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Two domains in alpha interferons influence the efficacy of the antiviral response

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Summary: Murine interferon-α1 and murine interferon-α4 share 80% of their amino acids, yet the proteins differ considerably in their ability to protect mouse or hamster cells against viral infection. With the aim of localizing areas within these proteins which influence the biological response we have constructed hybrid α1α4 genes by means of homologous recombination of the parent genes. When the antiviral activities of these proteins were compared, it appeared that there are at least two domains that affect the biological response to these proteins: area A (amino acids 10 - 20) and area B (amino acids 55 - 67). These areas are presumably involved in the interaction between ligand and receptor. Most interestingly, hybrids in which area A from IFN-α1 is combined with area B from α4, have antiviral activities on homologous cells that are one to two orders of magnitude higher than those of the parent proteins.

Interferon-α (IFN-α) is a group of closely related proteins which are able to protect cells against viral infection, can inhibit cell growth and posses immunomodulatory activities (1). These effects are mediated through binding to a specific cell surface receptor (2), followed by a change in the expression of several genes (3). The IFN-α proteins are encoded by a large gene family, which presumably arose by repeated duplication of an ancestral IFN-α gene (4).

Despite the homology between the IFN-α proteins, they can differ considerably in their biological activities (5, 6). For instance, murine IFN-α1 is 100 times as active in the protection of hamster cells against viral infection as is murine IFN-α4 (6), yet the two proteins differ in only 20% of their amino acids (7). Several groups have studied this phenomenon by the investigation of hybrid proteins, constructed by the use of common restriction enzyme sites within the genes. From these studies the rough picture emerged that several parts of an IFN-α molecule influence the magnitude of the biological response (6, 8-11). Due to the nucleotide sequence homology between the IFN-α genes it is also

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possible to obtain hybrid genes by means of homologous recombination between two parent genes in Escherichia coli (12, 13). We have used this approach for the construction of hybrids between the murine IFN-α1 and -α4 genes and we have analysed the behaviour of the corresponding hybrid proteins.

MATERIALS AND METHODS

Construction of hybrid genes. These experiments were performed largely according to methods given in Maniatis et al., (14). The exact cross-over point was established by nucleotide sequencing of fragments inserted into M13 vectors (15), using the enzymatic method described by Sanger et al., (16).

Cell culture and transfection. COS cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% foetal calf serum, penicillin and streptomycin. The cells were seeded in 35 mm Petri dishes and grown to 30% confluence. Transfection was performed using the DEAE-dextran technique (17). To this end the medium was removed and replaced by DMEM (1 ml), containing 1 μg PvuII- and HpaII-digested plasmid DNA and 100 μg DEAE-dextran (6, 18). After 2 h the mixture was removed and the cells were treated with 0.1 mM chloroquine in DMEM for 2 to 4 h. They were subsequently fed with DMEM plus serum. This was changed once 24 h after transfection. After 72 h the medium was removed and the cells were washed extensively with Hanks' balanced salt solution and incubated for 16 h with DMEM with one-tenth the normal methionine concentration plus 50 μCi [^{35}S]-methionine but without serum. This medium was used for polyacrylamide gel analysis and IFN assay.

IFN analysis. 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 hamster cells were calculated relative to the activity of murine IFN-α1 (6). Proteins secreted by transfected COS cells were separated on a 12.5% polyacrylamide gel according to the procedure described by Laemmli (19). The proteins were visualized by fluorography (20). Total radioactivity incorporated into 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. The approach followed is shown in Figure 1. First a PvuII-EcoRI fragment from pSVα1 containing the murine IFN-α1 coding region (6) was inserted into pBR328, resulting in pAGA. This plasmid was subsequently used for the construction of pAGB, by the insertion of a BamHI-SalI fragment from pSVα4 (6), which contains the α4 coding sequences. These cloning steps were carried out in a recA$^-$ host strain. Plasmid pAGB was subsequently linearized with ClaI and the linear construct was introduced into a recA$^+$ Escherichia coli strain. Resulting colonies which carried hybrid α1α4 genes were identified by their failure to hybridize to a BglI-NcoI fragment derived from pBR328 which is located next to the ClaI site in pAGB. In total 48 hybrid α1α4 genes were isolated, they were called α14-1, α14-2 etc. The cross-over points in these hybrids were established by analysis with restriction enzymes. From a preliminary analysis of the hybrid proteins 10 hybrid genes (α14-4, -6, -8, -10, -11, -12, -15, -16, -17 and -60) were selected for further experiments. With the exception of hybrids α14-4 and -8 the

