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Biochemistry

NH_2-Terminal amino acid sequence of human fibroblast interferon

(high-performance liquid chromatography/amino acid analysis/fluorescamine)

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ABSTRACT **The purification of human fibroblast interferon by chromatography on Blue Sepharose and high-performance liquid chromatography is described. The amino acid composition and a partial sequence of the homogeneous protein are reported. The NH_2 terminus was determined to be NH_2-Met-Ser-Tyr-Asn-Leu-Leu-Gly-Phe-Leu-Gln-Arg-Ser-Ser-Asn-Phe-Gln-X-Gln-Lys.**

Other laboratories have reported on the purification and partial structural analysis of human fibroblast interferon (1, 2). We present a novel purification and some additional sequences of human fibroblast interferon. Interferon used in our studies was prepared in serum-free medium and was purified by a procedure based on the combination of affinity chromatography and high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

Interferon Production and Assay. Crude fibroblast interferon was produced as described by Havell and Vilček (3), except that serum was omitted from the overnight induction medium. Interferon titers were determined by a cytopathic effect inhibition assay that was modified so that the entire assay could be performed within 16 hr (4). All interferon titers were expressed in terms of reference units/ml, calibrated against the reference standard for human leukocyte interferon (G-023-901-527) provided by the Antiviral Substances Program of the National Institute of Allergy and Infectious Diseases, Bethesda, MD.

Purification of Human Fibroblast Interferon on Blue Sepharose. Sodium chloride was added to medium containing interferon to a final concentration of 1 M, and the solution was then pumped onto a 25-ml Blue Sepharose CL-6B (Pharmacia) column (1, 5) at room temperature at a rate of 2.5 ml/min. The unfractionated interferon was maintained on ice during the loading process. The column was washed with 250 ml of sodium phosphate buffer (50 mM Na_2HPO_4 adjusted to pH 7.2 with HCl) containing 1 M NaCl and 30% (vol/vol) ethylene glycol. The interferon was eluted with the same solution containing 50% (vol/vol) ethylene glycol. Peak fractions of activity were pooled and stored at 4°C until used (Fig. 1). Activity appeared to be stable for at least 3 mo at 4°C or in liquid nitrogen.

In preparations having a low initial titer, a second passage through Blue Sepharose was required. In these instances, when a total of 25 liters of culture medium containing interferon had been chromatographed, the peak fractions from five columns were pooled and adjusted to 10% (vol/vol) ethylene glycol, 2 M NaCl, and 50 mM Na_2HPO_4 (pH 7.2). This material was then applied to another Blue Sepharose column. The column was then washed with 250 ml of the sodium phosphate buffer containing 2 M NaCl and 30% ethylene glycol. Some interferon was eluted at this step. The remaining interferon was eluted with the same solution containing 50% ethylene glycol. Interferon that eluted from the second Blue Sepharose column with both 30% and 50% ethylene glycol was satisfactory for use in the next step of the purification. The specific activity of the interferon that was deemed satisfactory for the HPLC step ranged from 30 to 300 megaunits/mg of protein. The Blue Sepharose was discarded after each run.

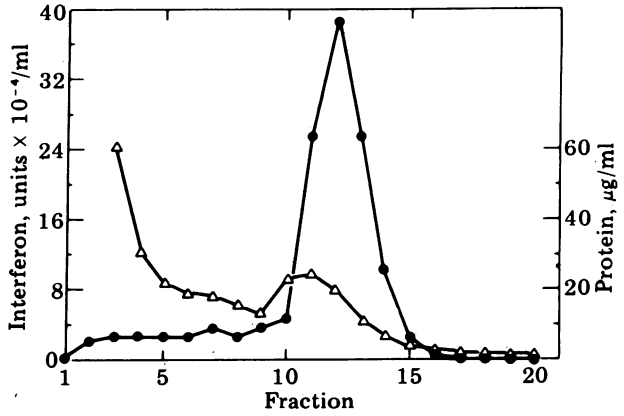

FIG. 1. Blue Sepharose chromatography of crude human fibroblast interferon. Fractions 1–9 are eluted with 30% ethylene glycol, whereas the remaining fractions are eluted with 50% ethylene glycol. The protein concentrations in fractions 3 and 4 may be high due to possible carryover of amino acids from the medium. The volume per fraction is 25 ml. ●—●, Interferon; △—△, protein.

