Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 4872–4876, July 1989
Biochemistry

Identification of critical regions in mouse granulocyte–macrophage colony-stimulating factor by scanning-deletion analysis

(expression/structure–function/mutagenesis)

ARMEN B. SHANAFELT AND ROBERT A. KASTELEIN†

Department of Molecular Biology, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304-1104

Communicated by Charles Yanofsky, March 29, 1989 (received for review February 28, 1989)

ABSTRACT **Structure–function relationships for mouse granulocyte–macrophage colony-stimulating factor were examined by generating a series of small deletions scanning the entire length of the molecule. Deletions of three amino acids were introduced at intervals of five amino acids by site-directed mutagenesis of the mature mouse granulocyte–macrophage colony-stimulating factor gene. The mutant proteins were expressed in *Escherichia coli* and assayed for biological activity. This procedure identified four regions critical to activity. These critical regions were further delineated by additional three-amino acid deletion mutants. Larger deletions at each terminus were also made, as well as changes of specific amino acid residues. The four critical regions span amino acid residues 18–22, 34–41, 52–61, and 94–115. The disulfide bridge between Cys-51 and Cys-93 was also shown to be essential for activity, whereas that between Cys-85 and Cys-118 could be removed without loss of activity. The possible structural and/or functional roles of the critical regions are discussed.**

The proliferation and differentiation of granulocytes and macrophages from progenitor bone marrow cells is mediated by a group of protein hormones known as the colony-stimulating factors (1). Four distinct colony-stimulating factors have been identified in both mouse and human; one of these is granulocyte–macrophage colony-stimulating factor (GM-CSF). It is produced by various cell types, such as T lymphocytes and monocytes (2), and has multiple activities *in vitro*. It not only stimulates the formation of granulocytes and macrophages from bone marrow progenitor cells (1) but also can activate mature eosinophils, neutrophils, and macrophages (3).

Both mouse (m) and human (h) GM-CSF have been characterized by cloning, sequencing, and expression (4, 5). The mGM-CSF polypeptide (124 residues; M_r, 14,346) is 54% identical to the hGM-CSF polypeptide (127 residues; M_r, 14,650). Despite the high degree of homology, the two polypeptides are species specific (5).

Limited information is available on structure–function relationships for GM-CSF. Proteolytic digestion of both proteins identified two disulfide bonds, linking the first and third, and second and fourth cysteine residues (6). Using chemically synthesized hGM-CSF protein fragments and analogs, Clark-Lewis *et al.* (7) identified residues 1–13 and residues 122–127 as being not critical and residues 14–25 as being critical for biological activity. Mutagenesis of mGM-CSF by Gough *et al.* (8) revealed that residues 11–15, 24–37, 47–49, and 81–89 are obligatory for function. Physical and biochemical studies indicate that both the murine and human proteins have similar secondary structures containing α-helical and β-sheet segments (9).

In this report we describe an analysis of a set of deletion mutants that together span the entire length of mGM-CSF. Characterization of 42 deletion proteins, expressed in *Escherichia coli*, has allowed us to identify and delineate four regions within mGM-CSF that are critical to the activity of the protein. Additional deletion and substitution mutants further define the importance of these and other regions of the molecule.

MATERIALS AND METHODS

Bacterial Host Strains and Vectors. The *E. coli* K12 strain JM101 (10) was used as host for the propagation and maintenance of M13 DNA. CJ236 (11) was used to prepare uracil-DNA for use in site-directed mutagenesis. AB1899 (12) was used as the host for expression of wild-type and mutant mGM-CSF. pINIIIompA2 (13) was used as the expression vector for all mGM-CSF genes. Elsewhere, we have described the expression of biologically active, mature GM-CSF with this *E. coli* secretory expression system (14). The mGM-CSF coding region was synthetically reconstructed using a series of oligonucleotides. Unique restriction sites were introduced where possible without altering the protein sequence and high-bias *E. coli* codons (15) were preferentially used. From this construct, an *Xba* I–*Bam*HI fragment containing the *ompA* leader sequence and the entire mGM-CSF gene was cloned in M13mp19 (replicative form) and used as the template for site-directed mutagenesis.

