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# Recombinant murine GM-CSF from *E. coli* has biological activity and is neutralized by a specific antiserum

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**We report the production and characterization of a mouse granulocyte-macrophage colony stimulating factor (mGM-CSF) made in *Escherichia coli*. The synthesis of mGM-CSF was directed by a plasmid containing a gene isolated from the EL-4 cell line. After induction of expression and accumulation of the protein in *E. coli*, mGM-CSF accounted for 10% of total cellular protein. This recombinant mGM-CSF was purified to 90% homogeneity by chaotrope extraction and gel filtration. Recombinant mGM-CSF, like the native molecule, stimulates the growth of granulocyte and macrophage colonies in serum-free cultures of mouse bone marrow cells. Antibodies raised against recombinant mGM-CSF not only reacted with the recombinant protein but also neutralized the biological activity of both native and recombinant mGM-CSF. These results indicate that the functional structure of the recombinant protein is similar to that of native mGM-CSF.**

*Key words:* colony stimulating factor/mGM-CSF neutralizing antiserum/procaryote expression vector/recombinant mGM-CSF/serum-free clonal assays

## Introduction

The discovery of conditions for clonal assay of hemopoietic precursor cells *in vitro* marked a major advance in the study of blood cell growth and differentiation (Pluznik and Sachs, 1965; Bradley and Metcalf, 1966). These assays for bone marrow colony formation allowed the identification and purification of four distinct murine hemopoietic growth factors, called colony stimulating factors (CSFs). One of these factors, interleukin-3 (Ihle *et al.*, 1982; Metcalf, 1984) or multi-CSF, stimulates the formation of colonies composed of granulocytes, erythrocytes, megakaryocytes and mast cells in various combinations. Activity of the other three factors appears to be more restricted to the granulocyte and macrophage lineages. G-CSF (Nicola *et al.*, 1983; Metcalf and Nicola, 1983) primarily gives rise to granulocyte colonies, M-CSF (CSF-1; Stanley and Heard, 1977; Shadduck *et al.*, 1980) to colonies composed mostly of macrophages, while GM-CSF (Burgess *et al.*, 1977) gives rise to mixed granulocyte-macrophage colonies in addition to pure colonies of both types. Unfortunately, detailed studies on the mechanism of action of GM-CSF have been hampered by the availability of only small quantities of pure material.

Recent attempts to identify and isolate the nucleotide sequence encoding both murine GM-CSF and human GM-CSF have proven successful (Gough *et al.*, 1984, 1985; Wong *et al.*, 1985). This information has provided the means to study the organization and regulation of the GM-CSF gene. Another important use of the gene is the synthesis of a recombinant protein in a cell not already producing GM-CSF. However, as an expression system for producing GM-CSF in the quantities and purity necessary for biological and structural studies, transfected animal cells are disappointing. They generally synthesize low levels of these proteins, of the order of 1 μg/ml (Gough *et al.*, 1984, 1985; Weisbart *et al.*, 1985; Wong *et al.*, 1985). Moreover, purification can be complicated by the presence of serum proteins in the medium used to grow the cells plus the potential presence of CSFs secreted by the mammalian cells themselves.

These problems can be avoided by using an *Escherichia coli* expression system to produce large quantities of recombinant mGM-CSF. We have produced, in *E. coli*, pure unglycosylated mGM-CSF and tested its biological activity *in vitro*. The recombinant mGM-CSF protein was used to raise antiserum which specifically immunoprecipitated GM-CSF and neutralized its activity.

## Results

### *mGM-CSF gene isolation and expression*

A cDNA library generated from the mRNA of induced EL-4 cells was screened by hybridization with oligomer DNA probes synthesized on the basis of the published cDNA sequence for mouse lung cell-derived GM-CSF (Gough *et al.*, 1984). The sequence of the longest positive insert was determined and found to be similar to that of GM-CSF from mouse lung cells (Figure 1). Over the coding region there were three base changes (at positions 186, 213 and 482), representing one of the two variants reported for different isolates of mGM-CSF (Gough *et al.*, 1985). Only the base change at position 482 generates an amino acid substitution, yielding valine instead of glycine.

The poly(A) sequence at the 3′ end of the molecule indicates that the cDNA covers the mRNA to its 3′ end. However, precise determination of the sequence at the 5′ end of the mRNA was not possible due to the poly(G) tail. The largest open reading frame extends 423 nucleotides from nucleotide 67 to 489. This region encodes a putative pre-mGM-CSF of 141 amino acids. The first 18 residues contain a series of hydrophobic amino acids followed by a frequent cleavage site for signal peptides (serine-alanine; Lingappa and Blobel, 1980; Watson, 1984). Thus, the most likely amino-terminal residue of mature mGM-CSF is alanine, in agreement with that found for human GM-CSF (Wong *et al.*, 1985).

