J. Microbiol. Biotechnol. (2001), **11**(6), 986–993

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Translation Initiation Factor IF1-Dependent Stimulation of 30 S Preinitiation Complex Formation: Rapid Isolation and fMet-tRNA Binding Activity of IF1

CHOI, SANG-YUN*, HYUN-JUNG KIM, JUNG-IK YANG, AND HYO-IL CHANG

Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

Received: July 2, 2001
Accepted: September 3, 2001

Abstract Translation initiation in prokaryotes involves the formation of a 30 S preinitiation complex, in which translation initiation factors play a role in the stimulation of fMet-tRNA (fMet) binding. However, the specific function and precise mechanism of initiation factor IF1 are still unclear. One reason hindering the study of IF1 is the difficulty in obtaining a functionally active factor with a high purity. In the present study, a large quantity of active IF1 was rapidly purified, obtained by the overexpression of the *infA* gene, and then used for a functional study. The induction of *infA* did not appreciably affect the growth rate of the protease-deficient strain *E. coli* AR68 harboring the IF1 overproducing plasmid. The level of IF1 obtained was approximately 1–2% of the total cell protein, which enabled the yield of highly purified IF1 (>98% pure) to be increased to 0.15 mg of IFl/g of cells. The IF1 was isolated within one day by the centrifugation of the ribosomal washed fraction, by ammonium sulfate fractionation, chromatography on batch of phosphocellulose, and FPLC Mono S. The overexpressed IF1 was found to be comparable to the factor isolated from normal cells, as determined by migration in NEPHGE/SDS 2-D gels. For binding of fMet-tRNA(fMet) to the 30 S ribosomal subunits, relatively high levels of binding were obtained when IF2 was present. The addition of IF1 up to 110 pmol proportionally stimulated the binding to a variable extent. This IF1-dependent stimulation of the 30 S preinitiation complex formation demonstrated that IF1 would appear to be exclusively essential for promoting the initiation phase of protein synthesis.

Key words: IF1, *infA*, ribosome, fMet-tRNA binding, NEPHGE/SDS 2-D gel

Studies on the process of protein synthesis have long been an important aspect of biology. For the last three decades, many advances in our knowledge on the major components and stages in protein synthesis have been made. However, the precise regulation of translation and the mechanism by which each component acts are not yet fully understood. Since protein synthesis is a key aspect of the overall pathway of gene expression and is the major metabolic process coupled to bacterial growth, elucidation of the mechanism of translation and its control is ultimately important. To understand the mechanism, the components involved in protein synthesis is needed along with how these components interact with each other.

In bacterial cells, protein synthesis involves the interaction of a large number of translational components, including three translation initiation factors [7]. Three initiation factors, IF1, IF2, and IF3, promote the formation of a 30 S preinitiation complex composed of the ribosomal 30 S subunit, initiation factors, GTP, mRNA, and N-formyl-Met-tRNA(fMet). Initiation factor IF2 is involved in the binding of the fMet-tRNA(fMet) of the 30 S subunit, and possesses a ribosome-dependent GTPase activity. The affinity of IF2 for the 30 S subunit is enhanced in the presence of IF1 and IF3 [29], while IF3 is unaffected by the other factors [28]. It has also been reported that the N-terminus of IF2 has an affinity per se to bind the 30 S subunit, while its C-terminal domains bind fMet-tRNA [11, 17, 27]. As the binding affinity to tRNA varies with the components in the complex, the specific binding of IF2 is involved in the stimulation of fMet-tRNA(fMet) [16]. IF3 prevents the association of ribosomal subunits, and catalyzes the translation of mRNA bound to ribosomes [5, 15, 26]. The molecular basis for the IF3 function is thought to be a conformational change of the 30 S subunit due to the binding of the factors [1].

The role of IF1 is less clear, yet it is known that IF1 is a basic protein (pI=8.9) and the smallest of the three initiation factors, with a mass of 8,119 Daltons. It has been reported that IF1 enhances the rates of both dissociation

*Corresponding author
Phone: 82-2-3290-3441; Fax: 82-2-927-3091;
E-mail: esychoi@korea.ac.kr

and association [4], increases IF2 binding to the 30 S subunit [14, 30], which may also stimulate the binding of fMet-tRNA(fMet) to the 30 S subunit, and increases the binding affinity of the AUG in the mRNA to the 30 S preinitiation complex. The gene for IF1, *infA*, has already been cloned [24] to examine the regulation of its expression and to determine whether or not this factor is required for cell viability [2]. This also facilitates the construction of overproducing strains to prepare large quantities of IF1 for biophysical studies, as described in this study. The gene *infA* maps at about 20 min on the *E. coli* genome, and encodes a protein of 72 amino acids. The native protein (71 amino acids) has no N-formyl-methionine [22]. A moderately strong Shine/Dalgarno sequence, GAGG, lies only 5 nucleotides upstream from the initiation codon. The transcription of *infA* is controlled by two promoters located at 245 bp and 65 bp upstream from the structural gene. S1 nuclease mapping indicates that *infA* is expressed as a monocistronic mRNA, and that a strong terminator lies 65 bp downstream from the *infA* termination codon, UGA, with a structure resembling rho-independent terminators [24].

