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Domain of *E. coli* translational initiation factor IF2 homologous to lambda cI repressor and displaying DNA binding activity

Gilles Vachon[a], Joel Raingeaud[b], Benoît Dérijard[a], Raymond Julien[b] and Yves Cenatiempo[a]

[a]*Institut de Biologie Moléculaire et d'Ingénierie Génétique, URA CNRS 1172, Université de Poitiers, 40 Avenue du Recteur Pineau, 86022 Poitiers Cedex, France* and [b]*Institut de Biotechnologie, Université de Limoges, 123 Avenue Albert Thomas, 87060 Limoges, France*

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The carboxy-terminal region of translational initiation factor IF2 is a common region to the three active forms of the factor (α, β and γ) but its function is still unknown. We report here that this region of IF2 carries at least one domain which is homologous to the N-terminal and middle part of the cI repressor of lambda phage. The IF2 homologous domain harbors functionally important features of the lambda repressor, e.g. the helix-turn-helix motif and some of the residues essential for the structure of the hydrophobic core of the repressor. This homologous domain of IF2 was fused to the β-galactosidase protein. The hybrid protein, as well as IF2 itself, shows a consistent DNA binding activity in nitrocellulose filtration assays but does not display the specificity of the cI repressor for the P_R operator. The implication of this domain in the transcriptional activity of IF2, reported by others, is discussed.

Initiation factor; *E. coli*; Lambda repressor; Protein–DNA interaction; Fusion protein

1. INTRODUCTION

IF2 is one of the three translational factors involved in the formation of the first peptide bond in *Escherichia coli*. IF2 promotes the binding of fMet-tRNA$_f^{Met}$ to the 30 S ribosomal subunit leading to the formation of the 30 S initiation complex. It is also responsible for GTP hydrolysis during elaboration of the 70 S initiation complex (for reviews, see [1–4]).

Furthermore, IF2 is suspected to participate to two cellular processes unrelated to protein synthesis, respectively, activation of transcription [5] and translocation of proteins across the bacterial membrane [6].

IF2 is present as two forms in the cell: α, a full length protein of 890 amino acids, and β lacking the first 157 N-terminal amino acids of the α form. These two forms result from the use of two independent and in-phase translational start sites on IF2 mRNA [7–10]. A smaller form, γ, lacking the first 269 amino acids of IF2α, arises artefactually by limited proteolysis during the purification process. The three forms α, β, γ are able to catalyze in vitro reactions such as GTP hydrolysis or dipeptide synthesis [11].

Correspondence address: G. Vachon, Institut de Biologie Moléculaire et d'Ingénierie Génétique, URA CNRS 1172, Université de Poitiers 40, Avenue du Recteur Pineau, 86022 Poitiers, Cedex, France. Fax: (33) 49 45 35 03.

Abbreviations: fMet-tRNA$_f^{Met}$, formylmethionine-tRNA$_f^{Met}$; EF-Tu, elongation factor Tu; IPTG, isopropyl β-D-thiogalactoside; DMSO, dimethyl sulfoxide; kbp, 1,000 base pairs.

Only recently, molecular studies were performed to identify the different regions of fMet-tRNA$_f^{Met}$ and rRNA protected by IF2 against chemical and enzymatic modifications [12,13]. However, molecular analysis of the factor itself and of the domains implicated in the different processes have to be strengthened, although initial attempts to crystallize IF2 have failed [4].

The IF2α protein sequence deduced from the *infB* gene [14] reveals at least three structural domains: the N-terminal part (residues 1–214) which is characterized by an unusual amino acid composition, then the central part of IF2 molecule (residues 350–600) which shows extensive homologies with well-known G proteins such as EF-Tu [11,14–16], and the C-terminal part of IF2 (residues 600–890). The latter has so far been less subject to investigations compared to the two other domains, and it did not seem to carry any particular structural features. However, like the central G domain, this segment is common to the three forms α, β, γ and encompasses a region possibly implicated in one or several functions of the factor.