To allow efficient expression of mGM-CSF in *E. coli*, we constructed a plasmid which placed the coding sequence behind a promoter-ribosome binding site sequence: the leftward transcription promoter of bacteriophage lambda ($P_L$) and the ribosome binding site from the *ner-1* gene of bacteriophage Mu (Figure 2). A portion of the naturally occurring nucleotide sequence had to be altered to introduce a restriction enzyme site so that translation would begin at the first amino acid of the mature protein, alanine. An oligonucleotide linker was synthesized encoding a methionine for translation initiation as well as the first 10 amino acids of mature mGM-CSF (Figure 2). In designing the linker

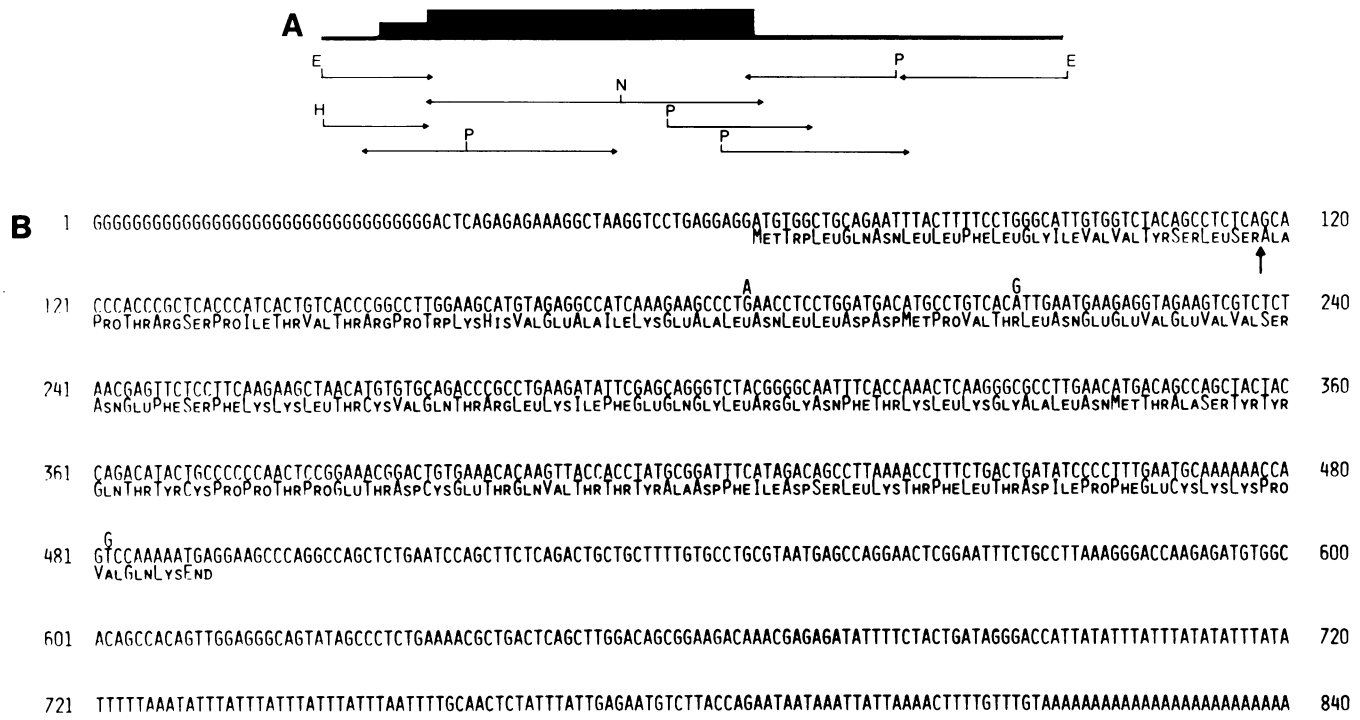


**Fig. 1.** The sequencing scheme, cDNA and deduced protein sequence for EL-4 derived mGM-CSF. **Panel A** indicates the signal and mature peptide coding regions of the cDNA by lines of different thickness. The region and direction of the sequence read are indicated by thin lines with arrows. The letters above these lines indicate whether an oligodeoxynucleotide primer (P) and chain termination was the method of sequencing or chemical degradation of a fragment labeled at a restriction endonuclease site (E=*Eco*RI, H=*Hind*III, N=*Nde*I) was used. **Panel B** shows the sequence of the cDNA, the alternative bases above those read, the deduced protein sequence below the nucleotide sequence, and the putative signal-peptide cleavage site marked with an arrow.

an attempt was made to minimize potential mRNA secondary structure which might make the ribosome binding site inaccessible. Computer modelling of RNA secondary structure was undertaken for the mRNA segment including the ribosomal binding site and the first 30 nucleotides of the coding sequence (Zuker and Stiegler, 1981). The difference in minimum free energy for the putative secondary structures between the naturally occurring sequence (−15.6 kcal) and that of the linker sequence (−4.6 kcal) was 11 kcal. Using primary sequence data alone, mRNA secondary structure cannot be predicted with certainty. However, from the calculated minimum free energies, the putative secondary structure of the naturally occurring sequence, and not that of the linker sequence, would be expected to form a stable stem and loop in this region (Tinoco *et al.*, 1971, 1973; Salser, 1977).

These elements were combined to form a mGM-CSF expression vector which was used to transfect an *E. coli* strain C600 (Maniatis *et al.*, 1980) that contained a plasmid encoding the thermosensitive repressor cI857 of $P_L$ [pcI857 (Remaut *et al.*, 1983)]. When the bacteria were shifted from the permissive to the non-permissive temperature (28° to 42°C), accumulation of a newly synthesized protein with an apparent mol. wt. of 14 500 was evident (Figure 3). Including the additional amino-terminal methionine residue, the calculated mol. wt. for the recombinant protein of 14 285 is in good agreement with that observed. Densitometer scanning of a Coomassie blue-stained gel of total *E. coli* cell protein from an induced culture indicated that mGM-CSF represented ~10% of the cellular protein.

