Characterization of recombinant-derived granulocyte-colony stimulating factor (G-CSF)

Paul WINGFIELD,*† Robert BENEDICT,*† Gerardo TURCATTI,*† Bernard ALLET,*† Jean-Jacques MERMOD,*† John DeLAMARTER,*† Marco G. SIMONA† and Keith ROSE†§

*Biogen S.A., P.O. Box 1211 Geneva 24, Switzerland, and †Département de Biochimie Médicale, Centre Médical Universitaire, 9 avenue de Champel, 1211 Geneva 4, Switzerland

Human granulocyte colony-stimulating factor (G-CSF), and a mutant having a Ser for Cys substitution at residue 18 were produced in *Escherichia coli* strain W3110. About 60 mg of pure protein was obtained from 50 g of wet cells with a recovery of about 20%. The proteins were characterized physically and chemically, including determination of disulphide bonds, which were found to exist between residues 37–43 and 65–75. Cys-18 is not involved in disulphide bond formation and was substituted by Ser with no effects on gross protein conformation or biological activity. Both the wild-type and the mutant recombinant-derived proteins, although not glycosylated, possess colony-stimulating activities. In a bioassay using the murine myelomonocytic leukaemic cell line WEHI 3B D⁺, activities were obtained which were similar to those of natural G-CSF and of a glycosylated recombinant-derived human G-CSF produced in monkey cells.

INTRODUCTION

Four glycoproteins which generate colonies of haemopoietic cells when present in cultures of bone marrow cells are called colony stimulating factors (CSFs; [1]). They can be further identified according to the lineages of haemopoietic cells that they stimulate in bone marrow cultures. Lineage specificities range from the broadly acting multi-CSF (or interleukin-3), which gives rise to many different colony types to the specific generation of granulocytic colonies by granulocyte CSF (G-CSF). The ability of the different CSFs to generate varying haemopoietic colonies *in vitro* suggests a potential role for the proteins *in vivo*. However, for nearly 20 years following the identification of CSFs, their putative role in normal haemopoiesis and disease could not be investigated, as their extremely low concentrations in body fluids and the very small quantities that could be purified from native sources precluded *in vivo* experimentation with pure factors.

Over the past four years the genes encoding each of the four known CSFs of both man and mouse have been isolated [2–4]. Characterization of the gene encoding G-CSF [5,6] has led to the production of the protein by recombinant techniques. Experiments *in vitro* have confirmed that pure G-CSF made by recombinant techniques gives rise to granulocytic colonies in bone marrow cultures, activates neutrophils, and differentiates certain myeloid leukaemic cells [6,7]. The ability of recombinant human G-CSF to elevate white cell counts in normal animals is also now well documented [8–10]. Elevation of peripheral blood concentrations of granulocytes in an animal by G-CSF has suggested a potential pharmacological role for this factor [11]. In cases of increased risk of infections due to myeloid depression there appears to be great potential for the beneficial administration of exogenous G-CSF.

In this paper we describe the preparation and properties of a non-glycosylated analogue of G-CSF, which has biological activity similar to that of glycosylated natural protein. Cys-18 in the analogue was shown to be in the reduced form and is not involved, unlike the other four cysteine residues, in disulphide linkages. As the presence in a protein of an unpaired cysteine residue can lead to disulphide exchange during structural analysis, we chose two approaches to avoid this. Firstly, in the wild-type protein we blocked Cys-18 by alkylation prior to digestion. Secondly, we prepared a mutant protein in which Cys-18 was substituted by site-specific mutagenesis by a Ser residue.

MATERIALS AND METHODS

Nucleic acid methods

The purification of plasmids, restriction digestions, isolation of DNA fragments, ligation of fragments and transfection of bacterial cells with plasmids were performed according to Maniatis *et al.* [12]. cDNA library construction followed the protocol described by Huynh *et al.* [13].