HPLC. This final step of purification by HPLC was similar to that used by Rubinstein *et al.* (6) for human leukocyte interferon. The solution of interferon from the affinity chromatography was pumped onto a 25 × 0.46 cm Lichrosorb RP-8 (10 μm) column at 22 ml/hr. The column was eluted at 22 ml/hr with 1 M formic acid/0.8 M pyridine, pH 4.2, for 10 min and then with increasing concentrations of *n*-propanol in the same buffer. The steps were 30% (vol/vol) *n*-propanol for 40 min, 32% for 40 min, 34% for 20 min, 41% for 20 min, and 60% for 40 min. Interferon activity was eluted only during the 32% *n*-propanol step (Fig. 2). In this particular chromatogram, the interferon had been passed through Blue Sepharose twice and, therefore, represented the major component. The purification is summarized in Table 1. The homogeneous interferon had a specific activity of 300 megaunits/mg of protein.

Slab Gel Polyacrylamide Electrophoresis. Slab gel polyacrylamide electrophoresis was performed on a 5–15% (wt/vol)

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Abbreviations: HPLC, high-performance liquid chromatography; >PhNCS, phenylthiohydantoin.

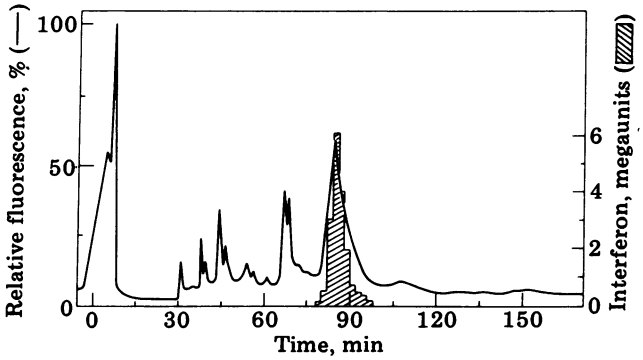

FIG. 2. HPLC of human fibroblast interferon. About 100 megaunits of interferon was applied to an RP-8 column. A step gradient of increasing *n*-propanol at constant pH (4.2) was pumped at 22 ml/hr. Interferon was eluted in a broad peak toward the end of the 32% *n*-propanol step. A portion (3%) of the column effluent was monitored with fluorescamine. The material purified in this chromatography was used for the gel in Fig. 4 and for the sequence analysis in Fig. 5.

polyacrylamide gradient or a 15% (wt/vol) polyacrylamide gel in a Tris·glycine (pH 8.3) buffer in the presence of 0.1% $NaDodSO_4$ as described (7). Protein standards and interferon were incubated in 2% (wt/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol for 30 min at room temperature prior to electrophoresis. Gels were stained with Coomassie blue. Alternatively, proteins were labeled with the fluorescent reagent fluorescamine. To samples dissolved in 20 μl of 50 mM lithium borate, pH 9.3/3% (wt/vol) $NaDodSO_4$ was added 10 μl of fluorescamine in acetone (1 mg/ml).

Detection of Amino Acids and Proteins by Fluorescence. Amino acid analyses were performed on a fluorescamine analyzer as described (8). Samples (0.5 μg) were hydrolyzed for 24 hr at 110°C in 200 μl of constant boiling hydrochloric acid containing 0.1% thioglycolic acid. An automated fluorescence detection system was used for monitoring peptides in column effluents (9). HPLC columns were obtained from EM Laboratories (Elmsford, NY). Pyridine, acetic acid, and formic acid were distilled over ninhydrin, and water was purified by passage through activated charcoal and mixed-bed deionizer cartridges (Hydro Service and Supplies, Durham, NC). Column eluents were degassed *in vacuo* and stored under argon. Polypropylene tubes and laboratory ware were used for fractions containing interferon. Proteins were assayed by injection of samples into the fluorescamine peptide/protein monitoring system; bovine serum albumin was used as a standard. This method gave excellent reproducibility and sensitivity with nanogram quantities and was in agreement with quantitation of the purified interferon by amino acid analysis.