Mutagenesis, Recombinant DNA, and Sequencing Protocols. Site-directed mutagenesis followed the protocol described by Kunkel *et al.* (11). Oligonucleotides, 20–28 nucleotides long, corresponding to mGM-CSF sequences incorporating the desired deletion or mutation were made complementary to the template DNA and used as primers in the mutagenesis reactions. Three (to five) plaques from each reaction were sequenced using the dideoxynucleotide method (16) with modifications described in the Sequenase (United States Biochemical) protocol. Where possible, the entire GM-CSF coding sequence was examined.

M13 (replicative form) DNA containing correct mutations was cleaved with *Xba* I and *Bam*HI (New England Biolabs). The digested M13 (replicative form) was combined in a 40-μl ligation mixture containing 0.2 μg of pINIIIompA2 (cleaved with *Xba* I and *Bam*HI) and incubated at 12°C for 4 hr. The ligation mixture was used to transform JM101 cells, and selection was made on Luria broth (LB) plates containing ampicillin at 50 μg/ml. Correct clones were transformed into AB1899 for protein expression.

Gel Electrophoresis and Immunoblotting. SDS/polyacrylamide gel electrophoresis (SDS/PAGE) was performed as described by Laemmli (17). Immunoblotting was performed essentially as described by Towbin *et al.* (18), except that

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: GM-CSF, granulocyte–macrophage colony-stimulating factor; m, mouse; h, human.

†To whom reprint requests should be addressed.

transfer occurred at 4°C for 16 hr at 30 V. The primary antibody was anti-mGM-CSF peptide hybridoma mg1.8.2 used at a 1:100 dilution of culture supernatant (19). Secondary antibody was ^{125}I-labeled mouse anti-rat IgG (Amersham) used at a 1:1000 dilution. Dried blots were exposed to Fuji RX film with an intensifying screen for 12–16 hr at −80°C.

Preparation and Quantitation of Protein Extracts. Five milliliters of LB containing ampicillin at 50 μg/ml were inoculated with 100 μl of medium from individual overnight cultures of AB1899 harboring the wild-type or a mutant expression plasmid. The cultures were grown to mid-logarithmic phase at 37°C, and protein expression was induced for 4 hr with 2 mM isopropyl β-D-thiogalactopyranoside, final concentration. Three milliliters of culture were centrifuged in a microcentrifuge (American Scientific Products, Stone Mountain, GA) for 5 min, and the pellet was resuspended in 500 μl of 10 mM sodium phosphate/150 mM NaCl, pH 7.0. The cell suspension was then lysed by sonication in a Branson Cell Disruptor 200 sonifier with 30 0.5-sec pulses at 40 W. SDS (0.1%, final concentration) was added to the lysates, and the mixtures were incubated for 5 min at room temperature and then centrifuged for 5 min in the microcentrifuge. The supernatant was recovered, frozen, then thawed, and centrifuged for 5 min in the microcentrifuge. Also, osmotic shock fractions of some of the mutants were prepared as described by Koshland and Botstein (20). This procedure does not require SDS. No difference in relative activity could be detected for mutant proteins prepared according to either procedure.

Aliquots of samples were analyzed by SDS/PAGE and Western immunoblotting. Autoradiograms were scanned by an LKB Ultroscan XL laser densitometer, and the concentration of wild-type and each mutant mGM-CSF protein was determined by comparing the area corresponding to the processed form of each protein with a standard curve generated with purified *E. coli*-derived recombinant mGM-CSF. A lane containing 30 ng of purified mGM-CSF was included on each gel for use as an internal calibration standard. The variation in protein concentration was found to be 3- to 5-fold based on analysis of extracts from four independent clones of the synthetic mGM-CSF construct expressed in AB1899 cells.