*Purification and biological activity of recombinant mGM-CSF*

The high concentration of mGM-CSF and its accumulation in refractile bodies in induced cultures of *E. coli* simplifies its purification. mGM-CSF aggregates from disrupted bacteria were

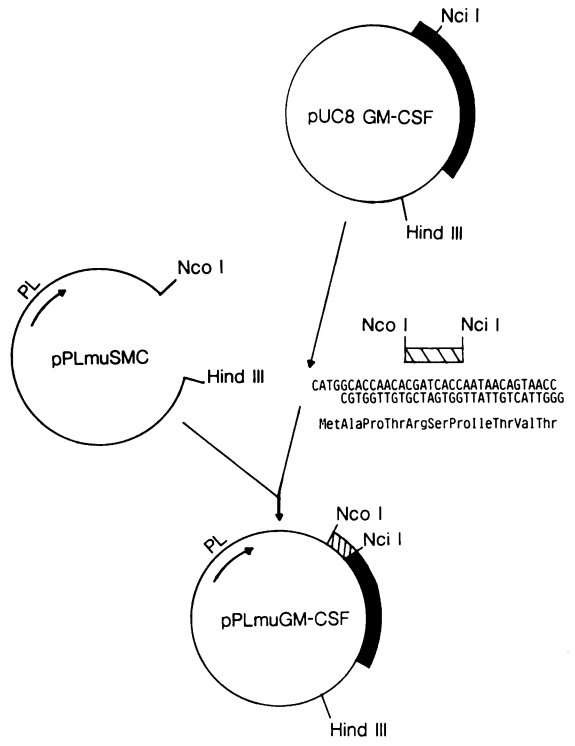


**Fig. 2.** Construction of a plasmid directing the expression of mGM-CSF in *E. coli*. A fragment of the subcloned mGM-CSF cDNA was isolated by *Nci*I and *Hind*III digestion of the plasmid. An expression vector opened by *Nco*I and *Hind*III digestion was ligated together with the synthetic DNA fragment (*Nco*I to *Nci*I) and the remaining mGM-CSF encoding cDNA sequence (*Nci*I to *Hind*III).

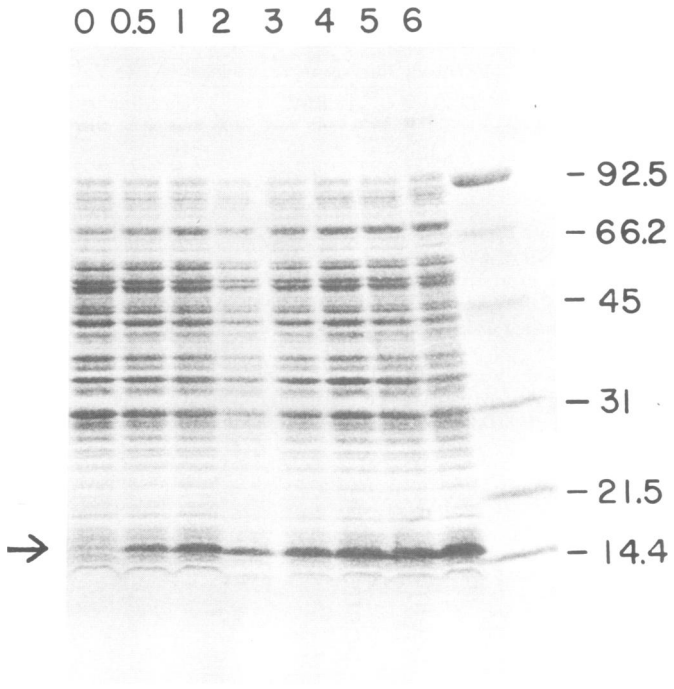


**Fig. 3.** Induction time course for mGM-CSF synthesis in *E. coli*. Aliquots of cultures were taken at the times indicated and diluted to a uniform cell concentration, lysed and analysed by gradient SDS-PAGE (8–25% acrylamide). Protein mol. wt. markers appear in the far right lane with their mol. wt. (in kd) indicated. Proteins were visualized by Coomassie blue staining. An arrow indicates the recombinant protein band accumulating after heat induction.

freed of most contaminants by low concentration chaotrope buffer washes, solubilized in 8 M guanidine hydrochloride, and isolated essentially free of other proteins by G-100 Sephadex chromatography in 8 M urea. Fractions from the column eluant were analyzed by SDS-PAGE. Those from the major peak appeared to be at least 90% pure (Figure 4, inset).

Every fourth eluant fraction was assayed for colony stimulating activity in serum-free cultures of mouse bone marrow cells. The fractions with highest activity corresponded to the major peak (fractions 44–56) of purified mGM-CSF as indicated by SDS-PAGE analysis (Figure 4). There was a minor peak of activity (fractions 20–28) in fractions containing higher mol. wt. proteins. This second peak probably represents aggregates of mGM-CSF not totally dissociated by the urea buffer.

The column fractions tested gave rise to clones composed of only granulocytes (G), only macrophages (M), or both (GM). The proportions of the different clone and colony types induced by each fraction are shown in Table I. There were no significant differences between the fractions, although there appeared to be a tendency for higher proportions of M clones in the less-purified fractions (16–32). The mix of granulocyte and macrophage colonies observed for fractions 44–56 was indistinguishable from that obtained with purified native mGM-CSF in this system (unpublished results).

