Published online 30 December 2011

Nucleic Acids Research, 2012, Vol. 40, No. 6 ***e45***
doi:10.1093/nar/gkr1262

In vitro experimental system for analysis of transcription–translation coupling

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Received October 19, 2011; Revised November 30, 2011; Accepted December 6, 2011

ABSTRACT

Transcription and translation are coupled in bacteria, meaning that translation takes place co-transcriptionally. During transcription–translation, both machineries mutually affect each others' functions, which is important for regulation of gene expression. Analysis of interactions between RNA polymerase (RNAP) and the ribosome, however, are limited due to the lack of an *in vitro* experimental system. Here, we report the development of an *in vitro* transcription coupled to translation system assembled from purified components. The system allows controlled stepwise transcription and simultaneous stepwise translation of the nascent RNA, and permits investigation of the interactions of RNAP with the ribosome, as well as the effects of translation on transcription and transcription on translation. As an example of usage of this experimental system, we uncover complex effects of transcription–translation coupling on pausing of transcription.

INTRODUCTION

In all living organisms, transcription is accomplished by multisubunit RNA polymerases (RNAPs). RNAP is a complex machinery which is subject to an intricate system of regulation. It interacts with a large number of other cellular components that assemble in its proximity and interact with either the same DNA template (DNA polymerases, topoisomerases, etc.), or on the nascent RNA (ribosomes, RNA processing enzymes, etc.). Recent studies have revealed complex mechanisms of inter-relations between RNAPs and these machineries. For instance, collisions of RNAP and the replication fork and their outcomes were investigated thoroughly both *in vivo* and *in vitro*. Elegant *in vitro* experimental systems revealed that the transcript synthesized by RNAP can be used by DNA polymerase as a primer for replication after the displacement of RNAP from DNA (1). The mechanism of primer formation was also described (2). Some collisions between replication forks and RNAP, however, were shown to impede replication and/or lead to a collapse of the replication fork (3–6).

In bacteria, the nascent mRNA synthesized by RNAP is immediately used by the translation apparatus. Interactions of the transcription apparatus with the translation machinery have been studied mainly in respect to the regulation of gene expression, rather than the physical interaction between ribosome and the RNAP. One of the classic examples of such regulation is when translation aids paused RNAP and restores its elongating state by melting the secondary structure of the nascent RNA behind RNAP (7). Another example of ribosome-mediated regulation of transcription is the case of the regulation of the tryptophanase (*tna)* operon of *Escherichia coli*, where the ribosome inhibits binding of the termination factor ρ, allowing RNAP to synthesize a full length mRNA (8,9). Transcription–translation coupling is also considered important to prevent the formation of R-loop structures (10). The most recent *in vivo* study focused on mechanistic aspects of the interactions of RNAP and the ribosome (11). This study suggested that the ribosome may 'push' RNAP and thus help it to overcome backtracking events. Most studies of the interactions of RNAP and the ribosome were performed *in vivo* or in crude extracts, which limits the possibility of understanding the mechanistic details of the cross-talk between the two machineries. In particular, other cellular components, such as transcription and translation factors, that may influence functioning of both machineries cannot be excluded in these experimental systems. Commercially available coupled transcription–translation systems are designed for the production of proteins *in vitro* (12–14). They contain all the components of both apparatuses and thus do not permit stalling of transcription or translation complexes, making it impossible to take a 'snapshot' of their interactions. Here, we report the development of

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in vitro coupled transcription–translation systems from purified components which allow the controlled movement of both machineries and investigation of the cross-talk between RNAP and ribosome.

MATERIALS AND METHODS

Protein purification

Purification of EF-G, EF-Tu, IF-1, IF-2, IF-3, MetRS and FTM. Plasmids (based on pCA24N, -gfp, cat) encoding 6XHis tagged IF-1, IF-2, IF-3, EF-Tu, EF-G MetRS and FTM, were obtained from the ASKA clone (-) collection (*E.coli* Strain National BioResource Project, Japan). Plasmids were transformed into T7 express Iq competent *E.coli* cells (High Efficiency, NEB). A 100 ml overnight culture was used to inoculate 4 l of LB media supplemented with 25 μg/ml chloramphenicol. Cells were grown in an orbital shaker at 37°C until an $OD_{600} = 0.4$ was reached. IPTG (0.250 mM final) was added and induction was 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 were pelleted and washed twice with translation buffer (TrLB) (10 mM Tris–HCl pH 7, 4, 60 mM NH_4Cl, 10 mM $Mg(OAc)_2$ and 6 mM β-mercaptoethanol). Cells containing over-expressed EF-Tu were washed with TrLB buffer containing 1 mM GTP to avoid precipitation of the enzyme. Pellets were resuspended in TrLB buffer + 10% glycerol and EDTA-free protease inhibitor cocktail (Roche), and incubated on ice with lysozyme (0.1 mg/ml) for 30 min. Cells were disrupted by sonication (Power output = 6 Duty cycle 60%) 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 was carried out in polycarbonate tubes for 22 h in a Ti-45 Beckman rotor at 33 000 rpm. The supernatants were then applied onto a His-Trap column (GE healthcare) connected to an AKTA Explorer FPLC (GE healthcare). Bound proteins were 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 were pooled and analyzed by SDS–PAGE (10%). Fractions containing the proteins of interest were dialyzed overnight against 2 l of TrLB buffer supplemented with 50% glycerol.

Purification of RNAP and σ^{70}. Purification of His-tagged RNAP (strain RL721, made and provided by R. Landick University of Wisconsin, Madison, Wisconsin, USA) and TAP-tagged RNAP (strain RpoBtap, made and provided by A. Emili, Department of Medical Research, University of Toronto, Toronto, Ontario, Canada) was performed exactly as described in Ref. (15). For the purification of σ^{70}, the rpoD gene was cloned into pET21 vector (Novagen) and transformed into BL21 DE3 cells. The purification was carried out exactly as in Ref. (16).

Purification of 70S ribosomes. Ribosomes were purified as described in Ref. (17) except that two high-salt washings were performed instead of one. 15 g of *E.coli MRE* 600 were resuspended in buffer A (20 mM Tris 7.6, 10 mM $MgCl_2$, 100 mM NH_4Cl, 6 mM 2 mercaptoethanol) and then lysed by two passes through a French press (C-019 constant systems UK) at 30 000 psi. DNAse I was then added to a final concentration of 20 μg/ml and the lysate incubated on ice for 30 min, before the volume was adjusted to 45 ml with buffer A. After two clearing spins, the supernatant was loaded onto two 35 ml sucrose cushions in 75 ml polycarbonate tubes. Ultracentrifugation was carried for 22 h at 35 000 rpm at 4°C in a Ti-45 Beckman rotor. The pellet was washed with buffer A and 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 was adjusted to 100 ml with buffer A containing 0.5 M NH_4Cl. After ultracentrifugation for 7 h at 22 000 rpm the pellet was washed again with buffer A and then resuspended in 100 ml of buffer A containing 0.5 M NH_4Cl, before a final ultracentrifugation step (7 h, 22 000 rpm). Purified ribosomes were resuspended in 1.6 ml of buffer A containing 50 mM Tris pH 7.6, frozen in liquid nitrogen and stored at −80°C

Preparation of DEAE purified S100 extracts

S100 extracts were obtained as described in Ref (18). *Escherichia coli MRE 600* cells were grown in LB media until an $OD_{260} = 0.6$ was reached. The cells were disrupted in grinding buffer (20 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 5% glycerol, 50 mM NaCl) by two 5 min rounds of sonication (Power output = 6 Duty cycle 60%) on ice. The crude extract was cleared by centrifugation at 15 000 rpm in a JA-25.50 Beckman rotor for 30 min. Then, ultracentrifugation of the supernatant was done at 30 000 rpm for 22 h in a Ti-45 rotor. The S100 crude extract was purified on a 16 ml DEAE-cellulose column (Whatman) equilibrated with buffer S100 (50 mM Tris–HCl pH 7.4, 70 mM NH_4Cl, 30 mM KCl and 7 mM $MgCl_2$). A linear gradient from 0 to 300 mM NaCl (in the same buffer) was applied and the eluted peak fraction was dialyzed against storage buffer (50 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 50% glycerol, 50 mM KCl).

