In vitro genetic reconstruction of bacterial transcription initiation by coupled synthesis and detection of RNA polymerase holoenzyme

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ABSTRACT

In vitro reconstitution of a biological complex or process normally involves assembly of multiple individually purified protein components. Here we present a strategy that couples expression and assembly of multiple gene products with functional detection in an *in vitro* reconstituted protein synthesis system. The strategy potentially allows experimental reconstruction of a multi-component biological complex or process using only DNA templates instead of purified proteins. We applied this strategy to bacterial transcription initiation by co-expressing genes encoding *Escherichia coli* RNA polymerase subunits and sigma factors in the reconstituted protein synthesis system and by coupling the synthesis and assembly of a functional RNA polymerase holoenzyme with the expression of a reporter gene. Using such a system, we demonstrated sigma-factor-dependent, promoter-specific transcription initiation. Since protein synthesis, complex formation and enzyme catalysis occur in the same *in vitro* reaction mixture, this reconstruction process resembles natural biosynthetic pathways and avoids time-consuming expression and purification of individual proteins. The strategy can significantly reduce the time normally required by conventional reconstitution methods, allow rapid generation and detection of genetic mutations, and provide an open and designable platform for *in vitro* study and intervention of complex biological processes.

INTRODUCTION

Advances in recombinant DNA technologies have allowed *in vitro* biochemical studies of recombinant proteins with important cellular functions. Often the most daunting and time-consuming studies involve purifying and assembling multiple, sometimes a large number of, recombinant proteins for *in vitro* reconstitution of a biological complex or process. Such have been the cases for *in vitro* reconstitution of replication (1), transcription (2,3), translation (4–7) and polyketide/non-ribosomal peptide synthesis (8) in bacteria and translation initiation in eukaryotes (9,10).

In this report, we present a strategy that experimentally reconstructs a biological complex or process (other than bacterial translation) by co-expressing multiple protein components from encoding DNA templates in an *in vitro* reconstituted protein synthesis system. Use of an *in vitro* cell-free protein synthesis system allows rapid co-expression of multiple proteins from encoding DNA templates without the potential limitations of heterologous protein expression in living cells, such as biohazard, toxicity and protein folding (11,12). Use of a reconstituted system instead of a cell-extract-based system allows synthesis of proteins in a largely nuclease- and protease-free environment that contains defined components and can be designed to favor protein folding and complex assembly (13,14). This strategy potentially can significantly save the time normally required by conventional *in vitro* reconstitution methods involving expression and purification of multiple proteins (1–3,8–10).

We apply this strategy to bacterial transcription initiation, the key step for gene regulation in bacteria (15). Transcription initiation in bacteria generally involves recognition of a promoter region by RNA polymerase (RNAP) holoenzyme, comprising RNAP core enzyme (two α subunits, one β subunit, one β' subunit and one ω subunit) and one of several σ factors (16–19). The primary σ factor (e.g. σ^{70} in *Escherichia coli*) is responsible for transcription initiation of genes expressed in exponentially growing cells, whereas alternative σ factors are responsible for transcription initiation of genes expressed during stationary phase or in response to specific

conditions (e.g. σ^{32} in *E. coli* for heat-shock response) (19–21). *In vitro* reconstitution of *E. coli* transcription initiation by conventional methods generally involves expression and purification of *E. coli* RNA polymerase (Ec RNAP) subunits and sigma factors and *in vitro* assembly of the initiation complex (2,3,22). Here we attempt to simultaneously express the subunits of Ec RNAP and an *E. coli* sigma factor from encoding DNA templates in a reconstituted protein synthesis system and demonstrate promoter-specific transcription in a single *in vitro* protein synthesis reaction.

The reconstituted protein synthesis system in this study is derived from PURESYSTEM (4), which comprises purified *E. coli* ribosomes and bulk tRNAs, purified recombinant *E. coli* aminoacyl-tRNA synthetases, translation factors and energy regeneration enzymes, and purified recombinant T7 RNA polymerase, which couples transcription from a T7 promoter of a DNA template to protein translation (4,13). Using this reconstituted protein synthesis system, we co-express Ec RNAP holoenzyme subunits from DNA templates under the T7 promoter, and in the same reaction, monitor the activity of the newly synthesized RNAP holoenzyme using a reporter expressed from an *E. coli* promoter. The reporter, firefly luciferase (Fluc), is encoded on an additional DNA template containing a sigma-specific promoter (Figure 1). Successful synthesis of a functional Ec RNAP holoenzyme leads to transcription of the reporter gene from the sigma-specific promoter and subsequent translation of the reporter protein from the encoding mRNA, resulting in a detectable signal (Figure 1).

MATERIALS AND METHODS

Reagents

All PCR reactions were performed using Phusion Hot Start High-Fidelity DNA polymerase (New England Biolabs). RNase inhibitor was from New England Biolabs. Primers were ordered from Integrated DNA Technologies (Coralville, IA). Purified Ec RNAP core enzyme and σ^{70}-saturated Ec RNAP holoenzyme were purchased from Epicentre Biotechnologies (Madison, WI), bulk tRNA and NTPs from Roche Applied Sciences (Indianapolis, IN) and other chemicals from Sigma-Aldrich (St. Louis, MO). Antibodies against Ec RNAP subunits were kindly provided by Dr Padraig Deighan (Harvard Medical School) and the antibody against Ec σ^{32} was purchased from Neoclone Biotechnology (Madison, WI).

