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Role of Carbohydrate in the Function of Human Granulocyte–Macrophage Colony-Stimulating Factor†

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ABSTRACT: cDNA clones for the human hematopoietic regulator granulocyte–macrophage colony-stimulating factor (hGM-CSF) were isolated from a λgt11 cDNA library prepared from RNA of COS cells transiently expressing the gene for hGM-CSF. As the RNA was a rich source of hGM-CSF mRNA, approximately 0.1% of the clones of this library contained hGM-CSF sequences. All of the clones analyzed were full length and were correctly processed. When subcloned into an expression vector and transfected into COS cells, the cDNA clones direct the synthesis of higher levels of the growth factor than the gene from which they were derived. The cDNA for native hGM-CSF was used to generate structural mutants which lack N-linked carbohydrate, O-linked carbohydrate, or both. Although the mutant proteins had differing specific activities, the nonglycosylated forms reproduce many, if not all, of the physiologic functions of authentic hGM-CSF. The role of carbohydrate in the secretion and function of hGM-CSF is discussed.

Colony-stimulating factors (CSFs) are acidic glycoproteins required for the survival, proliferation, and differentiation of hematopoietic progenitor cells in culture (Burgess & Metcalf, 1980). Functionally, the various CSFs are defined by the type of hematopoietic colony produced in semisolid culture. Hence, granulocyte–macrophage colony-stimulating factor (GM-CSF) stimulates the growth of progenitors which give rise to colonies containing granulocytes, macrophages, or a combination of both cell types (Wong et al., 1985). In addition to GM-CSF, granulocyte CSF (G-CSF or CSFβ), macrophage CSF (M-CSF or CSF-1), and multi-CSF (or IL-3) have been characterized and cloned from human sources (Souza et al., 1986; Kawasaki et al., 1985; Yang et al., 1986).

Recently, we obtained a genomic clone for GM-CSF from a human library in λ Charon 4A (Kaushansky et al., 1986). The gene is divided into four exons and three introns and contains the transcription- and translation-controlling elements typical of a eukaryotic structural gene. It is present in a single copy in the human genome and encodes a mature polypeptide of 127 amino acids. There are two N-linked glycosylation sites, and there are reported to be three O-linked glycosylation sites (S. C. Clark, personal communication) which account for approximately 6 kilodaltons (kDa) of the estimated 22 kDa of the mature growth factor.

Colony-stimulating factors are proteins of diverse physiologic function. We (Kaushansky et al., 1986) and others (Emerson et al., 1985) have found that recombinant hGM-CSF expressed in COS cells stimulates not only neutrophilic, eosinophilic, and monocyte–macrophage progenitor cells but also megakaryocyte colony-forming cells and, in the presence of erythropoietin, erythroid and mixed erythroid–nonerythroid colony-forming cells. Further, hGM-CSF has been shown to stimulate mature neutrophils to localize at sites of inflammation (Weisbart et al., 1985), mature eosinophils and monocytes to become activated and to enhance their killing of helminths (Handman & Burgess, 1979; Vadas et al., 1983), and mature monocytes and macrophages to enhance phagocytosis and tumor cell killing (Grabstein et al., 1986). In addition to these in vitro activities, recombinant hGM-CSF was recently demonstrated in primates to stimulate in vivo hematopoiesis (Donahue et al., 1986).

Despite the growing body of knowledge surrounding the in vitro and now in vivo physiology of hGM-CSF, little is known

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about the structural features responsible for the various functional properties of the growth factor. CSFs are heavily glycosylated molecules. Initially, carbohydrate was not thought to be necessary to support colony growth in vitro (Tsuneoka et al., 1981). Recently, however, it was reported that GM-CSF which lacks all carbohydrate failed to support erythroid progenitor cell proliferation (Burgess et al., 1987).

To investigate the structural features responsible for the various physiologic functions of hGM-CSF, structural mutants of the growth factor were prepared by in vitro site-directed mutagenesis. In order to facilitate this process, a cDNA clone for native human GM-CSF was first obtained. We describe here the generation of hGM-CSF cDNA clones by using the RNA from COS cells transiently expressing the genomic clone for hGM-CSF, the elimination of the two N-linked and all three O-linked glycosylation sites to produce variably glycosylated growth factors, and the physicochemical and functional characteristics of these structural mutants.

