Properties of Natural and Hybrid Murine Alpha Interferons

By MARGREET VAN HEUVEL,* I. JAAP BOSVELD, ARNO T. A. MOOREN, JAN TRAPMAN AND ELLEN C. ZWARTHOFF

Department of Pathology, Erasmus University, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

(Accepted 25 June 1986)

SUMMARY

Four natural murine interferon-α genes (MuIFN-α1, -α2, -α4 and -α6) and four hybrid genes (α1α4, α2α4, α4α1 and α4α2) were transiently expressed in monkey COS cells under the transcriptional control of the simian virus 40 early promoter. The proteins were labelled with [35S]methionine during a 16 h incubation and proteins secreted by the cells during this period were separated by polyacrylamide gel electrophoresis and subsequently visualized by fluorography. Under the conditions used, the IFNs represented 5 to 10% of the total amount of secreted proteins. All genes were found to encode biologically active IFN subspecies, including α4 which has a deletion of five amino acids. When the specific activities of the proteins were compared, it appeared that the specific antiviral activity of α4 on mouse cells was three- to sixfold higher than the activities of the other natural IFN subspecies. The specific activities of the hybrid proteins were similar to those of the natural proteins, except for the α2α4 hybrid which had a higher specific activity than the original proteins. The ability of the natural and hybrid subspecies to protect hamster cells against viral infection was determined using MuIFN-α1 as a standard. Large differences in activity were found, with α6 as the most and α4 as the least active subspecies.

INTRODUCTION

Type I interferons (IFNs) are proteins produced by mammalian cells upon induction with a virus or a double-stranded polyribonucleotide. Once secreted by the producing cells they are able to protect surrounding cells against viral infection. In addition to their antiviral activities, type I IFNs are able to inhibit cell growth and they can influence the immune system, e.g. by stimulating macrophages and natural killer cells (for reviews, see Finter, 1984). To elicit their biological properties it is necessary that the IFNs bind to specific surface receptors on the target cells. Subsequently, the expression of several genes within the target cells is affected (Fellous et al., 1982; Friedman et al., 1984; Jonak & Knight, 1984; Larner et al., 1984; Shulman & Revel, 1980). So far, however, little is known about the precise relationship between the different biological and molecular changes induced by IFNs.

In man and mouse, type I IFNs comprise a group of closely related proteins called IFN-α and a single antigenically distinct protein called IFN-β (for review, see Weissmann & Weber, 1986). The IFN-α proteins are encoded by the IFN-α gene family, consisting of over 10 members. These genes are tightly clustered in the genome. The single IFN-β gene is closely associated with the IFN-α gene family (Ohlson et al., 1985; Trent et al., 1982; Van der Korput et al., 1985). The IFN-α and -β genes do not contain introns and encode proteins of 186 to 190 amino acids, including a signal peptide of 23 amino acids. It is assumed that the IFN-α and -β genes share a common ancestor and that the IFN-α gene family arose by repeated duplications of the ancestral α gene. The IFN-α genes are relatively well conserved. Within a species the mean homology between two proteins is about 70%. Between human (Hu) and murine (Mu) IFN-α proteins the homology is 50 to 60%.
Despite these structural similarities, there are differences in the biological activities of the different IFN-α subspecies. They are for instance relatively species-specific: most HuIFNs have only a low activity on mouse cells (Weck et al., 1981a). Moreover, the relative efficacy of antiviral compared to antiproliferative or other responses may vary from one subspecies to another (Ortaldo et al., 1984; Rehberg et al., 1982). To investigate the properties of different IFN subspecies in vivo, we have chosen the mouse as a model organism. We have recently reported the isolation of four MulFN-α genes (Zwarthoff et al., 1985b). In this paper, we show that these genes encode biologically active IFN subspecies when introduced into a transient expression system. We found that the specific antiviral activity on mouse cells varied between the different proteins and that there are great differences in the abilities of the proteins to protect heterologous Chinese hamster ovary (CHO) cells against viral infection. In addition, we have constructed hybrid genes and compared the properties of their protein products to those of each parent.

**METHODS**

**DNA manipulations.** Plasmids were carried in *Escherichia coli* K12 strain DH1 and were purified from chloramphenicol-amplified cultures by the alkaline lysis method (Maniatis et al., 1982). Separation of plasmid DNA and cellular RNA was achieved on a Bio-Gel A-1.5m (Bio-Rad) gel filtration column. DNA fragments were isolated from agarose gels by electroelution. All cloning procedures were as described in Maniatis et al. (1982). Plasmid constructs were verified by restriction enzyme analysis.

