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# Structure-function studies of human interferons-$\alpha$: enhanced activity on human and murine cells

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## Summary

To identify functionally important regions of the human interferon (IFN)-$\alpha$ molecule, mutagenesis in vitro of human IFN-a genes was used to create analogs with deletions or specific amino acid replacements. These analogs were expressed in vitro using SP6 RNA polymerase and a rabbit reticulocyte lysate protein synthesis system. Deletion of 7 highly conserved hydrophilic amino acids from the C-terminus of human IFN-$\alpha$4 reduced, but did not abolish, antiviral activity on human cells. However, analogs with deletions of 15 or 25 amino acids from the C-terminus, or 28 amino acids from the N-terminus, had no measurable antiviral activity. The antiviral activity of human IFN-$\alpha$4 was increased by substitution of cysteine for serine at position 86, and lysine for arginine at position 121. However, other amino acid substitutions at positions 121, 122 or 123 reduced antiviral activity. The size of the side chain of the amino acid residue at position 130 was shown to be important. Replacement of the absolutely conserved leucine residue at position 131 with glutamine had little effect on antiviral activity. However, the introduction of a proline residue at this position abolished antiviral activity, probably due to the formation of a $\beta$ turn in the polypeptide chain. The antiviral activity of human IFN-$\alpha$4 on murine cells was increased by substitutions at positions 86, 121 and 133. This study illustrates the utility of the in vitro mutagenesis and rabbit reticulocyte lysate systems for the investigation of structure-function relationships, and extends our knowledge of the biologically active regions and species specificity of the human IFN-$\alpha$ molecule.

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## Introduction

Human interferons-$\alpha$ (IFNs-$\alpha$) are a family of at least 15 highly homologous proteins which bind to receptors on the cell surface (Aguet et al., 1984), induce an antiviral state, inhibit cell growth, stimulate the activity of natural killer cells and the cytotoxic activities of lymphocytes and macrophages, and induce cell differentiation in normal cells as well as some neoplastic cells (Pestka et al., 1987). Although the IFNs show species specificity, human IFNs-$\alpha$ are highly active on bovine cells and have low but detectable activity on murine cells. Human IFNs-$\alpha$ and murine IFNs-$\alpha$ show a high degree of sequence conservation with approximately 70% of amino acid residues conserved (Shaw et al., 1983).

Human lymphocytes, after infection with viruses, produce a mixture of IFN-$\alpha$ subtypes (Rubinstein et al., 1979; Allen and Fantes, 1980). The biological activities of those IFN-$\alpha$ subtypes which have been characterized vary significantly when assayed in vitro (Streuli et al., 1981; Weck et al., 1981; Rehberg et al., 1982; Fish et al., 1983). Differences in biological activities between the IFN-$\alpha$ subtypes may be due to differences in their affinities for the cell receptor (Yonehara et al., 1983; Aguet et al., 1984; Uze et al., 1985).

The human IFNs-$\alpha$ contain 166 amino acids, with the exception of human IFN-$\alpha$2, which contains 165 amino acids. Studies with hybrid molecules between IFN-$\alpha$ 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). Analogs of human IFN-$\alpha$ that lack 3 N-terminal amino acids retain full biological activity, indicating that at least part of the N-terminus is dispensable (Lydon et al., 1985; Edge et al., 1986). Similarly, the isolation of natural human leukocyte IFN which lacked 10 C-terminal residues but was biologically active (Levy et al., 1981) indicated that the highly-charged C-terminal sequence may not be absolutely required. This was confirmed by the removal by proteolytic cleavage of 13 C-terminal amino acids from human IFN-$\alpha$2 without greatly reducing biological activity (Wetzel et al., 1982). In addition, a truncated analog of human IFN-$\alpha$2, produced by removing 11 C-terminal amino acids by synthetic gene construction, retained some antiviral activity (Edge et al., 1986).