Materials and Methods

RNA Preparation and Analysis. COS cells transiently expressing the gene for hGM-CSF (Kaushansky et al., 1986) were grown to confluence. Human peripheral blood mononuclear cells were prepared as described (Kimura et al., 1984) and cultured in the presence of 1% phytohemagglutinin (PHA) for 24 h prior to harvest. RNA was prepared from cultured cells using guanidinium isothiocyanate (Chirgwin et al., 1979). Aliquots were denatured with formamide and formaldehyde and size-fractionated by electrophoresis through 1.4% formaldehyde–agarose gels (Rave & Boedtker, 1979), transferred to nitrocellulose (Thomas, 1980), and prehybridized at 42 °C for 4–6 h as described (Ulrich et al., 1984) with the addition of 10% dextran sulfate. A 540 base pair (bp) *Sst*I genomic fragment of hGM-CSF was purified from agarose gels and nick-translated to high specific activity [$(1\text{-}5) \times 10^8$ cpm/μg]. This probe begins in the promoter region of the gene and runs into the second intron. The probe containing 20 million cpm was added to 10 mL of prehybridization solution and the blot hybridized at 42 °C for 12–16 h. Blots were washed sequentially in 0.2× SSC/0.1% sodium dodecyl sulfate ($NaDodSO_4$) at 20, 40, and 65 °C and prepared for autoradiography.

cDNA Library Construction and Screening. Double-stranded cDNA was prepared by a modification of the RNase H/DNA polymerase I method (Gubler & Hoffman, 1983) adapted to the production of a λgt11 phage library (F. S. Hagen and C. Gray, unpublished results). The recombinant phage were plated at a density of 5×10^4 per 150-mm culture plate. Duplicate nitrocellulose lifts were prepared and prehybridized as for Northern blots, except that dextran sulfate was omitted. The filters were hybridized with 10^6 cpm/mL of nick-translated genomic probe and washed as for Northern blots. Several clones giving strong signals were plaque-purified, and phage DNA was prepared from liquid cultures (Maniatis et al., 1978). cDNA inserts were prepared by *Eco*RI digestion and gel electrophoresis. Agarose gel purified cDNA fragments were subcloned into pUC 13 for restriction analysis, into M13 for sequencing, and into the SV40 ori-based vector pDX (Busby et al., 1985) for transient expression of biologically active protein in COS cells.

Mammalian Cell Expression. cDNA restriction endonuclease fragments were blunted with T_4 polymerase, ligated to *Eco*RI linkers, digested with *Eco*RI, and subcloned into the unique *Eco*RI site of the mammalian cell expression vector pDX. Plasmid DNA was transfected into COS cells by calcium phosphate precipitation (Graham & Van der Eb, 1973). Culture medium [Dulbecco's modified essential medium, supplemented with antibiotics and either 10% fetal calf serum (FCS) or a serum-free supplement containing 1 μg/mL fibronectin, 10 μg/mL transferrin, 5 μg/mL insulin, and 15 nM selenous acid (Collaborative Research)] conditioned by the transfected COS cells was harvested after 3 days of incubation at 37 °C under 7% CO_2 and assayed for biologically active hGM-CSF.

Biological Assay for hGM-CSF. Bone marrow cells, obtained from normal volunteers with their informed consent, were fractionated on a Ficoll–hypaque density gradient (specific gravity 1.077). Low-density cells were depleted of adherent cells by double plastic adherence and of T cells by E-rosetting (Bagby et al., 1981). A total of 50 000–100 000 cells were cultured in α medium in the presence of 15% FCS, antibiotics, 0.9% methylcellulose, and up to 10% of the material to be assayed. Cultures were incubated for 13 days in a humidified atmosphere containing 5% CO_2, and granulocyte–macrophage colonies were enumerated by inverted microscopy. Each experiment described represents the mean of triplicate cultures. Fifty units of hGM-CSF are defined by the dilution which stimulates half-maximal colony formation, compared to an optimal concentration of phytohemagglutinin-stimulated lymphocyte conditioned medium (PHA-LCM). For the growth of megakaryocyte colonies, 25% human plasma was substituted for FCS (Kimura et al., 1984), and for the growth of erythroid bursts and mixed cell colonies (Powell et al., 1984), 1 unit of recombinant erythropoietin (Amgen, Inc.) was added on day 4 of culture. Optimal stimulation was provided by 1% PHA-LCM. For morphological analysis, cultures containing 50 units of the recombinant proteins were made semisolid with 0.3% agar, fixed, stained for chloroacetate esterase, and counterstained with toluidine blue. The colonies were enumerated and scored by direct microscopy.

