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High-level expression of a chemically synthesized gene for human interferon-γ using a prokaryotic expression vector

(gene synthesis/DNA cloning/promoter/ribosome binding site/biotechnology)

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ABSTRACT **A chemically synthesized gene for human interferon-γ has been cloned into a prokaryotic expression vector under the regulation of a synthetic constitutive transcriptional–translational control unit that contains a strong bacteriophage T5 early promoter and a strong ribosome-binding site. Cells harboring the recombinant plasmid express high levels (4×10^9 units per liter of culture) of antiviral activity specific for interferon-γ. Analysis of total cell lysates on NaDodSO$_4$/polyacrylamide gels revealed a 17,200-dalton protein, expected for the nonglycosylated form of human interferon-γ, that constitutes >15% of total cell protein.**

Human interferon-γ (HuIFN-γ) is an interesting protein that exhibits a number of different biological activities. In addition to its antiviral activity, HuIFN-γ has been shown to exhibit potent immunomodulation and cell proliferation-inhibition properties (reviewed in ref. 1). Recently, the nucleotide sequence of its gene has been determined (2, 3), and its expression in *Escherichia coli* has been achieved by using natural control signals from either the *trp* operon (2) or the *lac* UV5 operon (4). However, the reported yields were relatively low compared to those for IFN-α (5–7) and IFN-β (8, 9).

Among the large numbers of DNA fragments containing bacterial promoters that have been used for *in vitro* binding studies, the bacteriophage T5 early promoters (T5P25 and T5P26) have been found to far exceed other promoter fragments in the rate of complex formation with *E. coli* RNA polymerase (10, 11). Recently, we have reported the chemical synthesis and insertion of the T5P25 promoter in front of either the tetracycline-resistance (Tc^R) gene or the chloramphenicol acetyltransferase (*CAT*) gene, and have demonstrated that the synthetic T5P25 promoter is highly efficient *in vivo* (12).

We have also shown that the introduction of a synthetic ribosome-binding site (RBS) in front of the coding sequence for simian virus 40 small tumor antigen, and its subsequent insertion at the *Pst* I site within the pBR322 ampicillin-resistance (Ap^R) gene, resulted in the synthesis of authentic simian virus 40 small tumor antigen in *E. coli* (13). Using this model system, we were able to compare the efficiency of different synthetic RBS sequences *in vivo* (14).

In this communication, we describe the construction of a plasmid expression vector containing both the synthetic T5P25 promoter and a strong synthetic RBS; this vector (pJP_1R_3) is used for the efficient expression of a synthetic gene for human IFN-γ.

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MATERIALS AND METHODS

Construction of the *IFN-γ* Gene. The deoxyoligonucleotides (Fig. 1) comprising the entire *IFN-γ* sequence including initiation and termination signals were chemically synthesized using a modified solid-phase phosphite method. Details of the synthesis and construction of the gene will be published elsewhere.

Construction of the pJP_1R_3 Expression Vector. The pJP_1 plasmid DNA, a derivative of pBR322 in which the Tc^R promoter between the *Eco*RI and *Hin*dIII sites has been replaced by the strong synthetic bacteriophage early T5P25 promoter (12), was linearized with *Hin*dIII. The ends were repaired using DNA polymerase I (Klenow fragment) to which a DNA duplex containing a RBS sequence was inserted. The recombinant DNA was used to transform competent *E. coli* (LE 392) cells (15). The resulting pJP_1R_3 plasmid DNA was isolated and characterized by standard methods as described (12). The nucleotide sequence of the transcriptional–translational control region in pJP_1R_3 is shown in Fig. 2.

Cloning of the *IFN-γ* Gene into pJP_1R_3 Vector. The assembled synthetic *IFN-γ* gene (0.1 pmol) containing *Hin*dIII cohesive sequences at the termini was ligated to a 4-fold excess of pJP_1R_3 DNA that had been cleaved with *Hin*dIII and in which the terminal phosphate groups were removed by treatment with calf intestine alkaline phosphatase (16). The reaction was carried out at 12.5°C for 18 hr in a mixture (25 μl) of 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP/T4 DNA ligase (100 units/ml). The ligation mixture was used to transform competent *E. coli* (LE 392) cells. Clones resistant to both ampicillin and tetracycline were selected using standard methods (15) for characterization by restriction mapping.

