Expression of different coding sequences in cell-free bacterial and eukaryotic systems indicates translational pausing on *Escherichia coli* ribosomes

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Abstract Five different coding sequences of bacterial or eukaryotic origin in plasmids under the T7 promoter were expressed in a cell-free system derived from *Escherichia coli*. Translation on *E. coli* ribosomes resulted in a full-length product only in four of the five coding sequences tested. A unique pattern of less than full-length polypeptides was generated in each case. Many of these polypeptides on *E. coli* ribosomes reacted with a puromycin derivative, cytidylic acid-puromycin, which was radioactively labeled. Thus these incomplete polypeptides can be defined as nascent peptides bound to the ribosomal P site. Certain nascent peptides could be shifted into full-length protein indicating that they resulted from translational pausing. In contrast to these results, expression of the same coding sequences in a wheat germ or reticulocyte cell-free system resulted in a 80–90% full-length product with no evidence for nascent polypeptides and translational pausing.

Key words: In vitro protein synthesis; Nascent peptide; Translational pausing

1. Introduction

Data from both in vivo and cell-free experiments suggested that synthesis of proteins may proceed discontinuously. Translational pausing of nascent peptides has been hypothesized. Specific patterns of incomplete peptides that appear to be unique for each coding sequence expressed in vitro by coupled transcription–translation in an *Escherichia coli* system has been reported [1]. Different explanations for translational pausing have been offered as briefly reviewed in Tsalkova et al. [2]. Suggestions include mRNA secondary structure or possibly mRNA degradation. Alternatively, translational pausing may occur when rarely used codons are encountered with low abundance of cognate tRNA. An appealing hypothesis suggested earlier by Purvis et al. [3] was pursued by Thanaraj and Argos [4]. They proposed that translationally slow regions may coincide with domain termini and linking regions between domains thus facilitating cotranslational folding.

The purpose of the experiments reported here was to establish translational pausing on *E. coli* ribosomes and to demonstrate that this event was unique to the bacterial cell-free system. Expressing the same coding sequences in different in vitro systems led to the conclusion that translational pausing could not be demonstrated on wheat germ or reticulocyte ribosomes. A striking difference between the eukaryotic and prokaryotic systems was that with the five coding sequences tested, full-length protein was the predominant product formed on eukaryotic ribosomes. In the bacterial system, incomplete polypeptides that reacted with a puromycin derivative were seen in all cases.

2. Materials and methods

2.1. Materials

The following plasmids were used: (1) pUC18 into which the T7 promoter and the complete satellite tobacco necrosis virus (STNV) sequence was inserted [5]; (2) pUC18 as under (1) in which the coding sequence for the STNV coat protein was exchanged against the coding sequence for yeast binding protein for the E_3 subunit in the yeast pyruvate dehydrogenase complex (E_3BP) retaining the 5′ and 3′ untranslated region (UTR) of STNV [6]. (3) pGEM-3Z containing the rhodanese (RHO) coding sequence. (4) pGEM-3Z containing the chloramphenicol acetyltransferase (CAT) coding sequence. (5) pGEM-3Z containing the *E. coli* release factor 1 (RF-1) coding sequence. The coding sequences in all plasmids were under the T7 promoter. Plasmids 1 and 2 were kind gifts from Dr. Karen Browning (Department of Chemistry and Biochemistry, University of Texas at Austin, TX, USA). Plasmids 3–5 were prepared from plasmids provided originally by Drs. Paul Horowitz (Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX, USA), A.S. Spirin (Institute of Protein Research, Pushchino, Russia) and Warren Tate (University of Otago, Dunedin, New Zealand), respectively.

The following cell-free expression systems were used: *E. coli* S30 fraction which was prepared as described in Kramer et al. [7] from strain A19 (an RNase I-deficient K12 strain, originally provided by Dr. Knud Nierhaus, Berlin, Germany). Wheat germ S30 was kindly provided by Dr. K. Browning; rabbit reticulocyte lysate was bought from Ambion (Austin, TX, USA).

2.2. Methods

2.2.1. Transcription. Capped RHO mRNA was synthesized using an in vitro transcription kit (mMessage mMachine) from Ambion. The respective plasmid was linearized with a suitable restriction enzyme (*Hin*dIII). The transcription reaction was carried out according to the manufacturer's protocol followed by Sephadex G-100 chromatography.

2.2.2. In vitro expression of the coding sequences. In the *E. coli* system expression was carried out by coupled transcription–translation as described in detail in [7]. The total volume of the reaction

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Abbreviations: CAT, chloramphenicol acetyltransferase; C-puro, cytidylic acid-puromycin; E_3BP, binding protein for the E_3 subunit in the yeast pyruvate dehydrogenase complex; RF-1, release factor 1; RHO, rhodanese; STNV, satellite tobacco necrosis virus; TCA, trichloroacetic acid; UTR, untranslated region

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mixtures was 30 μl. They contained 5 μl of the *E. coli* S30 fraction, about 1 μg of the respective plasmid and 50 μM [^{35}S]Met (about 2 Ci/mmol). Incubation was for 30 min at 37°C.