In Vitro Mutagenesis. Two 22-base oligonucleotides were designed to replace the asparagine codons of residues 44 and 54 (Kaushansky et al., 1986) with glutamine codons, and one 35-base oligonucleotide was designed to replace the serine codons at positions 20, 22, and 24 (Kaushansky et al., 1986) with alanine codons. These serine residues have been shown to be the site of O-linked glycosylation in hGM-CSF (S. C. Clark, personal communication). For the production of cDNA which encodes polypeptides devoid of N-linked carbohydrate, mutagenesis was performed simultaneously with both oligonucleotides as described (Zoller & Smith, 1984) with the following modification. Screening for double N-linked mutants was performed by hybridizing duplicate lifts of phage to each ^{32}P end-labeled mutagenic oligonucleotide separately and washing with 3 M tetramethylammonium chloride (TMACl) at 62 °C (Wood et al., 1985). Clones hybridizing to both mutagenic oligonucleotides were plaque-purified and sequenced. For the production of O-linked carbohydrate-deficient cDNA, screening for the triple Ser → Ala mutant was performed by washing at 75 °C in 3 M TMACl. Each of the mutant cDNAs was sequenced by the dideoxynucleotide method (Sanger et al., 1977).

Concanavalin A–Agarose Chromatography. The serum-free supernatants from COS cells transiently expressing the native or mutant hGM-CSF cDNAs were concentrated by ultrafiltration and applied slowly to 5-mL columns of concanavalin A (Con A)–agarose (1 cm × 7 cm) previously equilibrated with 20 mM tris(hydroxymethyl)aminomethane (Tris), pH 7.4, 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. The columns were washed with 5 column volumes of

the same buffer, and bound glycoproteins were eluted with buffer containing 0.4 M methyl α-mannoside (αMM). Samples were dialyzed against several changes of phosphate-buffered saline (PBS) and assayed for biologically active hGM-CSF.

Generation of Anti-GM-CSF Antiserum. A 15-residue peptide (ESFKENLKDFLLVYC) matching residues 121–134 of hGM-CSF (Kaushansky et al., 1986) was synthesized (Pennisula Laboratories) and cross-linked with glutaraldehyde to keyhole limpet hemocyanin (KLH). This peptide matches a hydrophilic domain of hGM-CSF as predicted by the algorithm of Hopp and Woods (1981), is free of disulfide bonds or carbohydrate binding sites, and contains an additional tyrosine residue to allow radioiodination. The peptide–KLH conjugate was emulsified with complete Freund's adjuvant and used to immunize New Zealand White rabbits. Booster injections in incomplete Freund's adjuvant were given monthly. Antipeptide antibody titers were monitored by radioimmune precipitation of ^{125}I-labeled peptide. After 4 months, high antipeptide antibody titers were obtained, and the antisera were used for detection of denatured hGM-CSF by Western blotting.

Immunoblotting. Serum-free conditioned medium from COS cells transfected with either the native or the mutant hGM-CSF expression vectors was concentrated by ultrafiltration (Amicon YM-10). Native recombinant hGM-CSF was purified by gel filtration and reverse-phase high-performance liquid chromatography (HPLC) as described (Wong et al., 1985). N-Linked carbohydrate was removed enzymatically where indicated by overnight digestion with *N*-glycosylase (peptide:*N*-glycosidase F; Genzyme, Inc.) according to the manufacturer's recommendations. Protein samples were size-fractionated by reducing $NaDodSO_4$ gel electrophoresis and were transferred to nitrocellulose as described (Towbin et al., 1979) with the following modifications. Following electroblotting [transfer buffer (TRB) = 25 mM Tris–glycine, pH 8.3, in 20% methanol], protein was reduced in situ by treating with 25 mM β-mercaptoethanol in TRB at 80 °C for 30 min and then with 25 mM iodoacetic acid/400 mM Tris (pH 7.4)/50 mM NaCl at 20 °C for 30 min. The blot was incubated with the primary antiserum at 1:200 dilution in Western blot buffer [50 mM Tris (pH 7.4)/5 mM ethylenediaminetetraacetic acid (EDTA)/0.5% NP40/150 mM NaCl/0.25% gelatin] for 16 h at 4 °C, washed in buffer, treated with biotinylated goat anti-rabbit IgG (Vector Labs) at 1:1000 dilution in buffer for 60 min at 20 °C, and then washed and treated with an avidin–biotin–horseradish peroxidase complex (Vector Labs) for 60 min at 20 °C. Following a wash in buffer plus 0.4% Sarcosyl, the blot was developed in 4-chloro-1-naphthol (Bio-Rad) and washed with water. For quantitative analysis, ^{125}I-labeled goat anti-rabbit IgG was used in place of the biotinylated antibody. Following autoradiography of this blot, the immunoreactive proteins were removed and counted for γ emissions.