Automated Sequencing of Interferon. Automated Edman degradations were performed on a modified Beckman 890C sequenator. The modifications, which are similar to those described by Wittmann-Liebold (10) and Hunkapiller and Hood (11), included an improved vacuum system, improved reagent and solvent delivery systems, extensive solvent and reagent purification, and a device (12) that automatically converted anilinothiazolinone to phenylthiohydantoin (>PhNCS) derivatives of amino acids. Proteins were retained in the spinning cup with 6 mg of Polybrene that, together with 100 nmol of glycylglycine, had been subjected to seven precycles of Edman degradation. >PhNCS-amino acids were analyzed by HPLC on DuPont Zorbax ODS or CN columns with detection at 254 nm and 313 nm on a Waters Associates chromatograph. Peaks were integrated and gradient elution was controlled by a Spectra Physics SP4000 integration system. All >PhNCS derivatives were detected at 254 nm, except for those of serine and threonine which were detected at 313 nm.

Table 1. Purification of human fibroblast interferon

Steps	Total activity, megaunits	Total protein, mg	Overall recovery, %	Specific activity, megaunits/mg
Crude interferon	74	ND	100	ND
Ethylene glycol, 50%	37	1.08	43	34
HPLC	11	0.034	15	320

ND, not determined.

RESULTS

Polyacrylamide Gel Electrophoresis. Homogeneity of the preparation was determined by polyacrylamide gel electrophoresis in $NaDodSO_4$. A single protein band was obtained when the gels were stained with Coomassie blue (Fig. 3). An unstained track of the same slab gel was cut into 1-mm slices, and each slice was homogenized in 0.1 ml of complete Eagle's minimal essential medium containing 10% fetal calf serum, 12.5 mM Hepes, and 50 μg of gentamicin per ml. Each fraction was assayed for interferon activity. A single peak of activity coinciding with the protein band was obtained (Fig. 3).

For further proof of homogeneity, the purified interferon was labeled with fluorescamine. Again, a single (fluorescent) protein band was obtained (Fig. 4). This technique rules out the presence of peptide and protein contaminants that might go undetected by the Coomassie blue staining procedure, because the gels are photographed immediately after electrophoresis without any washing steps.

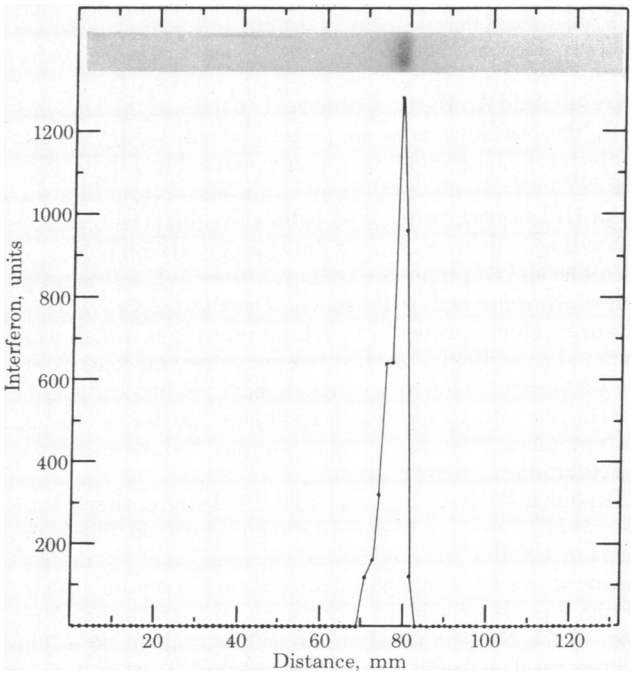

FIG. 3. $NaDodSO_4$/polyacrylamide gel electrophoresis of homogeneous human fibroblast interferon. A 5–15% polyacrylamide gradient slab gel was used. The gel was stained with Coomassie blue. Antiviral activity was determined on extracts from an unstained track. The points plotted as zero activity on the figure represent ≤40 units of interferon. The titers of interferon units shown represent units/ml in the 0.1-ml portions that were eluted from the homogenized gel fraction.

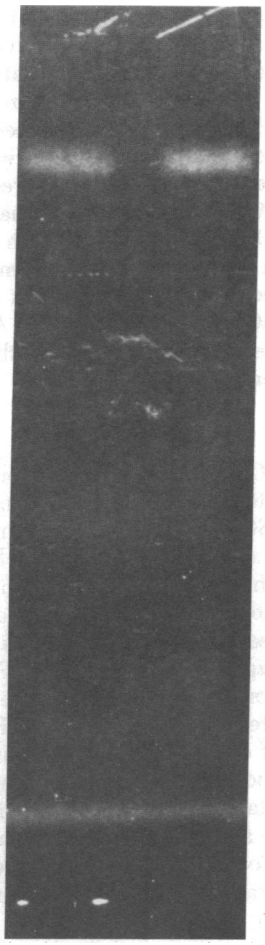

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoresis of interferon labeled with fluorescamine. A 15% polyacrylamide slab gel was used. Two fractions from the peak shown in Fig. 2 were analyzed.