Formylation and *N*-acetylation of tRNAfmet

Aminoacylation, formylation, *N*-acetylation and evaluation of aminoacyl-tRNAs were performed as described in Ref. (19) with the only difference that the aminoacyl-tRNAs were gel filtrated 4× using Bio-Rad Bio-Spin 6 columns equilibrated in 20 mM sodium acetate to remove traces of ATP. All tRNAs were purchased from Sigma and amino acids from USB. Pure S100 extract was used as source of aminoacyl synthetases to aminoacylate Phe-$tRNA^{phe}$, Val-$tRNA^{val}$, Tyr-$tRNA^{tyr}$ and Lys-$tRNA^{lys}$, being the only exceptions F-Met-$tRNA^{fmet}$ and *N*-Acetyl-Met-$tRNA^{fmet}$ where MetRS was used.

Preparation of DNA and RNA templates

DNA templates for promoter borne transcription and for *in vitro* mRNA synthesis were obtained by PCR using Phusion DNA polymerase (Finnzymes) and were subsequently purified from 2% agarose gels using the Gel

Extraction Kit (Qiagen). Oligonucleotides for PCR and for formation of assembled elongation complexes were purchased from IDT. RNA templates were prepared using T7 RNAP as described in Ref. (20).

Transcription elongation complexes

For experiments carried out on templates containing promoters, the transcription buffer (10 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$) differed only in the salt concentration: 50 mM KCl (TB50) for transcription initiation and elongation; 1 M NaCl (TB1000) for high salt washings. Transcription was carried out on solid phase by immobilizing the DNA which contained a biotin tag located at the 5′-end on streptavidin beads (Sigma). RNAP (1 pmol) was incubated in 20 µl TB50 with σ^{70} (4 pmol) in the presence of biotinylated DNA template (2 pmol) for 5 min at 37°C. Then, the transcription initiation mixture 15 pmol CpApUpC, 25 µM of GTP, ATP and CTP was added to a total volume of 40 µl and incubated for 7 min at 37°C (resulting in formation of elongation complex containing 11 nt long RNA; EC11). Streptavidin beads (10 µl, equilibrated in TB50) were added and the tube was shaken gently for 5 min. Two 1 ml washings with TB1000 containing 100 µg/ml heparin (Sigma) were performed, followed by five 1 ml washes with TB50. In order to translocate RNAP to the +56, +62, +72 and +80 positions (relative to transcriptional start site designated as +1), incomplete sets of NTPs were added to complexes to a final concentration of 25 µM, incubated for 5 min at RT and washed 5× with TB50. To walk EC11 to position +56-mer (labeling step), UTP, GTP and α-[^{32}P]-ATP (4 pmol) were added for 5 min at RT. After that, ATP was added, and incubated for another 5 min. To walk EC56 to position +62 CTP, ATP, and UTP were added; EC65 was walked to position +72-mer by addition of ATP, GTP and UTP; EC72 was walked to position +80 by addition of CTP, UTP and GTP. After washing of EC80, the volume of the reaction was reduced to 18 µl and transferred to the binding reaction (below).

Transcription in artificial elongation complexes (scaffolds) was performed as described in Refs (21–24) with some modifications. RNA (5 pmol), RNAP (7.5 pmol), and 5′-biotinylated template DNA (20 pmol) were incubated at 37°C in Low magnesium Low pH buffer (LmLpH) (10 mM Tris pH 7.4, 40 mM KCl and 5 mM $MgCl_2$) for 10 min, followed by the addition of 100 pmol of non-template DNA to a total volume of 40 µl. After 10 min incubation at 37°C, streptavidin beads (10 µl equilibrated in LmLpH) were added and gently shaken for 5 min at RT followed by two washings with TB1000 and five washings with LmLpH. Four picomoles of α-[^{32}P]-GTP were added and the mixture was incubated for 5 min at RT. The reaction was then washed 5× with LmLpH buffer and RNAP was translocated by addition of 25 µM GTP, ATP and CTP. The reaction was incubated at RT for 7 min and washed 5× with 1 ml of LmLpH buffer. As above, the reaction volume was reduced to 18 µl and used for the ribosome binding reaction.

For analysis of peptidyl transferase activity in the coupled system, 75 pmol of TAP-tagged RNAP was first immobilized on 10 µl of Ig-G beads (GE healthcare) equilibrated in TB100, by gentle shaking at 30°C for 30 min followed by five washings with LmLpH buffer. Transcription elongation complexes were assembled as above, except for 50 pmol of RNA, 200 pmol of template DNA and 1000 pmol of non-template DNA were used, followed by addition of translation machinery components (see below).

For the formation of 'translation first' artificial elongation complexes, translocated ribosomes (see below) were applied onto a sucrose cushion and subjected to ultracentrifugation as described in Ref. (25). The resulting pellet was washed 3× with TrLB and resuspended in 10 µl of the same buffer and then mixed with 60 pmol of template DNA and 30 pmol of RNAP. The reaction was incubated for 15 min at 37°C followed by the addition of 300 pmol of non-template DNA and subsequent incubation for 15 min at 37°C. α-[^{32}P]-GTP was used to label the 3′-end of the mRNA, and RNAP was walked by addition of 25 µM ATP and CTP. For Figure 3D, after ribosomes translocation (see below) transcription elongation complexes were chased by addition of 5 µM NTPs for times indicated in the figure.

All transcription reactions were terminated by addition of an equal volume of stop buffer (EDTA 20 mM, 7 M urea, 100 µg/ml heparin, 0.02% bromophenol blue, and 0.03% xylene cyanol) and the products were resolved by sequencing 6% PAGE and analyzed using ImageQuant software (GE-Healthcare).

Preparation of ternary complexes

In order to exchange the GDP bound to purified EF-Tu with GTP, EF-Tu•GDP (400 pmol) was mixed with GTP (600 pmol), phosphoenol pyruvate (800 pmol), and phosphoenol pyruvate kinase (200 µg/ml) in 30 µl ternary complex buffer (50 mM Tris–HCl pH 7.4, 40 mM NH_4Cl, 10 mM $MgCl_2$ and 1 mM DTT) and incubated for 10 min at 37°C. Equimolar amounts of aminoacyl-tRNA were then added and incubation at 37°C continued for a further 5 min. Six microlitres of the resulting ternary complexes were added in both, peptidyl-transferase and translocation assays (below).

