

## CLONING AND EXPRESSION OF A HUMAN INTERFERON $\alpha$ 2 GENE IN *E. COLI*

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### Abstract

The plasmid pALCA1SIFN containing cDNA that encodes the human interferon  $\alpha$ -2b was obtained from the ATCC (no. 531667). In this system the expression of the gene is under the control of an *alcA* promoter. *alcA* p is a specific promoter for expression of different genes in *Aspergillus filamentous*. In this plasmid the coding region of IFN $\alpha$ -2b is preceded by the coding region of a synthetic signal peptide. For direct expression of the IFN $\alpha$ -2b gene under the control of a *T7/lac* promoter of pET24d(+) expression vector, three subcloning steps were carried out which resulted in the construction of 3 new plasmids. pHA1, pHA2 and pHA4 in which the IFN $\alpha$ -2 gene is under the control of *lacp*, *lacuv5p* and *T7* promoter, respectively. Another plasmid, pHA3, was also constructed and is a modified version of pET24d(+). Provided there is a synthetic signal peptide preceding the coding region of IFN $\alpha$ 2 gene, the protein can be seen in either system, as shown by western blotting, albeit with a different level of expression. The best product can be seen in the pHA4 plasmid with a *T7p* as judged by dot and western blotting.

### Introduction

Human leukocyte interferon- $\alpha$ 2 is a protein with antiviral activity with a range of other biological activities [1-4]. It is one of the most widely studied of the various leukocyte (IFN- $\alpha$ ) interferon species in the laboratory and the clinic. This protein has common features in gene sequences with other leukocyte interferons [1-5-6]. However, the various human IFN- $\alpha$  subtypes have distinct antiviral properties in cell cultures [7] and differ in their

effects on cell growth and natural killer cell activity [2]. Interferon- $\alpha$  (IFN) genes cloned into the *psf1* site of pBR322 [8-9-10] have been shown to produce biologically active IFN. The native signal peptide of this protein can not be recognised and processed by *E. coli*. Presumably, the presence of the signal sequence may result in the rapid degradation of the expressed polypeptide [11]. Alternatively, the unprocessed IFN protein may be inherently inactive.

In the present study we have subcloned the IFN $\alpha$ 2 gene into three different expression vectors. The plasmids pHA1, pHA2 and pHA4 were made in order to compare the level of expression of the gene under the control of

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*lacp*, *lacuv5* and a very strong promoter *T7p*, respectively.

## Materials and Methods

### Microbiological Techniques

Bacterial strains: the three *E. coli* strains TG1, BL21 (DE3) and JM83 that were used, were stored at 4°C on Luria agar plates or at -80°C as overnight cultures in Luria broth containing 40% glycerol. The genotype of the *E. coli* JM83 strain is: *araδ* (pro-*Lac*) *rpsL*  $\Delta$  (80 *lacZ*  $\delta$  M/15). The genotype of TG1 is: *supE*, *hsdΔ5 thiΔ* (*Lac-proAB*) F' [*traD30 proAB+* *lacI lacZ sm 15*] and the genotype of BL21 (DE3) is: *hsd gal*( $\lambda$ CUts 87 *ind1 sam7 nin5 Lac uv5 T7 gene 1*).

This strain has an integrated *T7* RNA polymerase in its genome.

### Bacterial Plasmids

pALCA1SIFN, pUC19, pYZ4 and pET24d(+) were used in this study. pALCA1SIFN is a derivative of pDG6. It is an ampicillin resistant vector. pUC19 is a high copy number expression vector. It is also an ampicillin resistant vector. pYZ4 is a kanamycin resistant vector for the direct expression of eukaryotic genes in *E. coli* under *lacuv5* promoter control. PET24d(+) is a commercially available vector supplied by Novagen [12], every gene subcloned in this vector is expressed under the control of the very strong promoter provided it is transformed into an *E. coli* strain carrying a gene for *T7* RNA polymerase. *T7* promoter can only be recognised by *T7* RNA polymerase and almost all the protein synthesizing machinery of the bacterium will be involved in making the product of the gene, which is under the control of this promoter.