**Cell culture and transfection.** Cells were grown in Dulbecco's MEM (DMEM) supplemented with 5% foetal calf serum, penicillin and streptomycin. COS-1 cells (Gluzman, 1981) were seeded 1 to 2 days prior to transfection in 35 mm Petri dishes and grown to 30% confluence. Transfection was performed using the DEAE-dextran technique (McCutchan & Pagano, 1968). To this end, the medium was removed and replaced by DMEM (1 ml) containing 1 μg *Pvu*II- and *Hpa*I-digested plasmid DNA and 100 μg DEAE-dextran. Digestion of the plasmid with these enzymes removes practically all plasmid sequences leaving the eukaryotic DNA intact. This treatment results in a higher expression of the IFN genes (I. Self & J. De Maeyer-Guignard, personal communication). After 2 h, the mixture was removed and the cells were treated with 0-1 mM-chloroquine in DMEM for 4 h and they were subsequently fed with DMEM plus serum. The medium 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 45 μCi [35S]methionine but without serum. The resulting specific radioactivity of methionine was 63.5 × 10⁶ d.p.m./μg. This medium was used for PAGE and IFN assays.

**IFN assay.** IFN titres were determined in a cytopathic effect reduction assay, using vesicular stomatitis virus as a challenge. IFN titres on mouse cells were calculated relative to the NIH reference standard G002-904-511. Because no standard for hamster cells is available, titres on these cells were calculated relative to the activity of MulFN-αI on these cells, which was set at 100% of its activity on L cells.

**PAGE.** The ³⁵S-labelled proteins (40 μl culture medium) were separated on a 12.5% gel according to the procedure described by Laemmli (1970). The proteins were visualized by fluorography (Bonner & Laskey, 1974). Total radioactivity incorporated into secreted proteins was determined by gel filtration of the culture medium. These values and densitometric scans of the fluorogram were used to calculate the amount of label incorporated in each IFN band.

**RESULTS**

**Construction of expression plasmids**

All plasmids used for expression in COS cells were based on the expression plasmid pSV328A (Fig. 1). This plasmid was constructed from pSV10EF (Zwarthoff et al., 1985a) by removing the *HindIII*–*EcoRI* fragment containing the coding region of MulFN-10EF (= MulFN-α1) and by replacing it by the *HindIII*–*EcoRI* polylinker fragment of bacteriophage M13mp11 (Messing, 1983). The coding regions of the MulFN-α genes (Fig. 1) were subsequently inserted into the polylinker region of pSV328A, thus bringing them under the control of the simian virus 40 (SV40) early promoter. The polyadenylation signal is provided by the rabbit β-globin sequences.

For the construction of pSVα1, the *HindIII* site 20 nucleotides upstream of the ATG codon was converted into a *BamHI* site by the addition of a *BamHI* linker. Subsequently, the *BamHI*–*EcoRI* fragment containing the entire coding region was inserted into pSV328A, between the
Natural and hybrid murine alpha interferons

Fig. 1. Construction of expression plasmids containing IFN genes. (a) Physical maps of the IFN-α genes used. The portion coding for the signal peptide within the IFN coding region is hatched. Del, Position of the 15 nucleotide deletion in the α4 gene. (b) Outline of the expression vector pSV328A. The coding regions of the IFN genes are inserted in the polylinker of this plasmid. See text for details concerning the constructions. In all maps only the relevant restriction enzyme sites are shown: Ah, AhaI; Bg, BglII; Ec, EcoRI; Hi, HindIII; Mb, MboII; Nc, NcoI; Pv, PvuII; Ps, PstI; Rs, RsaI; Sa, SalI; Xm, XmnI. Ap, Ampicillin resistance gene.

For the construction of pSVα2 the MboII fragment spanning the left half of the coding region was isolated and treated with nuclease S1 to create flush ends. The fragment was then cut with BglII and the MboII–BglII fragment containing the 5' end of the gene was inserted in pSV328A between SmaI and BglII. This fragment was again isolated from the resulting plasmid by digestion with BamHI and BglII. It was subsequently ligated together with the BglII–AhaI fragment containing the 3' two-thirds of the α2 gene to pSV328A cut with BamHI and SmaI, thus creating pSVα2. For the construction of pSVα4, first the RsaI fragment spanning the left two-thirds of the gene was isolated and treated with nuclease Bal31 so as to remove approximately 60 nucleotides from the ends. After digestion with BglII the left part of the fragment was inserted in pSV328A between SmaI and BglII. The Bal31 digestion was analysed by nucleotide sequencing and a clone with 20 nucleotides left upstream of the ATG codon was selected for the subsequent construction of the expression plasmid pSVα4, which from this point on went analogously to the construction of pSVα2. The plasmid pSVα6 was created from pSVα1 by replacing the NcoI–EcoRI fragment of the latter for the analogous fragment from gene α6.

