Expressed protein ligation is a novel method for studying protein-protein interactions in transcription.

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Konstantin Severinov‡§ and Tom W. Muir¶

From the ‡Department of Genetics and Waksman Institute, the State University of New Jersey, Rutgers, Piscataway, New Jersey 08854 and the ¶Laboratory of Synthetic Protein Chemistry, The Rockefeller University, New York, New York 10021

Expressed protein ligation is a novel protein semi-synthesis method that permits the in vitro ligation of a chemically synthesized C-terminal segment of a protein to a recombinant N-terminal segment fused through its C terminus to an intein protein splicing element. In principle, the practical convenience of this method, combined with the expanded opportunities in protein engineering that it provides, makes it well suited for probing the molecular basis of complex processes such as transcription. Here we describe the successful application of expressed protein ligation to the ~600 amino acid σ70 subunit of Escherichia coli RNA polymerase. The resulting semi-synthetic σ70 constructs are shown to be fully functional and have been used to map the binding region of the bacteriophage T4 anti-sigma protein, AsiA, to within amino acids 567–600 of σ70. The success of these semi-synthesis studies sets the stage for the future generation of semi-synthetic σ70 molecules in which unnatural amino acids and biophysical probes are site-specifically incorporated in the RNA polymerase complex.

It is becoming increasingly clear that regulation of transcription involves protein-protein contacts between RNA polymerase and transcription factors (1). Characterization of these interactions, at the molecular level, is a prerequisite to fully understanding transcription mechanism and regulation. The ability to site-specifically incorporate unnatural amino acids (containing biophysical or biochemical probes) into the protein components of the transcription apparatus would greatly aid the study of these complex macromolecular machines.

Recent years have seen the development of a number of methods designed to allow the incorporation of unnatural amino acids into proteins (2–6). These approaches include in vitro protein expression (3), site-specific protein modification (4, 5), and protein total synthesis (6). Although powerful, each of these techniques has associated with it certain practical or synthetic limitations which have to some extent restricted their widespread application. Total chemical synthesis, which provides unparalleled freedom to manipulate protein structure, has been dominated in recent years by the use of chemical ligation techniques (7–13). Among these, the “native chemical ligation” approach by Kent et al. (14) has proven a particularly useful route to synthetic proteins. In this process, an N-terminal cysteine-containing peptide is chemically ligated to a peptide possessing a C-terminal thioester group with the resultant formation of a normal peptide bond at the ligation site. Despite the generality of the ligation chemistry, the strategy has been constrained by the need to generate the peptide building blocks using stepwise solid phase peptide synthesis (SPPS).1 The size limitations imposed by this requirement have restricted the application of native chemical ligation to the study of small proteins and protein domains (6).

Protein semi-synthesis, in which synthetic peptides and protein cleavage fragments are linked together, offers an attractive route to the generation of large protein analogs containing unnatural amino acids (15). The utility of existing semi-synthesis strategies is, however, tempered by the need to have unique chemical or enzymatic cleavage sites at the appropriate position within the protein of interest. The recently introduced expressed protein ligation technique directly addresses these limitations by providing a general way of chemically attaching synthetic peptides (via a putative native chemical ligation step) to the C terminus of recombinant proteins without the need to perform any intervening fragmentation steps (2). Here we report the results of model studies designed to explore further the chemical mechanism of expressed protein ligation. Insights gained from this preliminary work were then successfully applied to the generation of a functional semi-synthetic version of the 613 residue σ70 subunit of Escherichia coli RNA polymerase.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification of Proteins—The plasmid pCYB2_σ70-566, which expresses a 66-amino acid fragment of σ fused to intein-CBD from an isopropyl-1-thio-β-D-galactopyranoside-inducible trc promoter, was constructed by polymerase chain reaction amplification of the corresponding fragment of rpoD and recloning it in NdeI-Smal-treated plasmid pCYB2 (New England Biolabs). A linker of two non-natural amino acids (Ala-Gly) was inserted between the last amino acid of σ (Ile566) and Cys1 of the intein. pCYB2_σ70-566 was constructed similarly. The natural NdeI site at codon 452 of rpoD was removed by site-directed mutagenesis to facilitate the cloning. The protein sequence remained the same due to degeneracy of the genetic code. The plasmids were transformed into the E. coli XL1-blue; cells were grown to mid-log phase in 2 liters of LB medium plus 200 μg of ampicillin/ml and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside overnight. The expression level was low (~1 mg/liter), and we could barely detect the band of the overexpressed proteins on SDS gels.