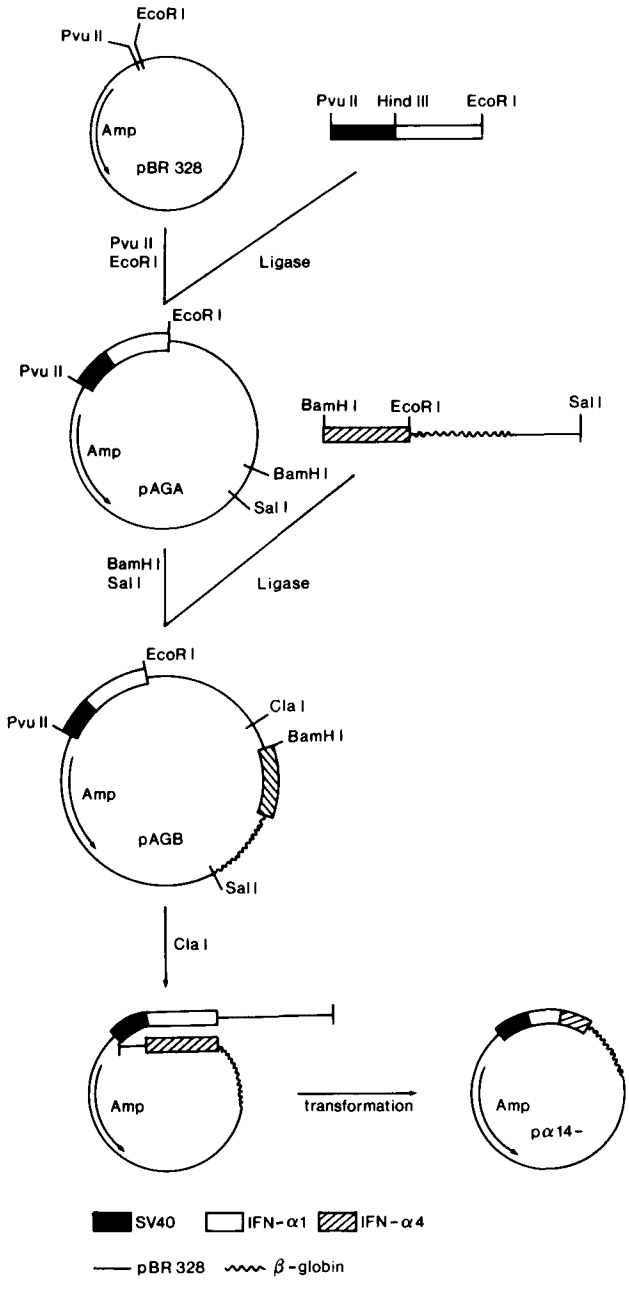

Figure 1. Construction of murine IFN-α1α4 hybrid genes. Only the relevant restriction enzyme sites are shown. Amp: ampicillin resistance gene.

cross-over points were further defined by nucleotide sequence analysis. An outline of the hybrids used throughout this study is shown in Figure 2, where the area in which cross-over has taken place is shown in black. Hybrid α1α4 was constructed using a common XmnI site and has been described before (6). As a source of the corresponding hybrid proteins we used the culture medium of monkey

