1. Recombinant DNA Technology and Characterization of Recombinant Alpha-2 Interferon

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Abstract

The strain of *E. coli,* KMAC-43, used for the large-scale production of alpha-2 interferon was engineered at Schering by Leibowitz and his co-workers. The alpha-2 interferon gene used in this construction was obtained from a cDNA clone, Hif-SN206, developed by Weissmann. The large-scale method developed in our laboratory for the purification of alpha-2 interferon from the bacterial extracts produces highly pure, crystalline alpha-2 interferon which is dissolved in 20 mM phosphate buffer at neutral pH to constitute bulk drug solution that is used in various formulations.

The protein has been thoroughly characterized using physico-chemical and biological methods. The average specific activity of clinical grade alpha-2 interferon is 1.7×10^8 u/mgP. The purity of the product has been established by reversed phase HPLC (>98% pure), amino acid analysis, amino acid sequencing, circular dichroism spectrometry, ultracentrifugation, SDS-PAGE and 2-D gel electrophoresis. Details of these methods and results will be discussed.

Introduction

In this article we review the collaborative works of several scientists from Schering Corporation, U.S.A. and Dr. Charles Weissmann's laboratory in Switzerland. The construction of a typical plasmid for the expression of human recombinant alpha-2 interferon in *E. coli* as well as the physico-chemical and biological properties of the protein are discussed.

Alpha-2 interferon producing strains

The strain of *E. coli* used for the production of recombinant human alpha-2 interferon for world-wide clinical trials was engineered at Schering Corporation, U.S.A. and contains the plasmid KMAC-43. The interferon gene in KMAC-43 was originally obtained from derivatives of an earlier construction, Hif-SN 206,

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of Weissmann and coworkers [1, 2]. The vector in Hif-SN 206 is pBR322. The IFN coding sequence of Hif-SN 206 is preceded by 51 nucleotides that are part of a signal sequence preceding the mature coding sequence in the human genome [2, 3]. Weissmann and his coworkers removed the remnant of the signal sequence of this interferon alpha-2 cDNA by fusing a lac promoter to the first codon of the mature coding sequence using a cloning strategy in which the sequence coding for the mature protein would be preceded by a translation initiation codon. This plasmid is designated AUG/206-1.

A brief summary of the construction of AUG/206-1 is depicted diagrammatically in Fig. 1. The 203 base pair Hae III fragment of the lac operon was digested with Alu 1 to obtain the regulatory region. This was ligated to a custom-made Eco RI linker fragment containing a translational start signal. After digestion with Eco RI and S_1, a promoter-operator fragment was generated that contained an initiator codon at its carboxy terminal end. The coding sequence of alpha-2 interferon cDNA was cleaved at the Sau 3a site located between the codon for the first and second amino acid of the mature interferon sequence, and the Hind III linker was ligated to the cleaved end, reconstructing the first codon and adding a Hind III cleavage site. The two ends, i.e. the promoter-operator fragment and the fragment containing the mature alpha-2 coding sequence were joined with DNA ligase to yield a sequence encoding mature interferon preceded by a methionine. The strain of *E. coli* containing the above plasmid, AUG/206-1, produced about 1 mg of alpha-2 interferon per liter of culture [4]. About 50 percent of the interferon molecules lost the methionine residue by post-translational cleavage [4].

In order to improve the yield of alpha-2 interferon, Weissmann and coworkers [4] constructed another strain, MISH-21b, with the alpha-2 interferon gene fused to the β-lactamase promoter of the pBR322 vector. The fusion was done in such a manner that the first ATG triplet was followed directly by the first codon of mature interferon (cysteine).

The yield of alpha-2 interferon in the MISH-21b fermentation was greatly improved over that of Aug/206-1 [4]. It was also found that greater than 90% of the interferon molecules did not contain N-terminal methionine [5] and that all of the intracellular interferon is soluble. KMAC-43 is a vastly superior producer, providing intra-cellularly soluble interferon without statistically meaningful levels of N-terminal methionine.

