from the pDA8 plasmid (3), using the oligonucleotides: 5'TGTGATCTGCCTCAAACCCACAGCCTG3' and 5'TCATTCC-TTACTTCTTAAACTTTCTTG3'.

The 495 bp coding fragment was phosphorilated and cloned into pNAO407 previously digested with NcoI-EcoRI and treated with the Klenow fragment of *E. coli* DNA polimerase I. *P. pastoris* MP36 HIS3$^-$ was transformed with ClaI-SalI digested pIFNPP according to Hinnen *et al. (10)*.

Culture conditions

Transformed yeasts were grown up to a cell density of 5 x 10^7 cells/mL of culture (OD_{600} 1.0) in 5 mL of G0 media, supplemented with 2% glycerol. The cells were then washed with sterile water and transfered to 100 mL of the YP medium supplemented each 24 hours with 0.5% methanol as the sole carbon and energy source. The growth was achieved at 30°C with constant vigorous shaking, during 120 hours.

Fermentations were carried out on 5 liter fermentors (B. E. Marubishi), with 3 liters of effective volume, containing G0 medium supplemented with 2% glycerol. The initial optical density was 0.3 OD_{600} and the fermentation conditions were 700 rpm, 1vvm of aeration, 30°C and pH 5.0. Interferon expression was induced after 12 hours of culture by addition of methanol and maintaining its concentration at about 0.4% during 120 hours.

Purification of biologically active IFN-α2

Induced cells were harvested by centrifugation at 3 500 g for 30 min. All steps were performed at 4°C. 80 g of cells were resuspended in 300 mL of lysis buffer (Tris HCl 0.1M pH 7.9, sucrose 10%, NaCl 0.3M, EDTA 5 mM). Cells were broken by mechanical shaking (Dyno Mill Model KDL, retention time 5 min) and the debris harvested by centrifugation at 3 500 g for 30 min. The pellet of broken cells (1.3 g of total protein) was solubilized with 90 mL of 30 mM Tris HCl, 30 mM NaCl, 5 mM DTT pH 7.2 and 6M GuCl at 4°C during 2 hours. The insoluble material was removed by centrifugation at 8 000 g for 30 min. IFN-α2 was recovered by slow dilution of the 6M GuCl solution with 1 200 mL of cold 30 mM Tris HCl, 30 mM NaCl, 80 μM $CuSO_4$ pH 7.0 over the course of 6 hours. IFN-α2 was purified from the diluted Guanidium chloride solution using a column of immobilized monoclonal antibody CB-IFN A 2.4 (11). The protein peak eluted from this column was concentrated 10 times by ultrafiltration (AMICON). Total protein concentration was determined by the Coomassie blue method (12). SDS-PAGE and Western blot were done according to Laemmli (13) and Towbin (14), respectively. The concentration of IFN in all samples was determined by ELISA (15).

Interferon-induced antiviral activity of the final preparations was determined through the inhibition of the cytopathic effect of Mengovirus on *Hep-2* cells (16).

RESULTS AND DISCUSSION.

Vector construction and methanol-regulated expression of IFN-α2 in *P. pastoris*

The vector pIFNPP (figure 1) is expected to direct the integration of the expression cassette by homologous recombination into the *P. pastoris* AOX1 locus.

This cassette contains: the methanol regulated *P. pastoris* alcohol oxidase I promoter (AOX1 p), the IFN gene, *S. cerevisiae* GAP transcriptional termination signal (GAP t), the *S. cerevisiae* HIS3 gene which provided a selectable marker for transformation of *P. pastoris* MP36, and the 2.1 kb DNA fragment from the downstream region of the chromosomal *P. pastoris* AOX1 gene, which together with the AOX1 promoter provide the chromosome-homologous ends needed for integration.

The ClaI-SalI digested vector was transformed into *Pichia pastoris* MP36. A transformant with the expression cassette integrated into the *P. pastoris* genome was selected by Southern blot and by expression analysis in a shake flask culture. This transformant was designated MP36 (pIFNPP).