The cloning of the gene for IF1 has facilitated a number of studies for the function of the factor. However, a precise understanding of how IF1 functions during the initiation phase of protein synthesis has not yet been achieved. One reason for the lack of knowledge is the difficulty in generating large quantities of the functional factor. As such, the development of an efficient process capable of purifying large quantities of the active factor would enable biophysical and functional studies. Accordingly, the current study presents a rapid purification procedure for IF1 in its native form, thereby allowing the characterization of the factor requirements in efficient protein synthesis judged by a sensitive activity assay.

MATERIALS AND METHODS

Bacterial Strain and Plasmids

E. coli strains JM101, which carries *lac*-i^Q; AR68, which is protease deficient *lon*$^-$, and IBPC 6321, which does not carry *lac*-i^Q nor a protease deficiency were examined as host cells for the IF-overproducing plasmid. *E. coli* strain AR68 was obtained from M. Rosenberg (Smith, Klein, and French CA, U.S.A.). Plasmid pTH2 carries *infA* on a 2.0 kb insert placed into the *Hin*dIII site of pBR322 [24]. To construct pCC-1 for IF1 overproduction, pTH2 was digested with *Bgl*I, and the 1,550 bp fragment containing the IF1 coding region was isolated. This fragment was treated with a Klenow fragment to create blunt ends, ligated to *Bam*Hl linkers, digested with *Mnl*I and *Bam*HI, and the resulting 250 bp fragment was purified. The 250 bp fragment was inserted into the *Sma*I and *Bam*HI sites of pUC19, and then excised by cleavage with *Eco*RI and *Pst*I. The isolated 290 bp fragment was inserted into the *Eco*RI and *Pst*I sites of pKK223 (Pharmacia) downstream from the *tac* promoter. The 290 nucleotide insert began 34 bp upstream from the AUG initiator codon and ended 37 bp downstream from the UGA terminator codon. The correct orientation of the insert in plasmid pCC-1 was confirmed by a restriction enzyme analysis.

Cell Growth

A single colony of freshly transformed *E. coli* AR68 bearing plasmid pCC-1 was used to inoculate 2×YT medium (16 g/l bactotryptone, 10 g/l bacto yeast extract, 5 g/l NaCl) supplemented with 100 μg/ml ampicillin, which was then grown with shaking at 37°C to the late exponential stage. For large-scale growth, the late exponential phase culture was used to inoculate 10 l of the same medium with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG) to give cell density of A_{595}=0.1. The 10 l culture was grown in a New Brunswick fermenter (Model MF-114) with aeration (1.5 volume of air/min) and shaking (800 rpm). Antifoam A (Sigma) was added up to a total of 0.03% when required. Growth was stopped in the early stationary phase when A_{595} was 6.6. The culture was immediately chilled to 0–4°C within 5 min by mixing with crushed ice. The cells were harvested by centrifugation at 5,000 rpm in a Sorvall HG-4L rotor, washed once with washing buffer (20 mM Tris-HCl, pH 7.5, 10 mM Mg-acetate, and 0.5 mM EDTA), and pelleted by centrifugation. The yields were typically 80–90 g of wet cell paste per 10 l batch. The cell paste was stored at −70°C.

Electrophoretic Analysis for Proteins

The analysis of the total protein preparations and chromatographic fractions was subjected to 15% SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) according to Laemmli [12]. The two-dimensional gel analysis using Nonequilibrium pH gradient electrophoresis (NEPHGE)-SDS PAGE was followed as described previously [9, 19] with minor modifications. The 50 S ribosomal proteins were extracted from the intact 50 S ribosome with glacial acidic acid as previously described [21]. The samples were applied on NEPHGE tube gels containing pH 3–10 ampholytes as previously described [19]. The electrophoresis was performed from the anode to the cathode at a constant voltage of 400 for 3.5 h. The gels were removed, soaked for 1 h in 5 ml of an SDS sample buffer [18], and then embedded on 15% SDS-PAGE gel. After running at a constant voltage, 100 volts for stacking and 150 volts for running, the gels were stained in 0.2% Coomassie blue in methanol/acetic acid/ water (5:1:5) and destained in the methanol/acetic acid/water solution. Photographs of the stained gels were scanned with a Quick Scan R&D microdensitometer.

Determination of Protein Level and Activity Assay

The protein concentrations were determined by the method of Schaffner and Weissmann [25] and Bradford using a Bio-Rad protein assay kit with bovine serum albumin as the standard. The relative level of IF1 produced from the cells was also quantitated by the immunoblotting method [8, 23]. The immunoblots were scanned with a microdensitometer. The IF1 activity was assayed by measuring the degree of formyl [^{14}C]methionyl-tRNA bound to either the 30 S subunit or the 70 S ribosomes with a given amount of initiation components in an *in vitro* assay system, as previously described [7] with minor modifications. Routinely, each IF1 activity assay contained 5.0 to 12 pmol of IF2, 30 pmol of either the 30 S subunit or the 70 S ribosomes, 20 μM A-U-G, 10 μg of unfractionated tRNA charged with 15 pmol [^{14}C]methionine in 20 mM Tris-HCl, pH 7.5, 100 mM NH_4Cl, 5 mM Mg-acetate, 1 mM dithiothreitol, and 1 mM GTP. After incubation for 5 min at 30°C, the reaction was terminated by adding 1 ml of a cold buffer (10 mM Tris-HCl, pH 7.5, 50 mM NH_4Cl, 10 mM Mg-acetate). Each assay mixture was filtered immediately through Whatman GF/C filters and washed. The filter was placed in 3 ml of scintillation cocktail and counted for radioactivity using a scintillation counter (Beckman LS-200).