DNA-template preparation

The plasmid pUCA105T7Fluc was derived from pUC19 containing the gene for Fluc under the control of a T7 promoter (14). The genes for Ec RNAP subunits (α, β, β' and ω) and *E. coli* sigma factors (σ^{70} and σ^{32}) were amplified from *E. coli* genomic DNA and cloned into pUCA108T7 vector, a derivative of pUCA105T7 vector. The expression of Ec RNAP subunits and sigma factors
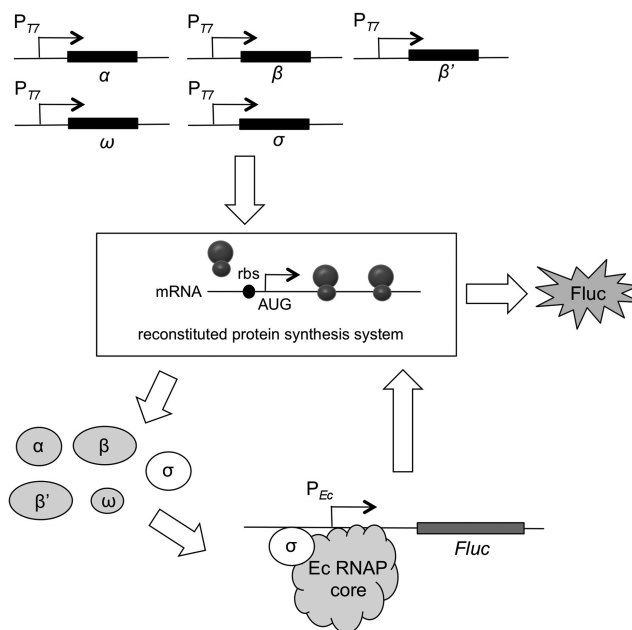

Figure 1. A scheme of coupled expression and detection of *E. coli* RNA polymerase (Ec RNAP) holoenzyme in a reconstituted protein synthesis system. *E. coli* RNAP subunits (α, β, β' and ω) and a sigma factor (σ) are encoded in separate linear or plasmid DNA templates under the control of a T7 promoter (P_{T7}). A reporter, firefly luciferase (Fluc), is encoded in an additional linear DNA template under the control of an *E. coli* promoter (P_{Ec}). The synthesized Ec RNAP subunits are assembled into RNAP core enzyme (Ec RNAP core) and subsequently form the holoenzyme with the sigma factor (σ) during transcription initiation. rbs: ribosome-binding site; AUG: start codon.

was under the control of a T7 promoter. The sequences of all cloned genes were verified by DNA sequencing.

For the expression of σ^{70} and σ^{32}, linear DNA templates were prepared by direct amplification from their cloning vector pUCA108T7 σ^{70} and pUCA108T7 σ^{32}, resulting in linear fragments containing the coding regions for σ^{70} and σ^{32} with an upstream T7 promoter and a downstream T7 terminator. All other linear templates containing non-T7 promoters were prepared by an over-lapping PCR strategy (23) involving two-step PCR reactions that added a promoter region to a gene of interest (Supplementary Figure S2A). More specifically, in the first step (first PCR), the reporter Fluc gene was amplified from pUCA105T7 Fluc to yield a DNA fragment containing the open reading frame (ORF) of Fluc, a 5′ UTR containing the ribosome-binding site and 3′ UTR derived from the vector. The promoter region of dnaKp1 (from −150 to +20) [...<u>TTGATG</u> (−35)..16 nt..<u>CATTTA</u>(−10) GTAGTCA(+1)] was amplified from *E. coli* genomic DNA and the promoter region of *tac* [...<u>TTGACA</u>(−35)..16 nt..<u>TATAAT</u> (−10)GTGTGGA(+1)] was amplified from pMalp2x vector (New England Biolabs). The amplification reactions generated a 3′ sequence that overlapped with the 5′ sequence of the reporter DNA fragment, allowing subsequent overlapping PCR reactions (second PCR) to yield the linear DNA templates encoding the reporter gene downstream of different promoters (Supplementary

Figure S2A). For best results, DNA fragments from the first PCR step were loaded on 2% agarose gel and then purified by QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified DNA fragments (∼50 ng) were used as the templates for amplification in the second PCR step using the promoter fw primer and 3′ UTR rv primer (Supplementary Figure S2A). DNA templates generated from the second PCR step were purified by QIAquick PCR purification kit (QIAGEN, Valencia, CA) and directly used for protein synthesis reactions.

To generate the linear DNA template for the expression of the wild-type Ec RNAP β subunit, the region containing the β gene, T7 promoter and T7 terminator was directly amplified from the cloning vector pUCA108T7β. To generate linear DNA templates for the expression of single-substitution mutants of the Ec RNAP β subunit, two-step overlapping PCR reactions were performed using mutagenic primer pairs containing the desired mutations (Q513P, Q513L and H526Y) (Supplementary Figure S2B) (23). In the first step (first PCR), pUCA108T7β was used as the template and two PCR reactions were performed for each single-substitution mutation using the upstream fw primer with the reverse mutagenic primer, and the downstream rv primer with the forward mutagenic primer, respectively (Supplementary Figure S2B). For best results, DNA fragments from the first PCR step were loaded on 2% agarose gel and then purified by QIAquick gel extraction kit (QIAGEN, Valencia, CA). The purified DNA fragments (∼50 ng) were used as the template for amplification in the second PCR step using the upstream fw primer and downstream rv primer (Supplementary Figure S2B). The resulting DNA fragments, each containing the β subunit gene with a single substitution, T7 promoter and T7 terminator, were used for *in vitro* protein synthesis reactions. The single substitution mutations were verified by direct DNA sequencing of the PCR-generated DNA fragments.

Reconstituted protein synthesis system

All recombinant proteins including translation factors, aminoacyl-tRNA synthetases, energy-regeneration enzymes and T7 RNA polymerase were kindly provided by Dr Dongxian Yue (New England Biolabs), which were also used for manufacturing PURExpress™, a commercial reconstituted protein synthesis system from New England Biolabs and derived from PURESYSTEM® (BioComber, Tokyo, Japan) (4). Ribosomes were purified according to established protocols (24,25). The reconstituted protein synthesis system was made following essentially the same protocol as Shimizu *et al*. (13) with a few modifications, including concentrations of aminoacyl-tRNA synthetases, possibly due to differences in specific activities, and potassium glutamate (200 mM instead of 100 mM).

