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# Expression of Glycosylated Human Interferon-Beta in High Levels in Chinese Hamster Ovary Cells

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**A novel plasmid (pNOW/CMV-A) for efficient expression of recombinant proteins was engineered. The human interferon-β (IFN-β) gene was incorporated into it to produce a vector (pNOW-hIFN-β) for expression in Chinese hamster ovary cells. The production of recombinant IFN-β increased with time up to 4 days of culture in the absence of any animal sera. The purified preparation of IFN-β possessed a sugar moiety and migrated to the same position as native human fibroblast IFN-β, either before or after treatment with glycosidase. The recombinant IFN-β possessed the same amino-terminal sequence as the product of the IFN-β gene. Since the recombinant IFN-β produced in Chinese hamster ovary cells is glycosylated and free from any fear of animal sera or neoplastic cells, such as fibroblasts, it may serve in treatment of patients indicated for IFN-β, such as those with multiple sclerosis and viral hepatitis.**

**Keywords:** Interferon, Expression system, Recombinant protein, Viral infection

Interferons (IFNs) are secretory polypeptides that protect cells from viral infection. Among them, IFN-β is secreted by fibroblasts in response to viral infection or exposure to synthetic double-stranded RNA. IFN-β is different from IFN-α in that it is glycosylated, which would contribute to biological activity characteristic of IFN-β. The gene encoding IFN-β is expressed in *Escherichia coli* for the production of recombinant human IFN-β [1, 2].

IFN-β produced in *E. coli*, however, is not glycosylated, and its physical properties and specific activity differ from those of native IFN-β considerably. Efforts have been directed toward the expression of recombinant IFN-β in host cells that may endow it with glycosylation for biological and biochemical properties comparable with those of natural IFN-β [3, 4]. The yield and activity of obtained recombinant IFN-β, however, have not been specified.

Chinese hamster ovary (CHO) cells confer adequate glycosylation on recombinant proteins expressed by them [5]. In this study, a new plasmid (pNOW/CMV-A) was engineered for efficient expression of recombinant proteins. The vector carrying the human IFN-β gene (pNOW-hIFN-β) was constructed and expressed in CHO cells for production of recombinant IFN-β in high yield and glycosylated form.

## Materials and Methods

*Construction of an Expression Vector (pNOW/CMV-A)*

A novel plasmid for an efficient expression of recombinant protein was engineered, and designated pNOW/CMV-A. In this vector, replication origin in *E. coli* was used as the backbone, and neomycin resistance gene as well as dihydrofolate reductase gene was employed as the region for selection, taking advantage of the dependence of a deletion mutant of CHO cells (CHO DG44) on dihydrofolate [6]. These genes were weakened (designated wNeo and wDHFR, respectively) for a stringent selection of CHO cells transformed with the vector, which is essential for efficient expression [7]. In essence, enhancer was removed from SV40 promoter, and 5'- and 3'-termini of the Kozak consensus sequence [8] were changed from A to C and from G to C, respectively. The region for expression comprised two elements; CMV immediate early (PCMV) was used as a promoter and bovine growth hormone (BGH) was utilized as a polyadenylation signal.

Figure 1 depicts the construction of a vector for the expression of human IFN-β (pNOW-hIFN-β). The 0.56-kilobase cDNA carrying the human IFN-β gene was amplified by reverse-transcription polymerase chain reaction (PCR) with primers 5'-AAT GCG GCC GCA CCA TGA CCA ACA AGT GTC TCC T-3' (sense) and 5'-GCT CTA GAG CTC AGT TTC GGA GGT AAC CTG-3' (antisense), which carry the *Not*I and *Xba*I restriction sites (underlined), respectively. The PCR was performed in a TP-cycler (Toyobo Co. Ltd., Osaka, Japan) for 30 cycles with each cycle consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 2 min (5 min in the last extension). PCR products were cloned into a TA cloning vector pT7Blue(R)T (TaKaRa Shuzo Co. Ltd., Shiga, Japan), and sequenced with use of M13 primer M1 and RV. The insert in the pT7Blue(R)T was then cut out by digestion with *Not*I and *Xba*I, cloned into the mammalian expression vector (pNOW/CMV-A), between PCMV and

