The EMBO Journal vol.8 no.1 pp.175-181, 1989

Molecular cloning of the chicken myelomonocytic growth factor (cMGF) reveals relationship to interleukin 6 and granulocyte colony stimulating factor

Achim Leutz, Klaus Damm[1], Esta Sterneck, Elisabeth Kowenz[2], Scott Ness, Rainer Frank, Heinrich Gausepohl, Yu-Ching E.Pan[3], John Smart[3], Michael Hayman[4] and Thomas Graf

European Molecular Biology Laboratory, 6900 Heidelberg, FRG, [1]The Salk Institute, La Jolla, California, USA, [2]German Cancer Research Center, 6900 Heidelberg, FRG, [3]Hoffman-La Roche Inc. Roche Research Center, Nutley, New Jersey and [4]The State University of New York at Stony Brook, Stony Brook, New York, USA

Communicated by T.Graf

Normal as well as retrovirally transformed avian myeloid precursor cells require the colony stimulating factor cMGF for their survival, proliferation and colony formation *in vitro*. cMGF has been shown to be a glycoprotein which is active in the picomolar concentration range. Co-expression of kinase type oncogenes in v-*myb* or v-*myc* transformed myeloid cells induces cMGF expression and confers factor independence via an autocrine mechanism. Here we describe the molecular cloning of cMGF from a myeloblast cDNA library and show that it is a 201 amino acid residue secretory protein which is modified by signal peptide cleavage and glycosylation during translocation into the lumen of membrane vesicles. A bacterially expressed trpE-cMGF fusion protein induces proliferation of E26 transformed myeloblasts in a cMGF bioassay suggesting that glycosylation is not absolutely necessary for biological activity. Sequence comparison reveals that cMGF is distantly related to G-CSF and IL-6.

Key words: cloning/colony stimulating factor/interleukins/myelomonocytic growth factor/oncogenes

Introduction

It is now well established that genes involved in growth control can be activated to become oncogenes following their deregulation or mutation. Genes of this family, also designated as 'proto-oncogenes', encode growth factors, receptors, signal transducing molecules or factor-induced *trans*-activating proteins. In the organism these genes play important roles in the regulation of embryonal tissue formation, wound repair and cell regeneration.

One of the best models to study growth control genes/proto-oncogenes and their transforming counterparts is the hematopoietic system. Cells of this system are continuously regenerated through a process of self-renewal and differentiation which is mediated by the action of hematopoietic growth factors (for review see Metcalf, 1984). Most hematopoietic growth factors of mice and humans have been identified based on their colony inducing ability of normal hematopoietic progenitor cells *in vitro* and are therefore also known as colony stimulating factors (CSFs).

We recently described an avian hematopoeitic growth factor with the ability to stimulate the growth of v-*myb* or v-*myc*-transformed chicken myeloid cells *in vitro*. This factor which we termed chicken mylomonocytic growth factor (cMGF) also stimulates macrophage and granulocyte colony formation in normal bone marrow cultures (Leutz *et al.*, 1984). cMGF was originally found to be produced by lectin-stimulated spleen cultures (Beug *et al.*, 1982) and later after LPS stimulation of normal or transformed macrophages (Leutz *et al.*, 1984). Interestingly, this factor was also released by v-*myb* or v-*myc*-transformed myeloid cells after superinfection with retroviruses carrying kinase-type oncogenes such as v-*src*, v-*erb*B, v-*fps*, v-*ros*, v-*yes* or v-*mil*. cMGF production by these cells leads to factor independence based on an autocrine growth mechanism, as could be demonstrated using cMGF specific antibodies (Adkins *et al.*, 1984; Graf *et al.*, 1986). In addition, maintenance of factor production depended on the continuous activity of the kinase oncogene, as shown with viruses containing a temperature sensitive kinase-oncogene (Adkins *et al.*, 1984; von Weizsäcker *et al.*, 1986). Experiments performed with the v-*myc* and v-*mil* containing MH2 leukemia virus indicated that cooperativity between proliferation inducing nuclear oncogenes and cMGF inducing cytoplasmic oncogenes is not restricted to *in vitro* systems: Both the v-*myc* and v-*mil* oncogene of this virus are required for high oncogenicity, presumably because v-*myc* induces myeloid cell proliferation while v-*mil* induces autocrine growth (Graf *et al.*, 1986). As a first step to elucidate how the cMGF gene is activated by kinase-type oncogenes and to determine whether it is related to any of the known CSFs we set out to molecularly clone the gene. Here we describe the isolation of a cMGF cDNA clone and demonstrate that its product expressed in bacteria is biologically active. Sequence comparisons with other genes indicate that cMGF is distantly related to interleukin-6 (IL-6) and granulocyte colony stimulating factor (G-CSF).

Results

Molecular cloning of cMGF by oligonucleotide screening of a chicken cDNA library

cMGF was purified from conditioned medium obtained after LPS stimulation of the macrophage cell line HD11 as described earlier (Leutz *et al.*, 1984). The amino acid sequences of the amino-terminus and of six individual peptides produced by trypsin treatment of the purified material were determined (Table I). Based on part of the sequence, we designed an oligonucleotide hybridization probe with the third base position of each codon replaced by inosine (see Materials and methods). We used the oligonucleotide to screen 1.6×10^5 recombinant clones from a chicken myeloblast cDNA library constructed in lambda phage gt10 and isolated a single positively hybridizing phage designated λFP1. The melting temperature

Table I. Sequences of cMGF peptides

	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Peptide 1:	Ala-Pro-Leu-Ala-Glu-Leu-Ser-Gly-Asp-His-Asp-Phe-Gln-Leu-Phe-Leu-His-Gly-Asn-Leu-Glu-Phe
Peptide 2:	Asn-Leu-Glu-Phe-Thr-Arg
Peptide 3:	Ile-Arg-Gly-Asp-Val-Ala-Ala-Leu-Gln
Peptide 4:	Gly-Phe-Gln-Ala-Glu-Val-xxx-Phe-Thr-Gln-Ile
Peptide 5:	Ala-Gly-Leu-His-Ala-Tyr-His-Asp-Ser-Leu-Gly-Ala-Val
Peptide 6:	Phe-Leu-Glu-Thr-Ala-Tyr-Arg

of the oligonucleotide-phage DNA hybrid was between 50 and 55°C. The recombinant phage DNA carried an *Eco*RI insert of ~1.2 kb which, when subcloned into a Bluescript plasmid, was designated pFP1. Insert DNA was radiolabeled and used as a hybridization probe in Northern blots revealing a 1.4 kb polyadenylated RNA from LPS stimulated HD11 macrophages. No such band was seen in unstimulated macrophages, v-*erb*B transformed erythroblasts or v-*myb* transformed myeloblasts (Figure 1 and data not shown). Southern hybridization to chicken genomic DNA digested with various restriction enzymes indicated that cMGF is encoded by a single copy gene (Figure 1B).

