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MODULATION OF EXPRESSION OF THE HUMAN GAMMA INTERFERON GENE IN E. coli BY SITE-DIRECTED MUTAGENESIS

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Plasmids expressing 2 forms of human immune interferon (IFN-γ) in E. coli have been constructed: 1) pIFNTacI which expresses IFN-γ with an N-terminal amino acid sequence of met-cys-tyr-cys-gln-, and 2) pIFNTacII which is a derivative of pIFNTacI from which the 9 base pairs (bp) coding for the cys-tyr-cys have been deleted. Quantitation of Western blots showed that approximately 10-fold more IFN-γ was produced in cells harboring pIFNTacII (7.5% of total cellular protein) as compared to pIFNTacI. The IFN-γ expressed in E. coli pIFNTacII is biologically active and routinely recoverable at 10^9 units per liter. When examined microscopically, IPTG induced E. coli harboring either plasmid construction contains prominent cytoplasmic inclusion bodies.

The complete cDNA sequence and the bacterial expression of human immune interferon (IFN-γ) have been previously reported by several laboratories (1-3). In these plasmid constructions, as well as those constructed in our laboratory, the amino terminus of native IFN-γ was assumed to be coding for cys-tyr-cys (1). The signal peptidase cleavage assignment gly-cys was based on a commonality of structure at the putative processing site between IFN-α and IFN-γ, since the 3 amino acids preceding the potential cleavage site (ser-leu-gly) are identical to those of a great majority of interferons (4,5). However, Rinderknecht et al. (6) demonstrated that the amino terminal amino acid of native IFN-γ is not a cysteine, but rather a blocked pyroglutamate, thereby necessitating the alteration of the primary IFN-γ DNA sequence in our E. coli expression vectors to reflect the native sequence. We report herein the deletion of the 9 bp DNA sequence which codes for the cys-tyr-cys residues (5) and subsequent alterations in expression efficiencies.

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An additional reason for making this specific 9 bp deletion was to investigate the potential role of the 2 cys residues in the formation of inclusion bodies within E. coli. The term inclusion bodies refers to the dense, insoluble aggregation of protein within E. coli where the products of foreign genes or truncated E. coli proteins are expressed at high levels (7,8). It has been postulated that improper folding of polypeptides could result in the inclusion body formation. Since 2 extra cys residues in cys-tyr-cys were not present in the mature native IFN-γ, this could alter intracellular linkage and subsequent inclusion body formation. We demonstrate that the deletion of cys-tyr-cys does not alter inclusion body formation.

MATERIAL AND METHODS

Bacterial Strains, Plasmids and Enzymes

E. coli strain 294 was obtained from ATCC (Rockville, MD) and E. coli strain JM103 was purchased from PL Biochemicals. Plasmids pGL101 and pEA108 were gifts from Dr. Tom Roberts. Bacteria were grown in M9 supplemented with thiamine, purified essential amino acids, and 0.5% glycerol as the carbon source. All DNA modifying enzymes were used according to the supplier's suggested protocols.

Library Construction and Identification of a Full-length IFN-γ Gene

Human peripheral blood leukocytes were obtained from buffy coats of normal donors. Cells were resuspended in RPMI-1640 containing 10% fetal calf serum and induced for 72 hr with the calcium ionophore A23187 and mezerein (9). RNA was extracted by homogenization in guanidine thiocyanate (10).

Poly(A)-containing RNA was isolated by poly(U)-Sepharose chromatography and size fractionated through a isokinetic sucrose gradient. Single-stranded cDNA (ss-cDNA) was synthesized with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, FL). Double-stranded cDNA (ds-cDNA) was synthesized from a ss-cDNA template with reverse transcriptase as described by Norgard et al. (11). The ds-cDNA was digested by incubation with S1 nuclease (11), and reaction products with a length of 500 bp or longer were tailed with dC and cloned into dG-tailed pBR322 at the PstI site.

