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Production of Human Interferon β in *Saccharomyces cerevisiae* Yeast Leads to a Decrease in Cytochrome c Oxidase Activity

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Abstract–The wild type yeast cells containing a multi-copy plasmid with human β-interferon gene under the control of the yeast PHO5 gene promoter exhibited a low growth rate, and the plasmid was eliminated in about 25% of these cells after 24 h of growth on nonselective medium. Pho80p and Pho85p genes are negative regulators of PHO5 gene expression. The transformants pho85/hIFN-β were practically nonviable and lost the plasmid. In mammalian cells, β-interferon is known to be responsible for abnormal mitochondrial function. To elucidate whether the intracellular production of β-interferon affect mitochondrial function in yeast, cytochrome oxidase activity was measured in mitochondrial extracts obtained from the strains pho80, pho85, wild type strain, and the strain wild type/hIFN-β. In mutant strain pho85 and the strain producer of human β-interferon, cytochrome oxidase activity proved to be 30% reduced. Based on these data, β-interferon is assumed to be toxic for the yeast cells because of abnormal mitochondrial function.

Key words: human β-interferon, loss of plasmid, MT function, viability, yeast recombinant cells.

INTRODUCTION

The endogenous immunomodulating agents not only regulate immunity, but represent the key factors which initiate inflammatory reaction, modify the functional states of nervous and endocrine systems, and eliminate tumor cells[2]. The nonpathogenic microorganisms, saccharomycete yeast that contain no toxic and pyrogenic factors can be used as producers of the recombinant human proteins applicable in clinical practice. However, overproduction of the heterologous protein may cause stress response of a cell, irreversible binding and inactivation of proteins involved in maturation of the heterologous protein, posttranslational modification and transport of the latter, and low viability of the strain-producer.

We have previously developed a strain synthesizing human β-interferon, VKPM Y-2286, in which the p-IFN gene is under the control of promoter for the PHO5 gene encoding the repressible acid phosphatase 2 [1]. This promoter is often used in biotechnology [2–5]. Both positive and negative protein regulators control PHO5-

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[2] Abbreviations: AP2, acid phosphatase 2, AT, antibodies; BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine tetrahydroxychloride; hIFN-β, human β interferon; MT, mitochondria; PMSF, phenyl methyl sulfonyl chloride; SDS, sodium dodecylsulfate.

gene expression which is inhibited by inorganic phosphate. Three positive transcription regulators are involved in AP2 biosynthesis. These are the proteins Pho4 responsible for basal level of gene expression, Pho2 enhancing the effect of Pho4, and Pho81 that mediates signaling on the content of inorganic phosphate in the medium and inhibits the effect of negative regulators. The negative regulators are represented by Pho85p cyclin-dependent proteinkinase and an associated cyclin Pho80p; both of them are involved in the control of Pho4 protein location and inorganic phosphate-dependent binding to PHO5 gene promoter [6]. Cofunctioning of the regulatory proteins ensures AP2 synthesis and that of the heterologous products in the medium with low inorganic phosphate concentrations.

Like some other cytokines, β-interferon accumulates in yeast cells in the aggregated state in the form of inclusion bodies [1]. The level of β-interferon production by the strain producer VKPM Y-2286 is an order of magnitude lower than that of other protein cytokines [1, 3, 5]. Human interferon might be involved in yeast cell metabolism or interfere with important cell functions.

In this study we aimed at determining the effect of hIFN on the mitochondrial function of the yeast cells and on cytochrome oxidase activity, in particular.

EXPERIMENTAL

Bacterial strains, plasmids, media. This study used plasmid pHBI [1] carrying hIFN-β gene under the control of promoter and terminator of the structural PHO5 gene that encodes repressible acid phosphatase. This plasmid has been constructed on the basis of pJDB207 plasmid carrying a selective marker, the truncated yeast LEU2 gene responsible for high number of plasmid copies in a cell. In addition, plasmid pRS426 [7] carrying yeast URA3 gene as a selective marker was used.