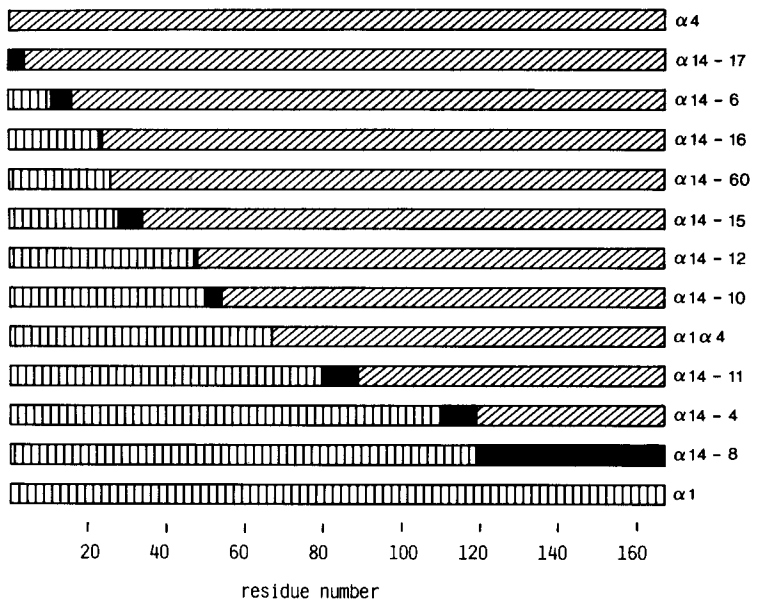

Figure 2. Outline of the murine IFN-hybrid genes used in this study. In each hybrid the area in which cross-over has taken place is shown in black. The cross-over areas of hybrids α14-4 and -8 were only roughly defined by restriction enzyme analysis. Hybrid α1α4 was constructed using a common XmnI site, as a consequence its cross-over point is exactly known. In all other hybrids the cross-over area was defined by nucleotide sequencing. The 5'-border of a cross-over area is defined by the last α1-specific nucleotide and its 3'-border by the first α4-specific nucleotide. Within the cross-over area both sequences are identical. The exact position of the cross-over with respect to the amino acid sequence is indicated by the amino acid residue number on the lower line.

COS cells (21) transfected with the hybrid IFN genes. Three days after transfection the culture medium was replaced with serum-free medium containing [^{35}S]-methionine. After a 16 h incubation this medium was collected and the proteins secreted during this period were separated by gel electrophoresis and visualized by fluorography (Figure 3). A band of approximately 21 kD, which represents the various (glycosylated) IFNs, is clearly visible in the lanes containing medium from COS cells transfected with IFN genes.

Activity of hybrid IFNs on chinese hamster ovary (CHO) cells. The specific antiviral activity of the different IFN species was determined from the antiviral titre and the amount of protein present as IFN (see Materials and Methods). The activities on CHO cells are given in Figure 4. We have previously shown that the specific activity of murine IFN-α1 on hamster cells is about two orders of magnitude higher than that of murine IFN-α4 (6). The activities calculated for the hybrid IFNs show that when the length of the N-terminal α1-fragment is increased from 16 (α14-6) to 22 amino acids (α14-16) the activity of the hybrid IFN increases 100-fold from the low α4-level (α14-6) to the high α1-level (α14-16). Due to the high degree of homology between the

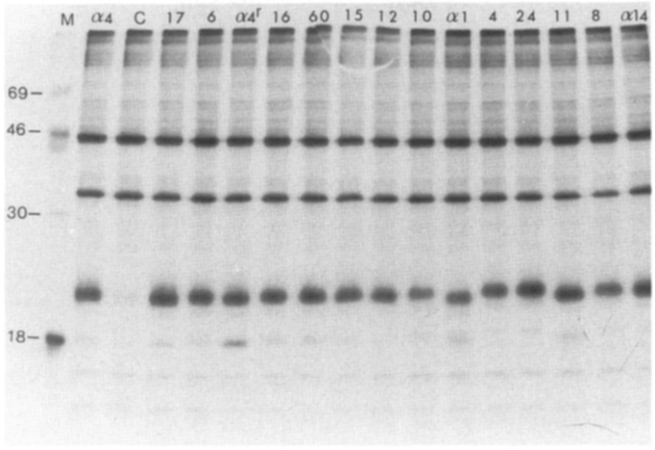

Figure 3. Fluorograph of a polyacrylamide gel containing ^{35}S-labelled proteins secreted by COS cells transfected with IFN expression plasmids. Lane M, molecular weight markers; lane C, medium from COS cells transfected with a plasmid not containing IFN sequences; other lanes, medium from COS cells transfected with (hybrid) IFN genes.

parent α1 and α4 proteins the actual difference between the two hybrids is only 3 amino acids (position 17, 19 and 20 (7); see also Figure 6). Thus, it is conceivable that these amino acids represent, or are part of, a domain that has a profound influence on the specific activity of the proteins. All other hybrids in which the length of the N-terminal α1-fragment exceeds 22 amino acids