After recovery by centrifugation, cells were resuspended in 40 ml of...
50 mM Tris-HCl, 500 mM NaCl, 10 mM EDTA, pH 7.9, and lysed by passage through a French press, and the lysate was cleared by low speed centrifugation. The overexpressed proteins were recovered from the cytosolic fraction by affinity chromatography on a 2-ml chitin column equilibrated in the same buffer as suggested by the manufacturer. The column was washed with 50 ml of buffer, and 25 ml of 0.2 M phosphate buffer, pH 7.3, 0.2 M NaCl and drained, and the beads were stored as a 50% suspension in the same buffer at 4 °C until further use. AsiA was purified as described by Severina et al. (16). Plasmid expressing AsiA genetically fused to a C-terminal promoter was provided by D. Hinton. The protein was overexpressed in BL21(DE3) cells and purified to homogeneity by IMAC. AsiA proteins were concentrated using a Centricon 3 centrifugal filter (Amicon) and stored at −20 °C in a buffer containing 50% glycerol.

Syntethic peptides—All peptides were chemically synthesized according to optimized 7-butyloxycarbonyl SPPS (17) and purified by preparative reverse-phase HPLC using a Vydac C-18 column. In all cases, peptide composition and purity were confirmed by electrospray mass spectrometry and analytical HPLC. Fluorescein was attached to the ε-amino group of the lysine residue in the peptide NH2-CED-NEYTARE-amincaproate-K-CO2H prior to the final cleavage/deprotection step using a 7-butyloxycarbonyl-Lys-e-(NH-Fmoc) orthogonal protection strategy. The construct His3-Cys-JSCH3-amincaproate-Asp500–566, was prepared by chemically ligating the purified, unprotected peptides NH2-His3-Cys-JSCH3-CO2H and bromo-acetyl (BrAc)aminocaproate-Asp500–566, using the previously described thioether-based chemical ligation strategy (11).

**Protein Ligation**—100–500 μl of 50% chitin bead suspension was combined with various cofactors in the presence or in the absence of 1 mM synthetic peptide. Cofactors were used at 100 μM concentration (dithiothreitol, mercaptoacetic acid, N-acetylcyesteine, and cysteine) and 1.5% v/v (thiophenol). Reactions were performed in 0.2 M phosphate buffer, pH 7.3, 200 mM NaCl (dithiothreitol, thiophenol, and cysteine), or 0.5 mM phosphate buffer, pH 7.3 (N-acetylcyesteine and mercaptoacetic acid). All reactions were incubated overnight with gentle agitation and then diluted 10-fold with transcription buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl2). Model experiments involving αδ160–566 were carried out at room temperature. Experiments involving αδ1–566 were carried out in the cold room, and all buffers were supplemented with 1 mM phenylmethylsulfonyl fluoride to minimize proteolytic degradation. The beads were allowed to settle, and the supernatant was dialyzed against two 1-liter changes of transcription buffer. The protein was then concentrated on a C-30 concentrator (Amicon) to ~1 mg/ml, diluted 2-fold with glycerol, and stored at ~20 °C.

**Ni-NTA Binding**—100–μl reactions contained 15 μl of Ni2+–NTAagarose (Quigen), 50–100 pmol of αδ160 or αδ160 derivative, 200 pmol of AsiAHis20, 20 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM MgCl2. Reactions were preincubated for 15 min at room temperature, after which the beads were pelleted by brief centrifugation, and the supernatant containing the unbound material was removed. The beads were then washed three times with the same binding buffer containing 10 mM imidazole, pH 8.0, resuspended in 50 μl of the buffer containing 100 mM imidazole, and incubated for an additional 15 min at room temperature. The supernatant containing the bound material was then withdrawn. Aliquots of the reactions were then analyzed on an 8–25% Phast gels (Amersham Pharmacia Biotech) and silver-stained. For transcription reactions, washed Ni2+–NTA-agarose beads containing αδ160 or the ligation product were purified through asiA, and 50 μl αδ160 or αδ160 derivative treated with an equal volume of 7.5 μg guanidine HCl (15 min at room temperature with agitation). 10 μl of the supernatants was removed, diluted to 100 μl with transcription buffer, and used for transcription reactions.

