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Tandem translation of *Bacillus subtilis* initiation factor IF2 in *E. coli*

Over-expression of $infB_{B.su}$ in *E. coli* and purification of α- and β-forms of $IF2_{B.su}$

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The protein synthesis initiation factor, IF2, in *Bacillus subtilis* has previously been characterized as being present in two forms, α and β, of molecular mass 79 and 68 kDa, respectively, on the basis of their cross-reaction with anti-*E. coli* IF2 antibodies and by the DNA sequence of the gene for IF2, $infB_{B.su}$. In this work we have cloned $infB_{B.su}$ in *E. coli* cells. Two proteins of molecular mass identical to the *B. subtilis* IF2α and -β were over-expressed and purified using a new three-step ion-exchange chromatography procedure. The N-terminal amino acid sequence of the two proteins was determined and the results confirmed that the two forms were IF2α and -β, both encoded by the *infB* gene. The N-terminal amino acid sequence determined for IF2β is Met^{94}-Gln-Asn-Asn-Gln-Phe. The presence of methionine at position 94 shows that this form is, in fact, the result of a second translational initiation in $infB_{B.su}$ mRNA, since the codon at amino acid position 94 is GUG, which is the normal codon for valine, but also known to be an initiator codon. This is a new example of the unusual tandem translation in *E. coli* of an open mRNA reading frame.

Bacillus subtilis; Translation initiation factor IF2; Recombinant protein expression; Protein purification

1. INTRODUCTION

The initiation of mRNA translation has been extensively studied in the Gram-negative bacterium, *Escherichia coli*. Three initiation factors have been identified, IF1, IF2 and IF3. The initiation factor, IF2, plays a major role; it promotes the binding of $fMet\text{-}tRNA_f^{Met}$ to the 30 S ribosome particle and hydrolyses GTP [1]. In *E. coli* IF2 exists in two size classes: IF2α (97.3 kDa) and IF2β (79.7 kDa). The β-form is translated from an in-frame translation initiation site at GUG^{158}, as suggested by nucleotide sequence analysis [2] and later by protein sequence analysis [3,4]. A third form, IF2β' (78.8 kDa) has recently been identified [5,6]. This form is also translated from an in-frame initiation site at AUG^{165}, seven codons downstream from GUG^{158}. Physiological levels of both forms of IF2 (IF2α and -β) are required for maximal growth rate of *E. coli* [6].

Little is known about the translational initiation apparatus of the Gram-positive bacterium, *Bacillus subtilis*, or, in particular, about initiation factor IF2. The IF2 gene, *infB*, has been located in the 145° map region [7] and its sequence has recently been determined [8]. The *B. subtilis infB* gene also encodes two proteins, IF2α (78.6 kDa) and IF2β (68.2 kDa), which are both expressed in *B. subtilis* and *E. coli* [8]. In the present work we provide direct proof that *B. subtilis infB* expresses IF2α and IF2β in *E. coli* from two in-frame initiation sites, as in the case of *E. coli infB*. This is a new example of the unusual tandem translation in *E. coli* of an open mRNA reading frame. We also describe an efficient method for the purification of *B. subtilis* IF2α and IF2β.

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2. MATERIALS AND METHODS

Tris[hydroxymethyl]-aminomethane (Tris) and *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) were from Sigma, USA. Acrylamide, *N,N'*-methylene-bis-acrylamide and *N,N,N',N'*-tetraethylenediamine (TEMED), were from Bio-Rad, USA, and all other chemicals were of analytical grade obtained from Merck, Germany.

Buffer H: 50 mM HEPES, pH 7.5, 10 mM $MgCl_2$ 7 mM 2-mercaptoethanol. Since buffer H was used with various concentrations of KCl, the millimolar concentration of KCl is shown in parentheses, e.g. H(100): [KCl] = 100 mM. Cracking buffer: 50 mM Tris-HCl, pH 6.8, 2 mM Na_2EDTA, 1% SDS, 140 mM 2-mercaptoethanol, 10% glycerol, 0.1% Bromphenol blue.

Q-Sepharose Fast Flow, S-Sepharose High Performance and Q-Sepharose High Performance were from Pharmacia-LKB, Sweden. Q- = quarternary amine- and S- = sulphonate-.