NFS-60 Proliferation Assay for mGM-CSF Activity. Extracts were assayed using the myeloid cell line NFS-60. Sample concentrations of mGM-CSF protein were adjusted to 36 ng/ml and titrated in quadruplicate to 0.2 pg/ml. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay described by Mosmann (21) was used to measure the extent of proliferation, and absorbance values were read with a V_{max} kinetic microplate reader (Molecular Devices, Palo Alto, CA). The concentration of each mutant and wild-type mGM-CSF that gave 50% maximum response was determined, and relative activity was calculated using the relationship: % activity = ($[\text{wild-type}]_{1/2}/[\text{mutant}]_{1/2}$) × 100%, where $[\text{wild-type}]_{1/2}$ and $[\text{mutant}]_{1/2}$ are the concentrations of wild-type and mutant mGM-CSF proteins, respectively, that gave 50% maximum response in the NFS-60 assay.

RESULTS

Systematic Introduction of Deletions Throughout mGM-CSF. The structural and functional importance of specific regions within the mGM-CSF polypeptide were determined by the systematic introduction of deletions along the entire length of the molecule (Fig. 1). Deletions of three amino acids were introduced every five amino acids on a synthetically generated mGM-CSF DNA sequence by site-directed mutagenesis. The positions of certain deletions (∇10 and ∇17) were chosen to preserve cysteine residues. In all cases the altered

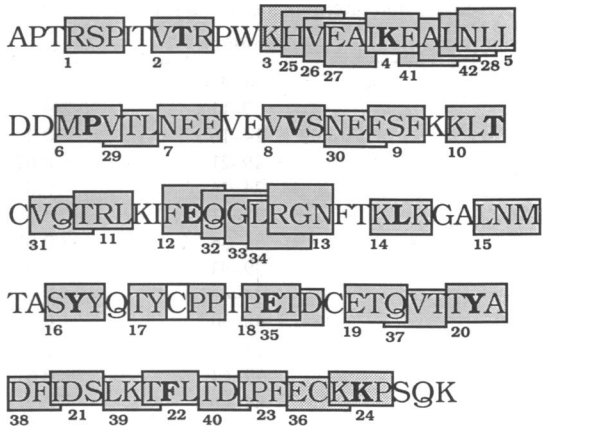

FIG. 1. Location of deletions. The primary amino acid sequence of mGM-CSF is shown with every 10th amino acid in boldface type. Each shaded box contains the amino acids deleted for a given mutant; the numerals below each box identify the respective mutant.

mGM-CSF coding regions were expressed using the plasmid vector pINIIIompA2 (13). The leader sequence of the *ompA* gene was fused to the mGM-CSF mature sequence, directing the mGM-CSF protein to the *E. coli* periplasm. Here the leader sequence is removed, generating mature GM-CSF (14). The *E. coli lon*$^-$ host AB1899 was used to minimize proteolysis in the hope that similar expression levels would be obtained for each mutant protein (12). The amount of each mutant polypeptide produced was determined by Western immunoblotting. Levels of altered GM-CSF in *E. coli* extracts were found to vary over a 10-fold range. We do not know whether this variation reflects differences in expression or stability of individual polypeptides in this *E. coli* host. Quantitative immunoblotting allowed us to correct for differences in the amounts of protein found in the extracts. Samples containing each mutant protein at 36 ng/ml were assayed for their ability to stimulate the proliferation of the mouse myeloid cell line NFS-60. Results were expressed as specific activity relative to that of native mGM-CSF produced and assayed under the same conditions (Table 1). The sensitivity of the assay was estimated to be 0.01% of wild-type activity. Any mutant protein exhibiting less than this activity was considered inactive, and the amino acid residues in a corresponding deletion mutant were referred to as critical to the activity of mGM-CSF. Mutants of >0.01% wild-type activity were considered active, and the deleted residues of such mutants were defined as noncritical. The term critical does not distinguish between those residues that are critical for the structural integrity of the polypeptide or those residues that are involved in a direct functional role—e.g., receptor binding, activation, or both.