Cloning and production of human G-CSF

A cDNA library to mRNA from human bladder carcinoma cell line, 5637, was constructed in the cloning vehicle λ gt10. This library was screened with oligonucleotides synthesized from the published coding sequence for human G-CSF [5].

The codons for the first four residues of mature human G-CSF were altered, without changing the amino acid sequence, to prevent potential formation of mRNA secondary structures. The altered gene was introduced into a bacterial expression vector under thermo-regulated transcriptional control of the phage λ pL promoter. The

Abbreviations used: G-CSF, granulocyte colony-stimulating factor; f.a.b.m.s., fast atom bombardment mass spectrometry.

† Present address: Glaxo Institute for Molecular Biology, 46 route des Acacias, 1211 Geneva 24, Switzerland

§ To whom correspondence should be addressed.

plasmid was then introduced into the *Escherichia coli* strain W3110. Fermentation conditions were essentially as previously described [14]. The expression level of human G-CSF was between 8% and 10% of the total cell protein as measured by densitometer scanning of Coomassie Blue-stained SDS/polyacrylamide gels.

Site-specific mutagenesis

Plasmid 1034 which contained the G-CSF coding sequence was cleaved by *Bgl*II and by a mixture of *Eco*RI and *Bam*HI. The resulting larger DNA fragments were purified and mixed to prepare the mutant plasmid Cys 1 by site-directed mutagenesis using the gapped technique described by Oostra *et al.* [15]. The synthetic oligonucleotide 5'-GCTCAAGTCGTTAGAGCA-3' was used to replace the sequence 5'-GCTCAAGTGC-TTAGAGCA-3' in the parent plasmid, thus substituting Cys-18 of G-CSF for a Ser residue.

Protein purification

The wild-type and mutant G-CSFs were both produced in *E. coli* as insoluble aggregates and were purified using the same method. The proteins were extracted with 6 M-guanidine hydrochloride as previously described [16]. The extract was fractionated by size-exclusion chromatography in the presence of 4 M-guanidine hydrochloride and 5 mM-dithiothreitol on Sephacryl S-200 (Pharmacia). Fractions containing G-CSF were pooled, and the protein renatured by dialysis under oxidizing conditions against a non-denaturing concentration (3 M) of urea [17]. The proteins were purified by cation-exchange chromatography at pH 5.3 using CM-Sepharose (Pharmacia) and gel filtration using Ultrogel AcA54 (LKB). From 50 g wet wt. of cells about 60 mg of pure protein was obtained, which represents an overall recovery of about 20%.

Protein determination

The molar absorption coefficients (ϵ) for the wild-type and mutant proteins were calculated from the amino acid composition [18]. The value used for both proteins was $16.76 \text{ mM} \cdot \text{cm}^{-1}$ and is based on an M_r of 18785.

Analytical separation methods

Gel electrophoresis in the presence of SDS was carried out on 15% (w/v) polyacrylamide gels using the procedure of Laemmli [19]. Electrofocusing on polyacrylamide gels was as previously described [20]. H.p.l.c. was performed as previously described [20] except that a 25 cm $\times$ 4 mm Nucleosil 300 Å 5 μm C₈ column (Machery Nagel, Düren, W. Germany) and shallower gradients of acetonitrile were used. The apparent molecular masses of the proteins were determined by size-exclusion chromatography using a column (45 cm $\times$ 1 cm) of Ultrogel AcA54. The column was equilibrated with 30 mM-Tris/HCl/0.1 mM-EDTA/0.5 M-NaCl, pH 7.5, and eluted at 0.25 ml/min. The column was calibrated using a gel-filtration calibration kit of low M_r proteins (Pharmacia).

Location of disulphide bonds

Wild-type protein (1.5 mg/ml) was incubated under N₂ in the dark in a buffer containing Tris (0.1 M, counter ion acetate), NaCl (125 mM), EDTA (5 mM) and iodoacetate (4 mM) at pH 8.0. After 30 min at room temperature (22 °C), solid urea was added to a final

concentration of 6 M and incubation continued for a further 60 min. The sample was diluted with 6 vol. of 50 mM-ammonium bicarbonate and dialysed extensively against the same buffer at 4 °C. Protein was recovered by lyophilization.

