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Multisubunit RNA Polymerase Cleavage Factors Modulate the Kinetics and Energetics of Nucleotide Incorporation: An RNA Polymerase I Case Study

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Abstract

All cellular RNA polymerases are influenced by protein factors that stimulate RNA polymerase-catalyzed cleavage of the nascent RNA. Despite divergence in amino acid sequence, these so-called “cleavage factors” appear to share a common mechanism of action. Cleavage factors associate with the polymerase through a conserved structural element of the polymerase known as the secondary channel or pore. This mode of association enables the cleavage factor to reach through the ‘secondary channel into the polymerase active site to reorient the active site divalent metal ions. This reorientation converts the polymerase active site into a nuclease active site. Interestingly, eukaryotic RNA polymerases I and III (Pols I and III, respectively) have incorporated their cleavage factors as bona fide subunits known as A12.2 and C11, respectively. Although it is clear that A12.2 and C11 dramatically stimulate the polymerase’s cleavage activity, it is not known if or how these subunits affect the polymerization mechanism. In this work we have used transient-state kinetic techniques to characterize a Pol I isoform lacking A12.2. Our data clearly demonstrate that the A12.2 subunit profoundly affects the kinetics and energetics of the elementary steps of Pol I-catalyzed nucleotide incorporation. Given the high degree of conservation between polymerase—cleavage factor interactions, these data indicate that cleavage factor-modulated nucleotide incorporation mechanisms may be common to all cellular RNA polymerases.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acs.bio-chem.7b00370.

Plots corresponding to global fits of WT and A12 to eqs 7-9(PDF)

All known cellular multisubunit RNA polymerases possess cognate protein factors that stimulate RNA polymerase-catalyzed cleavage of the nascent RNA.^{1,2} These so-called “cleavage factors” serve several purposes in transcription, including maintaining transcriptional fidelity and rescuing backtracked elongation complexes (ECs)³. Although there is little sequence homology among cleavage factors for different RNA polymerases, it appears that each cleavage factor contains a module capable of entering the polymerase active site through the polymerase secondary channel (or pore).¹ Consistent with this common mode of cleavage factor—polymerase interaction, cleavage factors all appear to stimulate RNA cleavage by modulating the positioning of the divalent metal cations in the polymerase active site.^{4,5} This reorientation converts the polymerase active site into a nuclease active site.

Although numerous studies have addressed multisubunit RNA polymerase nucleotide incorporation mechanisms, the majority of studies have been conducted in the absence of cleavage factors.^{6–10} Additionally, the studies that have included cleavage factors have largely focused on how a given cleavage factor affects polymerase pausing.^{11–13} The contributions of cleavage factors to the nucleotide incorporation reaction have not been described in detail.

Interestingly, eukaryotic RNA polymerases I and III (Pols I and III, respectively) have incorporated their cognate cleavage factors as bona fide subunits.¹ These subunits are termed A12.2 and C11 for Pols I and III, respectively. As a result of the A12.2 and C11 subunits, Pols I and III display levels of “intrinsic” cleavage activity much higher than those of their prokaryotic and eukaryotic polymerase counterparts. Although the genes that encode A12.2 and C11 in *Saccharomyces cerevisiae* (yeast) are not essential for cell viability, these subunits are critical for Pol I and Pol III nuclease activity.^{1,14}

The A12.2 and C11 subunits of Pols I and III copurify with the polymerases and are likely present at stoichiometric levels in all transcription elongation complexes. However, the extent of *in vivo* association between other polymerases and their cognate cleavage factors is unclear. Previous studies have shown that TFIIS occupies Pol II-transcribed loci, but the stoichiometry of this association is not known.¹⁵ Because other trans-acting factors occupy the same site on Pol II,¹⁶ it is unlikely that TFIIS is present on all Pol II transcription elongation complexes. However, genetic data from the Svejstrnp group demonstrated that a TFIIS point mutant that results in impaired cleavage activity has dominant negative effects on cell viability.¹⁷ These data indicate that *in vivo*, cleavage factors and their cognate

polymerase may be tightly associated. Thus, there is a need to carefully define the effects of cleavage factors on the nucleotide incorporation properties of their cognate polymerase.