Physico-chemical characterization of human recombinant alpha-2 interferon

The large-scale method for the selective extraction of alpha-2 interferon from the cells and its purification was developed at Schering Corporation U.S.A.

Figure 3 shows crystals of alpha-2 interferon obtained from a typical purification scheme. The shape and size of the crystals vary depending upon the conditions of crystallization [7].

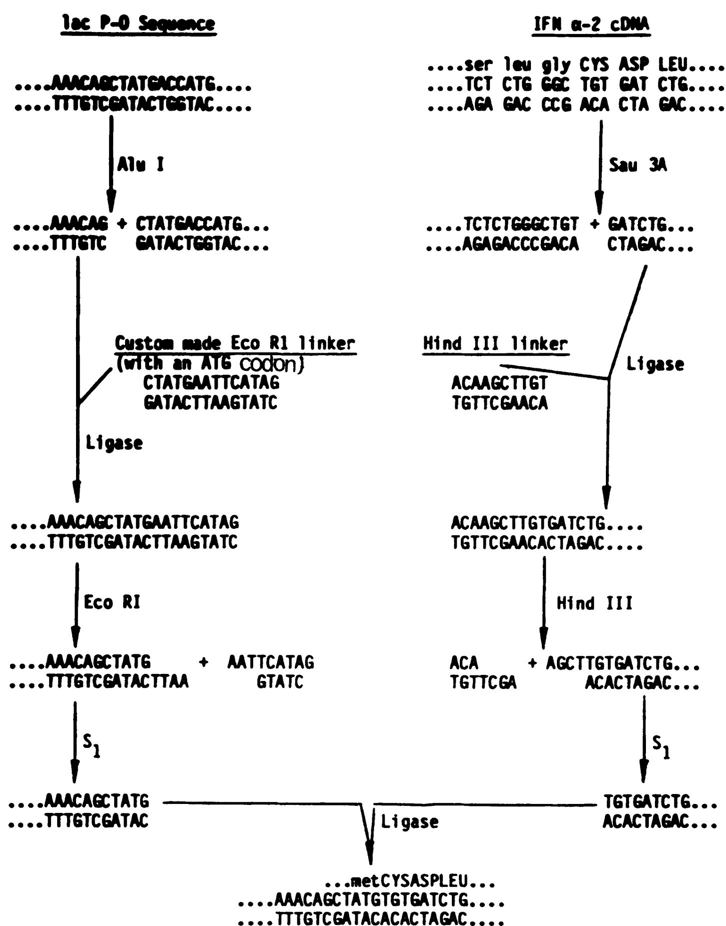

Figure 1. Summary construction of AUG/206-1.

The purity of interferon by HPLC analysis is shown in Fig. 4. The analysis was performed using a Waters Associates u-Bondapak C-18 column (30 cm x 3.9 mm) and a gradient of 31.4% to 62.7% aqueous acetonitrile (90%) containing O.O1M TFA at 1 ml/min, over 30 min [8]. The protein was detected by fluorescent monitoring after post-column derivatization with ortho-phthalaldehyde [9].

Table 1 shows typical results for amino acid analysis of alpha-2 interferon and in this particular case we have taken the average of 15 batches. As seen, the agreement between experimental and theoretical values on a mole percent basis is excellent. The method used for determining the amino acid composition involved acid hydrolysis of the protein with 6.7N hydrochloric acid *in vacuo* for 18 h at 160°C followed by chromatographic analysis of the digest on an ES Industries C-18 column (15 cm × 4.6 mm) utilizing paired ion chromatography as modified by Hatch and Radjai [10]. The amino acids were detected by fluorescent monitoring of ortho-phthalaldehyde post-column derivatives. Cysteine (cys-