In vitro protein synthesis reactions

The protein synthesis reactions (25 μl) were typically set up by mixing the reconstituted protein synthesis system (20 μl) with RNase inhibitor (2 U), DNA templates and/or purified Ec RNA polymerase (core or holoenzyme)

(1.1 pmol in Figure 2A and B or various amounts of the core enzyme in Figure 2C), and then incubated at 37°C for 4 h. The amounts of DNA templates per reaction for all experiments were as follows: 13 nM for linear templates of Fluc (*tac*-Fluc and dnaKp1-Fluc), 0.07 nM for the linear template of σ^{70} (P_{T7}-σ^{70}) (except in Supplementary Figure S4, in which various amounts were used), 0.25 nM for the linear template of σ^{32} (P_{T7}-σ^{32}), 0.07 nM for the linear templates of the wild-type and mutant β subunits, 0.05 nM for the plasmid templates of RNAP β and β′ subunits, 0.30 nM for the plasmid template of RNAP α subunit, 0.03 nM for the plasmid template of RNAP ω subunit and 4.0 nM for the plasmid template of pUCA105T7Fluc (Supplementary Figure S3). To examine the effect of small-molecule inhibitors, rifampicin (1.2 μM in Figure 4; 0.6 and 1.2 μM in Supplementary Figure S3 and various amounts in Supplementary Figure S5), mupirocin (Mup) (20 μM in Supplementary Figure S3) or chloramphenicol (Cm) (30 μM in Supplementary Figure S3) was added at the beginning of each protein synthesis reaction. The protein concentration of purified Ec RNA polymerase core enzyme was determined by Bradford assay.

Western blot analyses

Aliquots were taken from protein synthesis reactions after incubation at 37°C for 4 h and loaded on 10–20% Tris–glycine SDS–PAGE gels (Invitrogen, Carlsbad, CA). The gels were blotted for analyses with antibodies against Ec RNAP subunits and σ^{32} using Phtotope-HRP Western Blot Detection System (Cell Signaling Technology, Beverly, MA).

Luciferase assay

The activity of the Fluc reporter was assayed using the Luciferase Assay System (Promega, Madison, WI) in a microplate luminometer (Centro LB 640, Berthold Technologies, Oak Ridge, TN) according to manufacturers' instructions. Protein synthesis reactions were diluted 10-fold in 1× cell culture lysis reagent (Promega, Madison, WI) containing 1 mg/ml BSA. Aliquots (5 μl) were added in triplicate to a microplate for the luciferase assay in the luminometer.

RESULTS

Coupling Ec RNAP transcription to translation using purified enzymes

The reconstituted protein synthesis system contains T7 RNA polymerase to couple transcription to translation from a double-stranded DNA template containing a T7 promoter (4). A previous study has showed that the amount of synthesized protein in PURESYSTEM is largely correlated to the amount of the encoding mRNA (within a certain concentration range) transcribed by T7 RNA polymerase (26). We have previously demonstrated the detection of the activity of Fluc synthesized from a DNA template under a T7 promoter in PURESYSTEM (14). In this study, we first tested whether the synthesis and
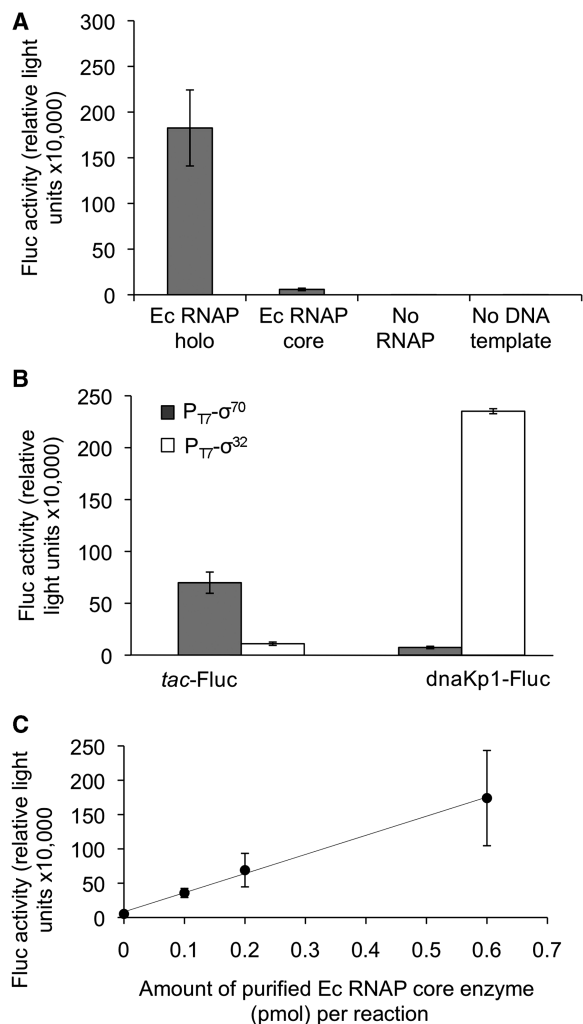
Figure 2. Coupling bacterial transcription to translation in the reconstituted protein synthesis system using purified Ec RNAP. (**A**) Fluc was expressed under the *tac* promoter in a linear DNA template in the presence of purified Ec RNAP holoenzyme (column 1, Ec RNAP holo) or Ec RNAP core enzyme (column 2, Ec RNAP core). As controls, the Fluc activity was measured in the absence of Ec RNAP holoenzyme (but in the presence of the Fluc template) (column 3, no RNAP) and in the absence of the Fluc template (but in the presence of purified Ec RNAP holoenzyme) (column 4, no DNA template). (**B**) Fluc was expressed under two different promoters in linear DNA templates (*tac*-Fluc and dnaKp1-Fluc) in the presence of purified Ec RNAP core enzyme. The Fluc activity was measured in the presence of the template for σ^{70} (P_{T7}-σ^{70}, shaded columns) or σ^{32} (P_{T7}-σ^{32}, white columns). (**C**) The Fluc activity was measured from the templates of dnaKp1-Fluc and P_{T7}-σ^{32} in the presence of various amounts (pmol per reaction) of purified Ec RNAP core enzyme. All data were obtained from at least two independent protein synthesis reactions.

activity of Fluc could be correlated to the sigma-factor-dependent transcription by purified Ec RNAP in the reconstituted protein synthesis system. Purified recombinant σ^{70}-saturated Ec RNAP holoenzyme or Ec RNAP core enzyme was added to the protein synthesis reaction containing a linear DNA template (*tac*-Fluc) encoding Fluc under the control of a *tac* promoter (27). As shown in Figure 2A, a significant increase in the activity of Fluc was observed in the

presence of σ^{70}-saturated Ec RNAP holoenzyme (Figure 2A, Ec RNAP holo) compared to Ec RNAP core enzyme (Figure 2A, Ec RNAP core). The data suggest that the σ^{70}-dependent transcription initiation from the *tac* promoter and the transcription of the full-length Fluc gene by Ec RNAP resulted in the translation of active Fluc from the encoding mRNA. No significant Fluc activity was detected when purified Ec RNAP was not added to the protein synthesis reaction (Figure 2A, no RNAP), suggesting the reconstituted protein synthesis system used in this study was largely free of contaminating Ec RNAP activity (also see data in Figure 3C). As another control, no Fluc activity was detected in the absence of the Fluc template even when purified Ec RNAP was added in the protein synthesis reaction (Figure 2A, no DNA template).