### Media

Luria broth (L broth) contains bacto-trypton, 5 g/l yeast extract and 10 g/l NaCl, adjusted to pH 7.5 with 1M NaOH and autoclaved. Luria agar (L agar) consists of L broth containing 1.2% agar.

All other methods regarding the growth of bacteria and transformation and recombinant techniques are derived from "Molecular cloning, A laboratory Manual" (Maniatis *et al.*, 1982) [13].

### Subcloning Procedures

pALCA1SIFN was cut with *SalI* and *EcoRI* and the interferon gene which appeared about 1 kb on a preparative low melting point agarose gel was removed and then purified. Subsequently, the gene was subcloned into pUC19 which was digested with the same enzymes. The ligation mixture was used to transform competent cells of TG1 and ampicillin resistant colonies were selected and further patched onto the plates containing

*Xgal* and IPTG. Because of the disruption of *lacZ'* gene, the recombinant colonies should appear as either white or pale blue colonies. Some of the white colonies were chosen and plasmids were extracted from such colonies. Then recombinant plasmids were digested with *SalI* and *EcoRI* to show a distinct band corresponding to the 1 kb of interferon gene (data not shown). Restriction map analysis was carried out with the following restriction enzymes: *EcoRI*, *SstI*, *HindIII*, *XbaI* and *NdeI* as shown in Figure 1.

### Subcloning of Interferon Gene into the pYZ4 Expression Vector

pHA<sub>1</sub> was digested with *EcoRI* and *Hind III* and the 1 kb band was purified from a preparative low melting point agarose gel and ligated to pYZ4 which was restricted with the same enzymes. The ligation mixture was used to transform TG1 competent cells. The recombinant colonies were selected and restriction mapping was carried out for such colonies with *BamHI*, *EcoRI*, *SalI*, *NdeI*, *SstI*, *XbaI* and *AvaI* as shown in Figure 2.

### Subcloning of Interferon Gene into pET24d(+)

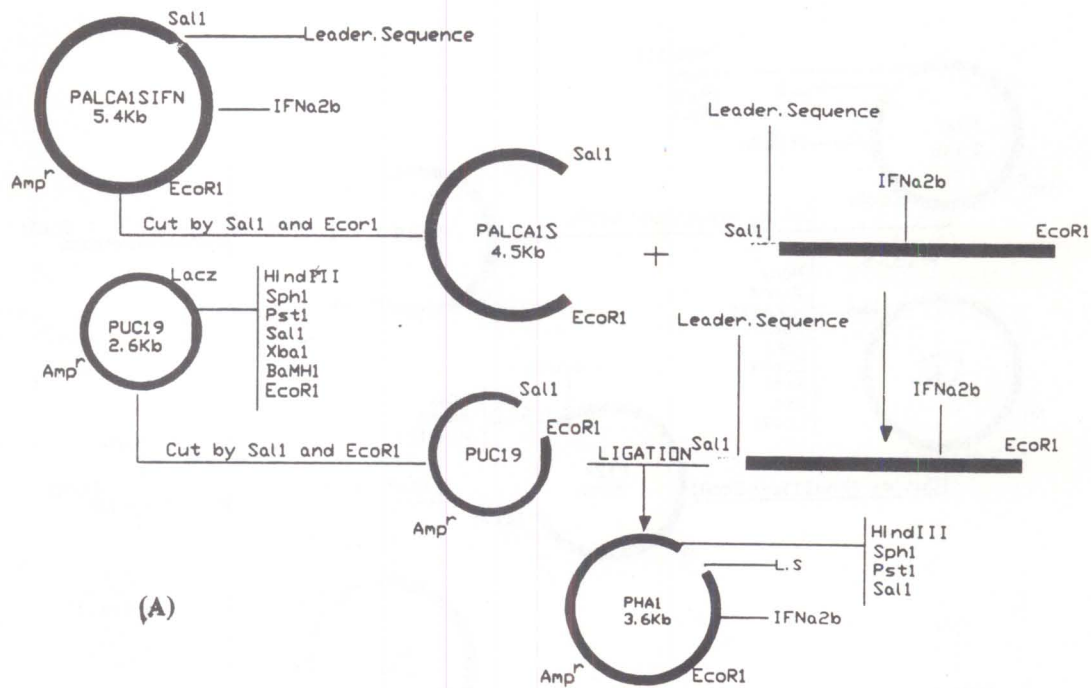
Because of the existence of a *SalI* site at the beginning of the IFN $\alpha$ 2 gene and a *SalI* site at the multiple cloning sites of pET24d(+) vector and considering that *SalI* and *XhoI* sites are compatible, pET24d(+) was cut with *SalI* and *XhoI* enzymes and religated with T4 DNA ligase. The resultant plasmid was named pHA3, (Fig. 3). By removing this *SalI* site in the vector, further gene manipulation would be easier. pHA2 was cut with *EcoRI* and *NcoI* and the band corresponding to 1 kb of interferon gene was purified from the low melting point preparative agarose gel and ligated to pHA3 which was cut with the same enzymes. Ligation mixture was used to transform BL21(DE3) competent cells. Because of the lack of *lacZ'* gene in pET24d(+), all recombinants were expected to be colorless. Therefore, a few colonies were randomly selected and restricted with *NcoI* and *EcoRI* to discriminate the recombinants from non-recombinants. pHA4 contains the human interferon alpha2 gene preceded by a synthetic signal peptide for periplasmic expression of the gene in *E. coli*. Restriction map analysis was carried out for this plasmid. pHA4 was restricted with *SstI*, *XbaI*, *EcoRI*, *SacI*, *HindIII* and *BamHI* as shown in Figure 4.