Results and Discussion

Overexpression of *infA* and its Effect on Cell Growth

The effective overexpression of *infA* requires a strong promoter and strong translational initiation signal. Since high levels of IF1 might be detrimental to cell growth, an inducible promoter appeared to be desirable. The Shine/Dalgarno region and translation initiator codon of *infA* are both favorable for strong protein synthesis and therefore required no change. The overexpression vector pKK223 was selected for the present study as it carries a *tac* promoter that is inducible by IPTG. A 290 bp fragment was isolated as described in Materials and Methods that contained the entire *infA* coding region, 34 bp of the flanking DNA upstream of the initiator AUG including the Shine/Dalgarno region, and 37 bp of the DNA downstream from the UGA terminator included a transcriptional termination signal. This fragment was then inserted downstream from the *tac* promoter to create the plasmid pCC-1.

pCC-1 was transformed into a number of different *E. coli* strains to determine which host was best for the overproduction of IF1: JM101, which carries *lac*-i^Q; AR68, which is protease deficient; and IBPC 6321, which does not carry *lac*-i^Q or a protease deficiency. The growth rate and IF1 production of the three transformed strains were compared when grown in the presence or absence of the *tac* promoter inducer, IPTG. All three transformed strains with pCC-1 overproduced IF1, as determined by immunoblotting with an anti-IF1 antibody [8], yet the highest levels were obtained with AR68/pCC-1 (Table 1). It was estimated that AR68/pCC-1 overproduced IF1 approximately 50-fold compared with the MRE strain. An SDS/PAGE analysis showed that AR68/pCC-1 overproduced a much higher level of IF1 compared to AR68/pKK233 (Fig. 1). Accordingly, all of the subsequent studies were performed using this protease deficient strain. To optimize the extent of IF1

Table 1. Productivity of IF1-producing cells.

Host strains	Induction[a]	Increase (fold) in IF1 synthesis[b]
MRE600	-	1.0
JM101/pCC-1	+	8.3±1.4
IBPC 6321/pCC-1	+	9.8±0.9
AR68/pCC-1	+	48.5±4.6

[a]"-" indicates uninduced cultures and "+" specifies induced cultures with IPTG.

[b]The band corresponding to IF1 in the immunoblots was scanned as described in Materials and Methods, and the increase (fold) in IF1 is given as the level of IF1 relative to that produced in MRE600. All results with experimental errors (S.E.M.) are averages of at least three separate experiments performed in duplicate.

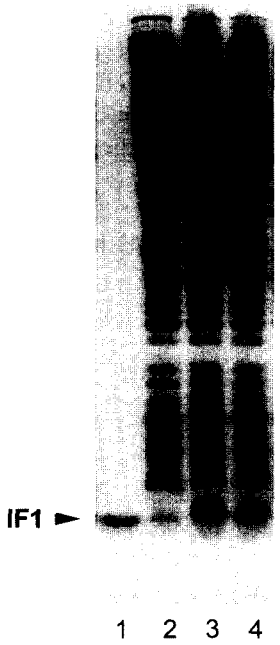

Fig. 1. Expression of IF1 upon induction with IPTG.
Stationary phase cells from 1 ml of culture were pelleted and lysed in a 100 μl gel sample buffer with sonication and boiling. The protein samples in the SDS buffer were subjected to electrophoresis in 15% polyacrylamide gels according to the method of Laemmli. Following electrophoresis, the gel was stained with Coomassie blue and photographed. Lane 1: classically purified IF1 from strain MRE600; lane 2: strain AR68/pKK233 with IPTG; lane 3: strain AR68/pCC-1 with IPTG; lane 4: strain AR68/pCC-1 without IPTG.

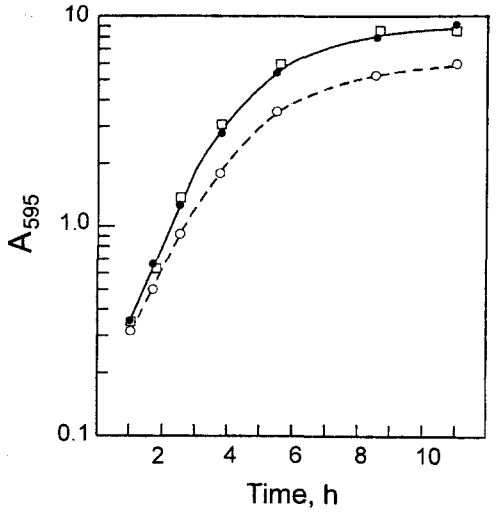

Fig. 2. Growth curves of *E. coli* AR68 cells with pCC-1 or pKK233.