Definition of Four Regions Critical to the Activity of mGM-CSF. Deletions in an initial set of 24 unique polypeptides defined four regions that were most critical to the activity of mGM-CSF (Table 1; ∇1–24). Additional deletion mutants (Table 1; ∇25–42) were generated and tested for activity to define more precisely the bounderies of the critical regions identified in the first set of mutants.

Critical region I. This region is defined by the mutants ∇4, -5, -25, -26, -27, -28, -41 and -42 and corresponds to amino acids Ala-18 to Ala-22. Mutants ∇5 and -28 cover amino acids Leu-23 to Leu-26. Although these deletions result in a 100-fold decrease of activity, Leu-23 to Leu-26 are considered expendable according to our definition of a critical residue. Such regions are undoubtedly important for a fully functional GM-CSF polypeptide but do not constitute critical residues.

Table 1. Activity and alterations of mutant mGM-CSF proteins

Mutant protein†	Alteration‡	% activity§
∇1	4–6	343
∇2	9–11	789
∇3	14–16	70.3
∇4	19–21	<0.01
∇5	24–26	0.8
∇6	29–31	4.6
∇7	34–36	<0.01
∇8	39–41	<0.01
∇9	44–46	1.9
∇10	48–50	0.3
∇11	54–56	<0.01
∇12	59–61	<0.01
∇13	64–66	572
∇14	69–71	5.7
∇15	74–76	1.1
∇16	79–81	3.0
∇17	83–84:86–87	9.9
∇18	89–91	0.07
∇19	94–96	<0.01
∇20	99–101	<0.01
∇21	104–106	<0.01
∇22	109–111	<0.01
∇23	114–116	<0.01
∇24	119–121	167
∇25	15–17	5.6
∇26	16–18	<0.01
∇27	17–19	<0.01
∇28	23–25	0.7
∇29	31–33	3.7
∇30	42–44	0.1
∇31	52–54	<0.01
∇32	61–63	<0.01
∇33	62–64	0.03
∇34	63–65	0.03
∇35	90–92	0.02
∇36	117–119	1.5
∇37	96–98	<0.01
∇38	102–104	<0.01
∇39	107–109	<0.01
∇40	112–114	<0.01
∇41	21–23	<0.01
∇42	22–24	<0.01
∇N19	2–19	<0.01
∇C94	94–124, Cys-85 → Ser	<0.01
∇C116	116–124	94.1
C118*	Cys-118 → Stop	158
K119*	Lys-119 → Stop	968
C51S	Cys-51 → Ser	<0.01
C85S	Cys-85 → Ser	15.4
C93S	Cys-93 → Ser	<0.01
C118S	Cys-118 → Ser	6.1
C51:93S	Cys-51 → Ser, Cys-93 → Ser	<0.01
C85:118S	Cys-85 → Ser, Cys-118 → Ser	129
mGM-CSF	Wild-type	100

†∇1 to ∇24 were the first series of deletion mutants tested.
‡Numbers indicate residues deleted in deletion mutants or residue changes.
§Activity is relative to that of wild-type mGM-CSF.

Critical region II. This region is defined by the mutants ∇7, -8, -29, and -30 and corresponds to amino acids Asn-34 to Ser-41. ∇7 and -8 are deletions interior to the critical region; ∇29 and -30 define the maximum N- and C-terminal borders, respectively. ∇29 has appreciable activity, ≈3.7%, and thus it is possible that this critical region does not begin until Glu-35 or Glu-36; however, it must contain Glu-36 as this is the most 3′ residue contained in ∇7. Conversely, ∇30 has very low activity, <0.1%, and thus the C-terminal border is most likely defined by Ser-41.

Critical Region III. This region is defined by deletion mutants ∇II, -12, -31,-32, -33, and -34 and comprises amino acids Val-52 to Gln-61. Both ∇11 and -31 are inactive and contain Thr-54, and thus this residue defines the minimum N-terminal boundary of this critical region. The use of additional three-amino acid deletion mutants at this junction is problematical, as they necessarily would include Cys-51 that was shown to be essential for function (see below). The mutants ∇32, -33, and -34 precisely define the C-terminal border of this critical region as Gln-61. In this overlapping series, ∇32 is inactive, but ∇33 and -34 have low activity (≈0.03%).