Results

RNA Analysis. To evaluate the level of hGM-CSF mRNA in transiently expressing COS cells, total cellular RNA was prepared and compared to RNA from peripheral blood lymphocytes which had been stimulated by 1% PHA for 24 h. These cells are one of the richest naturally occurring sources of hGM-CSF. Northern blot analysis, shown in Figure 1, demonstrated that transiently expressing COS cells contain significantly greater amounts of hGM-CSF-specific mRNA than PHA-stimulated lymphocytes. When the hybridizing bands were removed from the blot and counted, 9 times more

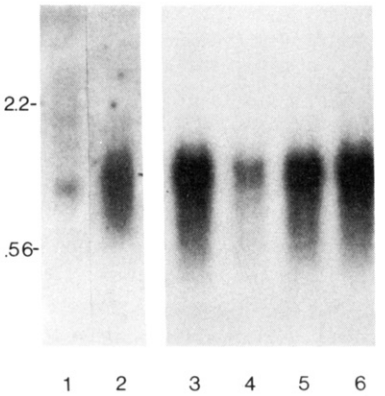

FIGURE 1: Northern blot analysis. Ten micrograms of whole cellular RNA obtained from lymphocytes stimulated for 24 h with phytohemagglutinin (lane 1) and from COS cells 2 days following transfection with the genomic GM-CSF expression vector pDgGM II (lane 2) was denatured with formamide and formaldehyde and size-fractionated by agarose gel electrophoresis. RNA was transferred to nitrocellulose and the blot probed for hGM-CSF-specific mRNA. In a separate experiment, 10 μg of RNA from COS cells transfected with pDcGMI (lane 3), pDcGMII (lane 4), pDcGMIII (lane 5), or pDcGMIV (lane 6) was denatured, size-fractionated, and probed for hGM-CSF-specific mRNA.

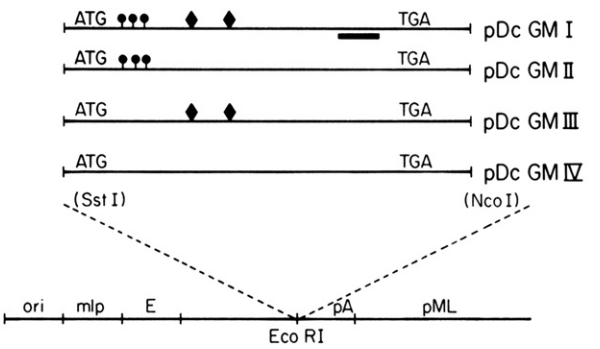

FIGURE 2: Expression vector pD3(X). The amp^R gene and *E. coli* origin of replication are derived from pML (Lusky & Botchan, 1981). The SV40 origin of replication (ori), the adenovirus major late promoter (mlp), the SV40 enhancer (E), and the adenovirus polyadenylation signal (pA) were assembled as unique restriction fragments (Busby et al., 1985). pDcGMI–IV were obtained by subcloning an *Sst*I/*Nco*I fragment of the cDNA derived from COS cells or the mutant cDNA derived from M13 into the *Eco*RI site of pDX. N-Linked glycosylation sites are indicated by (♦) and O-linked glycosylation sites by (●). The bar represents the location of the amino acid sequence used to prepare an anti-GM-CSF peptide antiserum. The locations of the initiation (ATG) and stop codons (TGA) are indicated.

probe hybridized to 10 μg of COS cell RNA than to 10 μg of RNA from stimulated lymphocytes. This blot also suggests which promoter is the more active in these COS cells. There are two alternative promoters, the hGM-CSF promoter and the expression vector adenovirus major late promoter (Figure 2). The hGM-CSF mRNA from COS cells migrates more slowly than the same mRNA from stimulated lymphocytes. As the adenovirus promoter and leader sequence of the expression vector add 253 bp to any inserted transcriptional unit, this analysis suggests that the major transcription initiation site is provided by the expression vector. This impression was confirmed by direct sequencing (see below).

cDNA Cloning and Expression. Poly(A+)-containing RNA was prepared from COS cells transiently expressing the gene for hGM-CSF and used to prepare a cDNA library in λgt11. Of the 5×10^5 recombinants prepared from 37 ng of cDNA, 3×10^5 were screened in duplicate by using a hGM-CSF genomic probe. Overall, 248 plaques hybridized strongly with a nick-translated genomic probe under very stringent wash

conditions, suggesting that approximately 0.1% of the clones contained cDNA for hGM-CSF. Six cDNA clones were plaque-purified, subcloned into M13, and sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977). All of the clones contained a single open reading frame, and their sequences matched that previously published (Wong et al., 1985; Lee et al., 1985) for hGM-CSF cDNA. In all six clones, the cDNA sequence begins approximately 20 bases downstream of the vector cap site. In contrast, the 3′ ends of all six cDNA clones correspond to the genomic polyadenylation signal.