In the wheat germ system, both STNV and E_3BP were expressed by coupled transcription–translation. The total volume of 50 μl contained 20 μl of the wheat germ extract (cf. [8]), about 1 μg of the plasmid and 50 μM [^{35}S]Met (2 Ci/mmol). Incubation was for 30–60 min at 30°C. Capped RHO mRNA was translated in the wheat germ system under similar conditions. The translation reactions were assembled as described by Lax et al. [8]; they contained 2–3 μg mRNA per 50 μl total volume and were incubated 30–60 min at 30°C.

The coding sequences were expressed in the reticulocyte system by linked transcription–translation using Ambion's Proteinscript II kit. Transcription was carried out in a total volume of 10 μl containing 0.5 μg plasmid for 1 h at 30°C. This was followed by translation for 30–60 min at 30°C. The translation reaction was carried out in a total volume of 50 μl containing 35 μl of reticulocyte lysate, 2 μl of the transcription reaction after synthesis of mRNA and 4 μl of [^{35}S]Met (1175 Ci/mol).

2.2.3. Analysis of the products formed. Aliquots of the reaction mixtures were withdrawn after incubation and protein precipitated by trichloroacetic acid (TCA). Incorporated [^{35}S]Met was determined by liquid scintillation counting. Another aliquot was analyzed by SDS–PAGE in tricine buffer according to Schägger and von Jagow [9].

Reaction with a puromycin derivative: cytidylic acid-puromycin (C-puro) (cf. [10]) was phosphorylated as described previously [11]. About 80 pmol of this preparation were added to each 10 μl of the *E. coli* transcription–translation reaction after its incubation for the indicated time at 37°C, then the incubation was continued for another 10 min at 37°C. An aliquot was then prepared for gel electrophoresis as in [9].

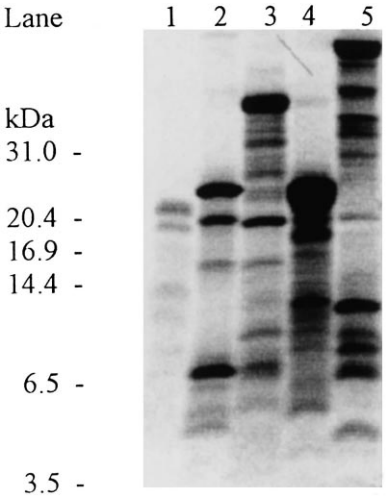

Fig. 1. Polypeptide pattern of STNV, E_3BP, RHO, CAT or RF-1 expressed in the *E. coli* cell-free system. The five coding sequences were expressed in the *E. coli* system in the presence of [^{35}S]Met as described in Section 2.2. Aliquots were withdrawn after 30 min of incubation, TCA-precipitated and analyzed by SDS–PAGE followed by phosphorimaging. The pattern of [^{35}S]Met-labeled polypeptides is shown. Lane 1 = STNV; lane 2 = E_3BP; lane 3 = RHO; lane 4 = CAT; lane 5 = RF-1. Molecular weight markers were run on the same gel; their positions are indicated on the left.

3. Results and discussion

3.1. Analysis of translation products obtained from expression of different coding sequences in the E. coli cell-free system

Five prokaryotic or eukaryotic coding sequences cloned into different vectors were expressed in the cell-free *E. coli* coupled transcription–translation system. The five coding sequences that were used in the experiments reported here are given in Table 1. Included in the table are some of the characteristics of the plasmid constructs and of the proteins which they encode. Most importantly, the coding sequences represent mRNAs from different sources. Two of the coding sequences, CAT and RF-1, are of bacterial origin. One was derived from yeast, E_3BP, which is a component of the mitochondrial pyruvate dehydrogenase complex. The STNV genome, encoding the STNV coat protein, and the coding sequence for bovine RHO, a mitochondrial sulfurtransferase, are naturally expressed on cytoplasmic ribosomes in higher eukaryotes.

These five coding sequences were expressed by coupled transcription–translation in the *E. coli* cell-free system as described under Section 2.2. After incubation, an aliquot of the reaction mixtures was analyzed by SDS–PAGE according to Schägger and von Jagow [9] followed by phosphorimaging of the gel to visualize the products formed. These results are presented in Fig. 1. Full-length protein was the major product formed when STNV, CAT, RF-1 or RHO was synthesized, whereas E_3BP gave only a very small amount of full-length protein that was barely detectable in the analytical system used. In the latter case, prominent bands are seen at about 7, 18 and 21 kDa. Less than full-length products are also seen for the other four coding sequences. In each case, these peptides gave a characteristic unique pattern.