Critical Region IV. This region is defined by the mutants ∇19, -20, -21, -22, -23, -36, -37, -38, -39, -40, and -C116 and comprises amino acids Glu-94 to Pro-115. It is the largest critical region in the molecule, spanning 22 amino acids or ≈18% of the mature mGM-CSF sequence. The N-terminal border is defined by ∇19 and, as in critical region III, is flanked by a critical cysteine residue, Cys-93 (see below). In this case, the amino acids N-terminal to Cys-93 appear to be fairly important for activity, as both ∇18 and -35 have very low activity (0.07 and 0.02%, respectively). The C-terminal end is defined by either Ile-114 or Pro-115, as both of these residues are contained in ∇23, and Pro-115 is the final residue in the active ∇C116 protein product.

Fig. 2 is an illustration of the relative biological activity of all 42 deletion proteins vs. the location of their deletions. This representation reflects the relative contribution of each segment of the molecule to the activity of intact GM-CSF, revealing both critical and noncritical regions. The four critical regions comprise 35% of the residues. The other 65% of the molecule can tolerate small deletions without complete loss of activity. It is important to note that, although 65% of the residues in GM-CSF seem noncritical, this does not imply that these regions can be deleted altogether; as is evident from Fig. 2, even small deletions, such as those generated here, often result in a substantial reduction in activity. Some mutant proteins have an activity that is considerably higher than that of native mGM-CSF. Deletions in these hyperactive

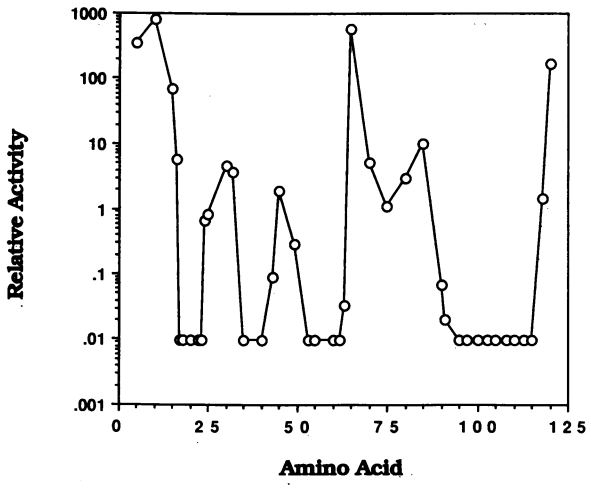

FIG. 2. Relative activity vs. location of deletion. Each mutant protein was assayed by its ability to drive NFS-60 proliferation. The percent of its half-maximal response relative to wild-type mGM-CSF is plotted against the location of the central amino acid of the deletion. The sensitivity of the assay was estimated to be 0.01% of wild-type activity; any mutant protein containing less than this activity was scored as inactive.

proteins seem to cluster at either terminus. At present we have no explanation for this phenomenon.

The N and C Termini Are Not Important for Activity. *N terminus.* Four N-terminal deletion mutants, ∇1, -2, -3, and -25, retain biological activity. All except ∇25, which lies at the border of critical region I, have close to, or greater than, full activity. ∇25 has appreciable activity (5.6%). These data suggest that the N-terminal 16 amino acids are expendable, and that Glu-17 is noncritical. The mutant ∇N19, which deletes Pro-2 to Ile-19, was generated to mimic active mutants of human GM-CSF (7). However, we found this mutant protein to be inactive, consistent with our observation that it contained the two N-terminal residues of critical region I.