At the end of methanol induction, IFN was detected as a band in Coomassie blue-stained SDS-PAGE (figure 2A, lane 1). This band comigrated with natural IFN (figure 2A, lane 5) and it was not present in the negative control (figure 2A, lane 2). The identity of the recombinant IFN was demonstrated by Western blot (figure 2B) using an anti-IFN polyclonal antibody developed in rabbits. As it can be observed, this recombinant IFN-α2b is insoluble, associated to cellular debris (figure 2B, lane 1) and it is not present in soluble cell extracts (figure 2B, lane 3).

High level expression of IFN-α2 is obtained using this system. According to an IFN-specific ELISA a concentration of 400 mg/L of culture was found to be associated to cellular debris. An IFN expression of 7% is obtained in *P. pastoris* in contrast to previous reports in other hosts for example *S. cerevisiae* 1-2% and *E. coli* 1% (3).

Purification and partial characterization of recombinant IFN-α2

The IFN-α2 was purified as described in materials and methods. Table 1 reports the percent recovery during the purification steps. According to the table, with 10 liters of fermentation it is possible to obtain 1 g of pu-

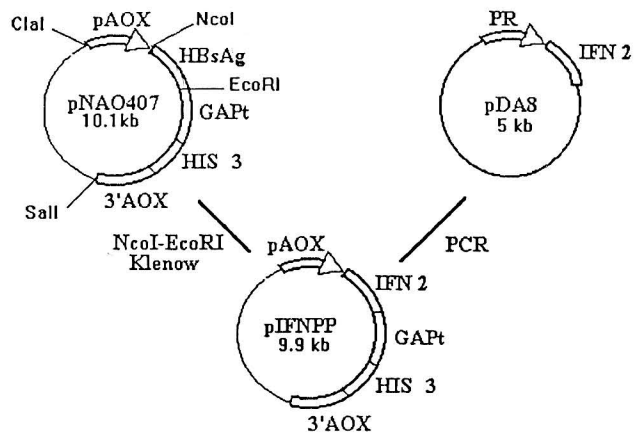

Fig. 1. Construction of the expression vector, pIFNPP.

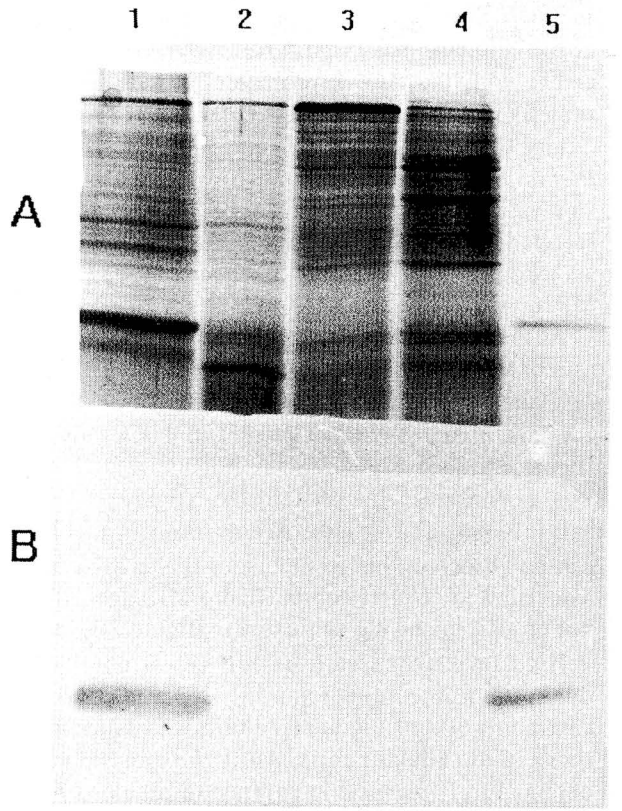

Fig. 2. SDS-PAGE on a 15% slab gel stained with Coomassie brilliant blue (A) and Western blot (B). Proteins were electrophoresed on a 15% slab SDS-PAGE, electroblotted onto nitrocellulose and an anti-IFN polyclonal antibody developed in rabbits used according to towin (1979). The samples were taken from grown and broken cells as explained in Materials and Methods. Lanes 1 and 2 show samples from cellular debris from induced and non induced cells respectively. Lanes 3 and 4, samples from soluble cell extracts from induced and non induced cells respectively, and Lane 5, purified IFN.

rified IFN-α2b to homogeneity. Figure 3 shows the chromatography profile of the immunoaffinity column used in the last purification step. The interferon eluted as a single peak from the immunoaffinity colum (figure 3A), in 15% SDS-PAGE the fraction number six (concentrated by ultrafiltration) gave a single band (figure 3B, lane 4) at an apparent molecular weight of 18 kD. Recombinant IFN appeared as a homogeneous species with the same molecular weight of the natural IFN-α2b.