We have recently reported an assay that simultaneously monitors Pol I nucleotide incorporation and nuclease activities.¹⁸ In that work, we proposed a simple model that describes yeast Pol I-catalyzed nucleotide incorporation and nucleolytic cleavage kinetics. In this study, we applied transient-state kinetic techniques to characterize a Pol I isoform lacking the A12.2 subunit (A12) and compared the results to those of wild-type (WT) Pol I. Our data clearly demonstrate that the A12.2 subunit profoundly affects Pol I nucleotide incorporation kinetics. Furthermore, by profiling nucleotide incorporation activation energy as a function of substrate adenosine triphosphate (ATP) concentration, we demonstrate that A12.2 specifically modulates the energetics of one or more elementary steps in the nucleotide incorporation pathway. Given the degree of conservation between multisubunit RNA polymerase mechanisms, these data call for similar characterizations of other RNA polymerase—cleavage factor systems as the effects we observe may be common to all multisubunit RNA polymerases.

MATERIALS AND METHODS

Buffers.

With the exception of electrophoresis components, all buffers were filtered through 0.22 μm Millipore express plus vacuum-driven filters unless specified otherwise.

Nucleotide incorporation measurements were performed in reaction buffer [40 mM KCl, 20 mM Tris-acetate (OAc) (pH 7.9) at 25 °C, 2 mM dithiothreitol, and 0.2 mg mL⁻¹ bovine serum albumin (BSA)]. This buffer was prepared fresh from concentrated stocks prior to each experiment. The pH values of Tris-OAc stocks were adjusted at the temperature corresponding to the nucleotide incorporation measurement. Note that nucleotides, nucleic acids, heparin, and BSA were prepared in reaction buffer that was pH-adjusted at 25 °C. Each of these reagents experienced a large dilution during assembly of reaction buffer.

Proteins.

Pol I was purified as described previously.¹⁹ Pol I is stored in 0.55 M K-OAc, 10 mM K-HEPES, 0.5 mM MgCl₂, and 45% (v/v) glycerol (pH 7.8) at -20 °C.

Nucleotides, Nucleic Acids, Heparin, and BSA.—Nucleotides, nucleic acids, heparin, and BSA were prepared as described previously.¹⁸ Briefly, nucleic acids were purchased from IDT (Coralville, IA), purified in house via polyacrylamide gel electrophoresis (PAGE), and dialyzed into dialysis buffer [40 mM KCl and 20 mM Tris-acetate (OAc) (pH 7.9 adjusted at 25 °C)]. Adenosine triphosphate (ATP) was purchased from Sigma-Aldrich as a sodium salt, dissolved with dialysis buffer, filtered through a 0.2 μm nylon filter (Fisher Scientific), and dialyzed into dialysis buffer. Lyophilized BSA was purchased from Fisher Scientific, dissolved with dialysis buffer, filtered through a 0.2 μm nylon filter (Fisher Scientific), and dialyzed into dialysis buffer. Heparin was purchased from Acros Organics as a sodium salt, dissolved with dialysis buffer, filtered through a 0.2 μm nylon filter (Fisher Scientific), and dialyzed into dialysis buffer.

Quenched-Flow Time Courses.—Quenched-flow time courses of single-nucleotide incorporation reactions were performed exactly as described previously.¹⁸ Electrophoresis and quantification of gels were performed as described previously.¹⁸

All AMP incorporation time courses were collected at 25 °C in triplicate. Each point in the plots depicted in Figure 2 represents the average of these measurements, and the error bars represent the standard deviation.

General Curve Fitting.—All data analyses were performed using custom-written scripts in Matlab. All fits were performed by weighting the residuals by the standard deviation obtained from replicate measurements. Optimizations were performed using the Matlab function *Lsqnonlin*. The error on each parameter value was calculated using a grid searching strategy as described previously.¹⁸ The reported error corresponds to a 68% confidence interval.

Fitting by Numerical Integration.—To globally fit the set of time courses shown in Figure 4, the system of coupled differential equations given by eq 1, defining Scheme 1, was numerically integrated using the Matlab program *Ode23s*.²⁰

$$\frac{d[EC_{10}]}{dt} = -[EC_{10}][ATP]k_1 + [EC_{10} \cdot ATP]k_2 + [EC_{10}^*]k_7 \quad (1A)$$

$$\frac{d[ATP]}{dt} = -[EC_{10}][ATP]k_1 + [EC_{10}ATP]k_2 \quad (1B)$$

$$\frac{d[EC_{10} \cdot ATP]}{dt} = [EC_{10}][ATP]k_1 - [EC_{10} \cdot ATP](k_2 + k_3) + [(EC_{11})_1]k_4 \quad (1C)$$

$$\frac{d[(EC_{11})_1]}{dt} = [EC_{10} \cdot ATP]k_3 - [(EC_{11})_1](k_4 + k_5) \quad (1D)$$

$$\frac{d[(EC_{11})_2]}{dt} = [(EC_{11})_1]k_5 \quad (1E)$$

$$\frac{d[EC_{10}^*]}{dt} = -[EC_{10}^*]k_7 \quad (1F)$$

The analytical jacobian, defined in eq 2, derived from eqs 1A—F was passed to Ode23s.

