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CLONING AND EXPRESSION OF HUMAN IFNα2B GENE IN SACCHAROMYCES CEREVISIAE

P. Mousavi Shafaee[*] and A.-A. Zia'ee

Institute of Biochemistry and Biophysics, University of Tehran, P.O. Box 13145-1384, Tehran, Islamic Republic of Iran

Abstract

Interferon is a protein secreted by eucaryotic cells following stimulation by viruses, bacteria, and many other immunogenes. Recent medical studies indicate that interferons have effective role in the treatment of virus infections, immunodeficiency and certain types of cancer such as hairy cell leukaemia (HCL). The aim of the present study is to apply yeast strain for secreting human IFNα2b following the use of yeast mating α factor signal peptide. On the other hand, cloning of IFNα2b gene without signal peptide and with non homologous signal peptide from Aspergillus was carried out as controls for comparison. First, human IFNα2b gene was amplified using mating α factor signal peptide codons and the first 20 bases of IFNα2b gene as a forward primer. This amplicon (700 bp) was cloned in pYZ4 vector and after using suitable restriction sites, the cleaved fragment was cloned in plasmid pYES2 as expression vector and named pPMSHβ2. Since the original construct of IFNα2b contained one of the Aspergillus signal peptides and flanked with Sal I and Eco RI sites, this gene (900 bp) was isolated and cloned in plasmid pET24d as an intermediate after using suitable sites. This insert was cleaved then cloned in pYES2 vector as pPMSHαB2. Also to construct IFNα2b without signal peptide, primers were designed to exclude the Aspergillus signal peptide from the original IFNα2b gene and the amplicon (500 bp) was cloned in plasmid pET23a then in pUC18 and finally in plasmid pYES2 and named as pPMSH2. Saccharomyces cerevisiae (INVSC1) was transformed with these three mentioned constructs and for interferon gene expression, galactose was used as an inducer. Primary results of western blotting analysis showed that IFNα2b gene with α factor signal peptide was produced inside the pPMSHβ2 transformed yeast cells. The Use of α Factor signal peptide is convenient for expressing the IFNα2b gene in yeast. Studies on growth condition optimization and IFN secretion are under consideration and application.

Keywords: *IFNα2b; Yeast; pYES2; Signal peptide; Plasmid construction*

Introduction

Interferons (IFN) are the body's most rapid defence against viruses, which are secreted by body cells following stimulation by viruses, bacteria, and foreign macromolecules. This secretion leads to the production of other proteins that may regulate virus multiplication, immune response, cell growth, and other cell functions [1].

All the 24 genes of the IFN family gene members

[*] *E-mail: mousavi_p@ibb.ut.ac.ir*

code for proteins with 165-166 amino acid that constitute at least 3 types of interferons; IFN α, IFN β and IFN γ [2].

Recent studies on IFNs indicate their effective role in the treatment of virus infections, immunodeficiency and certain types of cancer namely hairy cell leukaemia (HCL) [3]. Also, IFN shows an anti angiogenic effect in neoplastic diseases and has an anti-retroviral activity in both in vitro and in vivo. It inhibits both, virion production in persistently HIV infected cell lines and de novo infection. The cytopathic effects of HIV in macrophages were also inhibited by all classes of IFN [4].

As a therapeutic agent, IFN α is used for the treatment of AIDS-related Kaposi's sarcoma, where 30-50% response rate has been obtained. Therefore, the use IFN-α in the early stages of HIV infection may delay or stop its progression. In a randomised and controlled trial comparing IFN α with no treatment it has been shown that recombinant IFN α2b therapy delays progression to AIDS in a symptomatics seropositive HIV-infected individuals, with fewer opportunistic infections and other complications [5]. On the other hand, epidemiological and immunological studies demonstrated that the clinical and social improvement of patients with Schizophrenia who received intramuscular recombinant IFNα2b as needed, were greater than those in the control group who received placebo [6]. Both IFNα and β are anti-prolifrative agents and their absence on chromosome 9 in human tumors suggests the important role of these agents in natural tumor suppression [7]. Due to the large demand of applying IFNs, the idea of producing these products has attracted many biotechnologists. Human leukocytes IFN cDNA was the first one that cloned in E. coli using the pBR322 vector [8]. Some of the IFNα produced by cells received an O-link glycosylation but lack the sequence required for N-linked glycosylation. Expression of IFN gene in E. coli with different promoters gave different levels of IFN production. For instance, with β-lactamase promoter; 5×10^3 u/g cell, with Lac-uv5 promoter; 1.8×10^8 u/g cell and with trp synthetase; 1.8×10^7 u/g cell per litre of culture medium are obtained. Also, the yeast Saccharomyces cerevisiae has been widely applied as a host for production of heterologous proteins [9]. In 1988, Gal-1 promoter has been used to express IFN γ gene in yeast and showed 1000-fold growth in galactase as an inducer [10].

Both leader sequences of α-mating factor and Kex2 endoprotease for protein maturation were applied in S. cerevisiae for expressing heterologous recombinant proteins [11]. The native α-factor leader is composed of a prepropeptide followed by a dibasic kex2 protease maturation site and a peptide spacer consisting of 2 or 3 pairs of Glutamic acid or Aspartic acid and Alanin [12,13].

In two comparative studies, a spacer peptide has been reported to improve cleavage at the dibasic site, indicating that for some proteins a spacer peptide may lead to a better kex2 protease processing [14].

