Antiviral Research, 12 (1989) 37–48
Elsevier

AVR 00367

Functional significance of amino acid residues within conserved hydrophilic regions in human interferons-α

Martin J. Tymms, Beth McInnes, Gary J. Waine, Brian F. Cheetham and Anthony W. Linnane

Centre for Molecular Biology and Medicine and Department of Biochemistry, Monash University, Clayton, Victoria, 3168 Australia

(Received 17 February 1989; accepted 9 May 1989)

Summary

Site-directed in vitro mutagenesis was used to create analogs of human interferons (IFNs)-α1 and -α4. Analogs were expressed in vitro using SP6 RNA polymerase and a rabbit reticulocyte lysate cell-free protein synthesis system. Amino acid substitutions for the highly conserved residues at positions 33, 121, 122 and 123 greatly reduced the antiviral and antiproliferative activities on human cells of IFNs-α1 and -α4. In general, the amino acid substitutions had much less effect on the antiviral activities on bovine, compared with human, cells. Substitutions at positions 31, 41, 42, 124, 134, 135 and 136 had little or no effect on the biological activities of the IFN analogs. The abrogation of antiviral activity resulting from amino acid substitutions for the arginine residue at position 33 suggests that this arginine residue is required for binding to the IFN-α receptor on the cell surface.

Interferon-alpha; In vitro mutagenesis; Antiviral activity; Antiproliferative activity

Introduction

Human interferons-α (IFN-α) are a family of more than 14 highly homologous proteins which bind to receptors on the cell surface (Aguet et al., 1984). The IFN-α induce an antiviral state, inhibit cell growth, stimulate natural killer cell activity

Correspondence to: M.J. Tymms, Centre for Molecular Biology and Medicine and Department of Biochemistry, Monash University, Clayton, Victoria, 3168 Australia.

and cytotoxic activities of lymphocytes and macrophages, and induce cell differentiation in normal cells as well as some neoplastic cells (Pestka et al., 1987). Human lymphocytes, on infection with viruses, produce a mixture of different IFN-α subtypes (Rubinstein et al., 1979; Allen and Fantes, 1980). The biological activities of those IFN-α subtypes which have been characterized vary significantly when assayed in vitro (Weck et al., 1981; Fish et al., 1983). For example, the antiviral and antiproliferative activities on human cells of IFN-α1 are 20-fold lower than those of IFN-α2 (Streuli et al., 1981; Weck et al., 1981; Rehberg et al., 1982). The differences in biological activities between the IFN-α subtypes have been attributed to differences in their affinity for the cell receptor (Yonehara et al., 1983; Aguet et al., 1984; Uze et al., 1985). Studies with hybrid molecules between IFN-α subtypes suggest that the N-terminal portion of the molecule determines high antiviral activity on human cells (Weck et al., 1981; Rehberg et al., 1982). However, with this type of study it is not possible to delineate which amino acid residues are important for activity. Site-directed in vitro mutagenesis allows the assessment of the roles of individual amino acids within the IFN-α molecule. This technique has been used to show that some of the amino acids which are conserved in the IFN-α sequence can be replaced without loss of biological activity (Valenzuela et al., 1985).

Other conserved residues, such as tyrosine 123 (Nisbet et al., 1985) and arginine 33 (Camble et al., 1986) were shown to be critical for full biological activity. The amino acid residues 33 and 123, which are important for activity, fall within conserved hydrophilic regions of IFN-α. Strongly hydrophilic domains in globular proteins, particularly those containing charged amino acids, are predominantly found on the surface of the molecule, rather than being internal (Ghelis and Yon, 1982). Conserved residues within hydrophilic domains are therefore likely to be functionally important in a protein such as IFN, which interacts with a cell surface receptor. This paper examines the biological role of conserved residues within hydrophilic regions of IFN-α.