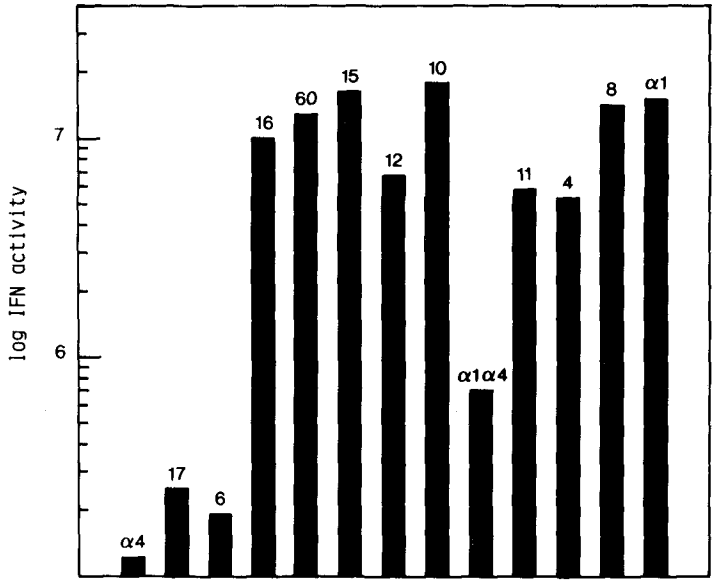

Figure 4. Specific antiviral activity of (hybrid) IFNs as measured on hamster CHO cells. The activities are represented as the logarithm of the specific antiviral activity (units per mg IFN).

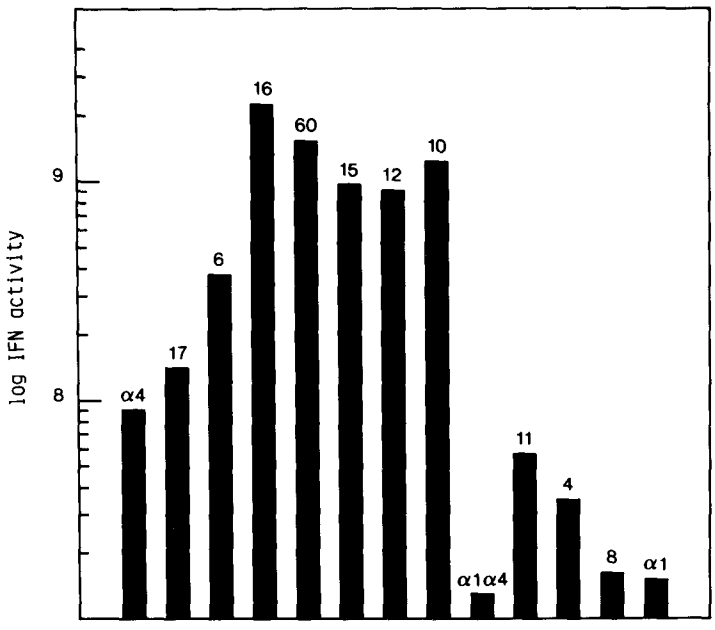

Figure 5. Specific antiviral activity of (hybrid) IFNs as measured on mouse L929 cells. The activities are represented as the logarithm of the specific antiviral activities (international units per mg IFN).

were found to have activities similar to that of α1, with the exception of the previously described hybrid α1α4. To exclude any possible errors made while constructing this hybrid, we assembled it once more and analysed two independent

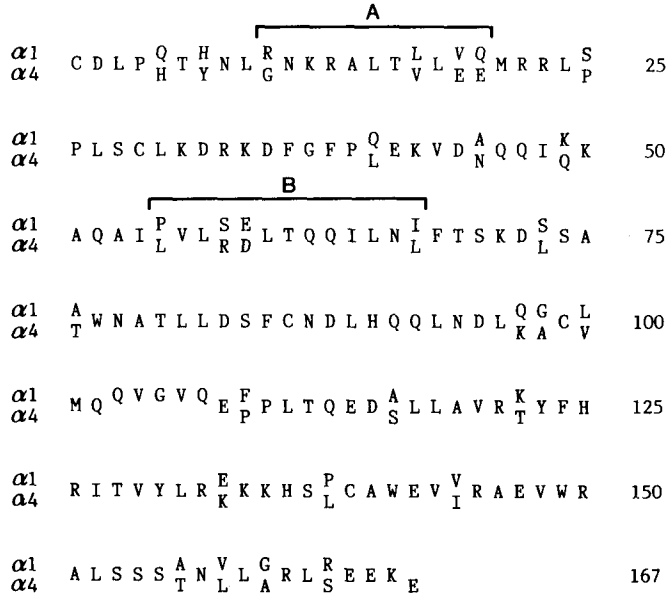

Figure 6. Amino acid sequence of murine IFN-α1 and -α4 as described previously (7). Differences between α1 and α4 are presented as follows: upper line, α1 residues; lower line, α4 residues. All other amino acids are identical for the two proteins. Areas A and B which influence the antiviral response are marked.

