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Review Article

Methodology for the analysis of transcription and translation in transcription-coupled-to-translation systems in vitro

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ABSTRACT

The various properties of RNA polymerase (RNAP) complexes with nucleic acids during different stages of transcription involve various types of regulation and different cross-talk with other cellular entities and with fellow RNAP molecules. The interactions of transcriptional apparatus with the translational machinery have been focused mainly in terms of outcomes of gene expression, whereas the study of the physical interaction of the ribosome and the RNAP remains obscure partly due to the lack of a system that allows such observations. In this article we will describe the methodology needed to set up a pure, transcription-coupled-to-translation system in which the translocation of the ribosome can be performed in a step-wise manner towards RNAP allowing investigation of the interactions between the two machineries at colliding and non-colliding distances. In the same time RNAP can be put in various types of states, such as paused, roadblocked, backtracked, etc. The experimental system thus allows studying the effects of the ribosome on different aspects of transcription elongation and the effects by RNAP on translation.

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1. Introduction

Transcribing RNA polymerase (RNAP) is involved in different types of interactions with other cellular entities and with other molecules of RNAP. In bacteria, transcription and translation are coupled [27] thus translation takes place co-translationally. The interaction of the transcriptional apparatus with the translational machinery has been studied over decades, focusing mainly on gene expression regulation and mechanisms such as attenuation (as described for the trp, his, le, and pheA operons in enteric bacteria), and polarity [24,22,23,10,42,47,44,15,43,2,16,46,45]. Transcription–translation coupling has also been proposed to be important preventing the formation of R-loop structures [26,17].

Although aforementioned mechanisms highlight the importance of the coupling between transcription and translation, they do not describe the outcome of direct, physical interaction between the two machineries. Most recently, research has been done on the mechanistic effects of the ribosome–RNAP interaction in vivo. It has been proposed that the rate of transcription depends on the rate of translation. This was observed using 70S ribosome mutants, which are slower than the wild type counterpart [30]. Under such conditions, transcription was observed to slow down significantly [1]. Another observation was that the translation apparatus could aid backtracked transcriptional complexes by a “pushing” them forward [13,30]. It was proposed that the ribosome was not only capable of rescuing RNAP from its backtracked state but it also exerted enough force onto the RNAP to aid it overcoming the DNA-bound Lac. It has been shown lately that backtracked RNAP can cause DNA double-strand breaks through collisions with replication forks [13]. It was suggested that the ribosome impedes backtracking and also rescues RNAPs stalled in the backtracked state linking transcriptional backtracking to translation in order to maintain genome stability. However, such proposed mechanisms do not explain previous observations where the rate of transcription of untranslated RNAs (i.e. rRNA) is nearly twice as fast compared to mRNA [39]. Furthermore, the present studies look only at the outcome on the backtracking phenomenon, leaving different pausing mechanisms such as pre-translocated stabilized pausing [3] without characterization. Understanding the interface needed for the correct cross-talk between RNAP and the ribosome could reveal new mechanisms of regulation and functions of both machineries and could be important for the development of antibiotics which would target these interactions. Efforts to evaluate this topic have been done using nuclear magnetic resonance and molecular modeling [8] but no biochemistry has been performed due the lack of a system to do so. There is also no information on the effects of such interactions on the ribosome. For example, it is possible that strongly paused RNAP could be a regulatory signal for the ribosome translating behind it, for
instance, the possibility of translational frameshifting caused by collisions with RNAP have not been addressed.

Most studies of the interactions of RNAP and the ribosome have been performed in vitro or in cell-free crude extracts or in commercially available coupled transcription–translation systems designed for the production of proteins in vitro [31,34,33,20,19,11,35,38]. Although such approaches can provide insight into global mechanisms of such interactions, they fail to give the possibility to analyze, in finer detail, the cross-talk between the two machineries, as other cellular components, i.e. transcription and translation factors, that may influence functioning of both machineries, usually cannot be excluded from such experimental systems, and/or stalled transcription or translation complexes (to make a snapshot of interactions) cannot be obtained due to uncontrolled presence of all substrates for all processes involved.

To analyze the interactions between RNAP and the ribosome, we developed two in vitro transcription-coupled-to-translation systems reconstituted from purified components. In both systems, by using specific sequences of nucleic acids RNAP can be positioned in various types of transcriptional complex such as paused, roadblocked, backtracked, pre-translocated, post-translocated, etc, allowing the study of the interaction in pre-defined transcriptional complexes. Also both systems permit the translocation of the ribosome towards RNAP in a step-wise manner, defined number of codons at a time, allowing the observation of the outcomes of interactions between the two machineries at colliding and non-colliding distances. The two systems differ on how they are set-up and are designed to monitor either of the two molecular machineries. The first system, “transcription-first” coupled transcription translation system (TR-CTT) was designed to analyze the outcome on translation by observing the synthesized peptide directly whereas the second system, “translation-first” coupled transcription translation system (TL-CTT) follows the outcome on transcription by monitoring the RNA transcript [9]. The methodologies for the setting up of these systems will be discussed throughout this article.