$$\frac{\partial \left\{ \frac{ds_i}{dt} \dots \frac{ds_n}{dt} \right\}}{\partial \{s_i \dots s_n\}} = \begin{bmatrix} -[ATP]k_1 - [EC_{10}]k_1 & k_2 & 0 & 0 & k_7 \\ -[ATP]k_1 - [EC_{10}]k_1 & k_2 & 0 & 0 & 0 \\ [ATP]k_1 & [EC_{10}]k_1 & -(k_2 + k_3) & k_4 & 0 & 0 \\ 0 & 0 & k_3 & -(k_4 + k_5) & 0 & 0 \\ 0 & 0 & 0 & k_5 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & -k_7 \end{bmatrix} \quad (2)$$

where s_i denotes the concentration of species i . All concentration terms in eqs 1A-F and 2 are time-dependent. Parameter optimization was accomplished in Matlab using a custom-built genetic algorithm described previously by us.¹⁸ As with our previous analysis of WT-catalyzed single-nucleotide addition, the data require the presence of two starting populations, EC^*_{10} and EC_{10} (see Scheme 1). Thus, the boundary conditions for solving eq 1 are such that at time zero there are populations of EC^*_{10} and EC_{10} denoted as $[EC^*_{10}]_0$ and $[EC_{10}]_0$, respectively. However, EC_{10} -ATP, $(EC_{11})_1$, and $(EC_{11})_2$ are considered to be zero at time zero because neither binding of ATP nor extension can occur before rapid mixing with ATP.

In our experimental design, we use ~10 nM Pol I. As previously described, Pol I is used to radiolabel the 3' end of the RNA. Thus, the observed extension product is determined as fraction extended relative to the total labeled RNA given by eq 3.

$$\text{frac}_i(t) = \frac{\frac{[RNA_i](t)}{\sum_i [RNA_i](t)} - \frac{[RNA_i](0)}{\sum_i [RNA_i](0)}}{1 - \frac{[RNA_i](0)}{\sum_i [RNA_i](0)}} \quad (3)$$

where $\text{frac}_i(t)$ is the fraction of RNA in state i at time t and $[RNA_i](t)$ refers to the phosphor counts obtained from phosphorimaged gels at time t . The concentrations of EC^*_{10} and EC_{10} at time zero are calculated according to fractions of the total EC concentration. Thus, the concentration of $[EC_{10}]_0$ is replaced with $x[\text{Pol I}]_{\text{total}}$, and the concentration of $[EC^*_{10}]_0$ is replaced by $(1 - x)[\text{Pol I}]_{\text{total}}$, where x is given by eq 4.

$$x = \frac{[EC_{10}]_0}{[EC^*_{10}]_0 + [EC_{10}]_0} \quad (4)$$

In fitting the data to eq 1A—F, x is a global floating parameter. In the quench-flow experiment, each reaction is arrested when the solution is rapidly mixed with 1 M HCl to stop the reaction. Upon separation of products and reactants on denaturing PAGE gels, we detect all 11-mer formed from a radiolabeled 10-mer. Thus, we detect 11-mer whether it has

been released or remains bound to the enzyme. Therefore, the data were subjected to analysis using the summation of the time- dependent functions for $(EC_{11})_1$ and $(EC_{11})_2$ obtained from the numerical solutions of eq 1A—F. In addition, it is observed that for A12 single-nucleotide incorporation time courses, eq 3 does not reach a value of one. Therefore, in fitting, the numerical solutions of eq 1 are multiplied by a global scaling factor. Given these considerations, the time course data in Figure 4 are fit to eq 5.

$$\text{frac}_{11}(t)_{[ATP]_j} = \alpha \left\{ \frac{[(EC_{11})_1](t)_{[ATP]_j} + [(EC_{11})_2](t)_{[ATP]_j}}{\sum s_i(t)_{[ATP]_j}} \right\} \quad (5)$$

where $\text{frac}_{11}(t)_{[ATP]_j}$ gives the fraction of labeled RNA in the 11- mer state at time t measured at the j th [ATP]. The sum in the denominator of eq 5 is taken over the time- dependent solutions of eq 1, with the exception of the solution of eq 1B, at time t and the j th [ATP] and therefore represents the total EC concentration. α is a global scaling factor that, as noted in eq 5, carries no [ATP] or time dependence.