Human immune interferon cDNA clones were identified by hybridization with a synthetic oligonucleotide (20-mer) which corresponded to published immune interferon sequences (1). Two positive clones were identified. A clone containing the 5' terminus was used as a probe to identify additional clones containing immune interferon sequences. A full-length clone was sequenced and used for further study.

Construction of IFN-γ Expression Plasmids

The plasmid pIFNTacI was constructed as follows (Figure 1). First, the full-length IFN-γ cDNA clone was digested with PstI and the 1.18 kb insert-containing fragment was isolated. This fragment was further digested with BstNI, and the 960 bp BstNI fragment was isolated by preparative acrylamide gel electrophoresis. The recessed 3' ends of the BstNI sites were filled in with the Klenow fragment of DNA polymerase I using conditions essentially as described in Maniatis (12). Synthetic oligonucleotides, prepared as described by Ito et al. (13) and Miyoshi et al. (14), were subsequently ligated to the IFN-γ cDNA. The phosphorylated 14-mer (400 pmol) was first annealed to 400 pmol of 16-mer and then blunt-end ligated to 13.6 pmol of the BstNI fragment of IFN-γ cDNA. Following overnight ligation at $4^{o}C$, the reaction mixture was supplemented with deoxyribonucleotides, and the Klenow fragment of DNA polymerase I was used to fill in the recessed 3' end as described above. The modified 960 bp BstNI fragment was subsequently purified by preparative acrylamide gel electrophoresis and ligated to equimolar amounts of PvuII-digested and alkaline phosphatase-treated pGL101. The resulting ligation mixture was used to transform E. coli MM 294. Transformants containing the appropriately modified IFN-γ cDNA were identified by duplicate hybridization with [^{32}P]-labeled 16-mer and with a [^{32}P]-labeled IFN-γ cDNA insert labeled by nick-translation. E. coli recombinants containing the correct orientation of the INF-γ cDNA insert were identified by hybridization analyses and restriction