These data establish preliminary evidence that transcription (including transcription initiation) by Ec RNAP was compatible with, and could be coupled to, translation in the reconstituted protein synthesis system, and the activity of Fluc was correlated to the *in vitro* transcription activity of Ec RNAP.

Synthesis of functional *E. coli* sigma factors and demonstration of promoter-specific expression

We next tested whether the reconstituted protein synthesis system could be used to produce *E. coli* sigma factors from the encoding DNA templates to allow promoter-specific transcription in the presence of purified Ec RNA core enzyme.

The protein synthesis reactions contained linear DNA templates encoding Fluc under an *E. coli* promoter, *tac* (27) or dnaKp1 [an *E. coli* σ^{32}-specific promoter (28)] (*tac*-Fluc or dnaKp1-Fluc), an *E. coli* sigma factor σ^{70} or σ^{32} under the T7 promoter (P_{T7}-σ^{70} or P_{T7}-σ^{32}) and the purified Ec RNAP core enzyme (the same amount as in Figure 2A, Ec RNAP core). A significant increase in the activity of Fluc was observed from the *tac*-Fluc template in the presence of P_{T7}-σ^{70} (Figure 2B, *tac*-Fluc, shaded column) compared to in the absence of P_{T7}-σ^{70} (Figure 2A, Ec RNAP core). Though the Fluc activity in the presence of P_{T7}-σ^{70} was still lower than that of σ^{70}-saturated Ec RNAP holoenzyme (Figure 2A, Ec RNAP holo), the data suggest that a functional σ^{70} was produced from P_{T7}-σ^{70} in the protein synthesis reaction, forming a holoenzyme with the purified Ec RNAP core enzyme to allow transcription initiation from *tac*-Fluc. As further evidence, in the presence of σ^{70}, a higher Fluc activity was observed from the *tac*-Fluc template than from the dnaKp1-Fluc template (Figure 2B, shaded columns). In comparison, in the presence of σ^{32}, a higher Fluc activity was observed from the dnaKp1-Fluc template than from the *tac*-Fluc template (Figure 2B, white columns). The data are consistent with the cellular roles of σ^{70} as the housekeeping sigma factor and σ^{32} as the sigma factor for heat-shock response (18,29), further supporting that functional *E. coli* sigma factors were synthesized and assembled into Ec RNAP holoenzymes capable of initiating transcription from specific *E. coli* promoters. In spite of the σ^{70} consensus sequence in the *tac*

promoter, a higher Fluc activity was observed from the dnaKp1 promoter in the presence of σ^{32} than that from the *tac* promoter in the presence of σ^{70}. One possible reason was the effect of the concentration of potassium glutamate in the reconstituted protein synthesis system on *in vitro* transcription (19,30). High concentrations of potassium glutamate have been shown to inhibit *in vitro* transcription by σ^{70} (30), whereas lowering the concentration of potassium glutamate (from 200 to 100 mM) decreased the Fluc activity from the σ^{32}/dnaKp1-Fluc templates by ~50% (data not shown). Another possible factor was the folding of the synthesized σ^{70}, which might result in a lower specific activity than that of σ^{32}.

Using the fixed amounts of the P_{T7}-σ^{32} and dnaKp1-Fluc templates, the Fluc activity was determined in the protein synthesis reactions containing increasing amounts of purified Ec RNAP core enzyme. As shown in Figure 2C, the Fluc activity increased proportionally to the amount of Ec RNAP core enzyme. The data provide a basis for the correlation between the Fluc activity and the amount of active Ec RNAP core enzyme in the reconstituted protein synthesis system. If the detection limit is defined as a signal-to-noise ratio (the ratio of the two Fluc activities in the presence and absence of the core enzyme, respectively) = 2, the data in Figure 2C suggest that as few as ~0.025 pmol (~1 nM) of Ec RNAP core enzyme (presumably also RNAP holoenzyme) can be detected in the reconstituted protein synthesis reaction.

Coupled expression and detection of Ec RNAP holoenzymes using the encoding DNA templates

To demonstrate the synthesis of the holoenzymes, the protein synthesis reactions contained the P_{T7}-σ^{32} and dnaKp1-Fluc templates and separate plasmid templates encoding all four (P_{T7}-$\alpha\beta\beta'\omega$) or three out of four subunits of Ec RNAP (P_{T7}-$\beta\beta'\omega$, P_{T7}-$\alpha\beta'\omega$, P_{T7}-$\alpha\beta\omega$ or P_{T7}-$\alpha\beta\beta'$). No purified Ec RNAP core enzyme was added in the protein synthesis reactions. Synthesis of all four Ec RNA subunits and σ^{32} from their DNA templates and assembly of a functional Ec RNAP holoenzyme would lead to transcription of Fluc from the σ^{32}-specific dnaKp1 promoter and detection of the Fluc activity.

