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Inducible Expression of Human Gamma Interferon

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ABSTRACT

Background: The premature termination of high producer clones, which will be killed due to cell proliferation and proteins production antagonism, is one of the basic drawback in recombinant proteins technology. Furtheremore, it is supposed some toxic proteins like interferon which we intended to clone and express, inhibit host cells' proliferation. So, it is necessary to tightly control IFN-γ production during growing and selecting of highly producing clones. **Methods:** In the present study, we constructed an expression vector, pMPGB43P2(6)K containing the cumate-regulatable expression cassette to high production of human IFN-γ in Chinese Hamster Ovary- Cumate Transactivator (CHO-CTA). The clones were selected in the cumate and without cumate treated medium. **Results:** Our results showed, induced IFN-γ expression level was about 5 of magnitude higher than the constitive transgenic system. **Conclusion:** Application of cumate-regulatable expression cassette, which can be switch off and on by cumate, is useful to production of high producer clones and express toxic proteins in animal cells. *Iran. Biomed. J. 10 (4): 197-202, 2006*

Keywords: Inducible expression of IFN-γ, IFN-γ Cloning, Cumate-regulatable expression cassette, Chinese hamster ovary-cumate transactivator (CHO-CTA)

INTRODUCTION

The premature termination of high producer clones, which will die due to cell proliferation and protein production antagonism, is one of the basic problems in recombinant protein technology. Therefore, tightly controlled expression of foreign proteins greatly aids selection of highly producer clones. A number of inducible systems are available. One of them is the cumate-regulatable expression cassette that its background backs to the pseudomonas cumate operon [1].

In *Pseudomonas putida F1*, the degrative pathway for p-cymene (*cym*) to its benzoate derivative p-Cumate consists of 6 genes organised in an operon (*cym*). The *cym* operon is followed by the *cmt* operon that is responsible for the further degradation of cumate. The expression of the genes in both operons is regulated by a 28 kDa repressor molecule (Cymene repressor [*cymR*]) that binds operator sequence downstream of the start site of promotor. *cymR* is in a DNA-binding configuration only in the absence of *cym* or cumate, the effector molecules. But, in modified inducible cumate expression cassette, *cymR* is used with an activation domain of the vp16 protein of herpes simplex viruse which can switch off in cumate-treated medium to control gene expression [1-4].

IFN-γ is secreted by lymphocytes and is a homodimeric glycoprotein containing two 21 to 24 kDa subunits. The size variation of the subunit is caused by variable degrees of glycosylation, but each subunit contains one identical 18 kDa polypeptide encoded by the same gene. IFN-γ has a single gene located on chromosome 12. This gene includes four exons and three introns and there is low polymorphism [5, 6]. IFN-γ has several functions including: activation of mono/polynuclear phagocytes, increase in expression of MHC molecule, differentiation of T-lymphocytes to Th1 subset, inhibition of cell division and etc [7-9].

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Todays three schemes of IFN-γ are used: therapeutic, prophylactic and mixed. Therapeutic scheme is used for the treatment of infectious diseases like *Leishmania donovani*, mycobacterium leprae, toxoplasma gondii etc. The scheme for prophylaxis is used for the prophylactic purposes to defend against opportunistic infections in Aids, for the prophylaxis of infective complications in chronic granulomatouse and in born T-cell immune deficiency. The mixed scheme of IFN-γ injection is used mainly in treatment of oncological diseases [10].

In the present study, we constructed an expression vector, pMPGB43P2(6)K containing the cumate-regulatable expression cassette for high production of human gamma interferon (huIFN-γ) in CHO (Chinese Hamster Ovary) cell line which stably produces CTA (Cumate Transactivator), a fusion protein composed of *cymR* and *vp16* (activating domain).

MATERIALS AND METHODS

Lmphocyte isolation and stimulation by PHA (phytohaemagglutinin). Sterile blood (10 ml) was drained from a healthy donor and mixed by 1 ml 10% EDTA and diluted with 22 ml Hank's solution. PBL were isolated by ficol and washed two times with Hank's solution. lymphocytes (5×10^6) were cultured as monolayer culture in 10 ml $RPMI_{1640}$ medium, supplemented with 15% heat inactivated FBS, 100 µg/ml of streptomycin and 100 U/ml of penecillin. PBL were stimulated to produce IFN-γ by treatment of 10 µg/ml PHA (Sigma, USA). The cells were collected 4 h after induction and used to prepare total RNA [9,11].

