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www.Fenyolab.org
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Rapid Isolation and Identification of Bacteriophage T4-Encoded Modifications of *Escherichia coli* RNA Polymerase: A Generic Method to Study Bacteriophage/Host Interactions

Lars F. Westblade,† Leonid Minakhin,† Konstantin Kuznedelov,† Alan J. Tackett,†,§ Emmanuel J. Chang,†,‡ Rachel A. Mooney,† Irina Vvedenskaya,‡ Qing Jun Wang,‡ David Fenyo,† Michael P. Rout,† Robert Landick,† Brian T. Chait,† Konstantin Severinov,‡ and Seth A. Darst*†

The Rockefeller University, New York, New York 10065, Waksman Institute for Microbiology and Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, Piscataway, New Jersey 08854, Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205, Department of Natural Sciences, York College/City University of New York, Jamaica, New York 11451, and Departments of Biochemistry and Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin 53706

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Bacteriophages are bacterial viruses that infect bacterial cells, and they have developed ingenious mechanisms to modify the bacterial RNA polymerase. Using a rapid, specific, single-step affinity isolation procedure to purify *Escherichia coli* RNA polymerase from bacteriophage T4-infected cells, we have identified bacteriophage T4-dependent modifications of the host RNA polymerase. We suggest that this methodology is broadly applicable for the identification of bacteriophage-dependent alterations of the host synthesis machinery.

**Keywords:** *Escherichia coli* • bacteriophage • RNA polymerase • bacteriophage infection • affinity isolation • MALDI MS

Introduction

In the bacterium *Escherichia coli* (*Ec*), transcription is dependent upon a single ~400 kDa multisubunit RNA polymerase (RNAP) that can exist in two forms: the catalytic core enzyme (α₂ββ′ρ′ω) and the promoter-specific holoenzyme (α₂ββ′ωσ). Transcription can be divided into three distinct stages: initiation, elongation, and termination. Regulation of RNAP can occur at each stage of transcription by protein, RNA, and small molecule trans-acting factors that associate with the enzyme to modulate its activity.

Bacteriophages (phages) are bacterial viruses dependent upon a host organism to propagate; therefore, phages are endowed with mechanisms that subvert the host’s cellular processes to serve the needs of the virus. Consequently, bacteriophages offer an exciting and tractable system by which to study developmental processes. Recently, a resurgence in bacteriophages as a tractable system to study developmental processes to serve the needs of the virus. Consequently, bacteriophages offer an exciting and tractable system by which to study developmental processes. The recent resurgence in the use of bacteriophages for the treatment or prophylaxis of bacterial infectious diseases—bacteriophage therapy—has increased the demands for understanding bacteriophage/host interactions. In particular, identification of phage proteins that bind to and inhibit essential host enzymes should allow the identification of novel drug targets. Therefore, it is highly desirable to have efficient methods for the identification of phage-encoded proteins that associate with host proteins.

Many phages encode proteins that directly bind to and regulate the activity of the bacterial host RNAP, and some phages encode enzymes that covalently modify the host transcriptional apparatus. These phage-induced modifications ensure coordinated temporal transcription of the phage genome and inhibition of host transcription.²

Bacteriophage T4 (T4) does not encode its own RNAP and relies on *Ec* RNAP for expression of its genes. To appropriate the host RNAP, T4 employs several redundant mechanisms that target *Ec* RNAP to the T4 genome² and promote the transcription of T4 DNA. One such mechanism involves the T4-encoded Alc protein that functions to increase the pool of host RNAP available for transcribing T4 DNA by selectively terminating RNAP transcription on Ec DNA without affecting transcription of T4 DNA.³–⁵

In this paper, we present a method for the rapid isolation of RNAP from phage-infected *Ec* cells, followed by matrix-assisted laser desorption ionization mass spectrometric (MALDI MS) analysis of proteins co-isolating with RNAP. This method allowed unequivocal identification of most of the known T4-encoded proteins that bind directly to host RNAP and identification of Alc whose binding to RNAP had not previously been reported, and also identified covalent modifications of the host RNAP by T4-encoded enzymes. This general procedure should be broadly applicable for the identification of phage-encoded proteins that interact with components of the host macromo-
lecular synthesis machinery and should become increasingly important in characterizing the many bacteriophage/host systems.