Alkylated wild-type G-CSF and native mutant protein (0.8 mg/ml) were cleaved with CNBr (2 mg/ml) in 70% (v/v) formic acid at 22 °C in the dark for 24 h. After removal of reagents *in vacuo*, samples were digested at 1.2 mg/ml in 1% (v/v) formic acid with pepsin (enzyme: substrate ratio, 1:100, w/w) for 16 h. The digests were examined directly by h.p.l.c. Portions of major fractions which were found to be susceptible to reduction with dithiothreitol (see [20]) were examined by amino acid analysis and fast atom bombardment mass spectrometry (f.a.b.m.s.).

The following techniques were as previously described: f.a.b.m.s. [21]; amino acid analysis and N-terminal analysis [20]; protein thiol analysis [22]; and protein disulphide analysis [23].

Circular dichroism

Spectra were measured using a Jobin-Yvon Dichrographe IV linked to a BBC microcomputer for recording data. Spectra were recorded at 20 °C using a 0.005 cm-pathlength cell with a 2 nm band-width. Measurements were made with 0.38 mg/ml (wild-type) and 0.23 mg/ml (mutant, Cys-18 $\rightarrow$ Ser) in 30 mM-Tris/HCl/0.5 M-NaCl/0.1 mM-EDTA, pH 7.5.

Urea-gradient electrophoresis

The method described by Creighton [24] was used. The electrophoresis buffer was 50 mM-Tris/phosphate, pH 7.5. Protein (about 100 μg) in 100 μl of electrophoresis buffer was subjected to electrophoresis at 20 °C for 18 h at 15 V/cm. Protein was visualized by Coomassie Blue staining.

Bioassays

Purified G-CSF was assayed for its biological activity using three bioassays. Firstly, it was assayed on a murine myelomonocytic cell line WEHI 3BD⁺ [25] grown in soft agar, for which 1 unit of activity is defined as the amount of G-CSF inducing 50% of colony differentiation. Secondly, in the human bone marrow colony assay, performed in 0.3% agar supplemented with 20% foetal calf serum [26,27], colonies over 40 cells were scored. In this assay 50% of maximal colony formation corresponds to 50 units of activity/ml. Finally, a proliferation assay based on the method of Mossmann [28] was used. NFS-60 cells, which require G-CSF for growth [29], were plated at 10^5 cells/ml on a microwell plate in the presence of varying concentrations of G-CSF. After 15 h cells were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide and absorbance was measured at 590 nm. One unit of activity is defined as the amount of G-CSF inducing 50% of maximal absorbance.

RESULTS AND DISCUSSION

Size and charge homogeneity

SDS/polyacrylamide-gel electrophoresis of wild-type G-CSF and G-CSF Cys-18 $\rightarrow$ Ser (Fig. 1) showed single bands of the same M_r (19000) similar to that predicted by the gene sequence (M_r = 18750). The M_r of both

Fig. 1. Results of SDS/polyacrylamide-gel electrophoresis

a, Standards (M_r indicated on the left); b, G-CSF; c, G-CSF Cys-18 $\rightarrow$ Ser.

Fig. 2. Amino acid sequence of G-CSF derived from the gene sequence

The sequence is numbered from the unprocessed Met at position 1. The authentic sequence is predicted to start with Thr at position 2. Peptides 18–23, 33–47 and 50–75 are indicated. These peptides were isolated during characterization of the disulphide linkage pattern (see the text).

proteins under native conditions was estimated by size-exclusion chromatography to be about 21 500, indicating that the proteins are monomers. Isoelectric focusing of the wild-type G-CSF indicated a main species (pI 5.9),

which constituted about 95–97 % of the Coomassie Blue-stained proteins (results not shown).