Yeast strains from the collection of the Laboratory of Biochemical gGenetics [8] served as recipient ones. Their genotypes are shown in Table 1. The strain CSG 55 kindly provided by Dr. D. S. Gross (Louisiana University, United States) bears the reporter gene lacZ under the control of HSP82 gene promoter, which is integrated in HSP82 locus of chromosome 16 [9]. Strain genotypes were designated using the following symbols: MATa and MATα, alleles of the copulation type loci; his3-11,15, leu2-3,112, and ura3-52, mutations leading to histidine, leucine, and uracyl demands, respectively; smol-1, a mutation that modifies the colony shape; cyh2, a recessive mutation that confers resistance to cycloheximide; pho3, a mutation in the structural AP1 gene leading to enzyme inactivation; pho80 and pho85, mutations in the regulatory genes leading to constitutive synthesis of the repressible AP2.

Table 1. Genotypes of yeast strains used in this study

Strain	Genotype
1-GRF18	MAT α his3-ll,15 leu2-3, 112 pho3-l
c21-1-GRF18	MAT α his3-ll,15 leu2-3, 112 pho3-l pho85-21
c10-1-GRF18	MAT α his3-ll,15 leu2-3, 112 pho3-l pho80-10
CSG55	MAT α ura3-52 leu2-3, 112 smol-l cyh2 LEU2::HSP82-lacZ

Yeast were grown on minimal medium containing per 1l of distilled water the following compounds: KH_2PO4, 0.85 g; $K_2HPO_4 \cdot 3H_2O$, 0.20 g; $MgSO_4 \cdot 7H_2O$, 1.03 g; $CaC1_2$, 0.10 g; NaCl, 0.10 g; $(NH_4)_2SO_4$, 5.0 g; thiamin, 200 μg; β-alanine, 500 μg; biotin, 2 μg; glucose, 20 g. The complete media, PEP and PEPPO, which contained peptone, 20 g and glucose, 20g per 1l of distilled water, were also used. The

PEPPO medium contained also 1 g of KH_2PO_4. Transformants were identified on the selective medium (the minimal medium that additionally contained histidin, 20 mg/l).

Plasmid DNA was amplified using the strain *E. coli* DH5α (F'/endAl hsdR17 (r_k^- m_k^+) supE44 thi-1 recAl gyrA (Nal^r) relAl Δ(lacZYA-argF)U169 deoR (ϕ80dlacΔ(lacZ)M15).

Bacteria were grown on LB medium. Transformants were selecteded on LB medium containing ampicillin (50 mg/l). The medium in Petri dishes contained agar (20 g per 1l). Yeast was grown at 30°; bacteria, at 37°.

DNA isolation. Plasmid DNA was isolated from *E. coli* by a conventional method [10]. Chromosome DNA of *S. cerevisiae* was isolated according to Fujimura method [11].

Transformation. Ca^{2+}-dependent transformation of bacteria was performed using techniques described by Sambrook et al. [12]. Yeast transformation was conducted using a technique described in [13].

Isolation and analysis of recombinant β-interferon. The strain-producer was grown on the phosphate-containing selective medium until the optical density of 1.0 at 550 nm. Afterwards, the cells were transferred into PEP medium and grown additionally for 24 h. The lack of inorganic phosphate in this medium leads to derepression of PHO5 gene promoter and induction of the recombinant protein synthesis. Then the cells were harvested by centrifugation at 5000 rpm, suspended in a buffer containing 50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 1 M NaCl and disrupted in a Braun desintegrator. After PMSF addition to crude cell extract to a final concentration of 2 mM, the suspension was centrifuged at 12 000 rpm for 10 min. Disintegration results in dissolving of most soluble cell proteins, whereas the aggregated IFN-β remains in precipitate. The latter was treated with 4 M urea for 30 min to remove contaminating proteins and centrifuged at 14 000 rpm for 15 min. After this procedure, the precipitate was treated with sodium dodecylsulfate (5% final concentration) to dissolve IFN-β. Protein electrophoresis was conducted in 17% separating and 6% concentrating PAG in the presence of SDS in Tris-glycine buffer according to the Laemmli method [14]. In control, the proteins from the original strain 1-GRF18 were used. Marker polypeptides (Sigma, United States) served as reference ones to estimate the protein molecular masses.