Preparation of Bacterial Extracts for Bioassay. The preparation of bacterial extracts and the bioassay of IFN-γ activity was carried out similar to the method described by Gray *et al.* (2) and as detailed in Tables 1 and 2.

Polyacrylamide Gel Analysis of Total Bacterial Extracts. Bacterial extracts of clones carrying the recombinant plasmids with the *IFN-γ* gene in either the correct or incorrect orientation were prepared by standard methods as detailed in Fig. 4 and analyzed on 15% NaDodSO$_4$/polyacrylamide gels according to Laemmli (17). Electrophoresis was carried out until the bromophenol blue dye had migrated 14 cm. The gels were stained with Coomassie brilliant blue R250 for analysis.

RESULTS AND DISCUSSION

Construction of pJP_1R_3-IFN-γ Plasmids. The chemical synthesis and enzymatic ligation of the complete *IFN-γ* gene is

Abbreviations: HuIFN, human interferon; RBS, ribosome-binding site.

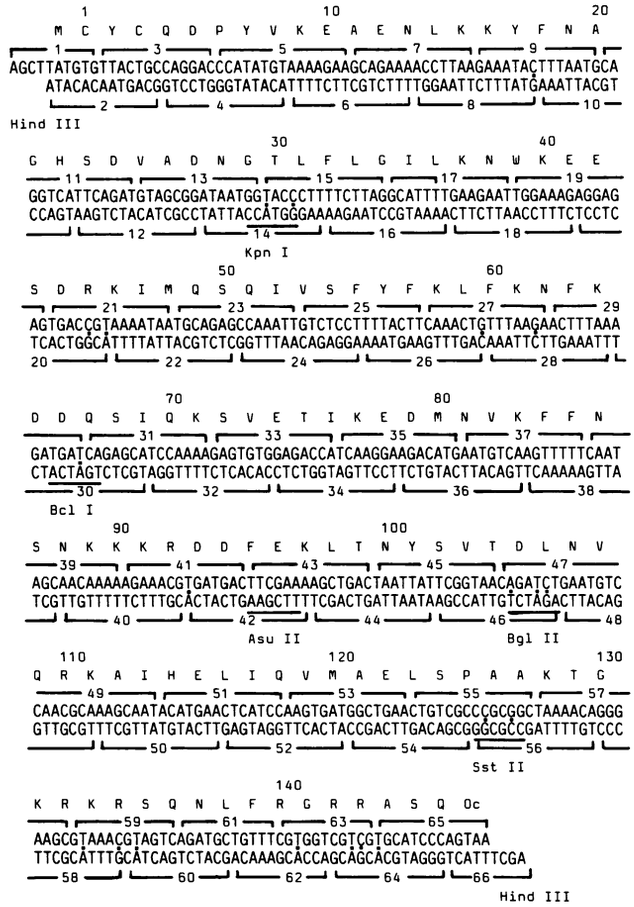

FIG. 1. Nucleotide and amino acid sequences of the synthetic *HuIFN-γ* gene. Individual synthetic oligonucleotides (numbered 1–66) are shown in brackets. Dots in between the duplex indicate changes with respect to the native sequence (2, 3). Numbers appearing above the amino acid sequence (represented by standard one-letter abbreviations) refer to the amino acid position in the native HuIFN-γ molecule. Single-cut restriction sites that have been introduced into the gene sequence are underlined. The DNA duplex is terminated with *Hin*dIII cohesive ends for insertion into the expression vector.

summarized in Fig. 1. Individual oligonucleotides were synthesized using a modified solid-phase phosphite method and were ligated together using T4 DNA ligase. Specific restriction endonuclease cleavage sites and base substitutions aimed at increasing the efficiency of translation in *E. coli* were introduced into the synthetic gene. Details on the design and construction of the gene will be published elsewhere.