Materials and Methods

Manipulation of IFN genes for expression

IFN-α1 and IFN-α4 were isolated from a λ Charon 4a-based human genomic library (Lawn et al., 1978; Linnane et al., 1984) and subcloned into appropriate plasmid vectors. In order to allow synthesis of leaderless mature IFN, DNA manipulations were performed in the region 5′ to the first cysteine codon of mature IFN. For the IFN-α1 gene the appropriate sequences were obtained by a single in vitro mutagenesis step. In the case of IFN-α4 an adapting linker was made and ligated to a partial digest of the gene to create a *Bam*H1 site and a yeast 5′ consensus sequence prior to the ATG initiation codon (Devenish et al., 1987). Following manipulation both genes had the following 5′ sequence:

$$\underset{\textit{Bam}\text{HI}}{\overline{\text{G G A T C C}}}\text{A A A C A}\underset{\text{Met 1}}{\overline{\text{A T G}}}$$

In vitro mutagenesis reactions

Oligonucleotide-directed mutagenesis reactions and DNA sequencing were performed as described previously (Nisbet and Beilharz, 1985).

In vitro expression of IFNs

IFN-α1, IFN-α4 and modified genes were cloned as *Bam*HI fragments into the *Bgl*II site of pSP64T (Kreig and Melton, 1984). The in vitro expression system used for the synthesis of IFNs-α which uses SP6 polymerase and rabbit reticulocyte lysate (RRL) has been described in detail elsewhere (Tymms and McInnes, 1988).

Expression of IFN analogs in yeast

IFN-α1 and modified IFN-α1 genes were cloned into the *Bam*HI site of the yeast expression vector pRJ55 (Devenish et al., 1987) and expressed in *S. cerevisiae* strain 8960-14B (*MATα*, *kar1-1*, *ura3-52*, *leu2-3*, *cir*$^+$). The growth of yeast cells and the purification of IFN have been described previously (Tymms and McInnes, 1988).

Polyacrylamide gel analysis of IFNs

Yeast and RRL-derived IFNs were separated on 9–15% SDS-polyacrylamide gels (Shoeman and Schweiger, 1982). The protein concentrations of yeast expressed IFN was quantified by laser densitometry of gels stained with Coomassie blue G250 (Serva, Heidelberg, F.R.G.), using bovine serum albumin as a standard. [^{35}S]methionine-labelled IFN in RRL was visualized by soaking gels in fluorography enhancer (Amplify, Amersham), drying and exposing to X-ray film at −80°C.

Biological assays

Monoclonal antibody-purified IFN-α or RRL preparations containing IFN-α were assayed for antiviral activity on human HEp-2 cells and bovine MDBK cells challenged with Semliki Forest virus as described previously (Jilbert et al., 1986). Antiproliferative activities of IFNs against cells of the human lymphoblastoid Daudi line were determined essentially as described by Mosmann (1983). Daudi cells in exponential growth in supplemented media (RPMI 1640, 15% FCS, 1% Na pyruvate) were seeded in U-bottomed 96-well microtitre plates at 10^4 cells per well. Interferons in supplemented media were added to give a total volume of 200 μl per well, and cells incubated at 37°C for three days. Viable cells were then stained with MTT, solubilized, and the absorbance at 540 nm recorded using a microtitre plate reader.

A plot of absorbance versus interferon dilution was constructed for each interferon sample to determine the dilution which gave 50% inhibition of cell growth.

Calculation of relative specific activities and statistical analysis

The antiviral or antiproliferative activities of unmodified IFN-α and IFN-α analogs synthesized in rabbit reticulocyte lysate were determined in several assays. The incorporation of [^{35}S]methionine, and hence the protein content, varied slightly between different translation mixes.

Activities were corrected for differences in [^{35}S]methionine incorporation. The mean of the corrected values was determined, and biological activities were then expressed as a percentage of the mean activity of unmodified IFN-α, from the same translation mix, to give a relative specific activity (RSA). RSA values for unmodified IFN-α and analogs were compared by Student's *t*-test.