Total RNA isolation. The cells were lysed with 4 M guanidinthiocyanate and extracted with phenol-chloroform after the addition of 0.2 M sodium acetate. RNA was precipitated twice by ethanol and dissolved in diethylpyrocarbonate water [12, 13].

Reverse transcription (RT)-PCR. Extracted RNA was used in RT-PCR. Briefly, RNA was reverse transcribed to make a DNA copy for use in PCR. RNA (0.5 µg) was added to a tube containing 5 mM $MgCl_2$, 1 mM of each dNTP, 50 mM Kcl, 10 mM Tris buffer, pH 8.3, 2.5 µM $OligodT_{(18)}$, 20 units of RNase inhibitor and 50 units of M-Mulv-reverse transcriptase (Fermentas, USA). The mixture was incubated at 45ºC for 35 min, heated at 95ºC for 5 min and placed on ice until using for PCR [13]. The cDNA was amplified by PCR using the following oligonucleoties as primers: 5′-GGC TTA ATT CTC TCG GAA ACG-3′ and 5′-AAA TTC AAA TAT TGC AGG CAG G-3′. The PCR was performed with an initial denaturation step at 94ºC for 5 min, followed by 40 cycles of 45 s 94ºC, 60 s 58ºC, 60 s 72ºC, and end step 5 min 72ºC. Following this primary amplifiction, a second PCR was performed using two specific nested primers containing Bam*HI* restriction sites.

Construction of recombinant plasmid (pMPGB 43P2(6)K-IFN-γ. The resulting PCR product, the 558 base-pair (bp) of HuIFN-γ coding sequence containing restriction sites of Bam*HI* enzyme and expressing vector pMPGB43P2(6)K (Kindly provided by Prof. Massie, Biotechnology Research Institute, National Research Council, Canada) was simultanously digested by Bam*HI* and purified by phenol/chloroform. The construct was constructed by inserting the entire HuIFN-γ coding region (Bam*HI* fragment) into pMPGB43P2(6)K.

Transformation of E. coli by the construct. The resulting construct was transfered into the competent *E. coli* (strain: DH5α) using $CaCl_2$ method and selected by plating on a medium containing ampicillin [13].

Screening of transformed bacterial colonies for the insert by PCR. To confirm the existence of the insert in the construct, PCR was performed for grown colonies in the ampicillin-treated medium, using a pair of primers amplifying 273 bp sequnces: 5′-AAT GCA GGT CAT TCA GAT G-3′ and 5′-AAC TGA CTT GAA TGT CCA A-3′. Briefly, small amount of each colony was added to separate tubes containing all amount of PCR reagents except the enzyme (Taq) and heated for 10 min. The Taq was then added to each tube and PCR was performed [13].

Sequence analysis of the insert in the construct. The complete nucleotide sequence analysis of the inserts in the 6 constructs derived from 6 different colonies which were grown and screened was performed and one of the inserts were found to be exactly identical to the published sequence in Genebank. So, the construct containing the correct insert was selected for the following procedure [13].

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Establishment of CHO-CTA cells producing HuIFN-γ. CHO-CTA cells (15× 10^6) were plated on 15 ml of CD medium (chemical defined, 1 × hypoxanthin/thymine supplemented and 4 mM L-glutamine) in a 100-mm Petridish. After 3-4 hours, 9 µg linearized construct (cut by XbaI enzyme) and 90 µl Lipofectamine 2,000 were separately mixed in 750 and 660 µl fresh CD medium, respectively and incubated at RT for 5 min. The two mixture were combined, incubated at RT for 20 min and slowly added to the cells. The next day, the cells were divided into two same groups each group containing 768 wells and supplemented with 600 µg/ml of Hygromycin (Life Technologies, USA). One group received in addition 3 µg/ml of cumate. Hygromycin resistant single clones were appeared after approximately two weeks in some wells of a 96-well plate and picked up. The single clones were separately subcultured in a 24-well plate and then transferred to a 6-well plate. The cumate of the medium was removed when the cells reached to confluence, and stable HuIFN-γ expressing clones were identified by ELISA and Western-blot analysis [14, 15].