In the present work, we reinvestigate the role of the C-terminal segment of IF2. We found that it displays an important sequence homology to the middle and N-terminal domains of the cI repressor of phage lambda, an extensively studied DNA binding protein. This domain corresponds to a region of the cI repressor harboring important functional and structural features, e.g. a helix-turn-helix motif.

We then sought for experimental data supporting the hypothesis that IF2 could be a DNA binding protein.

We used gene fusion technology to synthesize an IF2 C-terminal fragment–β-galactosidase hybrid protein. This fused protein was purified by affinity chromatography. In vitro binding assays performed with various DNA fragments showed to assess this hypothesis.

2. MATERIALS AND METHODS

2.1. *Reagents and materials*

Restriction endonucleases and all DNA modifying enzymes were from either Boehringer-Mannheim or Pharmacia and used as directed by suppliers.

2.2. *Strain and media*

The *E. coli* strain used to express the hybrid protein was SE 5000 (F−, *araD139, Δlac169, rpsL, thiA, recA*). The strain was grown on L-broth [17] and L-broth supplemented with ampicillin (50 μg/ml) when bearing the plasmid used in this study.

2.3. *IF2 protein*

Purified IF2 protein was kindly provided by S. Laalami (IBPC, Paris). A mixture of both forms, IF2α and IF2β, in a ratio of about 2/1 was used in this study.

2.4. *Construction and purification of the fusion protein*

The pGV233-2 vector was constructed from pKK233-2 (purchased from Pharmacia) as described elsewhere [16] by insertion of *lacZ* and *lacI* genes.

In Fig. 3, the stippled region, in the *infB* gene, shows the (*Dra*III)–*Hpa*I segment coding for an IF2 peptide encompassing the homologous part. The hybrid protein was constructed by isolating the *Dra*III–*Hpa*I fragment from pB16-1. The 3′ over hang *Dra*III site was digested with T4 polymerase and the fragment was then inserted into pGV233-2 between *Nco*I-filled and *Pvu*II. Purification of the fusion protein synthesized from pGJ3 (Fig. 3) was performed according to Ullmann [18] with previously reported modifications [16] except that induction was realized with only 0.02 mM IPTG over 1 h to avoid formation of inclusion bodies.

2.5. *DNA binding assay*

DNA fragments from pJLA501 [19] were labelled by random priming using d[α-^{32}P]ATP.

DNA fragments from pKK3535 were labeled by end repairing of the *Bam*HI site by the large fragment of DNA polymerase I using d[α-^{32}P]ATP as the radioactive nucleotide.

DNA binding was assayed as previously reported [20] except that binding buffer was composed as follows: 10 mM Tris-HCl, pH 7, 2 mM $CaCl_2$, 0.1 mM EDTA, 50 mM KCl, 5% DMSO, 100 μg/ml bovine serum albumin, labelled DNA (10^7–10^8 cpm/μg) $2 \cdot 10^{-12}$–$2 \cdot 10^{-11}$ M.

The total volume of the reaction was 300 μl. The mixture was incubated at 4°C for 30 min and 280 μl of the reaction was loaded on nitrocellulose filters (BA85, Schleicher and Schuell) under gentle vacuum. The filters were washed twice with 300 μl of cold binding buffer, dried and the radioactivity was measured in a scintillation mixture (Optiphase, Beckman).

3. RESULTS

The region of homology between IF2 and cI is presented in Fig. 1. It extends from Leu^{709} to Gly^{820} in IF2, corresponding to a segment of cI from Leu^{29} to Gly^{147} [21]. Considering only strictly identical amino acids upon sequence alignment, a 31.7% identity score is obtained. When substitutions of closely related amino acids are taken into account the degree of homology reaches 40%.

The homologous segment of the cI repressor contains the helix-turn-helix motif involved in the binding of the repressor to lambda operators O_R and O_L. It can be seen in Fig. 1 that the sequence Gly-Gln-Ser-Gly-Val-Gly of cI helix 3 [22,23] ('recognition helix'), has a highly conserved counterpart in IF2, where Gln is, however, missing.

