한국생물공학회지 제5권 제3호
Korean J. Biotechnol. Bioeng.
Vol. 5. No. 3. 293-298(1990)

Extracellular Production of Alpha-Interferon by Recombinant *Escherichia coli*: Part Ⅲ. Gene Expression for Product Formation

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유전자 재조합 대장균을 사용한 Alpha-Interferon의 생산과 분비: 제3부: Interferon 생산을 위한 유전자의 발현

노 갑 수 · 최 차 용

ABSTRACT

Alpha-interferon was produced by using recombinant *Escherichia coli* strains, which carry cloned alpha-interferon gene in plasmid vectors, pIF-Ⅲ-B and pIF-Ⅲ-C. With the aid of signal sequence of *E. coli* lipoprotein, which is placed right in front of the upstream of the cloned alpha-interferon gene of the plasmids, about 50% of alpha-interferon produced was excreted or secreted. Meanwhile, there was no extracellular production of alpha-interferon from the recombinant strain carrying the plasmid Hif-2h which lacks the signal sequence of lipoprotein.

INTRODUCTION

Recent advances in recombinant DNA technology have provided the means to produce many pharmaceutical proteins in bacteria that have not been obtainable in large quantity by any other techniques. In the meantime, bacterial host-vector systems developed for the production of various invaluable pharmaceutical proteins have encountered some problems, such as proteolysis, aggregate formation, plasmid instability and contamination with bacterial proteins.

In this report, as an attempt to improve the production of alpha-interferon(IFN) through solving some of the problems in relation with bacterial host-vector systems, we describe extracellular production of alpha-interferon by using pIN-Ⅲ plasmid vectors. The vectors carry the promoter created by combining of the DNA sequences from *lpp* and *lac*UV5 promoters and signal sequence for the outermembrane lipoprotein gene of *Escherichia coli*. Lipoprotein promoter is reported to be one of the most strong promoters in *E. coli* together with *tac*(1) and lambda P_L (2-4) promoters. Secreted or excreted proteins could circumvent the attack of intracellular protease, resulting in the enhancement in recovery. Also the chance of inclusion body formation is possibly reduced because excess intracellular accumulation of gene products is prevented due to the excretion or secretion of the proteins. The vector system also makes it possible to control the expression of the cloned gene by an adding inducer, IPTG, to the medium because the vectors also carry *lacI*^{q} gene which code for repressor that can bind to *lac*UV5 operator.

If the expression of the cloned gene is controllable, metabolic competition between cell growth and expression of cloned gene can be reduced during growth phase, and then expression of cloned gene can be induced during production phase.

We have previously reported the construction of vectors for alpha-IFN gene expression, *i. e.*, pIF-Ⅲ-B and pIF-Ⅲ-B and pIF-Ⅲ-C, and the growth behavior of the recombinant cells in part I(5) and part Ⅱ, respectively.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture conditions

E. coli strains used throughout this study are listed in Table 1. The bacteria were used as host strains for plasmids pIF-Ⅲ-B, pIF-Ⅲ-C and Hif-2h, which carry alpha-IFN gene. Plasmid pIF-Ⅲ-B and pIF-Ⅲ-C were constructed by transferring alpha-IFN gene from Hif-2h(11) to plasmid pIN-ⅢB3 and pIN-ⅢC3(6), respectively, which were provided by Inouye(State Univ. of NY at Stony Brook, USA). Schematic diagrams of the plasmids pIN-ⅢB3 and Hif-2h are shown in Fig. 1. Structures of the plasmids pIF-Ⅲ-B and pIF-Ⅲ-C are presented in Fig. 2.

All of the *E. coli* strains were cultivated in LB medium at 37℃. A rotary flask shaker(New Brunswick Sci.) set at 180 rpm was used for the culture of the recombinant cells in order to investigate the production of alpha-IFN. Cell growth was followed by measuring absorbance with spectrophotometer at wavelength of 600 nm.