Site-directed mutagenesis in vitro allows the assessment of the roles of individual amino acids within the IFN-$\alpha$ molecule. Conserved residues within hydrophilic domains are likely to be functionally important in a protein such as IFN, which interacts with a cell surface receptor. Some of the amino acids that are conserved in the human IFN-$\alpha$ sequence can be replaced without loss of biological activity (Valenzuela et al., 1985) whereas other conserved residues, such as arginine at position 33 (Arg33) (Camble et al., 1986), Arg121 and Lys122 (Tymms et al., 1987; 1989a) and Tyr123 (Nisbet et al., 1986; McInnes et al., 1989) are essential for full biological activity.

The species specificity of human and murine IFNs has also been examined by site-directed mutagenesis in vitro of human IFN-$\alpha$ genes. Human IFN-$\alpha_1$ has relatively high antiviral activity on murine cells compared with other human IFN-$\alpha$ subtypes. Amino acid substitutions at residues in IFN-$\alpha$1 which are shared with

murine IFNs-$\alpha$ has identified a number of residues critical for the antiviral activity on murine cells of human IFN-$\alpha_1$ (Beilharz et al., 1988). In a complementary approach, residues which increase the antiviral activity on murine cells of human IFN-$\alpha_2$ have been identified (Weber et al., 1987). The results of these studies suggest that amino acids between positions 121 and 136 determine activity on murine cells. Substitutions at positions 121, 122, 123, 125 and 136 in IFN-$\alpha_1$ potentiate murine cell activity more strongly than human cell activity (Beilharz et al., 1988; McInnes et al., 1989). Similarly, in human IFN-$\alpha_2$, substitutions at positions 121, 125 and 132 also strongly potentiate activity on murine cells (Weber et al., 1987).

This paper examines the effect of deletions at the amino and carboxy termini of human IFNs-$\alpha$, and investigates the biological roles of several amino acids in the carboxy terminus. Several amino acid substitutions which greatly enhance the antiviral activity on murine cells of human IFN-$\alpha_4$ were identified. In addition, two amino acid substitutions which increase the biological activity of human IFN-$\alpha_4$ on human cells were found.

## Materials and Methods

### *Site-directed in vitro mutagenesis*

Oligonucleotide-directed mutagenesis reactions and DNA sequencing were performed as described previously (Nisbet and Beilharz, 1986).

### *Random mutagenesis*

Chemical mutagenesis procedures are described elsewhere (Tymms et al., 1989b). Mutagenesis with mixed oligonucleotides was performed essentially as described by Ner et al. (1988) except that the mutagenic oligonucleotide was doped with 1 in 100 of each of the other nucleotides in every cycle of the synthesis.

### *Construction of the SP6-based expression vector pBM1*

Vector pSP64T (Krieg and Melton, 1984) was cut at a unique *Pvu*II site, treated with alkaline phosphatase (Chaconas and Van de Sande, 1980), and an 874 base pair blunt-ended *Ball–Pvu*II fragment from M13mp8 (Messing, 1983) containing the origin for single-stranded DNA replication was cloned into this site. The *Bam*HI site in the polylinker was then removed by digestion with *Bam*HI, filling in the recessed 3′ termini using the Klenow fragment of DNA polymerase I and blunt end ligation in the presence of 7% w/v polyethylene glycol. A self-complementary 12 base oligonucleotide with the sequence 5′-GATCTGGATCCA-3′ was phosphorylated using T4 polynucleotide kinase, annealed and ligated into the *Bgl*II cloning site, resulting in a *Bam*HI site flanked by two *Bgl*II sites.