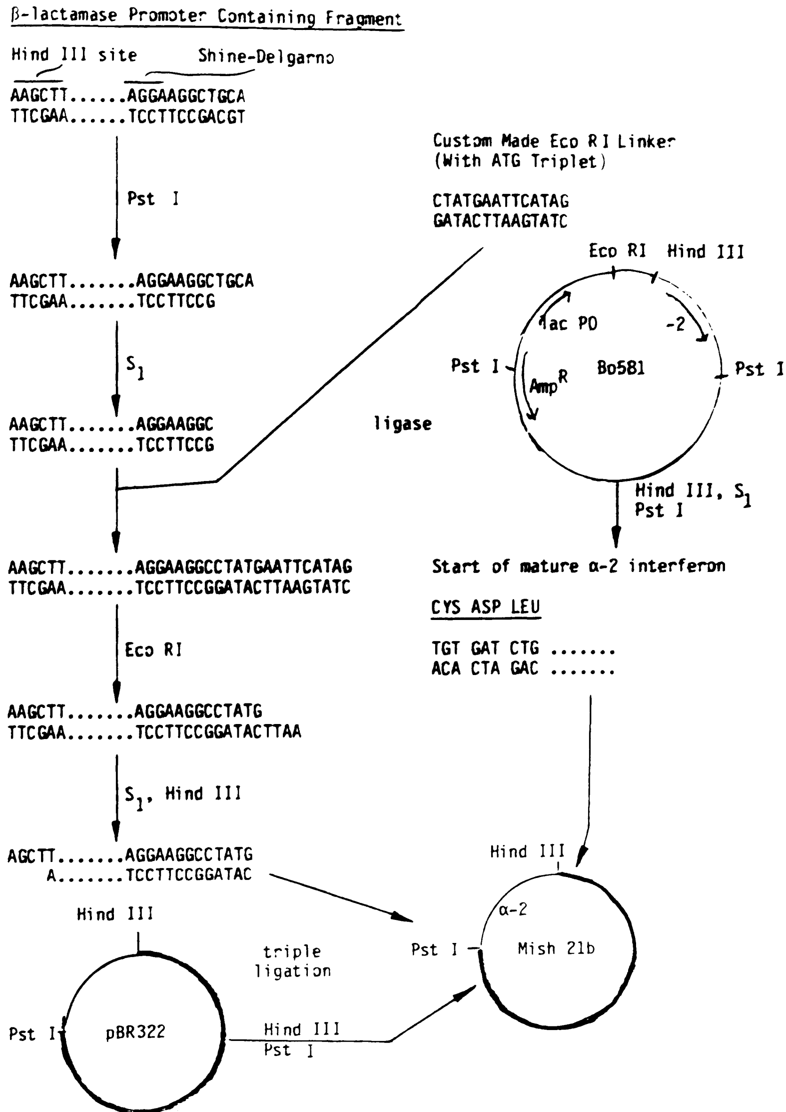

Figure 2. Summary construction of MISH 21b.

tine) and tryptophan were not quantitated due to their destruction during the hydrolysis. Proline was not detected since it does not form a fluorescent derivative with ortho-phthalaldehyde.

We have used two techniques for obtaining the amino acid sequence of the alpha-2 interferon protein. In the first, using a gas phase sequencer, Model 470A, Applied Biosystems Inc., sequencing was performed from the N-terminal through 57 amino acid residues [11]. The protein was subjected to reduction with dithiothreitol and alkylated with iodoacetamide to allow subsequent indentification of cysteine residues [12]. Sequencing was also performed without reducing