One of the gene's regions that seems to be essential for heterologous gene expression in the yeast, is the transcription termination site. A comparison of many yeast 3′-flanking sequences demonstrates a great variation in this sequence, but in most cases, the use of the PGK, and cyc-1 gene termination sequences lead to a higher level of expression [15].

Having all these criteria in mind, the present work has dealt with the concept of expressing IFNα in S. cerevisiae following the inclusion of α-MF. In this paper we present the first part of our studies including the technical manipulation that led to the designed construct. Transformation of S. cerevisiae with this construct gave the signal of interferon expression. For the next study we have planned to focus on the expression of these plasmids and the conditions that optimize the expression of the designed vectors in yeast.

Materials and Methods

Strains, Plasmids and Genes

Strains of Escherichia coli; Top10f`, DH5α, TG1 and NM522 and S. cerevisiae INVSC1 (MAT α his 3 Δ1 leu2 trp1-289 ura 3-52/ MAT α his 3 Δ1 leu2 trp1-289 ura 3-52) which were used in this study were purchased from Stratagene LTD. (Cambridge, UK). Plasmid pYES2 was purchased from Invitrogen Corp (Sant diego, Calif. USA), which is resistant to ampicilin and tetracycline for selection in E. coli. This plasmid also contains a URA3 gene for high-copy-number maintenance and selection in a Ura mutant of S. cerevisiae and a GAL1 promoter sequence. ATCC PALCA1S mother plasmid containing human IFNα2b gene proceeded by Aspergillus signal peptide. pET23a(+), pET24d(+) were purchased from Novagene Corp, pUC18 from HSRRB, Japan and pYZ4 was donated by Dr. F. Mehraein, Pasteur Institute, Tehran, Iran.

Construction of Plasmid Cassets

The original IFNα2b construct (pALCA1SIFN) which includes the Aspergillus Signal peptide was considered as a template for amplifying IFNα2b gene without the mentioned signal peptide. The forward (22 mer) and reverse (25 mer) primers were designed to include the NdeI and EcoRI sites, respectively (Table 1).

Table 1. *Oligonucleotide primers used for the amplification of PCR products for IFN expression*

Primer	***Sequences[1]***
SPMFα	*GGT ACC ATG AGA TTT TTT ACT CCT TCA ATT GCA GTT TTA TTC GCA GCA TCC TCC GCA TTA GCT GCT CCA .TGT GAT CTG CCT CAA ACC*
ECO	*GAA TTC CTC TGT AAG GGA CTA GTG CC*
IFN 22 mer	*TCA TAT GTG TGA TCT GCC TCA A*[*]
IFN 25 mer	*GAA TTC GTT ATC ATT CCT TAC TTC T*[*]
P1	*TAA TAC GAC TCA CTA TAG GG*
P2	*GCG TGA CAT AAC TAA TTA TTA CA*

[1] *KpnI, NdeI, and EcoRI sites are underlined.*
[*] *Dr. F. Mehraein, Pasteur Institute, Tehran, Iran, personal communication.*

Twenty five cycles under conditions of 93°C (denaturation), 62°C (annealing) and 72°C extension were considered for the amplification (Thermocycler Genius). The amplified product was digested with NdeI and EcoRI enzymes and cloned in pET23a, then in pUC18 as intermediate plasmid after selecting suitable restriction sites; XbaI and EcoRI. Finally this fragment was cloned in pYES2 choosing HindIII, EcoRI restriction sites (Figs. 1 and 2).

In the present study, our main strategy was to stimulate the yeast to secret interferon in its growing medium. Briefly, the signal peptide of α-factor of S. cerevisiae was designed to be added to the 5' end of the above mentioned 22 mer forward primer. The nucleotide sequence of the forward primer (SPMFα) and the reverse primer (ECO) are shown in Table 1.