## Results

### Expression Studies

The expression of the human interferon alpha2 gene was verified in pALCA1SIFN(*alcap*), pHA1 (*lacp*), pHA2(*lacuv5*) and pHA4 (*T7p*).

Plasmid pALCA has an *alcA* promoter which was



(A)

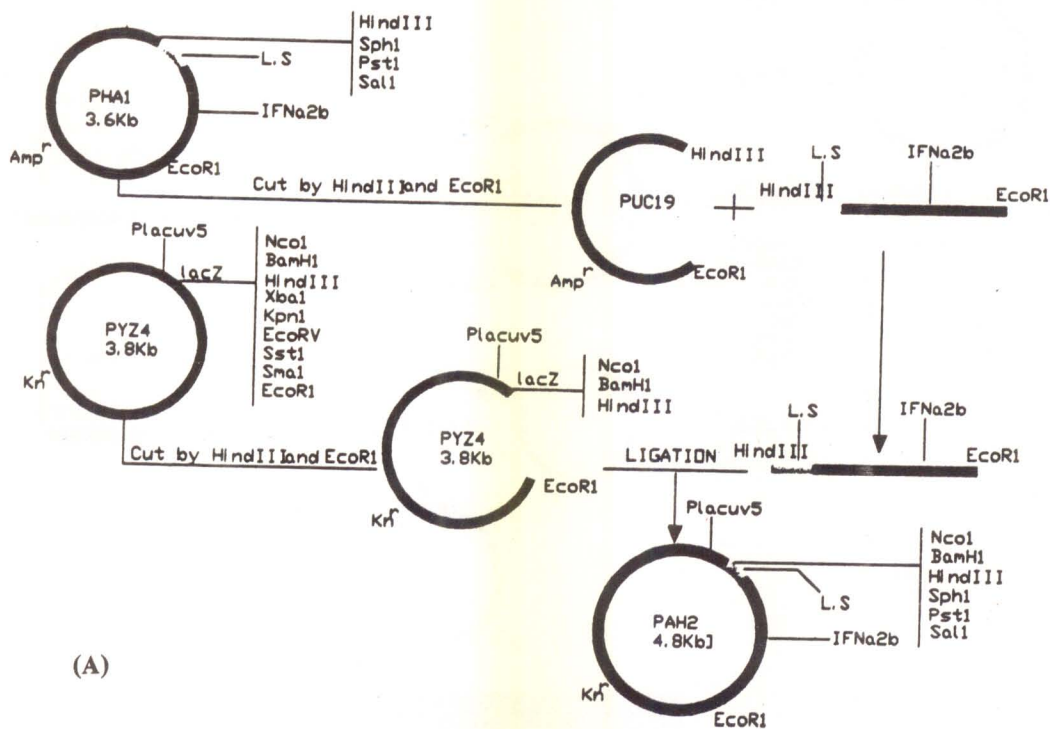


(B)

**Figure 1.** (A): Outline of the subcloning steps of IFN gene into pUC19, (B): Restriction map analysis of pHA1; lane (1) is pHA1 cut with *XbaI* and *NdeI* (partial digestion); lane (2) pHA1 cut with *XbaI* and *SstI*; lane (3) pHA1 cut with *EcoRI* and *NdeI* (arrow shown the 200bp); lane (4) pHA1 cut with *EcoRI* and *HindIII*; lane (5) DNA size marker; lane (6) pHA1 cut with *EcoRI* lane (7) pUC19 cut with *EcoRI*.

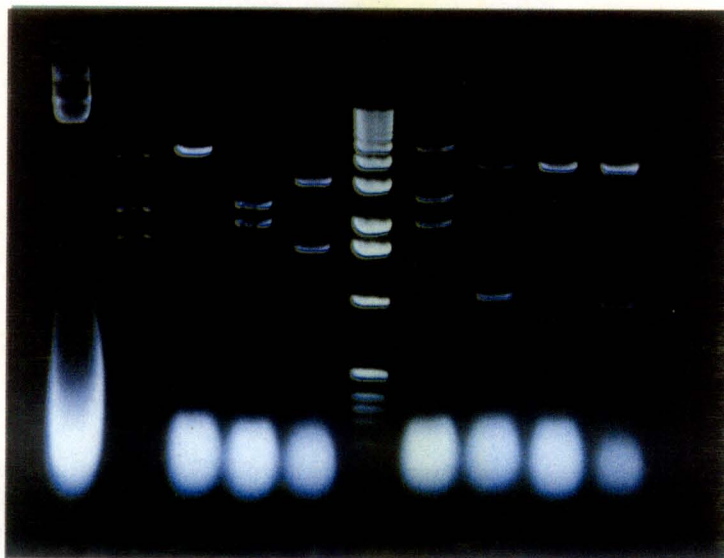
induced with threonine in JM83 cells. Threonine was used at 1mM, 10mM, and 100 mM concentration after 0.5, 1,2,3,4,5 and 6 hours separately. 3mls of culture was centrifuged and the pellet was dried. pHA1 has *lac* promoter; therefore, it was induced with IPTG at 1mM

concentration and the culture was centrifuged and the pellets were collected after 0.5, 1,2,3,4,5 and 6 hours. pHA2 has *lacuv5* promoter, TG1 cells which contain this plasmid were induced by 1mM IPTG and 3mls of culture were collected after 0.5, 1,2,3,4,5 and 6 hours. BL21(DE3)



(A)

1 2 3 4 5 6 7 8 9 10



(B)

**Figure 2.** (A): Outline of the subcloning steps of IFN gene into pYZ4; (B) Restriction map analysis of pHA2; lane (1,2) uncut pHA2; lane (3): pHA2 cut with *AvaI*; lane (4) pHA2 cut with *AvaI* and *XbaI*; lane (5) pHA2 cut with *AvaI* and *SstI*; lane (6) DNA size marker; lane (7) pHA2 cut with *NdeI* and *SalI* (partial digestion); lane (8) pHA2 cut with *NdeI* and *EcoRI*; lane (9) pHA2 cut with *XbaI* and *SstI*; lane (10) pHA2 cut with *BamHI* and *EcoRI*.

