LeuHisGlyAlaProLeuAlaGluLeuSerGlyAspHisAspPheGlnLeuPheLeuHisLysAsnLeuGluPheThrArgLysIleArgGlyAspValAlaAlaLeuGlnArgAlaVal

  424  GTGAGTGCCCCGATAACGCCACCTTCCCCTCCCAGCCTTATCGGCACCGCGGGGCCGTGGCCGGCGGCCTCCCCGCACCCCAGACATCATCCCTGGACTGCTCGGTCCTCCCCACGCTGT   543
  544  GCTCCATCCCCGCTCCATGCGGTGAGCAGCGGGTCGTTGTCCTGCGCTCCTGCAGACGGTGGGGGGGGTCCCGCATCCCCCGGGGTGTCGCAGGGCTATGGCCGTCTCACGGGGCGTCG   663
                                             t                                g
  664  GGCTCTGCAGCTCTGACCGTGTGCCCTCTCCCGCAGTGCGACACCTTCCAGCTGTGCACAGAGGAAGAGCTTCAGCTGGTGCAGCCGGACCCCCACCTCGTGCAGGCGCCGCTGGATCAG   783
                                      -.   CysAspThrPheGlnLeuCysThrGluGluGluLeuGlnLeuValGlnProAspProHisLeuValGlnAlaProLeuAspGln

  784  TGCCACAAGCGCGGCTTCCAGGCGGTGAGTGCGGGCACCCACCCGTGACACGAGCTGCCCGGCCTCAGCCCGGCTGGGGCTCCGAGCACCGAACCCCGATGCCGTTCCCTCGCAGGAGGT   903
       CysHisLysArgGlyPheGlnAla                                                                                            GluVa

  904  GTGCTTCACTCAGATCCGCGCCGGGCTGCACGCGTACCACGACTCGCTGGGCGCCGTCCTGCGGCTGCTGCCCAATCACACGACGTTGGTGGAGACGCTGCAGTTGGACGCCGCCAACCT  1023
       lCysPheThrGlnIleArgAlaGlyLeuHisAlaTyrHisAspSerLeuGlyAlaValLeuArgLeuLeuProAsnHisThrThrLeuValGluThrLeuGlnLeuAspAlaAlaAsnLe

 1024  CTCCTCCAACATCCAGCAGCAGGTGAGGAGGGGCGGCACACGGGGGGGGGGGGGGTTGGGGGCAGCCCGGACCCCACGGTGACACCGCCGCTCTGTGCCGCAGATGGAGGACCTGGGCC  1143
       uSerSerAsnIleGlnGlnGln                                                                                   MetGluAspLeuGlyL

 1144  TGGACACGGTGACGCTCCCGGCCGAGCAGCGCAGCCCCCCGCCCACCTTCTCCGGCCCCTTCCAGCAGCAGGTGGGGGGATTCTTCATCCTGGCCAACTTCCAGCGCTTCCTGGAGACGG  1263
       euAspThrValThrLeuProAlaGluGlnArgSerProProProThrPheSerGlyProPheGlnGlnGlnValGlyGlyPhePheIleLeuAlaAsnPheGlnArgPheLeuGluThrA
                                                            c
 1264  CGTACCGCGCGCTGCGGCACCTCGCCCGCCTCTGACGCCCCCCCACCCCCCC-ACCCCTCCCCGCTTATTTAATATTTATGGCTATTTAATATTTATCCGGCCGGGGGGGTCCCGCTGCC  1383
       laTyrArgAlaLeuArgHisLeuAlaArgLeu
                                             g                                                                           -   c-
 1384  CCCCCGCCCCCACGCAGGGTGCCGGCCCTGCGCTGCCATCCGTGCACGGGTCTCTGTGCAGCACCAGCCATGTTTAATATTTAAACACAAGCGTATCTCTGCTACGAGGTTTGCAGCCGG  1503
       -       gccgc     -     -                                                           - cg         c
 1504  GCTGAGCTCGGCGACCCCATGGGGAACCCCCACCCGCACCGAGGATGCTCCGAGCAGCACCGGGTGGGTGCGGGGATGGAGCTGCGGCGCTCCGTCTGGGATCGCTTCCAGGAGCGGTAT  1623
                       c           c  ---      -                -     -    a
 1624  TTATTCGCTGCTGGGTAAGGAGGGAGGGGGGCGCAGCCCACGATGGGGTCCCACGGTTGGGGCCGAGGCTCCGCTCTGCCCACGCTGAGCTCCTATTTATTGCAGCGTAACTTATTTGTG  1743
 1744  CCCCCACGCCTCCGGTGGTGTCTCAGAAACTCACAGCTGCTCCGGTCCTCCGGGCGGCGTTTTGTACTGTATTTATTCGTGCAGCTGGGTCAGCCCCACAGCAGCGCGCGCTGGTTTTTA  1863
 1864  TATAAGTCACAACGATGGAAATAAACTCTATTTTGCCTCCAACATTTGTGCTGTGTGCGCTTCCATGGGATGGGGCTGCCGTGGGGGGGGGGGGGGGCTGCAGCTCTGGGATGGCCACCG  1983
                                   *