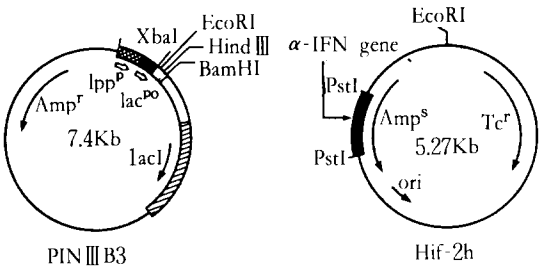

Fig. 1. Schematic diagrams of plasmids pIN-ⅢB3 and Hif-2h.

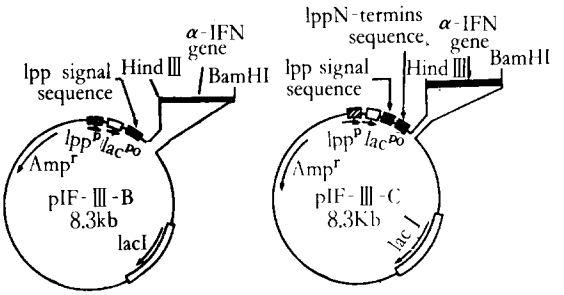

Fig. 2. Structure of plasmids pIF-Ⅲ-B and pIF-Ⅲ-C

Cell line and virus

Human larynx carcinoma cells, Hep2, were purchased from American Type Culture Collection (Rockvile, Mar-

Table 1. *E. coli* strains and genotypes

Sample	Genetic Markers	Reference
	F^-, thi−1, thr−1, leuB6	
C600	lacY1, tonA21, supE44, λ^-	7
	F^-, hsd20(r_B^-, m_B-), recA13,	
HB101	ara−14, proA−2, lacY−1, galK−2,	8
	rpsL20(Sm^r), xyl	
JE5505	F^-, lpp−2, pps, his, proA, argE,	9
	thi, gal, lac, xyl, mtl, tsx	
RB791	W3110 $lacI^qL8$,	10
	endA1, gyrA96, thi, hsdR17	

yland, USA). Vesicular Stomatitis Virus (VSV), Indiana serotype, was kindly provided by H. W. Park (Mass. Inst. Tech., USA). Hep2 cells were used as host cells of VSV challenge for IFN assay. They were cultured in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS, GIBCO) in disposable tissue culture flasks (25㎠, 75㎠) purchased from Nunc and Costar, USA. One hundred fold stock solution (10mg/ml) of kanamycin was used to prevent the culture from bacterial-contamination. VSV's were prepared according to Goorha (12). VSV was amplified in tissue culture flask, where Hep2 cells were densely monolayered, with MEM supplemented with 2% FBS. The amplified VSV's were dispensed in aliquots and kept at −70℃ for use in IFN assay. Optimum VSV dilution ratio for the IFN assay was determined in 96 well plate seeded with 9×10^4 Hep2 cells per well.

Induction of gene expression

For the induction of alpha-IFN gene expression in the recombinant *E. coli* strains, 1 mM of IPTG was added to the media. One hundred milli molar IPTG was prepared by dissolving it in distilled water and sterilized with membrane filtration. The stock solution was kept at −20℃ in aliquots.

Transformation

Transformation of *E. coli* strains with plasmid DNA was performed as described by Morrison(13). Transformants were screened on LB plate supplemented with 50 ㎍/ml of ampicillin.

Determination of alpha-IFN activity

Various methods for alpha-IFN assay were described in detail by Pestka(14). We followed the method described by Rubinstein(15). The assay is based on the measurement of a parameter associated with cytopathic effect on host animal cells.