2. Setting up an in vitro translation system

Briefly, both of the CTTs are assembled purified mRNA that at the 3’ end contains short sequence for assembly of artificial transcription elongation complex with purified RNAP, and closer to the 5’ end the RBS for initiation if translation with purified ribosomes and factors. The ORF of the mRNA codes for specific sequence that directs ribosome towards transcription elongation complex. TR-CTT and TL-CTT contain same components but differ in a way of their assembly.

2.1. mRNA design and synthesis

For the design of an mRNA template to be used for in vitro translation, there are several points that may affect the efficiency of the system should be taken into account. The translation initiation region (TIR) is the region composed of the Shine–Dalgarno sequence (SD), the sequence between the SD and the initiation codon (also known as spacer) and the initiation codon. Planning of this region is required to ensure that the ribosome will be able to efficiently bind to it and initiate translation. For the TL-CTT and TR-CTT systems the TIR of the gene 32 of the phage T4 which has been widely used and characterized in other translational systems has been used [28,18,49,14,37]. When designing an in vitro translation experiment, it is necessary to take into account that the mRNA, which will be used to program the ribosomes, will be added to the system already synthesized; therefore it will be uncoupled from transcription. This means that the mRNA will be folded into its secondary structure upon addition to the translation reaction. For this reason, it is desirable to design mRNA templates that have a weak secondary structure, especially in the (TIR). Secondary structure in the TIR will cause ribosome binding and translation initiation to fail because at this step the intrinsic helix-case activity of the ribosome cannot take place [37]. ORF of the mRNA has to contain codons for individual tRNAs that are commercially available (or can be isolated to high purity). The sequence of ORF must also allow easy analytical separation of the desired peptides by TLE in order to monitor translation. The 3’ end of mRNA would be used by RNAP during assembly of artificial transcription elongation complex, and the sequence thus needs to be adjusted to achieve desirable behavior of RNAP.

In order to synthesize mRNA, a DNA template carrying the T7 promoter, the gene 32 TIR and an open reading frame composed of the codons for M, F, V, Y and K, is first obtained by PCR. Note that sequences used here are explanatory and any sequence can be used depending on the experimental needs and/or availability of the purified tRNAs. For the in vitro synthesis of mRNA, 30 pmol of template DNA are mixed with 12 pmol of T7 RNAP (Promega) in a final volume of 500 μl of T7 transcription buffer (200 mM Tris–HCl (pH 7.9 at 25 °C), 30 mM MgCl2, 50 mM DTT, 50 mM NaCl, 10 mM spermidine, 2 mM all four NTP’s) at 37 °C for 6 h. 10 μl of RQI DNase I (Roche) are added and the reaction is incubated for 30 min at 37 °C. To stop the reaction, 50 μl of 0.5 M EDTA pH 8.0 and 50 μl of 3 M sodium acetate pH 5.2 are added. The RNA is partially purified by the addition of an equal volume of chloroform followed by vortexing for 30 s and subsequent centrifugation in table-top centrifuge (three chloroform extractions in total). The RNA is precipitated by the addition of 100% ethanol and incubated overnight at –80 °C. After centrifugation at 4 °C for 30 min, the pellet is desalted by one washing with 70% ethanol. The RNA pellet is dissolved in 60 μl of 2X RNA buffer loading dye (90% formamide + 0.02% bromophenol blue) and loaded on a 6% polyacrylamide denaturing gel. The electrophoresis is run at 50 W constant. The gel is then placed between two sheets of cling film and placed on top of a fluorescent TLC plate (Merck). UV light is used to detect the RNA, which is excised from the gel using a clean razor blade. The gel strip containing the RNA is put in a 1 ml tube and 600 μl of 0.3 M sodium acetate pH 5.2 are added. The mixture is vortexed overnight at 4 °C. The eluted RNA is extracted by the addition of 300 μl of chloroform and vortexing for 10 min. The sample is spun down at max speed for 2 min. The liquid is then transferred to a new tube being careful of not transferring any gel pieces. 600 μl of chloroform are added and a total of 3 chloroform extractions are performed. The RNA is then ethanol precipitated, desalted and its concentration and purity is assessed by spectrophotometry. PAGE purification of the synthesized mRNA is a crucial step. Because this procedure removes incompletely synthesized mRNA species that lack a correct 5’ and/or 3’ ends, which would lead to uncoupled transcription or translation species in the reaction, and would obscure interpretation of the results on coupling. The following step towards the set up of the translation system consists of the purification of all minimal components needed by the translational machinery to function, and the preparation of substrates for it.