Chemical analysis

There was good agreement between the predicted and observed amino acid compositions of both wild-type and mutant G-CSFs (Table 1). The *N*-terminal amino acid sequence of the first 20 residues of both proteins was the same and as predicted from the respective gene sequences (Fig. 2). Both proteins contained 90–95 % unprocessed *N*-terminal methionine. Similar results have been observed for other recombinant-derived proteins ([30] and refs. therein).

Secondary structure and conformation

The far-u.v. c.d. spectra for wild-type and G-CSF Cys-18 $\rightarrow$ Ser were similar (results not shown) and indicate that the two proteins have a closely similar backbone conformation. Calculation of secondary structure using the CONTRIN program [31] suggests 66 % alpha helix and 17 % beta sheet. The experimental estimates are similar to those predicted using the method of Chou & Fasman [32], namely, 50 % alpha helix and 18 % beta sheet. The secondary structure of G-CSF thus more closely resembles GM-CSF, which also contains both alpha helix and beta sheet structures [33], than that of some other lymphokines, for example, interleukin-1 β , which only contains beta-sheet structure [34].

The conformation of the G-CSF wild-type and mutant protein was studied by urea-gradient electrophoresis (see [24] for discussion of theory). Both proteins showed

Fig. 3. Urea-gradient electrophoresis of G-CSF

The buffer for electrophoresis and sample application was 50 mM-Tris/phosphate, pH 7.5. Electrophoresis in the direction indicated was towards the anode. (a) Wild-type G-CSF; (b) mutant G-CSF Cys-18 $\rightarrow$ Ser.

Table 1. Results of amino acid analysis

Values are expressed as molar ratios, with ratios predicted from the gene sequence in parentheses. Abbreviation: n.d., not determined.

Residue	Composition (residues/molecule)					
	Wild-type protein	Mutant protein	Peptide 33–47		Peptide 50–75	
			Wild-type	Mutant	Wild-type	Mutant
Cys*	5.03 (5)	4.06 (4)	1.38 (2)	1.35 (2)	1.93 (2)	2.48 (2)
Asx	4.11 (4)	4.20 (4)				
Thr	6.53 (7)	6.51 (7)	0.82 (1)	0.84 (1)		
Ser	11.60 (14)	12.86 (15)			3.45 (4)	3.37 (4)
Glx	25.96 (26)	26.07 (26)	4.00 (4)	4.00 (4)	2.14 (2)	2.30 (2)
Pro	12.79 (13)	11.23 (13)	0.68 (1)	0.71 (1)	2.60 (3)	1.99 (3)
Gly	13.97 (14)	14.08 (14)			3.24 (3)	3.54 (3)
Ala	18.80 (19)	18.73 (19)	0.56 (1)	0.68 (1)	2.93 (3)	2.85 (3)
Val	6.99 (7)	6.99 (7)				
Met	3.63 (4)	3.62 (4)				
Ile	3.87 (4)	3.82 (4)			0.94 (1)	0.95 (1)
Leu	33.10 (33)	31.97 (33)	2.15 (2)	2.07 (2)	5.71 (6)	5.41 (6)
Tyr†	3.05 (3)	3.13 (3)	1.01 (1)	1.04 (1)		
Phe	5.97 (6)	6.10 (6)				
His	4.97 (5)	5.02 (5)	1.14 (1)	1.14 (1)	0.85 (1)	0.88 (1)
Lys	4.04 (4)	4.05 (4)	2.16 (2)	2.09 (2)		
Arg	5.00 (5)	5.20 (5)				
Trp	n.d. (2)	n.d. (2)			n.d. (1)	n.d. (1)

* The cysteine content of proteins was estimated as carboxymethylcysteine from proteins reduced and alkylated with iodoacetamide (see the Materials and methods section). The cysteine content of peptides was estimated as cysteic acid from peptides oxidized with performic acid.