Peptidyl transferase and translocation assays. For translocation of the ribosome in both coupled and uncoupled systems, a method similar to Ref. (26) was used. For ribosome binding and translation initiation: 2 µM 70S ribosomes were mixed with 2 pmol of either mRNA (gene 32 −41 +81) or transcription elongation complexes in a final volume of 49 µl of TrLB. The mixture was incubated for 10 min at 37°C followed by the addition of either *N*-acetyl-met-tRNA^{fmet} (non-enzymatic initiation) or F-met-tRNA^{fmet}, IF-1, IF-2, IF-3 to final concentration of 5 µM and 200 µM GTP (enzymatic initiation) to final volume of 60 µl. After 10 min incubation, 10 µl aliquots were withdrawn and transferred to tubes containing 1.2X TrLB buffer, 5 µM EF-G, 200 µM GTP and 1 µM ternary complexes (translocation reaction). This mixture

was incubated for 7 min at 37°C. For RelE printing of the translocated complexes, 12 pmol of RelE (kindly provided by K. Gerdes, Newcastle University, UK) were added and the reactions incubated for 10 min at the same temperature.

To detect translocation by the inhibition of reverse transcription (toeprinting), a radiolabeled primer was annealed to the 3′-end of the mRNA prior to translation initiation by heating at 65°C for 10 min in TrLB without magnesium followed by quick cooling in the presence of Mg^{2+}. After translation initiation, 10 µl aliquots were added to the translocation mix that had the same components described above plus four dNTPs (to final concentration of 300 µM). After translocation, 1 U of SuperScript III reverse transcriptase (Invitrogen) (diluted in the buffer provided by supplier) was added and the reaction was incubated for another 10 min at 37°C. The products from both RelE cleavage and toeprint reactions were terminated, resolved and analyzed as described above.

The peptidyl transferase assay was conducted as described above (translation initiation and translocation) with the following differences. [^{35}S]-F-met-tRNA^{fmet} synthesized as above, was used to visualize the peptides. The concentration of ribosomes and mRNA (for the analysis of peptide synthesis in the absence of transcription) or immobilized transcriptional complexes (to analyze peptidyl transferase activity in the presence of the transcriptional machinery) was ~1 µM and all components were scaled up accordingly. After the translocation reaction, KOH was added to a final concentration of 100 mM to deacylate the tRNA, and incubated at RT for 15 min. The products were resolved by thin layer electrophoresis as described in Ref. (27).

RESULTS AND DISCUSSION

The aim of this study was to assemble a coupled *in vitro* transcription–translation system (CTT), which would allow investigation of interactions of RNAP and the ribosome and would exclude possible interference from other cellular components. In order to investigate potential interactions between the ribosome and RNAP, we must be able to walk and stall both machineries at desired positions of their respective templates. The system should allow the monitoring of both RNA and protein synthesis. Importantly, a CTT system should ensure that when transcription is analyzed, all RNAPs are coupled to translation, and, vice versa, when translation is monitored, all ribosomes are coupled to RNAPs. This would exclude the possibility of only a subset of molecules interacting with each other, which can generate partial effects and may lead to wrong conclusions.

In vitro translation system suitable for coupling to transcription

For coupling we used the *in vitro* translation system designed in the laboratory of H. Noller (26) with several modifications. For initiation and elongation of translation we purified ribosomes, IF-1,-2,-3, EF-Tu and EF-G, mixed aminoacyl synthetases (present in purified S100 extract), methionyl-tRNA synthetase and methionyl-tRNA formyltransferase. To be able to direct ribosome movement one codon at a time and to stall the ribosome at a desired position, we aminoacylated (with S100 extract) and purified individual aminoacyl-tRNAs. To decrease unwanted effects of impurities and eliminate traces of mRNAs and tRNAs, two high salt washings were used during the purification of 70S ribosomes. To avoid incorporation of ATP, required for aminoacylation, by RNAP in CTT system (which may influence interpretation of effects of coupling), purified aminoacyl-tRNAs were further subjected to four runs of gel filtration, which removed traces of ATP.

The translation system was first characterized in the absence of RNAP. A conventional way to characterize stepwise translation is toeprinting. The technique is based on the inhibition of the processivity of reverse transcriptase by ribosome(s) bound to the template RNA (Figure 1A). The comparison of the length of the cDNA provides a measurement of the ribosome's location with single-nucleotide resolution.

To test the activity of our translation system, we programmed the ribosomes with the T4 gene 32 mRNA (28), containing the sequence −41 to +81, relative to the translation initiation site. The first five codons in the mRNA code for M, F, V, Y and K. In the presence of initiation factors, EF-G, GTP and ternary complexes, but without ribosomes, reverse transcriptase synthesized a full length cDNA (Figure 1B Lane 1). Translation initiation was performed in two ways: enzymatic, using f-met-tRNA^{fmet}, initiation factors IF-1, IF-2, IF-3, and GTP, and non-enzymatically, using *N*-acetyl-met-tRNA^{fmet}, which binds to the P site independently of initiation factors. Non-enzymatic initiation is useful when the presence of GTP during translation initiation is not desired. Toeprints of the same intensity located at 15 nt downstream from the ribosome's A site were obtained for both translation initiation complexes (Figure 1B, lanes 2, 5). Translocation by the ribosome to defined positions was observed after adding limited sets of ternary complexes of the cognate aminoacyl-tRNA(s) (EF-Tu•GTP-aminoacyl-tRNA) in the presence of EF-G and GTP (Figure 1B, lanes 3, 4 and 6, 7). Movement of the ribosome was strictly EF-G dependent (Figure 1B lanes 8–11), indicating that no slippage of the ribosome had taken place. We concluded that the translation system was active and that we could control the movement of the ribosome.

During coupling of translation to transcription, the 3′-end of the RNA will be occupied by the transcribing RNAP (29). This implies that RNA in a CTT system will be inaccessible to a reverse transcription primer thus toeprinting is not suitable for assessing translation in CTT systems. *Escherichia coli* possesses several mRNA interferases that belong to toxin–antitoxin systems (30). One such mRNA interferase, the toxin RelE, cleaves mRNA in a ribosome-dependent manner. RelE binds in the vacant A site of a stalled ribosome and cleaves RNA between the first and second nucleotide of the codon (31), thus marking the position of the ribosome on the RNA