E. coli AR68 cells carrying either pCC-1 or pKK233 and growing exponentially in 2× YT without IPTG were transferred to 25 ml 2× YT supplemented with ampicillin (100 μg/ml) and IPTG (0.1 mM) to give an A_{595} value of 0.1, as described in Materials and Methods. The cultures were incubated at 37°C in a New Brunswick rotary shaker, aliquots were taken at the indicated time points, and the A_{595} values were determined and plotted semi-logarithmically. (●), pCC-1 with IPTG; (□), pCC-1 without IPTG; (○), pKK233 with IPTG.

overproduction, the accumulation of IF1 was followed by SDS-PAGE from the exponential to the stationary phases. The apparent level of IF1 was maximal in the stationary phase and was only slightly enhanced by the presence of IPTG (results not shown). Therefore, strain AR68/pCC-1 was grown to the stationary phase in the presence of 0.1 mM IPTG. However, since the *tac* promoter in pCC-1 was not completely repressed, some IF1 was still produced in the absence of IPTG (lane 4 in Fig. 1). The presence of IPTG did not appreciably affect the growth rate of any of the strains. The growth rate of the strains carrying pCC-1 was either slightly higher or equal to that of the strains carrying pKK223 without the *infA* insert (see Fig. 2 for AR68). These results indicate that the induction of *infA* expression was neither detrimental nor beneficial to cell growth.

Purification of IF1

The procedure employed to purify IF1 deviated considerably from that devised for normal cells [7]. Briefly, the salt concentration of cell lysates were adjusted to 1 M before pelleting the ribosomes, and the post-ribosomal supernatant was fractionated by ammonium sulfate precipitation, batchwise adsorption and elution from phosphocellulose, and FPLC chromatography on a Mono S column.

Frozen *E. coli* AR68/pCC-1cells (20 g wet weight) were ground with 30 g of alumina, 15 μg of Dnase, and 30 ml of a grinding buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride) in a chilled motar for a total of 20 min [10]. The alumina was removed by centrifugation at 8,000 rpm for 10 min in a Sorvall SS34 rotor, and resuspended in 10 ml of the grinding buffer, and centrifuged again. The supernatants were combined and centrifuged at 16,000 rpm for 20 min in the Sorvall SS34 rotor. The supernatants were then removed and saved as the S30 fraction. The S30 was brought to 1 M KCl and centrifuged for 2.5 h at 55,000 rpm in a Beckman type Ti60 rotor. The post-ribosomal supernatant was fractionated with ammonium sulfate, and the precipitate obtained between 40 and 80% saturation was dialyzed against Buffer A (20 mM KH_2PO_4, pH 7.5; 7 mM β-mercaptoethanol; 0.5 mM PMSF) containing 150 mM KCl (called Buffer A-150). The dialysate was mixed with 8 ml phosphocellulose equilibrated in Buffer A-200 and stirred for 5 min, and the resin was packed in a sintered glass filter (2.2×5 cm) over a 2 ml pad of the equilibrated resin. The resin was washed with 40 ml of Buffer A-200, and the adsorbed protein was eluted with Buffer A-1000. The eluted protein was concentrated by precipitation with 80% saturated ammonium sulfate for 45 min, followed by dialysis of the resuspended precipitates for 3 h against 2×4 l of Buffer B (HEPES, pH 7.5; 7 mM β-mercaptoethanol; 0.5 mM PMSF) plus 150 mM KCl (Buffer B-150). The dialysate was clarified by centrifugation to yield an average of 7.2 mg protein in 36 ml.

As shown in Fig. 3A, a fairly large part of the proteins, except IF1 (lane 2), was removed after the phosphocellulose step, and the remaining eluted proteins contained IF1,

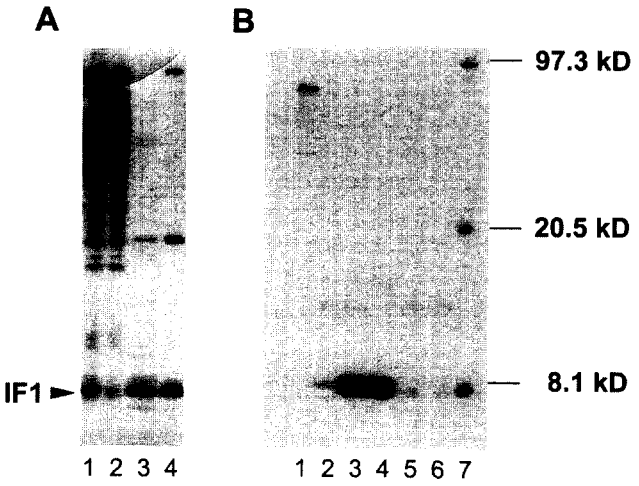

Fig. 3. Protein fractions following phosphocellulose and FPLC steps.