As shown in Figure 3A, a significant Fluc activity was observed in the presence of the templates encoding all four or the α, β and β' subunits of Ec RNAP (P_{T7}-$\alpha\beta\beta'\omega$ and P_{T7}-$\alpha\beta\beta'$). In the absence of one of the templates for α, β and β', the Fluc activity was reduced to the background levels (Figure 3A, P_{T7}-$\beta\beta'\omega$, P_{T7}-$\alpha\beta'\omega$ and P_{T7}-$\alpha\beta\omega$). The data suggest that a functional Ec RNAP core enzyme consisting of at least three subunits (α, β and β') was produced in the reconstituted protein synthesis system and all three subunits (α, β and β') were required for the assembly of a functional Ec RNAP core enzyme. The expression of the α, β and β' subunits and σ^{32} was further confirmed by western blot analyses using antibodies against each of the subunits and the sigma factor (Supplementary Figure S1). The similar Fluc activities observed in the presence and absence of the P_{T7}-ω template (Figure 3A, P_{T7}-$\alpha\beta\beta'\omega$ and P_{T7}-$\alpha\beta\beta'$) suggest that the ω subunit was not required
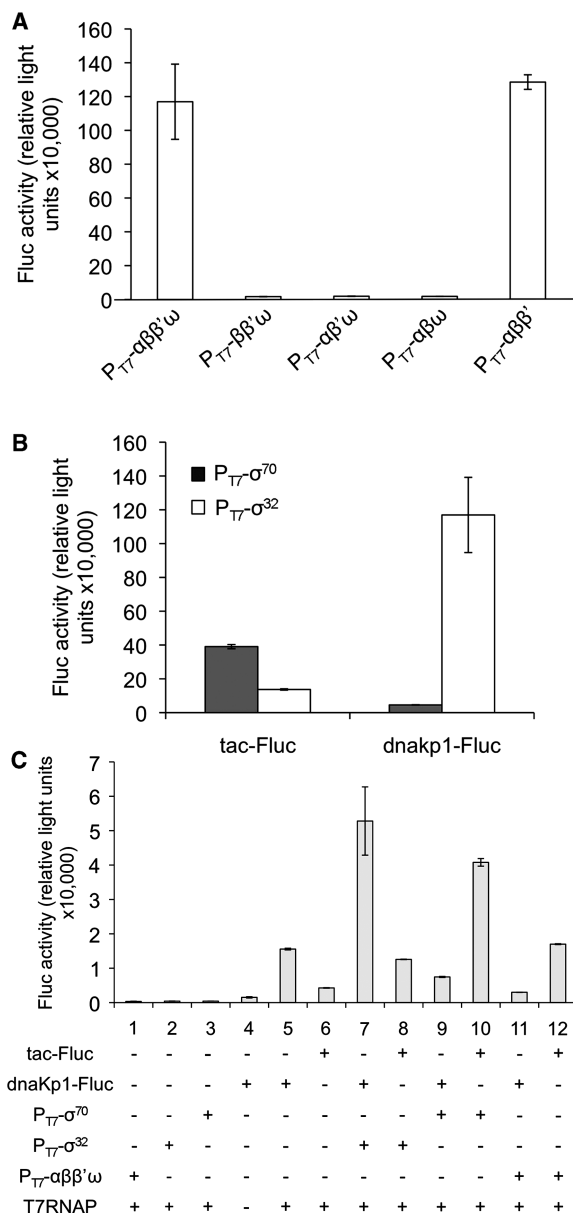
Figure 3. Coupled expression and detection of Ec RNAP holoenzymes in the reconstituted protein synthesis system using the encoding DNA templates. (**A**) The Fluc activity was measured in the protein synthesis reactions containing the linear DNA templates of P_{T7}-σ^{32} and dnaKp1-Fluc and the separate plasmid templates encoding all four (P_{T7}-$\alpha\beta\beta'\omega$) or three out of four subunits of Ec RNAP (P_{T7}-$\beta\beta'\omega$, P_{T7}-$\alpha\beta'\omega$, P_{T7}-$\alpha\beta\omega$ or P_{T7}-$\alpha\beta\beta'$). (**B**) The Fluc activity was measured in the protein synthesis reactions containing the linear DNA template encoding Fluc under *tac* or dnaKp1 promoter (*tac*-Fluc or dnaKp1-Fluc), the linear DNA template encoding σ^{70} or σ^{32} (P_{T7}-σ^{70}, shaded columns; P_{T7}-σ^{32}, white columns) and the separate plasmid templates encoding all four Ec RNAP subunits (P_{T7}-$\alpha\beta\beta'\omega$). (**C**) Control experiments to determine the background levels of the Fluc activity in the reconstituted protein synthesis system in the presence or absence of various DNA templates. The reconstituted protein synthesis system containing T7 RNA polymerase (T7 RNAP) were used for all experiments except in column 4 in which T7 RNAP was absent. All data were obtained from at least three independent protein synthesis reactions.

for the *in vitro* transcription activity of the synthesized Ec RNAP core enzyme, consistent with previous studies (31,32). However, since the expression of a functional ω subunit from its template was not confirmed in this study, we cannot rule out the possibility of the contaminating ω subunit in the reconstituted protein synthesis system. More analyses are needed to distinguish these scenarios.

The synthesis of a functional Ec RNAP core enzyme was further tested in the protein synthesis reactions containing the templates encoding all four subunits, the sigma factor template (P_{T7}-σ^{70} or P_{T7}-σ^{32}) and the Fluc template (*tac*-Fluc or dnaKp1-Fluc). As shown in Figure 3B, in the presence of P_{T7}-σ^{70}, a significantly higher Fluc activity was observed from the *tac* promoter than the dnaKp1 promoter (Figure 3B, shaded columns). In contrast, in the presence of P_{T7}-σ^{32}, a much higher Fluc activity was observed from the dnaKp1 promoter than the *tac* promoter (Figure 3B, white columns). The data are consistent with the known functions of Ec RNAP core enzyme during transcription initiation at different promoters (18,28), further supporting that a functional Ec RNAP core enzyme was indeed synthesized to form Ec RNAP holoenzymes with the sigma factors, capable of initiating transcription from specific *E. coli* promoters.