**In Vitro Transcription**—Abortive initiation reactions were performed in 20 μl of transcription buffer containing 20 mM of either the 123-base pair T7 A2 promoter containing DNA fragment (18) or the 150-base pair gal P1 fragment (19), 40 mM RNAP core enzyme (β′, β, α25-5-dimer), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM UTP (gal P1), and 50 μM αδ160 or αδ160 derivative (100 pmol), with 20 μg of DNA template. Reactions were performed with 5 μl (~10 pmol) of αδ160 or ligation product prepared as described above. The amount of cleavage product added to the reaction was equal to that of αδ160 or ligation product based on visual inspection of stained SDS gels. Reactions proceeded for 15 min at 37 °C and were terminated by addition of an equal volume of loading buffer containing 6 M urea. Transcription products were analyzed by urea-polyacrylamide gel electrophoresis (7 M urea, 20% polyacrylamide), followed by autoradiography.

### RESULTS

**Rationale**—The long-term objective of our studies is to explore transcription mechanism through the generation of semi-synthetic versions of the various subunits of *E. coli* RNA polymerase. In the current work, we are interested in systematically chemically modifying the C-terminal region of the α57 subunit. Our approach is based around the chemical ligation of a synthetic peptide (corresponding to the C-terminal region of α57) to a recombinant protein constituting the remainder of the α57 sequence. Our previous studies have shown that the commercially available pCYB expression plasmids (New England Biolabs) offer a way of generating recombinant proteins that are able to participate in ligation reactions with synthetic peptides possessing cysteine residues at their N terminus (2). Since pCYB expression vectors make use of a genetically manipulated protein splicing system (20), a process which has been shown to involve the intermediary of a thioester (21), it is presumed that the reaction of the synthetic peptide with the recombinant protein goes via native chemical ligation (14).

Before attempting the generation of a full size semi-synthetic α70 subunit, a series of model studies were carried out using a smaller recombinant fragment of the protein more amenable to accurate mass spectrometric analysis. The purpose of these studies were 2-fold: (i) to study the chemical mechanism of expressed protein ligation, and (ii) to explore the feasibility of applying the semi-synthesis approach to the α70 system.

**Preliminary Studies**—As a model system, a short recombinant fragment corresponding to amino acids 500–566 of the 613-amino acid long *E. coli* RNA polymerase α70 subunit was cloned into the pCYB vector. Subsequent overexpression in *E. coli* resulted in the generation of a protein chimera in which the desired α70 fragment was C-terminally fused to an intein protein splicing element itself linked to a chitin binding domain (CBD), included to allow rapid purification by affinity chromatography on chitin beads (20).

Our initial investigations focused on the release of the desired α70 fragment from the immobilized chimera through the treatment with various thiol-containing molecules. As can be seen from Table 1, N-acetylcyesteine was the only agent tried that failed to support detectable cleavage of the polypeptide from the support. In contrast, dithiothreitol, cysteine, mercaptoacetic acid, and thiophenol all caused efficient cleavage of the chimeric protein. Analysis of cleavage supernatants by reverse-phase HPLC revealed, in all cases, the presence of two main peaks. Electrospray mass spectrometry indicated that the first HPLC peak (~75% of the total product) corresponded to α amino acids 501–566 plus the two C-terminal linker amino acids.
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acids Ala-Gly (see “Experimental Procedures”) and thus had the first methionine residue removed. The second, minor peak contained the unprocessed product with the first methionine in place. The molecular weights of the major processed products of the different cleavage reactions are shown in Table I. The products of the cysteine, mercaptoacetic acid, and thiophenol cleavages had masses consistent with C-terminally derivatized polypeptides, whereas the major product of the dithiothreitol-mediated cleavage had a free C-terminal carboxyl group.