**Experimental Section**

**Ec Strains and Growth Conditions.** A strain of *Ec* MG1655 encoding two Fc-binding repeats of the Protein A (2PrA) affinity tag6 appended to the 3’ end of the *rpoC* gene (which encodes the RNAP β’ subunit) was constructed as previously described.7 Both the MG1655 *Ec* wild-type and MG1655 *Ec rpoCΔ:2PrA* strains exhibited almost identical growth curves in Luria–Bertani broth (LB; data not shown) and doubling times (*Ec* wild-type, 25.3 ± 1.6 min; *Ec rpoCΔ:2PrA, 28.5 ± 2.1 min).

All Ec strains were grown in LB at 37 °C with shaking. To prepare T4-infected biomass, wild-type *Ec* and *Ec rpoCΔ:2PrA* strains (4 L of each) at an *A600 nm* of 0.5–0.6 were infected with T4 (alt-) at a multiplicity of infection of 10. Infection was halted 15 min postinfection by rapidly cooling the samples in icy water baths. Cells were harvested by centrifugation, washed once with ice-cold 10% (v/v) glycerol, and frozen as pellets in liquid nitrogen. Cells were cryogenically disrupted with a Retsch MM301 mixer mill (Retsch) that was maintained at liquid nitrogen temperature and stored at −80 °C.8

**Affinity Isolation and Mass Spectrometric Identification of Protein Complexes.** Cryogenically lysed cells (1 g) were suspended in 5 mL of extraction buffer (20 mM Hepes (pH 7.4); 2 mM MgCl2; 150 mM NaCl) supplemented with one protease inhibitor cocktail tablet (Roche Diagnostics). Suspended lysate was treated with 0.002% (w/v) DNase I (Sigma-Aldrich) with agitation. The soluble fraction was isolated by centrifugation at 27,200 *g* for 10 min, and the supernatant was decanted and subjected to another 10 min centrifugation step. The supernatant was incubated with 25 mg of Dynabeads (Invitrogen) cross-linked to rabbit IgG (MP Biomedicals) with gentle agitation for 10 min. For β’–2PrA, and other affinity tagged proteins, rapid affinity isolations are optimal (data not shown).10 Dynabeads were collected with a magnet and washed with wash buffer (20 mM Hepes (pH 7.4); 150 mM NaCl). The β’–2PrA-tagged protein and copurifying proteins were eluted from the IgG Dynabeads with 0.5 M NH4OH and 0.5 mM EDTA. The eluted proteins were frozen in liquid nitrogen and evaporated to dryness in a SpeedVac (Thermo Savant). Dried protein samples were dissolved in SDS-PAGE loading buffer and heated at 95 °C. Samples were resolved by SDS-PAGE with 4–12% (w/v) Bis-Tris polyacrylamide gels (Invitrogen), and bands due to proteins were visualized by Coomassie blue staining. The entire gel lane was sliced into 1 mm slices, and the proteins in each gel slice were destained with a mixture of 50% (v/v) acetonitrile and 50 mM ammonium bicarbonate. After destaining, the proteins were reduced with 10 mM TCEP-HCl (Pierce) for 30 min and then alkylated with 50 mM iodoacetamide (Sigma-Aldrich) for 1 h. Finally, proteins in each gel slice were digested with Trypsin (Roche Diagnostics) at 37 °C for 4 h. The resulting peptides were extracted on reverse phase resin (Poros 20 R2, PerSeptive Biosystems), eluted with 50% (v/v) methanol, 20% (v/v) acetonitrile, and 0.1% (v/v) trifluoroacetic acid, and subjected to MALDI MS analysis. Proteins were identified by MALDI-QqTOF single-stage MS and MALDI-Ion-trap MS2 as previously described.10,11 Single-stage and multistage mass spectrometric data were used for protein identification with the programs ProFound12 and Sonar MS/MS,13 respectively.