C terminus. The mutant ∇C116, which deletes the C-terminal 9 amino acids, is fully active. As the number of deleted amino acid residues decreases, the activity of each successive molecule increases: C118* increases to 158%; K119*, encoding Ala-1 to Cys-118, increases to 968%. ∇24 and -36 have deletions in the expendable C-terminal domain and both yield active molecules, but they have significantly less activity than the corresponding C-terminal "complete" deletion mutants (1.5% for ∇36 vs. 94% for ∇C116; 167% for ∇24 vs. 968% for K119*).

Sequences Between Critical Regions Are Not Essential for Activity. Segments of GM-CSF that could tolerate small deletions without a total loss of activity were defined as noncritical. Three groups of active mutants can be identified: (*i*) those of greater or equal activity as wild-type; (*ii*) those having appreciable, but reduced activity (1–10%); and (*iii*) those of greatly diminished activity (<1%) (see Fig. 2).

The first group is comprised of almost all N- and C-terminal mutants: ∇1, -2, and -3 at the N terminus, and ∇24, K119*, C118*, and ∇C116 at the C terminus. Only mutant ∇13 at amino acids 64–66, which falls immediately after critical region III, results in a fully active molecule with an internal deletion. The intermediate class includes ∇5, -6, -9, -14, -15, -16, -17, -25, -28, -29, and -36 (∇5 and -28 have close to 1% activity). Although clearly important to the activity of the molecule, the amino acids defined by these deletions are not critical. The last group includes ∇10, -18, -30, -33, -34, and -35. All fall on the borders of critical regions and have greatly reduced activities. Such mutant proteins are probably of low activity because they may perturb the integrity of the critical regions (see *Discussion*).

The Disulfide Bridge from Cys-51 to Cys-93 Is Critical for Activity. Protease digestion analysis of h- and mGM-CSF (6) suggests that the four cysteines in GM-CSF pair 1–3 and 2–4. We generated mutant proteins that changed the four individual cysteines to chemically similar serine residues, as well as double mutants that changed both cysteines of the proposed pairs to serines. The activities of each of these mutant proteins are summarized in Table 1.

Changes of either or both Cys-51 and Cys-93 eliminated biological activity. These results show that the formation of this disulfide bond is absolutely required for an active molecule. In contrast, changes of Cys-85 or Cys-118 resulted in active molecules, albeit with lower than wild-type activity. The double mutant C85:118S, in which both Cys-85 and Cys-118 were replaced by Ser, retained full activity. As one or both of these cysteines could be removed without eliminating activity, the disulfide bridge from Cys-85 to Cys-118 is evidently nonessential. The reduction of activity by an unpaired cysteine, as observed for mutant proteins C85S and C118S, in which Cys-85 was replaced by Ser and Cys-118 was replaced by Ser, respectively, may result from formation of inappropriate disulfide linkages.

DISCUSSION

The aim of our work was to delineate regions critical to the activity of mGM-CSF. Four regions of this nature, comprising 35% of the mature protein, were identified using systematic incorporation of deletions into the wild-type sequence. The decision to set the size of the deletions at three amino acids was based on the fine structural deletion map of the lymphokine mouse interleukin 2 (22). In this study, three regions critical to activity were identified by a variety of techniques. A hypothetical deletion analysis of mouse interleukin 2 with various deletion sizes revealed that all three critical regions could be identified using deletions of three amino acids incorporated as infrequently as every 10 amino acids. Larger deletions would require fewer mutants but present the risk of introducing gross structural changes causing inactivation independent of removing functionally important regions. Smaller deletions, although increasing the precision in delineating boundaries, begin to require large numbers of mutants to effectively locate all important regions. The series of deletions used here left not more than two adjacent amino acids unperturbed, making it unlikely that any critical regions was overlooked.

Identification of critical regions exposed by the deletion approach used here requires careful consideration. By deleting any three-residue region, neighboring residues may be forced to occupy the space vacated by the missing residues. This transposition of residues may sometimes result in an inactive mutant as important residues are dislocated from their natural position. This type of secondary effect would result in the identification of these residues as being critical, even though they are not directly responsible for biological function. We cannot rule out this possibility. However, the removed residues are justifiably defined as critical since they are required for an active molecule. With respect to this issue, it is noteworthy that all highly defective mutant proteins (<1% activity) fall on the borders of critical regions. Presumably these proteins have severely reduced activities because they perturb the neighboring critical regions.