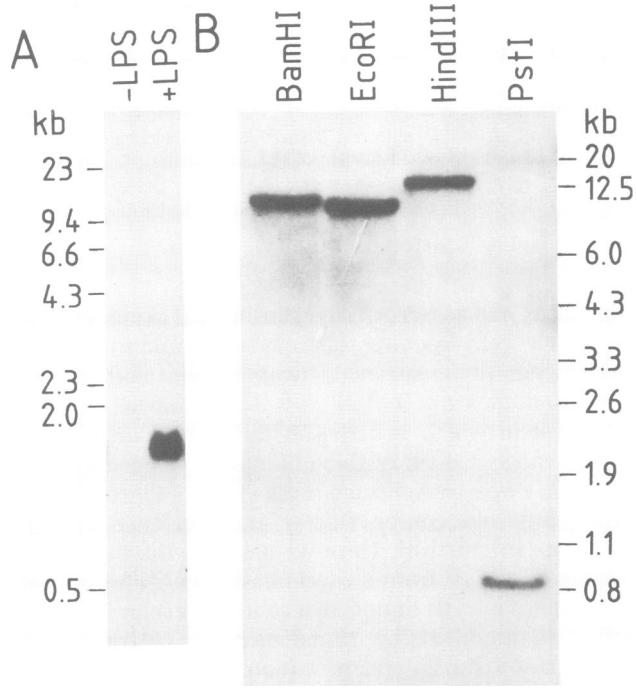

Fig. 1. Hybridization analyses of chicken RNA or DNA using the pFP1 insert as a probe. ^{32}P-radiolabeled cMGF insert was hybridized to fractionated nucleic acids immobilized on nylon membranes. Positions and sizes of DNA marker fragments are indicated. (**A**) Northern blot with polyadenylated RNA isolated from HD11 macrophages without LPS treatment (−LPS) and 3 h after LPS stimulation (+LPS). (**B**) Southern blot with chicken DNA digested with the indicated restriction endonucleases before electrophoresis.

Sequence of cloned cMGF

As shown in Figure 2 nucleotide sequencing of the pFP1 cDNA clone revealed a single long open reading frame which could encode a protein containing all of the six cMGF peptides of known sequence. The only discrepancy was at position 18 of the N-terminal peptide where glycine was determined by amino acid sequencing and lysine by DNA sequencing. Re-evaluation of the HPLC profile of the corresponding amino acid sequencing experiment indicated a minor lysine peak in addition to glycine. Glycine at this position therefore probably represents a protein sequencing artefact. This difference could explain the relatively low melting temperature observed for the oligonucleotide-cMGF clone hybrid.

The cMGF coding region is embedded between a short 5′ and a long 3′ untranslated region. The fact that the cMGF mRNA found by Northern hybridization has a size of 1.4 kb while the phage insert size is 1238 bases indicates that the cDNA clone is almost complete. This conclusion is supported by the finding that a polyadenylation signal was found near the 3′ end of the insert, even though the clone contained no poly(A) tail. The 3′ untranslated region has several remarkable features. (i) The cMGF coding region is followed by a C-rich (nucleotides 657−687), an AT-rich (nucleotides 688−719) and a GC-rich region (nucleotides 720−784). The sequence motif ATTTA occurs four times in the 28 nt long AT rich region and an additional four times before the end of the clone. Interestingly, the first four ATTTA repeats are part of the two larger repeats with the sequence 5′-ATTTA ATATTTAT-3′. Further experiments will be required to determine whether these repeats destabilize the cMGF mRNA as has been suggested with the mRNAs from hematopoietic growth factors, interferons, interleukins, PDGF and growth factor inducible nuclear oncogenes (Shaw and Kamen, 1986).

We have shown previously that cMGF is a secretory glycoprotein which is post-translationally modified in the endoplasmic reticulum and in the Golgi apparatus, that the factor is *N*-glycosylated at two sites and that cMGF is probably synthesized from a precursor containing a signal peptide (Leutz *et al.*, 1988). Indeed the protein encoded by the open reading frame of the cDNA clone contained two *N*-glycosylation sites (amino acid residue 100 and 114; marked by triangles in Figure 2). In addition, a stretch of 23 amino acids upstream of the determined N-terminal peptide appears to function as a signal peptide (van Heijne, 1983) which would be cleaved in front of the Ala in position 1 (Figure 2). This amino acid is followed by Pro, a motif which was found earlier as part of a cleavage signal in several secretory proteins as well as hematopoietic growth factor precursors (Watson, 1984; Schrader *et al.*, 1986). The nucleotide sequence also predicts a methionine residue 23 amino acids upstream of the cleavage site as a likely initiation codon since it is preceded by a Kozak-type consensus sequence (Kozak, 1984). Thus the calculated molecular weight of the cMGF precursor protein would be 22.345 kd and that of the mature protein 20.014 kd, or ~4 kd smaller than that determined by SDS−PAGE (Leutz *et al.*, 1988).