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Abbreviations: CHO, Chinese hamster ovary; DHFR, dehydrofolate reductase; IFN, interferon; I-MDM, Iscove's modified Dulbecco's medium; 2-ME, 2-mercaptoethanol; MTX, methotrexate

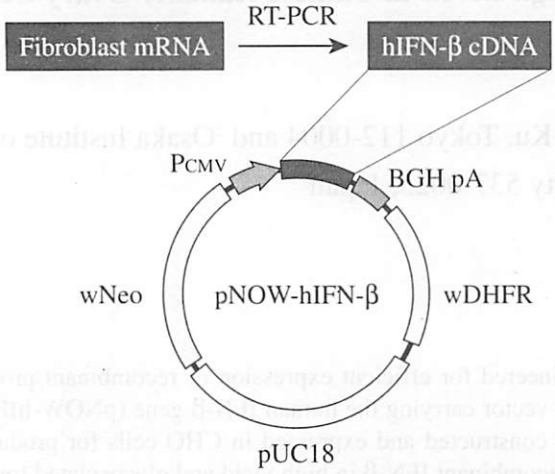


**Fig. 1.** Construction of the vector for the expression of human recombinant IFN-β. The gene for human IFN-β was amplified by reversed-transcription PCR, and inserted into a high-expression vector (pNOW/CMV-A) to engineer the vector for the expression of human rIFN-β (pNOW-hIFN-β).

BGH pA, and a novel plasmid designated pNOW-hIFN-β was engineered (Fig. 1).

*Expression of Human IFN-β in CHO Cells*

CHO DG44 cells were transfected with pNOW-hIFN-β and selected using neomycin and methotrexate (MTX; Shigma-Aldrich Inc., Missouri, USA). Transfection was carried out with DOTAP transfection reagent (Boehringer Mannheim GmBH, Mannheim, Germany). CHO DG44 cells [6] were plated at a density of 2 x $10^5$ cells per 6 ml in 60-mm plastic petri dishes in Iscove's modified Dulbecco's medium (I-MDM; GIBCO BRL, Life Technologies Inc., Maryland, USA) supplemented with 100 mM hypoxantine, 10 mM thymidine (GIBCO BRL) and 10% (vol/vol) dialyzed fetal calf serum (FCS; JRH Bosciences, Oklahoma, USA), and incubated at 37 °C for 20 h. Then, they were transfected with the pNOW-hIFN-β plasmid complexed with DOTAP. Transformants resistant to 0.4 mg/ml neomycin (GIBCO BRL) were grown for two weeks. They were selected and subcloned in a 96-well culture cluster (Corning Inc., New York, USA) in the presence of 0.4 mg/ml neomycin. The concentration of IFN-β in the supernatant of culture medium was determined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Toray Fuji Bionics, Tokyo, Japan). Transformed CHO cell lines secreting IFN-β in high level were then selected by MTX in concentrations increasing from 50 to 500 nM, in I-MDM supplemented with 10% dialyzed FCS and 0.4 mg/ml neomycin. The cell liens selected with 500 nM MTX plus 0.4 mg/ml neomycin were cultured further in I-MDM containing 10% dialyzed FCS, 500 nM MTX and 0.2 mg/ml neomycin.

The antiviral activity of IFN-β was determined by a modification of the method of Finter [9]. A 75-μ portion of serial dilutions of culture medium in minimal essential medium (MEM, GIBCO BRL) was delivered to wells of a microtiter plate, and sterilized by the UV light. Approximately 1.2 x $10^5$ human fibroblasts (GM2504) were added to each well, followed by vesicular stomatitis virus at a dose of 1 plaque forming unit per cell. The cytopathic effects were scored after 18 to 24 h, and the titer was estimated in comparison with IFN-β of the international standard tested in parallel.