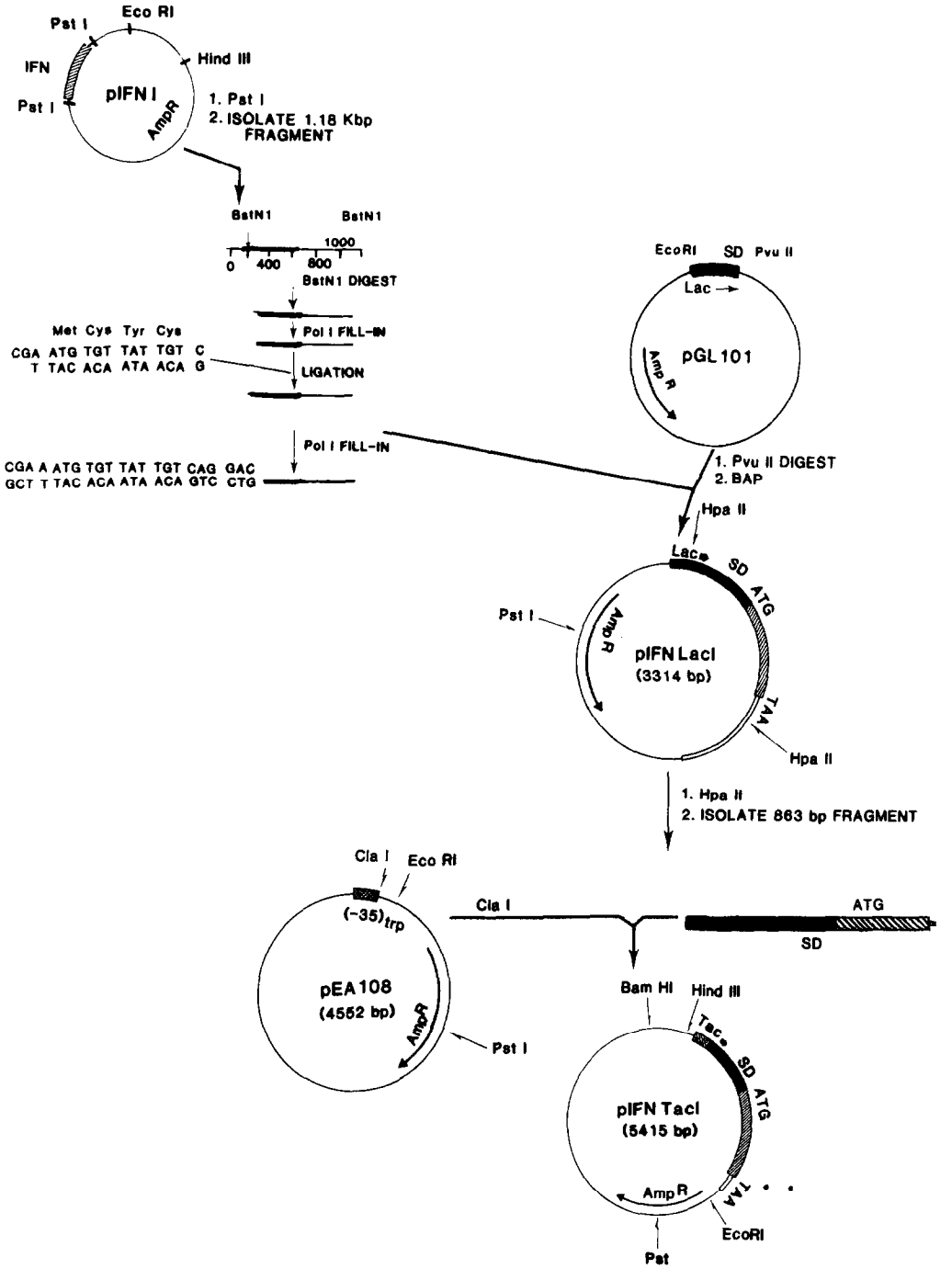

Figure 1. Construction of plasmid pIFNTacI.

endonuclease cleavage analyses. A single recombinant pIFNLacI was further characterized by nucleotide sequence analysis using the method of Maxam and Gilbert (15). The nucleotide sequence verified the presence of the lac promoter, the ribosome binding site (RBS) (16), a spacer of 8 bp between the RBS and the initiation codon, followed by the expected sequence at the 5' terminus of the coding region of IFN-γ messenger RNA (mRNA).

The plasmid pIFNTacI was constructed from pEA108 and pIFNLacI essentially as described by Bikel et al. (17). Briefly, the plasmid pEA108 (18) is a pBR322 derivative that contains a unique ClaI site adjacent to the -35 region of the E. coli trp promoter. An 863 bp HpaII fragment from pIFNLacI, containing the IFN-γ gene, ribosome binding site, lac operator, and lac promoter through base-21, was removed from pIFNLacI and cloned in the ClaI site of pEA108. In the resulting plasmid, pIFNTacI, the -35 consensus sequence of the trp promoter (cross-hatched area), is joined to the above desribed pIFNLacI sequence. Thus, the lac promoter was converted to a hybrid tac promoter with no modification of the IFN-γ coding sequence. The resulting plasmid was used to transform an E. coli $lacI^q$ strain, JM103.

Oligonucleotide Site-directed Mutagenesis of IFN-γ Gene

Plasmid pIFNTacI (10 μg) was digested with EcoRI and HindIII. The 1078 bp fragment containing the tac promoter, RBS and IFN-γ coding region was purified by preparative agarose gel electrophoresis and ligated into M13mp11 RF DNA (PL Biochemical) which had been previously digested with EcoRI and HindIII. After transformation of E. coli JM103, ss-DNA was prepared from clear plaques by established procedures (19).

A 19-base oligonucleotide was prepared by phosphotriester chemistry for use in generating the 9 bp deletion. The oligonucleotide sequence ATGGGTCCTGCATTCGCTG is complementary to the 9 bases immediately 5' to the desired deletion and 10 bases after the deletion. A stable DNA-DNA hybrid was formed between the ss-phage DNA containing the IFN-γ gene and the oligonucleotide, thus creating a 9-base loop in the complementary phage DNA strand. The standard procedures of Zoller and Smith (20) were used to create the desired mutation (Figure 2). A single recombinant, pIFNTacII, was subjected to nucleotide sequence analysis to confirm the 9 bp deletion.