Results

Mutagenesis and expression of IFN-α analogs

IFN-α1 has low antiviral activity on human cells, 2×10^7 U/mg, while the antiviral activity of IFN-α4 is high, 2×10^8 U/mg. A comparison of the sequences of IFN-α1 and IFN-α4 reveals that there are 30 amino acid differences between these two subtypes (Fig. 1). Of the 135 amino acid residues shared by these subtypes, 82 are conserved in all the IFN-α subtypes described (Weissmann and Weber, 1986). In this study we have examined the biological roles of amino acids in the highly conserved regions between residues 31 and 42, and between residues 121 and 136.

```
          1          11         21         31         41         51
          * *  ****   **      * *  **  ***  **  *  *  * *   ****    *   **
IFN-α1    CDLPETHSLD NRRTLMLLAQ MSRISPSSCL MDRHDFGFPQ EEFDGNQFQK APAISVLHEL

IFN-α4    ....Q....G ...A.I.... .G...HF... K........E .....H.... .Q.......M

          61         71         81         91         101        111
           ** **** *   **        **     *   ** *  *     *          **  * ** *
IFN-α1    IQQIFNLFTT KDSSAAWDED LLDKFCTELY QQLNDLEACV MQEERVGETP LMNADSILAV

IFN-α4    ...T....S. E......EQS ..G..S.... .......... I..VG.E... ...E......

          121        131        141        151        161
           *** ***** *   *** ** ********** * * *       *  *
IFN-α1    KKYFRRITLY LTEKKYSPCA WEVVRAEIMR SLSLSTNLQE RLRRKE

IFN-α4    R...Q..... .......... .......... ...F.....K .....D
```

Fig. 1. Aligned amino acid sequences of mature IFN-α1 and IFN-α4. The 30 amino acids at which IFN-α4 differs from IFN-α1 (Linnane et al., 1984; Weissmann and Weber, 1986) are given. Amino acids absolutely conserved throughout the 14 IFN-α subtypes are indicated with an asterisk (*).

Previous studies using IFN-α1 and an M13 expression system which synthesizes a fusion protein with an 8 amino acid leader indicated that substituting tyrosine 123 with a glycine or a serine resulted in a loss of antiviral activity on human cells (Nisbet et al., 1986). The expression of IFN-α1 carrying glycine or serine substitutions at position 123 was very poor in both the M13 expression system and a yeast expression system. For this reason we developed an in vitro expression system (Tymms and McInnes, 1988) which gives in excess of 1 μg ml^{-1} for all IFN-α analogs described in this paper (data not shown).

IFN-α1 and IFN-α4 proteins with one or two amino acid substitutions were made in vitro by site-directed mutagenesis. DNA sequencing was used to verify the fidelity of the mutations. Modified IFN-α genes were then transferred to the plasmid vector pSP64T into a cloning site downstream from the promoter for SP6 RNA polymerase.

Our previous work has established that IFN-α RNA generated by transcription of linearized template with SP6 polymerase is efficiently translated in a rabbit reticulocyte lysate cell-free protein synthesizing system (Tymms and McInnes, 1988). The fidelity of all of the IFN-α1 and IFN-α4 analogs described was assessed using SDS-PAGE analysis. In all cases a single radioactive band of the expected size was seen (data not shown).

Amino acid substitutions in the region between residues 121 and 136

To test whether substitutions at 123 affect the activity of both low and high activity IFN-α subtypes, tyrosine to serine substitutions were made in IFN-α1 and IFN-α4. This substitution resulted in a loss of 89% and 92% of the antiviral activ-

TABLE 1

Relative antiviral activities of modified IFN-α1 and IFN-α4 on human and bovine cells