2.2. Purification of 70S ribosomes

The standard ribosome purification protocol [28] involve one high salt wash to remove loosely associated non ribosomal proteins, tRNA and mRNA. However, we have observed that the use of two high salt washings, gives as a result, purer, more vacant, 70S ribosomes.

Briefly, 15 g of Escherichia coli MRE 600, an E. coli mutant strain which lacks RNase A which reduces ribosome turnover therefore

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increases the yield of 70S ribosomes is resuspended in buffer A (20 mM Tris 7.6, 10 mM MgCl₂, 100 mM NH₄Cl, 6 mM β-mercaptoethanol) and then lysed by two passages through a French press (C-019 constant systems UK) at 30,000 psi. In order to avoid contamination with genomic DNA, DNase I (Roche) is added to a final concentration of 20 μg/mL and the lysate is incubated on ice for 30 min and then the volume is adjusted to 45 mL with buffer A. After 2 clearing spins in Beckman JA-25.50 rotor at 15,000 rpm, the supernatant is loaded onto two 35 mL sucrose cushions (1.1 M sucrose, 20 mM Tris–HCl, pH 7.5, 500 mM NH₄Cl, 10 mM MgCl₂, 0.5 mM EDTA) in 75 mL polycarbonate tubes. Ultracentrifugation is carried out for 22 h at 35,000 rpm at 4 °C in a Ti-45 Beckman rotor. The translucent ribosome pellet is washed gently with buffer A and then the pellet is resuspended in 5 mL of the same buffer. After a clearing spin at 15,000 rpm in a JA-25.50 Beckman rotor, the volume is adjusted to 100 mL with buffer A containing 0.5 M NH₄Cl. After ultracentrifugation for 7 h at 22,000 rpm the pellet is washed again with buffer A and then resuspended in 100 mL of buffer A containing 0.5 M NH₄Cl before a final ultracentrifugation step (7 h, 22,000 rpm). Purified ribosomes are resuspended in 1.6 mL of buffer A containing 50 mM Tris pH 7.6, frozen in liquid nitrogen and stored at −80 °C. Ribosome concentration is calculated according to: A₂₆₀ = 1 equals to 23 pmol of 70S ribosomes/mL.

2.3. Purification of translation initiation and elongation factors

Purification of EF-G, EF-Tu, EF-Ts, IF-1, IF-2, IF-3, F-Met-tRNAfMet-aminocoyl tRNA synthetase (MetRS) and methionyl-tRNAformyltransferase (FTM).

Because the Histidine tag does not affect the activity of translation initiation or elongation factors, N-terminal (His)_₆-tagged proteins can be purified and used to set-up the translational system.

For expression and purification, plasmids (based on pCA24 N, -gfp, cat) encoding (His)_₆-tagged IF-1, IF-2, IF-3, EF-Tu, EF-G MetRS and FTM, can be obtained from the ASKA clone collection (E. coli Strain National BioResource Project, Japan). The plasmids are transformed into BL21 competent E. coli cells (NEB). A 100 mL overnight culture is used to inoculate 4 L of LB media supplemented with 25 μg/mL chloramphenicol. Cells are grown in an orbital shaker at 37 °C until an OD₆₀₀ = 0.4 is reached. IPTG (0.250 mM final) is added and induction is carried out at 30 °C for IF-3, EF-G and EF-Tu and at 37 °C for other proteins for a total of 4 h. After induction, cells are pelleted and washed twice with translation buffer (TrLB) (10 mM Tris–HCl pH 7.4, 60 mM NH₄Cl, 10 mM Mg(OAc)₂ and 6 mM β-mercaptoethanol). Cells containing over-expressed EF-Tu are washed with TrLB buffer containing 1 mM GTP to avoid precipitation of the enzyme [4]. Pellets are resuspended in TrLB buffer + 10% glycerol and EDTA-free protease inhibitor cocktail (Roche), and incubated on ice with lysozyme (0.1 μg/mL) for 30 min. Cells are disrupted by sonication in stainless steel tubes in an ice-water bath for 15 min, followed by two clearing centrifugation steps at 15,000 rpm in a JA-25.50 Beckman rotor. An ultracentrifugation step is done in polycarbonate tubes for 2 h in a Ti-45 Beckman rotor at 33,000 rpm. The supernatants are then applied onto a 5 mL His-Trap column (GE healthcare) connected to an AKTA Explorer FPLC (GE healthcare). Bound proteins are eluted with a linear gradient of imidazole (from 10 mM to 200 mM) in elution buffer (20 mM Tris pH 7.4 600 mM NaCl). Peak fractions are pooled and analyzed by SDS–PAGE (10%). Fractions containing the proteins of interest are dialyzed overnight against 2 L of TrLB and loaded onto a 1 mL MonoQ (GE healthcare) ion exchange column. A gradient from 5% to 100% TrLB containing 1 M NaCl is performed. The corresponding fractions are pooled and dialyzed overnight against 2 L of TrLB buffer supplemented with 50% glycerol. All proteins are purified to homogeneity of at least 90% and is recommended to checked for the lack of DNAse and RNase activity. The purified proteins are tested in the ribosome walking experiment, which will be described later.