**Cloning and Overexpression of Alc.** Due to the toxicity of T4 Alc to *Ec* cells, a T7 RNAP-directed Alc overexpression plasmid was constructed in several steps. First, using purified T4 genomic DNA as a template, the DNA encoding Alc was amplified by the polymerase chain reaction (PCR). The resultant PCR product was blunt-end cloned into pT7Blue (Novagen) in an antisense orientation relative to the T7 RNAP ϕ10 and Ec RNAP lacUV5 promoters to reduce basal expression of Alc, creating pT7-alcrev. Second, pT7-alcrev was cleaved with AfIII, treated with Klenow DNA polymerase to generate blunt ends, and digested with *BamHI* to obtain a fragment encoding Alc and the ϕ10 and lacUV5 promoters. The resultant fragment was cloned into pET28a (Novagen) cleaved with HindIII, treated with Klenow to generate blunt ends, and then cleaved with *BamHI* to create PET28-alc(T7-lacUV5). Finally, a DNA fragment encoding only the lacUV5 promoter was generated by digesting pUC19 with AfIII, followed by incubation with Klenow to generate blunt ends, and then cleaved with HindIII. The resultant lacUV5 fragment was cloned between the NoI site, also treated with Klenow, and the HindIII site of pET28-alc(T7-lacUV5), thus creating pET28-alc(lacUV5). Thus, Alc overexpression is under the control of the plasmid-encoded T7 RNAP ϕ10 promoter, while the Ec RNAP lacUV5 promoter drives transcription antisense to Alc reducing Alc toxicity.

The expression plasmid pET28-alc(lacUV5) was transformed into Ec cells harboring the paf *rpoB* allele3 (which partially nullifies the toxicity of Alc) and the λ (DE3) lysesogen encoding T7 RNAP, and transformants were selected in the presence of 50 µg/mL of kanamycin. Cultures were grown at 37 °C to an *A600 nm* ~ 0.6 and induced with 1 mM IPTG for 14 h at 37 °C. Cells containing overexpressed proteins were harvested by centrifugation and stored at −80 °C.

**Protein Purification.** Recombinant Alc formed inclusion bodies and was solubilized by adding urea to a final concentration of 8 M. A polyhistidine tag (derived from the vector) was appended to the amino terminus of Alc, thus Alc was purified by Ni2+-charged affinity chromatography (GE Healthcare) with 8 M urea present in all the column buffers. Purified (His)6-Alc was exchanged into renaturation buffer (40 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 0.5 mM EDTA, 2 mM β-mercaptoethanol, 5% (v/v) glycerol) by dialysis and concentrated using centrifugal concentration units (Pall Life Sciences). Finally, the protein was exchanged into storage buffer (20 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA, 0.5 mM DTT, 50% (v/v) glycerol) by dialysis and stored at −20 °C.

Wild-type Ec ϕ70 was purified as previously described.14 The Ec core enzyme and σ70-associated holoenzyme were reconstituted in vitro14 and fractionated by anion-exchange chromatography (Mono Q; GE Healthcare). Fractions containing core and σ70-associated enzymes were pooled separately and stored as described previously.15

**Analysis of Alc/RNAP Interactions.** Reactions (8 µL) containing 0.75 µg of Alc and either 0.125 µg of core RNAP or 0.07 µg of σ70-associated holoenzyme in transcription buffer (30 mM Tris-HCl (pH 7.9), 40 mM KCl, 10 mM MgCl2, 1 mM β-mercaptoethanol) were incubated at room temperature for 10 min.