Amino acids that determine the structure of the hydrophobic core of the repressor have been identified previously [24]. Out of 7 buried residues identified in cI, 6 are located in the homologous region between IF2 and cI. Among them, 3 are strictly conserved in IF2 (Val^{716}, Val^{725} and Leu^{735}) and 2 others (Ile^{720} and Ser^{739}), replacing Met^{40} and Leu^{65} in cI, were found in partially functional cI mutants.

Another homologous sequence block appears in the Ser^{783}–Lys^{791} subdomain in IF2 (corresponding to Ala^{111}–Arg^{119} in cI), which overlaps the RecA cleavage site of cI (Ala^{111}–Glu^{112} peptide bond) [25]. IF2 is not known to be a target of RecA protein in vivo.

Another important function of lambda repressor is the activation of transcription of its own gene via contact with the RNA polymerase. The acidic residue Glu^{34} seems to play a key role in this process [26] since any other residue but an Asp at the same position drastically reduces the corresponding position.

These observations based solely on primary sequence analysis are not sufficient per se to demonstrate that IF2 harbors the structural motifs previously identified in lambda cI, particularly the helix-turn-helix motif. Therefore, this theoretical study was carried on by calculation of the distribution of the hydrophobic amino acids along the putative helix-turn-helix motif of both IF2 and cI homologous regions. As shown in Fig. 2A and B, the overall distribution of hydrophobic residues is similar in CI and IF2 homologous regions. This observation is consistent with the similarity of the hydrophobicity profiles [27] that is observed in the first two-third of the homology (data not shown).

To experimentally investigate the properties of this domain of IF2, we decided to design a strategy that potentially allows its isolation from the original context in IF2. We therefore subcloned a defined IF2 gene segment in an expression vector, in frame with an ATG on its 5′ side and the eighth codon and the *lacZ* gene on its 3′ side.

Fig. 3 shows the construction of that gene fusion (see section 2). The *infB* region fused to *lacZ* encompasses Val^{689}–Val^{852} and thus is long enough to carry the overall region homologous to cI (Leu^{709}–Gly^{820}). The resulting gene fusion encodes then for a hybrid protein IF2′–β-galactosidase under the control of the inducible P_{trc} promoter that can be purified by affinity chromatography [16] (insert in Fig. 3).

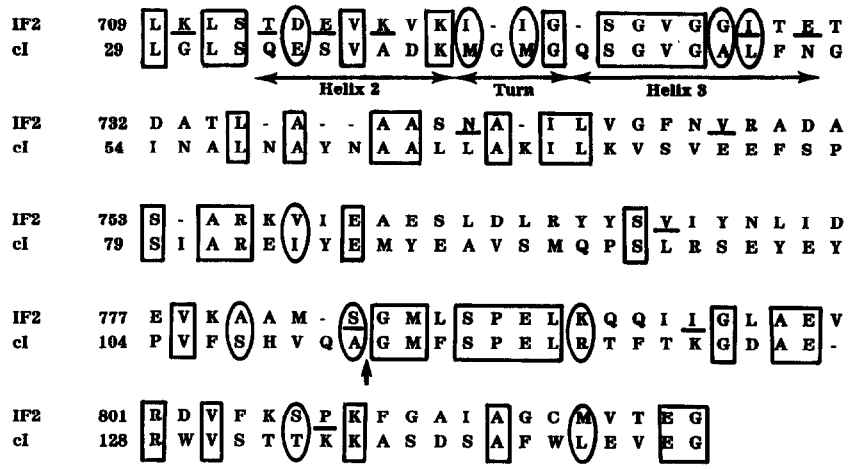

Fig. 1. Sequence homologies between IF2 and cI. The IF2 amino acid sequence [14] was compared to that for lambda cI [21] and one region of homology was detected. Sequence similarity alignments were done in accordance to Beckman Microgenie software. Exact amino acid homologies are boxed, near-identical residues are in rounded boxes, and IF2 residues found at the same position in other repressors [30] are underlined. The vertical arrow shows the RecA cut site in cI. Residues absent along the IF2 strand are replaced by dashes.