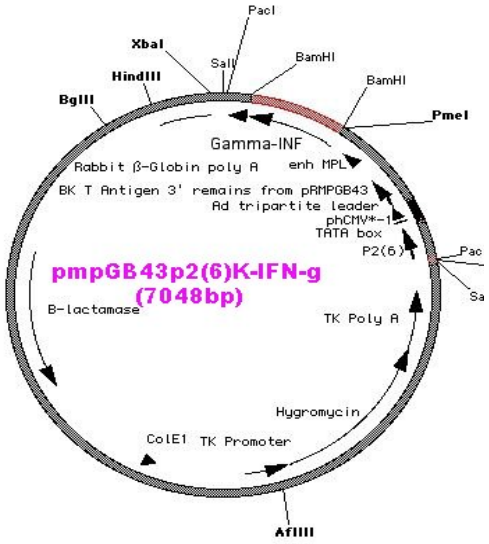


Fig. 1. The construct (pMPGB43P2(6)K-IFN-γ).

RESULTS

A full length cDNA encoding the signal and mature HuIFN-γ sequence derived from PBL of a healthy donor was amplified by PCR and followed by nested PCR containing Bam*HI* enzyme restriction sites. The resulting PCR product

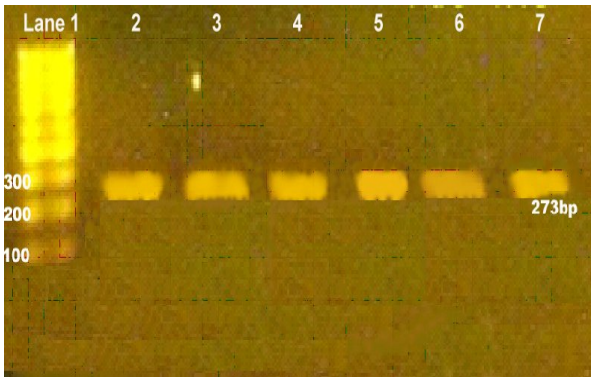


Fig. 2. Screening of transformed bacterial colonies for the insert sequences by PCR. Lane1, molecular size marker; Lanes 2, 3, 4, 5, 6 and 7, bacterial colonies miniprep results.

(558 bp) and expression vector pMPGB43P2(6)K (6490 bp) were digested and ligated (Fig. 1). The construct (7048 bp) was transferred to *E.coli* strain DH5α in a medium containing ampicillin and 33 bacterial colonies were grown. But PCR screening of the grown colonies showed only 6 colonies had inserted sequence (Fig. 2).

After complete nucleotide sequence analysis of all inserts, the construct which were derived from colony number 4 had exactly identical sequence in compare with the published ones in Genebank. This clone was chosen and tranfected CHO-CTA cells (Fig. 3). After 48 hours, transient expression of IFN-γ in the supernatant of the cells was 186 ng/ml/10^6 (Fig. 4). For permanent expression, the cells were divided equally into two groups, each group containing 768 wells. Clones (n = 93) in the cumate treated and 216 clones in untreated medium were grown. Test was done for all 93 cumate treated and only 48 of untreated clones ELISA. The results

ATGAAATATACAAGTTATATCTTGGCTTTTCAGCTCTG
CATCGTTTTGGGTTCTCTTGGCTGTTACTGCCAGAACC
CATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTT
TAATGCAGGTCATTCAGATGTAGCGGATAATGGAACT
CTTTTCTTAGGCATTTTGAAGAATTGGAAAGAGGAGAG
CGACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTT
ACTTCAAACTTTTTAAAAACTTTAAAGATGACCAGAGC
ATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATGA
ATGTCAAGTTTTTCAATAGCAACAAAAAGAAACGAGA
TGACTTCGAAAAGCTGACTAATTATTCGGTAACTGACT
TGAATGTCCAACGCAAAGCAATACATGAACTCATCCA
AGTGATGGCTGAACTGTCGCCAGCAGCTAAAACAGGG
AAACGAAAAAGGAGTCAGATGCTGTTTCGAGGTCGAA
GAGCATCCCAGTAA

Fig. 3. Complete nucleotide coding sequence of the insert (clone no. 4).

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showed, only 4 clones in the first group and 6 clones in the later group produce IFN-γ more than 100 ng/ml/10^6cell (Fig. 4). But, the average amounts of IFN-γ production with the clones that were growing with cumate were 1337 ng/ml in compare with 248.83 ng/ml produced with clones without cumate (Table 1).

