Production, purification and biological properties of an *Escherichia coli*-derived recombinant porcine alpha interferon

François Lefèvre,* René L’Haridon, Francisco Borras-Cuesta† and Claude La Bonnardière

Institut National de la Recherche Agronomique, Laboratoire de Virologie et Immunologie Moléculaires, Centre de Recherches de Jouy-en-Josas, Domaine de Vilvert, 78350 Jouy-en-Josas, France

Recombinant plasmids for intracellular synthesis of mature porcine interferon alpha 1 (IFN-α1) in *Escherichia coli* were constructed. High amounts of antiviral activity were obtained [up to $4 \times 10^5$ international units (IU) per ml of bacterial culture]. Recombinant porcine IFN-α1 (rIFN-α1) was purified to homogeneity by monoclonal antibody immunoaffinity and was found to have the expected $M_r$ (17-5K) and N-terminal sequence (except for the apparent lack of an N-terminal methionine). Its specific antiviral activity was $5 \times 10^7$ to $10 \times 10^7$ IU/mg MDBK cells. *In vitro* biological properties of this purified rIFN-α1 were compared to those of virus-induced porcine leukocyte interferon: the two interferons shared similar antigenic determinants and had the same ability to induce a cytocidal effect on primary cultures of pig kidney epithelial cells. However, rIFN-α1 was at least six times more active in inducing an antiviral state on homologous porcine cells. These properties are discussed in the light of a possible *in vivo* use of the purified recombinant molecule.

**Introduction**

Since the discovery of interferon (IFN) in 1957 (Isaacs & Lindenmann, 1957), much evidence has been given on its involvement in the pathogenesis of and recovery from viral diseases, which strongly supports its use as an *in vivo* antiviral agent in man. Until recently, however, all studies showing a clear beneficial effect of endogenous or exogenous IFN in experimentally induced viral diseases were performed in a limited set of laboratory animal species. Extending the study of the IFN system to other species such as domestic animals should provide further information about its role in other viral disease models. With the help of the recent advent of recombinant DNA technology, IFN could also represent a new therapeutic agent with a broad antiviral spectrum and immunomodulatory properties in veterinary medicine, as recently shown in cattle (see Bielefeldt Ohmann *et al.*, 1987, for a review).

The pig represents an economically important animal species for food and hence the development of such a new therapeutic and/or prophylactic agent against infectious diseases in this species is strongly desirable. Moreover, the pig is an interesting model for investigating the role of endogenous IFN in viral infections and for studying the pharmacology and toxicology of exogenous homologous IFN (La Bonnardière *et al.*, 1984).

Recently we showed that, as in the human, bovine and equine species (Capon *et al.*, 1985; Hauptmann & Swetly, 1985; Velan *et al.*, 1985; Himmler *et al.*, 1986), the porcine IFN-α (PoIFN-α) multigene family can be divided into two homologous, but distinct classes of genes. The class I subfamily, located on porcine chromosome 1 (Yerle *et al.*, 1986) contains at least 11 loci, of which nine have been cloned and two sequenced: PoIFN-α1 and PoIFN-α2 (Lefèvre & La Bonnardière, 1986; F. Lefèvre, unpublished results). The class II subfamily, which appears to be tightly linked to the class I subfamily, contains at least six distinct loci (F. Lefèvre, unpublished results). The low level expression of the PoIFN-α1 gene in *Escherichia coli* revealed that its potential product, a preprotein of 189 amino acids with a putative N-terminal signal peptide of 23 amino acids, had an IFN-α biological activity (Lefèvre & La Bonnardière, 1986).

In this article, we describe the construction of plasmids allowing the intracellular synthesis of large amounts of mature porcine recombinant IFN-α1 (rIFN-α1) in *E. coli* cells, its subsequent purification to homogeneity and some of its *in vitro* biological properties.