The purified IFN-α2 was analyzed by FAB-MS in order to detect mutations or posttranslational modifications. A modification at the N-terminal end was found. The N-terminal methionine was efficiently removed but the cysteine at this end was acetylated in about 70% of the final product (17), differing from natural human IFN-α2 which has not been found to have a blocked N-terminus (2). It is apparent that IFN-α2 shows a special susceptibility to acetylation because it has also been observed in *E.coli* and *Pseudomonas* (about 15%) (17).

IFN-α2b was biologically active as shown by the cytopathic effect inhibition assay. The specific activity was found to be 1 x 10^7 I.U./mg of IFN, different to natural

Table 1
IFN-α2b recovery during purification steps.

Step	IFN-α2b (mg)	Recovery %
Fermentation	400	-
Disruption	280	70
Extraction/Renaturation	140	50
Immunoaffinity	112	80
Concentrate	100	89

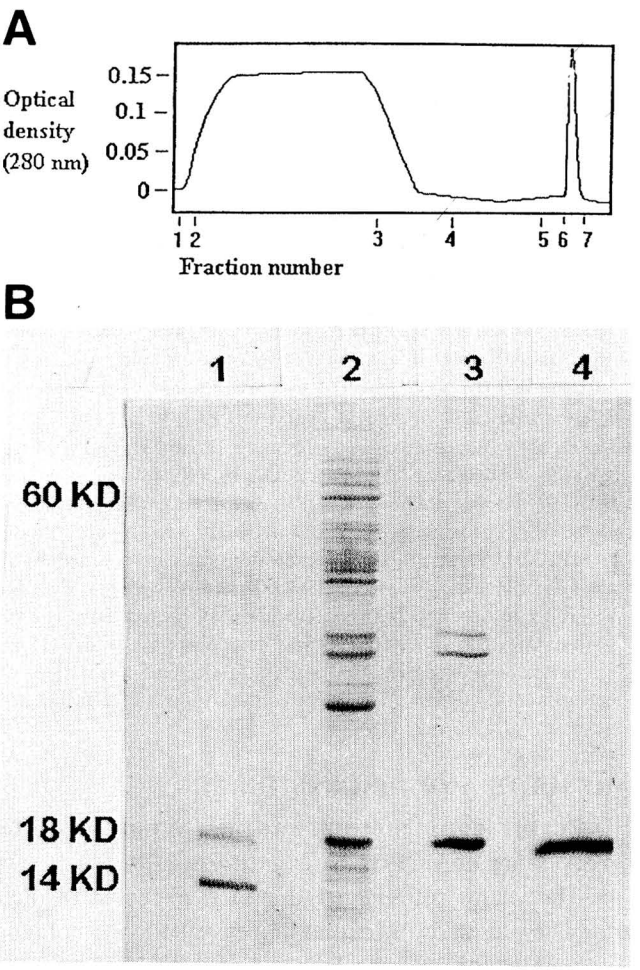

Fig. 3. (A): Immunoaffinity chromatographic profile of renaturalized samples. (B): 15% SDS-PAGE gel, Coomassie blue stained. 1, molecular weight markers. 2, solubilized broken cells. 3, aliquot before chromatography. 4, aliquot of the peak fraction.

human interferon (2 x 10^8 IU/mg) (2). This difference could be explained by the high level of acetylation of the recombinant IFN. The amino terminal acetilation occurs following the incorporation of the first amino acids into the polypeptide chain, this phenomenon could be avoided by expressing the protein fused to another protein, for example ubiquitin (18). This hybrid protein could be processed *in vivo* by specific hydrolases in the cytoplasm of yeast (18) and the resultant product would have a non blocked N-terminus.

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