Panel A: Proteins before (lane 1) and after (lane 3) the phosphocellulose step were loaded on 15% SDS/PAGE. The preparation of the samples was performed as described in Materials and Methods. Panel B: Proteins after the phosphocellulose step was applied to an FPLC Mono S column. Lanes 3 and 4 show a highly purified IF1 fraction eluted at 210–230 mM KCl. Lanes 4 in panel A and 7 in panel B: IF2α (97.3 kD), IF3 (20.5 kD), and IF1 (8.1 kD) purified from MRE600.

which was approximately 50% pure (lane 3). Almost all the remaining impurities were removed by chromatography on an FPLC Mono S [20]. Figure 3 demonstrates that substantial purification was accomplished in the phosphocellulose step (panel A), and essentially homogeneous IF1 was obtained following Mono S chromatography (panel B). Batches of 1.2 to 1.8 mg protein were diluted with one volume of Buffer B-0 to reduce the KCl concentration to 75 mM, and then applied to FPLC Mono S HR 5/5 column, previously treated with 5 mg of crude *E. coli* proteins followed by 1 M KCl wash and re-equilibration in Buffer B-75. After the sample injection, the column was washed with 15 ml of Buffer A-75, and then developed with a 30 ml linear gradient of 75–500 mM KCl in Buffer B. The eluate was monitored for absorbance at 280 nm, and the fractions (0.8 ml) were examined for the presence of IF1 by SDS-PAGE (15%) and immunoblotting. It is noteworthy that the Mono S column became saturated with as little as 0.6–0.8 mg of pure IF1. When more than 1.2 mg of the IF1 preparation was applied, the IF1 eluted as a broad peak, presumably the result of overloading. However, with less than 1.2 mg of sample, the IF1 eluted as a single sharp peak. An HEPES buffer at pH 6.8 gave a somewhat better resolution than that at pH 7.5. The purified IF1 was then concentrated on mini-phosphocellulose columns, as previously described [7]. As shown in Fig. 3B, the major peak fraction eluted at 210–230 mM KCl contained highly purified IF1 (>98% pure). An overall yield of 3 mg IF1 was obtained from 20 g of wet cell paste. The yield of proteins following various purification steps is shown in Table 2. This rapid and efficient procedure enabled the production of a large quantity of IF1 sufficient to determine its function and structure.

Characterization of Overexpressed IF1

A rapid and facile method for purifying IF1 from overproducing cells has been developed. Such preparations may therefore be suitable for biochemical and biophysical studies of the factor. An analysis of the purified IF1 by one-dimensional SDS-PAGE (Figs. 1 and 3) indicated that a full sized IF1 protein was obtained rather than a partially degraded one. However, it was uncertain whether the protein was active and it was physically indistinguishable from classically prepared IF1 and from IF1 in crude cell lysates. Accordingly, a more sensitive physico-chemical comparison of the purified forms from overproducing and nontransformed cells (classically purified) was made by employing two-dimensional NEPHGE/SDS-PAGE (Fig. 4). To improve the comparison of where the IF1 preparations migrated, the proteins were co-electrophoresed with total 50 S ribosomal proteins, which provided internal standards according to their isoelectric points. The overproduced IF1 migrated to the same spot where the pI was 8.9 in the gel (panel D), as the classically purified IF1 (panel C) plus

Table 2. Yield of purification of IF1[a].

Purification step	Protein (mg)
S-30 fraction	1,100±155
High salt washes (1 M)	380±46
Ammonium sulfate (40–80%)	93.2±35
Phosphocellulose	7.2±0.9
Mono S	3.0±0.6

[a]The purification and quantification of the proteins were performed as described in Materials and Methods and Results. The values shown with experimental errors (S.E.M.) are derived from a typical preparation involving a 20 g wet weight of cells, and are averages of five separate experiments.