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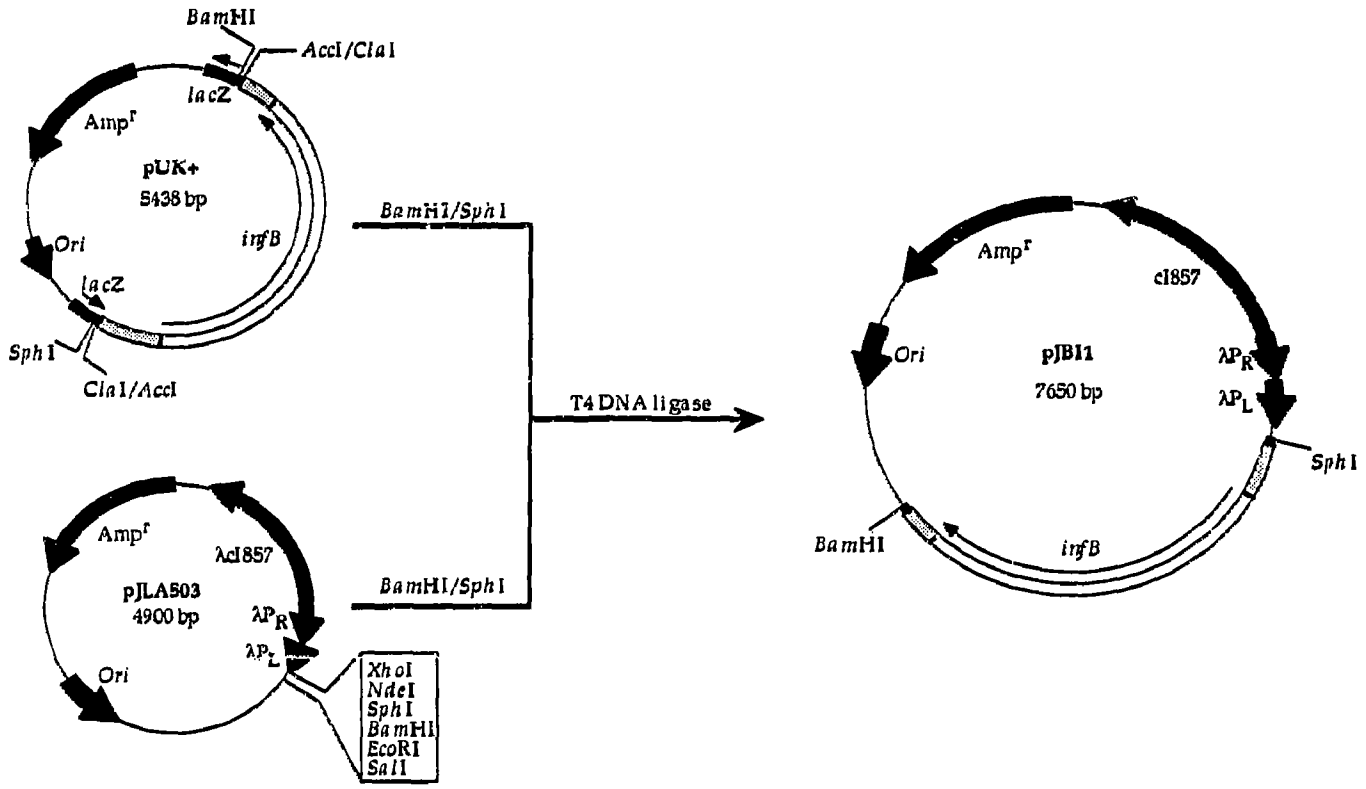

Fig. 1. Construction of the $IF2_{B.su}$ expression vector pJBI1. In pUK^+ the coding region for $IF2_{B.su}$ (white box) is surrounded by 379 bp upstream and 223 bp downstream (shaded box). This gene box was excised by restriction endonuclease digestion by *Bam*HI and *Sph*I. The fragment was subsequently ligated with the expression vector, pJLA503, previously cut with the same two enzymes.