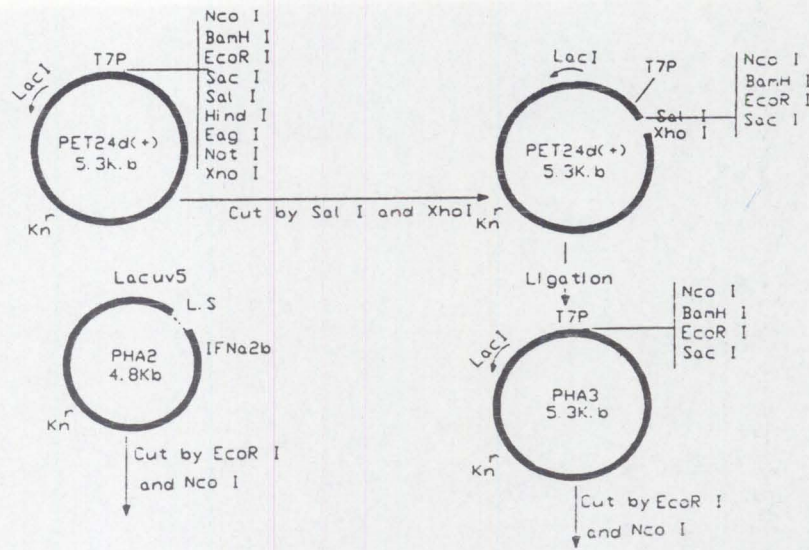


Figure 3. Outline of making plasmid pHA3 (see text for details)