**Methods**

*Bacterial strains, plasmids and growth conditions.* Plasmid and bacteriophage M13 constructions were cloned in *E. coli* strain RR1 and JM103, respectively. For experiments in recombinant IFN-α expres-
Fig. 1. Construction of plasmids allowing the expression of porcine rIFN-α1. For more details, see Methods. Blackened box and open box represent the thermosensitive repressor gene (cR857) of the bacteriophage λ and the ampicillin resistance gene, respectively. Sequences encoding the rIFN-α1 signal peptide and mature protein are indicated by stippled and light grey boxes, respectively. The dark grey box represents the cro-β-gal' coding sequence and the hatched box the Shine–Dalgarno sequence. The black arrow indicates the rightward promoter (PR) of bacteriophage λ. Thin lines mark pBR322 or bacteriophage λ sequences; bold lines show the porcine genomic non-coding sequences. PolI K, the Klenow fragment of *E. coli* DNA polymerase I.

DNA manipulation, plasmid constructions and nucleotide sequence analysis. All enzymes were used according to the manufacturer's recommendations. Oligonucleotides were synthesized on a Biosearch 8600 DNA synthesizer and purified further as described (Sambrook et al., 1989). DNA manipulations and plasmid constructions were done according to standard procedures (Sambrook et al., 1989) and are summarized in Fig. 1. Plasmid pD1A9225 (generously provided by Dr Antoine Danchin, Institut Pasteur, Paris, France) was the basic vector used for porcine rIFN-α1 expression. It allowed thermoinducible expression of heterologous coding sequences fused to the C terminus of a cro-β-galactosidase peptide (cro-β-gal', 49 amino acids) (Leplatois & Danchin, 1983). Plasmid pD1APoIFN-α1 was constructed by inserting, at the unique *Pvu*II site of pD1A9225, the *Pvu*II–*Hpa*I fragment of the PoIFN-α1 gene (containing sequences encoding part of the signal peptide and the complete mature protein) in the orientation allowing in-frame fusion with the cro-β-gal' coding sequence. Plasmid pLD67 was constructed by cloning in the proper orientation (Lathe et al., 1984) a 49 bp synthetic blunt-ended DNA fragment at the *Pvu*II site of pD1A9225. Due to this insert, translation of the cro-β-gal' sequence stopped at an in-frame TAA codon which was followed, 13 bp downstream, by the consensus Shine–Dalgarno sequence 5' AAG-GAGGT 3' (Gold, 1988) leading to a translational (re)initiation at a coding region inserted in the neighbouring unique *Xba*I site. Oligonucleotide-directed mutagenesis in bacteriophage M13 (Zoller & Smith, 1982) was used to construct a gene encoding methionyl IFN-α1 (MetIFN-α1): the sequence 5' CATATG 3' containing a methionine initiator codon and a unique *Nde*I restriction site was introduced just before the first codon (TGT, cysteine) of the putative mature IFN-α1 coding sequence. Plasmid pLD67MetIFN-α1 was constructed by inserting the MetIFN-α1 coding sequence between the *Xba*I and *Sal*I sites of pLD67 as described in Fig. 1. The nucleotide sequence extending between the cro-β-gal' and MetIFN-α1 coding regions was controlled according to Chen & Seeburg (1985) using a synthetic DNA primer.

Monoclonal antibodies (MAbs). A peptide corresponding to the 15 C-terminal residues of PoIFN-α1 (Lefèvre & La Bonnardiére, 1986) was synthesized by the method of Merrifield using the Fmoc-polyamide mode (Atherton & Sheppard, 1985) and coupled to ovalbumin via glutaraldehyde. Fifty μg of conjugate was injected twice into 3 month old BALB/c mice with a 20 day interval. Boosted splenocytes were fused with selectable murine SP2/O myeloma cells and hybridomas...
were selected in medium containing azaserine (Galfre & Milstein, 1981). Hybridomas were screened using a direct ELISA in plastic plates coated with the conjugate. Five MAbs which recognized both the synthetic peptide and the denatured form of rIFN-α1 (not shown) were isolated. One of them, C8-18, was used for immunoblotting the synthetic peptide and the denatured form of rlFN-α1 (not shown). Fusion between splenocytes and SP2/O cells was performed 4 days after the last injection, and growing hybridomas were screened for IFN reactivity by an immunosorbent bioassay set up in our laboratory (R. L'Haridon et al., unpublished results). Eight independent anti-IFN-α MAbs were isolated; one of them, C22, was used for the immunofluorimetry purification of rlFN-α1.