IFN-α analog	Relative antiviral activity	
	Human cells (HEp-2)	Bovine cells (MDBK)
IFN-α1	(100)	(100)
Lys_{121}, Lys_{122} → Leu, Leu	3.1 ± 0.5[a]	75 ± 11
Tyr_{123} → Ser	11.5 ± 3.6[a]	141 ± 20[b]
Phe_{124} → Ser	62 ± 13[a]	160 ± 30[b]
Arg_{125} → Gln	25.5 ± 4.8[a]	140 ± 30
Lys_{134}, Lys_{135} → Leu, Leu	32.6 ± 4.2[a]	93 ± 15
Tyr_{136} → Gly	102 ± 13	145 ± 17[b]
IFN-α4	(100)	(100)
Arg_{121}, Lys_{121} → Leu, Leu	8.2 ± 4.1[a]	28.6 ± 8.0[a]
Tyr_{123} → Ser	7.9 ± 2.8[a]	30.3 ± 5.3[a]
$Lys_{134,135}$ → Leu	31.4 ± 3.6[a]	70 ± 17[b]

Results shown are the mean of the percentage of the activity of unmodified IFN-α ± standard error of the mean, from at least five determinations.

[a]Difference from activity of unmodified IFN significant at $P < 0.01$.

[b]Difference from activity of unmodified IFN significant at $P < 0.05$.

ity on human cells in both IFN-α1 and IFN-α4 respectively (Table 1).

The tyrosine at 123 is in a highly hydrophilic region of the IFN molecule, flanked by charged residues. The tyrosine at 136 is also flanked by charged residues at 133, 134 and 135. Sequence comparisons show that the tyrosine at 136 is conserved in all human IFNs-α. At this position, murine IFNs-α have a histidine residue, which, like tyrosine, is very bulky. To test the importance of the size of the residue at position 136, the smallest possible amino acid, glycine, was substituted for tyrosine. This substitution did not alter the antiviral activity on human cells (Table 1). This suggests that the side chain character of the amino acid at position 136 is not critical for the antiviral activity of IFNs-α.

Two basic residues, either a lysine or an arginine, are conserved at positions 121 and 122 in all the human IFNs-α. Hydrophobic leucine residues were substituted for Arg_{121} and Lys_{122} in IFN-α4 and for Lys_{121} and Lys_{122} in IFN-α1. Both of these IFN analogs had reduced antiviral activity on human cells relative to the wild type IFN. Specifically, the substitutions in IFN-α4 reduced activity to 8% and the substitutions in IFN-α1 reduced activity to 3% (Table 1).

In addition to the two basic residues at positions 121 and 122 there are also two conserved lysine residues at positions 134 and 135 in human IFNs-α. Substituting leucines for lysines in both IFN-α1 and IFN-α4 resulted in modest, statistically significant, decreases in antiviral activity on human cells (Table 1), indicating that these residues are less important than the residues at positions 121 and 122. The phenylalanine residue at position 124 is conserved in all human IFNs-α. A modified IFN-α1 with the substantially smaller and more polar serine residue at position 124 was made. The antiviral activity on human cells of this analog was slightly lower than that of wild type IFN-α1 (Table 1). IFN-α1, in contrast to all other IFN-α subtypes, has an arginine residue at position 125. All other subtypes have glutamine at this position. This glutamine residue is not, however, a critical determinant of high antiviral activity on human cells, since the substitution of the arginine in IFN-α1 with glutamine results in a 75% decrease in antiviral activity on human cells (Table 1).

The data for the amino acid substitutions between positions 121 and 136 indicate that the residues 121, 122, and 123 in particular are important for antiviral activity on human cells. None of the substitutions in the 121-136 region changed the antiviral activity of IFN-α1 and IFN-α4 on bovine cells to the same extent as they changed the antiviral activity on human cells (Table 1). Only the Arg_{121}, $Lys_{121} \rightarrow$ Leu, and $Tyr_{123} \rightarrow$ Ser substitutions in IFN-α4 resulted in modest, but statistically significant, decreases in bovine antiviral activity. This indicates that the antiviral activity on bovine cells of IFN-α4 is more sensitive to structural change than that of IFN-α1. It is interesting to note that a number of the amino acid substitutions in IFN-α1 resulted in small increases in bovine antiviral activity.