 1984  GGACCCCCAGACGGCACAGAACCCCCGAGCGCAGCGGGGGTGGGGATGTGGGGATGCCGACACGGAGGGCTGCGCGATCTCGGCGTGGCAGTGGGGAGGGGACGCGTGGGGTGCAGGATG  2103
 2104  TGATCCGGGG
```

FIG. 3. Genomic structure and sequence of cMGF. (A) Schematic representation of the cMGF locus as determined by restriction analysis of three λ clones as well as by DNA sequencing. Exons are indicated as boxes; the noncoding regions within them are shown as open boxes. The 3′ boundary of exon 5 was not determined precisely. Sites for restriction enzymes are indicated by the following one-letter code: A, *Apa*I; B, *Bam*HI; H, *Hin*dIII; M, *Sma*I; P, *Pvu*II; S, *Sac*II; X, *Xho*I. (B) Nucleotide sequence of the cMGF gene and translation of the coding regions. The sequence was determined from the insert of one of three independent genomic clones. Nucleotides are numbered starting with +1 at the adenosine of the cap site (see Fig. 3). Asterisks (below the sequence) mark the nucleotides equivalent to the first (position +14) and last (position +1891) nucleotides of the cDNA clone (32). Nucleotides of the cDNA clone which differ from those of the genomic sequence are given in small letters above the genomic sequence (a dash denotes a missing nucleotide). The TATA box and polyadenylation signal are underscored.

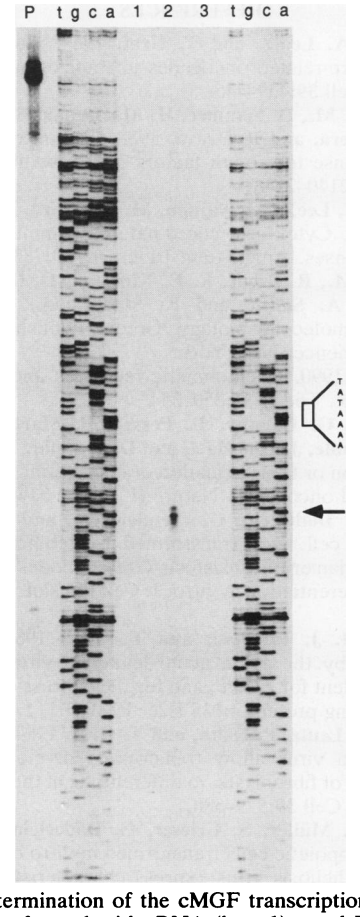

FIG. 4. Determination of the cMGF transcription start site. S1 analysis was performed with tRNA (lane 1), total RNA extracted from HD11 cells stimulated for 2 h with LPS (5 μg/ml) (lane 2), or RNA from unstimulated HD11 cells (lane 3). As a protecting probe, a 1,745-bp fragment comprising 1,680 bp upstream of the start codon and a 65-bp coding region derived from pMGF-F (P) (see Materials and Methods) was used. Samples corresponding to 8 μg of RNA were loaded on the gel. a, c, t, and g, dideoxy-sequencing products of pMGF-F, generated by using dITP nucleotide mix and the same primer as that used for the generation of the protecting probe. The blank box indicates the location of the TATA box, and the arrow marks the adenosine nucleotide of the cap site (assigned position +1 in the genomic sequence) (Fig. 2B).

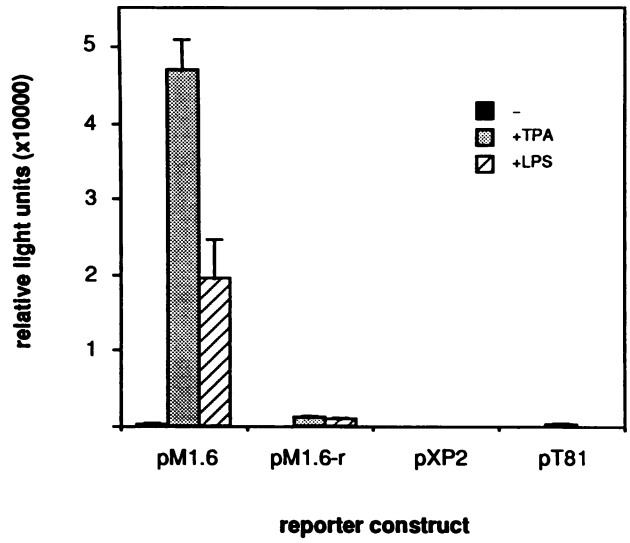

FIG. 5. A 1,680-bp cMGF genomic fragment contains a directional and inducible promoter. HD11 macrophages were transfected with the indicated luciferase reporter constructs (see text). An aliquot of the transfected cells was treated with TPA (200 nM; stippled bar) or LPS (5 μg/ml; hatched bar) for 12 to 14 h before extracts were prepared. The mean RLU, obtained from triplicate determinations under standard assay conditions, are shown (see Materials and Methods).

scriptional activation of cMGF expression is the crucial mechanism by which transformed myeloid cells become factor independent.

We found that expression from a cMGF promoter-reporter construct in different hematopoietic cell types parallels the activity of the endogenous cMGF gene. cMGF expression and promoter activity are induced in myelomonocytic cells by oncogenes coding for serine/threonine or tyrosine kinases and in macrophages after activation by LPS or TPA. The human EGF receptor kinase activates the cMGF promoter in myelomonocytic cells of two different differentiation stages (myeloblasts and macrophages). Upon removal of EGF from the medium of CRmyb-HER-transformed cells, cMGF promoter activity and expression