The primary structural features of the IF2 segment homologous to the cI repressor indicate that this region may be implicated in a nucleic acid binding function. Among the ligands of IF2 containing nucleic acids (rRNA and tRNA initiator), the tRNA initiator may be a potential candidate since ribosome–IF2 interaction does not seem to involve rRNA [13]. Our attempts to measure binding of the hybrid protein with the initiator tRNA were unsuccessful (data not shown), under conditions otherwise allowing an IF2 binding [12].

Alternatively, it is also conceivable that this region of IF2 could be implicated in DNA binding and activation of transcription, a function already suspected [5].

To investigate possibility that IF2 had the same target DNA, if any, as the cI repressor, we performed DNA binding assays on nitrocellulose filters using two different fragments purified from the plasmid pJLA501 [19]. The two fragments differ by the presence or absence of the P_R promoter. The larger fragment (1.2 kb) carries the three O_R target operators of cI, followed by the cI gene itself, while the smaller fragment (0.8 kb) carries only the truncated cI gene.

Results obtained from the 1.2 kb fragment are presented on Fig. 4A. IF2 and the hybrid protein exhibit a DNA binding activity with this fragment, 80–85% of DNA is bound with an IF2 concentration of 10^{-7} M and a hybrid protein concentration of 10^{-6} M, giving an apparent K_d of about $8 \cdot 10^{-9}$ to 10^{-8} M and $3\text{–}5 \cdot 10^{-8}$ M, respectively. In contrast, no binding could be observed with β-galactosidase alone or the IF2 G-domain–β-galactosidase hybrid protein under identical conditions.

DNA binding assays performed with the shorter fragment result in very similar patterns with the 4 proteins (data not shown). Thus, it is likely that (i) IF2 has a consistent affinity for DNA, (ii) the IF2-C terminal segment fused to β-galactosidase is involved in this DNA

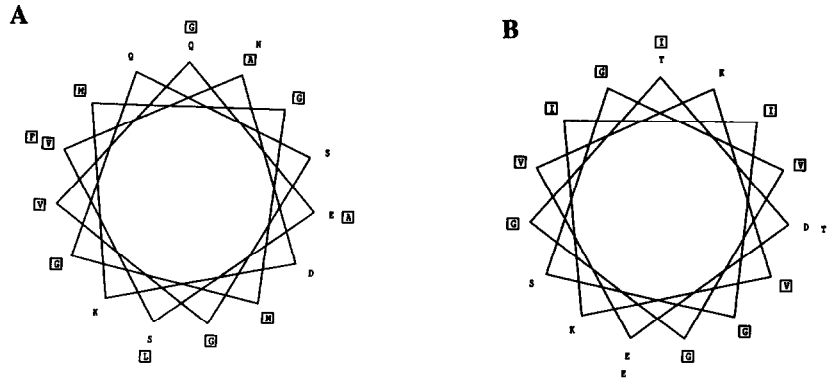

Fig. 2. Amphiphilic region distribution between IF2 and cI helix-turn-helix regions. The plots were obtained with the Helicalwheel program from the Genetics Computer Group Inc. (Madison, WI, USA) using a rotation angle of 96°. (A) The helical representation extends from Glu^{33} to Asn^{52} of cI. (B) The helical representation extends from the corresponding homologous region of IF2, i.e. from Thr^{713} to Glu^{730}. The boxed residues indicate hydrophobic residues.