Hybridization of the recombinant protein with antibodies to human β-interferon [15]. After electrophoresis in SDS-PAG, the gel was placed successively in the buffer for renaturation (4 M urea, 10 mM Tris-HCl, 20 mM EDTA) and in buffer for transfer (25 mM Tris-HCl, 190 mM glycine, pH 8.3, 20% methanol) for 15 min into each. Electrotransfer continued for 1.5 h at 30–40 V. The nitrocellulose filters with immobilized proteins were incubated in TBST buffer (10 mM Tris-HCl, pH 8.0; 150 mM NaCl, 0.05% Tween-20, 1% BSA) for 2 h at 37°C and then treated for 2 h at 37°C with 1: 1000 diluted with TBST polyclonal horse antibodies to human IFN-β (Boëhringer, Germany). After washing three times with TBST buffer, the filters were incubated with peroxidase-labeled 1: 7000 diluted rabbit antibodies to horse immunoglobulins (Sigma) for 1 h at 37°C. Afterwards, the filters were washed twice with PBST (58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 0,1% Tween-20) and once with 10 mM Tris-HCl, pH 7,5. Then the filters were developed with 0.02% DAB solution in 10 mM Tris-HCl, pH 7.5, with several drops of H_20_2.

Isolation of mitochondrial fraction from yeast cells [16]. Yeast were grown on PEP medium until mid-logarithmic growth phase. Cells were treated with lyticase (Sigma) to obtain spheroplasts which were washed with 1.2 M sorbitol, suspended in 10 mM Tris-HCl (pH 7.5) containing 0.6 M mannitol and 1 mM PMSF, and disrupted in a homogenizer. The resultant homogenate was centrifuged at 5000 rpm to

precipitate mitochondria from the supernatant at 14 000 rpm for 20 min.

Measurement of cytochrome c oxidase activity [17]. Enzyme activity was measured at 22°C in 1 ml of 40 mM K-phosphate buffer, pH 6.2, which contained 0.1% Triton X-100 and 25 μM of reduced cytochrome c (Sigma). The reaction was triggered by addition of the mitochondrial fraction. Cytochrome c oxidation was determined from the optical density at 550 nm. Specific activity was measured from a decrease in optical density for 1 min per 1 mg of protein.

β-Galactosidase activity was measured according to the Miller's method [18].

RESULTS AND DISCUSSION

The effect of recombinant β-interferon production on viability of the strain--producer VKPM Y-2286.

The strain VKPM Y-2286 is a wild type strain with respect to regulation of AP2 synthesis. It is isogenic to strain 1-GRF18 and carries pho3 mutation. Inorganic phosphate is known to repress transcription of all genes cloned under the control of PHO5 gene promoter. This particular feature of the expression system makes it possible to reduce the metabolic load on yeast cells by the two-stage growing of the strains producers. During the first stage of growth in phosphate-containing selective medium, biomass accumulates. Further cell transferring into PEP medium that contains no inorganic phosphate results in derepression of the recombinant protein synthesis.

Recombinant β-interferon was isolated from the strain-producer and identified using monoclonal antibodies against human β-interferon. β-Interferon production comprised 1 mg/l, which is significantly lower than the production of bovine γ-interferon and human α-interferon in the similar expression system [3, 5]. In addition, the onset of stationary growth phase of the strain-producer occurred earlier as compared to 1-GRF18 strain grown on PEP medium (Fig. 1).