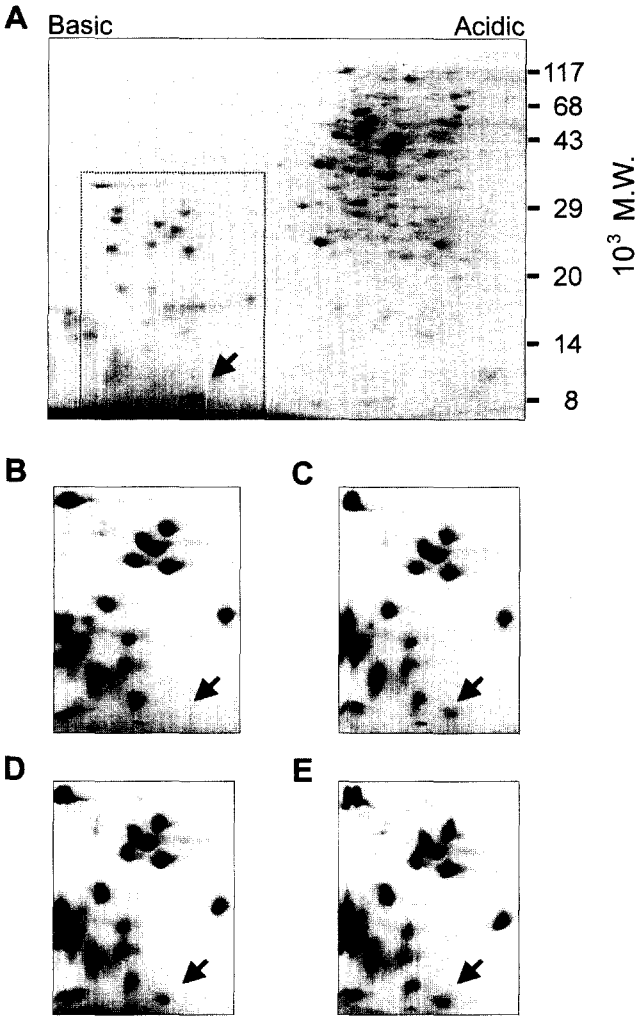

Fig. 4. Two-dimensional NEPHGE/SDS PAGE analysis.
The protein samples were analyzed by NEPHGE/SDS PAGE, stained with Coomassie blue, and photographed as described in Materials and Methods. Panel A: 100 μg S30 lysate of strain AR68/pCC-1; Panel B: 50 S ribosomal proteins alone; Panel C: 50 S ribosomal proteins plus classically purified IF1; Panel D: 50 S ribosomal proteins plus purified, overproduced IF1; Panel E: combined samples from panels C and D. Panels B-E indicate the boxed area in panel A. The arrows point to the IF1 spots.

the two IF1 preparations were not resolved from each other when co-electrophoresed (panel E). It is thus highly likely that the purified, overproduced IF1 molecule was intact and identical to native IF1. To detect more subtle differences in the covalent structure, more powerful analyses such as sequencing or mass spectroscopy would be required.

IF1 Activity in Stimulation of 30 S Preinitiation Complex Formation

The determination of the association properties for the 30 S subunit and initiation factors provided both a qualitative and quantitative analysis of the interaction of IF1 with the 30 S subunit. A group of 30 S ribosomal proteins cross-linked to IF1 were identified using a bifunctional protein cross-linking technique [13]. Recently, a more precise interactiing region on a ribosomal A site was identified by the use of chemical probes [3]. Besides the localization of the IF1 binding site, the kinetics of association of IF1 were also studied using covalent fluorescent-labeled IF1 prepared as a high purity [30]. The association constant for the (30 S:IF1) complex showed that IF1 bound weakly to the 30 S subunit without the other initiation factors, whereas the affinity of IF1 was significantly enhanced in the presence of IF2 and IF3. However, the role of IF1 in stimulated binding activity is not clear. A 50 S subunit joined with the 30 S preinitiation complex to form a 70 S initiation complex with the hydrolysis of GTP and the release of the initiation factors, resulting in a transition from the initiation to the elongation phases.

In order to determine the factor required for the initiation complex formation, activity assays were performed with different combinations of the initiation factors in the reaction mixture for their stimulation of fMet-tRNA(fMet) binding to either the 30 S subunit or the 70 S ribosomes. Table 3 predicts the effect of the initiation factors on the binding of formylmethionyl-tRNA. As expected, IF2 was critical, especially in the association with the 30 S subunit. However, it was noticeable that IF1 alone had no effect, yet it stimulated the IF2 activity appreciably. When IF2 was tested separately, the binding of [^{14}C] fMet-tRNA to the 30 S subunit was stimulated to some extent even in the absence of IF1. When 70 S ribosomes were used for the initiation complex formation, a different pattern of stimulation was observed. The binding of fMet-tRNA(fMet) with the 70 S ribosomes was generally higher, yet decreased depending on either IF1 or IF2 when compared to that with the 30 S subunit. A comparison of the activity between the overproduced IF1 and the classically purified IF1 was also performed simultaneously to verify their identities. The activities of the overproduced IF1 were not different from those of the classically purified IF1. The similar activities and points of saturation thus indicated that the proteins had identical specific activities. Accordingly, the proposed simple purification procedure would appear to be capable of providing a large quantity of highly pure factors, which were indistinguishable from those obtained by the laborious classical purification procedure.

Table 3. Requirement of initiation factors for the initiation complex formation.

	Formylmethionyl-tRNA binding[a]	
	30 S	70 S
None	120±11	335±8.9
IF1	125±3.5	330±6.7
IF2	720±14	615±19
IF1+IF2	1165±32	860±21
IF1[b]+IF2	1155±30	-

[a]The formylmethionyl-tRNA binding assays were carried out as described in Materials and Methods. The amounts of the factors added were: overexpressed IF1, 62.5 pmol; IF2a, 12.6 pmol; 30 S subunit or 70 S ribosomes, 30 pmol. The incorporation of formyl [^{14}C]Met-tRNA was determined by trichloroacetic acid precipitation and filtration. All results are represented as counts per minute. Experimental errors represent the S.E.M. over three independent experiments.

[b]Classically purified IF1 was used in this assay.