Amino acid substitutions in the region between residues 31 and 42

Since our results indicated that the region around tyrosine 123 is important for at least one of the biological activities of human IFNs-α, we next examined the

region between residues 30 and 42, which is adjacent to tyrosine 123 in the 3-dimensional structural model proposed by Sternberg and Cohen (1982).

An examination of the IFN-α sequence between residues 31 and 43 shows that this region is predominantly hydrophilic, as is the region around residue 123. The sequence conservation in this region is very high; 5 of the 13 residues between 31 and 43 are conserved in all IFNs-α (Weissmann and Weber, 1986). This region is characterized by a large number of charged residues, which is also a feature of residues 121 to 136. To test the importance of the side-chain characteristics of some of these charged residues between residues 31 and 43, two IFN-α4 analogs, each with a double amino acid substitution of amino acids with an opposite charge, were made. By changing the charge of the residues, the importance of the side-chain could be assessed without significant change to local hydrophilicity. Substituting acidic glutamic acid residues for Lys_{31} and Arg_{33} in IFN-α4 resulted in a dramatic loss of antiviral activity on both human and bovine cells (Table 2). However, substituting basic lysine residues for Glu_{41} and Glu_{42} resulted in only a small decrease in antiviral activity (Table 2). In the case of the $Glu_{41,42}$→Lys,Lys substitution but not the $Lys_{31,}Arg_{33}$→Glu,Glu substitution, maintenance of local hydrophilicity is apparently sufficient for the maintenance of most of the antiviral activity.

The marked loss of activity resulting from the $Lys_{31}Arg_{33}$→Glu,Glu substitution implies that either these residues are directly involved in receptor binding or that major structural changes in the IFN molecule occur when two glutamic acid residues are introduced.

One possible consequence of a large structural perturbation could be that the disulfide bond between residues 29 and 139, which is essential for full biological activity (Nisbet et al., 1985), may not form. Treatment of native IFN-α4, which has two disulfide bonds, with 2-mercaptoethanol results in the loss of the disulfide bonds and decreased mobility on SDS-polyacrylamide gels (data not shown). This presumably results from a conformational change due to unfolding. Polyacrylamide gel analysis of IFN-α4 and its analog, Lys_{31}, Arg_{33}→Glu,Glu indicates that the substituted protein displays the same mobility change upon reduction with 2-mercaptoethanol as does unmodified IFN-α4 (data not shown). This suggests that the

TABLE 2

Relative antiviral activities of modified IFN-α4 on human and bovine cells

IFN-α[4] analog	Relative antiviral activity	
	Human cells (HEp-2)	Bovine cells (MDBK)
Lys_{31}, Arg_{33} → Glu, Glu	<0.003[a,b]	<0.001[a,b]
Lys_{31} → Glu	16.4 ± 5.9[a]	72 ± 17
Arg_{33} → Glu	<0.1[a]	<0.05[a]
Arg_{33} → Lys	<0.1[a]	5.3 ± 2.1[a]
Glu_{41}, Glu_{42} → Lys, Lys	46.9 ± 5.1[a]	92 ± 12

Results shown are the means of the percentage of the activity of unmodified IFN-α ± standard error of the mean, from at least five determinations.

[a]Difference from activity of unmodified IFN significant at $P < 0.01$.

[b]Value for analogue expressed in yeast. When expressed in vitro, the activities were <0.1%.

Lys_{31}, Arg_{33}→Glu,Glu analog is probably capable of forming both disulfide bonds.

To investigate further the characteristics of the Lys_{31}, Arg_{33}→Glu,Glu substitution, IFN-α4 analogs with single amino acid substitutions were made (Table 2). The substitution of glutamic acid for lysine at residue 31 resulted in a 83% loss of antiviral activity on human cells. There was a small decrease in antiviral activity on bovine cells, which was not statistically significant. The substitution of glutamic acid for arginine at residue 33 resulted in a dramatic loss in antiviral activity on both human and bovine cells.