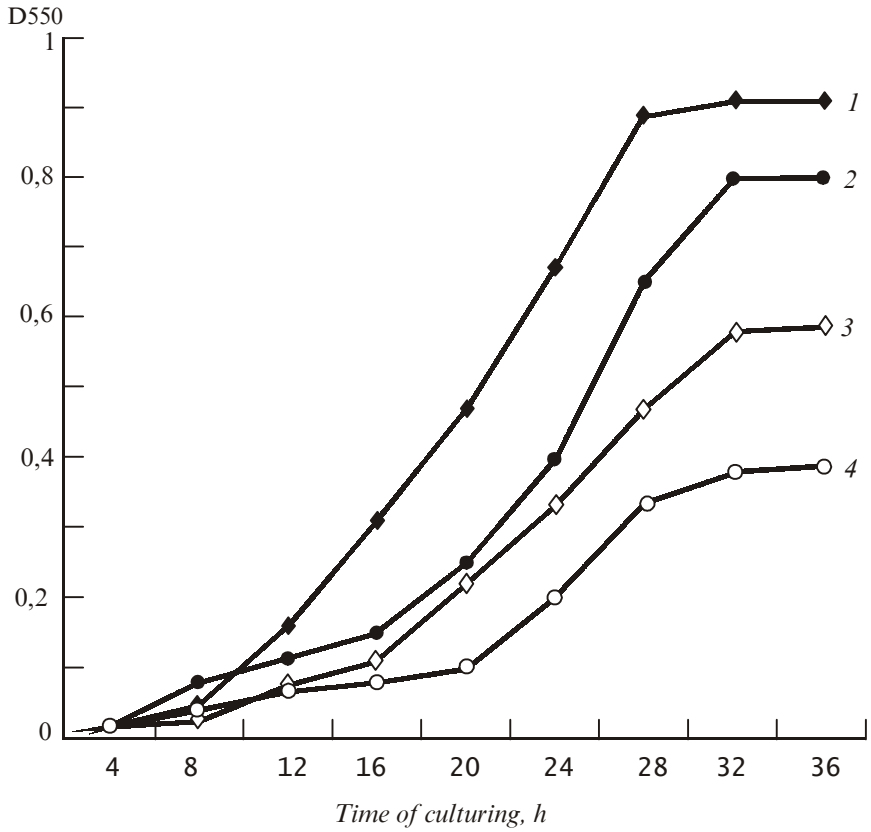

Fig. 1. Growth on PEP medium of various recipient and transformant strains bearing

pHBI plasmid; (*1*), 1-GRF18; (*2*), c10-1-GRF18; (*3*), VKPM Y-2286; (4), c-10-1-GRF18.

The loss of plasmids pHBI and pJDB207 at the frequencies of 25 and 3%, respectively, after 24-h growth under nonselective conditions suggested that the presence of β-interferon gene in a plasmid reduces the mitotic stability of the latter (Table 2). Heterologous protein toxicity for yeast cells seems to account for the high frequency of the recombinant plasmid loss.

Table 2. The frequency of mitotic stability of plasmid pYGIB in strains with different genotypes after 24-h growth under nonselective conditions

Strain	The number of analyzed clones	Percentage of clones after the plasmid loss
c10-l-GRF18 (pHBI)	500	29±2,0
l-GRF18 (pHBI)	500	25±1,8
ВКПМ -Y2286	500	27±2,0
c21-l-GRF18 (pHBI)	500	100

Hydrophoby of β-interferon that leads to high aggregation of this protein and irreversible binding to protein-chaperons might also account for toxicity. For example, overproduction of GroEL/ES chaperon proteins in bacteria is known to enhance 30 times the yield of recombinant γ-interferon [19].

The effect of recombinant β-interferon on the synthesis of Hsp82p heat-shock protein.

As mentioned above, intense synthesis of a heterologous protein may act as a stressful factor inducing the chaperon protein synthesis. One of the chaperon proteins was chosen for the purpose of modeling. It is encoded by HSP82 gene responsible for inactive state of some proteins until they acquire a proper conformation and cellular location [20]. Expression of this chaperon is induced in response to a temperature increase, changes in concentration of free oxygen radicals, and the content of nutrient substance [21].

The effect of β-interferon on induction of Hsp82p synthesis was assessed from β-galactosidase activity in strain CSG 55; its genotype is shown in Table 1. This strain had been transformed with pRS426 plasmid [7] carrying human β-interferon gene and promoter and terminator of PH05 gene. In CSG 55 (pRS426-IFN-β) transformants grown under the conditions similar to those used for VKPM Y-2286 strain (a producer of hIFN-β) β-galactosidase activity was measured. In control, the original strain CSG 55 and CSG 55 (pRS426) transformant were used. The results obtained show that the level of β-galactosidase activity remained unchanged in transformant CSG 55 (pRS426-IFN-β) and hence, β-interferon itself is not a stressful factor stimulating HSP82 gene expression in a cell.