E. coli strain UT5600 was a kind gift from Dr. M. Uhlen, Royal Institute of Technology, Stockholm, Sweden. Strain JM 105 was from Boehringer, Germany. The *E. coli* strains were grown in LB media (1% tryptone (Difco), 0.5% yeast extract, 1% NaCl), and in the same media containing 100 μg/ml ampicillin when transformed with plasmids containing the β-lactamase gene. During over-expression the 0.2% glucose was added.

The plasmids used were: pUK^+ (pUC19 containing *infB* gene of *B. subtilis* [7]); pJLA503 (expression vector containing P_L and P_R promotors from λ phage and the temperature-sensitive repressor cI_{857}, from Medac Inc.); pJBI1 (*infB* inserted into the expression vector jJLA503; this work). All plasmids were isolated by the SDS/NaOH method described in [9]. Restriction enzyme digestions with *Sph*I and *Bam*HI were performed as recommended by the respective manufacturers. Ligation with T4 DNA ligase (Biolabs Inc.) was carried out at 16 or 20°C for 9 h. Protruding 5' ends were trimmed with Klenow enzyme (Boehringer, Germany) at 20°C for 30 min in the recommended buffer. Isolation of DNA fragments from agarose gels was performed as described in the protocol from the Gene-clean Kit (Bio 101 Inc).

2.1. *Over-expression*

An *inoculum* of UT5600 transformed with pJBI1, started from a fresh LB plate containing 100 μg/ml ampicillin, was grown overnight at 28°C. The overnight culture was used to inoculate the expression culture essentially as previously described [10]. The culture was left at 28°C until a cell density of A_{550} = 1.0 was reached (usually after 4 h). The over-expression was induced by diluting the culture with an equal volume of warm, fresh medium at 56°C followed by 1 h growth at 42°C and 2 h at 37°C. The cells were harvested by centrifugation for 10 min at 8,000 rpm and 4°C in a Sorvall GS3 rotor. Expression of $infB_{B.su.}$ was detected by SDS-PAGE and Western immunoblotting analysis as previously described [8].

2.2. *Purification*

The harvested cells were resuspended in H(300) and lysed by passing through a French pressure cell at 1,000 psi. The cell extract was clarified by centrifugation at 18,000 rpm at 4°C for 1 h in a Sorvall SS34 rotor. The S30 supernatant was diluted with H(0) to give a KCl concentration of 150 mM and used directly in the following chromatographical step on Q-Sepharose Fast Flow. After sample application, the column was washed with H(150) and the proteins were eluted with a gradient from 150–500 mM KCl. The elution profile was followed by monitoring the A_{280} of the effluent and by SDS-PAGE. The fractions containing IF2 were pooled and diluted with H(0) to give a KCl concentration of 100 mM. This pool was applied to an S-Sepharose High Performance column. The column was washed and eluted with a gradient from 100–400 mM KCl. The elution profile was monitored as before. IF2-containing fractions were pooled, diluted to 100 mM KCl and loaded onto a final Q-Sepharose High Performance column. The column was washed and eluted as in the previous step, and the elution profile was monitored as described above. The fractions containing IF2 were pooled and dialysed against H(200) containing 50% glycerol.

3. RESULTS

3.1. *Over-expression of* Bacillus subtilis infB *in* E. coli

The gene *infB*, is part of a complex operon in *E. coli* that comprises *metY*, *pISA*, *nusA*, *infB*, *pISB* and *p35*.

