Constitutive Expression of a Murine Interferon Alpha Gene in Hamster Cells and Characterization of Its Protein Product

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SUMMARY

The coding part of a murine interferon alpha (MuIFN-α) gene was cloned into an expression plasmid containing the simian virus 40 early promoter and the rabbit β-globin polyadenylation signal. This construct was transfected into Chinese hamster ovary cells, together with a plasmid containing the EcoGpt gene as a selection marker. Resulting colonies were assayed for constitutive interferon production and analysed for integration of MuIFN-α genes. There was no obvious correlation between the number of genes integrated and the amount of interferon produced. The highest producer, designated CHO-pSV10EF-3, contained four copies of the mouse gene and constitutively secreted up to 100000 International Units of interferon per ml per day. The MuIFN-α subspecies produced by this clone was characterized by analysis of its antiviral activity on heterologous cells, heparin-Sepharose affinity chromatography and chromatofocusing. The results obtained indicate that it is identical or closely related to a minor component present in conventional MuIFN-α preparations.

INTRODUCTION

Interferons are a family of proteins with, by definition, antiviral activity (Stewart, 1979). In addition, interferons exhibit a great variety of other biological functions. For instance, they are able to inhibit cell proliferation and to modulate the immune system (Stewart, 1979). On the basis of antigenic properties, three distinct types of interferon, generally known as interferon-α, -β and -γ (IFN-α, -β and -γ) can be distinguished (Interferon Nomenclature, 1980). Most of our knowledge so far has been gained with human (Hu) IFNs (Pestka, 1983). However, several aspects of the interferon system can only be evaluated in an animal model. For these studies, the mouse is very suitable because of the broad knowledge that already exists on its genetics. Since IFNs in general are highly species-specific these experiments have to be carried out with murine (Mu) IFNs. Therefore, knowledge of the properties of mouse IFN (sub)species is important.

MuIFN-β and MuIFN-γ are each encoded by a single gene (Higashi et al., 1983; Gray & Goeddel, 1983) and MuIFN-α by a family of nine or more distinct genes (Owerbach et al., 1981; Shaw et al., 1983). Both MuIFN-β and -γ are probably glycosylated (Higashi et al., 1983; Gray & Goeddel, 1983). The properties of the MuIFN-α proteins (Fujisawa & Kawade, 1981), as well as the amino acid sequence deduced from the MuIFN-α genes sequenced so far (Shaw et al., 1983; Zwarthoff et al., 1985) indicate that some of the MuIFN-α subspecies are also glycoproteins. MuIFN-α, produced by mouse L cells, can be separated into at least five subspecies by chromatofocusing (Lemson et al., 1984). This rather laborious procedure gives rise to limited amounts of these proteins. For more extensive characterization of their properties, both in vitro and in vivo, larger amounts of the individual MuIFN-α proteins are required. This can be achieved by expression of isolated genes in suitable prokaryotic or eukaryotic cells. Since (most of) the MuIFN-α subspecies are probably glycoproteins, their expression in mammalian cells might be most suitable for this purpose.
Recently, we have molecularly cloned five MulFN-α genes from a genomic library (Zwarthoff et al., 1985). In the present paper we describe the subcloning of one of these genes into a eukaryotic expression vector, the subsequent constitutive expression of this construct in Chinese hamster ovary (CHO) cells, and the properties of the MulFN-α subspecies produced.

**METHODS**

**Cell culture and transfection.** Cells were grown in Dulbecco's MEM (DMEM, Flow Laboratories) supplemented with 5% foetal calf serum, penicillin and streptomycin.

CHO cells for transfection were cultured in a 1:1 mixture of DMEM and Ham's F10 medium supplemented with 3% foetal and 7% newborn calf serum and antibiotics. Approximately $5 \times 10^5$ cells were seeded per 9 cm Petri dish 1 day prior to transfection. Transfection was performed by the calcium phosphate precipitation technique (Graham & Van Der Eb, 1973) using 2 µg pSV10EF (see Fig. 1) and 0.2 µg pSV3gptH per Petri dish. The pSV3gptH plasmid, a derivative of pSV3gpt (Mulligan & Berg, 1981) was a gift from J. Hoeymakers. After 5 h the cells were washed twice with culture medium, treated for 2 min with culture medium containing 10% glycerol, washed again, and further incubated in medium. Selection for Ecogpt expression was started 48 h after transfection. To this end, the medium was supplemented with $0.2 \mu g/ml$ aminopterin, $5 \mu g/ml$ thymidine, $10 \mu g/ml$ xanthine, $15 \mu g/ml$ hypoxanthine, $25 \mu g/ml$ mycophenolic acid and $2.5 \mu g/ml$ deoxyctydine. After 14 days colonies were isolated.