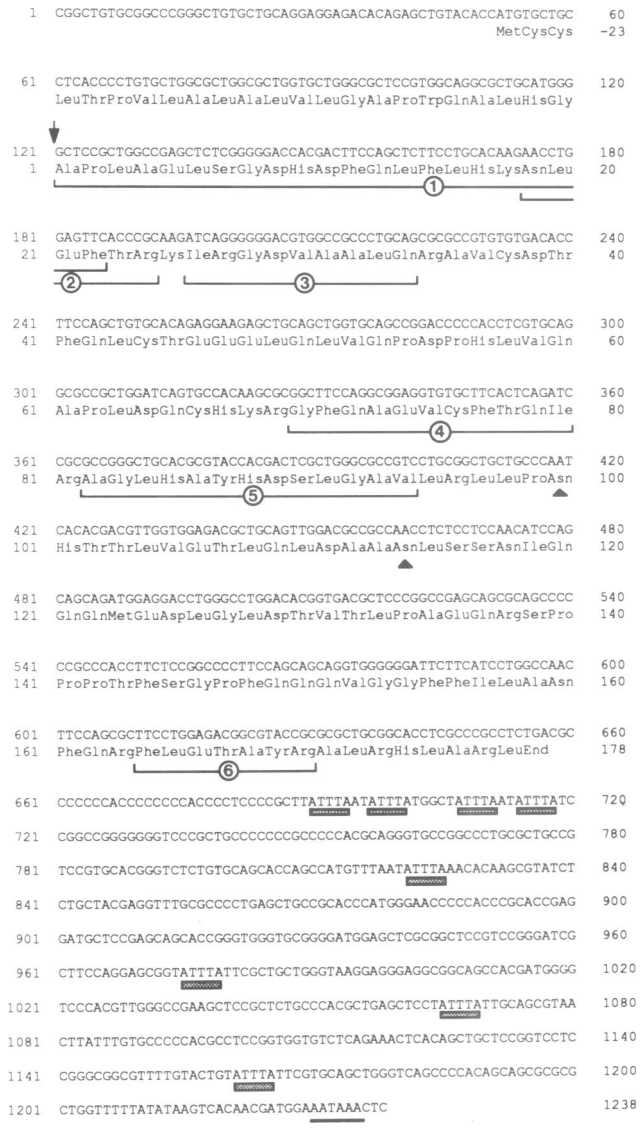

Fig. 2. Nucleotide sequence and predicted amino acid sequence of cMGF cDNA. The start of the mature cMGF protein is designated as amino acid number 1 and the cleavage site of the amino terminal leader peptide is indicated by an arrow. Brackets indicate the sequences corresponding to the peptides shown in Table I. *N*-glycosylation sites are marked by arrowheads. A polyadenylation signal is underlined by a solid bar. AT sequence motifs, which have been implicated in messenger destabilization, are underlined by stippled bars.

In vitro translation of cloned cMGF

To determine whether molecularly cloned cMGF is indeed translated into a precursor that can be cleaved into the mature factor, RNA was first transcribed *in vitro* from the cMGF insert and then subjected to *in vitro* translation. This was done in the presence and absence of canine pancreas membranes along with polyadenylated RNA from LPS stimulated HD11 macrophages as a control. The *in vitro* translated products were immunoprecipitated by antibodies raised against purified HD11 derived cMGF and then analyzed by SDS−PAGE. As shown in Figure 3, essentially identical results were obtained with the two types of RNA. In the absence of membranes, *in vitro* translated proteins from either RNA had a M_r of ~26.5 kd, that is, 2.5 kd larger than the smallest cMGF precursor detectable after

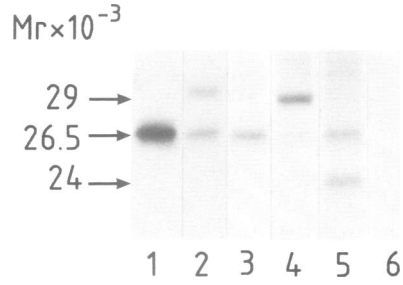

Fig. 3. *In vitro* translation of cMGF RNA templates. *In vitro* transcribed RNA from the pFP1 insert (lanes 1 and 2) or polyadenylated RNA from LPS stimulated HD11 macrophages (lanes 3−5) were subjected to *in vitro* translation without (lanes 1, 3 and 6) and with microsomal membranes (lanes 2, 4 and 5) and immunoprecipitated with cMGF antiserum (lanes 1−5) or preimmune serum (lane 6). Immunoprecipitated products applied to lane 5 were treated with endoglycosidase F before electrophoresis.

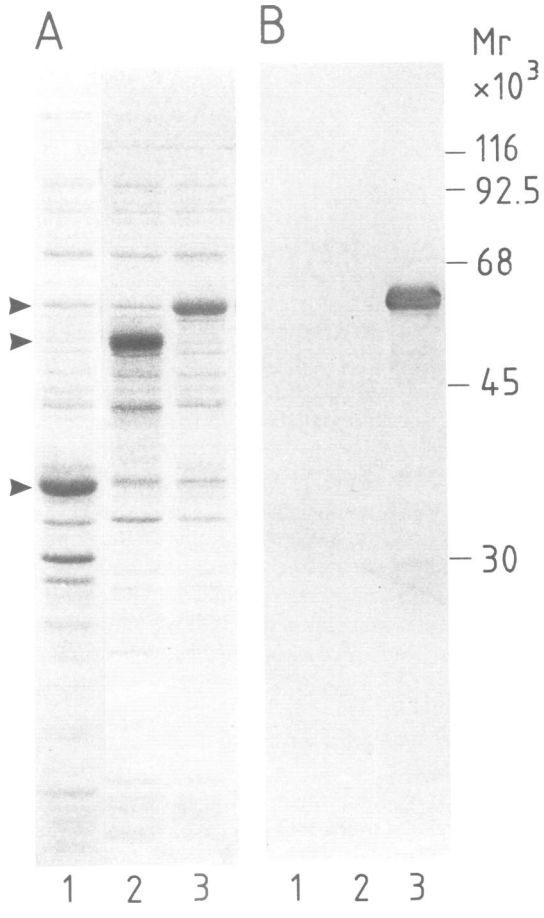

Fig. 4. Bacterial expression and antigenicity of rcMGF. Proteins from total bacterial lysates were separated by SDS−PAGE and stained with Coomassie Blue (**A**) or with cMGF antibodies in a Western blot procedure (**B**). Arrowheads indicate positions of the trpE protein or fusion proteins. Positions of mol. wt markers are indicated on the right. **Lane 1**, pATH1 lysate; **lane 2**, pATH1-12 lysate; **lane 3**, pATH3-3 lysate.