One cDNA was subcloned into pDX and used to transiently express hGM-CSF. A 593 bp *Sst*I/*Nco*I fragment from the cap site to the middle of the 3′ untranslated region was removed from pUC 13 containing the entire cDNA insert and was subcloned into the mammalian cell expression vector (to produce pDcGMI, Figure 2). When compared to the genomic expression vector and controlled for time, vector concentration, and cell number, the cDNA vector directed the synthesis of 4-fold more recombinant hGM-CSF, or about 5×10^4 units/mL of serum-containing culture medium.

In Vitro Mutagenesis. To prepare cDNA coding for a polypeptide which could not be N-glycosylated, two 22-base oligonucleotides which hybridize to the cDNA for hGM-CSF were synthesized. One of these oligonucleotides contained the codon for glutamine in place of the asparagine codon of residue 44, the other for asparagine residue 54. These oligonucleotides were used for in vitro site-directed mutagenesis of a hGM-CSF cDNA clone in M13. One thousand recombinant phage were screened in duplicate with each ^{32}P end-labeled oligonucleotide using wash conditions stringent enough to allow only 21 or 22 base matches to retain a hybridization signal (Wood et al., 1985). Approximately 60 phage hybridized to either probe independently, while only 3 hybridized to both probes. In each of these mutant phage, both Asn → Gln mutations occurred as shown by DNA sequencing. Next, the native hGM-CSF cDNA and the N-linked mutant cDNA clones were mutated to replace the three serine residues known to contain O-linked carbohydrate (S. C. Clark, personal communication) with alanine codons. In this way, mutant cDNA clones which contain only O-linked, only N-linked, or none of the known carbohydrate binding site codons were generated. A 593 bp *Sst*I/*Nco*I restriction fragment was removed from the replicative form of each of the mutant M13 phage and was subcloned into pDX (to produce pDcGMII, pDcGIII, and pDcGMIV, Figure 2).

Analysis of Recombinant Proteins. To determine if biologically active hGM-CSF was expressed by the altered cDNA, pDcGMI, pDcGMII, pDcGMIII, and pDcGMIV were transfected separately into parallel cultures of COS cells. RNA was obtained from cells 2 days later, and culture medium was obtained from separate cultures after 3 days. As shown in Figure 1, the GM-CSF-specific mRNA levels were equivalent for all cDNA species except for pDcGMII, which contained half the amount of specific message. When culture supernatants were evaluated by marrow colony-forming assays, similar levels of colony-stimulating activity were detected, except for pDcGMII, which again contained half the activity of the other conditioned media (Table I).

To biochemically demonstrate the elimination of carbohydrate in the mutant polypeptides, the recombinant hGM-CSF preparations were analyzed by affinity chromatography. Serum-free COS cell supernatants were applied to a Con A–agarose column. After the column was washed, the bound glycoproteins were eluted with 0.4 M αMM. As shown in Table II, a significant amount of native hGM-CSF and protein which no longer contains O-linked carbohydrate bound to Con A–agarose and could be eluted from the column. In contrast, both mutant hGM-CSF species lacking N-linked carbohydrate failed to bind to Con A–agarose.

Table I: Expression of Nonglycosylated hGM-CSF[a]

medium	colony-stimulating activity (units/mL)	medium	colony-stimulating activity (units/mL)
saline	0	pDcGMII (–N)	1.0×10^4
sham-transfected	0	pDcGMIII (–O)	1.9×10^4
pDcGMI	2.2×10^4	pDcGMIV (–N–O)	2.0×10^4

[a] Serial dilutions of culture medium conditioned by COS cells transfected with the native expression vector pDcGMI and by the mutant vectors pDcGMII–IV were plated in marrow colony-forming assays. The results represent the mean of four separate experiments plated in triplicate.

Table II: Con A–Agarose Chromatography[a]

medium	nonbound	bound and eluted
pDcGMI (native)	70	30
pDcGMII (–N)	100	0
pDcGMIII (–O)	71	29
pDcGMIV (–N–O)	100	0

[a] A 2-mL column (6 mm × 80 mm) of Con A–agarose was equilibrated in 20 mM Tris (pH 7.5)/150 mM NaCl/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$. Media conditioned by COS cells transiently expressing the cDNA for hGM-CSF or its mutants were applied slowly (0.2 mL/min), and the column was washed with equilibration buffer. The column was eluted with 0.4 M methyl α-mannoside in equilibration buffer. The starting material, pass-through and wash, and elution fractions were dialyzed against PBS, filter-sterilized, and assayed for GM-CSF by colony formation. The results are expressed as the percentage of units applied recovered in the pass and wash and eluted fractions.