2.4. Preparation of substrates for translation

One of the major characteristics of our coupled system is that it allows the translocation of ribosomes in a step-wise manner. Therefore, the preparation of individual, pure substrates for translation, aminoacyl-tRNA(s) (AA-tRNAs), is a requirement. For this reason, along with the MetRS and FTM (for preparation of F-Met-tRNAfMet) a mixture of other aminoacyl tRNA synthetases are DEAE purified from S100 extracts. Deacylated tRNA's purified from E. coli: tRNAfMet, tRNAAsp, tRNAArg, tRNAVal and tRNAPro can be obtained from Sigma. To reduce the amount of components present in the coupled system, the aminoacylation procedure is performed in a separate experiment independent from the translation reaction itself. The purification of resulting AA-tRNA includes phenol–chloroform extraction, ethanol precipitation, desalting and gel filtration to completely remove traces of ATP to circumvent the possibility that RNAP would incorporate those NTPs into the nascent RNA, affecting the interpretation of the effects on transcription–translation coupling itself. Efficiency of aminoacylation is then assessed as described below.

There are two ways to initiate translation in vitro. The enzymatic pathway in which initiation factors IF-1, IF-2 and IF-3 and GTP are responsible for 70S ribosome assembly (on the translation initiation region of the mRNA) and for positioning of the initiator tRNA f-met-tRNAfMet in the ribosome's P site. The alternative way does not require initiation factors and f-met-tRNAfMet. Instead, the peptidyl analogue N-acetyl-met-tRNAfMet that is capable of binding to the ribosome's P site unaided by enzymatic factors (henceforth, this type of initiation will be referred to as non-enzymatic initiation) is used to initiate translation.

2.4.1. Purification of S100 extracts as a source of aminoacyl tRNA synthetases

In order to prepare AA-tRNA, which will be used to translocate the ribosome, DEAE-S100 extracts can be used as a source of aminoacyl-tRNA synthetases, avoiding the necessity of purifying each AA-tRNA independently. This extract contains all soluble, cytoplasmatic proteins (including all aminoacyl-tRNA synthetases) and is free from ribosomes, membrane, cell wall, chromosomal DNA and tRNA.

S100 extracts are obtained as described in [6]. Briefly, E. coli MRE 600 cells are grown in LB media until an OD₆₀₀ = 0.6 is reached. The cells are disrupted in grinding buffer (20 mM Tris–HCl pH 7.4, 10 mM MgCl₂, 5% glycerol, 50 mM NaCl) by two rounds of sonication in an ice bath. The crude extract is cleared by centrifugation at 15,000 rpm in a JA-25.50 Beckman rotor for 30 min. Then ultracentrifugation of the supernatant is done at 30,000 rpm for 22 h in a Ti-45 rotor (Beckman). The resulting S100 crude extract is purified on a 16 mL DEAE-cellulose column (Whatman) equilibrated with buffer S100 (50 mM Tris–HCl pH 7.4, 70 mM NH₄Cl, 30 mM KCl and 7 mM MgCl₂). A linear gradient from 0 to 300 mM NaCl (in the same buffer) is applied and the eluted peak fraction is dialyzed against storage buffer (50 mM Tris–HCl pH 7.4, 10 mM MgCl₂, 50% glycerol, 50 mM KCl). Note that two peaks are eluted. The first peak corresponds to S100 extract and the second peak is tRNA eluting from the column.

2.4.2. Preparation of the NS-N10–methenyltetrahydrofoleric acid as precursor of N10-formyl-tetrahydrofolate

All tRNA charging methods have been optimized from [40]. In order to obtain the initiator aminoacyl-tRNA F-Met-tRNAfMet, the
compound N10-formyl-tetrahydrofolate donates the formyl group to the Met-tRNAfmet in a FTM dependent manner. Due to the chemical instability of the donor compound, a stable precursor is prepared (N5,N10-methenyltetrahydrofolate acid). This precursor is then transformed into N10-formyl-tetrahydrofolate just before the N-formyltransferase reaction takes place. To prepare the stable form, 25 mg of folinic acid (Ca salt (Sigma)) is dissolved in 2 mL 50 mM β-mercaptoethanol. Then, 220 μL of 1 M HCl is added and the samples incubated for 3 h at room temperature. When the incubation is finished, the reaction is diluted with 1 mL of 100 mM HCl and stored at −20 °C in 200 μL aliquots. Right before the preparation of F-Met-tRNAfmet by formylation and aminoacylation reactions (which are conducted simultaneously), N5,N10-methenyltetrahydrofolate is neutralized by the addition of 10 μL 1 M Tris–HCl pH 7.9 and 20 μL of 1 M KOH. After 15 min of incubation at room temperature, the neutralized form (N10-formyl-tetrahydrofolate) is added to the charging/formylation reaction.