*Affinity Purification of rIFN-β*

Sepharose 4B was conjugated with monoclonal antibodies to human IFN-β (mixture of 4008 and 4020) produced by the method described previously [10]. The column was washed with 5 ml of glycine-HCl buffer (0.1 M, pH 2.0) and then with 10 ml of phosphate buffer (5 mM, pH 7.4). To the affinity column of 1 ml in volume, 10 ml of culture medium containing rIFN-β (total: 11.7 x $10^5$ IU) was applied at a flow rate of 0.3 ml/min. The column was washed with 10 ml of phosphate buffer, and substances retained by it non-specially were washed out with 6 ml of glycine-HCl buffer (0.1 M, pH 4.0) supplemented with 50% (wt/wt) ethylene glycol. Then bound rIFN-β was eluted with 10 ml of 15 mM HCl (pH 2.0) at a flow rate of 0.15 ml/min, and 0.5-ml fractions were collected. Unbound and eluted fractions were made to a neutral pH with Tris-HCl buffer (1 M, pH 9.1). The yield of rIFN-β was 4.6 x $10^5$ IU with a recovery at 39%.

*SDS-PAGE*

SDS-PAGE was carried out by the method of Laemmli [11] on 12.5% (wt/vol) polyacrylamide gel. Three preparations of IFN, *i.e.*, rIFN-β expressed by CHO cells, rIFN-β produced in *E. coli* and natural IFN-β were made to 1% (wt/vol) with SDS. For the purpose of reduction, they received 2% (wt/vol) 2-mercaptoethanol (2-ME), heated at 100 °C for 5 min, and then they were subjected to electrophoresis.

*Digestion of Various IFN Preparations with Glycopeptidase F*

Solution containing rIFN-β expressed by CHO cells (1 μg/4 μl), rIFN-β produced in *E. coli* (2.5 μg/2.5 μl) or natural IFN-β (0.4 μg/4 μl) were made to a volume of 5 μl with Tris-HCl [1 M, pH 8.6]) containing 1% SDS and 0.2 M 2-ME. Each of them received glycopeptidase F (2 μl, [20 U/ml]: TaKaRa Shuzo), 13 μl of stabilizing reagent and 5 μl of distilled water, to a final volume of 25 μl, and then incubated at 37 °C for 17 h. The digest was transferred to buffer containing SDS and 2-ME. After electrophoresis, proteins in gel were visualized by a silver staining kit (Daiichi Seiyaku Co., Ltd., Tokyo, Japan) according to the manufacturer's instruction.

*Amino Acid Sequence of rIFN-β*

The final product of rIFN-β was separated on SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF; Bio-Rad, California, USA). Proteins were blotted, recovered and subjected to sequencing for amino-terminal amino acids in a HP G1005A Protein Sequencing System (Hewlett Packard, California, USA).

## Results

*Expression of rIFN-β by CHO Cells Transfected with pNOW-hIFN-β*

CHO cells were transfected with pNOW-hIFN-β (Fig. 1) for efficient expression of human IFN-β. Transformants resistant to neomycin were selected, and they were grown to confluence in 24-well plates. Out of 2.4 x $10^6$ transformants, 84 were selected by resistance to neomycin. Culture supernatants from the 84 neomycin-resistant transformants were tested for IFN-β by ELISA and biological activity. Four transformants were identified which secreted IFN-β in high concentrations, and they were propagated further. Figure 2 illustrates the distribution of 84 CHO clones with various levels of IFN-β secreted into media after 4 days of culture. Of them, 26 (31%) clones secreted IFN-β into culture media in levels higher than 1 x $10^4$ IU/ml; the highest level reached 14.9 x $10^4$.

Cell clones secreting IFN-β in high levels were derivatized for resistance to MTX by exposure to concentrations increasing from 50 to 500 nM, and then they were evaluated for the production of IFN-β. Several derivatives were obtained from clone 72 (the second highest producer), which secreted IFN-β into culture media in levels much higher than the parental clone. The clone that secreted the highest activity of IFN-β (1 x $10^6$ IU/ml in the medium) after 4 days of culture, in the presence of 500 nM MTX (initial cell concentration: 6 x $10^5$ cells/ml), was propagated further. Time courses of IFN-β produced by the three clones, which were resistant to neomycin plus 500 nM MTX, neomycin plus 50 nM MTX and neomycin alone, respectively, are shown in Fig. 3. Levels of IFN-β in media kept increasing for the transformant resistant to neomycin plus 500 or 50 nM MTX. Initially, levels of IFN-β were higher for the transformant resistant to neomycin plus 500 than 50 nM MTX, but they became comparable at 4 days of culture, when they had not reached a plateau and were still increasing. By contrast, levels of IFN-β in media harboring the transformant resistant to neomycin alone stayed low, albeit they slightly increased during 4 days of culture.