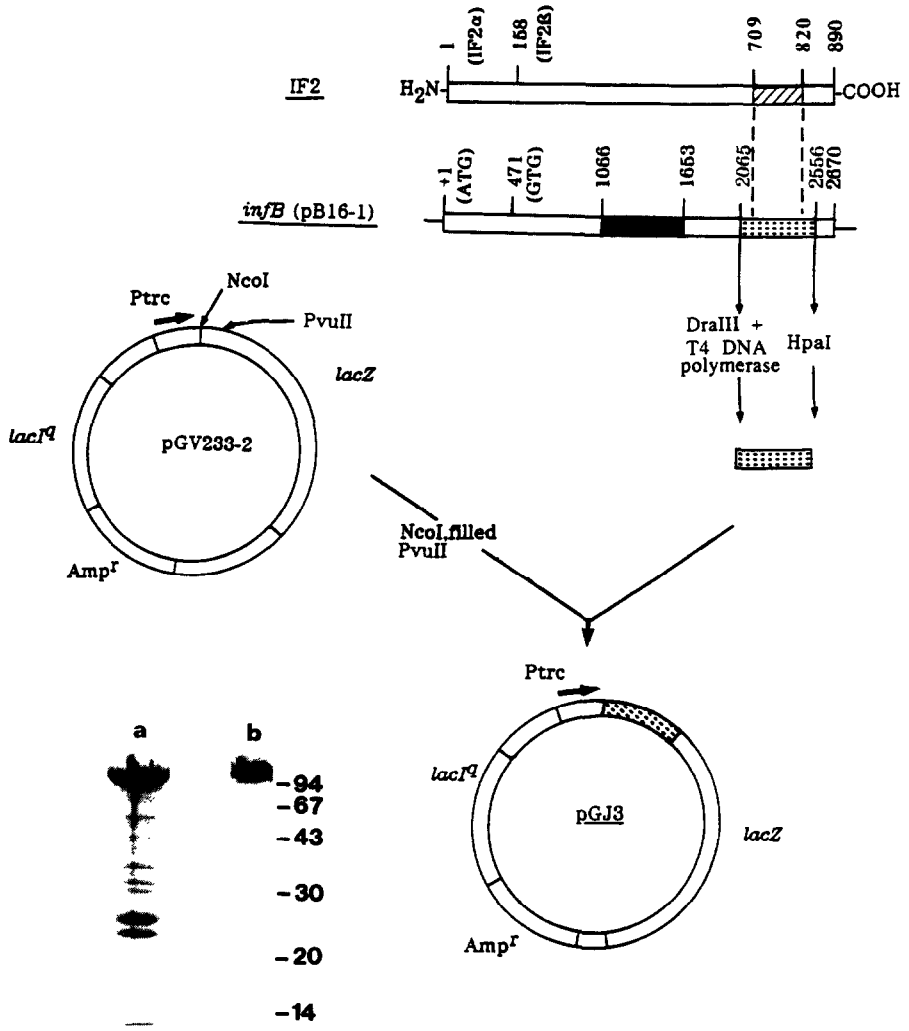

Fig. 3. Schematic map of IF2 and construction of the IF2′–β-galactosidase hybrid protein. The first amino acid positions of IF2α and IF2β, 1 and 158, respectively, are also indicated. The cross-hatched region represents the domain of IF2 homologous to lambda cI repressor (see section 2 for details of the fusion gene construct). The filled region represents the G-domain coding part of IF2, extending from Ala^{391} to Gly^{540}. The insert shows the expression of the hybrid protein in SE5000 *E. coli* cells (lane a) and the affinity-purified fusion protein (lane b) analysed by SDS-PAGE and stained with Coomassie blue R-250.

binding property, and (iii) IF2 does not display any specific affinity for the P_R promoter.

Previous in vitro experiments [5] report the ability of IF2 to activate transcription of the *rrnB* operon. However, it is not clear from this study if a DNA binding step, specific or non-specific, is required to activate RNA polymerase, or if a protein–protein interaction between IF2 and the RNA polymerase is responsible and sufficient to provide activation.