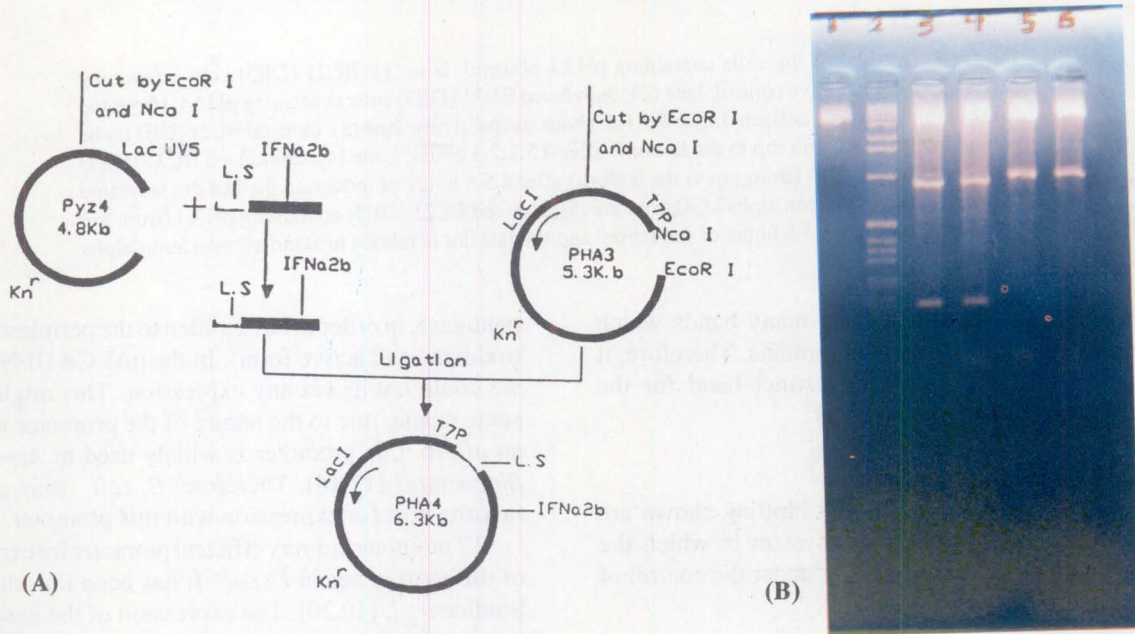


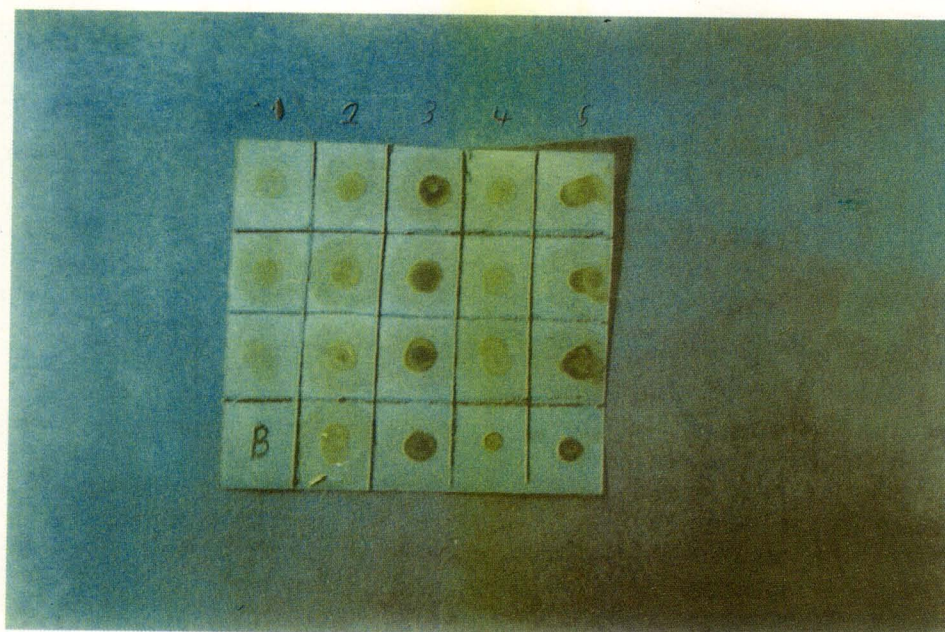
Figure 4. (A) Outline of subcloning of IFN gene into pHA4; (B) Restriction map analysis of pHA4; lane (1) pHA4 cut with *Sst*I; lane (2) size marker; lane (3) pHA4 cut with *Xba*I and *Sst*I; lane (4) pHA4 cut *Xba*I and *Sac*I lane (5) pHA4 cut with *Eco*RI and *Bam*HI lane (6) pHA4 cut with *Sac*I and *Hind* III.

cells containing pHA4 were induced by 1mM IPTG and the cells of culture were collected after 0.5, 1,2,3,4,5 and 6 hours.

**SDS-PAGE**

Cell lysates with equal concentration (10µl) from

cell lysate containing 7mg/ml protein, the concentration of proteins was determined by the Bradford method [14], were loaded from each culture onto a 15% SDS-PAGE and stained with either Coomassie blue or AgNO<sub>3</sub>. No distinct band corresponding to IFN protein was observed in the area range of 14 to 25kDa. In fact, at the same



**Figure 5.** Dot blot of the cells containing pHA4 plasmid: lane (1); BL21 (DE3) cells containing pET24d(+) as a negative control: lane (2); uninduced BL21 (DE3) cells containing pHA4: (from top to the bottom) cultures collected after 0.5.1.2.3 hours respectively: lane (3); induced BL21 (DE3) cells containing pHA4 (from top to the bottom) after 0.5.1.2.3 hours; Lane (4): uninduced BL21 (DE3) cells containing pHA4 (from top to the bottom) after 4.5.6 hours of induction the last dot is related to the standard interferon alpha2 (90ng); lane (5); induced BL21 (DE3) containing pHA4 (from top to the bottom) after 4.5.6 hours of induction and the last dot is related to standard interferon alpha 2 (180 ng).

molecular weight area there were many bands which were related to the *E. coli*'s own proteins. Therefore, it was not possible to observe a distinct band for the interferon protein (data not shown).