Amino Acid Analysis. Amino acid analysis of the homogeneous fibroblast interferon was performed with 0.5-μg samples of interferon. These analyses are summarized in Table 2. The analyses were essentially the same for samples from four different HPLC preparations and from individual fractions corresponding to the center and sides of an interferon peak.

Proline and tryptophan were not determined on these samples, which were used for the sequence analysis reported below. However, other samples of fibroblast interferon, which were prepared by induction in the presence of serum, were extensively analyzed.[§] They were found to have essentially the same composition and, in addition, 3 residues of proline, 3 residues of tryptophan, 3 residues of glucosamine, and no residues of galactosamine or mannosamine.

Sequence Analysis. Fig. 5 shows the NH_2-terminal sequence analysis of 5.9 nmol of human fibroblast interferon. Maximum yields of 1.8 nmol of methionine at cycle 1 and 1.5 nmol of leucine at cycle 5 were obtained. Because the routine yield of >PhNCS-methionine in this laboratory is about 80%, the overall yield of the NH_2-terminal amino acid was about 40%. This figure agreed well with the yields of >PhNCS-leucine at cycles 5, 6, and 9 by assuming a repetitive yield of 92%. The high yield of NH_2-terminal methionine and the finding of a single sequence through 19 cycles strongly suggested that this was a single pure protein. The assignments of serine at positions 2, 12, and 13 were based on the detection of the "dehydroserine" derivatives on a DuPont Zorbax CN column. Although there appeared to be a tyrosine peak at position 17, a varying background peak was often seen in the >PhNCS-tyrosine area of the chromatograms.

Beyond cycle 19, a high unexpected background of

[§] Details of the induction in the presence of serum, the purification by affinity chromatography and HPLC, and the analysis of fibroblast interferon will appear elsewhere.

Table 2. Amino acid composition of human fibroblast interferon

Amino acid	Residues
Aspartic acid (Asn and Asp)	15.1 ± 0.7
Threonine*	7.0 ± 0.5
Serine*	7.5 ± 0.5
Glutamic acid (Gln and Glu)	22.2 ± 0.5
Proline	Not determined
Cysteine	3.0 ± 0.3
Glycine	6.9 ± 0.7
Alanine	7.4 ± 1.0
Valine	5.7 ± 0.5
Methionine	4.5 ± 0.7
Isoleucine	9.4 ± 0.1
Leucine	22
Tyrosine	8.8 ± 0.2
Phenylalanine	8.0 ± 0.3
Histidine	4.5 ± 0.1
Lysine	11.0 ± 0.6
Arginine	11.9 ± 0.7
Tryptophan	Not determined

Samples (0.5 μg) were hydrolyzed in 200 μl of constant boiling hydrochloric acid containing 0.1% thioglycolic acid for 24 hr at 110°C. Average deviations are for nine samples. Values are normalized to leucine = 22.
* Not corrected for loss during hydrolysis.