A series of control experiments were performed to further quantify the background-level expression of Fluc in the absence of sigma-factor templates and/or RNAP subunit templates. In the first set of controls (Figure 3C, columns 1–3), the Fluc templates (*tac*-Fluc and dnaKp1-Fluc) were absent in the protein synthesis reactions. No Fluc activity was detected, indicating that there was no contaminating Fluc gene in the DNA templates of Ec RNAP subunits and sigma factors. In the second set of controls (Figure 3C, columns 4–6), the Fluc activity was measured from two Fluc templates (*tac*-Fluc and dnaKp1-Fluc) in the absence of other DNA templates. The Fluc activity observed for the dnaKp1-Fluc template (columns 5) was reduced significantly in the absence of T7 RNA polymerase (T7 RNAP, column 4), suggesting that T7 RNA polymerase present in the reconstituted protein synthesis system was partially responsible for the background transcription activity from the Fluc templates even though the expression of Fluc was under the control of *E. coli* promoters. Promoter-independent transcription by T7 RNA polymerase has been previously reported (33). Though no Ec RNAP subunits were detected in the absence of their DNA templates by the western blot analysis (Supplementary Figure S1, lane 1), we cannot rule out that a low level of endogenous RNAP from *E. coli* was present in the reconstituted protein synthesis system and was responsible for some of the background transcription activity from the Fluc templates. The notion of contaminating Ec RNAP activity in the reconstituted protein synthesis system was also suggested by the next set of controls that included the templates for the sigma factors but not for Ec RNAP subunits (Figure 3C, columns 7–10). In the presence of the σ^{32} template (P_{T7}-σ^{32}), a higher Fluc activity was observed from the dnaKp1 promoter (column 7) than the *tac* promoter (column 8). Similarly, in the presence of the σ^{70} template (P_{T7}-σ^{70}), a

higher Fluc activity was observed from the *tac* promoter (column 10) than the dnaKp1 promoter (column 9). As the last set of controls, the Fluc activity was measured in the absence of the templates for the sigma factors (Figure 3C, columns 11 and 12). Addition of the templates of Ec RNAP subunits did not result in significant further increase in the Fluc activity (Figure 3C, compare columns 11 and 12 with columns 5 and 6). Only when the templates for the sigma factors and Ec RNAP subunits were all present, a significant increase in the Fluc activity was observed from both Fluc templates (Figure 3B, note the Fluc activity at a different scale from that in Figure 3C). The signal-to-noise ratio (defined as the ratio of the two Fluc activities in the presence and absence of the RNAP subunit genes, respectively) is ~20 for both σ^{70}/*tac*-Fluc and σ^{32}/dnaKp1Fluc templates. A higher signal-to-noise ratio (>100) was obtained for Ec σ^{28} when Fluc was expressed from a σ^{28}-specific promoter, largely due to a very low Fluc activity (noise) in the absence of the RNAP subunit genes (data not shown).

Taken together, above data suggest that Ec RNAP subunits (at least α, β and β') were indeed produced from encoding DNA templates in the protein synthesis reactions and subsequently assembled into a functional Ec RNAP core enzyme capable of catalyzing sigma-factor-dependent, promoter-specific transcription initiation.

Rapid generation and functional detection of Ec RNAP mutants using PCR-generated DNA templates

Coupled expression and detection of Ec RNAP holoenzyme in the reconstituted protein synthesis system presents a potential platform for rapid *in vitro* analyses of bacterial transcription initiation and RNAP holoenzymes. Since DNA templates can be used for the expression of Ec RNAP subunits, the method would be particularly useful for characterization of Ec RNAP mutants. Previous studies have identified Q513 and H526 of the β subunit of Ec RNAP as two of key residues for the binding of the small-molecule inhibitor rifamycin (34–36). Single substitutions of either residue have been found to confer rifamycin-resistance both *in vitro* and *in vivo* (34,36). We made three such single substitutions in the β subunit of Ec RNAP (i.e. Q513P, Q513L and H526Y) (34) using an overlapping PCR strategy (23) (Supplementary Figure S2B). The resulting linear DNA templates, encoding the β mutants under a T7 promoter [P_{T7}-β(Q513P), P_{T7}-β(Q513L) and P_{T7}-β(H526L)], were directly used for the protein synthesis reactions in which other Ec RNAP subunits were expressed from separate plasmid templates (Figure 4, P_{T7}-αβ'ω). As a control, the wild-type β subunit was also expressed from a linear DNA template [P_{T7}-β (WT)] generated by direct amplification from its plasmid template. For comparison, the wild-type RNAP core enzyme was expressed from all-plasmid templates (Figure 4, P_{T7}-αββ'ω). To monitor the activity of the synthesized Ec RNAP, the P_{T7}-σ^{32} and dnaKp1-Fluc templates were used.

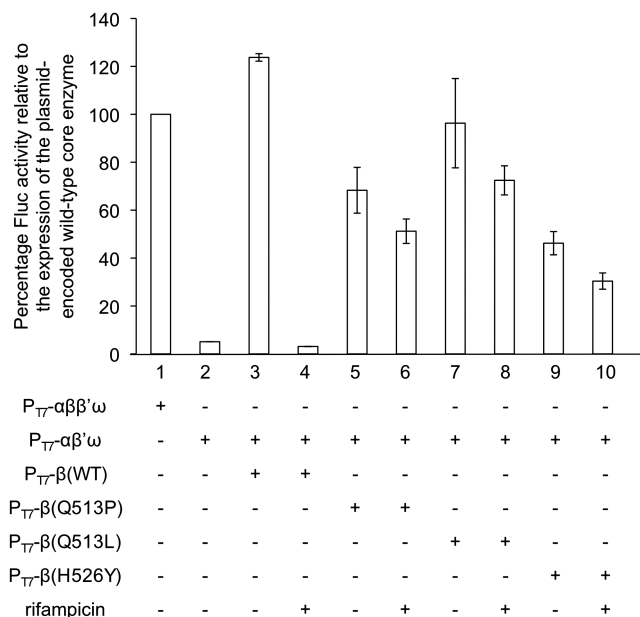As shown in Figure 4, the β subunit expressed from the linear template [P_{T7}-β (WT), column 3] or the plasmid

	1	2	3	4	5	6	7	8	9	10
P_{T7}-αββ'ω	+	-	-	-	-	-	-	-	-	-
P_{T7}-αβ'ω	-	+	+	+	+	+	+	+	+	+
P_{T7}-β(WT)	-	-	+	+	-	-	-	-	-	-
P_{T7}-β(Q513P)	-	-	-	-	+	+	-	-	-	-
P_{T7}-β(Q513L)	-	-	-	-	-	-	+	+	-	-
P_{T7}-β(H526Y)	-	-	-	-	-	-	-	-	+	+
rifampicin	-	-	-	+	-	+	-	+	-	+