Of particular relevance to the mechanism of expressed protein ligation is the exact chemical nature of the C-terminal group present on the cleavage product following treatment with a thiol agent. To investigate this, the major product from the thiophenol cleavage reaction was isolated and subsequently exposed to elevated pH. This resulted in its clean conversion into an earlier eluting form (by HPLC) with a mass exactly 92 Da less than before. Such a mass change is indicative of the hydrolysis of the phenyl \( \alpha \)-thioester derivative of the sigma polypeptide back to the free acid. Note, the product of the cysteine cleavage reaction was stable to high pH, evidence that the initial thioester product had, as expected, rearranged to a stable amide.

Next we evaluated whether the immobilized \( \sigma^{70} \) fragment could be chemically ligated to a short synthetic peptide (NH\(_2\)-Cys-Glu-Asp-Asn-Glu-Tyr-Thr-Ala-Arg-Glu-aminocaproate-Lys-\( \varepsilon \, \text{[fluorescein]CO}_2\text{H}) containing an N-terminal cysteine to facilitate ligation and a C-terminal fluorescein reporter group. Initial studies in which the beads were simply treated with a solution containing 1 mM peptide at pH 7.3 were unsuccessful, and neither ligation nor protein cleavage was detected, even after prolonged incubations. The presence of thiol cofactors can appreciably accelerate native chemical ligation reactions both in solution (22) and on solid phase (23). Consequently, we investigated the effect of thiol co-factors on our model ligation reaction (Table I). Consistent with our previous findings, thiophenol was the only cofactor that supported efficient ligation of the synthetic peptide to the recombinant \( \sigma^{70} \) fragment. Inclusion of 1.5% v/v of thiophenol in the ligation mixture at pH 7.3 containing the synthetic peptide (1 mM) resulted in extremely efficient ligation (>90% after overnight incubation) as indicated by HPLC and electrospray mass spectrometry analysis. The ligation product had a mass of 9023 Da and thus corresponded to the desired semi-synthetic polypeptide (expected mass = 9024 Da since the masses of \( \sigma \) fragment and synthetic peptide were 7215 and 1827 Da, respectively). The stability of the ligation product to the high concentration of thiophenol present in the ligation buffer confirms the presence of an amide linkage (and not a labile-thioester bond) at the ligation junction (14, 22). Note that no ligation was detected using a control peptide that did not contain an N-terminal cysteine residue.

The absolute requirement of thiophenol as a cofactor taken together with the results of our initial cleavage studies, indicates that the phenyl \( \alpha \)-thioester derivative of the \( \sigma \) polypeptide is being generated \textit{in situ} during the reaction and is then reacting with the synthetic peptide via a native chemical ligation reaction. Interestingly, both thiophenol-mediated cleavage and ligation reactions were found to follow similar kinetics (each taking several hours to go to completion) and are appreciably slower than is typically observed when using synthetic fragments (22, 23). The slow kinetics of the thiophenol-mediated cleavage/ligation probably reflects the position of the equilibrium in the initial A → S acyl transfer within the chimera. Based on the data from these model studies we propose the ligation mechanism shown in Fig. 1 in which the highly reactive phenyl \( \alpha \)-thioester derivative of the recombinant polypeptide is produced \textit{in situ} during the ligation process. Once generated this derivative will quickly and irreversibly react with the synthetic peptide to give the final product, thus generating a reaction sink.

\textbf{Preparation of a Semi-synthetic \( \sigma^{70} \) Derivative—} We next investigated whether a functional semi-synthetic \( \sigma^{70} \) could be obtained by expressed protein ligation. Genetic and biochemical data indicate that in the context of bacterial RNA polymerase lambda element, protein-DNA contacts between the evolutionary conserved C-terminal region 4.2 of \( \sigma^{70} \) and the –35 promoter element are crucial for promoter recognition (24–26). In addition, protein-protein contacts between \( \sigma \) region 4.2 and transcription factors are crucial for transcription activation (27, 28). Thus, we decided to focus our efforts on the semi-synthesis of a \( \sigma^{70} \) analog containing a chemically synthesized region 4.2. A recombinant protein containing the first 566 amino acids of \( \sigma^{70} \) fused to intein-CBD was used in the ligation reaction. This fragment was chosen because the region of \( \sigma^{70} \) defined by residues 560–570 is evolutionarily variable in length and sequence (29) and is likely to tolerate a non-natural cysteine introduced as a result of ligation. Sequence comparisons also indicate that region 4.2 does not extend past \( \sigma^{70} \) His\(^{500} \) on the C-terminal side (29). Based on these data, a 34-residue peptide was synthesized that corresponded to amino acids 568–600 of \( \sigma^{70} \) with an additional cysteine residue at the
N terminus to promote ligation. Chemical ligation of the synthetic 34-mer to the 568-residue recombinant protein (residues 1–566 of σ70 + Ala-Gly) was carried out using the general conditions described above. As a control, a second reaction was performed in the absence of the synthetic peptide and thus should have contained the cleavage product only. Analysis of the crude reaction mixtures by SDS-PAGE showed the presence of the expected ~70- and ~65-kDa bands in the ligation and control reactions, respectively (Fig. 2A). As in the model studies, the crude ligation reaction was essentially free of unligated material, although the mixture did contain a contaminant band at around 55 kDa (labeled σ70X in Fig. 2A, see below).