Based on the results of our previous studies on promoters (12) and RBS (13, 14), we have constructed an expression vector containing both the synthetic T5P25 promoter and a synthetic RBS, as shown in Fig. 2. This plasmid vector (pJP_1R_3), a derivative of pBR322 that has the promoter for the Tc^R gene replaced by the T5P25 promoter (12), also contains a strong synthetic RBS located immediately downstream from the synthetic promoter. As seen in Fig. 2, insertion of the *IFN-γ* gene at the unique *Hin*dIII site in pJP_1R_3 would place it under the control of the synthetic transcriptional and translational signals.

Previous attempts to directly clone natural DNA fragments containing T5P25 promoter into plasmid vectors had not been successful (18). In anticipation that the introduction of strong constitutive control signals might affect the viability of the cell or result in an unstable plasmid vector, we have purposely placed both the synthetic *IFN-γ* gene (with the synthetic RBS) and the pBR322 Tc^R gene (with its natural RBS), arranged in tandem, under the control of the same synthetic T5P25 promoter (Fig. 2). Maintenance of recombinants in the presence of tetracycline would then ensure the expression of not only the Tc^R gene, but also the concomitant expression of the *IFN-γ* gene from the same promoter as a dicistronic mRNA. Such a selective pressure is expected to prevent mutations in the control region that would turn off both genes and render the transformants incapable of growth in the presence of tetracycline.

Since the *IFN-γ* gene could be inserted in either of two orientations, individual clones were screened for proper insertion of the gene by restriction cleavage using double digestion with *Bam*HI and *Kpn* I (Fig. 3, *Left*). Those recombinants that contain the gene in the desired orientation would give two fragments, the smaller of which is 698 base pairs long. Those that contain the gene in the wrong orientation would have a smaller fragment 435 base pairs long. Several clones containing the gene in the proper orientation were further characterized by digestion with restriction enzymes whose recognition sites were specifically introduced into the synthetic gene. Fig. 3 (*Right*) shows the digestion patterns for clone pJP_1R_3-IFN-γ171, which is in agreement with the expected pattern shown in Fig. 3 (*Left*). The nucleotide sequence of the complete insert has also been confirmed by the Maxam and Gilbert chemical cleavage method (19).

Expression of the *IFN-γ* Gene in *E. coli*. Total cell lysates of individual clones that contain the *IFN-γ* gene were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. Those clones that contain the *IFN-γ* gene in the correct orientation (clones 102, 171, and 172) expressed a new protein (Fig. 4, lanes 5–7), with an apparent molecular size of 17,200 Da, that is not present in clones that contain either the parental pJP_1R_3 vector (lane 3) or the recombinant plasmid (clone 110) with the *IFN-γ* gene in the wrong orientation (lane 4). The size of this new protein is exactly that expected for nonglycosylated IFN-γ (2). Its accumulation in rapidly growing cells amounted to ≈16.3% of the total cell protein. This level of steady-state accumulation is extremely high considering the relatively small size of the IFN-γ molecule. Previous reports on the expression of HuIFN-γ in *E. coli* have failed to detect any new protein in total cell lysates by $NaDodSO_4$/polyacrylamide gel electrophoresis (2, 4).

Expression and Characterization of the IFN-γ Produced in *E. coli*. To demonstrate that the IFN-γ synthesized by the bacterial clones containing the pJP_1R_3-IFN-γ recombinant plasmids is biologically active, cell extracts were tested for antiviral activity using a cytopathic effect-inhibition assay (Table 1). Each of the clarified cell extracts in 0.05 original culture volume was serially diluted in culture medium for an activity assay using the A549 human lung carcinoma cells challenged with encephalomyocarditis virus (2). All three of the pJP_1R_3-IFN-γ clones that contain the *IFN-γ* gene in the correct orientation produced high levels of antiviral activity (4×10^9 units per liter of culture), in correlation with the amount of 17.2-kDa protein detected by $NaDodSO_4$/PAGE analysis. This level of expression is at least 4–8 × 10^4-fold higher than those achieved using other prokaryotic expression vectors (2, 4). In contrast, clones harboring either the parental pJP_1R_3 vector or the pJP_1R_3-IFN-γ plasmid with the *IFN-γ* gene in the wrong orientation showed no detectable antiviral activity.