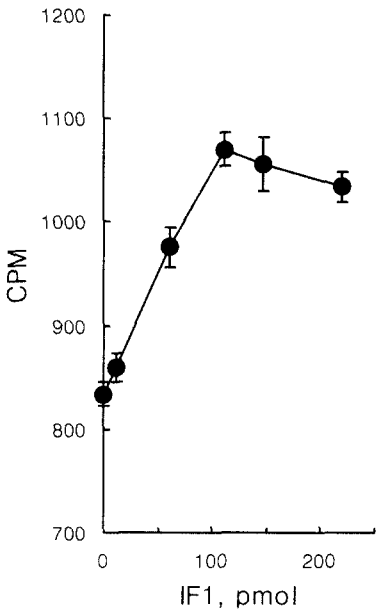

Fig. 5. IF1-dependent fMet-tRNA(fMet) binding to the 30 S ribosomes.

The IF1 stimulation of formylmethionyl-tRNA binding assays was carried out as described in Materials and Methods. The activity was assayed by measuring the degree of formyl [^{14}C]methionyl-tRNA bound to the 30 S subunit with a given amount of translation initiation components. After incubation for 5 min at 30°C, the reaction was terminated. Each assay mixture was filtered immediately. The filter was counted using a scintillation counter. The key ingredients per reaction mixture were: 30 S ribosomes (30 pmol); IF2α (12 pmol); 20 mM A-U-G: 1 mM GTP; 7 pmol of unfractionated tRNA charged with [^{14}C]methionine; purified overproduced-IF1 as indicated.

Although IF1 appeared to stimulate IF2 activity in the initiation complex formation, it was uncertain whether the initiation complex formation was dependent on the level of IF1. Therefore, activity assays were performed with different amounts of IF1 in the reaction mixture, then their stimulation of fMet-tRNA(fMet) binding to the 30 S subunit in the presence of IF2 and triplet A-U-G (Fig. 5) was assayed. In the binding assay, the formation of the 30 S preinitiation complex was moderately stimulated even in the presence of a low concentration of both IF1 and IF2. However, the addition of IF1 caused an additional stimulation of the complex formation through fMet-tRNA(fMet) with triplet A-U-G and ribosomes, which might explain the somewhat beneficial effect on the growth rate of cells overexpressing IF1. The IF1 proteins stimulated the binding with increasing amounts of IF1 up to 110 pmol of protein, after which no further increase in binding occurred. Even in the absence of IF1, the binding of [^{14}C] fMet-tRNA to the 30 S subunit was still stimulated to some extent. Taken together, these observations indicated that IF2 was the most critical factor for the basal level of initiation. However, the addition of IF1 was required for maximal stimulation of the binding. Therefore, these *in vitro* experimental results demonstrated that the formation of the 30 S preinitiation complex depends on the availability of both IF1 and IF2, and that IF1 is essentially required for promoting the initiation phase of protein synthesis.

Acknowledgment

This work was supported in part by a research fund from Korea University to S. Y. Choi.

References

1. Chiaruttini, C., M. Milet, and M. Springer. 1997. Translational coupling by modulation of feedback repression in the IF3 operon of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **94:** 9208–9213.
2. Cummings, H. S. and J. W. B. Hershey. 1994. Translation Initiation Factor IF1 is essential for cell viability in *Escherichia coli*. *J. Bact.* **176:** 198–205.
3. Dahlquist, D. D. and J. D. Puglisi. 2000. Interaction of translation initiation factor IF1 with the *E. coli* ribosomal A site. *J. Mol. Biol.* **299:** 1–15.
4. Godefroy-Colburn, T., A. D. Wolfe, L. Dondon, M. Grunberg-Manago, P. Dessen, and D. Pantaloni. 1975. Light-scattering studies showing the effect of initiation factors on the reversible dissociation of *E. coli* ribosomes. *J. Mol. Biol.* **94:** 461–478.
5. Gualerzi, C. O., C. L. Pon, R. T. Pawlik, M. A. Canonaco, M. Paci, and W. Wintermeyer. 1986. Role of initiation factors in *Escherichia coli* translational initiation, pp. 621–641. *In* Hardesty, B. and G. Kramer (eds.). *Structure, Function, and Genetics of Ribosomes.* Springer-Verlag, New York, U.S.A.
6. Hershey, J. W. B. 1980 The translational machinery: Components and mechanisms, pp. 1–68. *In* Prescott, D. M. and L. Goldstein (eds.). *Cell Biology: A Comprehensive Treatise.* Academic Press, New York, U.S.A.
7. Hershey, J. W. B., J. Yanov, and J. Fakunding. 1979. Purification of protein synthesis initiation factors IF1, IF2, and IF3 from *Escherichia coli*. *Methods Enzymol.* **60:** 3–11.
8. Howe, J. G. and J. W. B. Hershey. 1981. A sensitive immunoblotting method for measuring protein synthesis initiation factor levels in lysates of *Escherichia coli*. *J. Biol. Chem.* **256:** 12836–12848.
9. Howe, J. G. and J. W. B. Hershey. 1983. Initiation factor and ribosome levels are coordinately controlled in *E. coli* growing at different rates. *J. Biol. Chem.* **258:** 7228–7235.
10. Jung, H. J., H. Kim, and J.-I. Kim. 1999. Purification and characterization of CO^{2+}-activated extracellular metalloprotease from *Bacillus* sp. JH108. *J. Microbiol. Biotechnol.* **9:** 861–869.
11. Krafft, C., A. Diehl, S. Laettig, J. Behlke, U. Heinemann, C. L. Pon, C. O. Gualerzi, and H. Welfle. 2000. Interaction of fMet-tRNA(fMet) with the C-terminal domain of translational initiation factor IF2 from *Bacillus stearothermophilus*. *FEBS Lett.* **471:** 128–132.
12. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
13. Langberg, S., L. Kahan, R. R. Traut, and J. W. B. Hershey. 1977. Binding of protein synthesis initiation factor IF1 to 30 S ribosomal subunits: Effects of other initiation factors and identification of proteins near the binding site. *J. Mol. Biol.* **117:** 307–319.
14. Majumder, R. 1971. Studies on polypeptide chain initiation factors F1 and F2. *FEBS Letters* **18:** 64–66.
15. McCutcheon, J. P., R. K. Agrawal, S. M. Philips, R. A. Grassucci, S. E. Gerchman, W. M. Jr. Clemons, V. Ramakrishnam, and J. Frank. 1999. Location of translation initiation factor IF3 on the small ribosomal subunit. *Proc. Natl. Acad. Sci. USA* **96:** 4301–4306.
16. Meunier, S., R. Spurio, M. Czisch, R. Wechselberger, M. Guenneugues, C. O. Gualerzi, and R. Boelens. 2000. Structure of the fMet-tRNA(fMet)-binding domain of *B. stearothermophilus* initiation factor IF2. *EMBO J.* **19:** 1918–1926.
17. Moreno, J. M., J. Kildsgaard, J. Siwanowicz, K. K. Mortensen, and H. U. Sperling-Petersen. 1998. Binding of *Escherichia coli* initiation factor IF2 to 30 S ribosomal subunits: A functional role for the N-terminus of the factor. *Biochem. Biophy. Res. Comm.* **252:** 465–471.
18. O'Farrell, P. Z. 1975. The identification of prereplicative bacteriophage T4 proteins. *J. Biol. Chem.* **250:** 4007–4021.
19. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12:** 1133–1141.

