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ГЕНЕТИКА

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EXPRESSION OF GENE ENCODING MURINE INTERFERON ALPHA IN *ESCHERICHIA COLI* CELLS

V. Dotsenko[1*], S. Rybalko[2], M. Obolenska[1]

[1]Institute of Molecular Biology and Genetics
150, Zabolotnyi St., Kyiv 143, Ukraine
[2]Institute of Epidemiology and Infectious Diseases of L. Gromashevsky
5, Amosov St., Kyiv 038, Ukraine
e-mail: dotsdon@gmail.com

The goal of this study was to produce recombinant mouse IFNA11 in soluble form escaping the procedure of its refolding and maximally preserving its physiological activity. The mouse *Ifna11* was cloned into the *pET-24a(+)* vector and expressed in *E. coli*. The identity of IFNA was confirmed by antiviral activity test. The optimization of the temperature of cultivation and percentage of glucose in the medium has given a maximal output of soluble IFNA 35 mg per L.

Keywords: murine interferon alpha, protein production, procedure optimization, *E. coli* expression system.

Interferon alpha (IFNA) is the first cytokine that was discovered. Nevertheless it still keeps its own secrets – the peculiarity of its behavior in the absence of pathogen invasion, the mechanisms of its side effects during clinical application etc. Interferons comprise a large group of secreted cytokines implicated in innate and adaptive immunity. The three subgroups of IFNs (IFN I, IFN II and IFN III) are broadly classified and distinguished by their primary amino acid sequence homology, genetic locus, unique receptor complexes through which they transduce their signals and stimulus and cell type producing them [10, 18, 20]. The IFNs type I and type III are produced by nearly all nucleated cells while production of type II is restricted to natural killer, NK and T cells and by antigen presenting cells (monocyte/macrophage, dendritic cells) [20].

The IFNs exhibit a wide range of biological activities: antiviral, antiproliferative and immunomodulatory; they up-regulate MHC I and MHC II, the major histocompatibility complexes; activate immunoproteasomes; induce proapoptotic genes etc. [11, 19, 23]. The IFN type I that exhibits the strongest antiviral and antiproliferative activities has become an indispensible agent for the treatment of multiple diseases like hepatitis C [15, 16], hematological malignancies, chronic leukemia and lymphomas, melanoma [5], multiple sclerosis and many others [3].

The headache, malaise, fever, chills, fatigue, myalgia, nausea, backache and artralgia are typical side effects during IFNA treatment [4, 9]. The mechanisms of these side-effects as well as non-responsiveness to IFNA treatment of about 50% of patients chronically infected with hepatitis C virus are not clear and require additional studies [12].

IFNA interacting with its specific receptor activates the well-defined JAK-STAT signaling pathway with formation of an IFN-stimulated gene factor 3 (ISGF3) complex that moves into the cell nucleus and binds to specific IFN-stimulated response elements (ISRE3) in the promoters of certain genes known as IFN stimulated genes (ISGs). Binding of ISGF3 to these specific response elements regulates their transcription [10].

The bioinformatical genome-wide search for the genes containing ISRE in their promoters conducted in our lab has revealed three previously unknown potential target genes for IFNA that encode the proteins of nervous synapses [1, 2, 29]. We suggest that these genes may be responsible for genesis of side effects during IFNA treatment particularly those effects with deviant function of central nervous system. To check this idea we plan to carry out the *in vivo* and *in vitro* experiments and determine the responsiveness of these genes to IFNA. Large amount of IFNA is required for our experiments. Due to the high cost of IFNA produced by manufacturers the main goal of this study was to clone gene encoding murine IFNA and produce the protein. Here we describe a strategy for cloning and production of murine IFNA11 in *Escherichia coli* expression system.

Experimental procedures

Choice of *Ifna* gene for cloning.

The mouse genome contains 14 *Ifna* genes and three *Ifna* pseudogenes [18]. They are clustered on murine chromosome 4 [13]. The IFNAs of different subtypes consist of 165–208 amino acids, majority of IFNAs except IFNA14, IFNA6T and IFNAA (IFNA3) are glycosylated. The specific activities of subtypes are somewhat different, e.g. IFNA11 reveals the highest antiviral activity in assay with BALB 3T3 cells infected with Mengo virus [18] and that is why the *Ifna11* gene was selected for further cloning procedures.