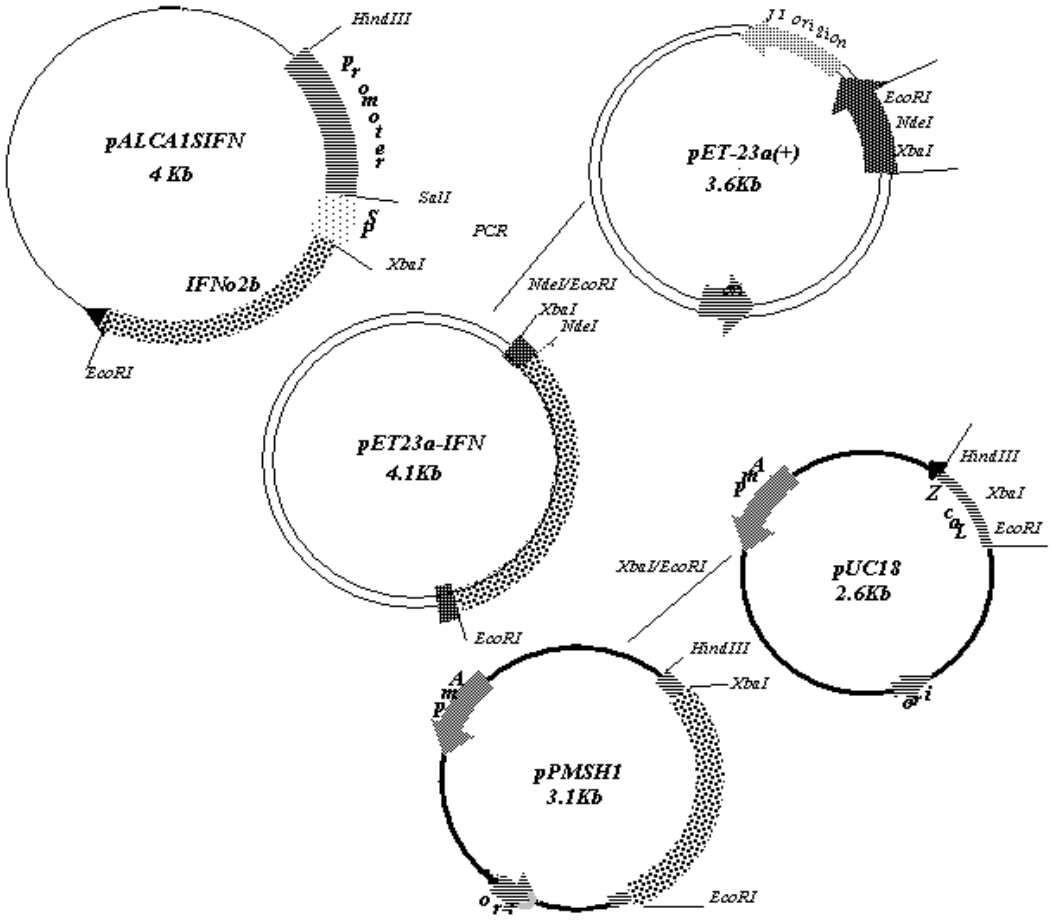

Figure 1. *Schematic representation of the pPMSH1 plasmid construct. To construct IFNα2b without signal peptide, primers were used to exclude the Aspergillus signal peptide from the original IFNα2b construct. The obtained amplicon (500 bp) was cloned in plasmid pET23a then in pUC18. Saccharomyces cerevisiae (INVSC1) was transformed with these three mentioned constructs and galactose was used as an inducer for interferon gene expression.*

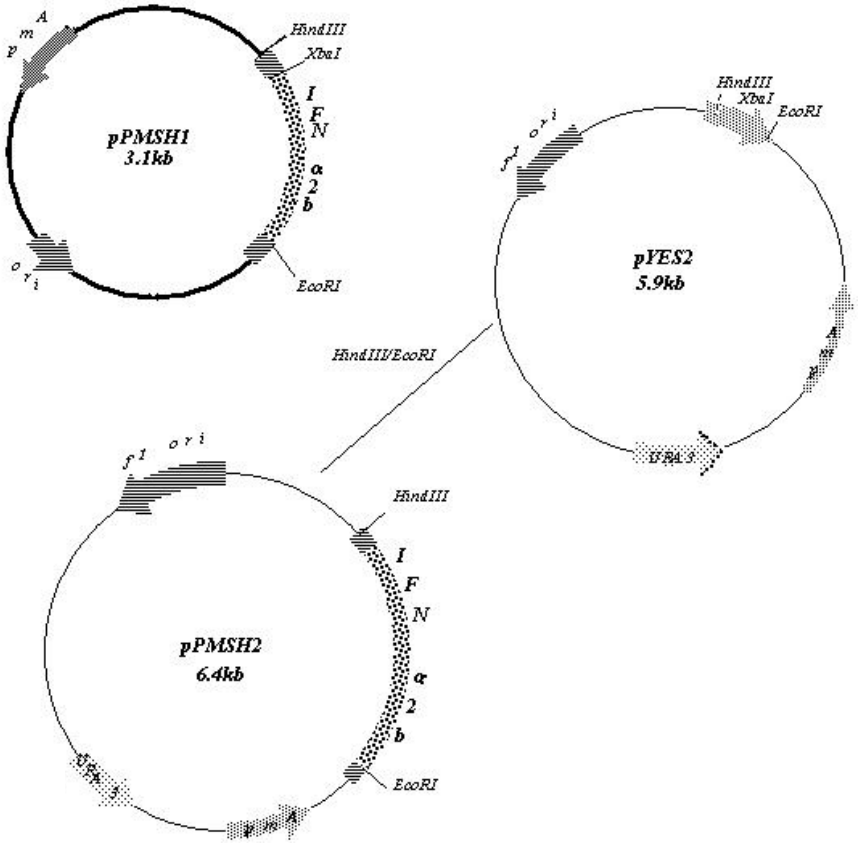

Figure 2. *Schematic representation of the pPMSH2 plasmid construct. pPMSH1 cleaved with HindIII and EcoRI and the fragment was inserted in plasmid pYES2 (pPMSH2).*

The pALCA1S plasmid containing IFNα2b was used as a template and the signal peptide was fused to IFNα2b gene during the PCR amplification. The PCR conditions were 30 cycles of 1 min at 93°C for denaturation, 1 min at 71°C for annealing and 1 min at 72°C for extention. PCR product was precipitated with ethanol, then cloned in EcoRV digested pYZ4. Suitable restriction sites were selected and the cleaved fragment was cloned in pYES2 vector (Fig. 3).

Since the original pALCA1S IFN plasmid contained the signal peptide sequence from Aspergillus, this sequence and the IFNα2b was isolated following SalI/EcoRI digestion and cloned in pET24d(+) as a control containing nonhomologous signal peptide sequence. DH5α strain of E. coli was transformed with this plasmid and plasmid extraction was followed. The extracted plasmid was digested with HindIII/EcoRI restriction enzymes. DNA fragment was purified, concentrated, then ligated to pYES2 plasmid using the same sites (Fig. 4).