Graphical Confidence Intervals.—The confidence intervals displayed graphically in Figure 3A—C were obtained in the following manner. Time course data were fit globally by eqs 7, 8, and 9. These fits resulted in four global parameters and 16 local parameters for the A12 data set and three global parameters and seven local parameters for the WT data set. The 68% confidence interval on each of these fitted parameter values was calculated by grid searching. Each data set was fit again by constraining a single parameter value to its upper or lower bound and allowing all other parameter values to float. This procedure resulted in $2N$ parameter sets, with N being the total number of parameters optimized in the original global fit. The $(2N + 2)$ $k_{\max}$ and $K_{1/2}$ (there are two $k_{\max}$ and two $K_{1/2}$ values governing the A12 data; these correspond to the two exponential phases of each time course) values were substituted into eq 9 to produce a $2N + 2$ simulated k_{obs} versus [ATP] data set. The k_{obs} versus [ATP] data sets corresponding to a given $k_{\max}$ and $K_{1/2}$ pair are combined into a set that includes a k_{obs} versus [ATP] data set obtained by substituting the best fit $k_{\max}$ and $K_{1/2}$ value obtained in the original global fit into eq 9. At each [ATP] in the sets, the maximum and minimum k_{obs} values are recorded and used as the upper and lower bounds, respectively, in Figure 3A—C.

Arrhenius Analysis. A12 AMP incorporation time courses collected at 1 mM ATP at 20 °C were collected in duplicate. A12 AMP incorporation time courses collected at 1 mM ATP at 15 °C were collected in triplicate. A12 AMP incorporation time courses collected at 50 μ M ATP at 20 and 15 °C were collected in duplicate. WT AMP incorporation time courses collected at 1 mM and 50 μ M ATP at 20 and 15 °C were collected in duplicate. k_{obs} values were obtained by fitting each time course to eq 7 or 8. The natural log values of the k_{obs} values from each replicate measurement were averaged, and their standard deviation was calculated. The average log values were fit to eq 6

$$\ln(k_{\text{obs}}) = \ln(A) - \frac{E_a}{RT} \quad (6)$$

where E_a is the activation energy, A is the Arrhenius prefactor, R is the gas constant, and T is the absolute temperature. Fits to eq 6 were performed by weighting the residuals to the standard deviations of the $\log k_{\text{obs}}$ values. The 68% confidence interval on E_a values was calculated by grid searching.

RESULTS

To characterize the influence of A12.2 on Pol I activity, we purified yeast Pol I from cells carrying a deletion of the *RPA12* gene. This polymerase was then assembled into synthetic transcription elongation complexes, and nucleotide addition and cleavage activities were measured as described previously for the WT enzyme¹⁸.

Figure 1A displays an example high-resolution denaturing PAGE separation of a A12-catalyzed single-nucleotide incorporation reaction performed at 50 μM ATP using the chemical quenched-flow approach.¹⁸ Figure 1B displays the equivalent measurement collected with WT Pol I. Consistent with previously published observations, in a comparison of the A12 and WT gels, it is readily apparent that A12 lacks detectable nuclease activity.¹⁴ Specifically, throughout the course of a single AMP incorporation reaction, WT Pol I removes a CA dinucleotide fragment from the 3' end of the RNA.¹⁸ As expected, this nucleolytic fragment is completely absent in the A12 gel. Furthermore, the GC dinucleotide that is generated during the labeling reaction is also absent in reactions with A12. These observations clearly demonstrate the essential role for A12.2 in Pol I's nuclease activity.

The time dependence of accumulation of the single-nucleotide extension product is also clearly influenced by the A12.2 subunit. Panels C and D of Figure 1 display quantifications of the single-nucleotide extension product abundance from triplicate measurements of A12 and WT nucleotide incorporation time courses, respectively, performed at 50 μM ATP. The time courses in panels C and D of Figure 1 bear little resemblance to one another. Although each time course is clearly biphasic, the phases of the WT time course are governed by amplitudes of opposite sign, whereas the A12 time course is governed by two rising phases. We have recently assigned the decay phase of the WT time course to WT's nuclease activity.¹⁸ Thus, it is not surprising that the A12 extension product does not exhibit a decay phase. Unexpected, however, is the fact that the two time courses occur on different time scales. Specifically, the rising phase of the WT time course is virtually complete within the first 0.1 s of the reaction, whereas the fraction of RNA in the extension product in the A12 time course continues to increase for >1 s. These data indicate that the A12.2 subunit has substantial effects on Pol I-catalyzed nucleotide incorporation.

