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THE BIOLOGICAL ACTIVITY OF INTERFERON ALPHA IS INFLUENCED BY TWO DISTINCT REGIONS IN THE PROTEIN

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Summary: With the aim to assign differences in activity between murine interferon-α1 and -α4 to specific amino acids, we have constructed hybrid genes and analysed the antiviral properties of the corresponding hybrid proteins. The hybrid genes were constructed by means of homologous recombination between the α1 and α4 genes in Escherichia coli. Hybrids in which the N-terminal part is derived from α1 show that two regions have a major effect on the activity: amino acid 10-20 and 55-67. When comparing hybrids with N-terminal α4 sequences, transitions in activity are found in the same regions. Interestingly, the curves for the two sets of hybrids are exactly each others mirror image.

One of the most striking properties of interferons (IFNs) is the ability to protect cells against viral infection (1). Besides this activity, IFNs were found to inhibit cell growth and to be able to induce certain cells of the immune system (2). To elicit these effects IFNs bind to a specific receptor on the target cells, whereupon the expression of several genes within these cells is affected (3,4). To date 3 distinct subtypes of IFN have been described, they are called IFN-α, IFN-β and IFN-γ (2). In mammals IFN-α proteins are encoded by a gene family with 15 to 20 members (5). Expression of several of these genes has been found to occur simultaneously (6,7). Molecular cloning of the individual genes and analysis of the properties of the corresponding proteins shows that, despite the homology, IFN-α proteins can differ considerably in their biological activity. Several groups have studied the relation between structure of the IFN-α proteins and their biological activity by using site-directed mutagenesis (8-14) and by the construction of hybrid IFN genes (13,15-24). A very elegant approach is construction of hybrid genes using homologous recombination in Escherichia coli. This results in large sets of hybrid genes with cross-over points throughout the coding region (13,20). As a consequence corresponding hybrid proteins with neighbouring cross-over points differ in

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only a few amino acids. We have used this approach on the murine IFN-α1 and -α4 genes (20 and this paper). Murine IFN-α1 and -α4 differ in their ability to protect mouse, hamster and rat cells against viral infection. The two proteins differ in 35 out of 167 amino acids (7). Here we describe the activities of hybrid proteins with N-terminal α1 sequences (α14-hybrids) and their counterparts, where the N-termini of the proteins are derived from α4 (α41-hybrids). In both sets of hybrids transitions in activity can be assigned to two distinct regions in the amino acid sequence. The activity curves for the α14-hybrids are the exact mirror image from those of the α41-hybrids.

MATERIALS AND METHODS

DNA manipulations. Plasmid isolations and enzyme incubations were mostly performed as described by Maniatis et al. (25). The exact cross-over points of the hybrid genes were established by sequencing plasmid DNA using synthetic oligonucleotides, complementary to the IFN sequence as primer for sequenase DNA polymerase (United States Biochemical Corporation, Cleveland, Ohio). Reaction conditions were as given by the manufacturer.

Production of IFN proteins. Monkey COS cells were grown in Duclbecco's MEM supplemented with 5% fetal calf serum and antibiotics. The cells were seeded in 35mm Petri dishes and grown to 30% confluence. Expression plasmids containing the (hybrid) IFN genes were transfected using the DEAE-dextran method as described before (20). 72 h after transfection the medium was replaced with medium containing ^{35}S-methionine. After a 16 h incubation this medium was used for polyacrylamide gelelectrophoresis and IFN assay.

Determination of specific antiviral activities. IFN titres were determined in a cytopathic effect reduction assay, using vesicular stomatitis virus as a challenge. IFN titres on mouse cells were calculated according to the NIH reference standard G002-904-511. Titres on chinese hamster ovary (CHO) cells were calculated relative to the antiviral activity of murine IFN-α1 (20). Titres on rat cells are given in laboratory units. Proteins secreted by transfected COS cells were separated on a 12.5% polyacrylamide gel as described before (20). The proteins were visualized by fluorography. Total radioactivity incorporated in secreted proteins was determined by precipitation with trichloroacetic acid. These values and densitometric scans of the fluorograph were used to calculate the amount of IFN in each sample.