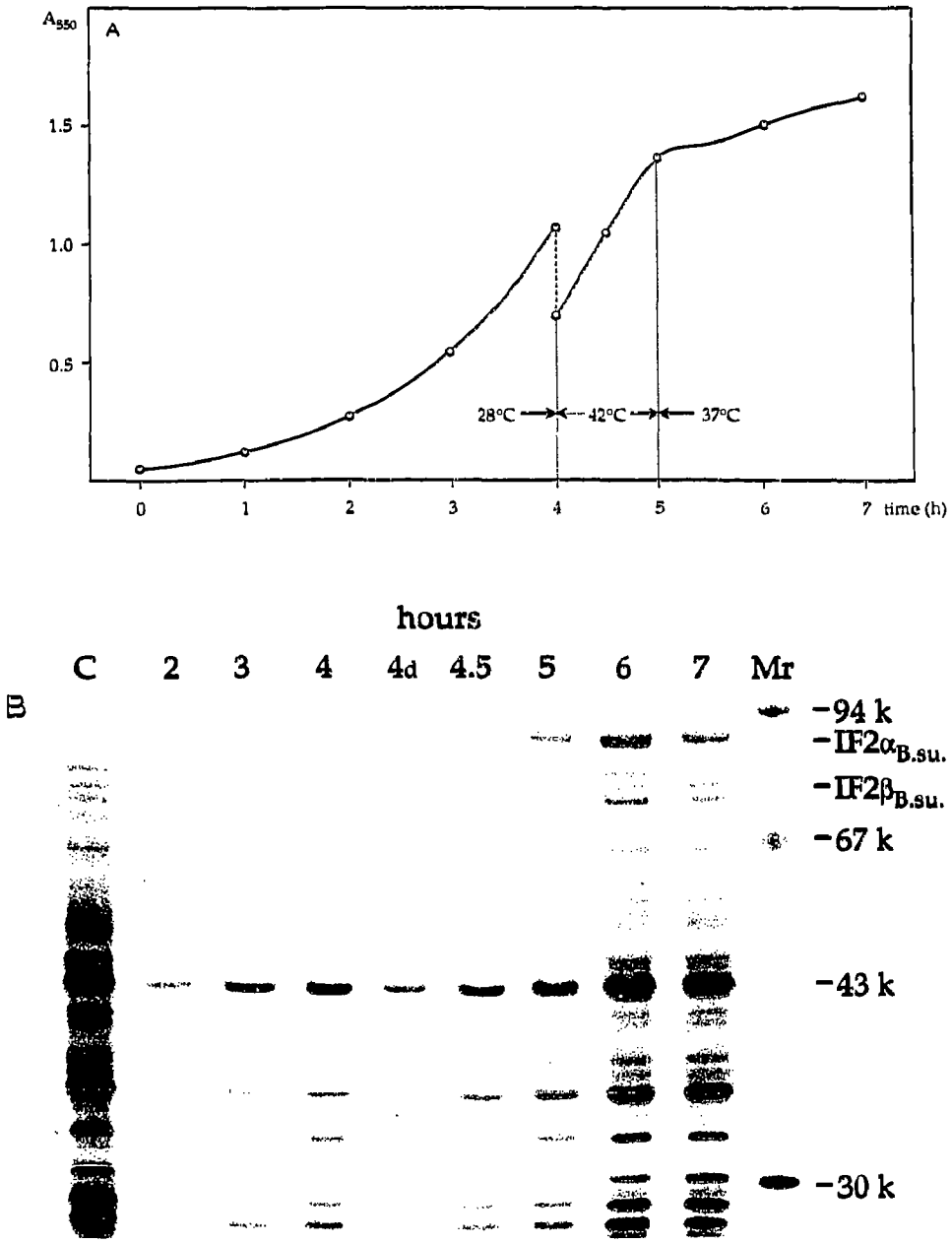

Fig. 2. (A) Growth curve for UT5600[pJBI1$infB_{B.su.}$] and (B) SDS-PAGE analysis of culture samples before and after induction showing the overexpression of *B. subtilis* IF2 in *E. coli*. Lane 1, UT5600 cells (without plasmid); lane 2–4, 2–4 h growth of UT5600[pJBI1$infB_{B.su.}$] at 28°C; lane 5,6, further 30–60 min growth at 42°C; lane 7,8, further 1–2 hours growth at 37°C; lane 6, Molecular weight markers. Each lane contains approximately 0.15 OD_{550} of cells incubated in cracking buffer. The gel was stained with Coomassie brilliant blue R.

The *B. subtilis infB* operon is similar except that *nusA* does not immediately precede *infB* but is separated from it by two short ORFs (p10 and p11) which may encode two proteins of 10 and 11 kDa [8].

The plasmid pJBI1 was constructed for expression in *E. coli* of the two forms of *B. subtilis* IF2. The coding region for *infB* of *Bacillus subtilis*, flanked up-stream by 379 bp containing the ORF for p11 and down-stream by 223 bp containing a 37 codon ORF, was excised from pUK^+ by *Sph*I and *Bam*HI and ligated with pJLA503 pre-cleaved with the same two restriction endonuclases as shown in Fig. 1. Potentially successfully ligated plasmid obtained from the transformation of JM109 with the ligation mixture was purified by the SDS/NaOH method and identified by restriction analysis. The ability of clones to over-produce IF2 was analyzed by SDS-PAGE.