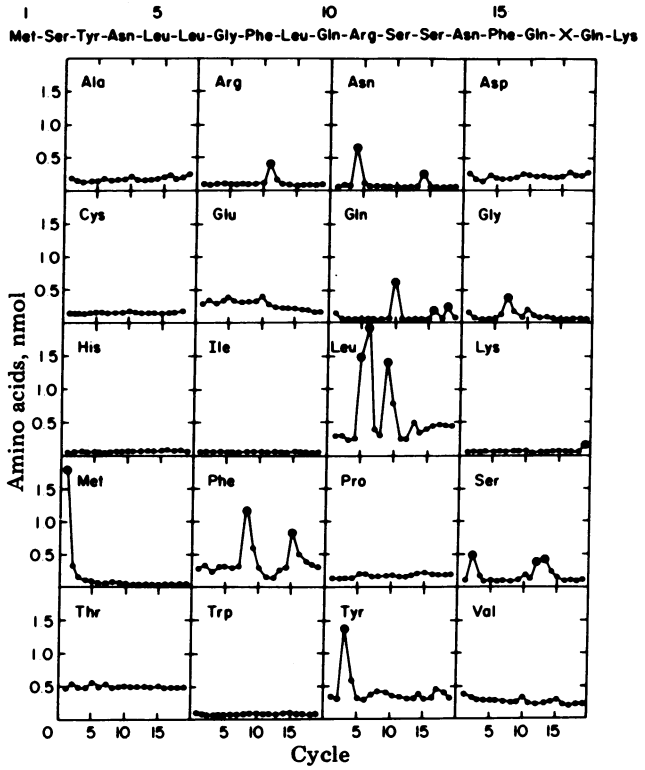

FIG. 5. NH_2-Terminal sequence analysis of 5.9 nmol of human fibroblast interferon. >PhNCS-amino acids were analyzed by HPLC and identified by comparison with standards. Values were corrected for injection loss by the inclusion of the methylthiohydantoin derivative of valine as an internal standard.

>PhNCS-leucine was encountered, but because the yields of >PhNCS-amino acids were only slightly above background at this point, it is unlikely that further identifications can be made with confidence from these results. A tentative sequence is possible for positions 20–27 but requires confirmation by further studies. In fact, the highly homologous sequence for mouse interferons A and B (13), which has Leu-Leu-Glu-Gln-Leu for positions 20–24, supports this tentative sequence. The excellent yields of >PhNCS-amino acids through position 10 were followed by a sudden decrease at position 14. The decrease in yields was apparently caused by the sequence Arg-Ser-Ser. A second more drastic decrease in yields was encountered beyond position 19, perhaps due to a hydrophobic stretch of amino acids.

The results presented here are identical to those reported by Knight *et al.* (1) and Tan (Y. H. Tan, personal communication) for positions 1–13 and extend the known sequence through position 19.

DISCUSSION

In this paper, we describe the purification of human fibroblast interferon to homogeneity. The affinity chromatography provides a high purification factor but results in a dilute solution of interferon in 50% ethylene glycol. The final product is then obtained in concentrated form, free of ethylene glycol and buffer salts, by HPLC. Because only volatile eluents are used, the interferon may be recovered salt- and solvent-free by evaporation. The specific activity of the purified protein is 300 megaunits/mg, similar to that of human leukocyte interferon (6, 14).

The purified protein formed a single band on $NaDodSO_4$/polyacrylamide gel electrophoresis (Figs. 3 and 4). The antiviral activity migrated with this protein band.

Our amino acid composition for fibroblast interferon differs significantly from that reported by Tan *et al.* (15) and that reported by Knight *et al.* (1). Those amino acids that are typically present as buffer contaminants (i.e., Asx, Ser, Glx, and Gly) are significantly higher in the report by Knight *et al.* (1). Our higher values for Cys, Met, and Trp may reflect avoidance of oxidative destruction of these residues during purification and acid hydrolysis.

The first 13 amino acids of the sequence we report for human fibroblast interferon is identical to that reported by Knight *et al.* (1) and Tan (Y. H. Tan, personal communication). The additional sequence from position 14 through position 19 is Asn-Phe-Gln-X-Gln-Lys. Position 17 was not identified with certainty. It is possibly Tyr or Cys. The slight increase in Tyr (Fig. 5) is probably due to background problems or the presence of a >PhNCS-cysteine derivative migrating in that area. With a recovery of 1.4 nmol of >PhNCS-tyrosine at position 3, we would have expected 0.34 nmol of >PhNCS-tyrosine (above background) for position 17 (at 92% repetitive yield).

Comparison of the first 19 amino acids of human fibroblast interferon with mouse interferons A and B (13) reveals four identical amino acids at positions 3, 6, 11, and 18. It is therefore likely that these proteins are related and have a common ancestral origin. However, the NH_2-terminal sequences of human leukocyte and fibroblast interferons have very little homology.

Unlike human leukocyte interferon, which has been isolated as several different species (6, 16; unpublished data), human fibroblast interferon appears to be a single molecular species. Essentially the same sequence was found for human fibroblast interferon produced in the presence of fetal calf serum (unpublished data); identical sequences for the first 13 residues were found for interferon from normal (ref. 1 and this paper) or transformed (Y. H. Tan, personal communication) fibroblasts. Accordingly, we conclude that a single gene for human fibroblast interferon is expressed, probably as a result of the existence of only a single structural gene. Little or no homology exists between the NH_2-terminal sequence of human fibroblast and human leukocyte interferon (14, 16) through positions 1–19. However, because both interferons appear to bind to the same cell receptor and have similar activities, some homology between the two in the area determining the active site has been postulated (17). Thus, as more sequence information becomes available, any homology between the two may become evident.

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