Transfection of COS-1 cells (Gluzman, 1981) with pSV10EF was done essentially as described for CHO cells. In this case 2 to 10 µg pSV10EF was used per Petri dish. Seventy-two h after transfection the medium was harvested for interferon assay.

Conventional MulFN-α (subspecies) preparations were prepared as described previously (Vonk & Trapman, 1983; Lemson et al., 1984). Conventional hamster interferon was obtained from the supernatant of CHO cells induced with Sendai virus.

**Interferon assay.** The interferon titre was determined in a cytopathic effect reduction assay, using vesicular stomatitis virus as a challenge. Unless otherwise indicated interferon titres on mouse L-929 cells are expressed in International Units (IU) per ml. Titres on heterologous cells are expressed in arbitrary units per ml.

**Southern blotting and hybridization.** Cellular DNA (10 µg) was digested with an excess of EcoRI. The DNA fragments were separated on a 1% agarose gel and transferred onto a Millipore HAHY nitrocellulose filter. The filters were hybridized in a solution containing 3 × saline sodium citrate (SSC), $100 \mu g/ml$ denatured salmon sperm DNA, $0.1\%$ sodium pyrophosphate, $0.1\%$ SDS, $10 \times$ Denhardt's solution and $9\%$ dextran sulphate. After overnight hybridization at $65^\circ C$ the filters were washed seven times, starting with $3 \times$ SSC and ending with $0.1 \times$ SSC at $65^\circ C$. The nick-translated HindIII-EcoRI fragment of pSV10EF (see Fig. 1) was used as a probe (sp. act. $2 \times 10^8$ d.p.m./µg). Exposure was for 1 to 2 days with a Kodak X-Omat AR film between two intensifying screens (Ilford) at $-80^\circ C$.

**Heparin-Sepharose affinity chromatography.** CHO-pSV10EF IFN in phosphate-buffered saline (0.02 M phosphate pH 7.3, 0.15 M NaCl; PBS) was layered onto a heparin-Sepharose column (1 × 9 cm, Pharmacia) equilibrated in PBS, and eluted with PBS at 4 °C at a flow rate of 12 ml/h and, subsequently, with 0.02 M-phosphate pH 7.3, containing 1.5 M NaCl. Four ml fractions were collected and assayed for interferon activity.

**Chromatofoocusing.** For analysis in the pH 7 to pH 4 range, CHO-pSV10EF IFN was dialysed overnight against 0.025 M imidazole- HCl pH 7.8. Next, the sample was applied to a 0.9 × 30 cm PBE 94 chromatofocusing column (Pharmacia) which was equilibrated with the same buffer. The column was eluted with 250 ml of Polybuffer 74 adjusted to pH 4 with HCl. For analysis in the pH 9 to pH 6 range, IFN was dialysed against a 0.025 M ethanolamine-acetic acid buffer, pH 9.4. In this case the column was eluted with Polybuffer 96 adjusted to pH 6 with acetic acid. All buffer solutions were degassed prior to use. The flow rate was 12 ml/h and fractions of 4 ml were collected. After elution was completed the pH of each column fraction was determined and indicated fractions were titrated for interferon activity.