2.4.3. Formylation, aminocacylation and N-acetylation of tRNAfmet

It is strongly suggested that formylation and aminoacylation of tRNAfmet are performed in the same reaction at the same time to prevent rapid oxidation of methionine into sulfoxide. 1 unit of tRNAfmet (Sigma) is mixed with 2 mM l-methionine (or 300 pmol of F[35S]-Met (1000 ci/mmol), 10 mM ATP, 50 mM HEPES, 10 mM KCl, 1 mM DTT, 20 mM MgCl2, 10 mM neutralized N5,N10-methenyltetrahydrofolate, 50 pmol MetRS, and 50 pmol FTM and incubated at 37 °C for 25 min in a final volume of 500 μL. The reaction is quenched by addition of 50 μL of 3 M sodium acetate pH 5.3 and 28 μL of 10% SDS. From this point onwards, the aminoacylated tRNA must be kept under acidic conditions pH < 5 to avoid spontaneous deacylation due to the instability of the ester bond that binds the amino acid to the tRNA at neutral and basic pH. The reaction is extracted by vortexing in the presence of 1 volume of phenol for 10 min. After a 5 min centrifugation at full speed in a table top centrifuge, the supernatant is separated from the phenol phase. The phenol phase is back-extracted by addition of 500 μL of 0.3 M sodium acetate pH 5.3, then the sample is vortexed and centrifuged. The supernatants are treated twice with chloroform, ethanol precipitated, desalted with washing with 70% ethanol and dissolved in 60 μL of 2 mM sodium acetate pH 5.3. To remove traces of ATP, the resulting F-Met-tRNAfmet is gel filtrated 4 times using Bio-Rad Bio-Spin 6 columns equilibrated in 2 mM sodium acetate to remove traces of ATP.

The preparation of the peptidyl analogue N-acetyl-met-tRNAfmet is carried out as described above, but the formylation step is omitted. The acetylation is done right after the ethanol precipitation step. The pellet is dissolved in 200 μL 200 mM sodium acetate followed by the addition of 2.5 μL of acetic anhydride (Sigma). After incubation for 1 h on ice, another 2.5 μL of acetic anhydride is added and the reaction incubated for one extra hour. The volume of the solution is then increased to 500 μL of 200 mM sodium acetate, precipitated, desalted, resuspended in 2 mM sodium acetate pH 5.3 and gel filtrated in Bio-Spin 6 column (Bio-Rad) as above.

2.4.4. General aminocacylation procedure (for tRNAs other than tRNAfmet)

The aminoacylation of tRNA is carried out the same way as for tRNAfmet. The difference is that the procedure does not require FMT or formyl group donor and also, SDS is not added at the end of the reaction. The reaction is catalyzed by aminocetyl synthetases present in the purified S100 extract. Briefly, 1 unit of tRNA (Sigma) is mixed with 2 mM amino acid, 10 mM ATP, 50 mM HEPES, 10 mM KCl, 1 mM DTT, 20 mM MgCl2, and 50 pmol S100 extract in 500 μL. The reaction is incubated at 37 °C for 25 min and quenched by addition of 50 μL 3 M sodium acetate pH 5.3. Purification of the aminoacyl-tRNA is carried out exactly as described for tRNAfmet.

The efficiency of aminoacylation, formylation (for enzymatic initiation) or N-acetylation of tRNAfmet (for non-enzymatic initiation) and the aminocacylation of all other tRNA’s can be evaluated by acid gel electrophoresis or thin layer chromatography (TLC) as described [41]. The migration in acid gel electrophoresis of deacylated tRNA differs from its aminocylated counterpart due to the presence of the amino acid, which retards the migration of the tRNA. Acid gel electrophoresis can also be used to assess the formylation and N-acetylation of Met-tRNAfmet. Following the protocols described above, the extent of aminoacylation, formylation and N-acetylation is generally greater than 90%.

2.5. Translation initiation and elongation

For initiation and translocation of the ribosome in both coupled and uncoupled systems a method similar to [37(b)] is used. For ribosome binding and initiation: 2 μM 70S ribosomes are mixed with 2 pmol of either mRNA or transcription elongation complexes (see below) in a final volume of 49 μL of TrLB. The mixture is incubated for 15 min at 37 °C followed by the addition of either N-acetyl-met-tRNAfmet (non-enzymatic initiation) or F-Met-tRNAfmet, IF-1, IF-2, IF-3 to final concentration of 5 μM and 200 μM GTP (enzymatic initiation) to a final volume of 60 μL and incubation at 37 °C for 10 min. During this step, the ribosome binds the SD sequence and accommodates the initiator tRNA in the P site of the ribosome.