Figure 4. Coupled expression and detection of Ec RNAP mutants in the reconstituted protein synthesis system. The Fluc activity was measured in protein synthesis reactions containing the linear DNA templates of dnaKp1-Fluc and PT7-σ^{32}, separate plasmid templates encoding all four or three wild-type Ec RNAP subunits (P_{T7}-αββ'ω or P_{T7}-αβ'ω) and the PCR-generated linear template for the wild-type β subunit [P_{T7}-β (WT)] or the single-substitution β subunit mutant [P_{T7}-β (Q513P), P_{T7}-β (Q513L) or P_{T7}-β (H526Y)] in the absence or presence of rifampicin (1.2 μM). The relative Fluc activity was presented with the activity from the template of P_{T7}-αββ'ω set as 100. All data were obtained from at least two independent protein synthesis reactions.

template (as in P_{T7}-αββ'ω, column 1) resulted in a similar Fluc activity, suggesting that both templates produced functional wild-type RNAP core enzyme. Addition of rifampicin (one of rifamycins) during the expression of the wild-type RNAP subunits significantly reduced the Fluc activity (Figure 4, column 4), to a level similar to when the template for the β subunit [P_{T7}-β (WT)] was absent in the protein synthesis reaction (Figure 4, column 2). Rifampicin at similar concentrations did not inhibit protein translation in the reconstituted protein synthesis system (Supplementary Figure S3). The data suggest that rifampicin inhibited the activity of the wild-type Ec RNAP core enzyme synthesized in the protein synthesis reaction, consistent with previous reports (36).

In the absence of rifampicin, the expression of the single-substitution mutants of the β subunit resulted in considerable Fluc activity compared to the expression of the wild-type β subunit [β (WT)] (Figure 4, columns 3, 5, 7 and 9), suggesting that the synthesized RNAP mutants were transcriptionally active. In contrast to β (WT) (Figure 4, column 4), all three single-substitution β mutants resulted in a significant Fluc activity in the presence of rifampicin (Figure 4, columns 6, 8 and 10), suggesting that the synthesized RNAP mutants were also rifampicin-resistant. The IC_{50}s for all three β mutants, defined as the concentrations of rifampicin necessary for the inhibition of the Fluc activity by 50%, were >10 μM, whereas the IC_{50} for β (WT) was ~0.2 μM (Supplementary

Figure S5). The data are consistent with previous *in vivo* studies and *in vitro* data using purified mutant enzymes (34–36).

DISCUSSION

In this study, we demonstrated *in vitro* reconstruction of basic *E. coli* transcription initiation from DNA templates in a reconstituted protein synthesis system. By adding purified Ec RNAP holoenzyme, we first established the reporter system and the reaction condition for coupled (Ec RNAP-initiated) transcription and translation. By adding purified Ec RNAP core enzyme, we then demonstrated the synthesis of the functional *E. coli* sigma factors from their encoding DNA templates. By using the DNA templates encoding Ec RNAP subunits and sigma factors, we finally demonstrated the synthesis and assembly of the functional *E. coli* holoenzymes. Successful synthesis of Ec RNAP subunits and sigma factors, and functional assembly of Ec RNAP holoenzymes for transcription initiation were supported by the sigma-factor-dependent, promoter-specific transcription of the reporter gene (Figures 2 and 3), the rifampicin-sensitive activity due to the expression of the wild-type β subunit, the rifampicin-resistant activity due to the expression of the single-substitution β mutants (Figure 4, Supplementary Figure S5) and western blot analyses of the expression of Ec RNAP subunits (Supplementary Figure S1).

By reconstructing *E. coli* transcription initiation, we also demonstrated the production of multiple proteins from their encoding DNA templates in the reconstituted protein synthesis system. In the case of Ec RNAP holoenzyme, at least five proteins (α, β, β', σ and Fluc) with molecular weights ranging from 32 kD to 155 kDa were produced from separate DNA templates in a single protein synthesis reaction. The synthesis of a functional Ec RNAP ω subunit was not confirmed in this study since no significant change in the Fluc activity was observed in the absence of the ω template (P_{T7}-ω) (Figure 3A). PCR-generated linear templates were used for the synthesis of Fluc and sigma factors, whereas circular plasmid templates were used for the synthesis of four Ec RNAP subunits (Figure 3). One of the plasmid templates for Ec RNAP subunits can be replaced by a separate PCR-generated linear template without significantly affecting the activity of the synthesized RNAP holoenzyme (Figure 4, column 3). In fact, all plasmid templates for Ec RNAP subunits can be replaced by PCR-generated linear templates without significantly affecting the activity of the synthesized RNAP holoenzyme (data not shown). Use of PCR-generated linear templates allowed rapid generation and analysis of mutants without cloning steps (Supplementary Figure S2).

Since the same protein translation machinery was responsible for the production of multiple proteins in the reconstituted protein synthesis system, it was necessary to balance the amounts of proteins synthesized for the assembly of the protein complex (e.g. RNAP holoenzyme) and for the detection of the reporter activity (e.g. Fluc

activity). In protein synthesis reactions, the amount of the template for Fluc was kept higher than the amounts of the templates for sigma factors and RNAP subunits (see 'Materials and Methods' section), due to the consideration that the expression of Fluc was under relatively weak *E. coli* promoters compared to the T7 promoter (37), which was used for the expression of sigma factors and RNAP subunits. Increasing the amount of the template for the sigma factor resulted in a decrease in the Fluc activity (Supplementary Figure S4). One limitation may be the protein synthesis capacity of the reconstituted protein synthesis system. The optimal ratio for the relative amounts of the templates encoding sigma factors and RNAP subunits was not determined in this study. A systematic optimization may be necessary to further increase the amount and specific activity of the synthesized RNAP holoenzyme as well as the signal generated by the Fluc activity.

In vitro reconstruction of *E. coli* transcription initiation demonstrated in this study can contribute to the study of not only known RNAP from model bacteria but also uncharacterized RNAP from other bacteria. Such approach has many potential advantages.

First, each RNAP subunit or sigma factor is encoded on a separate plasmid or linear DNA template, making cloning and site-specific mutagenesis a simple task and comprehensive mutational analyses a possibility. Using such strategy, it is possible to generate a large number of substitutions in the genes of RNAP subunits and sigma factors within a reasonable period of time, allowing high-throughput initial analyses of protein–protein and protein–small-molecule interactions. Using the conventional reconstitution methods, on the other hand, such tasks would be prohibitively laborious and time-consuming. Furthermore, RNAP or RNAP mutants that are toxic to the host cell due to interference with critical cellular functions or rapidly degraded due to low stability can not be produced *in vivo*.