20. Park, Y. S., M. H. Cha, W.-M. Yong, H. J. Kim, I. Y. Chung, and Y. S. Lee. 1999. The purification and characterization of *Bacillus subtilis* tripeptidase (PepT). *J. Biochem. Mol. Biol.* **32:** 227–232.
21. Pettersson, I., S. J. S. Hardy, and A. Liljas. 1976. The ribosomal protein L8 is a complex L7/L12 and L10. *FEBS Lett.* **64:** 135–138.
22. Pon, C. L., B. Wittmann-Liebold, and C. Gualerzi. 1979. Elucidation of the primary structure of initiation factor IF-1: Structure-function relationships in *E. coli* initiation factors. *FEBS Lett.* **101:** 157–160.
23. Ryou, C., J.-B. Kim, and M. Kwon. 2000. Immunochemical studies on expression of quinoproteins in *Escherichia coli*. *J. Microbiol. Biotechnol.* **10:** 95–98.
24. Sands, J. F., H. S. Cummings, C. Sacerdot, L. Dondon, M. Grunberg-Manago, and J. W. B. Hershey. 1987. Cloning and mapping of *infA*, the gene for protein synthesis initiation factor IF1. *Nucleic Acids Res.* **15:** 5157–5168.
25. Schaffner, W. and C. Weissmann. 1973. A rapid, sensitive, and specific method for the determination of protein in dilute solution. *Analyt. Biochem.* **66:** 502–514.
26. Shapkina T. G., M. A. Dolan, P. Babin, and P. Wollenzien. 2000. Initiation factor 3-induced structural changes in the 30 S ribosomal subunit and in complexes containing tRNA(f)(Met) and mRNA. *J. Mol. Biol.* **299:** 615–628.
27. Spurio, R., L. Brandi, E. Caserta, C. L. Pon, C. O. Gualerzi, R. Misselwitz, C. Krafft, K. Welfle, and H. Welfle. 2000. The C-terminal subdomain (IF2 C-2) contains the entire fMet-tRNA binding site of initiation factor IF2. *J. Biol. Chem.* **275:** 2447–2454.
28. Weiel, J. and J. W. B. Hershey. 1981. Fluorescence polarization studies of the interaction of *Escherichia coli* protein synthesis initiation Factor 3 with 30 S ribosomal subunits. *Biochemistry* **20:** 5859–5865.
29. Weiel, J. and J. W. B. Hershey. 1982. The binding of fluorescein-labeled protein synthesis initiation factor 2 to *Escherichia coli* 30 S ribosomal subunits determined by fluorescence polarization. *J. Biol. Chem.* **267:** 1215–1220.
30. Zucker, F. H. and J. W. B. Hershey. 1986. Binding of *Escherichia coli* protein synthesis initiation factor IF1 to 30 S ribosomal subunits measured by fluorescence polarization. *Biochemistry* **25:** 3682–3690.