*Expression of IFNs in vitro*

IFN-$\alpha_1$ and -$\alpha_4$ analogs generated in M13 vectors were cloned into the *Bam*HI expression site of plasmid vector pBM1, which contains a promoter sequence for SP6 RNA polymerase. Modified IFN genes were then transcribed using SP6 RNA polymerase, and the RNA translated using rabbit reticulocyte lysate (RRL; Promega Corporation, Madison, U.S.A.; Tymms and McInnes, 1988). $^{35}$S-labelled methionine (Amersham, Buckinghamshire, United Kingdom) was included in the translations to monitor the level of protein synthesis in individual translations. To assess the fidelity of RRL translations, samples were separated on 9–15% SDS-polyacrylamide gels and [$^{35}$S]methionine-labeled IFN was visualized by soaking gels in fluorography enhancer (Amplify, Amersham), drying and exposing to X-ray film at −80°C (Shoeman and Schweiger, 1982). Molecular size standards (protein test mixture 5, Serva, Heidelberg, West Germany) were $^{35}$S-labeled using general purpose $^{35}$S-labeling reagent (>800 Ci/mmol, Amersham).

*Biological assays*

RRL preparations containing IFN-$\alpha$ were assayed for antiviral activity on human WISH cells and bovine MDBK cells challenged with Semliki Forest virus as described previously (Jilbert et al., 1986) or on murine L929 cells challenged with encephalomyocarditis virus (Beilharz et al., 1988). Cells were grown to confluence, trypsinized, and distributed into 96 well microtitre plates ($10^4$ cells per well) in RPMI medium (Commonwealth Serum Laboratories, Melbourne, Australia) with 3% fetal calf serum. IFN samples were titrated in duplicate in half log steps down the plate. After incubation for 16 h, the medium was removed, replaced with medium containing Semliki Forest virus (100 $TCID_{50}$ per well) and the plates incubated for three days. The cytopathic effect (CPE) was scored by light microscopy on a scale of 0 (no CPE) to 4 (total cell destruction). The IFN titre, defined as the dilution giving 50% protection against virus-induced CPE, was determined by adding the scores of the duplicate wells for each sample. When this value was equal to 4, the IFN titre was the dilution corresponding to those sample wells. When this value was not equal to 4, the IFN titres were corrected by adding 0.1 to 0.4 log units to the dilution, according to the sums of the scores of the wells above and below the point at which the sum was greater than 4. Thus, IFN titres were determined to the nearest 0.1 log unit. The antiviral activity of all laboratory standards and samples was calibrated against reference human leukocyte IFN 023-901-527 (National Institutes of Health, Bethesda, U.S.A.). For assays on murine cells, the reference standard used was Cytimmune$^R$ mouse IFN-$\alpha/\beta$ (Catalogue number 20061, Lee Biomolecular, San Diego, CA, U.S.A.).

*Calculation of relative specific activities and statistical analysis*

The antiviral activity of unmodified IFN-$\alpha$ and IFN-$\alpha$ analogs was determined in several assays. The incorporation of [$^{35}$S]methionine, and hence the protein content, varied slightly between different translations. This variation did not usually exceed 20%. Thus, activities were corrected for differences in [$^{35}$S]methionine incorporation. The mean of the corrected values was determined, and biological activities were expressed as a percentage of the mean activity of unmodified IFN-$\alpha$ from the same translation mix, to give a relative specific activity (RSA). The significance of differences between RSA values for unmodified IFN-$\alpha$ and analogs was determined by Student's *t*-test.