To assess whether the C-terminal domain of IF2 could interact with upstream regulatory elements of the *rrnB* operon, we analysed IF2 affinity for these upstream sequences. The 7.5 kb *Bam*HI fragment carrying the *rrnB* operon was isolated from the pKK3535 plasmid [28]. This fragment harbors the tandem promoters P1 and P2 together with upstream sequences. In contrast, the 6 kb *Hin*dIII–*Bam*HI fragment lacks this proximal part of the operon.

The incubation of IF2 with the radiolabeled *Bam*HI fragment is shown in Fig. 4B.

The affinity of both IF2 and the fusion protein for the *rrnB* operon is similar, although lower, to the affinity observed for the lambda phage fragments. The apparent K_d deduced from these data is about $1–2 \cdot 10^{-8}$ M for IF2 and $1–2 \cdot 10^{-7}$ M for the fusion protein.

Again, IF2 harbors a higher affinity for the DNA than the fusion protein and the binding to the DNA is specific to the C-terminal domain of IF2.

By using the *Hin*dIII–*Bam*HI fragment a similar profile is obtained (data not shown), indicating that the tandem promoters and the upstream elements of the *rrnB* operon are not specifically bound by IF2.

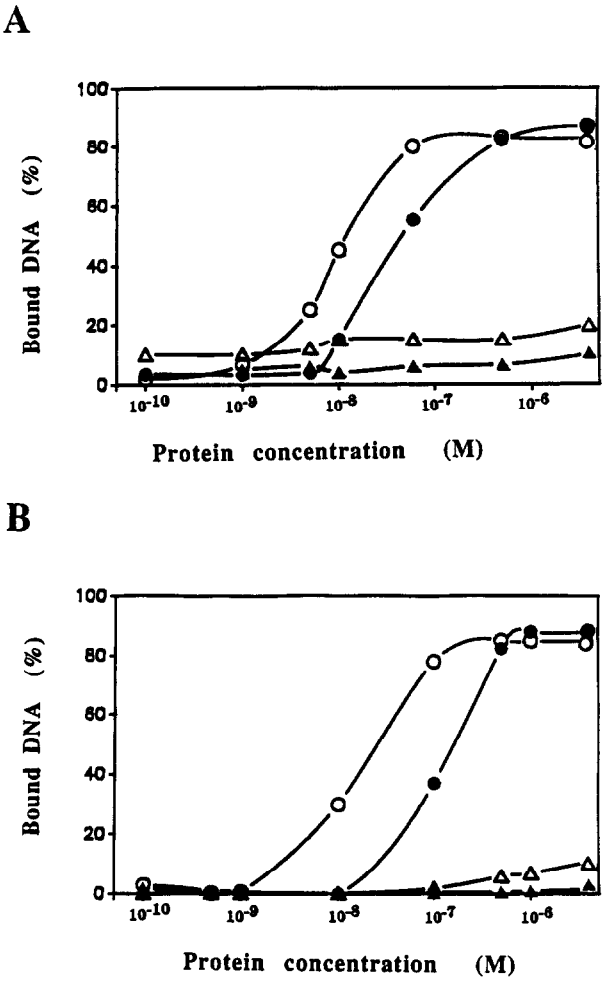

Fig. 4. DNA binding activity of IF2 and purified hybrid protein. (A) Incubation of IF2 and the purified hybrid protein with a 1.2 kbp-labelled DNA fragment isolated from pJLA503 plasmid (see section 2). The fragment carries the P_R promoter of lambda phage. (B) Incubation of the same proteins with a 7.5-kbp-labelled DNA fragment isolated from pKK3535 plasmid and carrying the entire *rrnB* operon. Protein–DNA complexes were isolated on nitrocellulose filters. The radioactivity was determined by scintillation counting. Both IF2 (○) and the hybrid protein (●) show a consistent non-specific DNA binding activity, although the fusion protein displays a lower affinity (see text). In contrast, β-galactosidase (▲) or a hybrid IF2 G-domain–β-galactosidase protein [16] (△) did not bind to DNA. The 100% value corresponds to the binding of about 1–5 fmol of labelled DNA. Each point represents the average of two independent experiments.