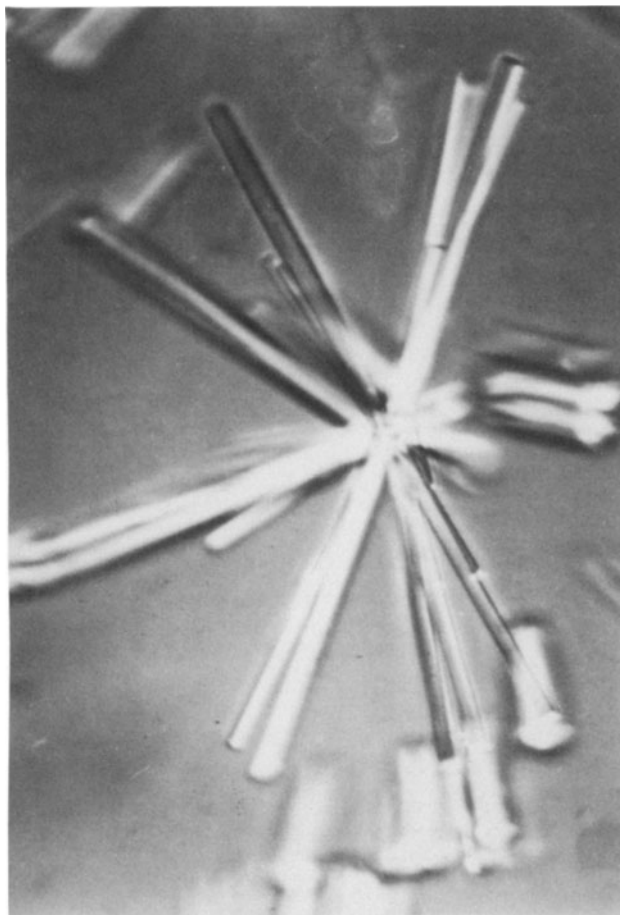

Figure 3. Crystals of alpha-2 interferon.

Figure 4. HPLC of alpha-2 interferon.

Table 1. Amino acid composition of alpha-2 interferon.

Amino acid	Theoretical mole percent	Average mole percent
Asx	7.74	9.06 ± 0.46
Ser	9.03	10.33 ± 0.48
Gly	3.23	3.80 ± 0.91
Glx	16.77	19.03 ± 0.79
Thr	6.45	5.54 ± 0.58
Ala	5.16	5.61 ± 0.58
Val	4.52	2.62 ± 0.23
Met	3.23	2.82 ± 0.59
Tyr	3.23	2.81 ± 0.45
Ile	5.16	3.52 ± 0.39
Leu	13.55	13.96 ± 0.46
Phe	6.45	6.51 ± 0.20
His	1.94	1.95 ± 0.15
Lys	7.10	6.53 ± 0.58
Arg	6.45	5.99 ± 0.70

the disulfide bonds and alkylating the protein. The phenylthiohydantoin amino acids were analyzed by HPLC on an IBM cyanopropyl silane column using 0.02 M sodium acetate, pH 6.2 with an organic eluting solvent, acetonitrile: methanol 4:1. The first 57 amino acids are in agreement with those predicted from the cDNA nucleotide sequence [2] (see Fig. 5).

Similar sequencing of fragments of alpha-2 interferon obtained after cleavage with cyanogen bromide [13] and isolation of the pure fragments by HPLC also confirmed the sequence.

Thus, we have determined the sequence of the protein for amino acids 1 through 57, 60 through 86, 88 through 97, 99–100, 102–103, 113 through 137, 139 through 146 and 149 through 164. Since the last amino acid (glu) could not be detected by the Edman degradation method, we performed tryptic digestion of the C-terminal cyanogen bromide fragment. Based on amino acid sequence and enzyme specificity [14], this treatment is expected to yield a peptide of 13 amino acids, a tripeptide (ser-lys-glu) and a free amino acid, arginine. The digest was fractionated by HPLC and the fraction containing the tripeptide and arginine was subjected to amino acid analysis. The only four amino acids detected were ser, lys, glu and arg which confirms the presence of glu in the terminal tripeptide.

In the second technique, which is still in a developmental stage we employed gas chromatography/mass spectrometry (GC/MS) sequence survey of alpha-2 interferon after subjecting the protein to non-specific cleavage with subtilisin followed by trifluoroacetylation and permethylation [15]. This experiment was done at the University of Geneva in Professor Robin Offord's laboratory under the supervision of Drs. Offord and Keith Rose. The sample was dissolved in