pulse labeling of HD11 cells (Leutz *et al.*, 1988). In the presence of membranes the M_r of the translation product was 28 kd, indicating that the protein is translocated through the membrane and modified by glycosylation. Endoglycosidase F treatment of immunoprecipitates obtained from HD11 RNA translated in the presence of membranes resulted

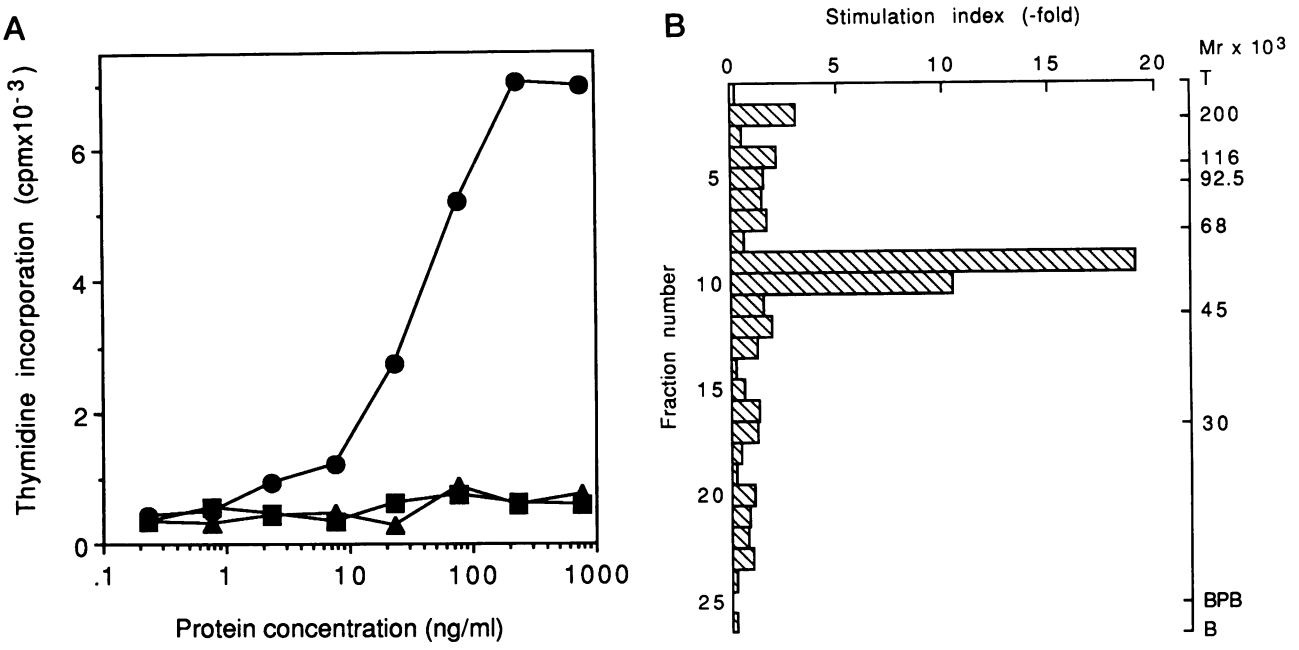

Fig. 5. Biological activity of recombinant cMGF. (**A**) Bacteria expressing proteins from various pATH plasmids were lysed and tested for biological activity in the cMGF bioassay on chicken E26 transformed myeloblasts. Stimulation of DNA synthesis determined by tritiated thymidine incorporation was plotted against the lysate−protein concentration. Recombinant cMGF (●, pATH 3-3), plasmid control (▲, pATH 1), frame shift control (■, pATH 1-12). (**B**) pATH3-3 lysate was subjected to non-reducing SDS−PAGE and the gel was fractionated in 5 mm slices. The slices were then eluted into culture medium and tested in the cMGF bioassay.

in an increased mobility corresponding to a decrease in size of 5 kd, that is, ~2.5 kd smaller than the product obtained without membranes. The seemingly more efficient processing of cMGF products from HD11 mRNA (lane 5) in comparison with products from *in vitro* transcribed RNA can be explained by the use of two different dog pancreas membrane preparations. Taken together these data indicate that the molecularly cloned insert consists of the complete cMGF coding region including a 23 amino acid signal peptide. During *in vitro* translation a precursor of 26.5 kd is synthesized whose signal peptide is cleaved off in the presence of membranes and which subsequently becomes glycosylated at two *N*-glycosylation sites, generating a 28 kd cMGF form.

Antigenic and biological properties of bacterially expressed cMGF

To determine whether bacterially expressed cMGF is antigenically related to the HD11 derived factor and possibly biologically active, the pFP1 insert was subcloned into the bacterial expression vector pATH-3, yielding the strain pATH 3-3. Expression of recombinant cMGF (rcMGF) fused to the bacterial trpE protein could be induced in bacterial growth medium without tryptophan. As controls either the plasmid alone (pATH 1) or a plasmid in which cMGF was cloned out of frame (pATH 1-12) were also tested. Proteins from bacterial lysates were separated by SDS−PAGE and bands visualized either by protein staining or by Western blotting with an antiserum to cMGF. As shown in Figure 4A, the different constructs expressed proteins of the expected sizes. The fact that only the fusion protein in pATH 3-3 lysates reacted with the cMGF antiserum (Figure 4B), demonstrates that bacterially expressed rcMGF is antigenically similar to macrophage derived cMGF.

In experiments aimed to test for biological activity, bacterial lysates were examined for their capacity to induce the proliferation of E26 transformed myeloblasts (Leutz *et al.*, 1984). As shown in Figure 5A, lysates from the pATH 3-3 strain stimulated DNA synthesis of chicken myeloblasts while lysates from controls were devoid of biological activity. It can also be seen that between 100 and 200 ng/ml of the total bacterial protein were needed to achieve a maximal effect on growth. In a further experiment we examined whether the trpE−cMGF fusion protein or a putative rcMGF cleavage product is the biologically active principle of pATH 3-3 lysates. Proteins were therefore separated on non-reducing SDS−PAGE, a procedure which has been shown not to destroy cMGF activity (Leutz *et al.*, 1984) and the gel lanes fractionated and eluted into growth medium. As shown in Figure 5B, the biological activity migrated in the position of the fusion protein (cf. Figure 4). When tested on normal bone marrow cells, rcMGF stimulated the formation of predominantly macrophage colonies in a concentration comparable to that which was active on E26 transformed myeloblasts (T.Graf and A.Leutz, unpublished observations).