Isolation and amplification of protein-coding fragment of *Ifna11 gene.*

Genomic DNA was extracted from murine liver using Genomic DNA Purification Kit (Thermo Scientific, USA). The full-size nucleotide sequence of *Ifna11* gene (ND: BC116870) corresponding to its coding part [27] was amplified using Pfu Polymerase (Thermo Scientific, USA) and forward primer with NdeI restriction site and reverse primer with XhoI restriction site. The primers for amplification were as follows: *Ifna11f* - 5'-ATC-**CAT-ATG**-TGC-GAT-CTG-CCT-CAC-ACT-TAT-AA-3' and *Ifna11r* - 5'-ATC-**CTC-GAG**-TCA-GGA-CTC-AAG-CCT-TCT-CTT-C-3'. The codons marked in bold correspond to the position of restriction sites and underlined codons – to the position of start and stop codons. The amplicon was purified using "GeneJET PCR purification kit" (Thermo Scientific, USA).

Cloning of *Ifna11.*

The purified PCR product and *pET-24a(+)* plasmid (Merck Millipore, USA) were consequently digested with NdeI and XhoI restriction endonucleases. Digested vector was gel-purified in order to remove residual supercoiled not digested plasmid using «GeneJET Gel Extraction Kit» (ThermoScientific, USA).

Inserts were cloned between the NdeI and XhoI sites of *pET-24a(+)* downstream of the T7 promoter and under control of the IPTG-inducible lac promoter (Fig. 1, 2).

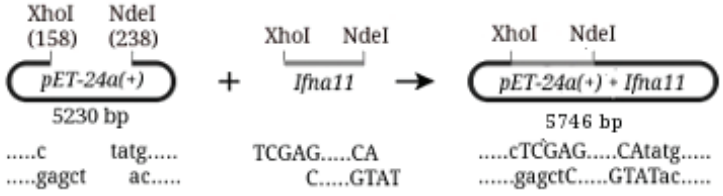

Fig. 1. Design of plasmid *pET-24a(+)* ligation with *Ifna11* insert.

E. coli XL1-Blue *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proA*$^{+}$*B*$^{+}$ *lacI*^{q} Δ*(lacZ)M15 zzf::Tn10 (Tet*^{R}*)*] (Stratagene, USA) cells were used for cloning procedure. Cells were transformed by a standard protocol of the heat shock method. Afterwards the cells were plated on solid LB agar containing 50 μg/ml kanamycin and incubated at 37°C for 16 h.

The germinated colonies were tested for the presence of built-in sequence via PCR using primers listed above. The presence of amplified inserts was tested by agarose gel electrophoresis.

Colonies that gave a positive signal for electrophoresis were cultured at standard conditions and used for plasmid preparation using standard alkaline lysis protocol. Restriction mapping analysis was performed using RsaI restriction enzyme. The nucleotide sequences of the selected clones and their identity with *Ifna11* coding sequence was confirmed on automated DNA sequencer with standard T7 promoter primer (Syntol, Moscow, Russia).

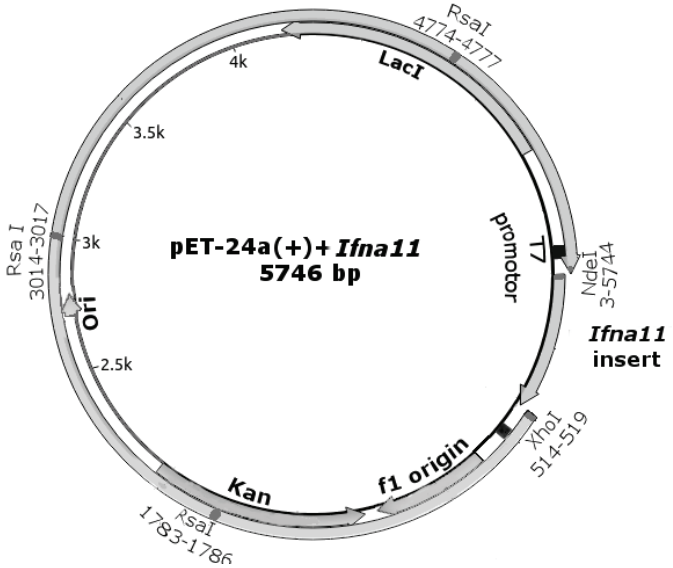

Fig. 2. Scheme of *pET-24a(+) – Ifna11* construction.