Second, use of an additional DNA template encoding a reporter protein under the control of a sigma-factor-specific promoter allows immediate detection of an active RNAP holoenzyme, eliminating the need for purification of RNAP and a separate *in vitro* transcription step, and saving, possibly by orders-of-magnitude, the time normally taken by the conventional reconstitution methods. Due to the presence of endogenous Ec RNAP and transcription factors, this coupled synthesis and detection strategy may not work for the expression of other bacterial RNAP in *E. coli* or in the *E. coli*-extract-based cell-free systems. Based on western blot analyses, the endogenous Ec RNAP and sigma factors (and possibly transcription factors) were not detectable in our reconstituted protein synthesis system (Supplementary Figure S1, lane 1). Therefore, the reconstituted system may provide more consistent and cleaner preparation of RNAP for functional studies, free of host binding proteins or host-hybrid enzymes (22). The drawbacks of *in vitro* synthesis of RNAP and sigma factors are that the amounts of synthesized proteins are low and not easily determined. Therefore, for detailed (follow-up) mechanistic studies, the conventional reconstituted methods may be preferred.

Third, we speculate that *in vitro* protein synthesis in the background of a low level of nucleases and proteases may benefit the folding and complex assembly of multi-component enzymes. The rate of the nascent chain elongation on ribosomes in a cell-free system is estimated to be ~2 residues/s (in contrast to ~20 residues/s in growing *E. coli* cells) (38), which may allow more time for proper folding and/or co-folding with other proteins in the reconstituted protein synthesis system than *in vivo*. This benefit is probably lost in the cell-extract-based systems as incomplete folded proteins may be degraded by proteases and unprotected mRNA may be degraded by nucleases before the productive assembly of a protein complex can be achieved.

Lastly, the *in vitro* synthesis and function of RNAP are correlated to the activity of a luminescent reporter protein commonly used in high-throughput assays. Therefore, the system is instantly ready for microplate-format high-throughput screening of small-molecule inhibitors of RNAP, a proven target for antibiotics (39). Since potential small-molecule inhibitors can be added during the synthesis, assembly and catalysis (transcription initiation) of RNAP, we speculate that our approach, resembling the natural processes inside the cell, can potentially lead to novel inhibitors that target the sites of RNAP not yet found by conventional *in vitro* screening methods in which purified and preassembled RNAP are normally used (40).

Reconstruction of *E. coli* transcription initiation in the reconstituted protein synthesis system may provide an initial step towards experimental reconstruction of more complex scenarios of bacterial transcription from genetic materials (DNA templates). For instance, additional sigma factors may be synthesized from their encoding DNA templates for functional analyses of multiple promoter regions, which can be linked to one or multiple reporter genes. Repressors and activators may also be synthesized in the same protein synthesis reaction for reconstruction of certain regulatory pathways in bacterial transcription. The ability of using multiple and PCR-generated DNA templates in the reconstituted protein synthesis system may allow rapid analyses of a large number of genes and promoters involved in bacterial transcription and its regulation. Future experiments will examine if low transcription by weak promoters or transcription activation/repression can be detected by, and correlated to, the activity of Fluc or other reporters in the reconstituted protein synthesis system. Another unanswered question is the maximal number of proteins that can be simultaneously synthesized from their DNA templates and detected as the result of their functions in the reconstituted protein synthesis system. Efforts to further enhance the synthesis and folding capacity of the reconstituted system are underway.

One can also envision experimental reconstruction of bacterial replication in the reconstituted protein synthesis system using DNA templates containing an origin of replication and genes encoding DNA polymerases and other components. In fact, our preliminary studies have suggested that coupled expression and detection of an *E. coli* DNA polymerase is possible in the reconstituted

proteins synthesis system and that the DNA-template-directed polymerization reaction is compatible with the condition of the protein synthesis reaction (S. Chong, unpublished). A reconstituted system capable of demonstrating replication, transcription and translation would facilitate *in vitro* study and intervention of three polymerization processes that are essential to all life.

In summary, reconstruction of *E. coli* transcription initiation in this study illustrates a general strategy for *in vitro* experimental reconstruction of a multi-component biological complex or process. The strategy couples expression of multiple gene products with detection of the resulting biological activities in a reconstituted protein synthesis system and allows rapid *in vitro* reconstruction of a functional biological complex or process from DNA templates instead of purified components. Since protein synthesis, complex assembly and enzyme catalysis occur in the same *in vitro* reaction mixture, this reconstruction process resembles *in vivo* biosynthetic pathways and avoids time-consuming expression and purification of individual proteins. The strategy can significantly reduce the time normally required by the conventional reconstitution methods and provide an open and designable platform for *in vitro* study and intervention of complex biological processes. The key for successful application of such strategy is the ability of the reconstituted protein synthesis system to produce multiple gene products in functional forms. The reconstituted protein synthesis system may be further modified by addition of new components, and/or optimization of existing components, in order to increase the capacity of protein synthesis and facilitate folding, assembly and detection of multi-gene products. Here we propose to name such a reconstituted system an 'Expressome'. Current reconstituted protein synthesis systems have largely been used as superior cell-free systems over cell-extract-based systems for synthesis of single proteins (11–14,41), incorporation of unnatural amino acids (4,42,43), study of nascent chain folding (44,45), *in vitro* protein evolution (41,46,47) and synthetic-cell applications involving few genes (48,49). In comparison, an Expressome contains, in addition to all the components of a reconstituted protein synthesis system, DNA templates designed to encode a set of genes involved in a particular biological complex or process and (if necessary) one or several reporter genes that facilitate detection. In other words, an Expressome is an *in vitro* reconstruction of a biological complex or process beyond protein translation and with higher complexity. A potential advantage of an Expressome would be to enable a 'cell-free genetic approach' to study biological functions, allowing genetic alterations to be rapidly generated and detected *in vitro*. Since *E. coli* has been extensively studied with widely available genetic tools and databases (50), the approach would be particularly valuable for the study of bacteria and other organisms with known genome sequences but for which no or limited genetic tools are available.

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