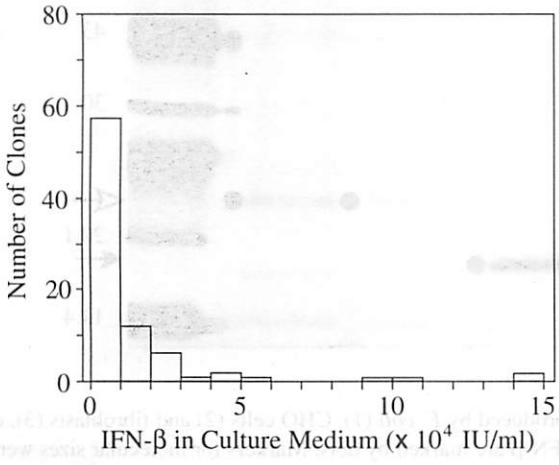


**Fig. 2.** Secretion of IFN-β into culture media by 84 CHO clones.

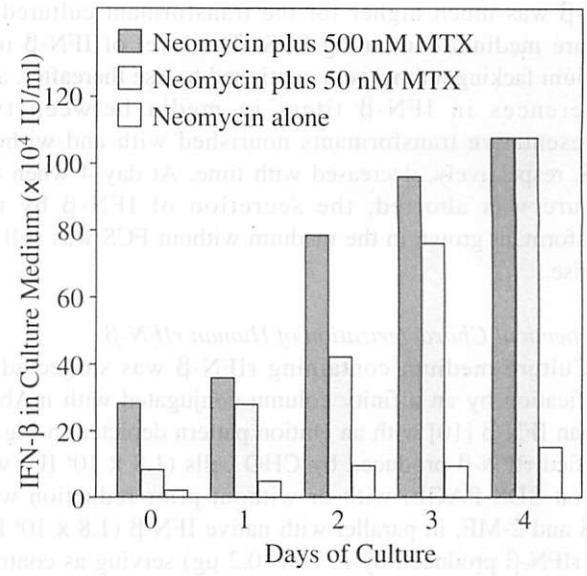


**Fig. 3.** Secretion of IFN-β by CHO clones derivertized with neomycin with or without MTX in two concentrations. Culture media contained CHO cells at a concentration of 3 x $10^5$/ml (same in the following experiments).

*Secretion of IFN-β by CHO Cells in the Absence of Animal Sera*

Of particular note were transformants that did not require animal sera for their growth; they retained the activity to secrete IFN-β into the culture medium. CHO cells were assimilated to culture medium without animal sera, transafected with pNOW-hIFN-β and selected with neomycin. The clone secreting the highest level of IFN-β in the absence of animal sera was compared with another clone similarly processed, in the presence FCS, and producing the highest IFN-β level (Fig. 4). Initially, the level of secreted

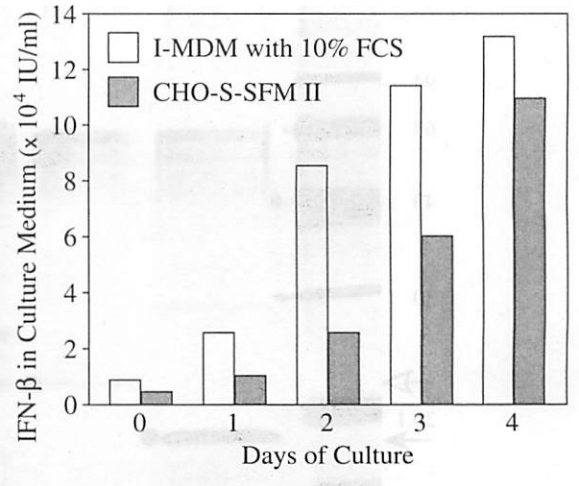


**Fig. 4.** Secretion of hIFN-β by CHO cells. CHO cells transformed with pNOW-hIFN-β were cultured in medium supplemented with animal serum (Iscove's modified Dulbecco's medium [I-MDM] added with FCS). They were compared for secretion of IFN-β with CHO cells assimilated to culture medium without any animal sera (Chinese hamster ovary suspension serum free medium [CHO-S-SFM II]; GIBCO BRL) and then transformed.