CYS ASP LEU PRO GLN THR HIS SER LEU GLY SER ARG ARG THR LEU

MET LEU LEU ALA GLN MET ARG ARG ILE SER LEU PHE SER CYS LEU

LYS ASP ARG HIS ASP PHE GLY PHE PRO GLN GLU GLU PHE GLY ASN

GLN PHE GLN LYS ALA GLU THR ILE PRO VAL LEU HIS GLU MET ILE

GLN GLN ILE PHE ASN LEU PHE SER THR LYS ASP SER SER ALA ALA

TRP ASP GLU THR LEU LEU ASP LYS PHE TYR THR GLU LEU TYR GLN

GLN LEU ASN ASP LEU GLU ALA CYS VAL ILE GLN GLY VAL GLY VAL

THR GLU THR PRO LEU MET LYS GLU ASP SER ILE LEU ALA VAL ARG

LYS TYR PHE GLN ARG ILE THR LEU TYR LEU LYS GLU LYS LYS TYR

SER PRO CYS ALA TRP GLU VAL VAL ARG ALA GLU ILE MET ARG SER

PHE SER LEU SER THR ASN LEU GLN GLU SER LEU ARG SER LYS GLU

Figure 5. Sequence of alpha-2 interferon.
(——— EDMAN ------ GC/MS)

deionized 8M urea containing 0.5M ammonium carbonate and 4% W/V SDS. The solution was dialyzed against water at 4°C and then lyophilized. 0.2 mg of the lyophilized powder was taken up in 50 μl subtilisin for 4 h at 37°C. The digest was lyophilized, then derivatized by trifluoroacetylation and permethylation. Approximately 1/5th of the product was used for GC/MS analysis. The peptides identified by this technique are shown in Fig. 5 which includes a similar survey of the C-terminal cyanogen bromide fragment.

The average apparent molecular weight of alpha-2 interferon was found to be 18,500 ± 600 from one-dimensional SDS-PAGE [16] (Fig. 6). The ± 600 standard deviation is a gel to gel variation. The apparent molecular weight obtained is in good agreement with 19,271 calculated from the amino acid sequence. The homogeneity of the protein is apparent from the single band seen even after staining with silver [17].

As seen in Fig. 7, there is excellent correlation between the location of bioactivity and the mobility of the major species on SDS-PAGE. In this case SDS-PAGE was conducted under non-reducing conditions.

The two-dimensional gel electrophoresis of alpha-2 interferon showed one major band and this is shown in Fig. 8 [18].

Circular dichroism (CD) spectra of alpha-2 interferon as obtained on a Jasco J-500A CD Spectropolarimeter with a DP-500N data processor are shown in Fig. 9. The spectra were run on samples diluted in 20 mM phosphate buffer at pH. 7 to yield a final concentration of 0.16 mg/ml, as determined by Lowry assay. The near and far UV spectra are corrected for baseline variation and are normalized

Figure 6. SDS-PAGE of alpha-2 interferon.

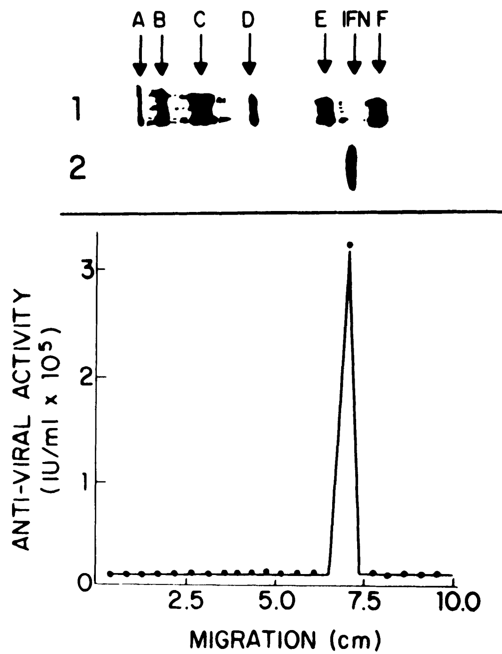

Figure 7. Correlation of biological activity of alpha-2 interferon with gel mobility.

to $^{\theta}$0.01%. The values for $^{\theta}$0.01% at five significant wavelengths are:

(nm)	1 cm	Chromophore
291	−1.22	Tryptophan
286	−1.16	Tryptophan
233	−14.3	Unknown
218	−139.0	α-helix
208	−144.0	α-helix

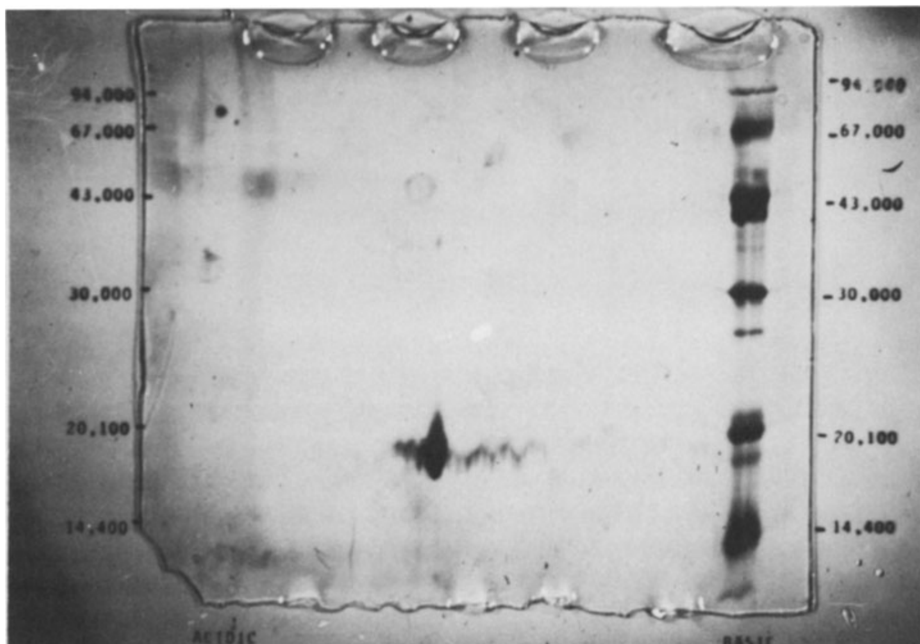

Figure 8. Two-dimensional gel of alpha-2 interferon.

The negative bands in the near UV at 291 and 286 nm are due to the highly asymmetric tryptophan chromophore. The 233 nm shoulder is characteristic of alpha-2 interferon; the nature of the chromophores that contribute to this dichroism is unknown.

The far UV data at 208 and 218 nm were evaluated using the methods of Chen [19, 20] and Greenfield [21] for computing protein conformation from CD spectra. The conformation parameters obtained are consistent with those predicted from the amino acid sequence using the Chou and Fasman rules [22–24]. Our results are very similar to the ones published by Bewley, Levine and Wetzel [25] for Genetech's cloned alpha interferon.

Fig. 10 shows the sedimentation velocity profile of alpha-2 interferon measured in the ultraviolet at 280 nm under two different solvent conditons. The experiments were performed at Cornell Medical Center using a Beckman Model E analytical ultracentrifuge. Alpha-2 interferon forms an aggregate in solution at a concentration of 0.7 mg/ml in phosphate buffer at neutral pH, as implied by its sedimentation coefficient of 4.82S (panel A). The aggregates are readily dissociated when the ionic strength of the medium is increased; for example, the sedimentation coefficient drops to 1.95S when 1.0 sodium chloride is included in the buffer, as observed in panel B.