**RESULTS**

**Construction of the expression plasmid pSV10EF**

We have isolated five MulIFN-α genes from a genomic library and determined their nucleotide sequences (Zwarthoff et al., 1985). A HindIII–EcoRI fragment containing the coding region of one of these genes (designated 10EF) was used to construct an expression plasmid. This 690 bp long fragment contains 20 bp of 5' non-coding and 104 bp of 3' non-coding sequences. Its nucleotide sequence is identical to the analogous fragment from the MulIFN-α1 gene described by Shaw et al. (1983). There is a putative N-glycosylation site in the amino acid sequence of the
protein encoded by this gene. In the plasmid pSV10EF (see Fig. 1) the 10EF gene is preceded by
the simian virus 40 (SV40) origin of replication and early promoter (PvuII–HindIII) and
followed by the 3' part (from the EcoRI site) of the rabbit β-globin gene (Van Ooyen et al., 1979).
The remainder of the plasmid consists of pBR328 sequences (BamHI–PvuII).

Integration and expression of pSV10EF in CHO cells

CHO cells were transfected with a co-precipitate of pSV10EF and the selection plasmid
pSV3gptH. Approximately 60% of the colonies analysed (24 out of 40) secreted interferon
constitutively. Control CHO cells did not produce interferon under these circumstances.
Interferon production was not related to the number of plasmids integrated in the different
clones. Fig. 2 shows a Southern blot analysis of DNA from six CHO-10EF clones. Under
the conditions used, there is only a little cross-hybridization between the hamster DNA and the
MuIFN-α probe, so the most prominent bands represent integrated MuIFN-10EF genes.
Although clones 1, 5 and 7 contained at least one IFN gene they produced hardly any interferon
(< 10 IU/ml); in contrast, clones 3, 8 and 9 were producers (clone 9: 500 IU/ml; clones 3 and 8:
> 2500 IU/ml).

A series of high producers was selected for further analysis. At first the cells grew slowly,
probably due to an anti-proliferative effect of the constitutively produced interferon. However,
Table 1. Antiviral activity* of different IFN preparations on mouse (L-929), hamster (CHO) and rabbit (RK13) cells

<table>
<thead>
<tr>
<th>IFN source</th>
<th>L-929</th>
<th>CHO</th>
<th>RK13</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-pSV10EF</td>
<td>192</td>
<td>192</td>
<td>8</td>
</tr>
<tr>
<td>COS-pSV10EF</td>
<td>192</td>
<td>192</td>
<td>ND†</td>
</tr>
<tr>
<td>Mouse-α</td>
<td>192</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Hamster</td>
<td>&lt;2</td>
<td>128</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Component 1</td>
<td>192</td>
<td>96</td>
<td>6</td>
</tr>
</tbody>
</table>

* Antiviral activity is expressed in arbitrary units.
† ND, Not done.

after a few weeks this effect was overcome. Clone CHO-pSV10EF-3 produced the highest amounts of interferon. This clone contains at least four copies of the 10EF gene (Fig. 2). It has now been kept in culture for 6 months during which time the number of integrated gene copies did not change (results not shown). It constitutively produces $0.2 \times 10^5$ to $1 \times 10^5$ IU IFN per ml per day, when titrated on mouse L-929 cells. For instance, a culture of 10 roller bottles of 1500 cm$^2$ produced about $6 \times 10^7$ IU of this IFN subspecies per day, which amounts to 1 IU per 60 cells per day.

Antiviral activity of CHO-pSV10EF IFN on heterologous cells

The antiviral activity of interferon produced by clone CHO-pSV10EF-3 was analysed on different types of cells (see Table 1). Surprisingly, CHO-pSV10EF IFN had a high antiviral activity on hamster cells. It also displayed some activity on rabbit cells. Similar results were found for pSV10EF IFN when the gene was transiently expressed in monkey COS cells (Table 1, COS-pSV10EF). Total MuIFN-α prepared from induced L cells always showed some activity on hamster and rabbit cells. Most of this activity on heterologous cells can be attributed to one subspecies (component 1; see Table 1 and Lemson et al., 1984). Interferon produced by control CHO cells after incubation with Sendai virus had no activity on mouse or rabbit cells.