cease, showing that continuous kinase activity is required for cMGF expression. This rules out the possibility that the kinase merely establishes a persistent phenotype constitutively expressing cMGF and suggests that a short-lived component is involved in the regulation of the cMGF promoter. Reporter expression from the cMGF promoter-reporter construct perfectly parallels the results obtained earlier in which we showed that cMGF activity (determined in a biological assay) can be induced by activation of conditional tyrosine (1) and serine/threonine kinases (47) or by stimulation of the human EGF receptor kinase (34).

However, when we tested the cMGF promoter/enhancer reporter construct in AEV-transformed erythroid cells which express the v-ErbB kinase, there was no significant expression. On the other hand, the v-ErbB kinase, a constitutively active mutated EGF receptor, does induce cMGF expression in myeloid cells (34). Similarly, TPA treatment of erythroid or lymphoid cells fails to induce the cMGF promoter (data not shown). The fact that kinases are primary transforming oncogenes in the erythroid lineage (28, 29) and that TPA does activate c-*fos* and c-*jun* in erythroid and lymphoid cells rules out the trivial explanation that kinase-inducible signalling pathways may be impaired in chicken erythroid or lymphoid cells. Instead, our data suggest that myeloid-specific cellular components are required for activation of the cMGF promoter. Since we examined only transformed myeloid cells, the nuclear oncogenes v-*myc* and v-*myb* appear at first sight to be candidates for such a component(s). However, this appears unlikely, since in myc-HER-transformed fibroblasts the cMGF promoter is not significantly induced by activation of the receptor kinase (data not shown). Furthermore, in myeloblasts transformed by the E26 v-*myb-ets* oncogene, cMGF expression is not induced by either LPS or TPA (data not shown).

The finding that the cMGF promoter exhibits lineage specificity and kinase inducibility in transient expression analysis should enable us to identify important *cis*-regulatory elements in the cMGF promoter and the factors involved in regulation of cMGF expression. In another study (41), we report that three proximal elements which bind AP-1- and

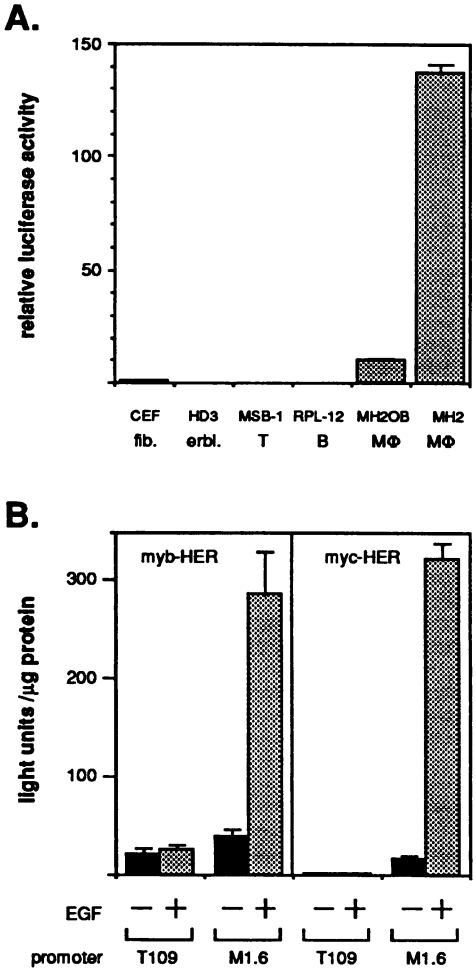

FIG. 6. The 1,680-bp cMGF promoter is myeloid specific and inducible by kinases. Transient expression from reporter constructs pM1.6 and pT109 are shown (see text). Means and standard deviations of triplicate determinations are shown. (A) Activity of pM1.6 in different chicken cell types, normalized to the activity obtained with pT109, is shown. fib, fibroblasts; erbl, erythroid cells; T, T-lymphoid cells; B, B-lymphoid cells; MΦ, macrophages. (B) CRmyb-HER and myc-HER transformed cells were transfected and seeded in the absence (solid bar) or presence (stippled bar) of murine EGF. The scale indicates RLU per microgram of protein of cell extracts (see Materials and Methods for experimental details and numerical data).

C/EBP-like factors appear to be crucial for myelomonocytic-specific and kinase-inducible reporter expression. We expect that understanding the regulation of the cMGF promoter will help in understanding the molecular mechanisms underlying lineage-specific oncogene cooperation.

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