In initial experiments we transformed strain JM105 with pJBI1. The cells were able to over-express both forms of *B. subtilis* IF2, but during purification substan-

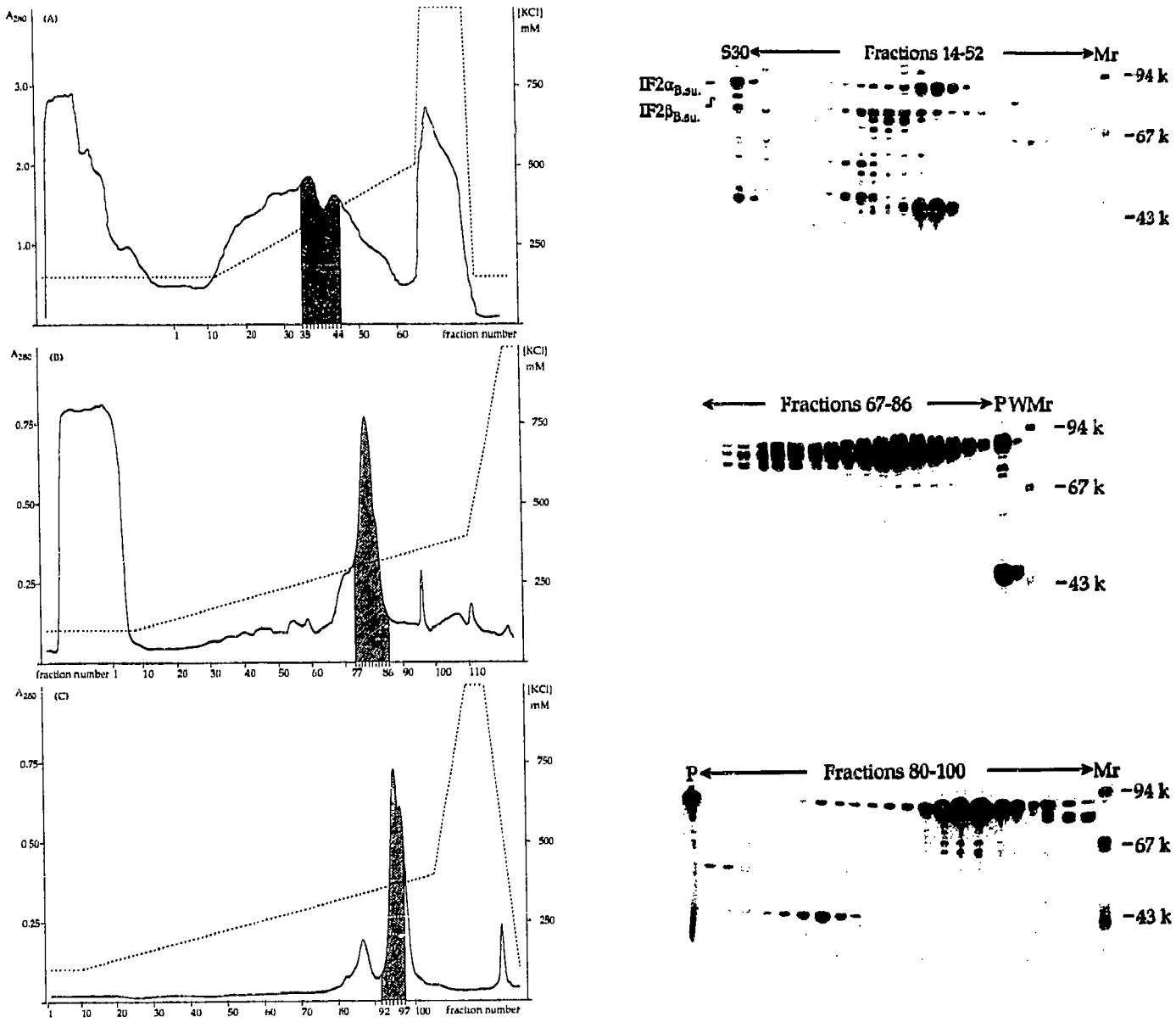

Fig. 3. (A) left panel, chromatography on Q-Sepharose FF of S30. Column dimensions, 5 × 5 cm (100 ml); sample, 200 ml S30 containing 2.1 g of proteins including ribosomes; flow rate, 5 ml/min; fraction size, 10 ml. The fractions which were pooled (35–44) are indicated as the hatched area. Right panel, SDS-PAGE analysis of the S30 sample applied to the column and of every second of fractions. 18–58. (B) left panel, chromatography on S-Sepharose HP of the pooled fractions from Q-Sepharose FF in A. Column dimensions, 1.6 × 5 cm (10 ml); sample, 32 ml containing 40 mg proteins; flow rate, 1 ml/min; fraction size, 1 ml. The fractions which were pooled are indicated as the hatched area. Right panel, SDS-PAGE analysis of the sample applied to the column (P) and of fractions 80–101.

tial amounts of the proteins were partially degraded. We therefore sought a strain where IF2 degradation would be minimal.

The *E. coli* strain UT5600 is defective in the outer membrane protease, OmpT, which has been shown to be the enzyme responsible for partial degradation of *E. coli* IF2 [11]. As seen from the purification results in Fig. 3, no