Transformation of E. coli and Propagation of Plasmids

Ligation reactions for the transformation of E. coli Top10F° were performed as described by Sambrook et al. [16]. Transformants were plated out on Luria-Bertani plates containing the required antibiotic. Colonies were picked up and grown overnight in Luria-Bertani medium containing the same antibiotic as before. Plasmids were purified as described (small-scale preparations of plasmid DNA) by Sambrook et al. [16].

Transformation of S. cerevisiae

The transformation procedure was carried out with slight modification of the previously reported method [17]. Briefly, 10 ml of YPD medium was inoculated with a colony of INVSC1 and shaked overnight in 30°C. The OD 600 of culture was determined, diluted to an OD 600 of 0.4 in 50 ml of YPD medium and grown for

further 2-4 h. Afterwards the cells were sedimented at

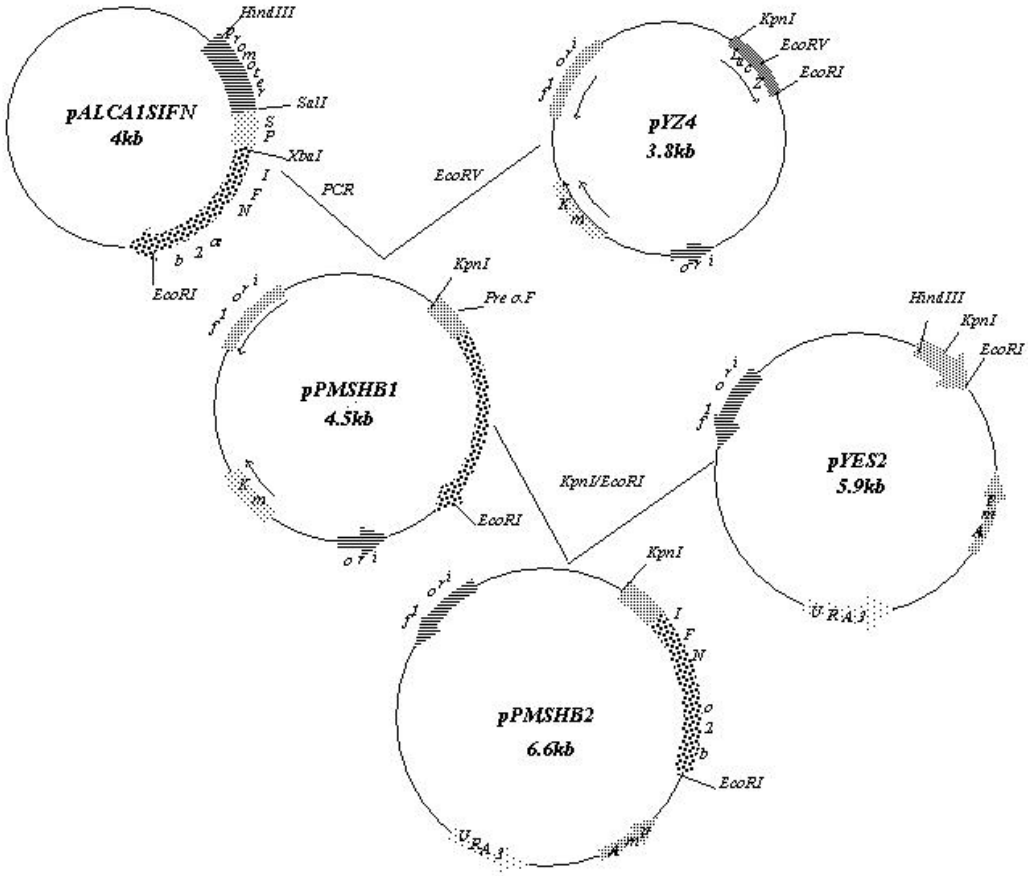

Figure 3. *Schematic representation of the pPMSHβ2 plasmid construct. Human IFNα2b gene was amplified by using mating α factor signal peptide codons added to the 5′ of the 20 bases of IFNα2b gene as a forward primer. This amplicon (700 bp) was cloned in pYZ4 vector and after using suitable restriction sites, the cleaved fragment was ligated with the digested pYES2 plasmid as expression vector (pPMSHβ2).*

2500 rpm then resuspended in 40 ml 1× TE. Again the cells were pelleted at 2500 rpm then resuspended in 2 ml of 1×LiAc/0.5×TE. The cells were incubated at room temperature for 10 min. For each transformation, 1 μg plasmid DNA and 100 μ of denatured sheared Salmon sperm DNA were added to 100 μl of yeast suspension and mixed. 700 μl of 1× LiAc/40%PEG/1× TE was added to this mixture, then mixed well and incubated at 30°C for 30 min. Thereafter, 88 μl DMSO was added, mixed well and heat shocked at 42°C for 7 min. The suspention was microcentrifuged for 10 s, the supernatant was removed and the cells were resuspended in 1 ml TE. The yeasts were repelleted then resuspended in 50-100 μl 1× TE and plated on a SC-U medium selective plate containing all nucleotides except Uracil. Under these conditions only the recombinant cells that gained the pYES2 plasmid grew up in this medium [17].

Protein Synthesis Induction

A single colony of transformed INVSC1 was inoculated into 15 ml of SC-U medium containing 2% glucose and grown overnight at 30°C with shaking at 225 rpm. Later, the OD 600 of the culture was determined and the volume of culture necessary for obtaining an OD 600 of 0.4 in 50 ml of induction medium (Gal 2%, Glc 1%, YNB 1× and Aminoacid mix 0.1 g/lit) was worked out. This volume was removed and the cells were pelleted. The pellet was resuspended in 1-2 ml induction medium (SC-U medium plus galactose) and left at 30°C with shaking for intervals of 0, 2, 4, 6, 8, and 10 h. For each time point, 5 ml of culture was removed from the flask and pelleted, then cells resuspended in 500 μl steril water. The pellets were

stored at −80°C until use [18].