Purification of rlFN-α1. E. coli strain CAG1139 harbouring the plasmid pLD67MetIFN-α1AHS was grown overnight at 37 °C in enriched medium to an optical density at 600 nm (OD600) of about 5. Extraction of rlFN-α1 from the bacterial pellet was conducted essentially as described by Valenzuela et al. (1985). The crude soluble extract was dialysed extensively against phosphate-buffered saline containing 0.02 mM-Met PMSF, and rlFN-α1 was purified from this dialysate by a single-step immunofluorimetry using specific anti-porcine rlFN-α1 MAb C22: monoclonal IgG was coupled to glutaraldehyde-activated amino-hexyl-Sepharose (Pharmacia) according to Cambiaso et al. (1975). Bound IFN was eluted from the column with 0.1 M-acetic acid, 0.3 M-NaCl, pH 2.5, extensively dialysed as above and analysed by SDS-PAGE and reverse-phase HPLC. About 200 pmol of protein was subjected to N-terminal amino acid sequencing by sequential Edman degradation using a gas-phase Applied Biosystem 470A acid analyser 120A.

Immunoblotting. Total bacterial proteins (0.5 OD600 unit of cell culture per gel slot) were separated by SDS–PAGE (Laemmli, 1970) followed by transfer to a nitrocellulose filter (Towbin et al., 1979). Immunodetection of rlFN-α1 was performed using a crude ascitic fluid prepared for an IFN-α antiviral assay by resuspending the bacterial pellet from 1 ml of culture in 200 µl of TNE buffer (10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 100 mM-NaCl), mixing the suspension with 200 µl of lysis solution (10 M-urea, 2% w/v SDS, 2% v/v 2-mercaptoethanol) and heating the lysate for 1 min at 100 °C. The IFN antiviral activity was assayed by inhibition of the c.p.e. of vesicular stomatitis virus on MDBK or other cell lines, as previously described (La Bonnardiére & Laude, 1981). Antiviral titres were expressed in IU/ml of bacterial culture on MDBK cells. In order to obtain constitutive expression of the recombinant protein, the HindIII–Smal fragment bearing the 3' end of the c857 thermosensitive repressor gene was deleted from pLD67MetIFN-α1 (Fig. 1). A saturation curve of E. coli CAG1139 harbouring this new construction (pLD67MetIFN-α1AHS) contained at least three times more antiviral activity (4 × 108 IU/ml of bacterial culture on MDBK cells) than the previous one. Western blot analysis performed for both constructs revealed that a single protein species with an apparent molecular mass of about 57 000 was immunodetected in E. coli cell extracts by the C8-18 MAb (Fig. 2c).

Purification and N-terminal sequencing of mature porcine rIFN-α1

Biologically active rIFN-α1 was extracted and purified as described in Methods. SDS–PAGE analysis revealed a single band with an Mr of 17.5 K comigrating with IFN-α antiviral activity and corresponding to mature porcine rIFN-α1 was immunodetected in E. coli cell extracts by the C8-18 MAb (Fig. 2c).

Results

High level expression of porcine rIFN-α1 in E. coli cells

To test its ability for being expressed at a high level in E. coli, the PoIFN-α1 coding sequence was inserted into the fused protein expression plasmid pD1A9225. The plasmid obtained, termed pD1APoIFN-α1, potentially codes for a 224 amino acid hybrid protein consisting of the entire mature IFN-α1 and remaining nine amino acids of the signal peptide (S15 to S23) fused to the N terminus to the 49 residues of cro–βgal. The pD1APoIFN-α1 construct was examined for protein synthesis in the protease-deficient strain (lon-) E. coli CAG1139. Large amounts of a protein species with the expected Mr (28K) was detected by SDS–PAGE of total E. coli proteins after heat induction (Fig. 2a). Western blot analysis of the gel showed that a band of similar size was the major protein species immunoreactive with the MAb C8-18 (Fig. 2b).