degradation occurs when UT5600 is used as host for the expression vector. Therefore, *E. coli* strain UT5600 was transformed with pJBI1. As shown in the SDS-PAGE insert in Fig. 2, two protein bands of apparent molecular masses of 84 kDa and 75 kDa appear after induction of UT5600[pJBI1] at 42°C for 2 h. The cross-reaction of the bands (marked IF2α and IF2β) with anti-*E. coli* IF2 antibodies was verified by Western blotting (not shown). Fig. 2 shows the growth curve resulting in a harvest of 40 g of over-producing cells (wet weight) (7 g dry weight) from 6.6 l of cell culture. An initial problem of precipitation of the recombinant protein was solved by decreasing the time of cell growth

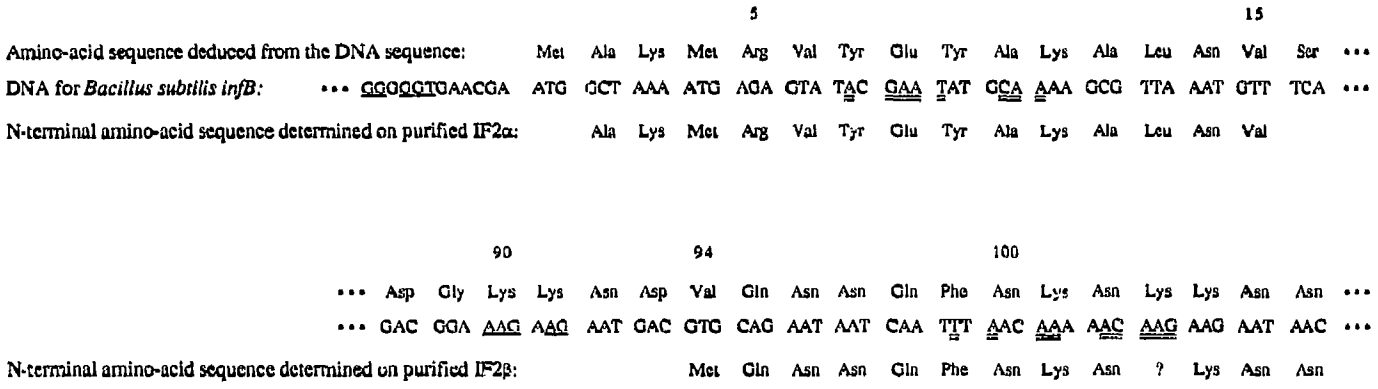

Fig. 4. *B. subtilis* IF2α and IF2β initiation regions. (Line 1) Deduced amino-acid sequence of *infB* encoded protein (residues 1–16 and 88–106); (line 2) nucleotide sequence of the *infB* gene of *B. subtilis* (the 5'-end and bases +264 to +318), from [5]; (line 3) N-terminal sequence of IF2α and IF2β as determined by protein sequencing. The numbers indicate the amino acid residue number in the IF2α sequence deduced from the DNA sequence of *infB*. The complementary S/D bases at the ribosome binding sites are underlined, and the complementary bases in the downstream box are double-underlined.