The substitution of lysine for arginine at residue 33, a conservative substitution, reduced antiviral activity on human cells to <0.1% of the activity of native IFN-α4. However, this analog maintained 5% of the antiviral activity of unmodified IFN-α4 on bovine cells.

Antiproliferative activity of IFN-α analogs

In addition to their antiviral activities, human IFNs-α inhibit the proliferation of a number of human cell lines. The antiproliferative activities of some of the IFN-α1 and IFN-α4 analogs were assessed using cells of the Daudi lymphoblastoid line (Table 3). For all the IFN-α1 and IFN-α4 analogs tested, the change in antiproliferative activity due to the amino acid substitution(s) was proportional to the induced change in antiviral activity. This indicates that these two biological activities are not potentiated with respect to each other for any of the IFN analogs described.

TABLE 3

Relative antiproliferative activities of modified IFN-α1 and IFN-α4 on human cells

Analog	Relative antiproliferative activity
IFN-α1	
Lys_{121}, Lys_{122} → Leu, Leu	2 ± 1
Tyr_{123} → Ser	9 ± 4
Phe_{124} → Ser	43 ± 8
Arg_{125} → Gln	17 ± 5
Lys_{134}, Lys_{135} → Leu, Leu	77 ± 9
IFN-α4	
Lys_{31}, Arg_{33} → Glu, Glu	<0.001
Glu_{41}, Glu_{42} → Lys, Lys	78 ± 20
Arg_{121}, Lys_{121} → Leu, Leu	9 ± 4
Tyr_{123} → Ser	6 ± 1
$Lys_{134,135}$ → Leu	53 ± 11

Results shown are the means of the percentages of the activity of unmodified IFN-α ± standard error of the mean, from at least three determinations.

Discussion

The results presented in this paper indicate that residue 33 and residues at and adjacent to 123 are required for full antiviral activity on human cells. The region around tyrosine 123 and the region around arginine 33 form antiparallel β-sheets in the Sternberg and Cohen model for human IFNs-α (Sternberg and Cohen, 1982). Regions within these two antiparallel β-sheets exhibit considerable sequence homology across all of the 14 IFN-α subtypes. These two regions are also critical for the antiviral activity of both human IFN-α1 and murine IFN-α1 on murine cells (Beilharz et al., 1988; Kerry et al., 1988).

The basic residues at 121 and 122 adjacent to the tyrosine at 123 are important for the full antiviral activity of IFN-α1 and IFN-α4 on human cells but are not critical for antiviral activity on bovine cells. However, the basic residues at 134 and 135 adjacent to tyrosine 136 are not as critical for antiviral activity on either human or bovine cells, when this activity is assessed in vitro. This is an unexpected result since these residues are conserved in all mammalian IFNs-α. Amino acids which appear relatively unimportant by in vitro analysis may nevertheless be critical for activity in vivo.

The double amino acid substitutions $Lys_{31},Arg_{33}\rightarrow Glu,Glu$ and $Glu_{41},Glu_{42}\rightarrow Lys,Lys$ were made in IFN-α4 to determine the effect of changing charge without changing hydrophilicity. In the case of the Glu_{41}, $Glu_{42}\rightarrow Lys,Lys$ substitution, the change in charge only slightly decreased biological activity. These hydrophilic residues might be important for stabilizing the structure of the IFN molecule. The substitution of hydrophobic residues could be made to test this hypothesis.

An IFN analog with the Lys_{31}, $Arg_{33}\rightarrow Glu,Glu$ substitution shows no detectable antiviral or antiproliferative activity on human, bovine or murine cells. Analysis of single amino acid substitutions showed that this loss of activity is probably attributable mainly to the $Arg_{33}\rightarrow Glu$ substitution. The magnitude of this loss in activity suggests that Arg_{33} may be particularly critical for interaction with the IFN receptor on the cell surface. Studies of IFN-α2 have shown that substitution of Arg_{33} with either alanine, lysine or methionine results in loss of antiviral activity on human WISH cells (Camble et al., 1986). These substitutions do not significantly affect antiviral activity on bovine MDBK cells (Edge et al., 1986). The arginine to glutamic acid substitution we have made does reduce antiviral activity on bovine cells, which implies that a negatively charged residue may be more disruptive to the IFN structure than the hydrophobic residues methionine and alanine.