Preparation of crude *E. coli* extracts for the assay of intracellular alpha-IFN

Cell extract preparation was based on Nagata(11). Harvested cells from 50ml of culture were washed with 5ml of washing buffer [50 mM Tris, pH8.0, 30 mM NaCl] twice or three times and resuspended in 5ml of lysozyme solution [75 mM EDTA, 75 mM Tris, pH8.0, 0.3 M Sucrose, 3㎎/ml of lysozyme]. After 30 min of incubation in ice bath the cells were frozen at −70℃ and thawed at 40℃ repeatedly for five or six times until complete lysis of cells was accomplished. After centrifugation at 200,000g for 1 hr the supernatant was taken as extract sample. The activity of the sample was regarded as the sum of the activities of cytoplasmic and periplasmic fractions.

The sensitivity of outermembrane to EDTA varied according to the hosts used. JE5505 was most resistant to EDTA and C600 was most sensitive to EDTA. When 50 mM EDTA was used, JE5505 was not lyzed at all while C600 was lyzed almost completely.

Preparation of samples for the assay of excreted alpha-IFN

The supernatant taken after centrifugation of the culture was sterilized with membrane filter of 0.2㎛ pore size to remove the remaining *E. coli* cells. In early days of sample preparation, the filtered supernatant was taken as a sample for the assay of the excreted alpha-IFN titer. This procedure appeared to be inadequate and cumbersome. Instead of membrane filtration, the supernatant was recentrifuged with Eppendorf centrifuge at 12,000 rpm for 10 min to remove even a trace of *E. coli* and used for the assay of excreted alpha-IFN successfully.

Preparation of samples for the assay of alpha-IFN from periplasmic space

Cells were harvested in an Eppendorf centrifuge for 2 min and the pellet was put on ice. The pellet was resuspended in 0.15ml of cold solution having a composition of 20% sucrose, 10mM Tris HCl(pH 7.5) and 5 ㎕ of 0.5 M EDTA(pH 8) was added. Then the resuspended cells were incubated in ice bath for 10 min. The cells were centrifuged for 5 min and the supernatant was quickly removed. The pellet was rapidly resuspended by vigorous vortex mixing in 0.1ml of cold distilled water. The resuspended cells were incubated for 10 min on ice and then centrifuged again for 5 min. The supernatant was taken as the sample for the assay of alpha-IFN remained at the periplasmic space of recombinant *E. coli* strain.

Preparation of standard alpha-IFN solution

Standard alpha-IFN used in this study was generously provided by Korea Green Cross Co.. It was dissolved in MEM in appropriate IU per ml of the medium and stored in aliquots at −70℃.

RESULTS AND DISCUSSION

Determination of optimal dilution ratio of VSV concentrate for the assay of alpha-IFN activity

To determine the optimal dilution ratio of VSV concentrate for the assay of alpha-IFN samples, Hep2 cell viability was investigated with varying concentrations of VSV in a 96well plate. Hep2 cell viability curve after 18 hr of VSV challenge is given in Fig. 3 and the picture of Hep2 cell is given in Fig. 4. As the data illustrate, 100-fold dilution of the VSV concentrate was optimal for the assay of alpha-IFN activity.

Production of alpha-IFN in various *E. coli* strains harboring various recombinant plasmid

Using *E. coli* strains C600, HB101, JE5505, and RB791 harboring recombinant plasmids Hif-2h, pIF-Ⅲ-B and

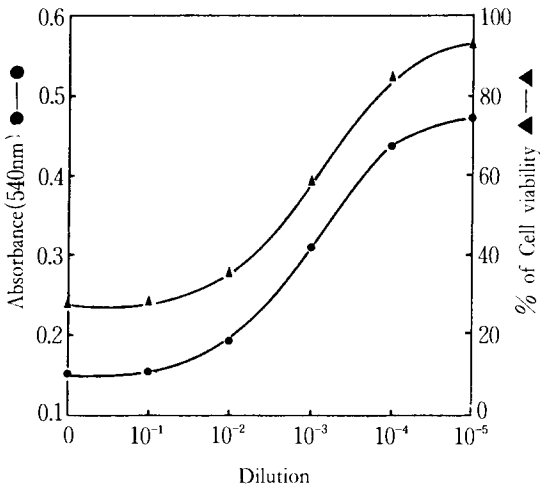

Fig. 3. Hep2 cell viability curve 18 hr after the challenge with varying dilution of VSV concentrate.