It is likely that the efficiency of the synthetic control unit can be further improved. The distance between the RBS and the ATG initiation codon in these pJP_1R_3-IFN-γ plasmids may not be optimal to allow the maximal rate of initiation at the level of protein synthesis. Preliminary evidence suggests that the efficiency may be significantly improved by simply increasing the distance between the promoter and the RBS (data not shown). It appears that the 5′-untranslated region

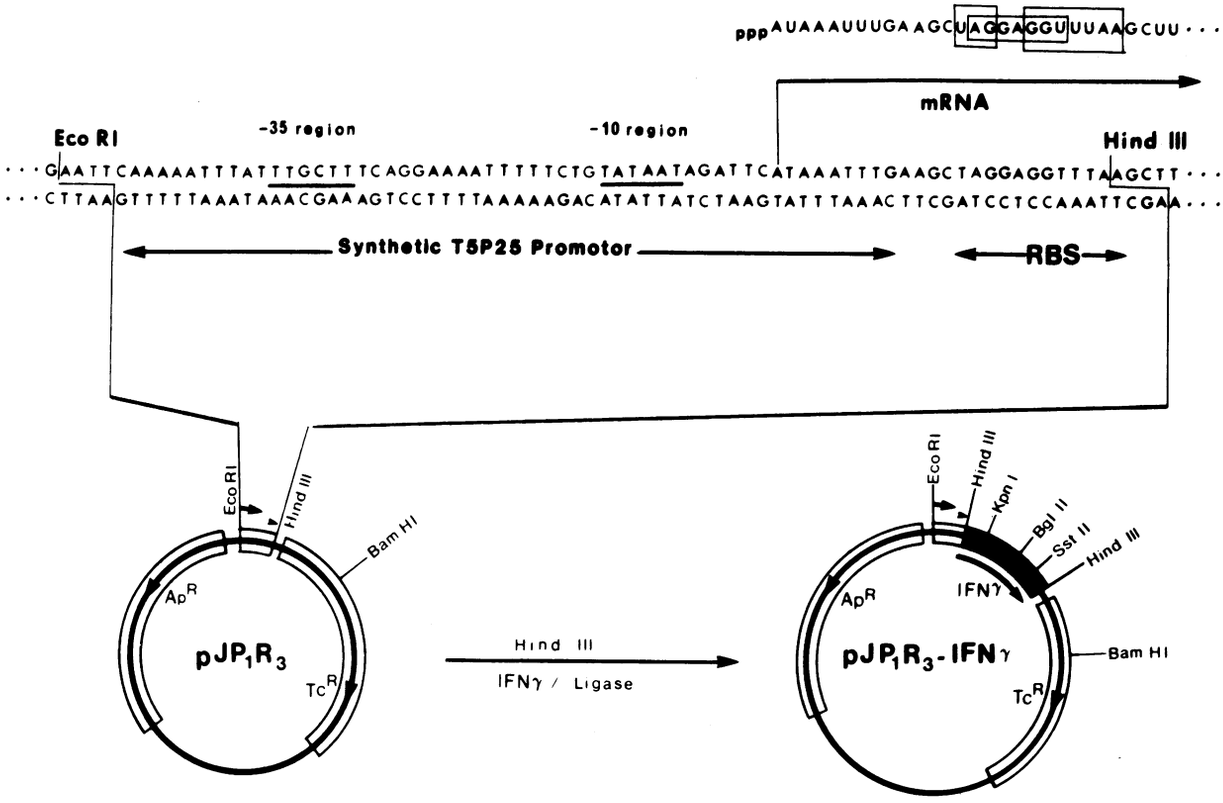

FIG. 2. Structure of the pJP_1R_3 expression vector and construction of pJP_1R_3-IFN-γ recombinant plasmids. The pJP_1R_3 vector is a derivative of pBR322 in which the Tc^R promoter, which lies between the *Eco*RI and *Hin*dIII sites, has been removed and replaced by a synthetic duplex containing both the bacteriophage T5 (T5P25) early promoter (12) and a strong RBS (13). Linearization of this plasmid with *Hin*dIII and insertion of the assembled *IFN-*γ gene would place the *IFN-*γ gene under the control of the synthetic regulatory signals.

of the mRNA in pJP_1R_3-IFN-γ may be too short to allow optimal binding of ribosomes during the initiation of translation.