4. DISCUSSION

Our results support the evidence that IF2 can also display a DNA binding activity mediated by a C-terminal domain homologous to the cI repressor of lambda phage.

The IF2 homologous domain shows a conservation of several important residues of the cI repressor (Fig. 1), such as residues essential for hydrophobic core stability, indicating that the IF2 domain could display a similar structure. Computational analyses are necessary to verify the structure of IF2 in this part of the protein. Crystallographic data obtained with the cI repressor may be used to build a computer model, as already realized for the IF2 G domain [11].

Although the first segment of cI recognition helix 3 (Ser-Gly-Val-Gly) is well conserved in IF2, the absence in IF2 of other important amino acids, which participate or cooperate to the recognition of specific bases by cI [23], indicates that the DNA recognition specificity of IF2 should differ from that of cI.

In order to analyze the DNA binding properties of the IF2 domain, an *infB* fragment encompassing this domain was fused to the β-galactosidase gene. The purified hybrid protein was used in nitrocellulose filtration assays. Our results confirm the hypothesis that IF2 and cI harbor different specificities.

Similar experiments performed with *rrnB* operon fragments lead to the conclusion that these *rrnB* upstream sequences are also not specifically recognized in vitro by IF2. However, it is important to note here that in vitro transcription activation of the *rrnB* operon occurs only at low ionic strength, i.e. KCl concentrations lower than 10 mM. Higher KCl concentrations (50 mM up to 100 mM) were used in our assays to ensure that binding was not a consequence of non-specific interactions between DNA and proteins. Furthermore, it was concluded, based on sedimentation experiments [5], that IF2 must be present prior to the initiation of transcription and influences the formation of the transcription initiation complexes. Since IF2 does not induce transcriptional activation from other promoters, the IF2 molecule is probably the selective component of the binary complex RNA polymerase–IF2 required to selectively activate the *rrnB* operon. Therefore it is possible that only the RNA polymerase–IF2 complex is capable of binding and specifically activating the ribosomal operon. The DNA binding activity of both IF2 and the fusion protein indicates that the C-terminal domain could be involved in this transcription activation process and the conservation of the acidic residue Asp^{714}, could indicate that this activation would occur by contact between this same domain of IF2 and RNA polymerase.

The fact that a fMet-$ARNt_f^{Met}$-hybrid protein binding was not observed under our experimental conditions does not imply that the fused IF2 domain is exclusively implicated in a DNA binding function. Indeed, it could possibly be involved in at least two cellular processes: initiation of protein synthesis via fMet-$ARNt_f^{Met}$ binding, and activation of transcription, via DNA binding. In the same way, one can note that fMet-$tRNA_f^{Met}$ binds in a highly specific fashion to polymerase holoenzyme [29].

During rapid exponential growth, *E. coli* produces no more ribosomes than are required for protein synthesis [30]. One hypothesis proposes that transcription and

translation are coupled by the factors involved in translation [31]. EF-TuTs [31] and fMet-tRNA$_f^{Met}$ [29] alter transcription selectivity in vitro.

One can imagine that the IF2 level regulates both initiation of protein synthesis and activation of transcription of ribosomal operon(s). According to this hypothesis, since the IF2 concentration in the cell is constant (10^{-6} M) [2], a low concentration of free IF2, occurring under high protein synthesis activity, could lead to activation of rDNA transcription. In contrast, high levels of rRNA would induce the dominant negative feedback control described before [32–34].

Our next goal will be to determine the DNA target sequence, if any, specifically recognized by the C-terminal IF2 domain. This could be achieved by rounds of incubation of IF2 with random sequence oligonucleotides and PCR amplification of the bound DNA, as already described [35]. Mutagenesis of conserved IF2 residues that are functionally important in the cI repressor will also be helpful to investigate the role of this domain of IF2 in *E. coli infB* mutants [10].

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