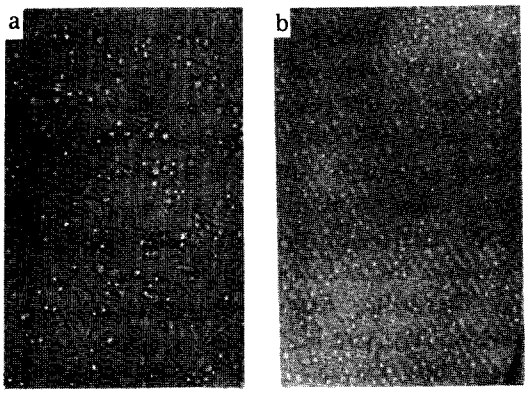

Fig. 4. Picture of Hep2 cell.
(a) Before VSV challenge
(b) 18hr after VSV challenge

pIF-Ⅲ-C, alpha-IFN production levels in cytoplasm, periplasmic space and supernatant were investigated. Hif-2h carries alpha-IFN gene at *Pst* I site of plasmid pBR322 and the gene is under the control of β-lactamase gene promoter. Plasmids pIF-Ⅲ-B and pIF-Ⅲ-C were constructed by transferring the IFN gene from Hif-2h to pIN-Ⅲ B3 and pIN-Ⅲ C3, respectively. Both of them carry *lpp* promoter, *lac*UV5 promoter and operator, $lacI^q$ gene and signal sequence of lipoprotein. The plasmid pIF-Ⅲ-C also carries N-terminus sequence of *lpp* in addition to its signal sequence. Cells were cultivated in 500ml flasks containing 100ml LB media supplemented wih 50㎍/ml ampicillin until the cultures reached stationary phase. The inducer, IPTG was added simultaneously with the inoculation. Three types of samples of each strain *i. e.*, supernatant, periplasmic fraction and extract, were taken and assayed. The picture of the 96 well plate after an assay is shown in Fig. 5 and the assay results are given in Fig. 6. As shown in Fig. 6, the total production level of alpha-IFN from JE5505 carrying plasmid pIF-Ⅲ-B was observed to be about 130 times as high as that from HB101 carrying plasmid Hif 2h. Stronger dual promoter of *lpp* and *lac*UV5 of pIF-Ⅲ-B seemed to be the primary reason for this improved production of alpha-IFN. Furthermore, about 50% of total alpha-IFN was secreted or excreted when pIF-Ⅲ-B was used, meanwhile secretion or excretion was

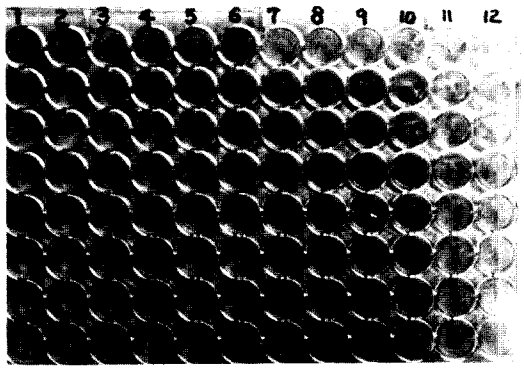

Fig. 5. 96 well plate after alpha-IFN assay via cytopathic effect inhibition method.
1,2: C600(pIF-Ⅲ-B) 3,4: HB101(pIF-Ⅲ-B)
5,6: Standard IFN 7,8: JE5505(pIF-Ⅲ-B)
9,10: RB791(pIF-Ⅲ-B)11,12: JE5505 (blank)