The antiviral activity detected in the extract of one of the three positive clones (pJP_1R_3-IFN 171) was further tested for several characteristic physical properties of HuIFN-γ (Table 2). As with natural human IFN-γ, the bacteria-produced HuIFN-γ is sensitive to treatment at pH 2, as well as with 1% $NaDodSO_4$. The antigenic properties of the bacteria-produced IFN-γ were also compared with those of authentic HuIFN-γ. Both the human and the bacterial IFN-γ activities were neutralized by an antiserum specific to HuIFN-γ but were unaffected by an antiserum specific to HuIFN-α. (Antiserum against HuIFN-β was not available for this

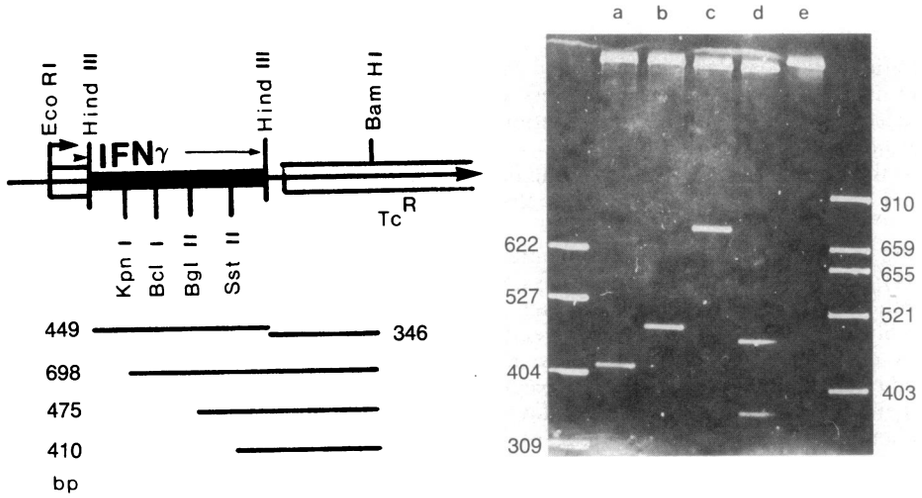

FIG. 3. Characterization of the pJP_1R_3-IFN-γ-171 recombinant plasmid by restriction mapping. Plasmid DNA from one of the pJP_1R_3-IFN-γ recombinant clones (number 171) that expresses IFN-γ activity was characterized by double digestion with *Bam*HI, which cleaves within the Tc^R gene, and one of several restriction enzymes whose cleavage sites have been introduced into the synthetic *IFN-*γ gene sequence. The digests were analyzed by electrophoresis in a 5% polyacrylamide gel. (*Left*) The restriction map of the region containing the *IFN-*γ gene. bp, Base pairs. (*Right*) The polyacrylamide gel-ethidium bromide pattern of the restriction enzyme digests of clone 171. Lanes: a, *Bam*HI/*Sst* II; b, *Bam*HI/*Bgl* II; c, *Bam*HI/*Kpn* I; d, *Bam*HI/*Hin*dIII; e, *Bam*HI alone. The molecular weight markers used were derived from pBR322 by digestion with either *Hpa* II (left lane) or *Alu* I (right lane).