A typical dose-response curve for colony formation stimulated by recombinant mGM-CSF (fraction 50) is shown in Figure 5. Previous experiments in which dilutions of up to 0.1 μl/ml of this fraction were tested indicated that a plateau for the number of colonies was reached with 1 nl/ml. Half-maximal colony numbers (50 colonies) were obtained with 0.11 nl/ml. Since the pro-

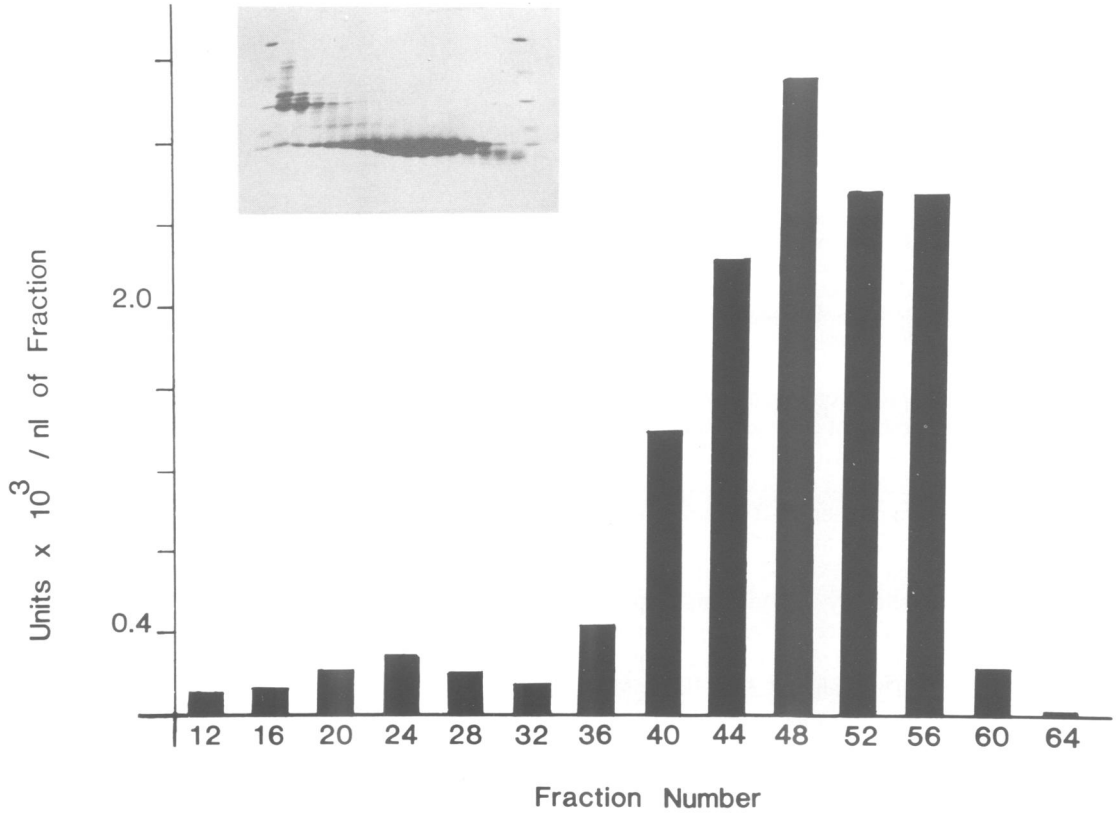


**Fig. 4.** Biological activity of purified mGM-CSF. The activity on bone marrow cells of different fractions from a G-100 Sephadex column used as a purification step for mGM-CSF is shown by solid bars. The histogram indicates the activity of the tested fractions expressed as units/nl. The inset shows a 10–27% (w/v) gradient SDS-PAGE analysis of even fractions from 32 to 62 inclusive from the same column run; proteins were visualized by Coomassie blue staining.

**Table I.** Composition of clones and colonies stimulated by recombinant mGM-CSF fractions eluted from a G-100 Sephadex column

| Fraction | Number of clones[a] | Percentage | | | Number of colonies[b] | Percentage | | |
|---|---|---|---|---|---|---|---|---|
| | | G | M | GM | | G | M | GM |
| 12 | 87 | 7 | 62 | 31 | 1 | 0 | 0 | 100 |
| 16 | 129 | 2 | 74 | 24 | 11 | 9 | 27 | 64 |
| 20 | 192 | 5 | 78 | 17 | 25 | 12 | 56 | 32 |
| 24 | 249 | 4 | 73 | 23 | 41 | 10 | 51 | 39 |
| 28 | 201 | 2 | 78 | 20 | 29 | 7 | 59 | 34 |
| 32 | 183 | 6 | 76 | 18 | 19 | 21 | 37 | 42 |
| 36 | 274 | 3 | 64 | 33 | 57 | 9 | 54 | 37 |
| 40 | 272 | 6 | 69 | 25 | 94 | 8 | 55 | 37 |
| 44 | 282 | 4 | 65 | 31 | 111 | 6 | 58 | 36 |
| 48 | 327 | 3 | 66 | 31 | 123 | 7 | 55 | 38 |
| 52 | 307 | 6 | 66 | 28 | 116 | 9 | 55 | 36 |
| 56 | 302 | 8 | 62 | 30 | 116 | 10 | 50 | 40 |
| 60 | 148 | 8 | 55 | 37 | 6 | 50 | 0 | 50 |
| 64 | 8 | 38 | 25 | 37 | 0 | 0 | 0 | 0 |

[a] > 10 cells.
[b] > 50 cells.