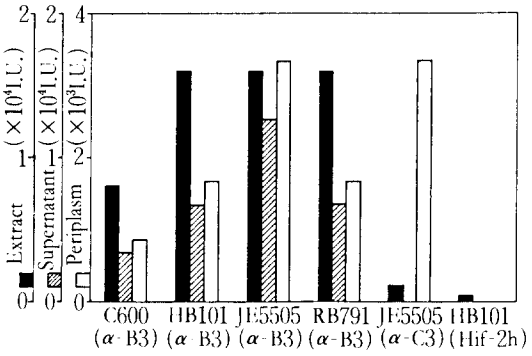

Fig. 6. Graph of α-IFN activity (IU/ml of LB medium) distribution in various *E. coli* host strains harboring plasmids, α-B3 (pIF-Ⅲ-B), α-C3 (pIF-Ⅲ-C) and Hif-2h.

not observed at all when Hif 2h was used. This difference was thought to result from the presence of the signal sequence of *lpp* which resides in the very upstream region of the cloned alpha-IFN gene of pIF-Ⅲ-B. The plasmid pIF-Ⅲ-C also showed similar periplasmic production of alpha IFN to the plasmid pIF-Ⅲ-B. However, in contrast to pIF-Ⅲ-B, pIF Ⅲ-C did not show any detectable activity in supernatant. This was thought to be a result from the fact that pIF-Ⅲ-C carries N-terminus sequence as well as the signal sequence of *lpp* at the upstream region of cloned alpha-IFN gene, which cause a formation of hybrid protein containing N-terminus of lipoprotein. When the proteins were transferred to the periplasmic space of the recombinant cell they form covalent bondings with fatty acids in the outermembrane. Excretion of alpha-IFN produced from pIF-Ⅲ-C across the outermembrane was thought to be prevented by these covalent bondings with fatty acids in the outermembrane.

Even in the case of plasmid pIF-Ⅲ-B, there were differences in the excretion level of alpha-IFN from strain to strain. Among them, JE5505 showed the highest excretion level of alpha-IFN and it was assumed that because JE5505 is a mutant which cannot produce lipoprotein, the structural protein of outermembrane, excretion of alpha-IFN through outermembrane was easier as compared with other host strains. Among all of the host strains used, JE5505 also showed the highest expression level. It was thought that because JE5505 is *lpp*$^-$, this characteristic possibly has some relationship with the efficient function of the *lpp* promoter in the host strain JE5505.

Conclusively, plasmid pIF-Ⅲ-B showed about 130 times higher expression of alpha-IFN gene than Hif-2h. It was probably due to the stronger dual promoter of *lpp* and *lac*UV5 from the plasmid pIF-Ⅲ-B. And approximately, 50% of alpha-IFN produced from recombinant cells carrying plasmid pIF-Ⅲ-B was secreted or excreted, implying that signal sequence of *lpp* played its role successfully. Finally, alpha-IFN production levels were strain specific and JE5505 showed the highest level, which means that selection or development of appropriate host strains is also an essential requirement in the successful production of eukaryotic proteins using bacterial host-vector systems.

ABBREVIATIONS

Amp^r: ampicillin resistant, IFN: interferon, IPTG: isopropyl-β-D-thiogalactopyranoside, IU: international unit, Kb: kilo base pairs, *lac*: lactose, *lpp*: lipoprotein, OD: optical density,
Tc^r: tetracycline resistant, VSV: vesicular stomatitis virus

요 약

대장균의 lipoprotein promoter, *lac* UV5 promoter 및 operator 와 lipoprotein의 signal sequence를 가지는 vector에 alpha-IFN 유전자를 cloning 함으로써 제작된 plasmid pIF-Ⅲ-B와 pIF-Ⅲ-C를 여러종류의 대장균 숙주 세포에 형질 전환하여 IFN의 생산성을 조사해 본 결과 lpp⁻형질을 가지는 JE5505가 가장 우수했으며, 기존 plasmid, Hif-2h에 비해 130배 높은 생산성을 보였다. 또한 생산된 IFN의 약 50%가 세포 외부로 분비됨을 확인하였다.

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(Received; October 19, Accepted; November 30 1990)