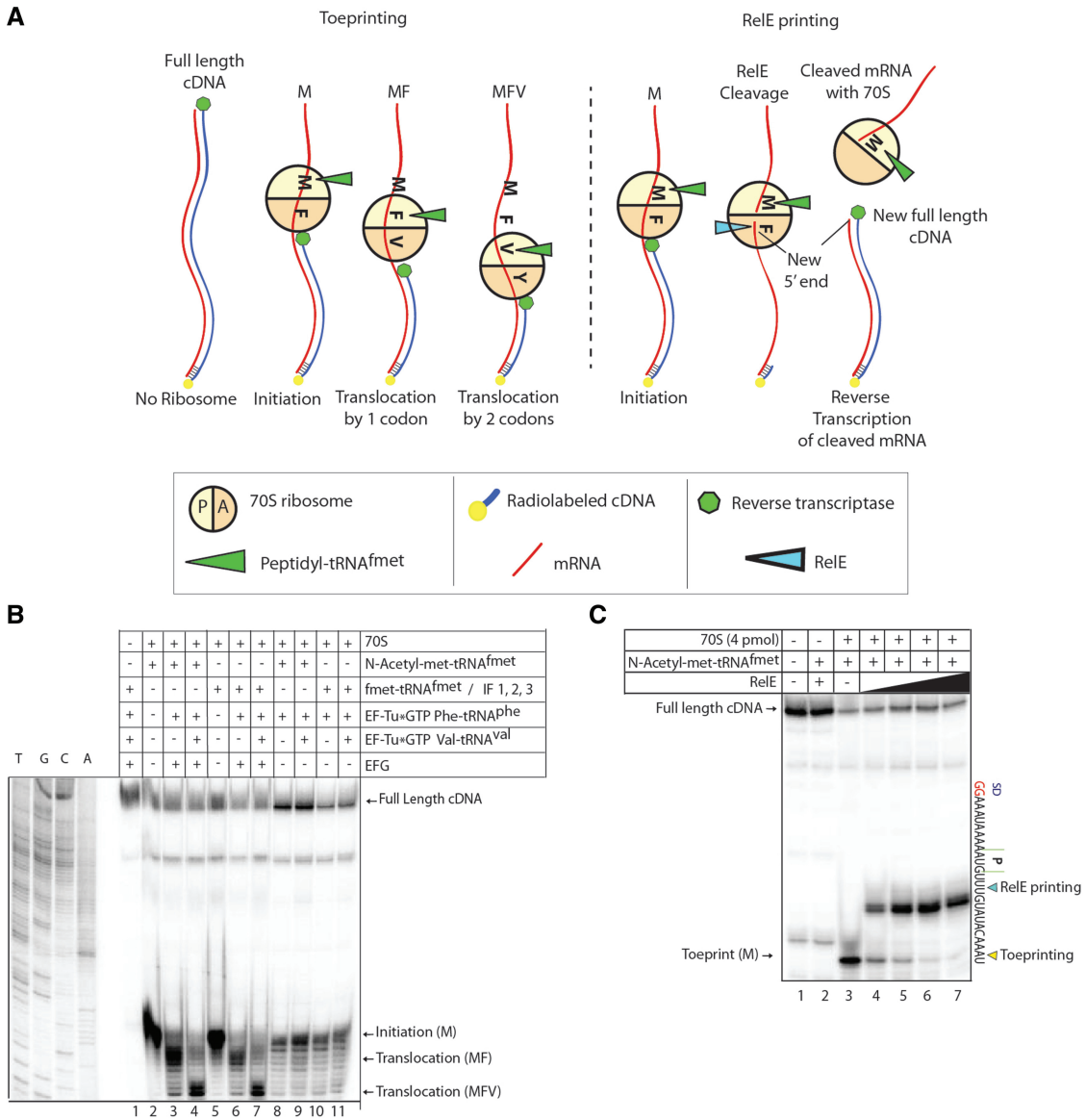

Figure 1. *In vitro* characterization of the translational machinery. (**A**) Schematic representation of the toeprinting of ribosome front edge and RelE cleavage site. Reverse transcription of mRNA (red line) results in synthesis of 5′-end labeled cDNA (blue line) of either full length in the absence of ribosomes (beige circle), and shorter cDNAs corresponding to stops of reverse transcriptase (green circle) in front of ribosomes or at the site of RelE (blue triangle) cleavage. Elongation by ribosome to specific positions (MF and MFV) towards the 3′-end of the RNA results in formation of shorter cDNA. RelE cleavage at the vacant A site of ribosome (dark beige half) results in dissociation of the ribosome and synthesis of cDNA to the cleavage site. (**B**) Toeprinting of initiating and translocating ribosomes on gene 32 mRNA. Shown is denaturing PAGE of toeprint cDNA's of ribosomes initiated in factor-independent (lanes 2–4, 8–9) or enzymatic (lanes 5–7, 10–11) manner and subsequently translocated in EF-G dependent (lanes 3, 4, 6, 7) and EF-G independent (8–11) manner to positions designated to the left of the gel. (**C**) Denaturing PAGE of cDNA's forming during titration of RelE (1, 4, 8, 14 pmol in lanes 4, 5, 6, 7, respectively) on translation initiation complex. Note that the lower bands present in lanes 1 and 2 are due to inhibition of reverse transcription by the secondary structure in the mRNA. This secondary structure melts when the ribosome is loaded onto the mRNA.

template. This method was successfully used for the analysis of paused and stalled ribosomes (32). We analyzed RelE cleavage by primer extension (Figure 1A) in the translation initiation complex, which had the phenylalanine codon UUU in the A-site. In the absence of ribosomes, no cleavage was detected, demonstrating the specificity and dependency of the cleavage event on the ribosome (Figure 1C lane 2). In the absence of RelE, a toeprint for the translation initiation complex was observed (Figure 1C lane 3). With increasing concentrations of RelE, we observed the diminishing of the toeprint and the appearance of cDNA corresponding to the location of the A-site, where RelE cleavage had occurred (compare Figure 1C lane 4 with lane 3). As higher concentrations of RelE were used, complete disappearance of the toeprint band was obtained (Figure 1C lane 7). We therefore used RelE as one of the tools to follow translation in the CTT system.

Coupling translation to transcription from DNA templates containing a promoter

Analysis of the effects of translation on transcription in a CTT required the ability to place RNAP in various transcription states such as paused, backtracked or pre-translocated. To be able to walk RNAP to these signals, we first used a biotinylated PCR product containing a strong T7A1 promoter utilized by *E. coli* RNAP (Figure 2A). A stable transcription elongation complex (EC) was obtained by transcription from T7A1 promoter in the presence of an RNA primer (CAUC), GTP, ATP and CTP, which allowed elongation only until position +11 (relative to transcription start site, +1). Transcription elongation complexes containing 11-mer RNA (EC11) were then immobilized on streptavidin beads and were washed to remove unused RNAP, σ^{70} and unincorporated NTPs from the reaction. From position +11 RNAP can be walked to any desired position on the template. RNA in transcription EC can be labeled in the body during 11-mer synthesis or during further walking, or alternatively at the 3′-end, by incorporation of radiolabeled NTPs. As a test, we translocated RNAP in a stepwise manner by using subsets of NTPs, which were washed away after each step, to position +80 (EC80) (Figure 2B). We used a template that coded for the same translation initiation region and first five codons as in the RNA templates used for the above RelE and toeprint experiments. EC80 were transferred into TrLB by washing the immobilized complexes, and 70S ribosomes were added. After ribosome binding, translation was initiated by the addition of *N*-acetyl-met-tRNA^{fmet}, followed by the addition of RelE (Figure 2A). In the presence of initiated ribosomes, we observed a 49-nt long RelE cleavage product, which corresponded to the expected position of the vacant A-site of the initiating ribosome (Figure 2C, lane 5). The intrinsic cleavage of the RNA in the EC80 by RNAP occurred due to a high concentration of phosphate in the RelE buffer, which was required to maintain the solubility of RelE. The extent of RelE cleavage demonstrated that the occupancy of the mRNA by the ribosome was greater than 50% (Figure 2C, lane 6). Ribosome translocation was also tested by adding a mixture of ternary complexes (allowing synthesis of MFVY tetrapeptide) in the presence of GTP and EF-G. We observed appearance of a 58 nucleotide long RelE cleavage product, corresponding to the A-site of the ribosome translocated by three codons (Figure 2D, lanes 3 and 7). Note however, that because RelE has a different affinity to various codons (32), the ratio between RelE cleavage products in initiated and translocated complexes does not reflect the actual proportion of ribosomes that escaped into elongation upon ternary complex addition. Note that some read-through of EC80 occurred in the presence of GTP used for ternary complexes formation and ribosome translocation (band above +80 in Figure 2D, lanes 3 and 4). This means that DNA template sequences that allow efficient read-through from stalled ECs in the presence of GTP may not be suitable for assembly of CTT.