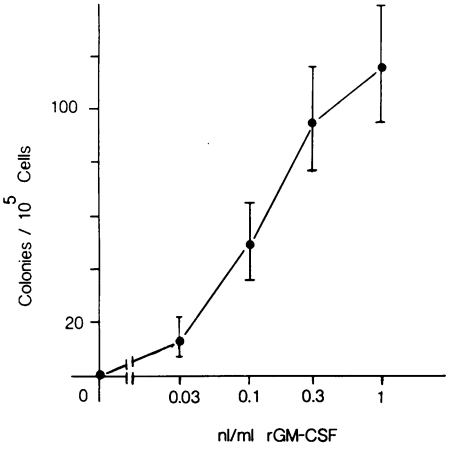


**Fig. 5.** A dose-response curve for recombinant mGM-CSF. Each point represents the number of colonies per $10^5$ bone marrow cells in serum-free cultures induced by the indicated dilutions of fraction 50 from the G-100 Sephadex eluant. The concentration range of recombinant protein tested was between 0.09 and 3 ng/ml. The horizontal bars show the 95% confidence limits assuming a Poisson distribution for colony counts.

tein concentration of the purified recombinant mGM-CSF in fraction 50 was 3 mg/ml, the number of colonies expected from 1 mg would be $\sim 1.5 \times 10^8$. A native mGM-CSF standard (Burgess *et al.*, 1977) required 5.6-fold the stated units to give equivalent numbers of colonies in our serum-free assay system. Thus, to compare published specific activities for native mGM-CSF with those for recombinant mGM-CSF, our calculated specific activity should be multiplied by 5.6 (yielding a specific activity of $8.4 \times 10^8$ units/mg). The recombinant-protein concentration necessary to yield half-maximal biological activity can also be deduced from Figure 5. This value, $2.3 \times 10^{-11}$ M, is sufficiently low to be consistent with that expected for pure mGM-CSF.

*Neutralizing antiserum to mGM-CSF*

Polyclonal antibodies to recombinant mGM-CSF produced in *E. coli* were raised by immunizing rabbits with the purified protein. An enzyme-linked immunosorbent assay (ELISA) showed

**Table II.** Antibody titre of anti-recombinant mGM-CSF serum

| Serum | Antibody titre versus recombinant mGM-CSF | | |
|---|---|---|---|
| | ELISA[a] | RIA[b] | Neutralization assay[c] |
| Pre-immune | < 10 | < 10 | < 10 |
| Post-immune | 10 000 | 2000 | 640 |

[a]ELISA titres are expressed as the reciprocal of the sample dilutions which yield an endpoint of $OD_{630} = 0.5$.
[b]RIA titres are expressed as the reciprocal of the sample dilution which caused 50% of the maximum quantity of radiolabeled mGM-CSF to be immunoprecipitated under the conditions described in Materials and methods.
[c]Neutralization titres are expressed as the reciprocal of the highest sample dilution which reduced by 50% the maximum biological activity observed wth 300 units of mGM-CSF on the FDC-P1 cell line.

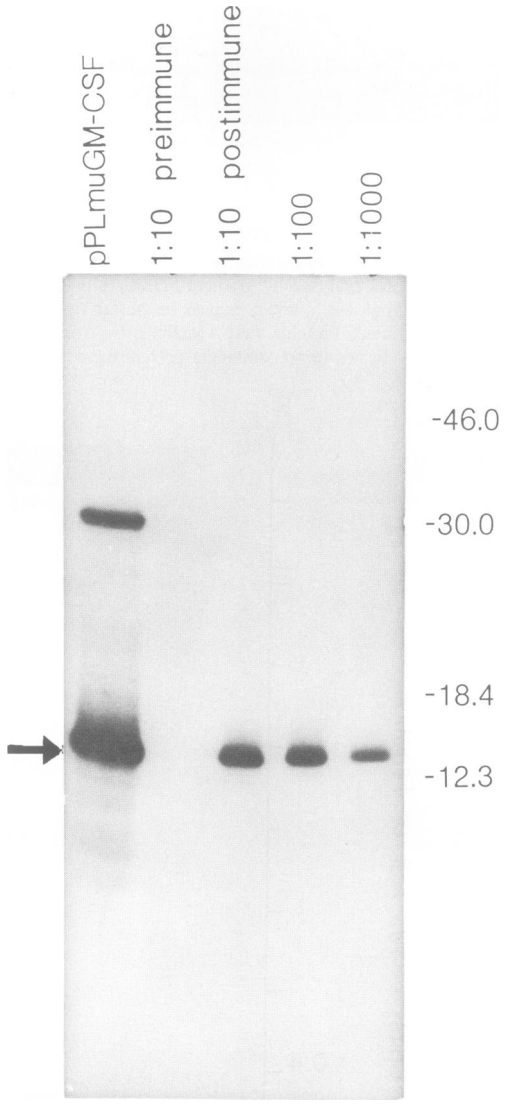


**Fig. 6.** Immunoprecipitation of *in vitro* synthesized recombinant mGM-CSF. Autoradiograph of the products of *in vitro* transcription-translation directed by the mGM-CSF expression plasmid. The [$^{35}$S]methionine-labeled proteins were either precipitated with pre-immune or post-immune antiserum at the dilutions indicated or the total labeled products analysed on 18% SDS-PAGE. The migration of radioactive mol. wt. markers in the gel are indicated by position and size (in kd) at the right. An arrow indicates the expected recombinant mGM-CSF product.