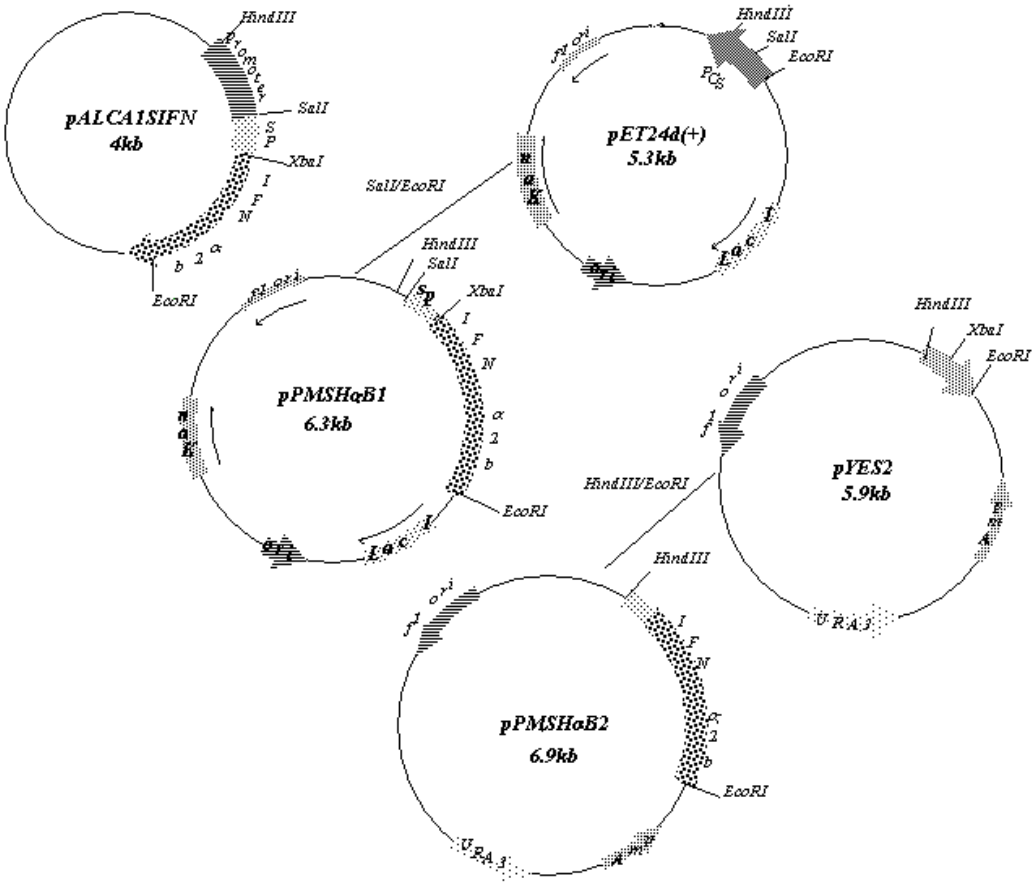

Figure 4. *Schematic representation of the pPMSHαB2 plasmid construct. Since the original construct of IFNα2b contained one of the Aspergillus signal peptides that flanked with Sal I/Eco RI sites, this fragment (900 bp) was enzymatically cleaved then cloned in plasmid pET24d after using suitable sites. Thereafter, this fragment was released and cloned in pYES2 vector (pPMSHαB2).*

Protein Extraction and Quantification

The frozen and fresh cells were resuspended in 500 µl of breaking buffer (50 mM sodium phosphate, pH:7.4, 1 mM EDTA, 5% glycerol, 1 mM phenylmethyl sulfonyl fluride) and centrifuged at 1500×g for 5 min at 4°C to harvest the cells. Supernatant was removed and the cells were resuspended in a volume of breaking buffer to obtain an OD 500 of 50-100. An equal volume of acid-washed glass beads was added and vortexed for 30 min to lyse the cells. The lysate was centrifuged for 10 min at maximum speed [17]. Supernatant was removed and transferred to a fresh tube and assayed for protein concentration following the application of Bradford assay and the use of BSA as a standard [19]. SDS-PAGE sample buffer was added to the lysate to a final concentration of 1× and then the mixture was boiled for 5 min. Afterwards 20 µg of the lysate was loaded on an SDS-PAGE gel and electrophoresed.

SDS-PAGE for Western Blot Analysis

Yeast clones were grown in SC-U medium to an OD 600 of 2 and grown further for intervals of 0, 2, 4, 6, and 10 h after the addition of galactose. Then, cells were removed by centrifugation and the supernatants concentrated by lyophilization. The lyophilysed yeasts were dissolved in 2× loading buffer (1:1) then subjected to SDS-PAGE (12.5% w/v) gel electrophoresis as described by Laemlli [20]. The electrophoresed proteins were transferred to nitrocellulose membrane at 4°C in 48 mM Tris-HCl (pH:8.3), 20% v/v methanol, 0.03% SDS and 39 mM glycin, using Trans Blot Cell. The transfer was done overnight at a constant 200 mA. The membrane was treated in the presence of mouse monoclonal Antibody against human IFNα2b protein and then with goat Anti-antibody that conjugated to

Alkalin phosphate [21,22].