clones. Both show exactly the same behaviour as the original hybrid, thus the specific activity must be accurate. This could point to two other domains which influence the antiviral response. The first is confined by the cross-over points of hybrids α14-10 and α1α4 and the second by the cross-over points of hybrids α1α4 and α14-11 (Figure 2). Also in these areas the actual difference between the active and less active hybrids is only a few amino acids (amino acids 55, 58, 59 and 67 in the first area and amino acids 73 and 76 in the second area; see Figure 6).

Activity of hybrid IFNs on mouse cells. In Figure 5 the specific activities of the (hybrid) proteins as measured on mouse (L929) cells are given. The natural α4 protein is about 6 times more active on mouse cells than α1 as we have shown previously (6). It now appears that with a combination of α1 and α4 sequences far more active proteins can be obtained. Thus, hybrid α14-16 has a 15-fold higher activity than α4 and hybrid α14-17. The amino acids responsible for this effect are thus confined by the cross-over points of hybrids α14-17 and α14-16 (amino acids 4 through 22). In this area positions 5, 7, 10, 17, 19 and 20 differ between the two hybrids. That amino acids 5 and 7 probably do not contribute to the observed difference is suggested by the the behaviour of an α4 mutant protein ($\alpha 4^r$) in which glycine at position 10 is replaced by arginine (Van Heuvel et al., to be published). The specific activity of this protein on mouse and hamster cells was found to be identical to the activity of α14-6 (results not shown). Thus, amino acids 10, 17, 19 and 20, or a subset of these, must be responsible for the observed difference in activity between α14-17 and α14-16. When the α1-fragment is further increased in length from 22 to 54 amino acids, the specific activities of the hybrid proteins stay high. However, a sudden 100-fold drop in activity occurs in the area between amino acids 55 and 67 (compare the activity of hybrids α14-10 and α1α4 in Fig. 5). This again points to an important role for amino acids 55, 58, 59 and 67, the amino acids in which these two hybrids differ. Past amino acid 67 no significant differences in specific activity were found.

DISCUSSION

So far it has been difficult to investigate the impact of certain amino acids on the biological activity of the relatively large IFN proteins. For instance, mutagenesis of conserved residues in human IFN-α2 did not lead to proteins with an altered behaviour (22). A comparison of sets of overlapping hybrid proteins as presented here, makes it possible to define two domains within the proteins (area A: amino acids 10 through 20 and area B amino acids 55 through 67) which affect the specific antiviral activity of these murine IFNs on mouse as well as on hamster cells. A combination of area A from α1 with area B from α4 even leads to hybrid IFNs which are far more active on homologous cells than the

natural IFNs. In both areas the actual difference between active and less active hybrids is only 4 amino acids (Figure 6). Thus identified it is possible to explore their impact separately by site-directed mutagenesis. It is reasonable to assume that more amino acids in area A and B than the ones that differ between active and less active hybrids influence the efficacy of these proteins. Now that these areas have been identified the importance of these amino acids can also be investigated. In view of the considerable homology between alpha IFNs from different species (4), it is conceivable that our findings are not restricted to the murine species but are also valid for alpha IFNs of other species.

The appearance of an antiviral state is a consequence of the interaction between IFN molecules and a cellular receptor (8). Indeed in many cases the biological effects of IFNs appeared to be directly proportional to receptor binding (2, 11, 23, 24). In view of these observations the most obvious explanation for the results presented here is that both area A and area B are involved in binding of the ligand to its receptor, either because they represent actual receptor binding sites or because they influence the tertiary structure of the molecules in such a fashion that a binding site is more (un)favorably positioned. A murine α1 fragment comprising the N-terminal 67 amino acids and similar fragments of other murine IFNs have a low but distinct antiviral activity (M. van Heuvel, unpublished results); this suggests that at least one receptor binding site is present in the N-terminal 67 amino acids. Experiments with hybrid IFNs, constructed by means of common restriction enzyme sites, by us (6; M. van Heuvel and J. Trapman, unpublished results) and others (8-11) show that more parts of the IFN molecule may contribute to the magnitude of the biological response. Thus, a comparison of other sets of overlapping hybrids could lead to the identification of additional domains that affect the efficacy of these proteins.

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