† For the peptides shown, the tyrosine content was determined by the separate analysis of hydrolysates of peptides not treated with performic acid.

essentially the same gel profile (Fig. 3) whether they were applied to the gel under native conditions (in sample buffer alone) or under denaturing conditions (in sample buffer plus 8-M-urea). The folding and unfolding transitions of both proteins occurred at about 5.3 M-urea with rates which were fast compared with the time of electrophoresis. From an analysis of the transition curves [35], the thermodynamic stability of both proteins was estimated to be -38 kJ/mol. Similar results have been recently described for recombinant-derived human granulocyte-macrophage-CSF [33].

The results of this section show that recombinant-derived G-CSF is a stable protein with a conformational stability typical of a normally folded protein, such as, for example, ribonuclease. Substitution of Cys-18 by a Ser residue does not change the secondary structure or the conformational stability of the protein.

Disulphide bonds

In wild-type G-CSF one thiol group/mol was titrated with 5,5'-dithiobis-(2-nitrobenzoate). The titration of this residue depended on the protein being unfolded with, for example, 1% (w/v) SDS or 6 M-urea. Modification of the free thiol group with iodoacetamide followed by titration of the protein with the disulphide reagent 2-nitrothiosulphobenzoic acid [23] indicated the presence of two disulphide bonds. Thus of five cysteine residues in G-CSF (Fig. 2) four are involved in disulphide linkages and one is free and is probably in a solvent inaccessible (buried) environment.

To locate the free cysteine residue we alkylated G-

CSF with [^{14}C]iodoacetamide, digested the protein with trypsin and separated the peptides by reverse-phase h.p.l.c. Only one radiolabelled peptide was detected and amino acid analysis identified the peptide as Cys, Leu, Glx, Asx, Val, Arg which corresponds to residues 18–23 of the G-CSF sequence (Fig. 2). Thus Cys-18 of G-CSF is not involved in disulphide linkages.

To locate the disulphide bonds between remaining cysteine residues, we examined both alkylated wild-type protein and the mutant having a Ser for Cys substitution at residue 18. By using techniques previously described [20] we isolated two major peptides, both reducible with dithiothreitol, from the wild-type protein. The mutant protein also gave rise to two major peptides of similar properties to those obtained from wild-type protein. Upon reduction, all four peptides shifted to later retention times, but none of them split into two components, suggesting the presence of a disulphide bond within a single peptide chain for all four fractions. The earlier eluting fractions from both proteins gave, upon amino acid analysis (Table 1), compositions consistent with the sequence from residues 33–47. This interpretation was supported by f.a.b.m.s. in positive ion mode, which showed, for the earlier eluting peptides from both proteins, strong signals at m/z 1772.5 (theoretical value 1772.8), consistent with a peptide containing residues 33–47 and with the *N*-terminal Gln dehydrated to pyroglutamyl and the two Cys residues in disulphide-bonded form (results not shown). The later eluting peptides from both proteins gave, upon amino acid analysis (Table 1), compositions consistent with the

sequence from residues 50–75 (or 51–76, both these sequences having the same composition). This interpretation was supported by f.a.b.m.s. in positive ion mode, which showed, for the later eluting peptides from both proteins, a strong ion envelope around m/z 2618 (results not shown). Such spectra would be expected from a peptide containing residues 50–75 (or 51–76, which has an identical composition) with the Cys residues partially reduced by the thioglycerol matrix used to obtain the spectra.

Given that no free thiol was present and that acid conditions were maintained during protein cleavage and peptide isolation, the pattern of disulphide bonding established is most likely to be correct.

It is worth noting that murine G-CSF has a very similar amino acid sequence to that of the human protein [37]. Both proteins contain five Cys residues, four of which are found in similar positions in the sequence. The Cys at residue 18 is the one not conserved, murine G-CSF having a Ser for Cys substitution in this position, thus resembling the mutant human protein studied here. We would expect the disulphide bonding pattern in murine G-CSF to be the same as we have determined for the human form.