Western Blot Analysis

Samples of bacterial culture harboring either pIFNTacII, induced or uninduced with IPTG (as described in the legend to Figure 3), were harvested by centrifugation, and the cell pellets were lysed in 100 μl of sample buffer. Samples were boiled for 5 minutes prior to loading, and 20-μl samples were analyzed by electrophoresis on duplicate 10-24% gradient SDS-polyacrylamide gels. At the end of the electrophoretic run, one gel was fixed and stained with Coomassie brilliant blue, and the relative mass of the stained protein was determined by scanning on a Beckman DU-8 densitometer. A second gel was processed for Western blot analysis. Protein was tranferred to a nitrocellulose membrane at 60 V for 5 h at 4°C. Western blot analysis was performed utilizing a specific IgG goat anti-human IFN-γ. Immune complexes were detected by employing the color development immunoassay of Bio-Rad which utilizes a horseradish peroxidase conjugated rabbit anti-goat IgG. Western blots were scanned for color intensity on a Kontes fiber optic scanner and plotted with a Hewlett Packard 3390A integrater.

Interferon Assays

Cells from IPTG-induced and uninduced cultures were pelleted and resuspended in 0.5 ml TS [50 mM Tris-HCl (pH 8.0); 10% sucrose] per 25 ml culture. Cells were lysed by adding 0.1 ml lysozyme (10 mg/ml freshly prepared in TS) and incubated at 4°C for 15 min. To the suspension was added 0.4 ml 0.25 M EDTA, 0.043 ml 5% human serum albumin, and 0.020 ml of 5% Triton X-100. The cells were sonicated on ice in three 10-second bursts. Cell lysates were centrifuged at 13,000 x g at 4°C to remove cell debris, and the supernatant removed. Immune interferon activity was assayed using serial dilutions of the lysate supernatants by quantitating 50% cytopathic effect reduction on human WISH cells challenged with vesicular stomatitis virus (9). Titers of IFN-γ were expressed in units based on NIH human reference IFN-γ (G-923-901-530) or human reference IFN-γ (G-093-901-529).

RESULTS

Analysis of the Expression in E. coli of IFN-γ which Differ in Their NH_2 Terminal Amino Acid Composition

The expression of met-cys-tyr-cys IFN-γ and met IFN-γ in E. coli from pIFNTacI and pIFNTacII was evaluated by immunochemical criteria and biological activity assays.