To test if RNAP remained active in the CTT after ribosome initiation and translocation, EC80 was allowed to elongate in the presence of all four NTP's (before RelE addition). As seen from Figure 2D, lanes 6 and 7, all transcription elongation complexes were active and resumed elongation. RelE cleavage products for both initiated and translocated ribosomes observed after chasing of transcription complexes were the same as in CTT assembled on EC80 (Figure 2D, lanes 6 and 7). Additionally, similar results were obtained when using f-met-tRNA^{fmet} and IF-1, IF-2, IF-3 or *N*-acetyl-met-tRNA^{fmet} for the initiation of translation (not shown), suggesting that both ways of initiation can be used according to experimental needs. Even though this experimental set-up confirmed that both transcription and translation were capable to co-exist in this CTT system, it also proved to be inefficient for analysis of interactions between the two machineries for the following reasons: (i) walking RNAP to remote positions may generate a lot of background noise (see Figure 2D), obscuring the analysis of interactions; (ii) the yield of mRNA generated in transcription from promoters is low making it impossible to analyze synthesized peptides. For these reasons, we decided to look for alternative experimental approaches that would allow us to overcome these obstacles.

'Translation first' CTT (TL-CTT) for analysis of effects of coupling on transcription

We explored a different approach to couple translation to transcription by using artificially assembled transcription elongation complexes. This technique is widely used for the investigation of RNAP properties during elongation (21,24,33). Transcription ECs assembled from synthetic complementary template and non-template DNA strands and synthetic RNA complementary to template DNA, are indistinguishable from transcription elongation complexes formed on double-stranded DNA of the same sequence by transcription from a promoter (21,34,35). The RNA used for transcription EC assembly in this study was synthesized using T7 RNAP and then purified by PAGE (see 'Methods' section). The RNA (mRNA) contained signals for translation initiation and a coding region similar to the templates described above (Figure 3A). The 3′-end of the mRNA contained a 9-nt long sequence complementary to a part of the template DNA oligonucleotide. The RNA–DNA hybrid formed between mRNA and the template oligonucleotide is recognized and bound by RNAP. The non-template oligonucleotide is then accommodated into the complex resulting in the formation of transcription EC. mRNA is then labeled by the addition of α-[^{32}P] NTP(s) encoded by the downstream template DNA.

In order to achieve complete coupling of RNAP to translation, transcription ECs were assembled with mRNAs, which were already occupied with an elongating ribosomes ['translation first' CTT (TL-CTT)]. To remove mRNAs unused by translation machinery, ribosomes were first allowed to elongate by one codon (MF dipeptide containing translation elongation complexes) and then were