As the aim of this work was to obtain a recombinant mature PoIFN-α1 devoid of its signal peptide, we chose to express the MetIFN-α1 protein (mature IFN-α1 with an additional N-terminal methionine) in E. coli. As pD1A9225 seemed to express the PoIFN-α1 coding sequence efficiently, we modified it to obtain pLD67 which permitted the expression of unfused coding sequences. The sequence encoding MetIFN-α1 was constructed by site-directed mutagenesis of the preIFN-α1 coding sequence and inserted into pLD67. The plasmid obtained, pLD67MetIFN-α1, enabled a expression of a cro–βgal peptide (of 51 residues) and MetIFN-α1 from a bicistronic mRNA molecule. E. coli CAG1139 cells bearing this construct grown and heat-induced as described in Fig. 2(c) contained a high antiviral activity (1.2 × 105 IU/ml of bacterial culture on MDBK cells). In order to obtain constitutive expression of the recombinant protein, the HindIII–Smal fragment bearing the 3' end of the c857 thermosensitive repressor gene was deleted from pLD67MetIFN-α1 (Fig. 1). A saturation curve of E. coli CAG1139 harbouring this new construction (pLD67MetIFN-α1AHS) contained at least three times more antiviral activity (4 × 108 IU/ml of bacterial culture on MDBK cells) than the previous one. Western blot analysis performed for both constructs revealed that a single protein species with an apparent Mr, of 17.5 K comigrating with IFN-α antiviral activity and corresponding to mature porcine rIFN-α1 was immunodetected in E. coli cell extracts by the C8-18 MAb (Fig. 2c).
subjected to the Edman degradation was not reduced or alkylated, the first N-terminal cysteine residue did not give a PTH derivative. We thus obtained: none-D-L-P-Q-T-H-S-L-A in the first 10 steps which clearly corresponds to the terminal sequence deduced from the DNA (Lefèvre & La Bonnardiére, 1986). On account of the yield of the first step of the Edman degradation (our result was 27%), which was within the normal range of yields (20 to 50%), and as no methionine-PTH was found, we think that at least a major fraction of rIFN-α1 was non-methionylated and unblocked at its N terminus; however we cannot exclude the existence of a minor fraction, methionylated and blocked at its N terminus.

In vitro biological properties of rIFN-α1

(i) Antiviral effect on different cells

The antiviral spectrum of rIFN-α1 was compared with that of PoIFN-Le obtained by infection of pig peripheral blood lymphocytes with influenza virus (La Bonnardiére et al., 1986). For that purpose, we used a panel of five cell lines belonging to four different species (Table 1). It appeared that, like IFN-Le, rIFN-α1 exhibited a broad spectrum of antiviral activity (10% activity on mouse cells as compared to MDBK cells). But interestingly, rIFN-α1 was clearly more active (by sixfold) than its natural counterpart on homologous porcine cells. This

Fig. 2. Characterization (by SDS-PAGE and Western blot analysis) and purification of porcine IFN-α1 expressed in E. coli CAG11399 cells harbouring various expression plasmids. (a) Cells bearing plasmid pD1APolIFN-α1 were grown at a restrictive temperature (30 °C) in the enriched medium described in Methods to an OD_{600} of 1.5. The culture was maintained at the same temperature (lane 1) or at an induction temperature (42 °C, lane 2) for 2 h. Proteins were analysed on a 12.5% SDS-polyacrylamide gel and stained with Coomassie blue. The arrow indicates the position of the 28K cro-βgal–IFN-α1 fusion protein. Other minor bands probably correspond to degradation products. (b) Western blot analysis of the gel in (a) using the C8-18 MAb. Lanes 1 and 2 refer to homologous samples of the gel in (a). (d) Purification of mature porcine rIFN-α1. Proteins were analysed on a 15% SDS-polyacrylamide gel and stained with Coomassie blue. Lane 1, total proteins from cells harbouring plasmid pLD67MetIFN-α1ΔHS; lane 2, approximately 2 μg of immunoaffinity-purified porcine rIFN-α1. Mr standards are indicated on the left of (a), (b), (c) and (d).
Table 1. Antiviral activity of porcine IFN-Le and rIFN-α1 on cell lines from different species