To identify amino acid residues that are most important for activity, only those residues deleted in mutant proteins with undetectable activity were scored as critical. A consequence of this arbitrary definition was that deleted residues of some highly defective mutant proteins were considered noncritical. This does not imply that these residues are unimportant: it simply means that these residues are of lesser importance than critical residues.

A synthetic mGM-CSF gene was used as the base template for generating all the mutants used in this study. Although it is not necessary for the methodology employed, we chose to make a synthetic gene because it facilitates examination through other mutational techniques. By identifying a limited number of regions important to an active molecule, it will be possible to focus on these areas using more intensive methods such as saturation mutagenesis and the random replacement of specific amino acids. The presence of unique restriction sites available in a synthetic gene permits convenient access to these regions of interest.

The nature of a critical region, whether it is functionally or structurally important, cannot be distinguished by the deletion method employed here. Extensive analysis of each critical segment will be required to determine their specific roles. However, some information can be obtained by a parallel analysis of the critical regions of m- and hGM-CSF. These proteins are similar molecules, both in primary sequence (≈54% identity) and conformation (9). Yet the two polypeptides are species specific. Comparison of the work described here and that of Clark-Lewis *et al.* (7) on synthetic peptide analogs of hGM-CSF shows that the two species share similar domains. The size and relative location of our critical region I coincide with that found in hGM-CSF. Similarities in amino acid composition around this region also imply that these respective critical regions are acting in a like manner. The strongly hydrophobic nature of this portion of

the molecule as well as the observation that Trp-13 (of mouse) is solvent-inaccessible (9) indicate the region is internal. This suggests that critical region I is structurally important. In apparent contradiction with our results, Clark-Lewis *et al.* (7) find that a hGM-CSF molecule extending to residue 96 (the third cysteine) retains residual activity; the corresponding mGM-CSF deletion mutant ∇C94 has no activity. This discrepancy could reflect a difference in the sensitivity of the assay.

Gough *et al.* (8) have identified residues 11–15, 24–37, 47–49, and 81–89 as required for generating a functional mGM-CSF. The present work and that of Zurawski and Zurawski (22) demonstrate the importance of correlating expression levels of mutant proteins with their corresponding activities. The inability of Gough *et al.* (8) to determine the mutant protein concentrations may explain why none of the critical regions identified in the present work coincide with their results; it is possible that insufficient expression of mutant proteins accounts for their negative assay results. Additionally, their use of large deletions to identify critical regions introduces the potential of gross structural changes that may have resulted in nonspecific inactivation of the mGM-CSF protein.

Circular dichroism measurements (9) and structure predictions using the algorithm of Chou and Fasman (23) suggest that m- and hGM-CSF are composed of both α-helical and β-sheet segments. We have compared the predicted locations of secondary structural components and the critical regions determined by this study. Interestingly, critical regions III and IV are predicted to be β-sheet segments, the borders of which coincide with the boundaries of these critical regions. These regions are each flanked on the N-terminal side by a cysteine residue involved in the formation of the critical disulfide bridge: Cys-51 for critical region III and Cys-93 for critical region IV (Fig. 3). Therefore, this disulfide bond might play a direct role in the arrangement of these critical regions with respect to one another. Since these regions are predicted to be β-sheet segments, it suggests that critical regions III and IV may form a parallel β-sheet structure.

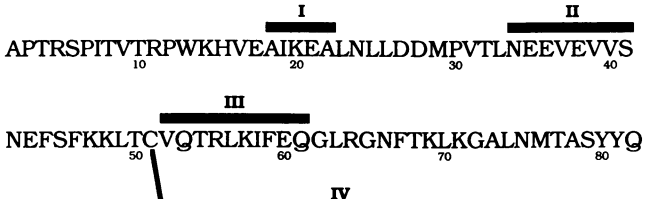

FIG. 3. Salient features of mGM-CSF. The four critical regions are indicated by bars above the primary amino acid sequence. The essential disulfide bridge is shown by the heavy line between the two cysteines that border the N-terminal sides of critical regions III and IV. The other nonessential disulfide bridge is represented by a broken line.