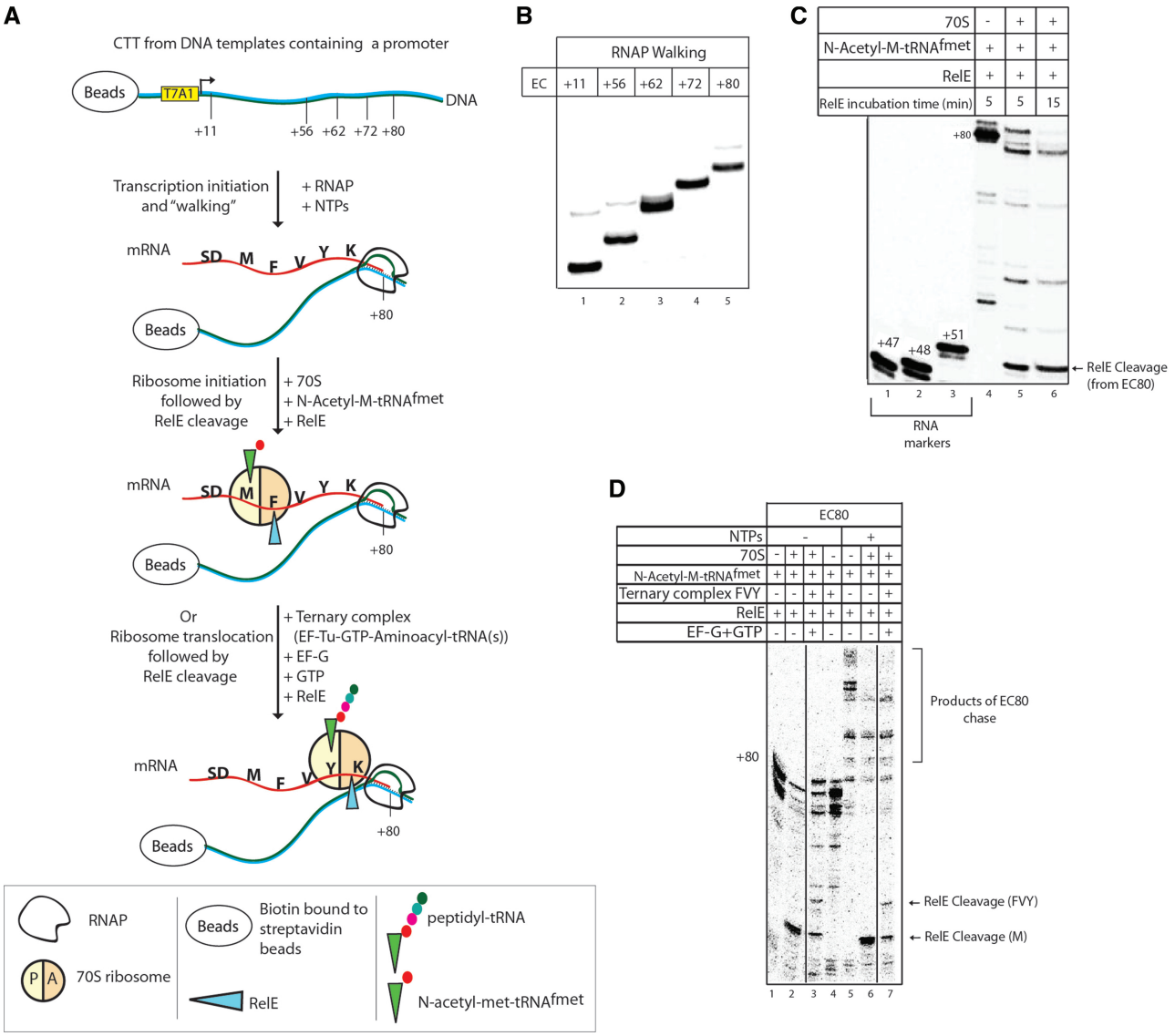

Figure 2. Characterization of promoter borne CTT. (**A**) Schematic representation of promoter borne CTT system assembly with non-enzymatic translation initiation. Biotinylated DNA template (for full sequence see Supplementary Data) containing T7A1 promoter utilized by *E. coli* is immobilized on streptavidin beads. *Escherichia coli* RNAP initiated from the promoter is 'walked' to a desired position. Translation initiation and elongation complexes formed on the mRNA can be analyzed by RelE cleavage. (**B**) Example of walking of RNAP. PAGE of the RNAs synthesized by RNAP during its walking to position +80 (to form EC80). (**C**) RelE mapping (for times designated above the gel) of translation initiation complexes in CTT system assembled with promoter borne transcription EC80 (lanes 4–5). EC80 was obtained as in panel B, and the transcript was radiolabeled at its 5′-end proximal part. RNAs from transcription ECs stalled at positions +47, +48 and +51 (lanes 1–3) were used as size markers to determine the size of the RelE cleavage product. Note that degradation of EC80 is caused not only by RelE cleavage but also by RNAP-dependent phosphorolysis by high phosphate of RelE storage buffer. (**D**) PAGE of radiolabeled mRNA of CTT (assembled with stalled EC80), in which translation initiation (lanes 2 and 6) and elongation (in the presence of EF-G; lanes 3 and 7) are probed by RelE cleavage. In lanes 5–7, EC80 was chased in the presence of all NTPs after translation initiation and elongation but before RelE cleavage. EC80 was obtained as in panel B, and the transcript was radiolabeled at its 5′-end proximal part. RelE cleavage products were identified as in panel C by walking RNAP to the corresponding positions and loading these RNAs as markers. A weaker band of RelE cleavage product after ribosome translocation is explained by different activity of RelE on various codons. Note, some transcription read through from EC80 (lanes 3, 4) in the presence of GTP required for translocation. Black vertical lines separate lanes originating from one gel which were brought together.

purified by ultracentrifugation through a sucrose cushion (25). During centrifugation, translation elongation complexes are separated from initiation and elongation factors, unused ternary complexes and unused mRNA. The resulting pellet containing only mRNA occupied by translocated ribosomes was washed to remove traces of supernatant and resuspended in TrLB. This mRNA, fully occupied with elongating ribosomes, was used for the assembly of the transcription EC.

First, we assessed the activity of the ribosomes in TL-CTT by testing for their ability to extend MF dipeptide to tetrapeptide MFVV, i.e. to translocate further by two codons, after addition of EF-Tu•GTP•Val-tRNA^{val} ternary complex, EF-G and GTP. The peptides,

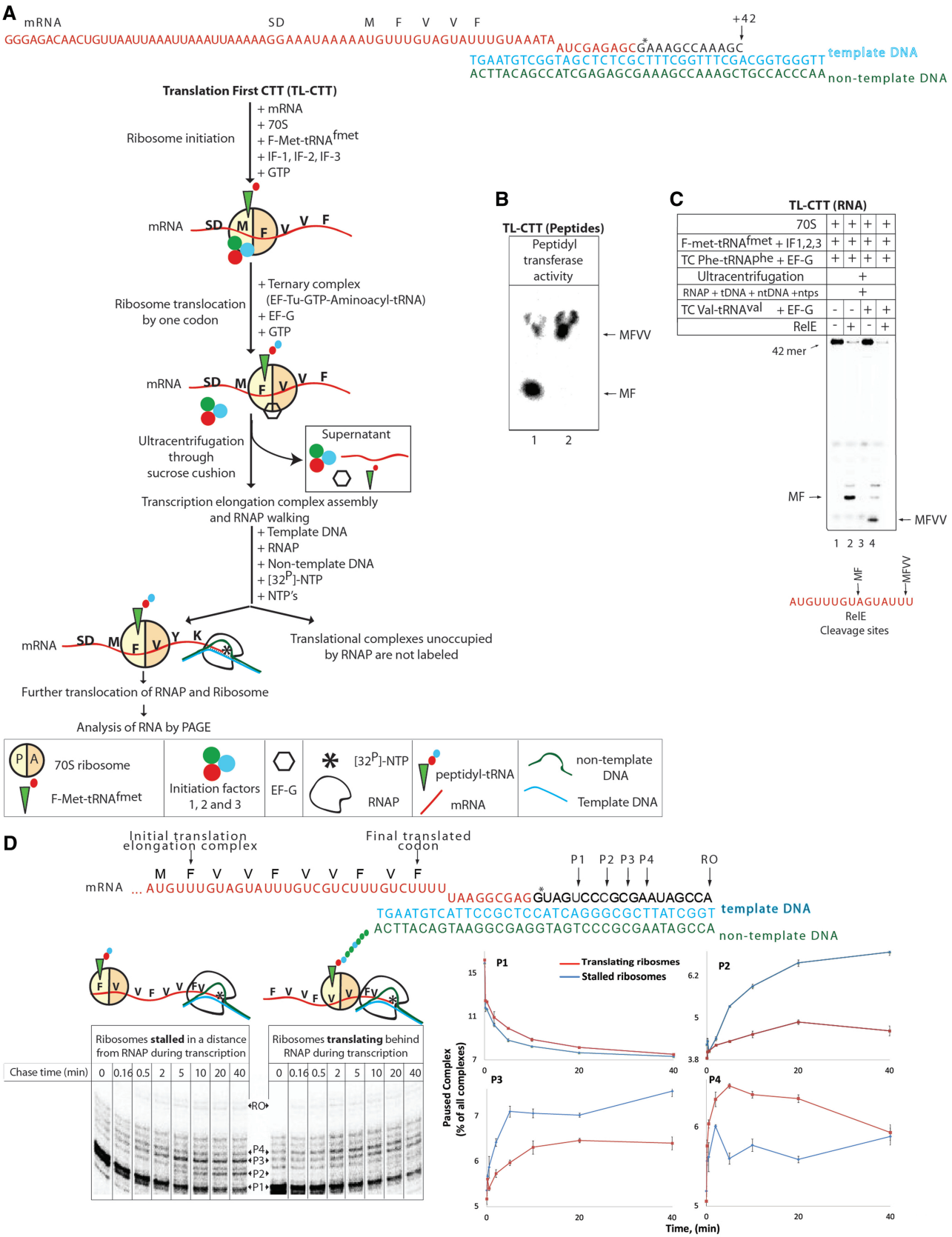

Figure 3. Characterization of 'translation first' TL-CTT. (**A**) Schematic representation of assembly of TL-CTT. Translation is initiated with f-Met-tRNA^{fmet} (green triangle) and initiation factors 1, 2 and 3 (green, blue and red circles). After translation initiation, ribosomes are allowed to elongate by one codon with synthesis of a dipeptide. The translation elongation complexes are separated from unused factors and unused mRNA by ultracentrifugation through a sucrose cushion. The mRNA carrying elongating ribosome is used in assembly of transcription EC. mRNA in

(continued)

radiolabeled at [^{35}S]-f-Met-tRNA^{fmet}, were analyzed by thin layer electrophoresis (TLE) (27). As seen from Figure 3B, the MF dipeptide was readily elongated into the MFVV tetrapeptide (Figure 3B, lane 2) demonstrating that the translational system was capable to endure not only the ultracentrifugation through the sucrose cushion but also the incubation times and components needed for the assembly of the EC. Note however that, although most of elongating ribosomes in TL-CTT set-up are active, some of them may not be coupled to RNAP, making TL-CTT unsuitable for following outcomes of coupling by peptides analysis.