Samples (4 µL of each reaction mixture) were resolved by nondenaturing PAGE on a 4–15% (w/v) gradient polyacrylamide gel, and the bands due to proteins were stained with Coomassie blue. To determine the composition of the bands due to proteins separated by gel electrophoresis under nondenaturing conditions, the bands were excised from the nondenaturing gel and equilibrated in SDS-PAGE loading buffer, and their composition was determined by electrophoresis on a 10% (w/v) SDS-PAGE denaturing gel.
Results and Discussion

Noncovalent Modifications of Ec RNAP: Affinity Isolation and Mass Spectrometric Identification of T4-Encoded Proteins that Coisolate with Ec RNAP. To identify T4-encoded Ec RNAP co-isolating proteins, we affinity isolated RNAP from T4-infected Ec cells (Figure 1, lane 2). Isolation was performed using the genomically 2PrA-tagged β’ subunit of RNAP. The conditions of affinity isolation were sufficiently gentle and rapid to maintain interactions with other cellular macromolecules. As a control, parallel isolation of proteins from T4-infected Ec wild-type cells infected with T4; lane 2, affinity isolated proteins from Ec rpoC::2PrA cells infected with T4. RNAP subunits in the 2PrA-mobility than the corresponding bands from the RNAP core proteins that are known to associate directly with RNAP: MotA, an activator of T4 middle promoters; gp55, a T4-encoded σ factor that controls the expression of T4 late genes; RpbA, a protein with unknown function; gp33, an activator of T4 late genes that coactivates T4 late genes with gp55; and AsiA, a potent inhibitor of host-dependent transcription, host RNAP appropriator, and coactivator of T4 middle genes.

We also identified the T4-encoded transcription termination factor Alc. Alc increases the pool of host RNAP available for transcribing T4 DNA by selectively terminating RNAP transcription on Ec DNA without affecting transcription of T4 DNA. On the basis of genetic studies, Alc is thought to bind directly to the β subunit dispensable region 1 (βDR1) of Ec RNAP, but no direct interaction between RNAP and Alc had been previously reported. Since the affinity isolation experiment indicated an Alc/RNAP interaction, we tested this result using nondenaturing PAGE (Figure 2A). Under our conditions, purified core RNAP migrated as a diffuse set of bands (Figure 2A, lane 1); the σ70-holoenzyme migrated as a single band (Figure 2A, lane 3); and purified Alc migrated with the dye front (Figure 2A, lane 5). From the gel, it is clear that the bands labeled c1 and c2 appear to have a slightly lower electrophoretic mobility than the corresponding bands from the RNAP core sample, suggesting an interaction between the core and Alc. With the mixture containing Alc and σ70-holoenzyme (Figure 2A, lane 4), two poorly resolved bands are observed: one (labeled h2) has the same mobility as the σ70-holoenzyme alone and the other (labeled h1) has a slightly decreased mobility compared to the σ70-holoenzyme alone.

The labeled bands were excised from the nondenaturing gel and their protein content established by denaturing SDS-PAGE (Figure 2B). It is apparent that the bands due to c1, c2, and h1 contained, in addition to the expected RNAP subunits, a band due to Alc (Figure 2B, lanes 4, 5, and 8, respectively), thus establishing that Alc binds both the core and σ70-holoenzyme. The material in bands c3, c4, and h2 contained little or no Alc (Figure 2B, lanes 6, 7, and 9, respectively). The h2 band contained the free σ70-holoenzyme; band c3 contained the RNAP assembly intermediate αβ′, and band c4 contained the free α subunit. Thus, it appears that Alc fails to bind to αβ′ despite the fact that the genetically identified determinants for Alc binding are located in the β subunit. This result suggests...
that Alc binding to RNAP may require determinants in both the β and β′ subunits.

**Covalent Modifications of Ec RNAP: T4-Dependent ADP-Ribosylation of the Ec RNAP σ Subunit.** ADP-ribosyltransferases catalyze the transfer of the ADP-ribosyl moiety from substrate ADP-ribosyl-nicotinamide onto residues of a target protein. T4 encodes two ADP-ribosyltransferases, Alt and ModA, that ADP-ribosylate Arg 265 of the RNAP σ subunit. Although ModA specifically modifies α-Arg 265 of both σ subunits, Alt modifies α-Arg 265 on only one of the two σ subunits, multiple sites within Ec RNAP, and other host proteins. The sites of Alt-dependent ADP-ribosylation within the β, β′, and σ70 subunits remain to be elucidated. Residue α-Arg 265 is involved in contacting both DNA and trans-acting factors, thus ADP-ribosylation of this key residue alters α/DNA and α/protein interactions necessary for transcription activation.