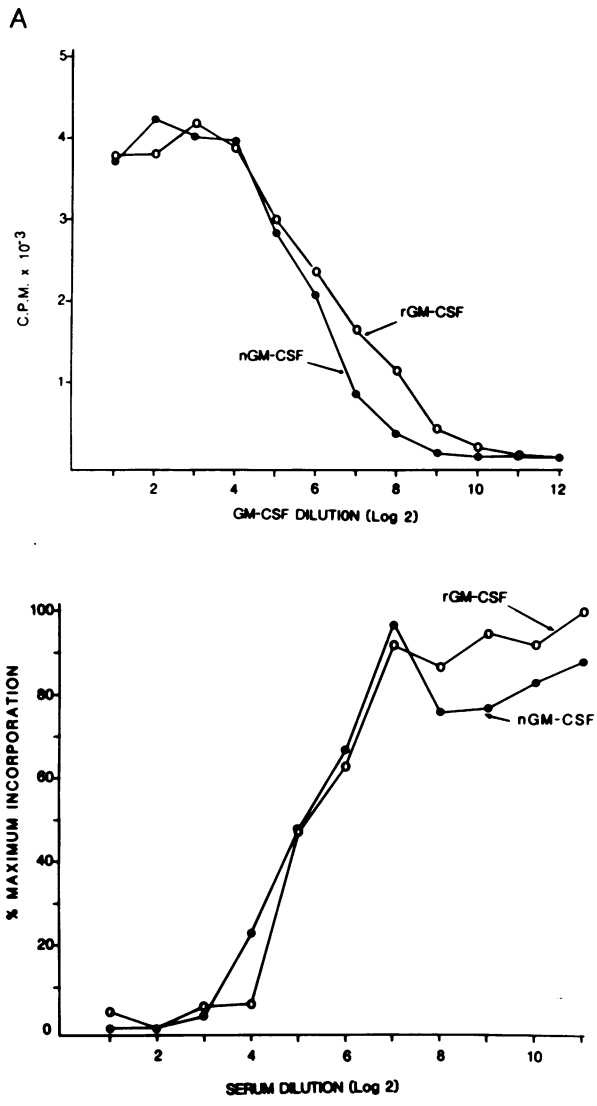


**Fig. 7.** Response of FDC-P1 cells to recombinant mGM-CSF in the absence or presence of neutralizing antiserum. **Panel A** FDC-P1 cells were incubated for 15 h with varying doses of recombinant mGM-CSF (○···○) or native mGM-CSF (●···●, starting concentration 60 000 units/ml) and labeled during the following 6 h with [$^3$H]thymidine. **Panel B** Varying concentrations of antibodies were incubated with a fixed amount of mGM-CSF. FDC-P1 cells were added to the complexes and incubated for 15 h and thymidine incorporation measured as in **Panel A**. Neither pre-immune serum nor antibodies against *E. coli* proteins inhibited mGM-CSF activity. Maximal incorporation with native mGM-CSF (●···●) was 1646 c.p.m. over background and 2217 c.p.m. with recombinant mGM-CSF (○···○).

that the rabbit serum had an antibody titre of 10 000 to recombinant mGM-CSF (Table II). These antibodies immunoprecipitated recombinant mGM-CSF which had been synthetically labeled with $^{35}$S in an *in vitro* transcription-translation system (Figure 6); this reaction was specific since pre-immune serum did not immunoprecipitate the protein. In a radioimmunoassay using the same $^{35}$S-labeled material, the antiserum showed a titre of 2000 (Table II). Pre-incubation of the antiserum with unlabeled recombinant mGM-CSF completely blocked the assay, indicating its specificity.

To test the effect of antibodies on the biological activity of mGM-CSF, we used the factor-dependent cell line FDC-P1 (Dexter *et al.*, 1980; Hapel *et al.*, 1984). This line proliferates in response to both native and recombinant mGM-CSF as shown by increased [$^3$H]thymidine incorporation to levels 5- to 10-fold above controls (Figure 7, panel A). Antibodies to recombinant mGM-CSF neutralized 50% of the activity of both native and recombinant proteins at a dilution equivalent to 1:640 (Figure 7, panel B). In addition, the antibodies inhibited the ability of recombinant mGM-CSF to stimulate colony formation by mouse bone marrow cells. These results indicate that the *in vitro* activity of native as well as recombinant mGM-CSF are specifically inhibited by the rabbit antiserum.

## Discussion

From the cell line EL-4 we have isolated a cDNA which encodes murine GM-CSF. The cDNA has one of each of the two alternative bases predicted for three sites in the coding region, but does not encode the extended pre-sequence reported by Gough *et al.* (1985). However, these authors have shown that enzymatic cleavage of their cDNA to the second methionine, which begins our putative pre-mGM-CSF, allows translation and secretion of mGM-CSF from transfected animal cells (Gough *et al.*, 1985). Since we sequenced only the isolate described here, it is not known if other EL-4 mRNAs contain different bases at the variant positions. Using the mature protein coding sequence, we have produced in bacteria recombinant mGM-CSF which is indistinguishable from its *in vitro* biological activity from native mGM-CSF.

Our approach to induce high level synthesis of mGM-CSF in *E. coli* was based on evidence that the secondary structure of the mRNA can be critical to translation (Iserentant and Fiers, 1980; Buell *et al.*, 1985). To prevent annealing of the ribosome binding site with the first codons of the message, an adenosine was substituted for all bases where degeneracy of the genetic code allowed the altered sequence to retain the correct amino acid. Although we have not compared expression of native and synthetic sequences in this study, we have done so for human GM-CSF where a significant increase in protein synthesis can be attributed to the altered 5′ end of the mRNA (DeLamarter *et al.*, in preparation).