To further confirm the occupancy of mRNA with ribosomes in TL-CTT, we performed RelE probing. mRNA in TL-CTT was labeled by incorporation of α-[^{32}P]-GTP by RNAP, ensuring that only mRNAs occupied with both, ribosome and RNAP, were monitored (Figure 3A). As can be seen from Figure 3C, lane 2, RelE cleaved most of mRNA in TL-CTT, indicating that almost all transcription ECs contained elongating ribosomes. RelE cleavage after translocation of ribosome by two codons (after addition of EF-Tu•GTP•Val-tRNA^{val}, EF-G and GTP) further revealed that most of these ribosomes were active (Figure 3C, lane 4).

To test the effects of translation on transcription in the TL-CTT, we used an artificially assembled transcription EC containing a sequence that makes RNAP prone to pause in a hairpin-independent manner (36). Note that the template is so pause-prone that, in low NTPs concentrations used (5 μM) RNAP hardly reaches the end of template (RO) even after several minutes (Figure 3D). The aim of this set-up was to analyze the effect of collision of ribosomes with transcription EC on pausing of transcription (Figure 3D). After formation of TL-CTT, RNAP was walked to the position of first pause (P1) by addition of an incomplete set of NTP's. The ribosome was either left in one codon translocated configuration after synthesis of MF dipeptide (stalled translation elongation complex, left panel in Figure 3D), which is unable to interact with RNAP due to the distance between the two machineries, or allowed to translocate until colliding with RNAP by addition of Phe-tRNA^{phe} and Val-tRNA^{val} ternary complexes, EF-G and GTP for 3 min (translating complex, right panel in Figure 3D). After that, all four NTPs were added permitting RNAP elongation (though, in low NTPs concentration used, most of RNAPs pause before reaching the end of this pause-prone template). The ribosome stalled in MF configuration remains in this position and does not follow transcribing RNAP (left panel in Figure 3D), while the translocated ribosome follows transcribing RNAP till decapeptide is synthesized (right panel in Figure 3D). Note, however, that decapeptide synthesis allows the front edge of the ribosome to travel as far as the rear edge of RNAP, which has reached the end of the DNA template. This is explained by the fact that the ribosome is known to protect around 15 nt of mRNA downstream of its A-site, while RNAP is known to protect around 15 nt from the 3′-end of the transcript.

As can be seen in Figure 3D, there was a complex response of transcription pausing to the presence of ribosome translating the mRNA co-transcriptionally (compare 'stalled' and 'translating' panels of Figure 3D). Pauses P2 and P3 were read-through by RNAP when ribosome was allowed to translate behind RNAP. In contrast, however, P1 was affected only weakly by coupling. Furthermore, P4 was slightly increased in the presence of co-transcriptionally translating ribosome. Given the absence of the secondary structure of the mRNA, the observed effects can be attributed to direct physical interactions between the two machineries. Decrease of pausing at P2 and P3 could be explained by recently proposed pushing of RNAP by the ribosome (11). However, these effects, as well as the opposite effect on P4 and weak effect on P1, require further investigation, which is now under way.

'Transcription first' CTT (TR-CTT) for analysis of coupling effects on translation

In order to monitor effects of ribosome/RNAP collision on peptide synthesis, it is important to have all ribosomes being coupled to RNAPs. However, as mentioned above, some elongating ribosomes in TL-CTT may remain unincorporated into transcription EC. Therefore, we modified CTT to be able to monitor peptide synthesis by ribosomes

Figure 3. Continued
TL-CTT is labeled by incorporation of radiolabeled NMP during RNAP walking, ensuring that mRNA is labeled only in coupled complexes. Sequences of the nucleic acids scaffold used for transcription EC assembly are shown at the top of the panel. Nucleotides incorporated after CTT assembly are in black, asterisk represents the radiolabeled nucleotide. The ribosome can then be translocated allowing collisions to occur between the two machineries, followed by the analysis of transcripts by denaturing PAGE. (**B**) Peptidyl transferase assay to analyze the activity of the ribosome after purification and TL-CTT assembly. MF dipeptide (formed prior purification with [^{35}S]-f-Met-tRNA^{fmet}; lane 1) was allowed to be extended to tetrapeptide (MFVV; lane 2). Products were resolved by thin layer electrophoresis. (**C**) Occupancy of mRNAs with ribosomes in TL-CTT revealed by RelE cleavage. Shown is PAGE of mRNA. Note that only coupled complexes are visible since mRNA is labeled during RNAP transcription after TL-CTT assembly. RelE cleavage was performed in the translation elongation complex containing dipeptide MF (formed prior purification) and after this complex was allowed to elongate by two codons (to tetrapeptide MFVV). The sequence below shows where RelE cleavage takes place. (**D**) Complex effects of coupling on pausing of transcription as an example of using TL-CTT. Shown is PAGE of mRNA. Sequences of oligonucleotides used for TL-CTT assembly are shown at the top of the panel. Nucleotides incorporated after CTT assembly are in black, asterisk represents the radiolabeled nucleotide, arrows show pauses P1, P2, P3 and P4 formed during chase (positions of pauses were determined by walking RNAP to each position; not shown). After TL-CTT formation, RNAP was walked to P1 by addition of incomplete set of NTP's. Then, translation elongation complex was either left with dipeptide MF ('stalled' complexes) or was allowed to elongate behind RNAP by addition of F and V ternary complexes in presence of EF-G and GTP for 3 min ('translating' complexes). After this step, RNAP was allowed to transcribe by addition of four NTPs. While the stalled ribosomes remain at their initial position, the translating ribosomes follow transcribing RNAP. Gel shows effects on transcriptional pausing by coupled ribosome. Plots show quantification of some pauses as a fraction (in percent) of all complexes in the lane versus time. Error bars are standard deviation from two independent experiments.

coupled with transcription ECs ['transcription first' CTT (TR-CTT)]. We used pre-assembled transcription ECs because the amount of RNA available to program the ribosome could be easily increased in these complexes as, for example, compared to the promoter borne CTT (above). To form assembled transcription ECs, a TAP-tagged RNAP (37) immobilized on sepharose immunoglobulin G beads was used. RNA not bound in transcription EC was washed away ensuring that, when translation is initiated, all of the translating ribosomes are coupled to transcription. To allow us to follow nascent peptide synthesis, translation was initiated with [^{35}S]-F-Met-tRNA^{fmet} (in the presence of translation initiation factors and GTP). After translation initiation, the TR-CTT was incubated with different sets of ternary complexes, EF-G and GTP, permitting the ribosomes to elongate by one or more codon(s) at a time (Figure 4A). The nascent peptides were then deacylated and visualized using TLE. Efficiency of peptide synthesis in the TR-CTT was similar to that observed in the uncoupled translation system in the absence of RNAP (Figure 4B). It is important to mention that, in TR-CTT some ECs remain unoccupied by ribosomes (as revealed by RelE cleavage of the mRNA labeled RNAP; Figure 4C), making TR-CTT unsuitable for analysis of effects of coupling on transcription.