Southern Blot Technique

To approve the insertion and maintenance of IFNα2b gene after several cloning in bacteria and yeast, the final constructs were used for Southern blotting and the application of labeled PCR product of PALCA1SIFN plasmid containing the cDNA of human IFNα2b gene as a probe. The probe labelling and membrane blotting procedures were done according to Amersham ECL Direct Nucleic Acid Labelling end Detection Systems, Catalog No. RPN 3000/3001/3005.

Results

The aimed strategy of the present work is to use yeast for interferon production in order to eliminate the toxic products that usually observed when E. coli is applied as a host for foreign gene expression. This strategy is not only simplifying the extraction procedures but also is a cost effective factor as well. This can be achieved by extracting the products directly from yeast or from their growing culture. Therefore, we first planned to construct a plasmid that its expression leads at least to the excretion IFNα2b in the growing medium, then the optimization of growth conditions was our second trial.

The first construct (pPMSHβ1) was obtained following the cloning of PCR product (using SPMFα and ECO as forward and reverse primers, respectively) in pYZ4 plasmid digested with EcoRV (Table. 1). DNA extraction from suspected clones containing the cloned amplicon was performed and the existence of insert with its proper size (700 bp) was tested by enzymatic digestion (Fig. 5). Clones with the right fragment of IFNα2b and the fused signal peptide sequence were first determined. Subsequently, such fragment was released from pPMSHβ1 by digestion with KpnI and EcoRI. This insert was cloned in pYES2 expression vector using the same restriction sites and pPMSHβ2 construct was achieved. To confirm the presence of this insert with its proper size (800 bp), PCR technique was applied using P1 and P2 primers (Fig. 6). The additional 100 bp in PCR product is due to the addition of about 100 bp of the forward primer containing the signal peptide sequence. Also two other pPMSH2 (without signal peptide) and pPMSHαB2 (with Aspergillus signal peptide) plasmids were constructed as mentioned before in materials and methods section.

To confirm the fragment size, direction and insertion of pPMSHβ2 construct, restriction mapping analysis was done with Eco0109I enzyme that produced DNA fragments of 2760, 2526, 800 and 480 base pairs. These fragments were an indication of the correct direction and size of the insertion (Fig. 7 A and B). Digestion of pYES2 plasmid as a control with the enzyme, produced fragments of 2760, 2567 and 480 base pairs. Comparison of the fragments in agarose gel showed that only the construct in lane 2 was with the correct direction and size (Fig. 7). Other constructs (lanes 1 and 3) were pYES2 without inserts.

In the case of pPMSHαB2, restriction mapping with Eco0109I produced fragments with 2760, 2560, 986 and 480 base pairs indicating that direction and size of the

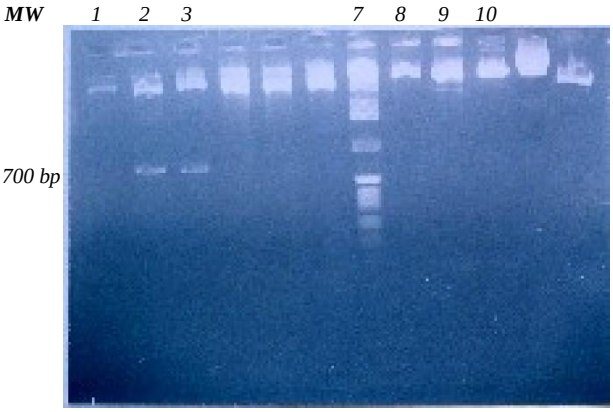

Figure 5. *The product of enzymatic digestion of pPMSHβ1 and pYES2 with KpnI and EcoRI (700 bp).*
Well 1: pPMSHβ1 was cleaved with EcoRI; Well 2: pPMSHβ1 was cleaved with EcoRI and HindIII; Well 3: pPMSHβ1 was cleaved with EcoRI and KpnI; Well 7: DNA molecular size marker X(Boehringer mannaheim); Well 8: pYES2 was cleaved with EcoRI; Well 9: pYES2 was cleaved with EcoRI and HindIII; Well 10: pYES2 was cleaved with EcoRI and KpnI.

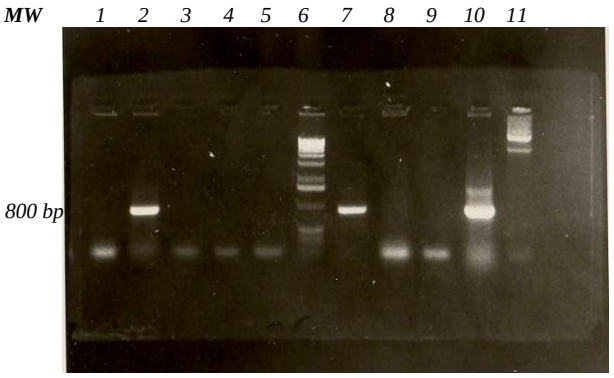

Figure 6. *The PCR products of pPMSHβ2 clones with P1 and P2 primers after electrophoresis on 0.8% agarose gel (800 bp).*
Well 1-5, 7, 8, 10: PCR products of suspected colony harboring pPMSHβ2 construct; Well 6: DNA molecular size marker X (Boehringer, Mannaheim); Well 9: negative control (without template); Well 11: negative control (with pYES2 as a template).

insertion were correct. pYES2 plasmid fragments under the same condition of digestion were 2760, 2567 and 480 base pairs. Therefore, constructs belong to lanes 4 and 10 were the correct ones (Fig. 8).