RESULTS

Construction and expression of hybrid IFN genes. Construction of the α14-hybrid genes has been described before (20). For the α41-hybrids essentially the same method was used. Briefly: first the two coding sequences were inserted into the same plasmid (see Figure 1). The plasmid was propagated in a recA$^-$ strain of E. coli. After verification of the construct by restriction enzyme analysis, it was linearized with the enzyme ClaI and introduced into a recA$^+$ E. coli strain. Resulting colonies were analysed by colony hybridization using a plasmid fragment located close to the Cla I site as a probe. Plasmid DNA of colonies that failed to hybridize was analysed with restriction enzyme digestions. In all cases these were found to

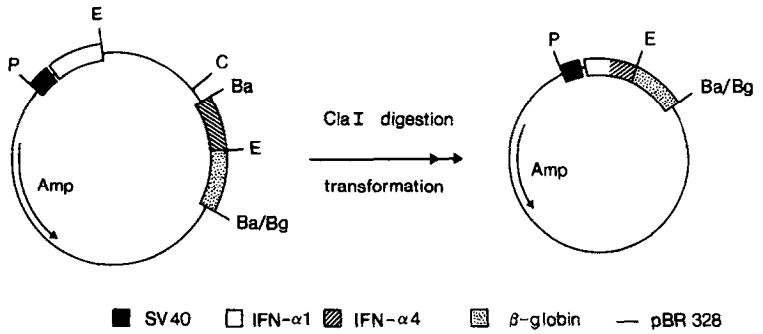

Figure 1. Construction of hybrid murine IFN-α14 genes. For construction of α41-genes the two IFN-coding sequences were reversed. Only relevant restriction enzyme sites are shown. P, PvuII; E, EcoRI; C, ClaI; Ba, BamHI; Bg, BglII. Amp, Ampicillin resistance.

contain genuine hybrid IFN genes. In total 48 α14 and 22 α41 hybrid genes were generated. Their cross-over points were established by nucleotide sequencing. Figure 1 also shows that the construction of the hybrid genes was such that after recombination they are preceeded by the SV40 origin of replication/early promoter. The rabbit β-globin sequences provide a polyadenylation signal. The plasmids were used directly for expression of the hybrid genes in monkey COS cells. Based on the position of the cross-over point and the antiviral activity measured in prelimenary experiments, a subset of hybrids was selected for further detailed analysis.

Antiviral activities of natural and hybrid IFNs. The specific antiviral activities of the natural murine IFN-α1 and -α4 proteins on mouse L929, hamster CHO and normal rat kidney (NRK) cells are presented in Table 1. IFN-α4 has an 8-fold higher activity on mouse and rat cells than α1. However, α1 has 100 fold higher activity on hamster cells.

In Figure 2 the antiviral activity of the hybrid IFNs on mouse (2A), hamster (2B) and rat (2C) cells is shown. When the α14-hybrids are aligned, such that the length of the α1 sequences increases (from left to right in the dashed curve), it appears that the antiviral activity of the proteins on mouse cells (Figure 2A) first increases from 10^8 U/mg to over 10^9 U/mg and afterwards decreases again to approximately 10^7 U/mg. With the α41-hybrids with increasing N-terminal α4 portions (from left to right, continuous line), the curve is a mirror image. The activity drops from 10^7 to 10^5 U/mg and increases again to 10^8 U/mg. The α14-hybrid with the highest activity on

Table 1. Antiviral activities of murine IFNs on different cells

IFN	mouse	hamster	rat
α1	150[a]	150	2
α4	1000	2	15

[a] activities are given in U/mg x 10^{-5}.