The biological activity of wild-type and mutant G-CSF was measured in three different bioassays. The most specific bioassay, the human bone marrow colony assay, gave a specific activity of $(1\text{--}3.6) \times 10^8$ units/mg with the wild-type and mutant G-CSF, which is identical to the activity of natural purified G-CSF [38]. In the cellular differentiation assay using the murine cell line WEHI 3BD⁺, no difference was found between the activities of wild-type and mutant G-CSF [$(2.7 \pm 1.3) \times 10^6$ and $(2.2 \pm 1.6) \times 10^6$ units/mg, respectively]. These activities are comparable with natural G-CSF [38] and with a glycosylated recombinant-derived human G-CSF produced in monkey cells [37]. Finally, a cellular proliferation assay on the murine cell line NFS60 gave activities of 2.6×10^7 units/mg for both wild-type and mutant G-CSF. All three bioassays thus indicated that mutant Cys-18 → Ser and wild-type G-CSF have similar activities, supporting the physicochemical analyses which indicated similar conformation and stability (see above).

Conclusions

(1) G-CSF can be produced in large quantities using methods described herein, with biological activity similar to natural protein, which is glycosylated [39]. (2) Cys-18 is not involved in disulphide linkages. It is probably located in a solvent-inaccessible environment and can be substituted by Ser with no effects on gross protein conformation or biological activity. (3) Disulphide bonds exist between cysteine residues 37–43 and 65–75. (4) From sequence comparison with murine G-CSF it is most likely that disulphide bonds in this protein exist between cysteines 42–48 and 70–80 (residue numbers are from the authentic *N*-terminal residue Val, although if this protein were to be produced in *E. coli*, the initiating methionyl residue would almost certainly be retained).

We thank Dr. Stuart Craig and Professor R. Pain for supplying the c.d. data, and thank the following for their support: Fonds National Suisse de la Recherche Scientifique; Schmidheiny Foundation; Stanley Thomas Johnson Foundation; Luzerner Krebsliga; Ligue Suisse Contre le Cancer.