IFN-β was much higher for the transformant cultured in culture medium containing FCS. The level of IFN-β in a medium lacking animal sera continued to rise thereafter, and differences in IFN-β titers in media between two representative transformants nourished with and without FCS, respectively, decreased with time. At day 4 when the culture was aborted, the secretion of IFN-β by the transformant grown in the medium without FCS was still on the rise.

*Biochemical Characterization of Human rIFN-β*

Culture medium containing rIFN-β was subjected to purification by an affinity column conjugated with mAb to human IFN-β [10] with an elution pattern depicted in Fig. 5. Purified rIFN-β produced by CHO cells ($1.8 \times 10^4$ IU) was run on SDS-PAGE, with or without prior reduction with SDS and 2-ME, in parallel with native IFN-β ($1.8 \times 10^4$ IU) and rIFN-β produced by *E. coli* (0.2 μg) serving as controls (Fig. 6). Essentially the same results were obtained for rIFN-β produced by CHO cells and native IFN-β, either with or without prior reduction. Both rIFN-β preparations produced by CHO cells and native IFN-β by fibroblasts migrated to a position of 24 kilodaltons (kDa), while rIFN-β produced by *E. coli* moved to that of 19 kDa.

When the three preparations of IFN-β were digested with glycopeptidase F and then subjected to SDS-PAGE, they all migrated to the position of 19 kDa (Fig. 7), thereby indicating that the difference in molecular size between rIFN-β produced by CHO cells as well as native IFN-β and rIFN-β produced by *E. coli* would be attributable to the lack of sugar moiety in rIFN-β of *E. coli*.

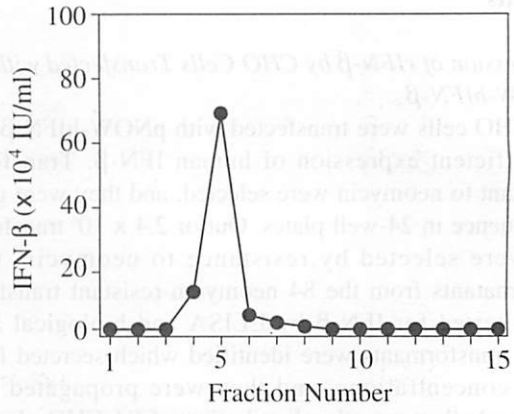


**Fig. 5.** Purification of rIFN-β in culture media by an affinity column of mAb. The pattern of rIFN-β eluted from the column is shown.

The N-terminal amino acid sequence of rIFN-β produced by CHO cells was determined. The first 21 amino acids of the product of the IFN-β gene has the signal peptidase cleaving sequence, and therefore, they are split and removed in the process of secretion. The sequence of nine amino acid residues of rIFN-β that followed, spanning 22nd to 30th positions, were determined to be MSYNLLGFL, which is identical to the sequence of the corresponding amino acids in the product of the IFN-β gene.