Analysis of CHO-pSV10EF IFN by chromatofocusing and heparin–Sepharose affinity chromatography

In an earlier study we characterized conventionally prepared total MuIFN-α by chromatofocusing and heparin–Sepharose affinity chromatography (Lemson et al., 1984). These experiments were carried out for CHO-pSV10EF IFN. Fig. 3(a, b) shows the analysis of CHO-pSV10EF IFN on two chromatofocusing columns, together covering the range between pH 9 and pH 4. In Fig. 3(a), using a pH 7.5 to 4 gradient the activity eluted from the column in the void volume. Using a pH 9 to 6 gradient (Fig. 3b) all antiviral activity eluted at pH 8.2. This indicates a pI value of 8.2 for the product of gene 10EF. When CHO pSV10EF IFN was analysed on the heparin–Sepharose column it was found that all antiviral activity appeared in the low-salt fraction, both when titrated on mouse cells (Fig. 3c: open symbols) and hamster cells (Fig. 3c: closed symbols). Comparison of the data presented here with those obtained with a conventional MuIFN-α preparation (Lemson et al., 1984) strongly suggests that the protein product encoded by gene 10EF is identical to a minor MuIFN-α subspecies (component 1) present in poly(I)-poly(C)-induced mouse L cell interferon (see also Table 1). The molecular weight of the 10EF protein is about 22K (data not shown), indicating that it is glycosylated (unglycosylated MuIFN-α has a molecular weight of 18K; Fujisawa & Kawade, 1981).

DISCUSSION

In this study we described the constitutive expression of a MuIFN-α gene (10EF or α1) in CHO cells. Recently, Shaw et al. (1983) described the transient expression of this gene in monkey COS cells. The interferon produced by the engineered CHO cells was analysed regarding its antiviral activity on different cells, chromatofocusing and heparin–Sepharose affinity chromatography. The results obtained suggest that the MuIFN-α gene codes for a sub-
species that is identical (or closely related) to component 1, one of the MuIFN-α subspecies present in mouse L cell interferon. This subspecies also has, as shown here for 10EF IFN, a high antiviral activity on heterologous cells, a pI value > 7.5 and no affinity for heparin (Lemson et al., 1984). The large amounts of interferon secreted by the CHO cells enabled us to establish the pI value of this component as 8.2. The other MuIFN-α subspecies have pI values between 5.6 and 6.5 (Lemson et al., 1984). The reason for this large difference remains unclear at the moment. Analysis of the protein products of other cloned genes may resolve this problem.
Co-transfection of CHO cells with the MuIFN-α 10EF gene under direction of the SV40 promoter and the EcoGpt selection gene resulted in Gpt+ colonies of which the majority constitutively secreted MuIFN. The amount of interferon produced differed considerably among the various clones analysed and was not related to the number of gene copies present. We presume that the site of integration in the host genome is of importance for the successful expression of a transfected gene.

The highest producer in our experiments, CHO-pSV10EF-3, secretes between $0.2 \times 10^5$ and $1 \times 10^5$ IU IFN per ml of culture medium per day. Similar amounts of interferon are produced by incubation of mouse L cells with a virus or poly(I)-poly(C) (Yamamoto et al., 1974; Trapman, 1979). However, these conventional interferon preparations contain 60 to 80% MuIFN-β (Yamamoto & Kawade, 1980; Trapman, 1980). The 20 to 40% of MuIFN-α activity can be separated in at least five different subspecies (Lemson et al., 1984). Component 1 accounts for approximately 5% of the MuIFN-α activity of induced mouse L cell interferon and thus for only 1 to 2% of the total MuIFN activity in these preparations. From these figures we conclude that expression of IFN genes in CHO cells gives three advantages over a conventional production of MuIFN-α. Firstly, interferon synthesis is constitutive, secondly, only one of the subspecies is produced and thirdly, its concentration is 50 to 100 times higher.

Constitutive expression of the HuIFN-γ gene and a HuIFN-α gene, both under direction of the SV40 early promoter in CHO cells, has been reported (Haynes & Weissmann, 1983; Scahill et al., 1983). In these cases the genes were amplified together with the dihydrofolate reductase gene by increasing concentrations of methotrexate, resulting in cells with up to 50 copies of the IFN genes each. The amount of interferon formed by the highest producers was between $0.2 \times 10^5$ and $1 \times 10^5$ IU per ml per day. A comparison with the experiments described here for a mouse IFN gene, however, remains difficult because 1 IU of mouse interferon is probably not identical to 1 IU of human interferon. They can only be compared if the number of molecules produced by the different cells can be determined.

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