#### Dot and Western Blotting

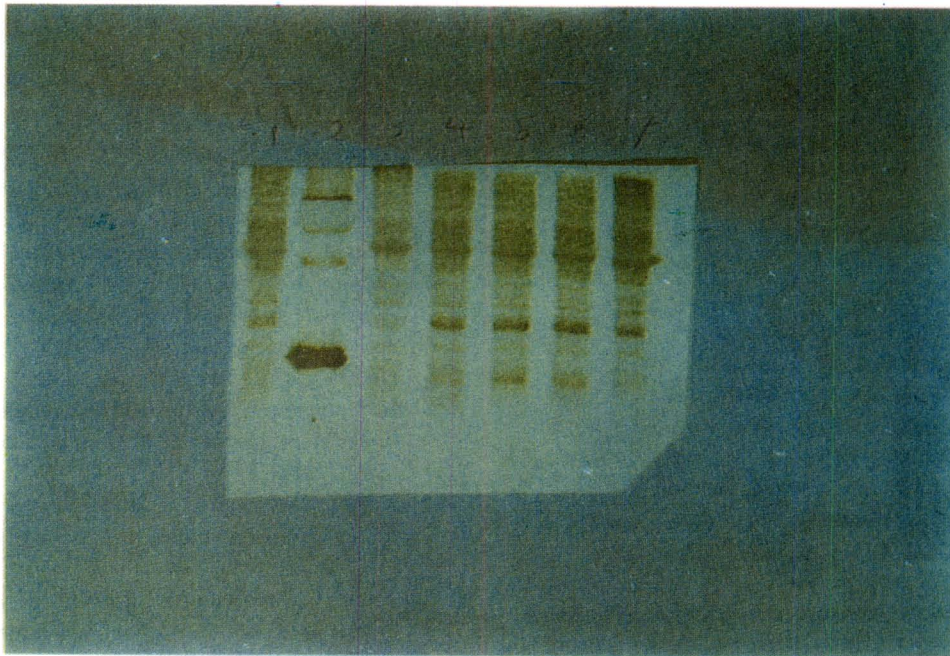
The results of western and dot blotting shown are corresponding to the pET24d(+) system in which the expression of the interferon gene is under the control of T7p (Fig. 5,6).

#### Discussion

Although the human IFN $\alpha$ 2 protein was among the first proteins produced by recombinant technology, its production as a processed and active form in *E. Coli* has been quite a "hit or miss" approach. Expression of this protein in the form of inclusion bodies (cytoplasmic and reduced form) was not considered because as an inclusion body it lacks any biological activity. Since the native signal peptide of this protein is not processed by *E. coli*, some attempts [15-16] have been made to replace its native signal peptide by a synthetic signal peptide which can then be efficiently processed by *E. coli* signal

peptidase, in order to be extruded to the periplasmic area (oxidized and active form). In the pALCA IIFN system we could not detect any expression. This might be, to some extent, due to the nature of the promoter which is an *alcAp*, this promoter is widely used in *Aspergillus filamentous* [17,18]. Therefore, *E. coli* may not be a favorite host for expression with this promoter.

T7 promoter is a very efficient promoter for expression of different genes in *E. coli*. It has been introduced by Studier *et al.* [19,20]. The expression of the gene under the control of this promoter is so high that almost all the protein synthesizing machinery of the *E. coli* gets involved. This promoter can only be recognised by an RNA polymerase of the T7 phage which has been integrated to the *int* gene of BL21, downstream of a *lacuv5* promoter. pET24d(+) is a commercially available vector supplied by Novagen [21] which has a T7 promoter and a *lacI* gene (Fig. 7). BL21(DE3) is a strain of *E. coli* in which the gene encoding the T7 RNA polymerase has been integrated to its genome [20,21]. The gene encoding the interferon alpha2 was cloned downstream the T7 promoter of pET24d(+). BL21(DE3) cells which have been transformed by this plasmid are expected to produce