Expression of IFNA11 in *E. coli* System

A number of different expression systems are available, and some of them like *Bacillus subtilis*, *Streptomyces lividans*, methylotrophic yeasts *Pichia pastoris,* murine myeloma NS0 Cells, baculovirus-infected insect cells and even plant system *Nicotiana tabaccum* were tested for IFNA production [21, 22]. These systems provide rapid scale-up, high control of the level of expression and post-translational modification. However, *E.coli* expression system still remains the system of choice for the synthesis of heterologous proteins and IFNA*,* particularly. The system is well established and produces proteins at a high level. It provides the fast growth of host cells to high cell density [6, 27]. There are available well elaborated genetically modified strains for safe and high-performance protein production.

Besides its advantages *E. coli* expression system has several disadvantages namely inability to post-translationally modify the proteins and to intensively synthesize eukaryotic proteins due to the presence of rare codons in bacterial genome. The first disadvantage is irrelevant to IFNA production as the activity of IFNA does not depend on its glycosylation [18]. Eight rare codons in *E.coli* and therefore low amounts of corresponding tRNAs are problematic for high expression of protein of interest [17]. Unlike *E. coli* the coding sequence of *Ifna11* contains 5 AGG (Arg), 5 AGA (Arg), 2 CCC (Pro), CGA (Arg), CUA (Leu) and AUA (Ile) codons. To overcome the second problem the co-production of the rare tRNAs are highly required. Rosetta (DE3) host strain meets a requirement as it contains pRARE encoding tRNA genes argU, argW, ileX, glyT, leuW, proL, metT, thrT, tyrU, and thrU. So the rare codons AGG, AGA, AUA, CUA, CCC, and GGA are supplemented.

Rosetta (DE3) cells were transformed with the *pET-24a(+) – Ifna11* plasmid using heat-

shock method and cultivated overnight at 37°C on Luria–Bertani (LB) agar plates containing 50 μg/ml kanamycin and 34 μg/ml chloramphenicol. One colony was inoculated in 5 ml LB medium with antibiotics and 1% glucose and the culture was grown overnight at 250 rpm, 37°C. Afterwards 1.5 ml of this start culture was added to 30 ml (ratio 1:20) of LB medium with antibiotics and further incubated at 37°C to OD_{600} ~ 0.5. Thereafter the cultures were induced by IPTG at final concentration of 1 mM and cultured for an additional 20 hours at 37°C. The culture that was not induced with IPTG served as control.

Monitoring of IFNA11 production.

The initial IFNA11 expression at zero time and in 3 and 20 hours after IPTG induction was controlled. Cells from induced and un-induced cultures were harvested by centrifugation (4000×g, 10 min, 4°C). Pellet was resuspended in lysis buffer (25 mM HEPES pH 7.0, 500 mM NaCl, 10% w/v Glycerol, 0.025% w/v NaAzide, 0.5% w/v CHAPS, 10 mM $MgCl_2$) and homogenized in Potter homogenizer. The homogenate was centrifugated at 10000 g for 20 min at 4°C, supernatant containing the soluble proteins was collected and the pellet containing the insoluble proteins was resuspended in the initial volume of lysis buffer. The concentration of protein in total homogenate and both fractions was measured with Bradford reagent and equal amounts of protein were loaded on 12% SDS–PAG and subjected to electrophoresis [14]. Gels were stained with Colloidal Coomassie [8] (Fig. 3, 4).