3.2. Are the less than full-length peptides nascent peptides on the ribosomes?

The intriguing observation of these specific peptide patterns leads to the question of their nature and the mechanism by which they are generated. One possibility is that the peptides are aborted after their synthesis, a phenomenon observed for short peptides in *E. coli* cells and cell-free systems (discussed and referenced in [1]). Alternatively, the peptides may be stalled leaving them bound as peptidyl-tRNA to the ribosomes in the A or P site. In this case, the peptides qualify as nascent peptides. They may be accumulated on the ribosomes because the mRNA has been fragmented at specific sites or because translation of the mRNA pauses at specific

Table 1
Description of the coding sequences and their products

Coding sequence	Source	5′ UTR	Shine–Dalgarno sequence	mRNA naturally capped	M_r of protein (kDa)
STNV	plant virus	STNV	no	no	21.7
E_3BP	yeast	STNV	no	no	45.4
RHO	bovine mitochondrion	RHO	yes	yes	33.3
CAT	*E. coli*	CAT	yes	no	25.7
RF-1	*E. coli*	RF-1	yes	no	40.5

The DNA sequences of the coding sequences were published in the following references: STNV: [13]; E_3BP: [6]; RHO: [14]; CAT: [15]; RF-1: [16].

points along its sequence. Only in the latter situation do they have the potential of being extended to full-length protein.

Whether the incomplete polypeptides were nascent chains, was evaluated by reactivity with a puromycin analogue. Puromycin binds to the ribosomal A site and acts as an acceptor substrate for the peptide held as peptidyl-tRNA in the ribosomal P site during the peptidyl transferase reaction [12]. Here we use a cytidylic acid derivative of puromycin [10] that can be phosphorylated and has a 1000 fold lower K_M value than puromycin itself.

Reaction mixtures containing newly synthesized (non-radioactive) polypeptides were incubated with the radioactively labeled puromycin derivative ([^{32}P]C-puro) immediately after coupled transcription–translation in the *E. coli* system. The results, presented in Fig. 2, show that on *E. coli* ribosomes a major portion of the less than full-length newly synthesized polypeptides are nascent peptides. Many of the bands representing incomplete polypeptides by amino acid radioactivity (Fig. 1) were reactive with radioactive C-puro (Fig. 2). There are, however, quantitative differences in the amount of radioactivity associated with the same-size peptides in Fig. 1 compared to Fig. 2. Nascent peptides were labeled with one radioactive puromycin at their C-terminus (Fig. 2), whereas polypeptides in Fig. 1 contained different amounts of methionine depending on their amino acid sequence. Also, there may be nascent peptides on the ribosomes bound into the A site; these would not react with C-puro.

3.3. Translational pausing on E. coli ribosomes?

Nascent peptides might result from degradation of the mRNA. This means that the peptidyl-tRNA is bound at the end of an mRNA fragment. Alternatively, the nascent peptides may result from slow or stopped elongation at certain points along the coding sequence, translational pausing.

The following experiments were carried out to evaluate whether the nascent peptides shown in Fig. 2 involve translational pausing. Protein synthesis was initiated on *E. coli* ribosomes with formyl-[^{35}S]Met-tRNA$_f$ in a reaction mixture that

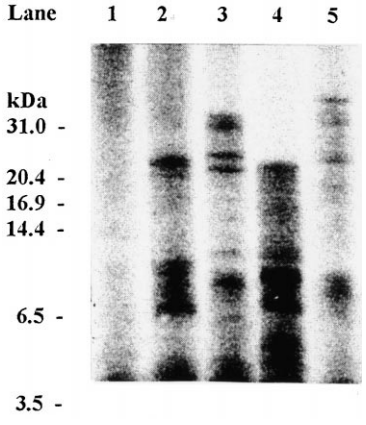

Fig. 2. Reaction of nascent peptides with C-puro. STNV, E_3BP, RHO, CAT or RF-1 were expressed in an *E. coli* cell-free system. Synthesis was carried out for 15 min at 37°C in the presence of unlabeled amino acids. Immediately after incubation, [^{32}P]C-puro was added to the reaction mixtures, and the incubation was continued for an additional 10 min. The reaction mixtures were TCA-precipitated and analyzed by SDS–PAGE and phosphorimaging. Samples were loaded in the same order as given for Fig. 1.