## Discussion

Many groups of investigators have attempted to express

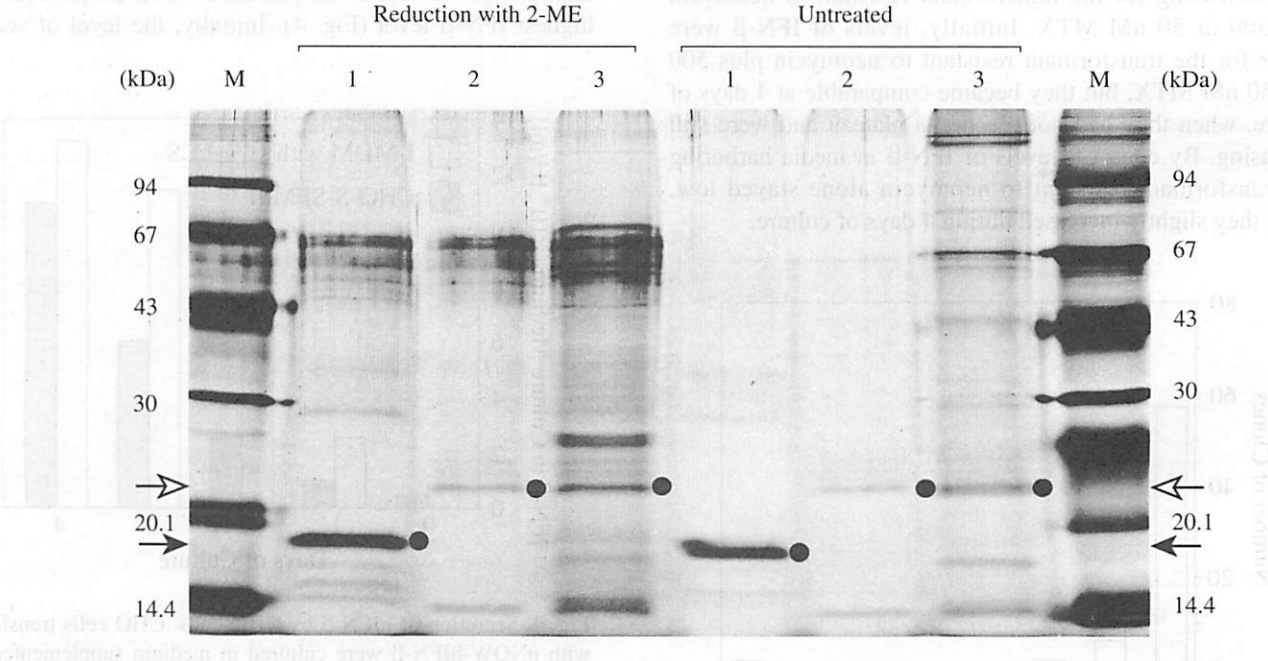


**Fig. 6.** SDS-PAGE of three preparation of IFN-β. Profiles are shown for IFN-β produced by *E. coli* (1), CHO cells (2) and fibroblasts (3), either with or without reduction by 2-ME in the presence of SDS. Bands representing IFN-β are marked by dots. Markers for molecular sizes were run in parallel on the both sides, and the position of small (19 kDa) and large (24 kDa) IFN-β are indicated by arrows with closed and open heads, respectively.

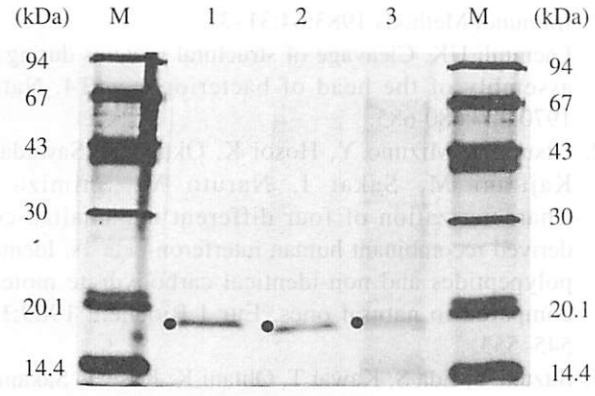


**Fig. 7.** Digestion of three preparation of IFN-β with glycopeptidase F. Profiles of SDS-PAGE are shown for IFN-β produced by *E. coli* (1), CHO cells (2) and fibroblasts (3). Bands representing IFN-β are marked by dots. Molecular markers were run on the both sides.

the IFN-β gene in host cells to obtain substantial quantities of rIFN-β in a glycosylated form, for the full expression of biological and biochemical characteristics. Thus, McCormick and his associates transformed mutant CHO cells dependent on dihydrofolate reductase (DHFR) [6] with a plasmid containing a human DNA sequence carrying the IFN-β gene linked to a DNA fragment encoding mouse DHFR [3]. Utsumi *et al.* reported the characteristics of recombinant human IFN-β expressed in four different mammalian cells [12]. Miyaji *et al.* documented the expression of recombinant human IFN-β in Namalwa KJM-1 cells [4].