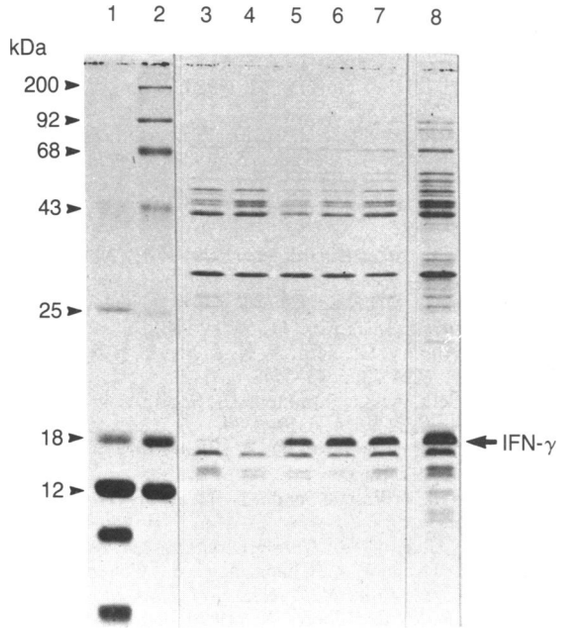

FIG. 4. Analysis of HuIFN-γ synthesized in bacteria. Transformants containing either the parental pJP_1R_3 expression vector or the pJP_1R_3-IFN-γ recombinant plasmid were grown in LB broth containing tetracycline (20 μg/ml). Overnight cultures were inoculated into fresh medium and grown to an OD_{600} of about 1.5. Cells corresponding to 3 A_{600} units were pelleted by centrifugation at 6000 rpm (Beckman JA20 rotor) for 5 min at 4°C. The cells were resuspended in lysing buffer (200 μl) containing 50 mM Tris·HCl, pH 6.8/2% $NaDodSO_4$/2% 2-mercaptoethanol/10% glycerol/0.01% bromophenol blue. After standing on ice for 7 min, the lysates were heated at 95–100°C for 5 min and clarified by centrifugation at 40,000 rpm (IEC A192 rotor) for 10 min at 4°C. The supernatants (10-μl aliquots) were analyzed on a 15% (acrylamide/bisacrylamide, 30:0.8) $NaDodSO_4$/polyacrylamide gel according to Laemmli (17). The gels were fixed and stained with Coomassie brilliant blue R250. Lanes: 1 and 2, molecular weight markers (Bethesda Research Laboratories); 3, lysate from pJP_1R_3; 4, lysate from pJP_1R_3-IFN-γ-110 in which the insert is in the wrong orientation; 5–7, lysates from pJP_1R_3-IFN-γ-102, -171, and -172, respectively, in which the inserts are in the correct orientation; 8, a darker exposure of lane 6 to reveal the less intensely stained bacterial proteins. The 17.2-kDa component is indicated by the arrow.

study.) We have shown that the bacteria-produced HuIFN-γ, which is presumably not glycosylated, is biologically active and exhibits biochemical and immunological properties similar to those of the natural protein.

It has been suspected that the insertion of efficient constitutive transcriptional–translational signals into expression vectors may result in unstable plasmids because of negative selection pressure (18). In our construction of the pJP_1R_3-IFN-γ recombinants, the *IFN-γ* gene was inserted upstream of the Tc^R gene in such a way that both genes would be under the control of the same synthetic T5P25 promoter. Transformants exhibiting Tc^R must, therefore, contain a functional T5P25 promoter, which allows the expression of a dicistronic mRNA that confers not only Tc^R but also encodes IFN-γ. Such a construction should assist in the selection and maintenance of cells that express high levels of *IFN-γ* or other genes cloned into this vector. Indeed, we have detected appreciably less IFN-γ either by $NaDodSO_4$ gel electrophoresis or by assaying for antiviral activity when recombinant clones were repeatedly cultured in medium containing ampicillin instead of tetracycline for extended periods. Preliminary analysis has suggested that the loss of expression of *IFN-γ* is the result of DNA insertions in the region of the synthetic promoter and RBS (unpublished results). This problem was not observed if the cells were maintained in the presence of tetracycline even after many passages, and we have no difficulty maintaining these recombinant clones. This strategy has thus allowed the cloning of efficient constitutive transcriptional–translational signals into expression vectors. Results suggest that the new pJP_1R_3 can be used as a general expression vector for high-level expression of genes cloned into the *Hin*dIII site.