at 42°C from 4 h to 1 h followed by growth at 37°C. From several experiments, it seems likely that prolonged growth at 42°C denatures the over-expressed protein. The solubility of the two over-produced proteins was improved further by increasing the concentration of KCl in the extraction buffer from 100 mM to 300 mM. As seen in Table I, the content of over-expressed *B. subtilis* IF2 in the S30 supernatant amounts to 10% of the total cytoplasmic protein including ribosomal proteins.

3.2. *Purification of* B. subtilis *IF2α and IF2β*

The two forms of *B. subtilis* IF2 were purified by using a new three-step high performance FPLC procedure involving ion-exchange chromatography on Q-Sepharose FF, S-Sepharose HP and Q-Sepharose HP. Figure 3 shows the chromatograms from the three ion-exchange fractionations with SDS-PAGE analyses of the fractions containing IF2α. IF2β was isolated by the same procedure.

Initiation factor IF2 has a net negative charge at pH 7.5. However, unevenly distributed charges on the IF2 molecule result in local strongly basic areas, which allows the use of physiological pH for both anion- and cation-exchange chromatography. A summary of the purification data is presented in Table I. Essentially pure IF2α and IF2β are obtained in the third chromatographic step. Approximately 20 mg of IF2α and 10 mg of IF2β were obtained from 40 g of cells. The purified IF2α and IF2β were dialysed against 50% glycerol in buffer H(200). The protein concentrations were determined by a modified Bradford Coomassie brilliant blue dye assay [12], and the solutions were stored at −20°C.

3.3. *N-Terminal amino-acid sequence of* B. subtilis *IF2α and IF2β*

The purified proteins were sequenced from their N-termini as previously described [13] and the resulting amino acid sequences are shown in Fig. 4. The N-terminal sequence of IF2α corresponds to the sequence predicted from the DNA sequence [8] with the exception of the terminal methionine, which must have been removed by methionyl-aminopeptidase [14].

We have previously suggested from the DNA sequence [8] that position 94 is the first amino acid residue in *B. subtilis* IF2β. As seen in Fig. 4 the codon at amino acid position 94 is GUG, which, as illustrated in Fig. 4, means that IF2α contains valine at this position. However, the sequence results show that methionine is the N-terminal amino acid in IF2β and that the succeeding amino acids correspond to the ones predicted from the DNA sequence at position 95 and onwards. The presence of methionine and not valine at position 94 therefore shows that IF2β is, in fact, the result of a second translational initiation in $infB_{B.su}$. Although it has been shown previously, immunochemically and by mutagenesis, to occur for IF2β in *E. coli* [3–6], this is the

Table I

A summary of the *B. subtilis* IF2 purification data

Step	IF2α purification			IF2β purification		
	Total protein (mg)	IF2α (mg)	Purification factor	Total protein (mg)	IF2β (mg)	Purification factor
S30 supernatant	2,100	145	1	2,100	73	1
Q-Sepharose FF	120	60	7	80	35	12
S-Sepharose HP	40	26	10	26	16	18
Q-Sepharose HP	20	20	14	10	10	29

The data are calculated from 6.6 liters of culture resulting in a harvest of 40 g wet weight of cells or 7 g dry weight of cells. The first step, ion-exchange chromatography on Q-Sepharose FF, separated the two forms of IF2, IF2α and IF2β, which were purified independently by the two following steps, ion-exchange chromatography on S-Sepharose HP and Q-Sepharose HP.

first direct proof for an internal translational initiation in *infB*.