## Results

*Generation and expression of IFN analogs*

Oligonucleotide-directed mutagenesis of the human IFN-$\alpha$4 gene was used to delete 7 or 15 amino acids from the C-terminus (analogs [1–158] and [1–151]) and 28 amino acids from the N-terminus ([29–165]); to replace Ser86 with Cys (Ser86Cys), Arg121 with Lys (Arg121Lys); to produce the double mutation Ser86Cys, Arg121Lys; to replace both Arg121 and Lys122 with glutamic acid (Arg121Glu,Lys122Glu) and to create a triple amino acid substitution Arg121Glu,Lys122Glu,Tyr123Ala. Similarly, Tyr130 in human IFN-$\alpha_1$ was replaced by phenylalanine (Tyr130Phe), arginine (Tyr130Arg) or glycine (Tyr130Gly). Random chemical mutagenesis of human IFN-$\alpha$4 produced the analogs Leu131Pro, Glu133Lys, and a deletion of 25 amino acids from the C-terminus ([1–141]). Mutagenesis using mixed oligonucleotides (Ner et al., 1988) generated the analog Leu131Gln,Thr132Lys. The mutations were confirmed by DNA sequencing. All analogs were expressed at high levels in the RRL expression system, which has been shown to be highly suitable for the expression of IFNs (Tymms and McInnes, 1988). Analysis of the translation products by polyacrylamide gel electrophoresis showed a single major radioactive band of the expected size. Previous experiments have established that this major band corresponds to the IFN product, by purification of the product on a YOK 5/12 monoclonal antibody affinity column (Tymms and McInnes, 1988). A sample of the electrophoresis data is shown in Fig. 1, which also confirms the truncation of the molecules in the [1–141] and [1–151] analogues. Amino acid substitutions did not noticeably alter the mobility of the IFN molecule (data not shown). A minor band of lower mobility than the major IFN band was occasionally seen after polyacrylamide gel electrophoresis (Fig. 1, lane A). Such bands always accounted for less than 5% of the total protein product, and probably represent premature termination of translation of the IFN gene. Since truncated products all show reduced activity, the presence of this minor species in low amounts would not affect the calculations of relative biological activities. The antiviral activities of the analogs were determined using human WISH cells, bovine MDBK cells and murine L929 cells.

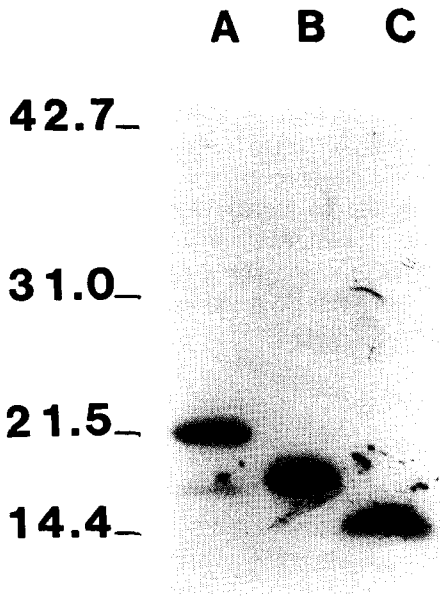


Fig. 1. Fluorograph of $^{35}$S-labeled IFN-$\alpha_4$ and analogs separated on a 9%-15% polyacrylamide/SDS gel under reducing conditions. Proteins were prepared by translation in vitro of mRNA in the presence of [$^{35}$S]methionine. Samples were IFN-$\alpha_4$ (A), IFN-$\alpha_4$ [1–151] (B) and IFN-$\alpha_4$ [1–141] (C). Numbers at the side refer to molecular mass in kDa of molecular weight size standards.

*C-terminal and N-terminal deletions*

The interpretation of studies which have used proteolytic fragments of IFN is complicated by possible contamination of the preparation with small amounts of undigested protein. Truncated IFNs have been produced by in vitro mutagenesis, but expression of the truncated proteins in *E. coli* has proven difficult, with very low yields obtained (Edge et al., 1986). The in vitro expression system used in this study was found to produce good yields of all the truncated proteins tested.

The deletion of 8 amino acids from the C terminus ([1–158]) reduced the antiviral activity on human cells to 3% of the activity of unmodified IFN-$\alpha$4 (Table 1). Removal of a further 7 amino acids ([1–151]) reduced the antiviral activity of IFN-$\alpha$4 to less than 0.2%. Similarly, no antiviral activity was detected in molecules from which 25 C-terminal amino acids were removed ([1–141]). Deletion of 28 amino acids from the N-terminus ([29–166]) also abolished antiviral activity as measurable in this assay system using IFNs synthesized in the rabbit reticulocyte lysate system (Table 1).