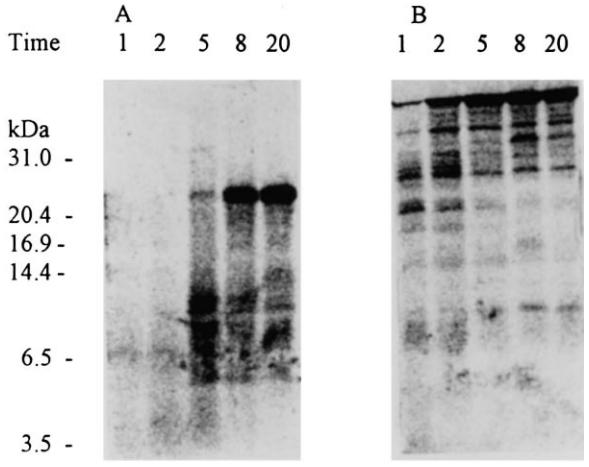

Fig. 3. Extension of nascent peptides to full-length protein on *E. coli* ribosomes. Synthesis of CAT (A) and RF-1 (B) was initiated with f[^{35}S]Met-tRNA (50 pmol/30 μl reaction mixture, 5 Ci/mmol) in the absence of folinic acid and methionine. After 2 min incubation at 37°C, methionine was added to give 300 μM. The incubation was continued and at the indicated time (min), an aliquot was removed and the reaction stopped by addition of TCA. The samples were prepared for SDS–PAGE, electrophoresis was carried out and the gel was scanned by phosphorimaging.

lacked folinic acid and methionine. After 2 min of incubation, a large amount of non-radioactive methionine was added to the reaction mixture and the incubation continued for different lengths of time. The results, shown for CAT and RF-1 in Fig. 3, indicate that the peptides initiated during the first 2 min of incubation, seen initially as low-molecular weight peptides, were elongated to the full-length polypeptides upon continued incubation. The amount and percentage of full-length polypeptides increased throughout the time of incubation. There is less radioactivity in the lanes of Fig. 3 representing the early time points. This is probably due to formation of initiation complexes and short peptides of insufficient length to be detected by the SDS–PAGE analysis. Most importantly, incomplete polypeptides seen in the early time points (5 min for CAT, 1 and 2 min for RF-1) appear to be of the same size as nascent peptides identified by reaction with C-puro (lanes 4 and 5, Fig. 2).

The type of experiment presented in Fig. 3 is preferred over a pulse-chase experiment with radioactive methionine to demonstrate that there was no proteolytic degradation from the N-terminal end of the molecule. However, a pulse-chase experiment was carried out with nearly identical results as shown in Fig. 3 (data not presented). The data demonstrate that most of the incomplete peptides observed on *E. coli* ribosomes as nascent CAT or RF-1 peptides (Fig. 2) were not the result of proteolysis or mRNA degradation but were actually caused by translational pausing.

3.4. Synthesis of full-length protein in eukaryotic cell-free systems

When the same coding sequences listed in Table 1 were expressed in the wheat germ or reticulocyte in vitro systems, 80–90% of the translation products were full-length protein. This is exemplified for STNV, E_3BP, and RHO in Fig. 4. These proteins were synthesized in the presence of [^{35}S]Met as described under Section 2.2. Expression in the reticulocyte lysate was carried out from the respective plasmids by linked

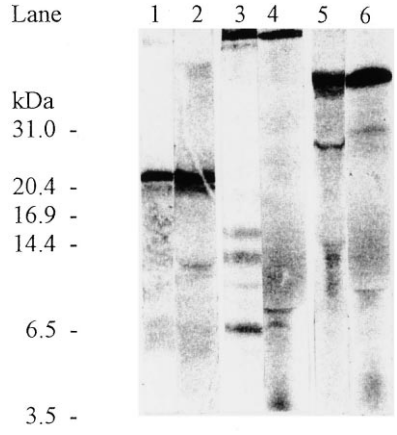

Fig. 4. Expression of STNV, E_3BP and RHO in the wheat germ and reticulocyte cell-free systems. The coding sequences for STNV, E_3BP or RHO were expressed in the presence of [^{35}S]Met for 30 min (wheat germ extract) or 60 min (reticulocyte lysate) at 30°C and processed as described in the legend to Fig. 1. Lanes 1 and 2 = STNV; lanes 3 and 4 = E_3BP; lanes 5 and 6 = RHO. Lanes 1, 3, and 5 = wheat germ system; lanes 2, 4 and 6 = reticulocyte system. Positions of proteins used as molecular weight standards are indicated on the left.

transcription–translation. For the wheat germ system, it was necessary that the RHO mRNA was capped. This was done by separate transcription in the presence of a cap analog (see Section 2.2). Even though the message had to be isolated, then added to the wheat germ translation assay, very few incomplete peptides are visible in the respective lane of Fig. 4. Less than full-length peptides generated in the wheat germ or reticulocyte cell-free systems did not react with C-puro (data not shown). Slow or stalled elongation leading to pause-site peptides appears not to be a feature of the ribosomes in the wheat germ extract or the reticulocyte lysate.

In conclusion, expression of all coding sequences including the one for E_3BP generated full-length protein as the predominant product in the eukaryotic systems. Nascent peptides could be identified only on *E. coli* ribosomes.

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