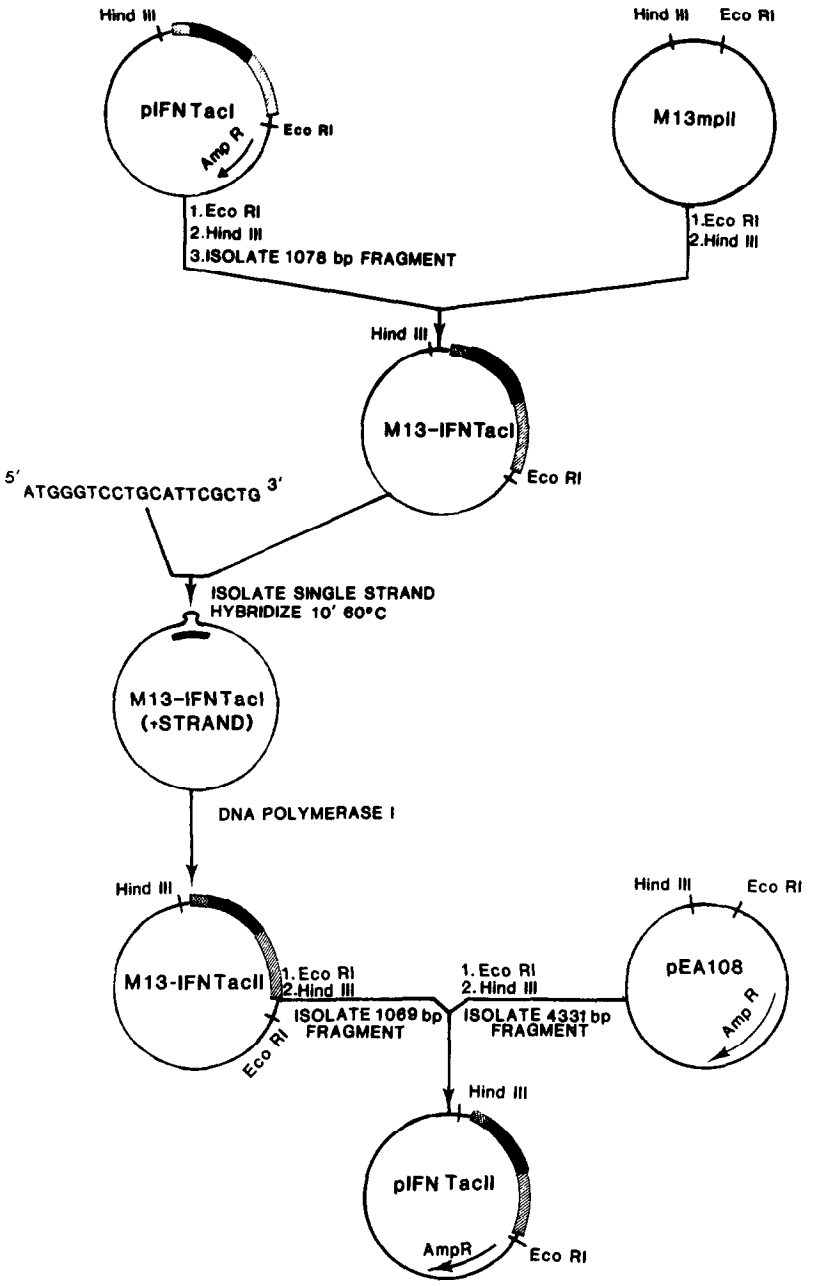

Figure 2. Construction of plasmid pIFNTacII.

Figure 3, Panel A, shows a comparison of the Coomassie blue staining pattern of proteins from cells carrying various plasmids which were either uninduced or induced with 5 mM IPTG. As expected, protein profiles of cells harboring the parental plasmid, pEA108, did not change significantly after the addition of IPTG (Figure 3, lanes 3 and 4). Lane 5 shows the protein staining pattern for JM103 with pIFNTacI grown uninduced (4 h at 30°C), while lanes 6 and 7 show the protein staining pattern of these cells following a 4 or 16 h induction with 5 mM IPTG. There is no appreciable change in the overall staining