*Replacement of serine at position 86 with cysteine*

With the exception of human IFN-$\alpha_1$, all human IFNs-$\alpha$ have serine at position 86. Human IFN-$\alpha_1$, which has a cysteine residue at position 86, has lower activity

TABLE 1

The relative antiviral activities on human and bovine cells of human IFN analogs with deletions at the amino or carboxy termini

| IFN analog | Relative antiviral activity | |
|---|---|---|
| | Human cells (WISH) | Bovine cells (MDBK) |
| Unmodified IFN | 100 | 100 |
| [1–141] | <0.2 | <0.05 |
| [1–151] | <0.2 | <0.05 |
| [1–158] | 3.2 ± 0.5[a] | ND |
| [29–165] | <0.2 | ND |

Results shown are the means of the percentages of the activity of unmodified IFN-$\alpha$ ± standard error of the mean from six to ten determinations.
[a]Difference from activity of unmodified IFN-$\alpha$ significant at $P$ less than 0.01.
ND = not done.

TABLE 2

The relative antiviral activities on human and bovine cells of human IFN-$\alpha$ analogs with amino acid substitutions

| IFN analog | Relative antiviral activity | |
|---|---|---|
| | Human cells (WISH) | Bovine cells (MDBK) |
| Unmodified IFN | 100 | 100 |
| Ser86Cys | 91 ± 23 | 125 ± 26 |
| Arg121Lys | 240 ± 33[a] | 115 ± 16 |
| Arg121Lys,Ser86Cys | 395 ± 80[a] | 116 ± 21 |
| Arg121Glu,Lys122Glu | 0.20 ± 0.05[a] | 0.054 ± 0.012[a] |
| Arg121Glu,Lys122Glu,Tyr123Ala | 0.059 ± 0.030[a] | 0.021 ± 0.009[a] |
| Tyr130Phe | 92.2 ± 24.7 | 67.5 ± 11 |
| Tyr130Arg | 15.7 ± 2.4[a] | 26.2 ± 7.3 |
| Tyr130Gly | 2.4 ± 0.5[a] | 9.4 ± 1.3[a] |
| Leu131Pro | <0.2 | ND |
| Leu131Gln,Thr132Lys | 77.3 ± 9.4 | 52.9 ± 8.9 |
| Glu133Lys | 143 ± 33 | 90 ± 10 |

Results shown are the means of the percentages of the activity of unmodified IFN-$\alpha$ ± standard error of the mean, from six to ten determinations.
[a]Difference from activity of unmodified IFN significant at $P$ less than 0.01.
ND = not done.

on human cells than most of the other human IFNs-$\alpha$. However, replacement of the serine residue at position 86 with cysteine had no effect on the antiviral activity on either human or bovine cells (Table 2). Thus, the presence of a cysteine residue at position 86 is not by itself responsible for the lower specific activity of human IFN-$\alpha_1$.

*Substitutions at positions 121, 122 and 123*

Our previous studies have shown that amino acid residues at positions 121, 122 and 123 are important for activity of human IFNs-$\alpha$ (Tymms et al., 1989a; McInnes et al., 1989). Specifically, we have shown that substitution of leucine residues for

the charged residues Arg121 and Lys122 results in a loss of antiviral activity on human cells, but little change in antiviral activity on bovine cells. Amino acid replacements at the residue Tyr123 of IFN-$\alpha_1$ have shown a requirement for a large hydrophobic residue at this position, so that replacement with an alanine residue results in loss of 92% of antiviral activity on human cells, but no change in activity on bovine cells.

Replacement of the residues at position 121 and 122 with negatively-charged glutamic acid to give the analog Arg121Glu,Lys122Glu reduces the antiviral activity on human cells of IFN-$\alpha_4$ to 0.2% of that of the unmodified IFN, and on bovine cells the activity is reduced to 0.05% (Table 2). These results indicate that the introduction of negative charge to this region is more detrimental than substituting hydrophobic residues such as leucine, since antiviral activities on human and bovine cells of the analog Arg121Leu,Lys122Leu are 8% and 29% respectively (Tymms et al., 1989a). The highly conserved positively charged residues Arg121 and Lys122 may play a specific structural role.