In the present study, we have used a strong constitutive promoter, as opposed to inducible ones (20–22), for high-level expression of exogenous genes in *E. coli*. It is not obvious by examination of the nucleotide sequence why the T5P25 promoter is so efficient. Although it contains a perfect consensus Pribnow box (T-A-T-A-A-T), it has only three of the six consensus nucleotides at the −35 region. Studies in our laboratory have suggested that, in addition to the Pribnow box and the −35 sequence, a *Hin*dIII-like (A-G-C-T-T) sequence present around the −35 region may also be

Table 1. Expression of IFN-γ in *E. coli* carrying pJP_1R_3-IFN-γ

Sample	Antiviral activity: Units per ml of lysate	Antiviral activity: Units per liter of culture
pJP_1R_3-IFN-γ-102	80×10^6	4×10^9
pJP_1R_3-IFN-γ-171	80×10^6	4×10^9
pJP_1R_3-IFN-γ-172	80×10^6	4×10^9
pJP_1R_3-IFN-γ-110	<2	
pJP_1R_3	<2	

Bioassay of the HuIFN-γ activity produced in bacteria. Bacterial lysates for bioassay were prepared by inoculating overnight cultures grown in LB broth containing tetracycline (20 μg/ml) into fresh medium and allowing the cultures to reach an OD_{600} of 1.5. The cells were harvested and suspended in 0.05 original culture volume of 50 mM Tris·HCl, pH 8.0/30 mM NaCl/bovine serum albumin (1 mg/ml)/lysozyme (1 mg/ml). After standing on ice for 30 min, the cells were disrupted by sonication, and the cell lysates were clarified by centrifugation at 120,000 × *g* for 30 min. Antiviral activity in the lysates were quantitated by the inhibition of the cytopathic effect of encephalomyocarditis virus on a human lung carcinoma cell line (A549). The assays were carried out by serial dilutions (1:2) of the bacterial lysates in a 96-well microtitre plate (Falcon). pJP_1R_3-IFN-γ-102, -171, and -172 are independent clones that contain the *IFN-γ* gene in the correct orientation. pJP_1R_3-IFN-γ-110 contains the *IFN-γ* gene in the reversed orientation. pJP_1R_3 does not contain the *IFN-γ* insert.

Table 2. Characterization of IFN-γ produced in *E. coli*

Sample	Antiviral activity after treatment: Control	Anti-IFN-α	Anti-IFN-γ	pH 2
IFN-α	1000	8	1000	1000
IFN-β	1000	1000	1000	1000
IFN-γ	1000	1000	<4	<4
pJP_1R_3-IFN-γ-171	1600	1600	<4	<4

Characterization of IFN-γ produced in bacteria. Neutralization reactions were carried out by incubating a known amount of antiviral activity from bacterial lysates with sufficient antiserum to neutralize 2 times the amount of activity of the corresponding type of human interferon. The reactions were carried out in Earle's salts with 10% fetal calf serum for 16 hr at 4°C. Treatment of samples at pH 2 was carried out by titrating a known amount of antiviral activity with HCl to pH 2. After 16 hr at 4°C, the samples were neutralized with NaOH. All samples were adjusted to twice the pretreatment volume with Earle's salts before being assayed for antiviral activity. The natural HuIFN-α (GO23-901-527) at 20,000 units/ml, HuIFN-β (GO23-902-527) at 10,000 units/ml, and HuIFN-γ (Gg23-901-530) at 4000 units/ml were obtained from the National Institute of Allergy and Infectious Diseases (Bethesda, MD) and were used as antiviral units references for the respective interferon species throughout this study. Anti-IFN-α and anti-IFN-γ were obtained from Interferon Research (New Brunswick, NJ).

important for the binding of RNA polymerase (23). Thus, the *Hin*dIII-like sequence (T-G-C-T-T), which overlaps the −35 sequence (T-T-G-C-T-T) found in the T5P25 promoter, may be partially responsible for its high efficiency.

Modifications of both the promoter and the RBS sequences are being carried out to obtain a better insight into the roles of these sequences in regulating gene expression. These studies should also provide for the construction of even more efficient expression vectors.

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