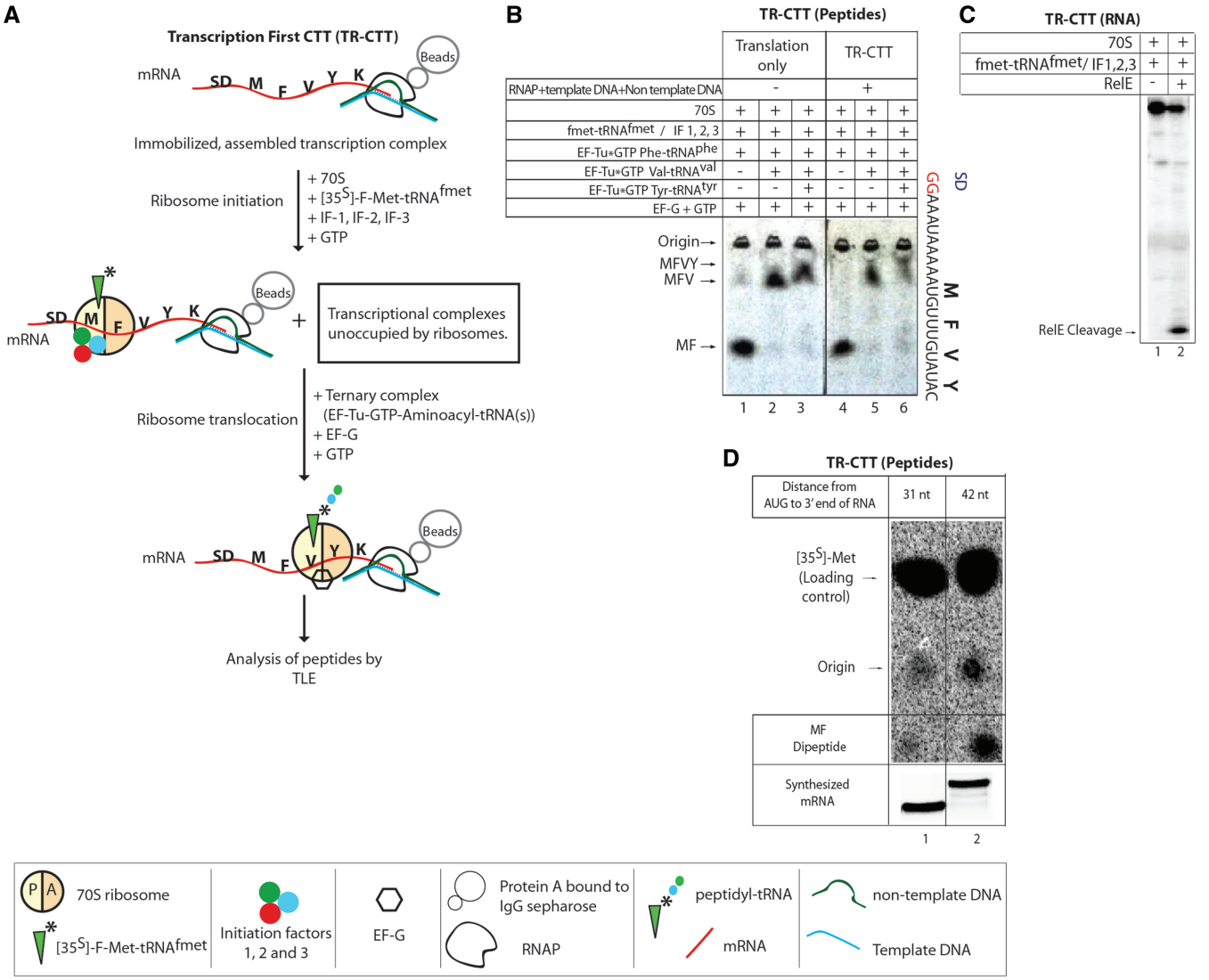

Figure 4. Characterization of 'transcription first' TR-CTT. (**A**) Schematic representation of assembly of TR-CTT. Solid phase immobilized transcription EC (for full scaffold sequence see Supplementary Data) is washed to remove unincorporated mRNA, after that translation is initiated and allowed to elongate on the mRNA of the EC. (**B**) Peptidyl transferase activity in TR-CTT. Ribosome elongation by F, FV or FVY codons is followed by thin layer electrophoresis (TLE) of synthesized peptides labeled with [^{35}S]-f-Met-tRNA^{fmet}. Comparison of the peptidyl transferase activity of the ribosome on naked (without transcription EC) mRNA (lanes 1–3) and in the TR-CTT (lanes 4–6). (**C**) Not all transcription ECs are coupled to translation in TR-CTT as evidenced by RelE cleavage after TR-CTT assembly. Shown is PAGE of mRNA labeled during RNAP walking as in Figure 3. (**D**) Rough estimation of mRNA length behind transcribing RNAP sufficient for translation initiation as an example of using TR-CTT. MF dipeptide formation was used as a measure of translation initiation efficiency (given that it does not require translocation) on mRNAs containing 31- and 42-nt spacers between its 3′-end occupied by RNAP and the AUG start codon.

We tested if TR-CTT can be used to determine the distance that would allow efficient initiation by ribosome. Note that in Figure 4B, RNAP was far enough from the AUG codon (42 nt between AUG and 3′-end of mRNA) to allow translation initiation as efficient as on the naked mRNA. As an example, we compared it to mRNA with AUG codon being 31 nt away from 3′-end of mRNA. As can be seen from Figure 4D, this distance was already too short for efficient translation initiation (compare lanes 1 and 2). Further investigation is under way to determine exact distances between elongating RNAP and elongating and initiating ribosomes.

CONCLUSIONS

The translation coupled to transcription systems developed in our study will, for the first time, allow direct assessment of the effects on both transcription and translation upon physical contacts of both machineries. The system permits investigation of the effects of the ribosome on various transcription complexes (paused, backtracked, etc.), as well as the possible control of the rate of translation by RNAP. While eliminating possible interference from other cellular components, the CTT systems described here will also allow exploration of the effects of individual transcription/translation or other factors on the cross-talk between the two machineries. CTTs will be used for measuring distances between transcribing RNAP and the ribosome translating the nascent RNA as well as the distance of RNAP from 5′-end of RNA required for efficient initiation of translation. CTTs will also be useful for understanding contact interfaces between the two machineries. These experiments are currently under way.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Methods.

ACKNOWLEDGEMENTS

We thank David Adams for critical reading of the manuscript, Kenn Gerdes for providing purified RelE, and Hani S. Zaher for help with the TLE assays.

FUNDING

UK Biotechnology and Biological Sciences Research Council, BBSRC under SysMO initiative and the European Research Council (ERC-2007-StG 202994-MTP). Funding for open access charge: European Research Council (ERC-2007-StG 202994-MTP).

Conflict of interest statement. None declared.

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