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell line</th>
<th>IFN-Le (IU/ml)</th>
<th>rIFN-α1 (IU/ml)</th>
</tr>
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<tbody>
<tr>
<td>Bovine</td>
<td>MDBK</td>
<td>3000</td>
<td>3000</td>
</tr>
<tr>
<td>Human</td>
<td>WISH</td>
<td>330</td>
<td>330</td>
</tr>
<tr>
<td>Murine</td>
<td>L929</td>
<td>440</td>
<td>250</td>
</tr>
<tr>
<td>Porcine</td>
<td>PD5</td>
<td>330</td>
<td>2200</td>
</tr>
<tr>
<td></td>
<td>ST83</td>
<td>110</td>
<td>660</td>
</tr>
</tbody>
</table>

* The antiviral titres of porcine IFN-Le and rIFN-α1 preparations having the same titre on MDBK cells (3000 IU/ml) were determined also on human, murine and porcine cell lines.

Table 2. Antiviral and cytocidal activities of porcine and human IFNs on low-passage porcine kidney cells (RPa 5495)*

<table>
<thead>
<tr>
<th>IFN</th>
<th>Anti-human IFN-α serum†</th>
<th>MDBC</th>
<th>RPa 5495</th>
<th>Log₃ ratio</th>
<th>CCA/AVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>PolIFN-Le</td>
<td>+</td>
<td>8</td>
<td>7</td>
<td>4</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
<tr>
<td>rIFN-α1</td>
<td>-</td>
<td>10</td>
<td>10</td>
<td>7</td>
<td>-3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>4.5</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HuIFN-Le</td>
<td>-</td>
<td>8.5</td>
<td>8.5</td>
<td>6</td>
<td>-2.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

* Confluent monolayers of MDBK or RPa 5495 cells were treated as for the antiviral assay with the same preparations of PolIFN-Le, rIFN-α1 or HuIFN-Le. Antiviral activity (AVA) of these preparations was expressed as log₃ of the highest dilution giving >50% protection of the cell sheet against virus challenge. Cytocidal activity (CCA) was expressed as the log₃ of the highest dilution at which cell sheets were 100% killed (see Laude & La Bonnardiére, 1984, for details).
† Sheep anti-human IFN-α serum diluted 100-fold in the titration medium.

Suggests that IFN-α1 could not be a major component, if any, of the virus-induced IFN-Le.

(ii) Cytocidal effect
It has been shown that IFNs from several species exert a toxic effect on primary cultures of pig kidney epithelial cells (Laude & La Bonnardiére, 1984). This so far unexplained phenomenon was observed specifically in porcine kidney cells, and only in primary or low-passage cells. We assayed rIFN-α1 on these cells by comparison with both PolIFN-Le and HuIFN-Le (Table 2). Clearly, the recombinant IFN-α was cytotoxic for low-passage pig kidney cells. This effect was caused by IFN itself because in this assay we used rIFN-α1 purified to homogeneity by immunoaffinity; also, the cytocidal as well as the antiviral effect were abolished by anti-IFN-α neutralizing antibodies. The ratio of antiviral to cytocidal titres for the three kinds of IFNs were similar and ranged around 3³, which means that 30 antiviral units were enough to cause cell death in this system (Table 2).

(iii) Antigenicity
It was of interest to determine whether rIFN-α1 shared antigenic determinants with PolIFN-Le. Previous work showed that anti-human IFN-α serum could neutralize PolIFN-Le efficiently (La Bonnardiére et al., 1986). It appeared that this serum neutralized rIFN-α1 to the same extent (Table 3). Recent results obtained with MAbs directed against rIFN-α1 confirm this high antigenic similarity between the cloned species and natural IFN (R. L’Haridon & C. La Bonnardiére, unpublished results).

Discussion
A system for large-scale production of purified mature recombinant porcine IFN-α was described. Quantitative optimization of this system is now in progress. However, the amount of recombinant protein already obtained is suitable for in vivo studies. The use of recombinant IFNs and other cytokines as immunomodulating and anti-infectious agents in domestic animals represents a growing field of interest. We have examined several in vitro biological properties of rIFN-α1 and found several reasons for its suitability as a molecule for in vivo experimentation.