While ribosome binding reaction takes place, EF-Tu-GDP must be exchanged into its EF-Tu-GTP form followed by the binding of the AA-tRNA to it thus forming the ternary complex EF-Tu-GTP-A-tRNA. In order to exchange the GDP to GTP, EF-Tu-GDP (400 pmol) is incubated with GTP (600 pmol) in the presence of EF-Tx (60 pmol) phosphoenol pyruvate (PepT) (400 pmol) and phosphoenol pyruvate kinase (PK) (200 μg/mL) in 30 μL ternary complex buffer (50 mM Tris–HCl pH 7.4, 40 mM NH4Cl, 10 mM MgCl2 and 1 mM DTT). The reaction is incubated for 10 min at 37 °C. Then, 6 μL final concentration of aminoacyl-tRNA is added and further incubated at 37 °C for 5 min (Fig. 1). 6 μL of the resulting ternary complexes are added in both, peptidyl-transfase and translocation assays (below).

In order to translocate the ribosomes by one or more codons the above components are mixed with addition of EF-G. For instance, if the ORF codes for MFVYK, and the ribosome is to be translocated by one codon (F), to tubes containing 1.2x TrlB buffer and 800 μM final concentration of GTP, EF-G is added first at a concentration of 20 μM final, followed by addition of 6 μL ternary complexes (in this case EF-Tu-GTP-Phe-tRNAphe) and then a 10 μL aliquot is withdrawn from the reaction containing initiated ribosomes and transferred to the elongation reaction (40 μL final volume). This mixture is incubated for 3 min at 37 °C. The reaction can be terminated in different ways according to what is to be analyzed. For instance, if the peptide is to be observed, the peptidyl-tRNA is dissociated from the complex by increasing the pH (to pH 14) by addition of 4 μL of 100 mM KOH (which is anyway required to disrupt the ester bond linking the peptide to the tRNA before TLE); the reaction can also be terminated by addition of 200 mM EDTA or by prolonged (>5 min) incubation on ice.

2.6. Assessing translation initiation and elongation by toeprint assay

Before attempting to use the reconstituted system in transcription–translation experiments, translation initiation and elongation need to be assessed to ensure that the system is working properly.
To do so, a technique known as toeprinting [28] can be used. Toeprinting is based on the inhibition of the progression of the reverse transcriptase (rt) by an obstacle in the RNA (in this case the ribosome). This method allows the detection of ribosomal complexes in initiation and elongation states with single nucleotide resolution. Briefly, a radiolabeled oligonucleotide is annealed to the 3’ end of the mRNA and rt is allowed to synthesize cDNA. In the absence of a bound ribosome, the rt will generate full length cDNA. If translation is initiated, the rt will collide with the ribosome generating a shorter cDNA compared to the full length control. If the ribosome is to be translocated by one or more codons, the cDNA synthesized by the rt will be even shorter compared to the cDNA generated in initiated translation complexes only.

The peptidyl transferase assay is done to visualize the synthesized peptides permitting its visualization. The amount of ribosomes and mRNA is 50 pmol per initiation reaction for the analysis of peptide synthesis. After the translocation reaction, KOH is added to a final concentration of 100 mM and incubated at 37 °C for 30 min to deacylate the peptidyl-tRNA. The products are resolved by thin layer electrophoresis [7].

Because the translational system has been designed to take small steps towards RNAP, the peptides that are synthesized are in the range from dipeptides to hexapeptides, excluding the use of SDS–PAGE because of the low resolution that it provides. Thin layer chromatography (TLC) has been routinely used as a tool for the identification and characterization of the primary structure of polypeptides [48,52,51]. For the resolution of peptides, the stationary phase is usually composed of cellulose or silica gel, layered on an inert backing such as plastic or aluminum. The mobile phase is composed of organic solvents mixed in different ratios depending on the sample that is to be analyzed. A TLC-based technique known as Thin Layer Electrophoresis (TLE) takes advantage of the affinity of the samples to the solid and mobile phases but also separates them according to the charge of the peptides. This technique has been used for the elucidation of the mechanism of translation fidelity [51]: on a plastic backed, cellulose TLE plate, the samples are spotted in the middle and a buffer (mobile phase) is allowed to run over the samples from the top and the bottom of the plate. The plate is then placed into an electrophoresis chamber and voltage is applied see below.

Cellulose chromatography plates (plastic backed) (Merck) are cut in rectangles measuring 17 cm L × 7 cm W. A line is drawn right in the middle of the plate. The samples (from peptidyl transferase assay) are spotted on this line (referred to as the origin). The plate is then placed in an electrophoresis chamber (with a footprint 15 × 9.5 × 22 cm) with separated buffer chambers. In each chamber Pyrac buffer (200 mL Acetic acid (Sigma) 5 mL pyridine (Sigma), in 1 L of water) is added (30 ml each). The buffer is allowed to run over the plate for 25 min. The Pyrac buffer is added to the plate slowly without touching the sample. Once the buffer has concentrated the samples, the plate is covered with Stoddard solvent (Sigma) and the electrophoresis is run at 1200 V, 13 mA, 13 W for 30 min. Note that the pyrac buffers added in the chambers should not be allowed to directly contact each other during electrophoresis due to risk of fire. This experiment should be always run under the fume hood on a tray. The buffers are discarded and the plate is dried for 3 h. Then it is carefully wrapped in cling film, exposed to a phosphorscreen and scanned using Typhoon scanner (Fig. 2).