To investigate the effect of simultaneous changes at positions 121, 122 and 123, the analog Arg121Leu,Lys122Leu,Tyr123Ala was created. The antiviral activity of this analog on both human and bovine cells was less than 0.06% of the antiviral activity of unmodified IFN-$\alpha_4$ (Table 2). The relative antiviral activities on human cells of the analogs Arg121Leu,Lys122Leu and Tyr123Ala are 8.2% and 8.3% respectively (Tymms et al., 1989a; McInnes et al., 1989). Thus, the antiviral activity of the analog with the triple substitution is much less than that of the Arg121Leu,Lys122Leu or the Tyr123Ala analogs, so amino acid replacements in this region have an additive effect. It is of interest that the antiviral activity on bovine cells of the analog with the triple substitution is reduced to the same extent as the antiviral activity on human cells; in contrast the antiviral activity on bovine cells of the analog Arg121Leu,Lys122Leu was reduced to a much smaller extent than the antiviral activity on human cells (Tymms et al., 1989a).

The conservative replacement of the arginine residue at position 121 in human IFN-$\alpha_4$ with lysine resulted in a modest, but statistically significant, increase in activity on human cells (Table 2). The double substitution of Arg121Lys with Ser86Cys resulted in a further increase in activity on human cells. Neither of these two analogs showed any change in antiviral activity on bovine cells. The enhanced activity of these analogs was unexpected, since the only human IFN-$\alpha$ subtype which has cysteine at position 86 and lysine at position 121 is IFN-$\alpha_1$, which has relatively low antiviral activity on human cells.

*Substitutions at positions 130, 131 and 133*

The tyrosine residue at position 130 is conserved in all mammalian IFNs-$\alpha$ (Weissmann and Weber, 1986). To investigate the importance of this residue, we made a conservative substitution, Tyr130Phe, and two radical substitutions, one introducing positive charges, Tyr130Arg, and one minimising the side of the side chain, Tyr130Gly. The conservative substitution with phenylalanine had no significant effect on antiviral activity on either human or bovine cells (Table 2).

Of the radical substitutions, introduction of a glycine residue had the most severe effect, reducing antiviral activity on human cells by 97.6% and on bovine cells by 90.6%. A smaller reduction in activity, of 84.3% on human cells and 73.8% on bovine cells, was seen with the arginine substitution (Table 2). These results suggest that the size of the side chain of the amino acid at position 130 is more important than its hydrophobicity.

The leucine residue at position 131 is absolutely conserved in all known mammalian IFNs-$\alpha$, while the amino acid at position 132 differs in different IFNs-$\alpha$ (Weissmann and Weber, 1986). Substitution of these two residues with glutamine and lysine respectively had little effect on the antiviral activity of human IFN-$\alpha_4$ (Table 2), and thus the precise nature of the side chain at position 131 is not important. However, replacement of the highly conserved leucine residue with proline reduces the antiviral activity by greater than 99.8% (Table 2). The $\beta$ turn index of this substitution, calculated according to the method of Chou and Fasman (1979) is $2.5 \times 10^{-4}$, indicating a very high probability of a $\beta$ turn, which would result in a severe disturbance of the secondary structure.

Glutamic acid at position 133 is highly conserved in human IFNs-$\alpha$ (Weissmann and Weber, 1986). Replacement of glutamic acid with lysine had no effect on the antiviral activity of human IFN-$\alpha$4 on both human and bovine cells (Table 2).