Recombinant IFN-α1 induced an efficient antiviral state in a panel of different cells. Moreover, it was at least six times more efficient than crude preparations of IFN-Le in triggering this state on porcine cells. This could be because the IFN-α1 subtype is less represented and exhibits a greater specific activity than other subtypes present in IFN-Le. Differences in the specific activities of IFN-α subtypes have been well documented in other species: for example, recombinant human IFN-α2 is 100
times more active than recombinant human IFn-α1 in inducing an antiviral state in homologous human cells and this ratio is correlated with a different affinity for the IFN receptor (Streuli et al., 1981; Aguet et al., 1984; Uzé et al., 1985). Alternatively, as an N-glycosylation site is present at positions 78 to 80 of PoIFN-α1 (Lefèvre & La Bonnardièref, 1986), it cannot be excluded that it is normally expressed in IFN-Le and that the glycosylated leukocyte-derived protein has a lower antiviral specific activity than its recombinant unglycosylated E. coli-derived homologue. According to Piascecky (1988), PoIFN-Le would mostly be unglycosylated. If so, this finding argues in favour of IFN-α1 being a minor component of IFN-Le. In any case the fact that rIFN-α1 is antigenically related to IFN-Le (Table 3) suggests that there are no major structural differences (in the active site of the molecule) between this IFN-α subspecies and those present in IFN-Le.

Amino acid sequencing revealed no methionine at the N-terminal end of rIFN-α1. This is an advantage for in vivo use of the molecule because the presence of an additional methionine at the N terminus of recombinant proteins might induce an immune response that could be deleterious to the host. Similarly, it was found by others that most human recombinant IFN-α2 molecules produced in E. coli are methionine-free at the N terminus (Nakagawa et al., 1987). However in our study, the presence of a minor fraction of MetIFN-α1 formylated or blocked in another way cannot be excluded.

Previous work in our laboratory showed that various preparations of human, bovine and porcine IFN-Le could specifically induce a cytotoxic effect in confluent monolayers of low-passage pig kidney epithelial cells (Laude & La Bonnardièref, 1984). The results presented here confirm these data and show that a recombinant porcine IFN-α subspecies has retained this biological property. It was recently shown that recombinant murine IFN-γ exhibits a very similar effect on low-passage mouse embryonic fibroblasts (Dijkmans et al., 1989). The ability of IFNs to induce active cell death in some normal tissues could represent a new property of these cytokines whose biological significance has not yet been established. The cytoid effect could have serious implications for the toxicity of rIFN-α1 and could prohibit its use for in vivo experiments, especially in the young animal. Clinical trials in man have clearly shown that recombinant IFNs can be responsible for some adverse reactions (Scott, 1983). Besides, the work of Gresser (1982) showed that large quantities of exogenous or endogenous IFN can induce toxic effects in newborn mice. However, preliminary data are now available to show that a single administration of rIFN-α1 did not induce any detectable toxic effect in newborn and adult pigs (C. La Bonnardièref, unpublished results).

We believe that the availability of the porcine rIFN-α1 molecule has opened a new field for in vivo investigations concerning the role of IFN in viral pathogenesis and its possible prophylactic use in pigs. During experimental infection of newborn pigs by the transmissible gastroenteritis coronavirus, which induces acute and often fatal diarrhea, it has been shown that early after infection and before the onset of clinical signs, high levels of IFN-α are produced in the intestinal tract, lungs, serum and urine (La Bonnardièref & Laude, 1981). Recombinant IFN-α1 was used to obtain MAbs which can neutralize PoIFN-Le antiviral activity (R. L’Haridon et al., unpublished results). These antibodies could be used to investigate the pathogenic significance of this IFN-α response by neutralizing it during the course of infection. The possible anti-infectious and/or immunomodulatory properties of rIFN-α1 will be studied by examining its in vivo effects on the outcome of experimental virus diseases in pigs.

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References