The effect of mutations pho80 and pho85 on viability of strains--producers of β-interferon.

Under the control of PHO5 gene promoter, β-interferon is synthesized only at the

second stage of growth. The negative regulator Pho85p is not only known to control expression of PHO5 gene, but also that of genes encoding many chaperons, in particular Hsp82p, Ssa2p, Ssa4p, etc. [6].

The strain c21-l-GRF18 with mutation in PHO85 gene had been transformed with pHBI plasmid. We used this strain to elucidate whether heterologous protein production increases with increasing the number of chaperon proteins in a cell. Mutation pho85 not only failed to increase the β-interferon yield, but the transformants carrying IFN-β gene proved to be nonviable because of plasmid loss in strain c21-1-GRF18 (pHBI) after 16-h growth under nonselective conditions.

Since the pho85 mutation leads to constitutive (independent of inorganic phosphate concentration) expression of the PHO5 and IFN-β genes during the entire period of growing, we used the isogenic strain cl0-l-GRF18 (pho80) to elucidate whether the constitutive synthesis of β-interferon accounts for low viability of the strain c21-l-GRF18 (pHBI). Like mutations in PHO85 gene, mutations in PHO80 gene lead to constitutive expression of PHO5 gene, but they do not affect the level of HSP82 transcription. After transformation of the strain cl0-l-GRF18 with plasmid pHBI, the frequency of plasmid loss was estimated in transformants (Table 2) and the growth curves were compared. Figure 1 shows that the mutation pho80 causes the earlier onset of stationary phase during the cl0-l-GRF18 (pHBI) strain growth. The c10-1-GRF18 (pHBI) transformants were similar in stability to the VKPM Y-2286 strain.

Thus, the constitutive synthesis of β-interferon has no effect on mitotic plasmid stability in the strain cl0-l-GRF18 and leads to earlier onset of stationary growth phase in a cell population. Although pho85 mutation is characterized by a higher number of chaperon proteins destined to improve β-interferon conformation, the negative effect of heterologous protein increases suggesting that both mutation in PHO85 gene and accumulation of β-interferon may disturb the same vitally important cell functions.

The effect of recombinant β-interferon and mutations in PHO85 gene on cytchrome c oxidase activity.

In mammalian cells, β-interferon causes mitochondrial disfunction and inhibits activity of many mitochondrial enzymes, in particular, that of cytochrome c oxidase [22]. Some mutations in PHO85 gene are cause damages in mitochondrial genome. Mutation pho85-21 does not lead to respiratory incompetence of strain c21-1-GRF18. Mitochondria were isolated from the VKPM Y-2286 strain that synthesizes human β-interferon and from strains c10- and c21-1-GRF18, to determine the activity of cytochrome c oxidase, a marker mitochondrial enzyme, and evaluate the state of mitochondria in these strains. The results are shown in Fig. 2.

It can be seen that mutation in PHO80 gene has no effect on specific activity of cytochrome c oxidase. At the same time, the enzyme activity is 30% reduced in strains VKPM Y-2286 and c21-1-GRF18. The complete lack of a subunit in cytochrome c-oxidase complex is known to lead to 5–15% reduced activity of this enzyme [23]. The cumulative effect of a mutation in PHO85 gene and heterologous β-interferon with respect to at least one of the respiratory chain enzymes accounts probably for low stability of the c21-l-GRF18 (IFN-β) transformants. Expression of a heterologous protein may play an unexpected (unclear *apriori*) role in yeast cell metabolism. Therefore, in a strain-overproducer, low cell viability or a loss of plasmid are expected.