*Enhanced antiviral activity on murine cells*

Murine IFNs-$\alpha$, unlike the majority of human IFNs-$\alpha$, have a cysteine residue at position 86. Replacement of the serine residue at position 86 in human IFN-$\alpha$4 caused a 14 fold increase in antiviral activity on murine cells (Table 3). A similar increase was seen when glutamic acid at position 133 was replaced by lysine (Table 3). Replacement of arginine 121 by lysine increased the antiviral activity on murine cells 20-fold, while the double substitution of Ser86Cys,Arg121Lys increased activity 40-fold. None of these substitutions had any effect on the antiviral activity of human IFN-$\alpha$4 on bovine cells (Table 2).

TABLE 3

The relative antiviral activities on murine cells of human IFN-$\alpha$ analogs with amino acid substitutions

| IFN analog | Relative antiviral activity |
|---|---|
| Unmodified IFN-$\alpha_4$ | 100 |
| Ser86Cys | 1367 ± 316[a] |
| Arg121Lys | 2034 ± 486[b] |
| Arg121Lys,Ser86Cys | 4667 ± 243[b] |
| Glu133Lys | 1502 ± 307[b] |

Results shown are the means of the percentages of the activity of unmodified IFN-$\alpha$ ± standard error of the mean, from six to ten determinations.

[a]Difference from activity of unmodified IFN significant at $P$ less than 0.05.

[b]Difference from activity of unmodified IFN significant at $P$ less than 0.01.

## Discussion

Eight highly conserved hydrophilic amino acids may be deleted from the C-terminus with retention of considerable antiviral activity in vitro. However, removal of 15 or more residues from the C-terminus abolished all measurable antiviral activity. This contrasts with a previous report that a fragment produced by deletion by proteolytic digestion of 56 amino acids from human IFN-$\alpha$ had 30% of the antiviral activity of the native molecule (Ackerman et al., 1984). This discrepancy might be explained by contamination of the N-terminal fragment preparation with undigested IFN. Alternatively, since the 110 amino acid fragment is derived from an intact IFN molecule, it could remain folded in a biologically active conformation, whereas the absence of the C-terminal amino acids in the deletion analogs could prevent the protein from achieving a suitable tertiary structure.

Amino acid residues which are both absolutely conserved and important for biological activity in one human IFN-$\alpha$ subtype have, where tested, also been found to be important in other human IFN-$\alpha$ subtypes, e.g. Arg33 in IFNs-$\alpha_2$ and -$\alpha_4$ (Camble et al., 1986; Tymms et al., 1989a), and Tyr123 in human IFNs-$\alpha_1$ and $\alpha_4$ (Tymms et al., 1989a). Thus, Tyr130, found to be important for biological activity of human IFN-$\alpha_1$ in this study, is likely to be important for biological activity of other IFN-$\alpha$ subtypes. Residues which are not conserved must be responsible for the differences in activity between the human IFN-$\alpha$ subtypes. Human IFN-$\alpha_1$, which has relatively low biological activity, has cysteine at position 86 and lysine at position 121. Our finding that replacement of Ser86 with cysteine and Arg121 with lysine increases, rather than decreases, the activity of human IFN-$\alpha$4 demonstrates that these residues are not responsible for the low activity of human IFN-$\alpha_1$. Thus, other residues which differ between these two subtypes must account for the differences in biological activities.

There is a disulfide bond between cysteine residues at positions 29 and 139 in human IFNs-$\alpha$. Regions around Arg33 and Tyr123, which are both important for full biological activities, are predicted in the tertiary structure to be juxtaposed and interactive by hydrogen or ionic bonding (Sternberg and Cohen, 1982). However, deletion of the 28 amino acids preceding Cys29 abolished antiviral activity, showing that these residues are not dispensable, and some may be required to maintain the conformation of the IFN molecule. The results of the C-terminal deletion studies suggest that the highly conserved region between residues 140 and 155 is also important for biological activity. Amino acids in this region may be required to establish the correct orientation of the amino acid residues linked by the disulfide bond between the cysteine residues at positions 29 and 139.