REFERENCES

1. Metcalf, D. (1984) *The Hemopoietic Colony Stimulating Factors*, Elsevier, Amsterdam
2. Metcalf, D. (1986) *Blood* **67**, 257–267
3. Clark, S. C. & Kamen, R. (1987) *Science* **236**, 1229–1237
4. Delamarter, J. F., Mermod, J. J., Johnson, M. J., Buell, G. N., Shaw, A. & Gough, N. M. (1987) *Ann. N.Y. Acad. Sci.* **511**, 50–64
5. Nagata, S., Tsuchiya, M., Asana, S., Kaazi, Y., Yamamoto, T., Hirata, Y., Kubota, N., Oheda, M., Numura, H. & Ono, M. (1986) *Nature (London)* **319**, 415–418
6. Souza, L. M., Boone, T. C., Gabrilove, J., Lai, P. H., Zsebo, K. M., Murdock, D. C., Chazin, V. R., Bruszewski, J., Lu, H., Chen, K. K., Barendt, J., Platzer, E., Moore, M. A. S., Mertelsman, R. & Welte, K. (1986) *Science* **232**, 61–64
7. Tsuchiya, M., Nomura, H., Asano, S., Kaziro, Y. & Nagata, S. (1987) *EMBO J.* **6**, 611–616
8. Cohen, A. M., Zsebo, K. M., Inoue, H., Hines, D., Boone, T. C., Chazin, V. R., Tsai, L., Ritch, T. & Souza, L. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2484–2488
9. Welte, K., Bonilla, M. A., Gillio, A. P., Boone, T. C., Potter, G. K., Gabrilove, J. L., Moore, M. A. S., O'Reilly, R. J. & Souza, L. M. (1987) *J. Exp. Med.* **165**, 941–948
10. Tamura, M., Hattori, K., Oheda, M., Kubota, N., Imazeki, I., Ono, M., Ueyama, Y., Nagata, S., Shirafuji, N. & Asano, S. (1987) *Biochem. Biophys. Res. Commun.* **142**, 454–460
11. Matsumoto, M., Matsubara, S., Matsuno, T., Tamura, M., Hattori, K., Nomura, H., Ono, M. & Yokota, T. (1987) *Infect. Immun.* **55**, 2715–2720
12. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, 1st edn., Cold Spring Harbor Press, NY
13. Huynh, T. V., Young, R. A., Davis, R. W. (1985) in *DNA Cloning* (Glover, D. M., ed.), 1st edn., vol. 1, pp. 49–78, IRL Press, Oxford
14. Wingfield, P., Payton, M., Tavernier, J., Barnes, M., Shaw, A., Rose, K., Simona, M. G., Demczuk, S., Williamson, K. & Dayer, J.-M. (1986) *Eur. J. Biochem.* **160**, 491–497
15. Oostra, B. A., Harvey, R., Ely, B. K., Markham, F. A. & Smith, A. E. (1983) *Nature (London)* **304**, 456–459
16. DeLamarter, J. F., Mermod, J. J., Liang, C. M., Eliason, J. F. & Thatcher, D. R. (1985) *EMBO J.* **4**, 2575–2581
17. Orsini, G. & Goldberg, M. E. (1978) *J. Biol. Chem.* **253**, 3453–3458
18. Wetlaufer, D. B. (1962) *Adv. Protein Chem.* **17**, 303–390
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
20. Schrimsher, J. L., Rose, K., Simona, M. G. & Wingfield, P. (1987) *Biochem. J.* **247**, 195–199
21. Savoy, L. A., Jones, R. M. L., Pochon, S., Davies, J. G., Muir, A. V., Offord, R. E. & Rose, K. (1988) *Biochem. J.* **249**, 215–222
22. Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
23. Thannhauser, T. W., Konishi, Y. & Scheraga, H. A. (1984) *Anal. Biochem.* **138**, 181–188
24. Creighton, T. E. (1986) *Methods Enzymol.* **131**, 156–172
25. Metcalf, D. & Nicola, N. A. (1982) *Int. J. Cancer* **30**, 773–780
26. Moonen, P., Mermod, J.-J., Ernst, J., Hirschi, M. & DeLamarter, J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 4428–4431
27. Iscove, N. N., Guilbert, J. & Weyman, C. (1980) *Exp. Cell. Res.* **126**, 121–126
28. Mossman, T. (1985) *J. Immunol. Methods* **65**, 66–73
29. Holmes, K. L., Palaszynski, E., Frederickson, T. T., Morse, H. C., III & Ihle, J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6687–6691

30. Rose, K., Savoy, L.-A., Simona, M. G., Offord, R. E., Wingfield, P., Mattaliano, R. J. & Thatcher, D. R. (1987) *Anal. Biochem.* **165**, 59–69
31. Provencher, S. W. (1982) *Comput. Phys. Commun.* **27**, 229–242
32. Chou, P. Y. & Fasman, G. D. (1974) *Biochemistry* **13**, 222–244
33. Wingfield, P. T., Graber, P., Moonen, P., Craig, S. & Pain, R. H. (1988) *Eur. J. Biochem.* **173**, 65–72
34. Craig, S., Schmeissner, U., Wingfield, P. & Pain, R. H. (1987) *Biochemistry* **26**, 3570–3576
35. Hollecker, M. & Creighton, T. E. (1981) *Biochim. Biophys. Acta.* **701**, 395–404
36. Pace, C. N. (1975) *CRC Crit. Rev. Biochem.* **3**, 1–43
37. Tsuchiya, M., Asano, S., Kaziro, Y. & Nagata, S. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7633–7637
38. Nicola, N. A., Metcalf, D., Matsumoto, M. & Johnson, G. R. (1983) *J. Biol. Chem.* **258**, 9017–9023
39. Normura, H., Imazeki, I., Oheda, M., Kubota, N., Tumura, M., Ono, M., Ueyama, Y. & Asano, S. (1986) *EMBO J.* **5**, 871–876

Received 12 May 1988/6 July 1988; accepted 12 July 1988