After the successful assessment of both ribosome translocation and peptidyl transferase activity, the system can now be coupled to transcription.

3. Coupling transcription to translation

In these article strategies for the assembly of two, coupled transcription to translation systems (CTTs) will be described. The first system to be explained can be used for the analysis of the outcome of the collision between the ribosome and RNAP on translation by looking at the synthesized peptides; the second CTT to be discussed has been aimed at the analysis of the outcome on transcription upon the collision of RNAP and the ribosome.

3.1. Formation of artificially assembled transcription elongation complexes

Artificially assembled transcription elongation complexes (AAEC) are formed with mRNA synthesized in vitro using T7

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RNAP in exactly the same way as mRNA was obtained for the characterization of the translational system. To assemble the AAEC, a DNA oligonucleotide containing a 8–9 nucleotide-long sequence complementary to the 3' end of the mRNA surrounded by non-complementary overhangs at its 3' and 5' ends is mixed with the mRNA in 1X transcription buffer, TB (10 mM Tris HCl pH 7.4, 5 mM MgCl₂, 40 mM KCl) (Fig. 3). This oligonucleotide serves as a "template DNA", because the sequence immediately downstream of the hybrid (5' end overhang) serves as template, which dictates the nucleotides that RNAP incorporates in the growing mRNA chain. Upon hybridization, RNAP (purified as described in [5]) is added into the reaction and the RNA–DNA hybrid is recognized and bound by RNAP positioning the 3' end of the mRNA in the active center. To complete the assembly of the AAEC, a second DNA oligonucleotide (non-template DNA), fully complementary to the template DNA, is allowed to anneal with the overhanging stretches of the template DNA and is then accommodated into the complex resulting in the formation of a fully active elongation complex. AAEC are indistinguishable from transcription elongation complexes formed on double-stranded DNA of the same sequence by transcription from a promoter [32,50,12].

Although the protocol for the assembly of artificial elongation complexes is the same regardless of the sequence, the RNAP walking reaction varies according to the sequence in the template DNA that is being used. For RNAP walking experiments methodology see [29]). AAEC brings flexibility to the system by allowing the ease of interchanging the transcribed region just by simply changing the template and non-template DNA oligonucleotides. This allows the exploration of interactions of ribosome with RNAP stabilized in different transcriptional states. The various transcription states of RNAP are mainly determined by the sequences of the RNA–DNA hybrid in the elongation complex, and the adjacent sequences in template and non-template strands [3,21,36,25]. Putting RNAP into different states is therefore achieved by simply changing the sequences of the oligonucleotides used in AAEC. Also AAEC allows the positioning of RNAP directly into the transcription elongation stage skipping the initiation step all together, and thus simplifies the system as compared to promoter-borne transcription. Additionally an advantage of using AAEC over promoter-borne transcription is that the concentration and homogeneity of mRNA does not depend on promoter strength, promoter escape and processivity of transcription elongation. All of these limiting scenarios are avoided by using purified mRNA.
3.2. “Transcription first” CTT (TR-CTT) for analysis of coupling effects on translation

To analyze the effects of interactions between the ribosome and RNAP on translation (i.e. its peptidyl transferase activity), the AAEC is formed as follows: 75 pmol of biotin-tagged RNAP is first immobilized onto 10 μL of streptavidin beads (GE healthcare) equilibrated in 100 μL TB containing 0.1 M KCl, by gentle shaking at 26 °C for 7 min. To remove RNAP, which has not been bound to the beads, 5 washings with 1X TB buffer are performed. To assemble AAEC, mRNA (50 pmol) and template DNA (200 pmol) are added to the immobilized RNAP and incubated at 37 °C for 15 min. After this step, non-template DNA (1000 pmol) is added and incubated for 15 min at 37 °C. It is important to wash the AAEC at least 5 times with TB to remove unused oligonucleotides and mRNA, which if left in the system may lead to formation of uncoupled translation complexes and thus obscure the analysis of the interactions in coupled species. At this point, the AAEC is ready for the RNAP to be walked and positioned in a desired transcriptional state. After walking, the AAEC must be washed to remove traces of NTPs and the volume is reduced to 18 μL.