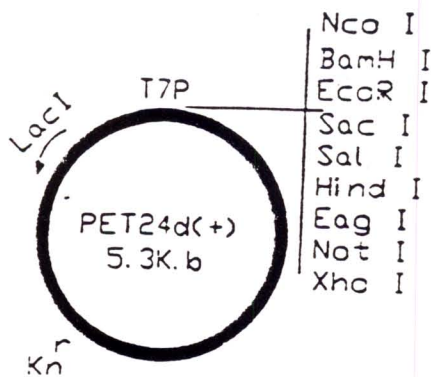
**Figure 6.** Western blot: lane (1). BL21 (DE3) cells induced for 0.5 hour by IPTG; lane (2); standard interferon alpha 2; lane (3): BL21 (DE3) cells containing pET24d(+) as a negative control; lane (4),(5). (6) and (7) BL21 (DE3) cells containing pHA4 induced by IPTG after 1.2.3.4 hours.

large amounts of the gene product. As has been shown, this system works efficiently for the expression of the human interferon alpha2 gene in *E. coli*.

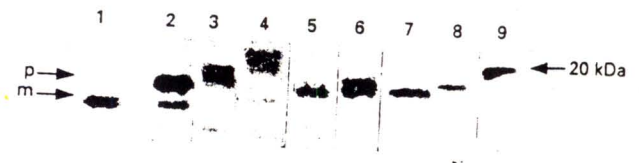
In western blot there appeared a band corresponding to 24 kDa which was about 5 kDa heavier in molecular weight than the standard IFN $\alpha$ 2 protein. Although we have used a synthetic signal peptide which should be processed by *E. coli* signal peptidase, we have not observed a processed product. Considering that the proteins were extracted from whole pellets, this 5 kDa difference is due to the unprocessed synthetic signal peptide. In 1994, Voss *et al.* [16] had introduced a new

signal peptide; STII, Heat-Stable enterotoxins. This signal peptide was recognized by *E. coli* and the periplasmic active product was shown to be identical to human IFN $\alpha$ 2 produced by leukocytes. In the data provided by Voss *et al.*, although they grew the cells in a fermenter, the processed band of the protein appeared quite faint in comparison to the unprocessed band. When they optimized the fermentation conditions they obtained two fold yield of the processed product (Figs. 8,9).

Since Voss *et al.*, have used a large culture in the fermenter (10L); they could observe the processed and unprocessed bands related to the IFN $\alpha$ 2 protein, but in

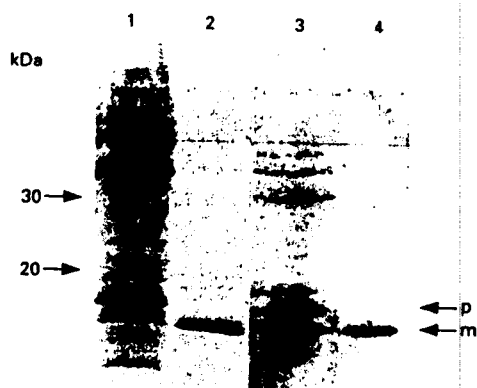


**Figure 7.** Plasmid pET24d(+) (see text for details)



**Figure 8.** Comparison of the processing efficiency of different leader sequences (Western blot) IFN $\alpha$ 2c was expressed with different leader sequences in the *E. coli* strain W3110: lane (1) purified IFN $\alpha$ 2; lane (2) IFN $\alpha$ 2 with STII; lane (4-9) IFN $\alpha$ 2 with unprocessed signal peptide; (M) size marker; (P) unprocessed IFN $\alpha$ -2C.

the present study we have used a small culture (3ml; in a flask) which is far from the ideal fermentation conditions. Therefore, for future work we plan to use the fermenter system and then optimize the fermentation conditions.



**Figure 9.** Western blot of biomass obtained from fermentation at pH 7.0 and pH 6.7 Lane (1) typical fermentation pH 7.0; Lane (3) fermentation at pH 6.7; Lanes (2) and (4) purified IFN- $\alpha$ 2c. Unprocessed precursor; the mature processed IFN- $\alpha$ 2C.

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