The recombinant mGM-CSF supported proliferation of a factor-dependent cell line and stimulated colony formation by mouse bone marrow cells. Use of a serum-free clonal assay system demonstrated that the biological activity observed for the recombinant protein was not due to enhancement of an activity present in serum. Stimulation of colony formation in bone marrow cultures following recombinant mGM-CSF addition might occur indirectly. For example, other cells present that are able to induce the growth of granulocytes and macrophages might be stimulated by recombinant mGM-CSF. However, the recombinant protein supports the growth of the factor-dependent cell line suggesting that the biological activity is a direct effect of recombinant mGM-CSF. Since *E. coli* lacks the machinery to synthesize glycoproteins, these results provide an unambiguous demonstration that the sugar groups found on native mGM-CSF are not required for activity *in vitro*. Similar results for the biological activities of the glycoproteins gamma-interferon (Le *et al.*, 1983; Thurman *et al.*, 1985) and interleukin-2 (IL-2; Rosenberg *et al.*, 1984; Ortaldo *et al.*, 1984) and their recombinant equivalents have been reported. Whether recombinant mGM-CSF is also active *in vivo* remains to be determined.

The high concentration of recombinant mGM-CSF produced in bacteria means that the protein can be isolated to a high degree of purity in a single chromatographic step. Although the recombinant protein is at least 90% pure, the formal possibility exists

that a contaminant is responsible for the observed biological activity. However, antiserum raised against *E. coli* lysates does not inhibit the purified recombinant protein's activity, nor do *E. coli* lysates stimulate FDC-P1 cell growth (data not shown). Furthermore, the specific activity for recombinant mGM-CSF of nearly $10^9$ units/mg is comparable with that reported for purified native mGM-CSF from endotoxin-treated mouse lung cells (Burgess *et al.*, 1977; Sparrow *et al.*, 1985).

Although antibodies to other CSFs, notably CSF-1 (Stanley, 1979) and IL-3 (Bowlin *et al.*, 1984), have been reported, none have previously been raised against GM-CSF. It may be that the absence of glycosyl residues on the recombinant mGM-CSF enhances the protein's antigenicity. Alternatively, the very limited amount of protein available previously may explain the lack of success at raising antisera to GM-CSF.

The production of large amounts of biologically active mGM-CSF means that a number of important questions related to the mechanism of action of GM-CSF can be addressed. The structure of the protein can be studied with more precision and the regions of the molecule responsible for biological activity identified. Furthermore, the role of circulating GM-CSF in health and disease can be tested by supplying additional protein or neutralizing that already present. One of the first benefits of having large quantities of mGM-CSF has been the development of a neutralizing antiserum which could be useful for studies on the localization of GM-CSF as well as its role *in vivo*.

## Materials and methods

*Nucleic acids*

mRNA isolated from phorbol-12-myristate-13-acetate (PMA) treated cultures of EL-4 cells was reverse transcribed into cDNA using the Okayama-Berg protocol (Okayama and Berg, 1982). The cDNA was inserted into the plasmid vector pHG327, a modified pKCR vector (O'Hare *et al.*, 1981), which has a unique *Sst*I site for cDNA cloning and two flanking *Bam*HI sites to allow convenient excision of the inserted cDNA molecule. The resultant library consisted of $\sim 2 \times 10^5$ individual cDNA molecules (a kind gift from Mr W.Boll and Dr C. Weissmann). 0.02% of these scored positive by hybridization with several oligodeoxynucleotide probes synthesized according to the available sequence of mGM-CSF (Gough *et al.*, 1984) using the solid-phase phosphotriester method (Ito *et al.*, 1982).

DNA was sequenced by the chain termination method (Sanger *et al.*, 1977; Messing and Vieira, 1982) where synthetic oligodeoxynucleotides were used as primers for the reaction. Otherwise restriction enzyme sites were filled out with a radioactive base using the Klenow fragment of polymerase I and the sequence determined by chemical degradation of the DNA chain (Maxam and Gilbert, 1980).

Restriction endonuclease digests, ligation of DNA fragments and transfection of vectors into *E. coli* were performed according to Maniatis *et al.* (1980).

*Clonal assay cultures*

Assays for colony stimulating activity were performed with femoral bone marrow cells from C57/B1 mice cultured in serum-free methylcellulose medium as previously described (Eliason and Odartchenko, 1985). The standard medium consisted of a 3:1 mixture of enriched Dulbecco's Modified Eagle's medium (EMED) and Ham's F-12 nutrient mixture (FMED). This was further supplemented with 1% w/v purified bovine serum albumin (BSA), 0.9% methylcellulose, human transferrin at 320 μg/ml, insulin at 9 μg/ml, hemin at 20 μM, linoleic acid at 5.6 μg/ml, cholesterol at 5.2 μg/ml, additional L-glutamine at 204 μg/ml, nucleosides each at 10 μg/ml and trace elements (Eliason, 1984). Bone marrow cells were cultured at a final concentration of $5 \times 10^4$ nucleated cells/ml and mGM-CSF samples were diluted in EMED containing 1% BSA to be 10% v/v of the final mixture.