Hybrids between IFNs α1 and α4 (α1α4 and α4α1) were constructed by ligating the left XmnI site in α1 to the only XmnI site in α4 (see Fig. 1). The crossover point in these hybrid genes is between amino acids 67 and 68. Hybrids between α2 and α4 (α2α4 and α4α2) were constructed using their single BglII site (see Fig. 1). In these the crossover point was at position 60. All hybrid genes were inserted into the polylinker of pSV328A (Fig. 1) in an analogous way to the constructions used for the parent genes.
Table 1. **Antiviral activity produced by expression plasmids as measured on mouse (L929) and hamster (CHO) cells**

<table>
<thead>
<tr>
<th>IFN</th>
<th>L929*</th>
<th>CHO†</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1</td>
<td>3200</td>
<td>3200</td>
</tr>
<tr>
<td>α2</td>
<td>2400</td>
<td>640</td>
</tr>
<tr>
<td>α4</td>
<td>25600</td>
<td>16</td>
</tr>
<tr>
<td>α6</td>
<td>3200</td>
<td>5120</td>
</tr>
<tr>
<td>α1α4</td>
<td>3200</td>
<td>256</td>
</tr>
<tr>
<td>α2α4</td>
<td>51200</td>
<td>640</td>
</tr>
<tr>
<td>α4α1</td>
<td>1600</td>
<td>32</td>
</tr>
<tr>
<td>α4α2</td>
<td>3200</td>
<td>64</td>
</tr>
</tbody>
</table>

* IFN titres on L cells are given in IU/ml.
† Titres on CHO cells were determined as described in Methods and are given in U/ml.

**Expression in COS cells**

The expression plasmids described in the preceding section were digested to remove all vector sequences and they were subsequently transfected into monkey COS cells. Seventy-two h after transfection the medium was removed and replaced with medium containing [35S]methionine but without serum, and 16 h later the medium was collected. The amount of antiviral activity secreted during this period was assayed on mouse and hamster cells in a cytopathic effect reduction assay. Titres on CHO cells were related to the activity of MuIFN-α1 on these cells, which is equal to its activity on L cells (Zwarthoff et al., 1985a). Table 1 shows the titres found in a representative experiment. It appeared that all the natural and hybrid IFN genes coded for IFN subspecies with biological activity on mouse as well as on hamster cells. Surprisingly, the α4 gene, which contains an in-phase deletion of 15 nucleotides (Zwarthoff et al., 1985b), also encoded a biologically active IFN. Moreover, α4 and the α2α4 hybrid produced considerably more antiviral activity on mouse cells than the other genes. The results obtained with hamster cells showed that α4 had only a low activity on these cells. In contrast, α6 was even more active on hamster cells than on mouse cells.

To determine whether the differences in activity found were indeed due to differences in specific activity of the various proteins we decided to compare the amount of IFN produced by the transfected COS cells. To this end the proteins secreted by the cells during the 16 h labelling period described above were separated on a polyacrylamide gel. Fig. 2 shows the fluorograph of such a gel. The genes used for transfection are indicated on top of each lane. Most IFNs can be seen as bands of approximately 22 000 mol. wt. (22K), moving slightly slower than the 18 K mol. wt. marker. IFN-α6 ran at 18K. In the control lane transfected with pSV328A no bands were visible in this area. The molecular weights observed were consistent with the nucleotide sequence data, which suggests that α6 lacks the N-glycosylation site at positions 78 to 80 in the protein that is present in α1, α2 and α4 and as a consequence also in their hybrids (Zwarthoff et al., 1985b). Densitometric analysis of the fluorogram indicated that 5 to 10% of the label incorporated into secreted proteins was present in the IFNs (results not shown). The amount of label in the IFN bands (except α6) varied slightly between the different lanes; this was partly due to differences in total incorporation and partly to actual differences in the amounts of IFN present. That the α6 band contained significantly less label than the other IFN bands was caused by the fact that α6 has only one methionine residue, whereas all the other subspecies contain two methionines.

**Specific activities of natural and hybrid IFNs**

Specific activities of the different subspecies were calculated from the antiviral titre of the medium and the amount of label present in IFN in the same sample as described in Methods. The specific activities are presented as units × 10³/d.p.m. in the second and fourth columns of Table 2. It should be noted that the specific activity of α6 when expressed in this way was overestimated because the protein contains only one methionine residue. Using the known specific activity of the added [35S]methionine, the specific activities were also calculated as
units/mg protein present in the IFN band. This also corrected the specific activity of α6. The values found are displayed in columns three and five of Table 2. The results show that the differences in antiviral activity produced by the different genes as displayed in Table 1 were largely due to differences in the specific activity of the corresponding proteins. Thus, the specific activity of α4 on mouse cells was considerably higher than that of the other natural subspecies. It
Table 2. Specific activities of natural and hybrid IFNs on mouse (L929) and hamster (CHO) cells