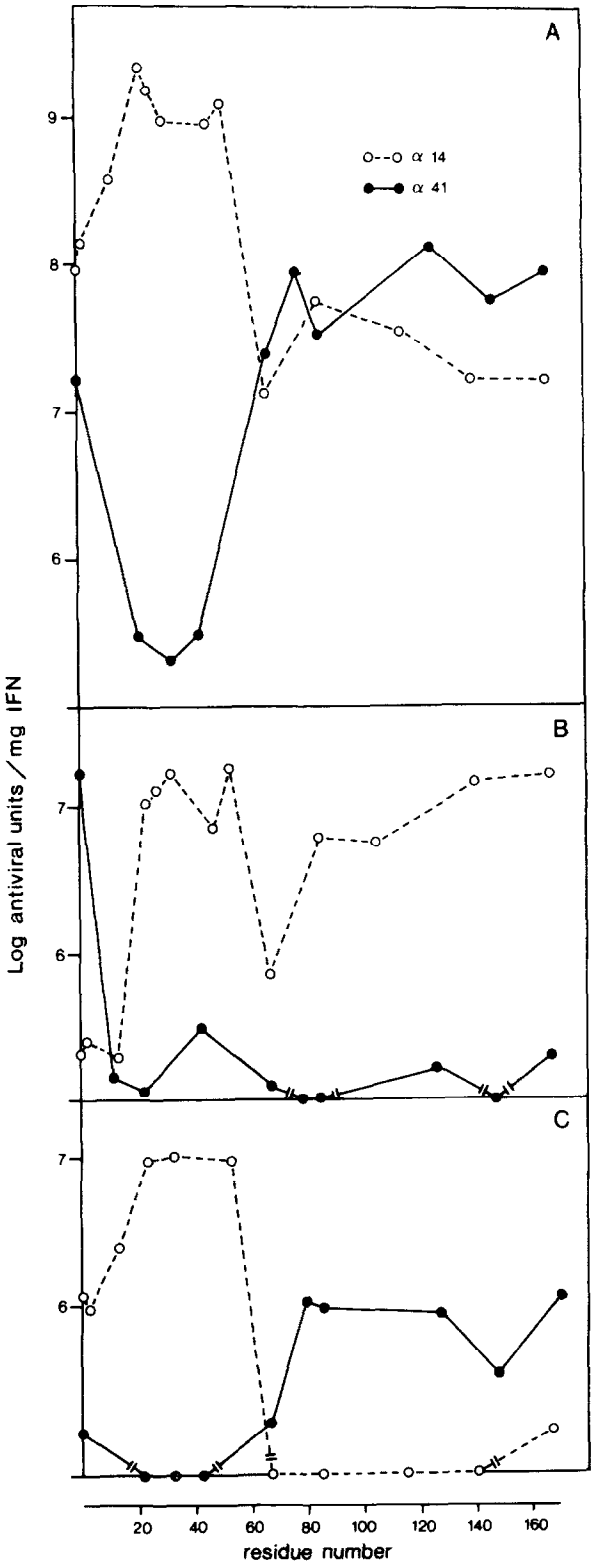

Figure 2. Specific antiviral activity of (hybrid) IFNs as measured on A: mouse L929, B: hamster (CHO) and C: rat (NRK) cells. The activities are plotted on a logarithmic scale against the cross-over point of the different hybrids.

mouse cells differs from α4 only in the N-terminal 22 amino acids. The decrease in activity shown in the curve for the α41-hybrids occurs exactly in the same area. The cause for the difference in activity between the proteins must consequently be assigned to amino acids in this region which differ between α1 and α4: residues 5, 7, 10, 17, 19 and 20. In an earlier report we showed that the α1 or α4 origin of residue 10 is important for the antiviral activity on mouse cells, but residues 5 and 7 are not (20). The transition in activity can thus be confined to amino acids 10 through 20. We have called this region: domain A. In the curve for the α14-hybrids a 100-fold drop in activity occurs between amino acids 54 and 68. In the α41-hybrids a 1000-fold increase in activity is found between hybrids with cross-over points at position 44 and 68. Combining these results identifies a second region, domain B, with a major effect on the activity and which is located between amino acids 54 and 68.

When the activities of both sets of hybrids on hamster cells are considered (Figure 2B) it appears that here domain A has the major influence on activity. The low α4-like activity of 2×10^5 U/mg increases 100-fold between residues 16 and 22 in the α14-hybrids, it decreases from 107 to 10^5 U/mg in the α41-hybrids between amino acids 1 and 21. Thus, only residues 17, 19 and 20 in domain A can be responsible for these transitions. If domain B has any influence on the antiviral activity on hamster cells is not clear. There is only one α14-hybrid (cross-over at 68) of which the activity diverges from the neighbouring hybrids.

The curve of the activities of the hybrid IFNs on rat cells (Figure 2C) looks very much like the one on mouse cells: an increase and subsequent decrease with the α14-hybrids and an decrease and increase with the reverse hybrids (activities $<10^5$ U/mg were below the detection limit). Thus, domains A and B also affect the antiviral activity of murine IFN-α on rat cells.