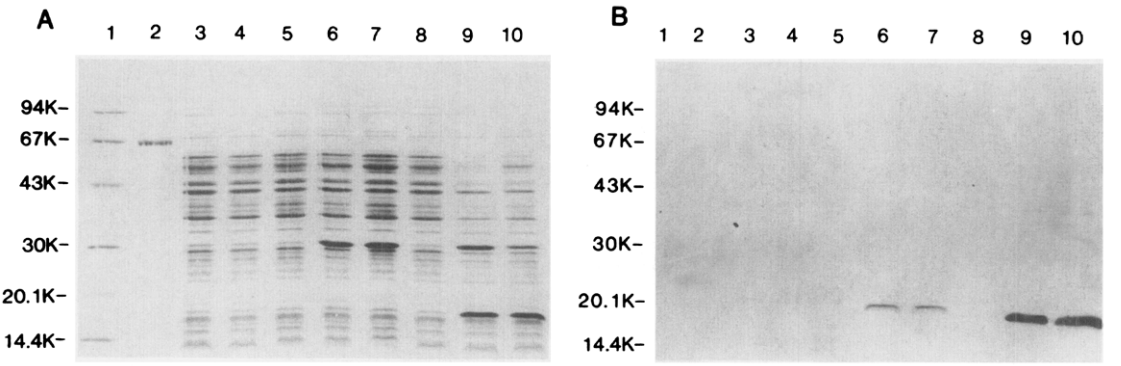

Figure 3. Detection of induced IFN-γ expression in JM103 hosts. Cultures harboring pEA108, pIFNTacI, and pIFNTacII were grown at 30°C in M9 medium, induced at an A_{550} of 0.8 with 5 mM IPTG, and further incubated for either 4 or 16 h. At these times, cells were collected by centrifugation and samples were prepared for electrophoresis as described in Materials and Methods. Uninduced samples of each were grown in parallel. Duplicate 10-24% linear gradient SDS-PAGE gels were electrophoresed in parallel at 100V for 11 h. Lane 1 in each gel contained molecular weight standards, and lane 2 contained 5 ng purified natural human IFN-γ (the only visible band, however, is human serum albumin, added to stabilize the purified IFN-γ). All other lanes contained 40 μg total cellular protein from the following samples: lane 3, pEA108, -IPTG, 4 h; lane 4, pEA108, +IPRG, 4 h; lane 7, pIFNTacI, +IPTG, 16 h; lane 8, pIFNTacII, -IPTG, 4 h; lane 9, pIFNTacII, +IPTG, 4 h; and lane 10, pIFNTacII, +IPTG, 16 h. The proteins in both gels were separated in parallel 11 h at 100V. Panel A--the gel was stained with Coomassie brilliant blue G, destained, photographed, and scanned at A_{565}. Panel B--from an identical gel, proteins were transferred to nitrocellulose and specific immunochemical detection for IFN-γ was performed as described in the Materials and Methods.

profile between the control strain and that carrying pIFNTacI. Figure 3A, lane 8, shows proteins from an uninduced 4-h culture of JM103 with pIFNTacII. As expected, the uninduced pattern of protein staining was indistinguishable from the preceding lanes. Incubation of this strain for 4 and 16 h following induction with IPTG (Figure 3A, lanes 9 and 10, respectively) results in the significant accumulation of a protein species with an approximate molecular weight of 18,000 daltons. This molecular weight estimation corresponds well with the value predicted for unglycosylated IFN-γ from the DNA sequence. Analysis of densitometric tracings indicate that the 18,000 dalton band represents approximately 7.5% of the total cellular protein. Maximum steady-state levels of the 18,000 dalton protein appear to be achieved by 4 h of induction, since little or no additional accumulation of IFN-γ occurs during the next 12 h of growth. In order to establish that the band which appears following IPTG induction was IFN-γ, a duplicate gel was processed for Western analysis and probed with goat anti-human IFN-γ IgG (Figure 3, panel B). In addition to detecting the 5 ng of natural IFN-γ standard (lane 2), met-cys-tyr-cys IFN-γ was detected in lanes 6 and 7, and an intense staining of met IFN-γ appeared in lanes 9 and 10. Although no IFN-γ band could be seen in induced pIFNTacI constructions (Figure 3A, lanes 6 and 7) by Coomassie blue staining, a Western blot analysis of these cells (Figure 3B, lanes 6 and 7) demonstrated immunologically detectable amounts of IFN-γ.