Taken together, these results demonstrate that recombinant bacterial cMGF, expressed as a trpE fusion protein, behaves very similarly to macrophage-derived cMGF.

Relationship of cMGF to other hematopoietic growth factors

To determine the relationship of cMGF to other known genes/proteins, several databases (including Swiss Prot, release 7; EMBL Data Bank, release 16; and Genbank, release 56) were searched. The only genes which exhibited significant sequence homologies with cMGF were the hematopoietic growth factors G-CSF and IL-6. These sequences were aligned with a newly developed computer

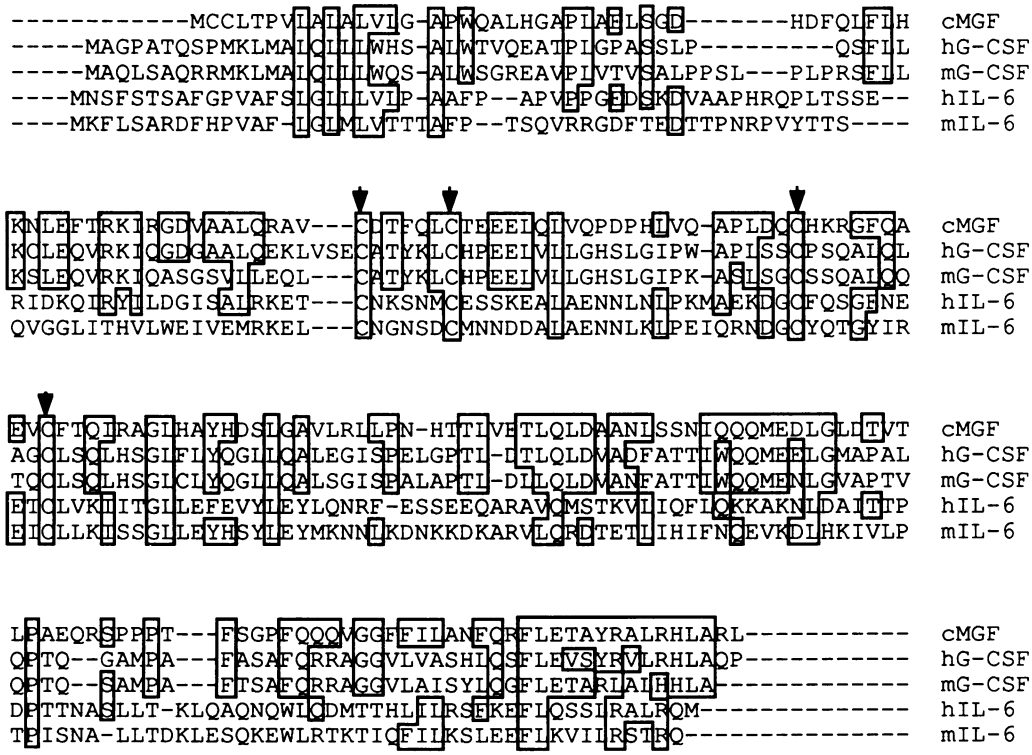

Fig. 6. Comparison of amino acid sequences of cMGF with other hematopoietic growth factors. Conserved amino acids are boxed. The arrowheads indicate conserved cysteine hG-CSF, human G-CSF; mG-CSF, mouse G-CSF; hIL-6, human IL-6; mIL-6, mouse IL-6.

program (Vingron and Argos, submitted for publication). As shown in Figure 6, the three factors share homology over the whole length of their sequence. Interestingly, the positions of all four cysteines were conserved between the protein sequences of cMGF and IL-6 and, allowing for a single amino acid gap, between cMGF and G-CSF as well. The human and murine G-CSFs each contain an additional cysteine residue which have not been conserved either between the mammalian G-CSFs or other factors. The numerical results of the alignment, summarized in Table II, indicate that cMGF is a distant relative of G-CSF and IL-6 and that it may be more closely related to G-CSF than to IL-6.

Table II. Homology between cMGF and G-CSF/IL-6[a]

	Nucleotide sequence (%)	Amino acid sequence (%)
Human G-CSF	56	56[b] (38)[c]
Mouse G-CSF	54	52 (35)
Human IL-6	41	41 (23)
Mouse IL-6	37	39 (18)

[a]Determined from amino acid alignment using UWGCG programs (Devereux *et al.*, 1984).
[b]Percentage including conservative amino acid changes.
[c]Percentage of identical amino acids.

Discussion

In this paper we describe the molecular cloning and sequencing of cMGF, a growth factor which stimulates the proliferation and colony formation of normal and transformed avian cells of the myeloid lineage. The most striking feature of the cMGF sequence is its structural relationship to the hematopoietic growth factors IL-6 and G-CSF but not to macrophage-CSF (M-CSF) or granulocyte/macrophage-CSF (GM-CSF). This finding is surprising in light of the fact that the biological activity is closer to the latter factors.

Is cMGF a new growth factor, is it the chicken homolog of IL-6 and G-CSF or is it their ancestral precursor? Murine G-CSF was originally described as a maturation factor which induced granulocyte colony formation and which induced the WEHI-3D$^+$ cell line to differentiate into granulocytes (Nicola *et al.*, 1983). However, recent experiments indicate that the primary effect of G-CSF on WEHI-3 cells is to allow the terminal differentiation of cells which would otherwise die (Böhmer and Burgess, 1988). Survival supporting effects of G-CSF have also been observed on multipotential hematopoietic progenitors and recently it has been shown that the factor synergizes with IL-3 by enhancing precursor cell proliferation (Ikebuchi *et al.*, 1988). IL-6 on the other hand was initially described as an activity which induces immunoglobulin secretion in mature B cells (Hirano *et al.*, 1986). It is now apparent that like G-CSF, this factor also augments the activity of IL-3 and supports the formation of granulocyte/macrophage type colonies (Ikebuchi *et al.*, 1987; Wong *et al.*, 1988). Furthermore, it has recently become clear that IL-6 is a highly pleiotropic factor acting on widely different cell types such as neurons and liver cells (Morrone *et al.*, 1988; Satoh *et al.*, 1988; for review see Wong and Clark, 1988).