The analysis presented here begins to unravel the different structural and/or functional elements of GM-CSF. Identification of critical regions provides the opportunity to rapidly analyze the essential components of the protein. The knowledge gained from these studies would lead to a better functional understanding of this lymphokine.

We thank Felix Vega for synthesis of DNA, John Abrams for supplying antibodies, and Warren Dang for technical support. We thank Charles Yanofsky for his valuable suggestions on the manuscript. Gerard Zurawski, Ken-ichi Arai, and Phil Hodgkin are thanked for critically reading the manuscript. We also thank Gary Burget for preparation of the manuscript.

1. Metcalf, D. (1986) *Blood* **67**, 257–267.
2. Metcalf, D. (1985) *Science* **229**, 16–22.
3. Lopez, A. F., Williamson, D. J., Gamble, J. R., Begley, C. J., Harlan, J. M., Klebanoff, S. J., Waltersdorph, A., Wong, J., Clark, S. C. & Vadas, M. A. (1986) *J. Clin. Invest.* **78**, 1220–1228.
4. Gough, N. M., Metcalf, D., Gough, J., Grail, D. & Dunn, A. R. (1985) *EMBO J.* **4**, 645–653.
5. Lee, F., Yokota, T., Otsuka, T., Gemmel, L., Larson, N., Luh, J., Arai, K. & Rennick, D. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4360–4364.
6. Schrimsher, J. L., Rose, K., Simona, M. G. & Wingfield, P. (1987) *Biochem. J.* **247**, 195–199.
7. Clark-Lewis, I., Lopez, A. F., To, L. B., Vadas, M. A., Schrader, J. W., Hood, L. & Kent, S. B. H. (1988) *J. Immunol.* **141**, 881–889.
8. Gough, N. M., Grail, D., Gearing, D. P. & Metcalf, D. (1987) *Eur. J. Biochem.* **169**, 353–358.
9. Wingfield, P., Graber, P., Moonen, P., Craig, S. & Pain, R. H. (1988) *Eur. J. Biochem.* **173**, 65–72.
10. Messing, J. (1983) *Methods Enzymol.* **101**, 20–78.
11. Kunkel, T. A., Roberts, J. D. & Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382.
12. Howard-Flanders, P., Simson, E. & Theriot, L. (1964) *Genetics* **49**, 237–246.
13. Ghrayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. & Inouye, M. (1984) *EMBO J.* **3**, 2437–2442.
14. Greenberg, R., Lundell, D., Alroy, Y., Bonitz, S., Condon, R., Fossetta, J., Frommer, B., Gewain, K., Katz, M., Leibowitz, P. J., Narula, S. K., Kastelein, R. & van Kimmenade, A. (1988) *Curr. Microbiol.* **17**, 321–332.
15. De Boer, H. A. & Kastelein, R. (1986) in *Biased Codon Usage: An Exploration of Its Role in Optimization of Translation and in Maximizing Gene Expression,* Biotechnology Series, eds. Resnikoff, W. & Gold, L. (Butterworth, Boston), pp. 225–283.
16. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
18. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
19. Miyajima, A., Otsu, K., Schreurs, J., Bond, M. W., Abrams, J. S. & Arai, K. (1986) *EMBO J.* **5**, 1193–1197.
20. Koshland, D. & Botstein, D. (1980) *Cell* **20**, 749–760.
21. Mosmann, T. R. (1983) *J. Immunol. Methods* **65**, 55–63.
22. Zurawski, S. M. & Zurawski, G. (1988) *EMBO J.* **7**, 1061–1069.
23. Chou, P. Y. & Fasman, G. D. (1978) *Annu. Rev. Biochem.* **47**, 251–276.