Southern blot analysis also confirmed the insertion of IFNα2b in pYES2 vector. Supercoil, relaxed and linear forms of the constructs were shown (Fig. 9).

To make sure that the whole cloning manipulation process did not affect the sequence (no mutation induced) of the IFNα2b final construct (pPMSHβ2), sequencing of pPMSHβ2 was carried out applying chain termination automated method (data was not shown). Fortunately, no mutation was observed and these plasmids were used for the transformation of yeast cells. A good number of transformed yeast colonies containing pPMSHβ2 construct were obtained and tested for IFNα2b production before and after galactose induction following SDS-PAGE and Western blot analysis.

The pattern of protein band of lanes loaded with these two samples showed that a protein band corresponding to molecular mass of 19 kD appeared after 6 h galactose induction. However, Western blot analysis showed that IFNα2b was produced in the yeast cell after 4 and 6 h of galactose induction (Fig. 10). Meanwhile, no such detection was observed when culture medium was considered for protein isolation and western blot analysis.

Discussion

Interferons are a family of polypeptides secreted by a large variety of eucaryotic systems upon exposure to several mitogenes and viruses. Recently, responsive effects of recombinant interferons have been reported in the treatment of specific virus infection, immuno-deficiency and certain types of cancer [1].

The production of recombinant IFN has been examined in different expression systems. At the present time, bacterial expression system is applied for mass production of the IFN, but some disadvantages have been observed in the treatment of patients with this recombinant product [23]. For example, E. coli produces endogenous byproducts which are toxic for human beings and even during the purification process of IFN, it was difficult to exclude these toxic impurities from this polypeptide, while no such toxic materials are produced by yeast [24].

On the other hand, IFN produced by E. coli is not effective as a therapeutic agent in all patients with the above mentioned types of diseases. In other words, a group of patients do not respond to this agent because the modification and folding of this recombinant IFN is

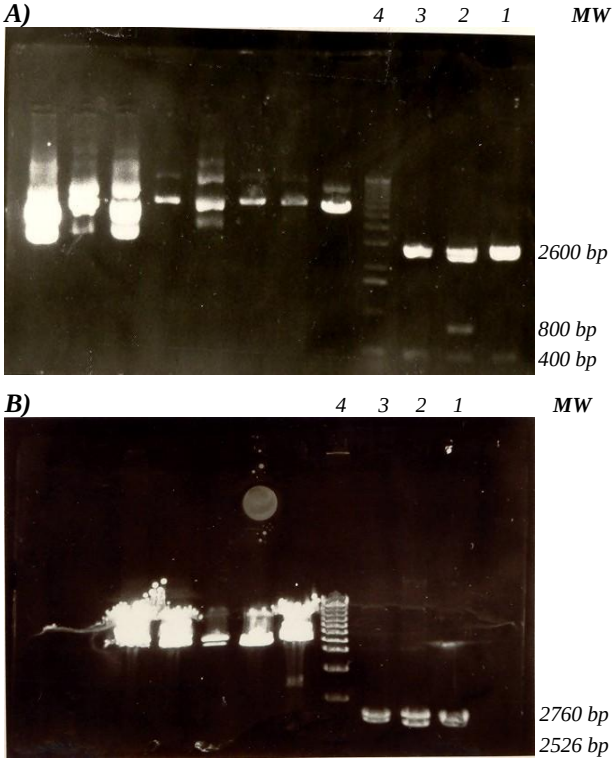

Figure 7. *(A and B). Restriction map of pPMSHβ2 construct. Restriction mapping analysis was done with Eco0109I enzyme which produces DNA fragments of 2760, 2526, 800 and 480 base pairs. pYES2 plasmid with Eco0109I enzyme produces fragments 2760, 2567 and 480 base pairs. A and B after 1 and further 3 h running, respectively.*
Well 1: pPMSHβ2 was cleaved with Eco0109I; Well 2: pPMSHβ2 was cleaved with Eco0109I; Well 3: pPMSHβ2 was cleaved with Eco0109I; Well 4: DNA molecular size marker X (Boehringer, Mannaheim).

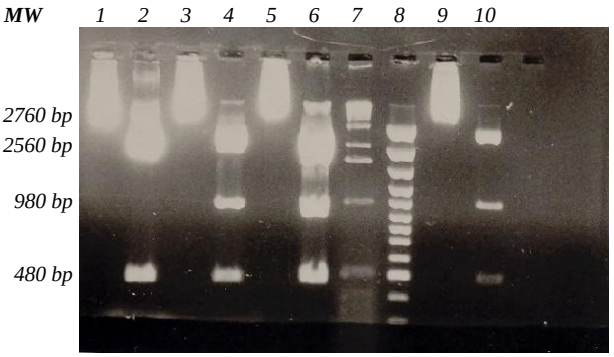

Figure 8. *Restriction mapping of pPMSHαB2 construct. Digestion of pPMSHαB2 vector with Eco0109I produces fragments of 2760, 2560, 986 and 480 base pairs. Digestion of pYES2 plasmid under the same condition gave fragments of 2760, 2567 and 480 base pairs.*
Well 1, 3, 5, 9: Supercoils of pPMSHαB2; Well 2, 4, 6, 10: pPMSHαB2 digested with Eco0109I; Well 7: DNA molecular size marker X (Boehringer, Mannaheim); Well 8: DNA molecular size marker 100 bp (Boehringer, Mannaheim).