Semi-synthetic σ70 Is Functional—The bacteriophage T4 anti-sigma protein, AsIΔ, engineered with a C-terminal hexahistidine tag was used in a Ni2+-NTA-agarose co-immobilization assay to investigate the binding of the ligation product, the cleavage product, and the full-length recombinant σ70 to AsIΔ (Fig. 2A). A mixture of AsIΔHis and recombinant σ70 was loaded onto Ni2+-NTA-agarose beads (lane 4), and the unbound material was removed (lane 5). The beads were subsequently washed with 10 mM imidazole buffer and then eluted with 100 mM imidazole buffer. Both σ70 and AsIΔHis were found in eluted fractions (lane 6). Since recombinant σ70 in the absence of AsIΔHis did not interact with the beads (lane 3), we conclude that σ70 was retained on the beads through direct protein-protein interaction with AsIΔHis, as expected (16). When a mixture of AsIΔHis and the cleavage product, containing the first 566 amino acids of σ70, was loaded onto Ni2+-NTA-agarose beads (lane 7), all of the σ70 fragment appeared in the unbound fraction (lane 8), whereas an analogous experiment with the ligation product indicated interaction of the semi-synthetic 602-amino acid long σ70 derivative with AsIΔHis (lane 12). In the absence of AsIΔHis, semi-synthetic σ70 did not interact with the Ni2+-NTA-agarose beads (lane 15).

The crude ligation and ligation reactions each contain an additional protein (labeled σ5 on Fig. 2A) that migrates faster than either the expected cleavage or ligation products. The appearance of this band is dependent on the addition of σ5 to protein overproducing lysates to the chitin beads, and we conclude that this band is probably a product of σ proteolysis. As this σ fragment may interfere with the function of the desired ligation product, we removed it using the AsIΔ immobilization method described above (see “Experimental Procedures”). When the purified ligation product and the corresponding amount of the cleavage product were combined with E. coli RNA polymerase core, the resulting holoenzymes were active in the galP1 promoter as was the holoenzyme reconstituted with full-length recombinant σ70 (Fig. 2B). The galP1 promoter belongs to the “extended −10” promoter class and is active even in the absence of sigma region 4.2 (30). From this experiment we conclude that both the cleavage and the ligation products retained their biological activity during the overnight incubation with thiophenol.

A similar experiment was repeated on the T7 A2 promoter. T7 A2 is a strong promoter of the “−10/−35” class and requires interaction between σ region 4.2 and the −35 box for its activity. As can be seen from the autoradiogram shown on Fig. 2C the holoenzyme reconstituted with the ligation product was almost as active as the holoenzyme reconstituted with the full-length recombinant σ70. In contrast, the holoenzyme reconstituted with the cleavage product was completely unable to
support transcription by the core enzyme on T7 A2 (lane 3). We conclude that the semi-synthetic, 602-amino acid long 70\(\sigma\) derivative is functional in promoter-dependent transcription. The results also establish, as expected, that the non-natural cysteine introduced at the ligation site does not interfere with 70\(\sigma\) function and, in agreement with the data of Kumar et al. (30), that the last 13 amino acids of 70\(\sigma\) are not necessary for unregulated transcription.