Next, to assess the relative specific activity of the various forms of GM-CSF produced by our mutant cDNAs in expression vectors, we analyzed the conditioned media immunologically. A rabbit antiserum was raised against a 15-residue synthetic peptide coupled to KLH. The antiserum failed to detect other growth factors, including hM-CSF, hG-CSF, PDGF, insulin, transferrin, or murine multi-CSF. This antiserum recognizes a region of GM-CSF far removed from the carbohydrate binding sites (Figure 2). To determine if the presence of carbohydrate affects antiserum binding, we enzymatically removed the carbohydrate present on native hGM-CSF. As shown in Figure 3, the antiserum binds as intensely to highly purified native GM-CSF as to the same amount of protein from which the carbohydrate has been removed enzymatically. Using this antiserum, we next analyzed the recombinant proteins present in equal volumes of medium conditioned by COS cells transfected with the native hGM-CSF expression vector and that of the various mutant cDNA vectors. As shown in Figure 3, substantially less immunoreactive protein is present in medium conditioned by COS cells transfected with DNA which lack N-linked carbohydrate binding sites than in that transfected with the native hGM-CSF. O-Linked carbohydrate does not affect the level of immunoreactive protein secreted by COS cells to a significant degree. Since similar amounts of biologically active protein (except half the amount of non-N-linked carbohydrate-containing protein) were analyzed in this experiment, these results suggested that the specific activities of the native and N-linked carbohydrate-deficient forms of hGM-CSF differed. To quantitate these findings, we used an ^{125}I-labeled second antibody on a Western blot containing equal biologi-

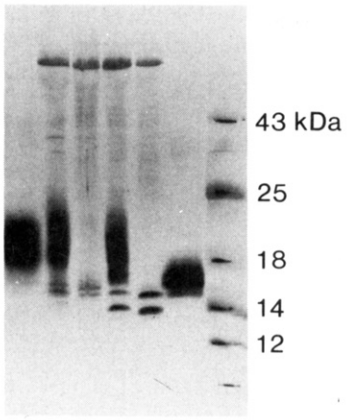

FIGURE 3: Immunoblotting of hGM-CSF. 2×10^4 units of HPLC-purified native hGM-CSF (lane 1) are compared to the same amount of material after complete digestion with *N*-glyconase F (lane 6). In lanes 2–5, equal volumes of concentrated serum-free medium conditioned by COS cells transfected with pDcGMI (lane 2), pDcGMII (lane 3), pDcGMIII (lane 4), and pDcGMIV (lane 5) are analyzed. The samples were size-fractionated by $NaDodSO_4$–polyacrylamide gel electrophoresis under reducing conditions, electrophoretically transferred to nitrocellulose, and probed for immunoreactive protein by Western blot as described under Materials and Methods. Molecular weight markers are shown.

Table III: Functional Evaluation of hGM-CSF Mutants[a]

medium: cell type	pDcGMI, native	pDcGMII (–N)	pDcGMIII (–O)	pDcGMIV (–N–O)
experiment 1				
neutrophil	67	71		
eosinophil	17	16		
monocyte	56	52		
experiment 2				
neutrophil	55		43	51
eosinophil	40		47	44
monocyte	32		29	39

[a] Bone marrow cells were prepared and cultured as described (Bagby et al., 1981) except they were made semisolid with 0.3% agar. Whole cultures were fixed, stained for chloroacetate esterase, and counterstained with toluidine blue as described under Materials and Methods. The data represent the results of triplicate plates containing a total of over 200 colonies for each form of hGM-CSF and are expressed as the percentage of colonies containing each cell type.

cally active amounts of the recombinant proteins. In four separate experiments, we found that 6 times more immunoreactive protein was present in 2×10^4 units of native hGM-CSF than in the same biologically active amount of the two forms which lack N-linked carbohydrate.

Functional Analysis of Recombinant Proteins. Limiting dilutions of serum-free COS cell supernatants were assayed for their ability to stimulate granulocyte and/or macrophage colony growth in semisolid culture. In five separate experiments, there were no significant differences between the maximal number of GM colonies produced by the native and the mutant forms of recombinant hGM-CSF. When the GM colonies were evaluated for cell type by cytochemistry, a similar distribution of neutrophils, eosinophils, and monocyte–macrophage-containing colonies developed (Table III). There were no significant differences when cultures were stimulated with varying concentrations of recombinant growth factor, or when colony size or cellular composition was analyzed.