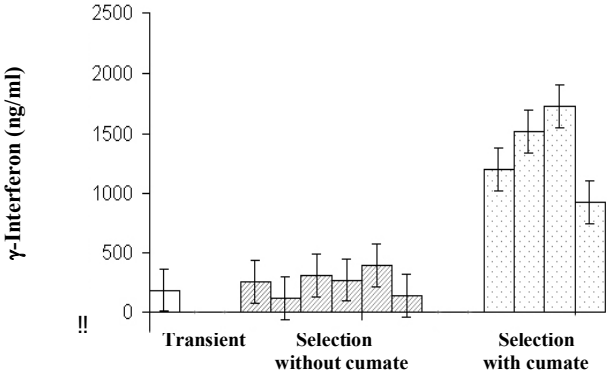


Fig. 4. Detection of gamma interferon in different clones which selected by cumate and without cumate. The difference of two groups was statistically significant (p<0.001). The mean of cumate groups were 1337 ± 18.67 and without cumate group 248.83 ± 10.16.

Table 1. Comparison of IFN-γ production in different clones which selected in/without cumate treated medium.

Expression	Clone no.	without cumate IFN-γ (ng/ml/0^6cell)	with cumate IFN-γ (ng/ml/0^6cell)
Transient	-----	186	-----
Stable	1	250	-----
	2	120	-----
	3	310	-----
	4	270	-----
	5	397	-----
	6	146	-----
	7	----	1200
	8	----	1506
	9	----	1719
	10	----	923
Mean	---	248.83	1337

DICUSSION

For the first time, Gray *et al.* [16] cloned and expressed HuIFN-γ in *E. coli* in 1983. But, the produced IFN-γ wasn't glycosylated. As we know, the glycosylation is very important and can change the antigenic, solubility, pharmaceutical, biological fuction of recombinant proteins [7, 8, 17-20].

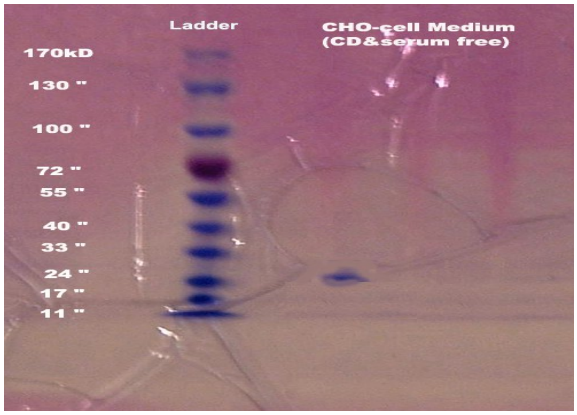


Fig. 5. SDS-PAGE analysis of IFN-γ in the medium of CHO-CTA cells (clone no. 9).

Recently, in order to produce recombinant proteins more similar to native ones, many scientists prefer to use mammalian cells [21-24]. So, CHO cells have been very popular mammalian host of the commercial production of the therapeutical important proteins like IFN-γ, human erythropoitin, human chorionic gonadotropin, coagulation factor VIII, Follicle Stimulating Hormone, HIV envelope proteins etc, in CHO-CTA cells [25-28].

As we mentioned in the introduction, IFN-γ is supposed to be a cytotoxic for cell proliferationat high concentration. Therefore, for cloning and expression IFN-γ, we used the cumate-regulatable expression cassette. As shown in Figure 4 and Table 1, induced IFN-γ levels were about five magnitude higher than the constitutive trangenic system. The clones selected with cumate treated medium produced IFN-γ more than the clones selected without cumate but the number of clones which grew in cumate containing medium (93 clones) were

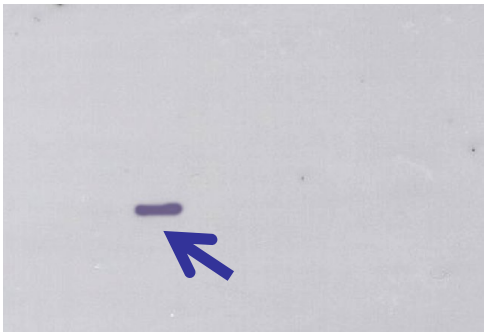


Fig. 6. Western-blot analysis of IFN-γ in the medium of CHO-CTA cells (clone no. 9).

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less than the clones grew without cumate (216 clones) due to little toxicity of cumate for cell growth. Finally, in addition to application of cumate-regulatable expression cassette to high production of proteins in animal cell culture, it will be useful for functional studies and gene therapy application.

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