Preliminary experiments in a chicken liver cell assay suggest that recombinant cMGF can act like IL-6 to induce acute response genes (G.Grieninger, New York, personal communication). Although this result indicates a possible relationship to IL-6, crosslinking experiments with iodinated cMGF revealed that the cMGF receptor has M_r of 120 kd

(A.Leutz, unpublished results) and is thus clearly distinct from the 80 kd murine IL-6 receptor (Yamasaki *et al.*, 1988) and the 175 kd murine G-CSF receptor (Nicola and Peterson, 1986). No biological activity of recombinant murine IL-6 or of human G-CSF could be detected with the chicken myelobast proliferation assay even when tested over a wide concentration range (A.Leutz, unpublished results). This is perhaps not surprising considering the large evolutionary distance between mammals and birds. More conclusive data were obtained from a Southern analysis of chicken DNA, using cMGF probes along with human IL-6 and murine G-CSF probes. Our preliminary results indicate the presence of distinct genes for the three growth factors, thus strongly suggesting that cMGF is encoded by a new gene and that it is not the avian homolog of the mammalian factors IL-6 or G-CSF.

Does a cMGF homolog exist in mammals? As perhaps expected, cMGF is devoid of biological activity in murine colony assays (N.Iscove, Toronto, personal communication) and in a human liver assay (G.Morrone, Heidelberg, personal communication). Hybridization of a cMGF probe to human and murine genomic DNA gives rise to several bands which do not appear to hybridize with G-CSF and IL-6 probes, suggesting that a mammalian homolog does exist. However, the issue will only be settled if a mammalian version of the cMGF gene can be molecularly cloned, work which is currently in progress in our laboratory.

The observation that hematopoietic growth factors such as IL-1 and IL-6 can exhibit a synergism with other factors and that they can affect cells of non-hematopoietic origin suggests that they act in a pleiotropic fashion, perhaps involving a paracrine-type network of cellular communication. Such a network can only be established and maintained by a tight regulation of factor expression in different cell types. Indeed, stimulation of fibroblasts with serum growth factors or tumor necrosis factor (TNF) induces genes which are themselves highly homologous to growth factors or cytokines (Linzer *et al.*, 1985; Kohase *et al.*, 1986; Anisowicz *et al.*, 1987; Wahl *et al.*, 1987; Rollins *et al.*, 1988; Ryseck *et al.*, 1988). Similarly, we have shown that cMGF can be induced in myeloid cells by kinase type viral oncogenes as well as by treatment with LPS or lectins (Adkins *et al.*, 1984; Leutz *et al.*, 1984). In this context it will be interesting to determine whether cMGF is regulated at the transcriptional or post-transcriptional level and whether the AT or the GC rich sequences in the 3′ non-coding region of the cMGF RNA play a role in the turnover of cMGF mRNA.

The finding that a fusion protein between cMGF and trpE expressed in bacteria is biologically active is surprising. Neither the trpE moiety nor the cMGF signal-peptide present in the fusion protein seems to impair the cMGF activity. Furthermore, the specific activity of the fusion protein is similar to that of macrophage derived cMGF assuming that the amount of recombinant protein is ~1–5% of total bacterial protein. This indicates that the trpE moiety does not interfere with the correct folding of the cMGF protein; an observation which has also been made with other recombinant fusion proteins (Desplan *et al.*, 1985; Angel *et al.*, 1988). Of course we cannot rule out the possibility that the trpE–cMGF fusion protein might be proteolytically cleaved after its addition to the culture medium. Further experiments will be required to determine whether intact fusion protein indeed binds to and activates the cMGF receptor.

Bacterially expressed cMGF is unglycosylated but active, suggesting that the extensive carbohydrate modifications found on the macrophage derived factor are not required for its biological activity. However, glycosylation of mammalian GM-CSF has been shown to modulate some of its biological activity, suggesting that the same might be true for cMGF (Donahue *et al.*, 1986). It is also possible that glycosylation might alter some of the biological properties of cMGF, such as its tissue distribution or its half-life *in vivo*. The availability of the recombinant material should enable us to investigate the role of the carbohydrates found on cMGF in more detail.

Materials and methods

Amino acid sequencing of cMGF

About 20 μg (~1 nmol) of HPLC purified cMGF (Leutz *et al.*, 1984) was subjected to electrophoresis on a 10% SDS gel (Laemmli, 1970). After staining with Coomassie Blue the band corresponding to cMGF was excised and the protein eluted from the gel slice as described by Hunkapiller *et al.* (1983). The eluted protein was lyophilized, the pellet was resuspended in 0.1 ml water and then 1 ml of ice cold methanol was added. The precipitate was collected by centrifugation and resuspended in 50 mM ammonium bicarbonate and 0.1 mM $CaCl_2$. One third of the sample was directly analyzed by N-terminal sequencing in a gas phase sequencer as described by Hewick *et al.* (1981). The rest of the sample was digested by trypsin and the peptides were separated by reversed-phase HPLC. The mobile phase was 0.1% trifluoroacetic acid. A linear gradient of 0–60% acetonitrile was used over a period of 120 min.

cDNA library construction

The library used had been constructed for another purpose, to be described elsewhere. Briefly, six *ts*21 E26 virus-transformed myeloblast clones were grown at 37°C and shifted to 42°C for 24 h, then back to 37°C for 4 h. The cells were pooled, washed twice with ice cold PBS and poly(A)$^+$ RNA was prepared using an SDS–proteinase K lysis procedure (Vennström and Bishop, 1982). Double-stranded cDNA was prepared (Watson and Jackson, 1985) and cloned into the λgt10 vector as described previously (Huynh *et al.*, 1985).