The interaction of IFNs-α with the human IFN-α receptor is probably dependent upon a number of amino acid interactions. Some of these interactions may be critical for the initial binding and other interactions may stabilize the advanced stages of the interaction. Thus, Arg_{33} may interact directly with the receptor, while residues at and adjacent to Tyr_{123} may be important for stabilizing the IFN-receptor complex. The nature of these interactions may be determined by either NMR or X-ray diffraction studies.

Acknowledgements

We wish to thank K. McIver and V. Lepper for excellent bioassays, Dr I.R. Mackay for review of the manuscript, and J. Oh and V. Chia for their typing. This work was supported by the National Health and Medical Research Council of Australia.

References

Aguet, M., Grobke, M. and Dreiding, P. (1984) Various human interferon α subclasses cross-react with common receptors: their binding affinities correlate with their specific biological activities. Virology 132, 211–216.

Allen, G. and Fantes, K.H. (1980) A family of structural genes for human lymphoblastoid (leukocyte-type) interferons. Nature (London) 287, 408–411.

Beilharz, M.W., Tymms, M.J., Chambers, P.J., McInnes, B., Pitha-Rowe, P.M. and Linnane, A.W. (1988) Amino acid substitutions which alter the antiviral activity of human IFN-α on mouse cells. J. Interferon Res. 8, 779–782.

Camble, R., Petter, N.N., Trueman, P., Newton, C.R., Carr, F.J., Hockney, R.C., Moore, V.E., Greene, A.R., Holland, D. and Edge, M.D. (1986) Functionally important conserved amino-acids in interferon-α2 identified with analogues produced from synthetic genes. Biochem. Biophys. Res. Commun. 134, 1404–1411.

Devenish, R.J., Maxwell, R.J., Beilharz, M.W., Nagley, P. and Linnane, A.W. (1987) The construction of expression vectors for the production of interferons in yeast. Dev. Biol. Stand. 67, 185–199.

Edge, M.D., Camble, R., Moore, V.E., Hockney, R.C., Carr, F.J. and Fitton, J.E. (1986) Interferon analogues from synthetic genes: An approach to protein structure-activity studies. In: I. Gresser (Ed.), Interferon, Vol. 7, pp. 1–46. Academic Press, New York.

Fish, E.N., Banerjee, K. and Stebbing, N. (1983) Human leukocyte interferon subtypes have different antiproliferative and antiviral activities on human cells. Biochem. Biophys. Res. Commun. 112, 537–546.

Ghelis, C. and Yon, J. (1982) Protein Folding. Academic Press, New York.

Jilbert, A.R., Hertzog, P.J., Burrell, C.J., Gowans, E.J., Linnane, A.W. and Marmion, B.P. (1986) Production of a monoclonal antibody to human interferon-α (IFN-α) and its use to identify IFN-α-producing cells in virus infection in vivo. Microb. Pathogen 1, 159–168.

Kerry, J.A., Tymms, M.J. and Linnane, A.W. (1988) Conserved amino acid residues arginine 33 and tyrosine 123 are critical for the antiviral activity of murine interferon-α1. Biochem. Biophys. Res. Comm. 155, 714–719.

Kreig, P.A. and Melton, D.A. (1984) Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. Nucleic Acids Res. 12, 7057–7070.

Lawn, R.M., Fritsch, E.F., Parker, R.C., Blake, G. and Maniatis, T. (1978) The isolation and characterization of linked γ- and β- globin genes from a cloned library of human DNA. Cell 15, 1157–1174.