To determine the facility of mass spectrometric analysis for elucidating posttranslational modifications of RNAP, we sought to identify the ModA-dependent ADP-ribosylation of the Ec α subunit. To do this, tryptic peptides of the α subunit affinity isolated from both uninfected and T4-infected Ec cells were prepared and analyzed by MALDI MS. Using a subtractive approach, the resulting mass spectra were scanned for peptides present in the T4-infected sample but absent from the uninfected sample. A peak due to a singly charged species at m/z 3838.0 was observed in the T4-infected sample but not the uninfected sample (Figure 3A) and thus is likely due to T4-dependent modification of the α subunit. The theoretical m/z for a singly protonated ADP-ribosylated tryptic peptide containing α residues 244–271 is 3837.8. The match of both the theoretical and observed masses suggests that the species at m/z 3838.0 is due to α residues 244 to 271, encompassing an ADP-ribosyl group. This interpretation is further corroborated...
by the missed cleavage at α-Arg 265, suggesting that ADP-ribosylation of this Arg residue may block tryptic cleavage.

Interrogation of this peptide ion using MALDI-ion-trap MS2 mass spectrometry yielded a family of strong peaks corresponding to fragmentation of the ADP-ribosyl modification. Peaks consistent with the neutral losses of adenosine monophosphate, adenosine diphosphate, and ADP-ribose, combined with multiple water/ammonia losses were observed (Figure 3B). MS3 of a peptide ion at \( m/z \) 3838.0 (loss of ADP-ribose + water) yields a fragmentation pattern consistent with the interpretation that the original peptide ion at \( m/z \) 3838.0 is indeed an ADP-ribosylated species. (C) MS3 analysis of the ions in the MS2 spectrum with \( m/z \) 3237.0 (corresponding to the neutral loss of ADP-ribose + HCN2 + water/ammonia) yielded backbone fragmentation providing partial sequence of the peptide. The fragmentation pattern is consistent with the interpretation that the original peptide ion at \( m/z \) 3838.0 is indeed derived from α residues 244–271. Observed are fragments corresponding to the preferential cleavage of the singly-protonated peptide ion carboxy-terminal to acidic residues and amino-terminal to Pro residues. Together, the MS, MS2, and MS3 spectra in panels (A), (B), and (C) provide strong evidence that α is ADP-ribosylated on Arg 265 within the tryptic peptide encoding residues 244–271. b+ indicates the loss of ammonia from a b-ion, and y+ indicates the loss of ammonia from a y-ion. ADP-ribosylated α-Arg 265 is shown as R+.

**Conclusion**

We have affinity isolated Ec RNAP from T4-infected cells and analyzed the T4-encoded co-isolating proteins using MALDI MS. The T4 phage system has been studied extensively, and T4-encoded proteins that interact with and regulate the activity of host RNAP have been identified over the years by painstaking genetic and biochemical approaches. Our results show that optimized RNAP affinity isolations, coupled with mass spectrometric identification, allow rapid identification of many T4 proteins known to interact with Ec RNAP, as well as the novel identification of Alc as a binding partner, whose low affinity for RNAP prevented earlier identification. In addition, this method is also suitable for the identification of phage-dependent covalent modifications of the host transcription machinery, as demonstrated by our comprehensive mass spectrometric analysis.
analysis of an ADP-ribosylated peptide. The ADP-ribosylation of proteins is an important process in both prokaryotes and eukaryotes. Indeed, many bacterial exotoxins are ADP-ribosyltransferases and function to manipulate and override host cellular processes. In eukaryotes, ADP-ribosylation is involved in regulating, among other things, the activity of histones and membrane traffic. Elucidation of the MS2 and MS3 fragmentation characteristics of ADP-ribosylated peptides should facilitate the definition of control mechanisms and signaling pathways that are dependent upon ADP-ribosylation. Finally, we suggest that this methodology, coupled with phage genomic sequencing, will become an important tool for probing novel host/phage systems.

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