The effect of β-interferon on mammalian cells is mediated by interaction of this protein with correspondent transmembrane receptor, which is followed by signal transduction through the JAK-STAT pathway [24]. In strain VKPM Y-2286, β-interferon is located within a cell. Although the yeast cell contains no receptors for

cytokinines, some yeast proteins are probably capable of receiving signal from intracellular interferon. The extracellular domains of many mammalian receptors, in particular, those of cytokine, insulin, and growth factor receptors, are known to include fibronectin repeats Fn-III [25]. Therefore, we have undertaken the search for the yeast proteins homologous to β-interferon receptors. Analysis of the Proteome database (http://www.proteome.com/datebases/YPD/reports), revealed three proteins containing Fn-III-domains, YKL071Wp, YEL043Wp и Chs5p, in *S. cerevisiae* yeast.

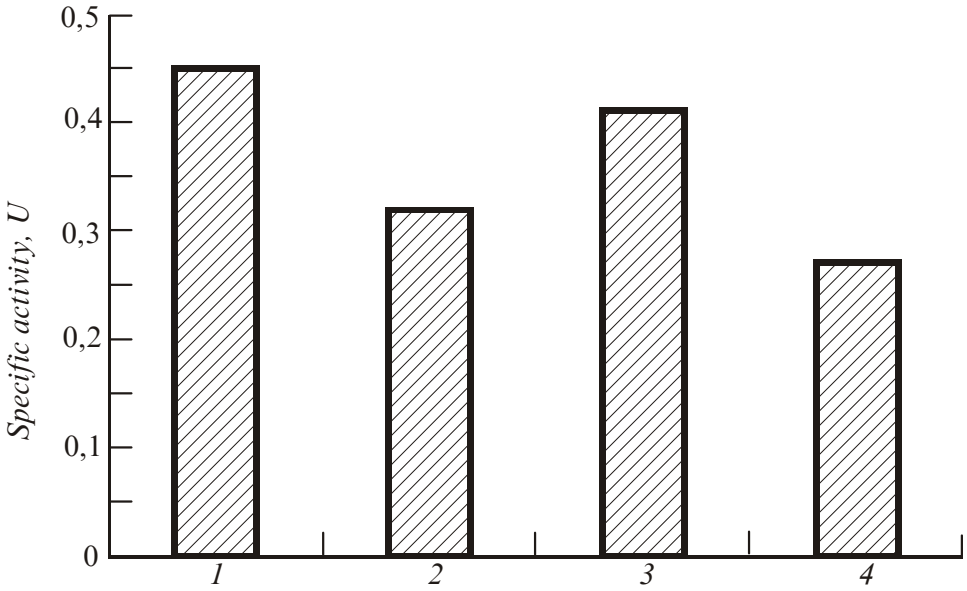

Fig. 2. Specific activity of cytochrom c oxidase in various yeast strains; (*1*), 1-GRF18; (*2*), c-21-1-GRF18; (*3*), c-10-1-GRF18; (*4*), VKPM Y-2286. Average values of three independent measurements of activity are represented (standard error is inferior to 10%).

As shown by studying the correspondent gene expression as dependent on phosphate concentration and the mutations affecting PHO5 gene regulation, the level of YKL071W and YEL043W mRNA is reduced in pho85 deletion mutants [6]. Expression of CHS5 gene was independent of pho85 mutation, but Pho85p regulates an activator of this gene, CSR2. The CHS5 gene encodes a protein required for chitin III synthase activity. The functions of two other genes remain unknown. Thus, expression of all three proteins that are homologous to extracellular domain of β-interferon receptor is inhibited in pho85 mutants. No effect of pho80 mutation on expression of these genes was observed.

Thus, the results obtained in this study suggest that like in mammalian cells, human β-interferon functioning in *S. cerevisiae* yeast cells affect the mitochondrial activity. No effect of the recombinant protein on the level of chaperons was observed in the strain-producer. Unlike the bacteria, in yeast cells, the negative effect of human β-interferon is probably caused by abnormal functioning of a major complex of mitochondrial respiratory chain rather than accumulation of the denatured protein aggregates.

One way to overcome the high toxicity of a heterologous protein in a yeast cell is the use of secreting producers. However, the carbohydrate component of proteins secreted by saccharomycetes contains mannose residues connected by α-1,3-bonds [26], which are strong antigenic determinants and therefore, they are not applicable for medical purposes. In our opinion, the use of another yeast species, for example, *Pichia pastoris,* is the most promising for development producers secreting human β-interferon.

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