The specific activity of alpha-2 interferon has been determined by two means. First, the anti-viral activity was determined by a cytopathic effect (CPE) inhibi-

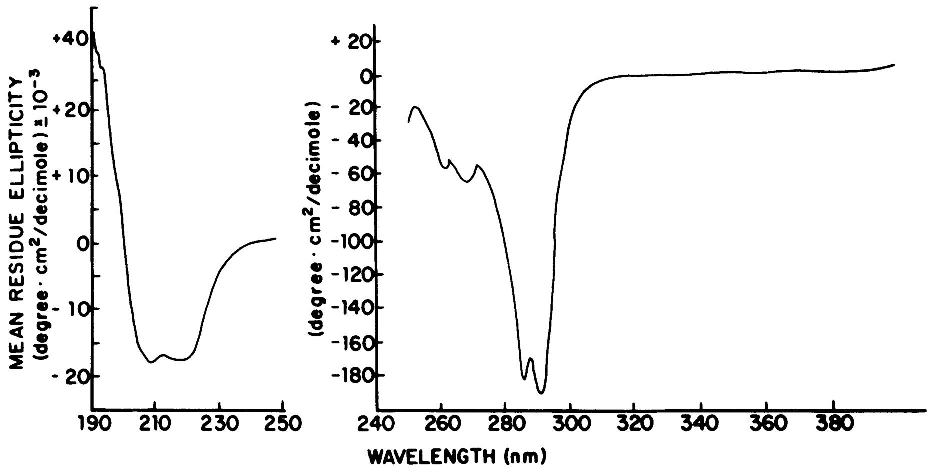

Figure 9. Circular dichroism of alpha-2 interferon.

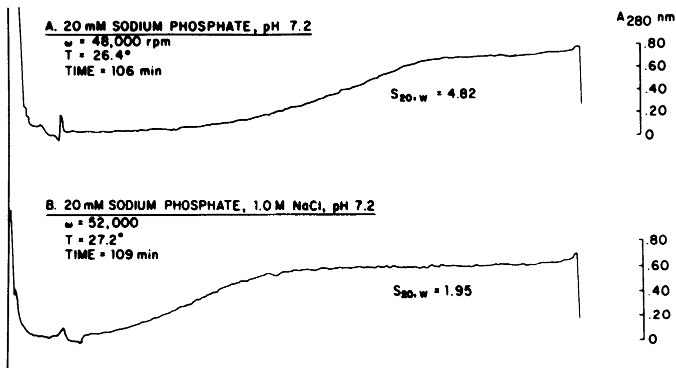

Figure 10. Sedimentation velocity of alpha-2 interferon.

tion assay using human foreskin diploid fibroblasts and EMC virus (ATCC-VR-129) and protein concentration was obtained by Lowry assay using human serum albumin as standard. Second, the specific activity was evaluated by radioimmunoassay (RIA) employing polystyrene bead immobilized polyclonal antibodies to α-interferon and iodinated monoclonal NK-2 antibodies [26]. Table 2 contains a summary of the values for specific activity calculated by CPE and RIA. The mean value for 35 batches was $(1.72 \pm 0.1) \times 10^8$ IU/mg protein by CPE and $(1.70 \pm 0.08) \times 10^8$ IU/mg protein by RIA.

Antiviral activity of recombinant human alpha-2 interferon

In Table 3 the *in vitro* antiviral activity of alpha-2 interferon against a number of viruses is given. As seen, alpha-2 interferon is most active against rhinovirus in

Table 2. Correlation of RIA-CPE-Protein Assays (17 Batches).

Test methods	Correlation coefficient
RIA-Protein	0.949
RIA-CPE	0.937
CPE-Protein	0.927

Table 3. In vitro antiviral activity of IFN alpha-2.

Challenge virus	Host cell	TCID50 units/ml
Rhinovirus	MRC-5*	$1.0 \times 10^1 - 6.2 \times 10^2$
Coronavirus	WI-38*	1.0×10^3
Respiratory syncytial	HEP-2**	1.0×10^3
Herpes simplex (I)	WI-38*	1.2×10^4
Vaccina	WI-38*	4.0×10^4
Herpes simplex (II)	WI-38*	3.5×10^5
Varicella-Zoster	WI-38*	5.0×10^5
Cytomegalovirus	WI-38*	3.0×10^6
Adenovirus	HEP-2**	5.0×10^7

* Lung
** Nasopharyngeal

the lung cell line. It is also very potent against coronavirus and respiratory syncytial virus. While its potency is moderate against the Herpes type viruses, it is inactive against adenovirus.

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