Finally, both the mutant and the native recombinant proteins were capable of supporting the growth of megakaryocyte colonies and erythroid bursts. As shown in Table IV, concentrations of either recombinant native or mutant forms of hGM-CSF which would half-maximally stimulate GM colony formation were equal in their ability to maximally stimulate the growth of megakaryocyte colonies and erythroid bursts.

Table IV: Hematopoietic Colony Response to Native and Mutant hGM-CSF[a]

medium	colony no. granulocyte–macrophage	erythroid bursts	mega-karyocytes
sham-transfected COS CM	3.0 ± 1.1	3.4 ± 0.9	0
PHA-LCM	43.0 ± 3.0	28.0 ± 2.0	2.0 ● 0
native cDNA	29.0 ● 3.0	26.0 ± 1.0	3.3 ● 0.7
mutant cDNA			
pDcGMII (–N)	31.0 ± 5.0	29.0 ± 4.0	2.3 ● 0.9
pDcGMIII (–O)	29.0 ● 3.0	19.0 ± 3.0	ND[b]
pDcGMIV (–N–O)	36.0 ● 2.0	22.0 ± 2.0	ND

[a] Spent culture medium from COS cells transfected with pDcGMI (native) or pDcGMII-IV (mutant) cDNA expression vectors were plated at 1% final concentration in bone marrow colony-forming assays. Controls include sham-transfected COS cell-conditioned medium and 1% PHA-stimulated lymphocyte-conditioned medium (PHA-LCM). The data represent the mean number of colonies formed (±SEM) in a typical experiment plated in triplicate and have been reproduced 3 times. [b] ND = not done.

Discussion

The human hematopoietic regulator hGM-CSF is a trace glycoprotein found in many tissues and is required for the survival, proliferation, and differentiation of granulocyte and macrophage progenitor cells in vitro. In the present studies, we have examined the structure–function relationship of hGM-CSF carbohydrate by in vitro mutagenesis. To facilitate the generation of mutants, we cloned the cDNA for hGM-CSF by using COS cells transiently expressing the gene as a rich source of specific mRNA.

By eliminating the translation initiation and stop signals upstream of the coding sequence for the amino terminus of hGM-CSF (Kaushansky et al., 1986), we were able to express biologically active protein from a genomic clone of hGM-CSF at high levels using an SV40 ori-based expression system in COS cells. We reasoned that this would be a rich source of hGM-CSF-specific mRNA. When compared directly by Northern blot analysis, transiently expressing COS cells have approximately 10 times higher levels of specific mRNA than lectin-stimulated lymphocytes. When a λgt11 cDNA library was prepared from the RNA of these COS cells, we found that approximately 0.1% of the independent recombinant cDNA clones were hGM-CSF specific. All of the clones assessed demonstrated proper intron splicing, and all of the clones sequenced contained a complete copy of hGM-CSF cDNA. Finally, the cDNA was able to direct the synthesis of higher levels of biologically active hGM-CSF than the genomic expression vector. Thus, using a genomic clone in a mammalian cell expression vector, we were able to efficiently generate a cDNA clone for hGM-CSF.

Once a complete cDNA for hGM-CSF was available, site-directed mutagenesis was facilitated. A double mutant was generated in a single reaction, resulting in cDNA coding for a polypeptide lacking N-linked glycosylation sites. Additional mutagenesis generated mutants which lacked the serine residues known to contain O-linked carbohydrate, and mutants which lacked both forms of carbohydrate. These cDNA were transcribed at levels similar to the wild-type cDNA, and the recombinant polypeptides produced were demonstrated to lack terminal mannose residues by chromatography over Con A–agarose. When the relative specific activity of the mutant recombinant proteins was assessed in vitro, we found that polypeptides lacking N-linked carbohy-

drate had approximately 6-fold higher specific activity. Two previous studies have commented on the relative specific activities of murine GM-CSF and forms lacking some or all of the carbohydrate present in the native molecule. Sparrow and co-workers (Sparrow et al., 1985) compared the specific activity of asialo-GM-CSF to a previously reported value for native murine GM-CSF and found significantly greater activity for the asialo form. Also, DeLamarter and co-workers (DeLamarter et al., 1985) report a specific activity of murine GM-CSF produced in *E. coli* higher than that reported for the native molecule.

Finally, the functional properties of the recombinant mutant proteins were compared to those of the native growth factor. Using whole agar cultures and cytochemistry, we have shown that the distribution of cell types stimulated by the native and nonglycosylated forms of hGM-CSF was similar. Furthermore, by dose–response analysis, both forms were equally able to stimulate the growth of megakaryocyte colonies and, in the presence of erythropoietin, to stimulate the growth of erythroid bursts.