4. DISCUSSION

We provide direct evidence that *E. coli* translates *B. subtilis* IF2 mRNA from two in-frame initiation sites into two protein forms of different molecular mass, IF2α and IF2β. The two forms correspond precisely to the two forms seen in crude extracts from *B. subtilis*. IF2β is translated starting at an internal GUG codon and has an N-terminal methionine. The presence of an N-terminal methionine shows that *B. subtilis* IF2β is the result of a translational initiation as is the case for *E. coli* IF2β and IF2β'. When predicted from the DNA sequence the second amino acids of the *E. coli* factors IF2α, IF2β and IF2β' are threonine, serine and threonine, respectively [5]. In this work we show that alanine is the N-terminus of IF2α in *B. subtilis*. The N-terminal methionine has never been seen when the purified forms of IF2 have been sequenced. The second amino acid in *B. subtilis* IF2β, in which the N-terminal methionine is present in the purified protein, is glutamine. These observations are in agreement with the rule of N-terminal methionine cleavage: the excision of N-terminal methionine by methionyl-aminopeptidase depends on the nature of the second amino acid, the probability of cleavage being higher the shorter the amino acid side chain [14].

It is unusual that an initiation site is located inside a translational region of an mRNA. However this occurs for two different mRNAs, both coding for IF2. There is little homology between the *infBβ* initiation regions of *E. coli* and *B. subtilis*. The Shine and Dalgarno sequence (S/D) in the IF2β mRNA of *B. subtilis* (underlined in Fig. 4) is not particularly strong although slightly better than that of *E. coli* [5,8]. It is a puzzle why the β-form is so efficiently expressed.

It has previously been hypothesized that a sequence (UCAUGAAUCACAAAG) downstream of the initiator codon complementary to part of the 16 S rRNA is important for expression [15]. A similar downstream box is found in the case of *E. coli*, with 10 nucleotides complementary to the 16 S RNA [6]. In the *infB* mRNA of *B. subtilis* 8 bases complementary to this 15-nucleotide sequence are found at the initiation site of IF2α and 9 complementary bases are found at the initiation site of IF2β. The complementary bases are double-underlined in Fig. 4. They are situated at approximately the same distance from the initiation codons: +22 to +34 for *E. coli infBβ* [6] and +17 to +30 for *B. subtilis infBβ*.

How can a translational initiation take place at an initiation site located in an open reading frame? Such a mechanism requires initiation (formation of initiation complex: binding of 30 S ribosome, fMet-tRNA and 50 S ribosome) simultaneously with translation (ribosomes moving) through this region of the mRNA. In other words it requires the sufficiently fast formation of a thermodynamically stable initiation complex during the time of passage across the internal initiation site of two successive ribosomes translating the complete open reading frame, in this case IF2α. Since it is well established that the initiation step is the rate-limiting step of translation, the translation must slow down in front of the internal initiation site in order to allow the formation of the internal initiation complex. In the *E. coli infB* gene several rare codons are found in the region just upstream the IF2β initiator codon, whereas no rare codons are found in the near downstream region. A similar distribution of rare codons is found in the *B. subtilis infB*. This finding may indicate that a pausing of the ribosome upstream the IF2β initiator codon may allow for efficient initiation at the IF2β initiator region of the *infB* mRNA.

It still remains an unanswered question why two forms of IF2 exist in the prokaryotic cell. It seems to be an advantage to the cell, since two organisms such as *E. coli* and *B. subtilis*, which have been separated early in evolution, both have the two forms. In *E. coli* it has been shown that the presence of both forms is needed for maximal growth, but it is not essential since *E. coli* is still able to grow with only one of the two forms [6].

How general is the existence of two IF2 forms in Gram-negative and -positive bacteria? In the bacteria investigated so far two forms have been found in several cases [16], with the exception of *B. stearothermophilus*. In a report of cloning and sequencing of *B. stearothermophilus infB*, only one form of initiation factor IF2 was found [17]. However, no immunochemical analysis was used in this work. Using monospecific polyclonal rabbit antibodies raised against *E. coli* IF2α in immunoblot experiments with fresh lysate of *B. stearothermophilus* cells, we have observed cross-reaction with two protein bands. When estimated from SDS-PAGE and immunoblot the two proteins are approximately 82 and 72 kDa (B. Søballe, K.K. Mortensen and H.U. Sperling-Petersen, unpublished). This observation strongly indicates the presence of two forms of IF2 in *B. stearothermophilus*. The proteins are now being purified for sequence identification.

The increasing evidence for tandem translation of prokaryotic *infB* has led us to elucidate the generality of this phenomenon. We are at present using specific antibodies for the screening of a number of different bacteria for multiple forms of initiation factor IF2.

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