70\(\sigma\) Amino Acids 568–600 Are Sufficient for Interaction with AsiA—The results presented in Fig. 2A demonstrate that 70\(\sigma\) amino acids 568–600 are necessary for AsiA binding. In order to show that 70\(\sigma\) amino acids 558–600 are also sufficient for interaction with AsiA, we performed a Ni\(^{2+}\)-NTA-agarose co-immobilization experiment with the synthetic 33-mer. Two different experiments were performed, and the same result was obtained. The first experiment was essentially a repetition of the experiment shown in Fig. 2A and demonstrated that the synthetic 33-mer can be immobilized on Ni\(^{2+}\)-NTA-agarose through AsiAHis (data not shown). The complementary experiment was done using wild type, untagged AsiA. Instead, a synthetic hexa-histidine tag was chemically ligated to the N terminus of 70\(\sigma\) amino acids 568–600 as described under “Experimental Procedures.” AsiA and His-tagged 70\(\sigma\) fragment were loaded on Ni\(^{2+}\)-NTA beads; the beads were washed and eluted with increasing concentrations of imidazole in the buffer. As can be seen, AsiA was found in the fractions containing elevated concentrations of imidazole, and the elution profiles of AsiA and His-tagged 70\(\sigma\) amino acids 568–600 from Ni\(^{2+}\)-NTA-agarose beads were identical, indicating strong interaction (Fig. 3). A control experiment showed that AsiA did not interact with Ni\(^{2+}\)-NTA-agarose (Ref. 16 and data not shown). We conclude that 70\(\sigma\) amino acids 568–600 are sufficient for interaction with AsiA.

**DISCUSSION**

Expressed protein ligation is a novel approach that allows synthetic peptides to be chemically ligated to the C terminus of recombinant proteins fused to an intein protein splicing element (2). Based on the requirement for an N-terminal cysteine within the synthetic peptide, as well as the known involvement of thiosteres in protein splicing (21), it is assumed that the process involves a native chemical ligation (14) step. This has been confirmed in the present study through a series of model studies which, moreover, indicate a phenyl alpha-thioester derivative of the recombinant protein to be the key reactive species in the process. This thioester derivative is also believed to be generated quite slowly during the process, meaning that the ligation reaction is probably pseudo-first order with the synthetic peptide being present in huge excess at any given time (Fig. 1).

We also describe the preparation, using this new approach, of a 602-amino acid long semi-synthetic 70\(\sigma\) protein with an intact biological function. This molecule was used to map the determinants of AsiA binding within amino acids 568–600 of 70\(\sigma\). Our result is in excellent agreement with that of Colland et al. (31), who used hydroxyl radical protein-protein footprinting to demonstrate that the only region of 70\(\sigma\) that is protected from radical cleavage by AsiA is located between residues 572 and 588. 70\(\sigma\) amino acids 568–600 comprise the functionally important region 4.2 of the asiA family of proteins. This region is thought to assume a helix-turn-helix conformation and to interact directly with the −35 box of the promoter. The present results, taken together with our finding that the binding of region 4.2 to the −35 box or AsiA is mutually exclusive,\(^2\) suggests that AsiA may inhibit transcription directly, by occluding the DNA binding surface region 4.2.

The results presented here illustrate the enormous potential of the expressed protein ligation technique for exploring the mechanism and regulation of complex biomolecular machines. In the case of E. coli RNA polymerase, we have demonstrated that the ligation conditions do not destroy protein function and that semi-synthetic 70\(\sigma\) subunits can be reconstituted with the RNA polymerase core enzyme to give a fully functional holoenzyme. The stage is thus set for future studies in which biochemical and biophysical probes are site-specifically introduced into the 70\(\sigma\) subunit. For example, we are currently site-specifically introducing cross-linkable probes into semi-synthetic 70\(\sigma\). Promoter complexes formed by RNA polymerase holoenzymes reconstituted with derivatized, cross-linkable sigmas will allow us to study protein-protein and protein-nucleic acids contacts that govern transcription activation and promoter recognition. Finally, it is worth noting that other semi-synthetic subunits of the E. coli RNA polymerase complex can be reconstituted in vitro,\(^3\) suggesting expressed protein ligation will have widespread utility in this multiprotein system.

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\(^2\) E. Severinova, K. Severinov, and S. A. Darst, submitted for publication.

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