Linnane, A.W., Beilharz, M.W., McMullen, G.L., Macreadie, I.G., Nisbet, I.T., Novitski, C.E. and Woodrow, G.C. (1984) Nucleotide sequence and expression in *E. coli* of a human interferon-α gene selected from a genomic library using synthetic oligonucleotides. Biochem. Int. 8, 725–732.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Nisbet, I.T. and Beilharz, M.W. (1985) Simplified DNA manipulations based on in vitro mutagenesis. Gene Anal. Techn. 2, 23–29.

Nisbet, I.T., Beilharz, M.W., Hertzog, P.J., Tymms, M.J. and Linnane, A.W. (1985) Single amino acid substitutions at conserved residues of human interferon-α can affect antiviral specific activity. Biochem. Int. 11, 301–309.

Nisbet, I.T., Beilharz, M.W., Hertzog, P.J., Tymms, M.J. and Linnane, A.W. (1986) Structure/function studies on human IFN-α1 by site-directed mutagenesis indicate amino acid residues determining antiviral activity and species specificity. In: W.E. Stewart II and H. Schellekens (Eds), The Biology of the Interferon System, pp. 19–22. Elsevier, Amsterdam.

Pestka, S., Langer, J.A., Zoon, K.C. and Samuel, C.E. (1987) Interferons and their actions. Ann. Rev. Biochem. 56, 727–777.

Pratt, J.M. (1984) Coupled transcription-translation in prokaryotic cell-free systems. In: B.D. Hames and S.J. Higgins (Eds), Transcription and Translation: A Practical Approach, pp. 179–209. IRL Press, Oxford.

Rehberg, E., Kelder, B., Hoal, E.G. and Pestka, S. (1982) Specific molecular activities of recombinant and hybrid leukocyte interferons. J. Biol. Chem. 257, 11497–11502.

Rubinstein, M., Rubinstein, S., Familletti, P.C., Miller, R.S., Waldman, A.A. and Pestka, S. (1979) Human leukocyte interferon: production, purification to homogeneity and initial characterization. Proc. Natl. Acad. Sci. USA 76, 640–644.

Shoeman, R.L. and Schweiger, H.G. (1982) Gene expression in Acetabularia. I: calibration of wheat-germ cell-free translation system proteins as internal references for two-dimensional electrophoresis. J. Cell. Sci. 58, 23–33.

Sternberg, M.J.E. and Cohen, F.E. (1982) Prediction of the secondary and tertiary structures of interferon from four homologous amino acid sequences. Int. J. Biol. Macromol. 4, 137–144.

Streuli, M., Hall, A., Boll, W., Stewart II, W.E., Nagata, S. and Weissmann, C. (1981) Target cell specificity of two species of human interferon-α produced in *Escherichia coli* and of hybrid molecules derived from them. Proc. Natl. Acad. Sci. USA 78, 2848–2852.

Tymms, M.J. and McInnes, B. (1988) Efficient in vitro expression of interferon-α analogues using SP6 polymerase and rabbit reticulocyte lysate. Gene. Anal. Techn. 5, 9–15.

Uze, G., Mogensen, K.E. and Aguet, M. (1985) Receptor dynamics of closely related ligands: 'fast' and 'slow' interferons. Eur. Mol. Biol. Organ. J. 4, 65–70.

Valenzuela, D., Weber, H. and Weissmann, C. (1985) Is sequence conservation in interferons due to selection for functional proteins? Nature (London) 313, 698–700.

Weck, P.K., Apperson, S., Stebbing, N., Gray, P.W., Leung, D., Shepard, H.M. and Goeddel, D.V. (1981) Antiviral activities of hybrids of two major human leukocyte interferons. Nucleic Acids Res. 9, 6153–6166.

Weissmann, C. and Weber, H. (1986) The interferon genes. Prog. Nucl. Acids Res. Mol. Biol. 33, 251–300.

Yonehara, S., Ishii, A. and Yonehara-Takahashi, M. (1983) Cell surface receptor-mediated internalization of interferon: its relation to the antiviral activity of interferon. J. Gen. Virol. 64, 2409–2418.