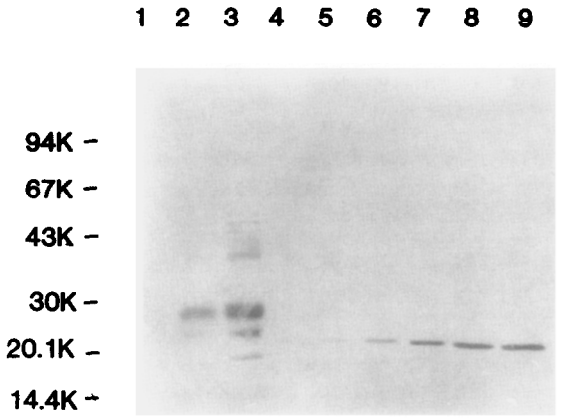

Figure 4. Quantitative Western blot analysis of IFN-γ. Panel A--natural human IFN-γ was loaded in lanes 1-3 of a 10-24% SDS-PAGE gel in amounts of 2 ng, 10 ng, and 40 ng, respectively. Increasing amounts of extracted proteins from pIFNTacI were loaded in lanes 4-7: 1.0 μg, 2.5 μg, and 5.0 μg, respectively. Total proteins from pIFNTacII were loaded in lanes 7-9: 1.0 μg, 2.5 μg, and 5.0 μg, respectively. Samples were separated through a polyacrylamide gel, transferred, and stained immunochemically as described in the text. Panel B--known amounts (1, 2, 5, 10, 40 ng) of IFN-γ were electrophoresed and processed for Western blot analysis as above (data not shown). Western blots were scanned and the area under each curve determined. The results are plotted as relative area versus the mass (ng) of IFN-γ.

Quantitation of Mass of IFN-γ Produced from pIFNTacI and pIFNTacII

In order to quantitate the amount of IFN-γ produced in these cells, a Western blot analysis was performed (Figure 4). Briefly, known amounts of IFN-γ and whole cell lysates of JM103 harboring pIFNTacI or pIFNTacII were separated by electrophoresis and processed for Western blot analysis. The Western blot analysis used for quantitation is shown in Figure 4. Individual lanes were scanned and areas under the peaks determined. Lanes 5-7 and 8-10 contain whole cell extracts from JM103-pIFNTacI and JM103-pIFNTacII, respectively, which had been induced for 4 h at 30°C. Analysis of these data indicate approximately 10-fold more IFN was produced in pIFNTacII when compared with pIFNTacI.

Biological Activity of the IFN-γ

The antiviral activity of the met-cys-tyr-cys IFN-γ and met IFN-γ in bacterial lysates was determined. E. coli harboring the pIFNTacI or pIFNTacII plasmids were grown at 30°C for 2 h and induced with IPTG and the cells were harvested at various times (Table 1). Biological activity of the IFN-γ in the cell supernatant was assayed by the inhibition of VSV-induced cytopathic effect on human WISH cells (see Materials and Methods).

Cells from either construction did not produce detectable amounts of IFN-γ prior to IPTG induction. The pIFNTacI-containing cells accumulated IFN-γ to a maximum level of 7×10^6 units per liter after 4 h of induction. In dramatic contrast, pIFNTacII-containing cells accumulated IFN-γ to levels greater than 10^9 units per liter. Depending on the time at which cells were harvested, the pIFNTacII-containing cells accumulated 50 to 500 times more units of biologically active IFN-γ. Prodution of IFN-γ to levels of

Table 1

IFN-γ Construction

Hours after induction	Cytopathic effect--units/liter		Relative increase II/I
	pIFNTacI	pIFNTacII	
2	2×10^6	1×10^8	50
4	7×10^6	8×10^8	115
6	3×10^6	10^9	300
8	2×10^6	10^9	500

10^9 units per liter were routinely observed using the pIFNTacII construction even without optimizing bacterial growth conditions.

Inclusion Body Formation

Microscopic examination of IPTG-induced E. coli carrying either pIFNTacI or pIFNTacII shows the formation of large opalescent inclusion bodies within the majority of cells. Cells not induced with IPTG appear normal. Inclusion body formation was observed as early as 0.5 h after induction and the size of these structures increased for at least 4 h. The majority of inclusion bodies form at one or both ends of the cells. An additional characteristic of the cells is that they become much larger and elongated relative to control cells. Since inclusion bodies are not disrupted by sonication or passage through a French press, inclusion bodies were purified from other cellular components based on differential centrifugation. Coomassie blue staining of proteins found within inclusion bodies revealed that greater than 50% of the mass was IFN-γ. The identity of this prominent band was confirmed by Western blot analysis.