To achieve high expression levels of recombinant proteins, it is necessary to improve the productivity per cell. In the present study, a novel vector pNOW/CMV-A was constructed for obtaining an efficient system for the expression of human IFN-β. This vector consisted of a weakened neomycin resistant gene (with enhancerless SV40 promoter and inefficiently transcribable neomycin phosphotransferase gene) and a weak DHFR gene (with enhancerless SV40 promoter). The CHO cells transformed with this vector usually exhibit a very weak resistance to neomycin. When such CHO transformants were selected in the medium with a usual concentration of neomycin, therefore, only the transformants can survive in which the construct is integrated into the transcirptional hot spot. Theoretically, primary transformants with this vector would produce large amounts of a foreign gene incorporated into this vector. The gene can be amplified readily by selecting derivatives resistant to MTX. Suzuki *et al.* expressed bovine conglutinin with a full biological activity by a similar system, and achieved a concentration of 18.6 mg/L after 4 days of culture [13]. Likewise, Ohtani *et al.* efficiently expressed human mannan-binding lectin by such a system and accomplished a concentration of 128 mg/L within a 4-day culture [7]. The novel expression vector (pNOW/CMV-A) represents a modification of the vector of Suzuki *et al.* [13] with substantial improvements for efficient production of recombinant proteins. As such, pNOW/CMV-A is hoped to be employed widely for the expression of many glycosylated proteins with desired biological activities.

In the present study, CHO cells transformed with the pNOW/CMV-A vector carrying the human IFN-β gene (pNOW-hIFN-β [Fig. 1]) secreted IFN-β in levels up to 1.5 x $10^5$ IU/ml in a primary culture during 4 days. Recombinant IFN-β expressed by CHO cells transformed with pNOW-hIFN-β migrated to the same position as native IFN-β on SDS-PAGE. When they were removed of their carbohydrate moieties, they both migrated to the same position, thereby indicating that they share the protein moiety, also. The N-terminal amino acid sequence was the same as that deduced from the nucleotide sequence of the IFN-β gene. Since recombinant IFN-β expressed in CHO cells, when it is freed of the carbohydrate moiety, migrated to the same position as that expressed in *E. coli*, the IFN-β gene would have been fully expressed in it. About 30% of cell clones secreted IFN-β in levels greater than 1 x $10^4$ IU/ml. A derivative resistant to 500 nM MTX produced IFN-β at the maximal concentration of 1 x $10^6$ IU/ml by 4 days of culture, which is 50-times higher than that of a native human IFN-β induced in human fibroblasts by poly(I)-poly(C) [12]. The efficiency of rIFN-β production achieved by CHO cells engineered with pNOW-hIFN-β was not to be compared with those reported by McCormick *et al.* [3] and Miyaji *et al.* [4], because the concentration of IFN-β in culture media was not given in IU/ml in their studies.

IFNs are widely used for treatment of viral infections in human beings. Although IFN-α is by far the most frequently used, IFN-β may have antiviral and immunomodulatory capacities distinct from those of IFN-α. Probably because of them, IFN-β is preferred in treatment of neurological disorders, typified by multiple sclerosis [14, 15]. Recombinant IFN-β may have the largest potential application in treatment of chronic viral hepatitis induced by hepatitis B virus [16] and hepatitis C virus [17]. In actuality, the patients who did not respond to IFN-α did respond to subsequent IFN-β [18]. Hepatitis B virus and hepatitis C virus are estimated to infect approximately 400 and 200 million people worldwide and can induce severe liver diseases ranging from chronic hepatitis through liver cirrhosis to eventual hepatocellular carcinoma. It is to be hoped that the indication of rIFN-β in a glycosylated form will be extended to many patients infected with hepatitis viruses over the world.

In conclusion, we have developed a system for the expression of recombinant human IFN-β in high levels and a glycosylated form in CHO cells. The production of rIFN-β by CHO cells clears concerns for contamination with genetic materials in commercial rIFN-β manufactured with fibroblasts. Furthermore, no animal sera were necessary to maintain CHO cells secreting rIFN-β, precluding fear associated with prions and other as yet unidentified pathogens in them. In the absence of animal sera, a high density culture of CHO cells in suspension will be feasible for enhanced yields, and enough amounts of human rIFN-β will be obtained for treatment of patients with a variety of viral infections.

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