Structure-function studies of the IFNs-$\alpha$ using in vitro mutagenesis have identified three classes of highly conserved amino acid residues. Residues in the first class, which includes Arg33, appear of critical importance for biological activity. The conservative substitution Arg33Lys profoundly reduces the activity of IFNs-$\alpha$ in a range of assays (Camble et al., 1986), and substitution of glutamic acid at this position reduces the antiviral activity to less than 0.003% of the activity of the unmodified IFN (Tymms et al., 1989a).

Residues in the second class potentiate the activity of the IFNs, but may be replaced by some other amino acids without resulting in large changes in biological activity. Thus, Tyr123 is structurally important, but substitutions with other large amino acids such as phenylalanine and histidine have little effect on activity (McInnes et al., 1989). Similarly, residues at positions 121, 122 and 125 potentiate the activity of human IFN-$\alpha$ on human cells. However, changes at these positions have a much greater effect on the antiviral activity of human IFN-$\alpha$ on murine cells (Beilharz et al., 1988).

Residues in the third class are highly-conserved, such as Leu131, Glu133, Lys134 and Lys135, but can be altered without significant loss of activity (Table 1; Tymms et al., 1989a). Many of these residues may be remote from the receptor binding domain and, as a consequence, single amino acid changes have only a minimal effect on the overall structure. However, even at these positions, some amino acid replacements, such as the substitution by proline at Leu131, can cause a considerable loss in biological activity. The absence of detectable antiviral activity of the analog Leu131Pro (Table 1) may be attributed to a gross structural change caused by the introduction of a $\beta$ turn.

Strong antibody-antigen interactions can be formed between as few as 5 or 6 amino acids (Geysen et al., 1988). In such interactions, one or two amino acids can be critical for binding affinity. Thus, Arg33 may form an essential part of the IFN receptor binding domain, either structurally or by direct ionic interaction with residues of the receptor. Amino acid residues in the C-terminus may then be necessary to maintain the geometry for the interaction.

IFN-$\alpha_4$ has a high specific antiviral activity, comparable to that of IFN-$\alpha_2$, which is being used extensively clinically (Galvani et al., 1988). The substitution of two amino acid residues at residues 86 and 121 in IFN-$\alpha_4$ results in a 4 fold increase in antiviral activity on human cells. This result was unexpected as the substitutions were designed to increase the antiviral activity on murine, not human, cells. Studies of the receptor binding of IFN-$\alpha$ subtypes and analogs (McInnes et al., 1989) has shown that antiviral activities generally correlate with binding affinity. Thus, the amino acid substitutions Ser86Cys and Arg121Lys probably increase the binding to the human receptor. It is of interest that these same substitutions apparently improve the interaction of human IFN-$\alpha_4$ with the murine receptor.

Positively charged amino acids in the region between residues 121 and 133 are important for activity on murine cells, since antiviral activity is increased by substituting lysine for glutamic acid at position 133, and decreased by replacing arginine 125 by glutamic acid (Beilharz et al., 1988). This may indicate that these residues have higher charge compatibilities with the residues which are proximal in the murine receptor. Weber et al. (1987) have reported that the substitution of threonine for arginine at position 131 in IFN-$\alpha_2$ results in a dramatic loss in activity on murine cells. This suggests that there is a general requirement for positive charge between positions 125 and 133.

*In conclusion*, mutagenesis in vitro of human IFN-$\alpha$ genes and expression of IFN analogs in the rabbit reticulocyte lysate system has successfully identified several amino acids of high importance for biological function. However, the results

presented in this paper indicate that predicting the functional consequences of amino acid substitutions is not straightforward. Another study has shown that even with X-ray crystallographic data, the functional effects of amino acid substitutions cannot be predicted reliably (Amit et al., 1986). Saturation random mutagenesis of IFN genes may allow the generation of a larger number of analogs, and should contribute to our understanding of the mechanism of action of this polyfunctional cytokine.

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