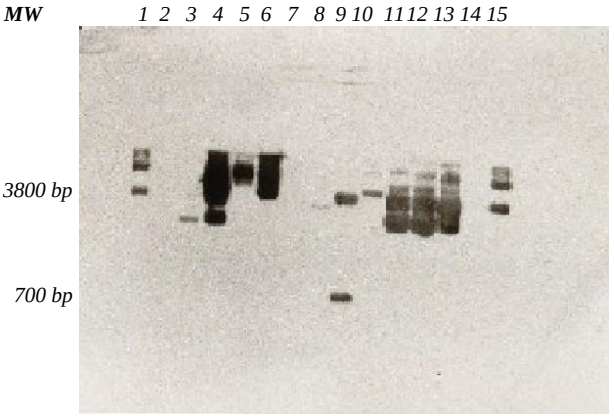

Figure 9. *Southern blot analysis of all constructs. labeled PCR product of IFNα2b from PALCA1SIFN plasmid was used as a DNA probe.*
Well 1, 15: pALCA1SIFN as a positive control; Well 2, 14: pYZ4 and pUC18 as a negative control; Well 3: pPMSH1 clone; Well 4: pPMSH2 clone; Well 5: pPMSH2 clone; Well 6: pPMSH2 clone; Well 7: DNA molecular size marker λ (digested with HindIII); Well 8: PPMSHβ1 clone; Well 9: PPMSHβ1 digested with KpnI and EcoRI; Well 10: pPMSHβ2 clone; Well 13: pPMSHαB1 clone.

somehow different from that produced by certain cells in human body. Therefore, yeast as a eukaryotic system would be a suitable choice for compensating what bacterial system is unable to prepare [25].

Yeast such as S. cerevisiae have widely been applied as a host for production of heterologous proteins. Sc α-factor leader sequence has usually been used for facilitating protein secretion and the endoprotease Kex2p has also been recommended for the presence of protein maturation [13].

In order to obtain efficient expression yeast, we considered the high-copy number 2-μm plasmid-based shuttle vector, pYES2, to achieve higher yields. Additionally, the yeast derived GAL1 promoter in this plasmid can be induced up to 1000 fold when galactose is used as an inducer [18].

Since the leader peptide may be important for obtaining correct folding and posttranslational modifications during secretion of fusion protein, we used α-factor leader sequence that was previously reported of having high efficiency in this regard [13]. As was indicated in the introduction, there are several strategies and efforts in connection with the design of proper and suitable aminoacid residues in the signal peptide sequence that have been considered in order to facilitate the secretion of cloned product.

To improve Kex2p processing and higher secretion of the α-factor leader/IFNα2b fusion, a prolin residue codon was inserted before the initiation codon to disrupt

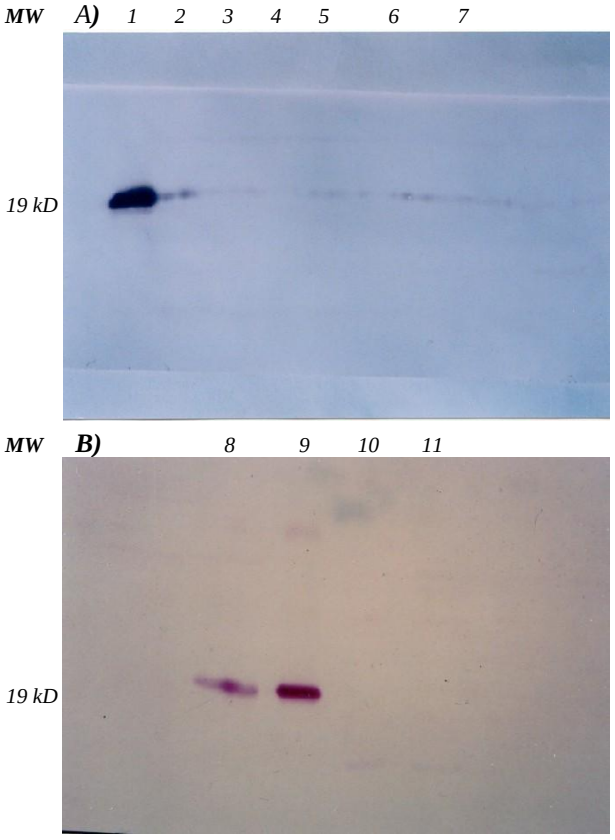

Figure 10. *(A and B). Western blot analysis of protein extract of pPMSHβ2 clone after 2, 4, 6 h of induction with galactose. Protein extract of the transformed yeast harboring pYES2 was used as a negative control.*
Well 1, 9: The standard IFNα2b protein; Well 2, 3, 8: Protein extract from pPMSHβ2 clone after 6 h galactose induction; Well 4, 5: Protein extract from pPMSHβ2 clone after 4 h galactose induction; Well 6, 7: Protein extract from pPMSHβ2 clone after 2 h galactose induction; Well 10, 11: Protein extract from pYES2 clone after 2 h as a negative control.

α helix conformation in the Kex2p cleaving site and facilitating its exposure to the signal peptidase enzyme[26].

Our western blot results (Fig. 10) confirmed the presence of Interferon α2b only in yeast cell extract and not in the growing medium. Detecting no IFN in the growing medium as a secreted product may be due to the presence of proteases in the medium cause degradate of a significant fraction of the secreted Interferon during growing and even storage period before carrying out the biochemical analysis.

To prevent such product degradation and digestion it is recommended to add sufficient protease inhibitor in the growing medium to suppress of more than eight putative yeast's proteases. Therefore, we suggest the optimization of growth conditions in order to decrease protease secretion and function in the growing media.

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