From these studies, it is clear that carbohydrate modification is not necessary to allow the full range of progenitor cell stimulation provided by native hGM-CSF. Although mature cell responses to nonglycosylated hGM-CSF were not fully assessed in this study, the recombinant mutant hGM-CSF were able to inhibit the migration of neutrophils in response to the chemoattractant fMet-Leu-Phe (data not shown). Thus, the role of the carbohydrate modification of hGM-CSF is uncertain. One potential role for carbohydrate modification was suggested by our studies. Despite equivalent efficiency of transfection, and nearly equal levels of transcription, the expression vectors containing the N-linked carbohydrate-deficient mutant cDNA consistently secreted far less immunoreactive protein than the native hGM-CSF expression vector. Thus, N-linked carbohydrate may play a role in the secretory process in COS cells. Other functions suggested for carbohydrate modification include enhanced survival in the circulation, augmentation of binding to plasma proteins for transport, or enhancement of protein solubility (Ashwell & Morrell, 1974).

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Stoichiometry of *lac* Repressor Binding to Nonspecific DNA: Three Different Complexes Form†

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ABSTRACT: The stoichiometry of *lac* repressor binding to nonspecific DNA was investigated by three different techniques. Four molecules of the fluorescent probe 5,5′-bis(8-anilino-1-naphthalenesulfonate) [bis(ANS)] bind to each repressor subunit with an average dissociation constant of 20 μM. Nonspecific DNA displaces most of this bound bis(ANS), reducing the fluorescence. Titrations of repressor with nonspecific DNA monitored with high [bis(ANS)] (5–15 μM) had end points at 8 base pairs per repressor. Lower [bis(ANS)] (0.1–1 μM) resulted in end points at either 15 or 26 base pairs per repressor, depending on the ionic strength. These end points correspond to complexes containing approximately one, two, or four repressors per 28 base pairs. Boundary sedimentation velocity experiments with saturating amounts of repressor revealed that five repressors can bind to 28 base pairs. By monitoring the circular dichroism as DNA was added to repressor, the sequential appearance of complexes containing approximately four, two, and one repressors per 28 base pairs was observed. The inability of repressor cores or iodinated repressor to bind to complexes containing one or two repressors per 28 base pairs implies that all of the repressors directly contact the DNA in the complex containing four repressors per 28 base pairs. It is proposed that while two subunits of each repressor contact the DNA in complexes containing one or two repressors per 28 base pairs, only one subunit of each repressor contacts the DNA in the complex with four repressors per 28 base pairs. These results suggest a novel mechanism for the one-dimensional diffusion of repressor along DNA.

The binding of the *lac* repressor both to its operator and to nonspecific DNA has been extensively studied and serves as a model for understanding specific protein–DNA interactions (Bourgeois & Pfahl, 1976; Muller-Hill, 1975; Wu et al., 1978). Many investigators have reported that the *lac* repressor binds to nonspecific DNA to form a complex containing two tetrameric repressor molecules per 28 base pairs of DNA, i.e., a complex with 14 base pairs per tetramer. This conclusion has been reached on the basis of circular dichroism studies (Butler et al., 1977; Durand & Maurizot, 1980), boundary sedimentation velocity studies (Revzin & von Hippel, 1977), thermal melting of the DNA in the presence of repressor (Wang et al., 1977), and studies with a fluorescent probe covalently bound to the repressor (Kelsey et al., 1979). A popular explanation for this result is that while each repressor spans 28 base pairs along the DNA, repressor molecules can bind along opposite sides of the same DNA segment, giving a complex with two tetramers per 28 base pairs. Zingsheim et al. (1977) have provided direct visual support for this model by observing a double layer of repressor tetramers bound to nonspecific DNA in electron micrographs. In contrast, Worah et al. (1978) have observed the formation of a complex containing only one repressor per 28 base pairs, using the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS).[1] This result implies that the first and second tetramers to bind per 28 base pairs are bound differently.

In this study, the stoichiometry of *lac* repressor binding to nonspecific DNA has been investigated in three different ways: by use of the fluorescent probe 5,5′-bis(8-anilino-1-naphthalenesulfonate) [bis(ANS)], by the boundary sedimentation velocity technique, and by circular dichroism. Bis(ANS), a covalent dimer of ANS (Farris et al., 1978), binds

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[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; bis(ANS), 5,5′-bis(8-anilino-1-naphthalenesulfonate); CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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