1-ml aliquots of the cell suspensions were plated in 35 mm Petri dishes and incubated at 37°C in a fully humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$. Duplicate cultures were prepared for each sample tested. After 7 days of incubation, all clones of >10 cells were counted by using an inverted microscope. Clones were typed as granulocyte (G), macrophage (M) or mixed (GM) based on the size of the cells and the colony morphology. In some experiments, the classification was confirmed by examination of Giemsa stained slides of cells from individual colonies. To compare unit values with published units from serum-containing assays, a dose-response curve for purified native mGM-CSF (kindly provided by Dr A.W.Burgess) was determined in a titration experiment.

*Cell proliferation assay*

The factor-dependent mouse cell line FDC-P1 (a kind gift from Dr T.M.Dexter) was maintained in alpha MEM supplemented with 10% fetal calf serum and 10% WEHI-3 conditioned medium. Thymidine uptake was performed according to the protocol of Hapel *et al.* (1984). $10^4$ washed FDC-P1 cells were incubated for 15 h with serial 2-fold dilutions of mGM-CSF. Cells were then pulsed for 6 h with 0.5 μCi [$^3$H]thymidine, harvested on glassfibre filters, and radioactivity determined by scintillation counting. For experiments testing antibody neutralization, the serum proteins were precipitated with 40% ammonium sulfate, dialysed and filtered. Two-fold serial dilutions of the ammonium sulfate fraction were incubated with 300 units of mGM-CSF for 1 h at 37°C in a volume of 100 μl, with a maximal serum concentration of 0.5 mg/ml in the assay. FDC-P1 cells were added to the antibody-mGM-CSF complex and thymidine uptake measured as above.

*Immunological methods*

New Zealand white rabbits were immunized at multiple sites intradermally with a suspension of purified recombinant mGM-CSF (200 μg in 1 ml plus 1 ml Freund's complete adjuvant) and were boosted twice at 1 month intervals. Serum was obtained 10–14 days after each boost. The antibody titre of the serum was determined by both ELISA and RIA.

Sera were tested by ELISA (Engvall, 1980) for antibodies against recombinant mGM-CSF which had been pre-adsorbed to microtiter plates. Serum was serially diluted 10-fold and the dilutions added to the pre-coated plates (50 μl/well). After incubation at 4°C for at least 2 h, the free immunoglobulins were removed by washing and the bound antibodies were treated with peroxidase-labeled goat anti-rabbit IgG (200 ng/ml in PBS containing 0.5% Tween 20). 50 μl of peroxidase substrate solution (containing 0.1% of ABTS and 0.03% of hydrogen peroxide in 0.1 M sodium citrate and 0.2 M sodium phosphate buffer solution, pH 4.0) was then added to each well. After 30 min the optical density of the reaction product was measured at 630 nm. Pre-immune serum and PBS were included as negative controls.

The RIA mixture contained 0.05 M sodium phosphate, pH 7.2, 1 mg/ml bacitracin, 0.025% Tween 20, 30 mg/ml of Protein-A Sepharose, serial dilutions of rabbit serum and $10^5$ c.p.m. of $^{35}$S-labeled recombinant mGM-CSF. After incubation at 4°C for 16–24 h, the mixtures were pelleted and washed and the bead-bound antigen-antibody complexes assayed by liquid scintillation counting.

*Protein purification*

30 g of recombinant mGM-CSF bearing *E. coli* cells were suspended in 100 ml of 0.1 M Tris, 0.05 M EDTA buffer, pH 7.5, using a Polytron homogenizer. The cell walls were structurally weakened by the addition of 25.6 g of sucrose and 30 mg of lysozyme and incubation at 30°C for 30 min. The cell suspension was lysed by passing twice through a french pressure cell at 8000 p.s.i. Soluble components of the cell were separated from the cell debris and mGM-CSF aggregates by centrifugation at 10 000 *g* for 30 min. The major membrane proteins and most of the oligopolysaccharide was extracted from the pellet by resuspending in the Tris-EDTA buffer containing 0.75 M guanidine hydrochloride and 1% (v/v) Tween 40. After three washes with this buffer, 3 g wet weight of cell debris and aggregate remained. The pellet was rinsed with PBS by suspension with the Polytron and centrifugation. The pellet was solubilized in 20 ml of 8 M guanidine hydrochloride, 5 mM dithiothreitol, using sonication. After clarification of the solution by centrifugation, it was applied directly to a 100 × 2.5 cm column of Sephadex G-100 equilibrated with 0.1 M Tris-HCl pH 7.8, 5 mM dithiothreitol and 8 M urea. 3-ml fractions were collected and the mGM-CSF fractions identified using SDS-PAGE (Laemmli, 1970).

*Other methods*

A cell-free transcription-translation system was used to synthesize the recombinant protein encoded by the expression vector (Zubay, 1973; Bottomley and Whitefeld, 1979). Lysates of bacterial strain D10 were prepared for *in vitro* protein synthesis and incubated together with plasmid DNA and [$^{35}$S]methionine to generate radiolabeled polypeptides (Gesteland, 1966). These were then separated by SDS-PAGE on 18% acrylamide gels which were dried and autoradiographed (Laemmli, 1970).

For immunoprecipitation, the radiolabeled polypeptides were mixed (1:10 v/v) with wash buffer (1% Triton X-100, 1% Aprotinin, 0.5% deoxycholate, 0.1% SDS in 0.1 M NaCl, 0.05 M Tris, and 1 mM EDTA) plus the antiserum. After incubation on ice for 90 min, Protein A-Sepharose beads were added. The bead-immunoprecipitate complexes were washed free of non-specifically bound polypeptides by repeated rinses with wash buffer. These complexes were then released from the beads by boiling and electrophoresed as above.

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