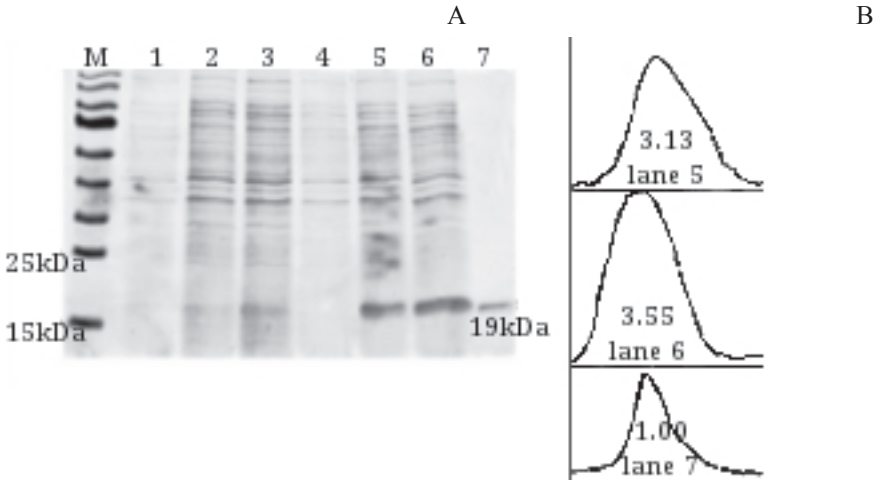

Fig. 3. Expression of *IFNA11* in transformed Rosetta (DE3) cells grown in LB medium at 37°C:
A: SDS-PAGE analysis of non-fractionated homogenate of transformed Rosetta (DE3) cells. Lanes: M – PageRuler Prestained Protein Ladder, 10 to 170 kDa (Thermo Scientific, USA); 1, 2, 3 –samples of not induced by IPTG cellular culture collected at 0, 3 and 20 hours of cultivation, respectively. 4, 5, 6 – samples of induced by 1 mM IPTG cellular culture collected at 0, 3, 20 hours after induction, respectively; 7 – 0.5 μg of human IFNA, Mr 19 kDa. B: densitogram of human IFNA-specific band (lane7) and the bands of similar molecular mass (lanes 5 and 6).

In 3 and 20 h after IPTG induction the products of the expected molecular mass were determined in the cultivated cells (Fig. 4A, lanes 5, 6). We have measured the density of these two bands and the band of IFNA with ImageJ software. Taking the area under the curve of human IFNA signal conventionally for one the corresponding values of two other signals is estimated as equal to 3.13 (3 h of cultivation) and 3.53 (20 h of cultivation) arbitrary units. It is clear that the most amount of protein is synthesized during first 3 hours of cultivation and only slightly

increases till 20th hour. The approximate yield of IFNA is ~ 35 μg/ml of culture medium. The specific antiviral activity of total lysate was verified and confirmed by resistance of L292 fibroblasts to the vesicular stomatitis virus [4].

Optimization of protein yield

We decided to get the desired product in the soluble form for to evade the laborious procedures with inclusion bodies and to preserve the biological activity of the final product. To realize this we varied the temperature of cultivation and the medium composition and controlled the yield of the product in the soluble form by SDS-PAGE. We have tested two parameters – the reduced temperature of cultivation (25°C) and the standard medium supplemented with 1% glucose.

The cultivation at lower temperature limits the *in vivo* aggregation of recombinant protein and promotes their correct folding and increases their solubility too [25]. It slows down the rates of transcription and translation and partially eliminates the heat shock proteases. Previously it was shown, that higher production of IFNA2 with better stability was obtained at 25°C [7]. The glucose addition as a carbon source is often used in order to reduce the time of cultivation and increase the yield of desired product in the soluble form [24]. Addition of 1% glucose represses the induction of lac promoter by lactose which is present in LB medium.