Deoxyoligonucleotide probe design and labeling

A 35 base oligonucleotide was custom synthesized using the phosphoramidite method (Beaucage and Caruthers, 1981). The oligonucleotide corresponded to part of the cMGF amino terminus (amino acid residues 8–14; nucleotides 142–176 in Figure 2), contained inosine at ambiguous wobble base positions (Ohtsuka *et al.*, 1984; Martin and Castro, 1985) and was complementary to the cMGF coding strand. The first nucleotides of two leucine codons (amino acid residues 14 and 16) were chosen according to codon usage. The oligonucleotide had the sequence 5′-TTICCITGIAGIAAIAGITGIAAI-TCITGITCICC-3′. The oligonucleotide was purified by denaturing gel electrophoresis on a 20% polyacrylamide gel containing 8% urea (Maniatis *et al.*, 1982) and 5′-end labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Arrand, 1985).

Library screening and phage purification

Plaque filter replicas of the cDNA library on nitrocellulose membranes (Schleicher and Schüll) were prehybridized and then incubated with the labeled oligonucleotide probe at 45°C for 16 h in hyridization buffer (see below) without formamide. Membranes were washed twice for 30 min each at room temperature and at 45°C in 5 × SSC plus 0.1% SDS before exposure. The positive phage was plaque purified and phage DNA was prepared and digested with *Eco*RI (Maniatis *et al.*, 1982). The recombinant *Eco*RI cDNA insert was isolated from agarose gel using Gene Clean (Bio 101) and ligated into Bluescript (Stratagene) and appropriate M13 vectors (Yanisch-Perron *et al.*, 1985).

RNA and DNA analysis

HD11 macrophages were stimulated with LPS as described (Leutz *et al.*, 1988) and RNA was extracted after 3 h by a guanidinium thiocyanate method (Chomczynski and Sacchi, 1987).

Polyadenylated RNA was selected by oligo(dT)–cellulose chromatography (Aviv and Leder, 1972) fractionated on 1% agarose–formaldehyde gels

and transferred to GeneScreen Plus (Du Pont) membrane. DNA was extracted from Spafas-chick liver, digested with restriction endonucleases, separated by electrophoresis on 0.8% agarose gels, transferred to Gene Screen membrane (Du Pont) and crosslinked by exposure to UV light (Southern, 1975; Reed and Mann, 1985; Ausubel *et al.*, 1987).

Membranes for DNA analysis were prehybridized at 42°C in hybridization buffer containing 5 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1% SDS, 50% formamide. For RNA analysis they were prehybridized overnight in 5 × SSC, 0.5% SDS, 5 × Denhardt's (1× Denhardt's is 0.2 g/l, of each polyvinylpyrrolidone, bovine serum albumin and Ficoll 400) 1 mM EDTA, 0.05% sodium pyrophosphate, 200 μg/ml of salmon sperm DNA and 50% formamide. Hybridization was performed overnight in the same solution supplemented with $5-7 \times 10^7$ c.p.m. of ^{32}P-labeled DNA probe prepared by random oligonucleotide priming (sp. act., 7×10^8 c.p.m./μg). Membranes were first washed twice at room temperature in 1 × SSC plus 0.5% SDS then twice at 65°C in 0.1 × SSC plus 0.1% SDS for 30 min each and were then exposed to X-ray film (Kodak X-Omat) with intensifying screens (Du Pont).

DNA sequencing by the chain termination method (Sanger *et al.*, 1977) was carried out from single- and double-stranded templates (Chen and Seeburg, 1985; Yanisch-Perron *et al.*, 1985) using the Sequenase sequencing kit (USB).

In vitro transcription, translation and immunoprecipitation

RNA was transcribed *in vitro* from the T3-promoter of the Bluescript-cMGF plasmid DNA in the presence of $M^7G(5')ppp(5')G$ (Boehringer; Konarska *et al.*, 1984). Approximately 100 ng of *in vitro* transcribed RNA and 5 μg of HD11 derived polyadenylated RNA were subjected to *in vitro* translation using a rabbit reticulocyte lysate exactly as described by the manufacturer (Promega). Canine pancreas membranes obtained from the same source were added to allow signal peptide cleavage and core glycosylation (Walter and Blobel, 1983). Following translation, samples were diluted with RIPA buffer, immunoprecipitated and subjected to endoglycosidase as indicated, followed by electrophoresis and fluorography as described before (Leutz *et al.*, 1988).

Expression of recombinant cMGF

The *Eco*RI cDNA insert was cloned into the bacterial expression vectors pATH1 and pATH3 (constructed by T.J.Koerner and A.Tzagoloff, gift from J.Lipsick). Fusion protein expression of ORF's fused to the N-terminal portion of the trpE gene was induced in M9 medium by tryptophan starvation. Bacteria were harvested and proteins released and renatured as described by Desplan *et al.* (1985). Protein concentration in bacterial lysates were determined with a protein assay kit (Bio-Rad).

Acknowledgements

The authors are grateful to Brian Sproat and Philipe Neuner for the synthesis of oligonucleotides, Martin Vingron for his help with protein sequence alignments and Hilary Davies for excellent secretarial assistance.