DISCUSSION

The results obtained with both sets of hybrids are compatible with the existence of at least two regions in the IFN-α proteins that have a major effect on the antiviral activity of these proteins. These regions, which we called domains A and B are confined by the cross-over points of hybrids between which large transitions in activity are found. In both domains only 4 amino acids differ between murine IFN-α1 and -α4 as is shown in Figure 3. These can now be analysed in more detail using site-directed mutagenesis.
It is interesting that different combinations of domains A and B result in IFNs with very divergent activities on both mouse and rat cells, this is illustrated in Table 2. Hybrids in which domain A is derived from α1 and domain B from α4 have activities on mouse and on rat cells that are much

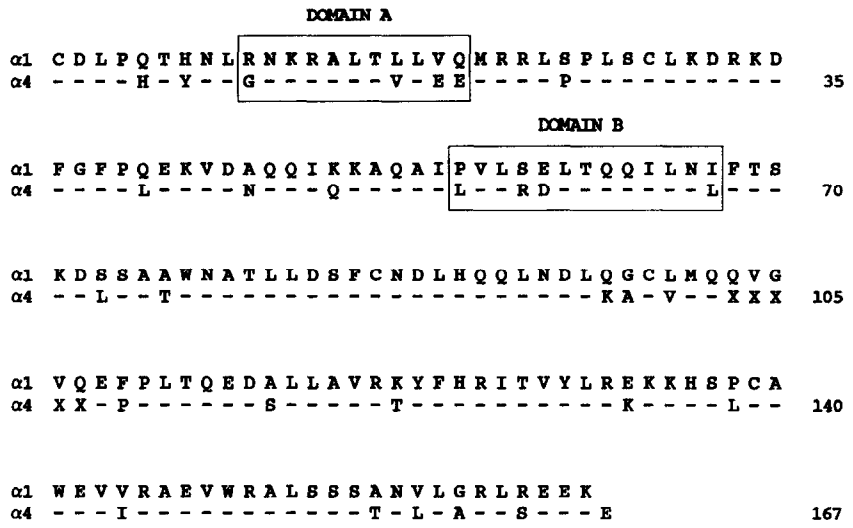

Figure 3. Amino acid sequence of murine IFN-α1 and -α4 as described previously (8). Differences in primary sequence are shown as follows: upper line: α1 residues, lower line α4 residues. Domains A and B, which affect the antiviral activity are indicated.

higher than the activities of the natural α1 and α4 proteins. Thus, mutagenesis of these areas can lead to IFN species with much higher activities on homologous (mouse) and heterologous (rat) cells than those of the parental proteins. Table 2 also shows that the reverse combinations of domains A and B leads to hybrids with extremely low acitivities.

In general it is thought that IFNs mediate their effect via binding to specific receptors, although a role within the target cell cannot be excluded. In many cases the biological effects of IFNs were found to be directly proportional to their affinity to the receptor (26,27). A logical explanation for our results would be that domains A and B are involved in the binding of IFN-α to its receptor, either directly or such that they influence the stereochemistry of the protein so as to position the receptor binding site more (un)favorably. In view of the considerable homology between IFN-α proteins from different species it is conceivable that these findings also hold for IFN-α's from other species. Results obtained by other groups with hybrids between human IFN-α subspecies show that the origin of the N-terminal 60 amino acids has a major impact on the activity on human as well as on

Table 2. Interaction between domains A and B

IFN	A from	B from	mouse	rat
α1	α1	α1	150[a]	2
α14	α1	α4	20000	150
α41	α4	α1	2	<2
α4	α4	α4	1000	15

[a] U/mg x 10^{-5}.

mouse cells (13,17,18,23). Data obtained with site-directed mutagenesis also stress the relative importance of the N-terminal part of the protein. For instance, mutations of amino acid residue 33 can completely abolish activity in human and murine IFN-α (11,14). That at least one (of the) receptor binding site(s) must be located in the N-terminal part of the protein is also suggested by the finding that a truncated murine IFN, containing only the N-terminal 67 amino acids, has a low but distinct antiviral activity (20). Secondary structure models for IFN-α have been calculated by several groups. All models calculate an α-helical conformation for regions that coincide largely with domains A and B (24,28-30). The fact that domains A and B seem to influence each other (Table 2) suggests that these helices co-operate together in presenting the necessary amino acids to the receptor.

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