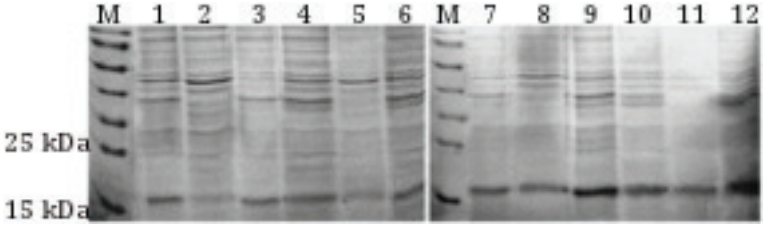

Fig. 4. The production of recombinant IFNA11 by the cells grown at 37°C and 25°C with or without 1% glucose. Lanes: M – PageRuler Prestained Protein Ladder, 10 to 170 kDa (Thermo Scientific, USA); 1, 2, 3 – total, soluble and insoluble fractions from the cells grown at 25°C with 1% glucose in the medium; 4, 5, 6 – total, soluble and insoluble fractions from the cells grown at 37°C with 1% glucose in the medium; 7, 8, 9 – total, soluble and insoluble fractions from the cells grown at 25°C without glucose in the medium; 10, 11, 12 – total, soluble and insoluble fractions from the cells grown at 37°C without glucose in the medium.

To assess the yield of INFA in soluble and insoluble forms under different conditions of cells cultivation the density of corresponding bands was measured using ImageJ software (Table 1).

Table 1

The percentage of soluble and insoluble INFA output at different conditions of cells cultivation

Concentration of glucose in the medium	Temperature of cultivation			
	25°C		37°C	
	Soluble	Insoluble	Soluble	Insoluble
1%	10.5	89.5	25.7	74,3
0%	11.8	88.2	36.3	63.7

The maximal output of IFNA in soluble form was obtained after cultivation of transformed Rosetta (DE3) cells at 37°C in the medium without glucose. The method may be applied to production of IFNA in the soluble form and in required amount. There is still a possibility to optimize the IFNA output by testing different additives to culture medium and lysis buffer. This task will be the challenge for the further experiments.

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ЕКСПРЕСІЯ ГЕНА, ЩО КОДУЄ МИШАЧИЙ ІНТЕРФЕРОН АЛЬФА В КЛІТИНАХ *ESCHERICHIA COLI*

В. Доценко[1], С. Рибалко[2], М. Оболенська[1]

[1]Інститут молекулярної біології та генетики
вул. Заболотного, 150, Київ 143, Україна
[2]Інститут епідеміології та інфекційних хвороб імені Л. Громашевського
вул. Амосова, 5, Київ 038, Україна
e-mail: dotsdon@gmail.com

Метою даного дослідження було отримання мишачого рекомбінантного IFNA11 у розчинній формі, оминаючи процедуру рефолдингу для максимального збереження його фізіологічної активності. Мишачий *Ifna11* клонували в *pET-24a (+)* вектор і продукували в *E. coli*. Ідентичність IFNA було підтверджено тестом противірусної активності. Максимальний вихід розчинного IFNA при модифікаціях температури культивування і вмісту глюкози в середовищі становив 35 мг/л.

Ключові слова: мишачий інтерферон альфа, продукція білка, оптимізація процесу отримання, система експресії *E. coli*.

ЭКСПРЕССИЯ ГЕНА, КОДИРУЮЩЕГО МЫШИНЫЙ ИНТЕРФЕРОН АЛЬФА В КЛЕТКАХ *ESCHERICHIA COLI*

В. Доценко[1], С. Рыбалко[2], М. Оболенская[1]

[1]Институт молекулярной биологии и генетики
ул. Заболотного, 150, Киев 143, Украина
[2]Институт эпидемиологии и инфекционных болезней имени Л. Громашевского
ул. Амосова, 5, Киев 038, Украина
e-mail: dotsdon@gmail.com

Целью данного исследования было получение мышиного рекомбинантного IFNA11 в растворимой форме, минуя процедуру рефолдинга для максимального сохранения его физиологической активности. Мышиный *Ifna11* клонировали в *pET-24a (+)* вектор и производили в *E. coli*. Идентичность IFNA была подтверждена тестом на противовирусную активность. Максимальный выход растворимого IFNA при модификациях температуры культивирования и содержания глюкозы в среде составлял 35 мг/л.

Ключевые слова: мишиный интерферон альфа, продукция белка, оптимизация процесса получения, система экспрессии *E. coli*.