DISCUSSION

The construction and evaluation of 2 plasmids which express 2 forms of IFN-γ has been presented. The purpose of altering a previously constructed IFN-γ plasmid (pIFNTacI) was both to delete DNA squences coding for the N-terminal amino acids cys-tyr-cys to more accurately reflect native IFN-γ and to test whether the 2 cys residues were the causative agent in inclusion body formation. Assay of biological activity from bacterial lysates results in a more striking comparison relative to the quantitative accumulation. Biologically active IFN-γ was recoverable from induced pIFNTacII cells at levels 50 to 500 times greater than from the pIFNTacI cells while the increase in IFN-γ mass was 10-fold. This difference may reflect that the met-IFN-γ protein is more soluble in E. coli, and therefore, more easily extracted than the met-cys-tyr-cys IFN-γ molecule. This could be explained by an alteration in the folding of the IFN-γ molecule lacking the 2 cys residues. Alternatively, the specific activity of the altered recombinant protein may be greater since it more closely resembles natural human IFN-γ.

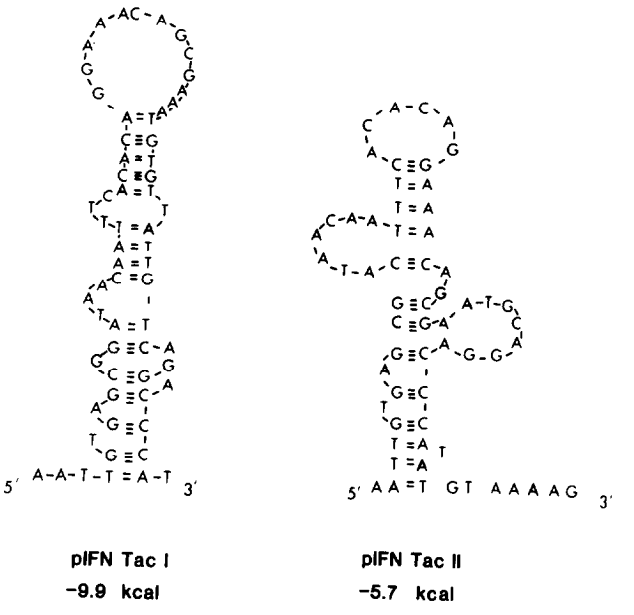

Figure 5. mRNA secondary structures which may form following transcription of pIFNTacI and pIFNTacII genes.

The absolute mass increase in IFN-γ accumulation in the pIFNTacII construction is most likely due to an increased efficiency of ribosome loading and subsequent translation. Tessier et al. (21) observed vast differences in production of IFN-γ when the sequence upstream of the initiator AUG codon was modified. A systematic comparison of mRNA secondary structure models for several variants suggested that the presence of stem-loop structures blocking translation initiation signals could drastically decrease the efficiency of IFN-γ synthesis. Computer-assisted analyses of the lowest free energy associated with secondary structure of the first 60 bases of mRNA made in pIFNTacI and pIFNTacII revealed significant differences in the possible stem-loop structures (Figure 5). As shown in Figure 5, pIFNTacI has a -9.9 kcal stem-loop structure while the mRNA from pIFNTacII has a -5.7 kcal structure. This difference suggests that the pIFNTacII mRNA is a relatively unstructured mRNA, and thus, translation initiation may be significantly more efficient than with pIFNTacI. Subsequent analysis of these constructions will require an investigation of mRNA stability and translational efficiency.

It was also of considerable interest to determine the effect of the removal of the cys-tyr-cys tripeptide on inclusion body formation. Microscopic analyses of induced cultures of E. coli containing either plasmid revealed the formation of inclusion bodies as quickly as 0.5 h after IPTG induction. This result strongly suggests that the cys-tyr-cys portion of the IFN-γ is not necessary for inclusion body formation since, with the removal of cys-tyr-cys, no other cysteines are left in the recombinant IFN-γ. Thus, aberrant disulfide bridge formation is not sufficient for inclusion body formation.

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