Translation initiation and elongation is done as in section 1.5 taking into account that the initiator tRNA is radiolabeled to permit the visualization of the peptides (Fig. 4). Once the experiment is finished, the ester bond in the peptidyl-tRNA is hydrolyzed by addition of KOH (to a final concentration of 100 mM) and incubation at 37 °C for 30 min. Although the samples can be loaded straight away on the TLE system, they can also be concentrated using an evaporator, keeping the temperature at or below 37 °C to avoid damaging the synthesized peptides.

Because in this system all translating ribosomes are coupled to the transcription elongation complexes (all free RNA is washed...
3.3. “Translation first” CTT (TL-CTT) for analysis of coupling effects on transcription

In order to be able to use AAEC for the analysis of the outcome on transcription by the translating ribosome, it is necessary to achieve complete ribosomal occupancy on the AAEC, as the presence of a mixed population of coupled and uncoupled transcriptional complexes would obscure the observed outcome of the collisions on RNAP. For this reason, this system requires the assembly of the translation system in the absence of the transcriptional machinery (hence “translation first” [TL-CTT]) followed by purification of translation elongation complexes from unused mRNA. This is followed by AAEC formation and translocation of both ribosome and RNAP to analyze the interactions on transcription. The system is set up as follows: translation is assembled and the ribosome is translocated by one codon as explained in section 1.5. To separate unused mRNA from translational complexes, the dipetidyl containing translation elongation complexes are purified by ultracentrifugation through a sucrose cushion (1.1 M sucrose, 20 mM Tris–HCl, pH 7.5, 500 mM NH4Cl, 10 mM MgCl2, 0.5 mM EDTA) and spun at 258,000 g in a TLA100.3 rotor for 2 h [51] (Fig. 5). The resulting pellet containing only mRNA occupied by translocated ribosomes is washed 3X with TrLB to remove traces of supernatant and resuspended in 10 μL of the same buffer. Then, the AAEC is formed by addition of 60 pmol of template DNA and 30 pmol of RNAP. The reaction is incubated for 15 min at 37 °C following the addition of 300 pmol of non-template DNA and subsequent incubation for 15 min at 37 °C in 1X TrLB. At this point, the system has been formed and the RNA can be labeled by incorporation of radiolabeled NMPs by RNAP. RNAP can be positioned in any desired transcriptional complex before or during ribosome translocation. From the main reaction, 10 μL aliquots are used for the elongation reactions as explained in 1.5. Reactions in this system are terminated by addition of an equal volume of stop buffer (EDTA 20 mM, 7 M urea, 100 mM NaCl, 0.02% bromophenol blue, 0.03% xylene cyanol in formamide) and the products are resolved by sequencing 6% PAGE (19:1) and analyzed by phosphorimaging and ImageQuant software (GE-Healthcare).

As all transcription elongation complexes are coupled in TL-CTT, this system can be used to assess the effects of ribosome approaching and colliding with RNAP on the behavior of the transcription elongation complex.

4. Conclusions

Lately, a major importance has been given to the interplay amongst cellular machineries such as transcription, translation and replication; the analysis of interactions of RNAP with the replication machinery has suggested the existence of complex regulatory mechanisms, which affect cellular activities ranging from gene expression to genome organization and stability. However, elucidation of the cross-talk amongst the major cellular machineries is incomplete due to the lack of characterization of the interactions between RNAP and the translational apparatus. Recently, in vivo approaches have been designed to study the effects on transcription elongation caused by the ribosome and impact of these effects on replication fork progression [13]. Nevertheless, the mechanistic interactions of the colliding ribosome with RNAP and its outcome on peptide synthesis and on transcriptional elongation complexes stabilized in different states remains obscure. Therefore, the use of reconstituted in vitro transcription–translation systems can be useful to elucidate precise mechanistic details of these interactions.

In this article, methodologies for the purification of all components needed for the assembly of both fully functional transcription and translation machineries in vitro, and the setup of transcription coupled to translation systems is described. The systems which were assembled and the outcomes monitored with them are:

1. “Translation first-CTT” (TR-CTT) system: In this system all translational complexes are coupled to transcription; therefore it can be used to analyze the outcomes of the collision between the ribosome and RNAP on transcription by direct observation of the nascent peptide synthesized in the CTT. The system can also be used to analyze the effect of transcription on translation initiation and elongation, depending on the distance between RNAP and the ribosome. In addition the system can be used to analyze if paused RNAP can function as a regulatory signal for the translational machinery.

2. “Translation first” CTT (TL-CTT) system: In this system all transcriptional complexes are coupled to translation; therefore, it can be used to study the effects on the collision between the ribosome and RNAP on transcription by following the nascent, radiolabeled mRNA. The system can be used to observe the effects on transcriptional pausing caused by the colliding ribosome. Moreover, TL-CTT could be also used to evaluate the effect of the phasing of the ribosome approaching RNAP (given that ribosome moves in triplets), on transcription. TL-CTT can be used to observe the effects of moving ribosome on diverse transcription elongation complexes and for analysis of the function of various transcription and translation factors on outcomes of coupling.

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References