<table>
<thead>
<tr>
<th>IFN</th>
<th>L929</th>
<th>CHO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IU x 10^3/d.p.m.</td>
<td>IU/mg</td>
</tr>
<tr>
<td>α1</td>
<td>48</td>
<td>5 x 10^7</td>
</tr>
<tr>
<td>α2</td>
<td>72</td>
<td>3 x 10^7</td>
</tr>
<tr>
<td>α4</td>
<td>284</td>
<td>1 x 10^8</td>
</tr>
<tr>
<td>α6</td>
<td>192</td>
<td>0.7 x 10^8</td>
</tr>
<tr>
<td>α1α4</td>
<td>84</td>
<td>9 x 10^7</td>
</tr>
<tr>
<td>α2α4</td>
<td>731</td>
<td>8 x 10^8</td>
</tr>
<tr>
<td>α4x1</td>
<td>50</td>
<td>5 x 10^7</td>
</tr>
<tr>
<td>α4x2</td>
<td>33</td>
<td>3 x 10^7</td>
</tr>
</tbody>
</table>

It is interesting to see that the α2α4 hybrid which also contained the 15 nucleotide deletion had an even higher specific activity. However, α1α4 in which the deletion was also present, had a specific activity that was only about one-third that of α4. The α6 subspecies displayed a considerable activity on mouse and an even higher activity on hamster cells. Thus, the absence of N-glycosylation had no obviously deleterious effect upon the biological activity of this protein. In contrast to the considerable activities of α1, α2 and α6 on hamster cells, the activity of α4 on these cells was at least 100-fold lower. The activities of the hybrid proteins on hamster cells were found to differ from those of their parents; instead of the high (α1, α2) or low activities (α4), intermediate values were found.

DISCUSSION

In this study, we have investigated the expression of several MuIFN genes in a transient expression system. Under the conditions used, the IFNs represent 5 to 10% of the proteins secreted by the COS cells. The data obtained demonstrate that all the genes analysed encode biologically active IFN subspecies. Moreover, we found that MuIFN-α4 has three- to sixfold higher activity on mouse cells than the other α proteins. This is a surprising result since the α4 gene contains a unique deletion of five amino acids. Expression of MuIFN-α4 has also been achieved in E. coli (Kelley & Pitha, 1985). However, the results obtained led the authors to conclude that the specific activity of this subspecies is 100-fold lower than that of MuIFN-α1 or an α4α2 hybrid IFN. We do not know at present what the reason for this discrepancy might be.

When mouse L cells are induced with Sendai virus and the individual IFN mRNAs are visualized in a S1 nuclease experiment, the mRNAs transcribed from the α4 and β genes are at least 10 times more abundant than the mRNAs from the α1, α2 and α6 genes (Zwarthoff et al., 1985b). When these results are combined with the relatively high specific activity of α4, it is highly probable that IFN-α4 represents the major part of the IFN-α activity produced by mouse fibroblasts. This hypothesis is supported by the results obtained in the experiments on CHO cells. An IFN-α preparation from L cells has only a low activity on CHO cells (Lemson et al., 1984). We now find that, not only IFN-α1, but also IFN-α2 and IFN-α6 are active on CHO cells, whereas IFN-α4 has only a very low activity (see Table 2). The finding that the MuIFN-α6 subspecies is highly active on hamster cells suggests that it could be useful in experiments in vivo with hamsters.

The hybrid genes were constructed with the objective of localizing the different phenotypic properties displayed by the different α proteins to certain regions within the genes. Studies using hybrids between HuIFN-α1 (D) and -α2 (A) show that this is, at least to some extent, possible (Rehberg et al., 1982; Streuli et al., 1981; Weck et al., 1981b). The behaviour of the hybrid IFNs on heterologous hamster cells shows that their activities are intermediate between those of either parent, but that hybrids with the N-terminal ends of α1 or α2 have significantly higher activities than constructs with the identical part of α4. This suggests that the origin of the N-terminal end has some influence on the behaviour of the hybrid with respect to hamster cells. When the...
activities of the hybrids on mouse cells are compared it is interesting to see that the α2α4 hybrid has an even higher activity on mouse cells than α4. The most obvious difference between α4 and the other subspecies in the C-terminal part of the protein is the 15 nucleotide deletion. However, since the α1α4 hybrid has a lower activity than α4, the absence of the five amino acids is apparently not sufficient by itself to explain the relatively high activity of α4 and α2α4. At present, we are studying the effect of the deletion in more detail using site-directed mutagenesis. The α2α4 hybrid shows that it is possible to create IFN subspecies with a specific activity that is higher than that of the natural proteins. Preliminary results obtained in our laboratory with other hybrids suggest that this is not an isolated case and that the construction of hybrids might be a way to 'improve' the IFNs.

The authors are indebted to Drs Seif and De Maeyer-Guignard for making available their results prior to publication. This work was sponsored in part by The Netherlands Cancer Foundation (KWF).

REFERENCES


(Received 21 January 1986)