References

Adkins,B., Leutz,A. and Graf,T. (1984) *Cell*, **39**, 439–445.
Arrand,J.E. (1985) In Hames,B.D. and Higgins,S.H. (eds), *Nucleic Acid Hybridization: A Practical Approach*. IRL, Press, Oxford.
Angel,P., Allegretto,E.A., Okino,S.T., Hattori,K., Boyle,W.J., Hunter,T. and Karin,M. (1988) *Nature*, **332**, 166–170.
Anisowicz,A., Bardwell,L. and Sager,R. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7188–7192.
Ausubel,F.M., Brent,R., Kingston,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (1987) *Current Protocols in Molecular Biology*. John Wiley, New York.
Aviv,H. and Leder,P. (1972) *J. Mol. Biol.*, **134**, 743.
Beaucage,S.L. and Caruthers,M.H. (1981) *Tetrahedron Lett.*, **22**, 1859–1862.
Beug,H., Hayman,M. and Graf,T. (1982) *EMBO J.*, **2**, 1069–1073.
Böhmer,R. and Burgess,A. (1988) *Int. J. Cancer*, **42**, 53–58.
Chen,E.Y. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Chomczynski,P. and Sacchi,N. (1987) *Anal. Biochem.*, **162**, 156–159.
Desplan,C., Theis,J. and O'Farrel,P.H. (1985) *Nature*, **318**, 630–635.
Devereux,J., Haeberli,P. and Smithies,O. (1984) *Nucleic Acids Res.*, **12**, 387–395.
Donahue,R., Wang,E., Kaufman,R., Foutch,L., Leary,A., Witek-Giannetti,J., Metzger,M., Hewick,R., Steinbrink,D., Shaw,G., Kamen,R. and Clark,S. (1986) *Cold Spring Harbour Symp. Quant. Biol.*, **51**, 685–692.
Graf,T., v. Weizsaecker,F., Grieser,S., Coll,J., Stehelin,D., Patschinsky,T., Bister,K., Bechade,C., Calothy,G. and Leutz,A. (1986) *Cell*, **45**, 357–364.
von Heijne,G. (1983) *Eur. J. Biochem.*, **133**, 17–21.
Hewick,R.M., Hunkapiller,M.W., Hood,L.E. and Dreyer,W.J. (1981) *J. Biol. Chem.*, **256**, 7990–7997.
Hirano,T., Yasukawa,K., Harada,H., Taga,T., Watanabe,Y., Matsuda,T., Kashiwamura,S., Nakajima,J., Koyama,K., Iwamatsu,A., Tsunasawa,S., Sakiyama,F., Matsui,H., Takahara,Y., Taniguchi,T. and Kishimoto,T. (1986) *Nature*, **324**, 73–76.
Hunkapiller,M.W., Lujan,E., Ostrander,F. and Hood,L.E. (1983) *Methods Enzymol.*, **91**, 227–236.
Huynh,T.V., Young,R.A. and Davis,R.W. (1985) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach*. IRL Press, Oxford, Vol. 1, pp. 49–78.
Ikebuchi,K., Wong,G., Clark,S., Ihle,N., Hirai,Y. and Ogawa,M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 9035–9039.
Ikebuchi,K., Clark,S., Ihle,J., Souza,L. and Ogawa,M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3445–3449.
Kohase,M., Henrikson-DeStefano,D., May,L.T., Vilcek,J. and Sehgal,P.B. (1986) *Cell*, **45**, 659–666.
Konarska,M.M., Padgett,R.A. and Sharp,P.A. (1984) *Cell*, **38**, 731–736.
Kozak,M. (1984) *Nucleic Acids Res.*, **12**, 857–872.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Leutz,A., Beug,H. and Graf,T. (1984) *EMBO J.*, **3**, 3191–3197.
Leutz,A., Beug,H., Walter,C. and Graf,T. (1988) *J. Biol. Chem.*, **263**, 3905–3911.
Linzer,D., Lee,S.-J., Orgen,L., Talamantes,F., Nathans,D. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 4356–4359.
Maniatis,T., Fritsch,E. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Martin,F.H. and Castro,M.M. (1985) *Nucleic Acids Res.*, **13**, 8927–8938.
Metcalf,D. (1984) *The Hemopoietic Colony-Stimulating Factors*. Elsevier, New York.
Morrone,G., Ciliberto,G., Oliviero,S., Arcone,R., Dentes,L., Content,J. and Cortese,R. (1988) *J. Biol. Chem.*, **263**, 12554–12558.
Nicola,N. and Petersen,L. (1986) *J. Biol. Chem.*, **261**, 12384–12389.
Nicola,N., Metcalf,D., Matsumoto,M. and Johnson,G.R. (1983) *J. Biol. Chem.*, **258**, 9017–9023.
Ohtsuka,E., Matsuki,S., Ikeharat,M., Takahashi,Y. and Matsubara,K. (1984) *J. Biol. Chem.*, **260**, 2605–2608.
Reed,K.C. and Mann,D.A. (1985) *Nucleic Acids Res.*, **13**, 7207–7221.
Rollins,B.J., Morrison,E.D. and Stiles,C.D. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3738–3742.
Ryseck,R.-P., Macdonald-Bravo,H., Mattei,M.G. and Bravo,R. (1988) *Exp. Cell Res.*, in press.
Sanger,F., Miklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Satoh,T., Nakamura,S., Taga,T., Matsuda,T., Hirano,T., Kishimoto,T. and Kaziro,Y. (1988) *Mol. Cell. Biol.*, **8**, 3546–3549.
Schrader,J.W., Ziltener,H.J. and Leslie,K.B. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2458–2462.
Shaw,G., Kamen,R. (1986) *Cell*, **46**, 659–667.
Southern,E.M. (1975) *J. Mol. Biol.*, **98**, 503–517.
Vennström,B. and Bishop,J.M. (1982) *Cell*, **28**, 135–143.
Wahl,S.M., Hunt,D.A., Wakefield,L.M., McCartney-Francis,N., Wahl,L.M., Roberts,A.B. and Sporn,M.B. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5788–5792.
Walter,P. and Blobel,G. (1983) *Methods Enzymol.*, **96**, 84–93.
Watson,C.J. and Jackson,J.F. (1985) In Glover,D.M. (ed.), *DNA Cloning: A Practical Approach*. IRL Press, Oxford, Vol. 1, pp. 79–880.
Watson,M.E.E. (1984) *Nucleic Acids Res.*, **12**, 5145–5164.
von Weizsäcker,F., Beug,H. and Graf,T. (1986) *EMBO J.*, **5**, 1521–1527.
Wong,G., Witek-Gionnotti,J., Temple,P., Kriz,R., Gerenz,C., Hewick,R., Clark,S., Ikebuchi,K. and Ogawa,M. (1988) *J. Immunol.*, **140**, 3040–3044.
Wong,G.G. and Clark,S.C. (1988) *Immunol. Today*, **9**, 137–139.
Yamaaki,K., Taga,T., Hirata,Y., Yawata,H., Kawanishi,Y., Seed,B., Taniguchi,T., Hirano,T. and Kishimoto,T. (1988) *Science*, **241**, 825–827.
Yanisch-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–109.

Received on September 29, 1988; revised on November 3, 1988