To characterize the single-nucleotide incorporation reaction catalyzed by A12 and compare it to that of the WT enzyme, we collected time courses at multiple ATP concentrations. Panels A and B of Figure 2 display extension product time courses collected across a range

of ATP concentrations (circles) with AA12 and WT, respectively. It is clear that at all ATP concentrations each polymerase isoform gives rise to very different nucleotide incorporation time courses. To begin characterizing and comparing the [ATP] dependence of A12- and WT-catalyzed nucleotide incorporation kinetics, we fit each time course by weighted nonlinear least-squares (WNLLS) analysis to a sum of two exponentials (eq 7 for A12 time courses and eq 8 for WT time courses) (solid lines in panels A and B of Figure 2).

$$\text{frac}_{11, \Delta A12}(t) = \alpha_{\text{fast}} \left(1 - e^{-tk_{\text{fast}}} \right) + \alpha_{\text{slow}} \left(1 - e^{-tk_{\text{slow}}} \right) \quad (7)$$

$$\text{frac}_{11, \text{WT}}(t) = \alpha_1 \left(1 - e^{-tk_{\text{rise}}} \right) - \alpha_1 \left(1 - e^{-tk_{\text{decay}}} \right) \quad (8)$$

Equation 7 defines four parameters for each A12 time course: $\alpha_{\text{fast}}(k_{\text{fast}})$ and $\alpha_{\text{slow}}(k_{\text{slow}})$ are amplitude(rate constant) pairs governing the fast and slow phases of the time courses, respectively. For the WT time courses, it was found that the two amplitude terms were within error and equal in magnitude but of opposite sign (fits not shown). Thus, the WT data were fit by constraining the amplitudes to be the same (α_1 in eq 8) but of opposite sign for each [ATP]. Consequently, there are three parameters determined from each time course from the WT data set: k_{rise} and k_{decay} defining the observed rate constants of the rising and decay phases of the time course, respectively, and α_1 . It is important to note that constraint was lost on the value of the faster of the two observed rate constants when fitting the time course collected with A12 at 10 μM ATP (see the green points in Figure 2A). Thus, that time course was fit to a single exponential to provide a single observed rate constant and amplitude parameter.

Panels A and B of Figure 3 display the fast and slow observed rate constants (k_{fast} and k_{slow} , respectively) obtained from the A12 data set (Figure 2A) plotted as a function of [ATP] (circles). For comparison, Figure 3C displays the observed rate constant governing the rising phase (k_{rise}) of the WT 11-mer time courses plotted as a function of [ATP] (circles). It is clear that neither k_{fast} nor k_{slow} (describing A12) corresponds to the values of k_{rise} (describing WT) (compare panels A and B of Figure 3 to panel C). The differences between the WT and A12 data sets presented in Figure 3 provide strong evidence of a role for A12.2 in nucleotide addition, beyond its role in nucleolytic activity.

Figure 3D displays the A12 and WT amplitude parameters obtained from the WNLLS analyses plotted as a function of [ATP]. It is clear from Figure 3D that the amplitudes of each phase (α_{fast} and α_{slow}) of the A12 time courses display a dependence on [ATP]. This is in sharp contrast to the WT time courses that are governed by an amplitude parameter with no [ATP] dependence. The results from Figure 3D provide further evidence that A12 differs from WT in its nucleotide incorporation properties.

Despite differing at each [ATP], both A12 (k_{fast} and k_{slow}) and WT (k_{rise}) observed rate constant values appear to be roughly hyperbolic when plotted against [ATP].¹⁸ We subjected the A12 and WT 11-mer time courses to global WNLLS to test for the hyperbolic dependence of each observed rate constant on [ATP] and obtain k_{max} and $K_{1/2}$ values defining each observed rate constant. The time courses collected with A12 were subjected to global WNLLS analysis using eqs 7 and 9 (Figure S1).

$$k_x([\text{ATP}]) = \frac{k_{\text{max},x}[\text{ATP}]}{K_{1/2,x} + [\text{ATP}]} \quad (9)$$

where $k_{\text{max},x}$ represents the maximum observed rate constant at saturating [ATP] and $K_{1/2,x}$ represents the midpoint of the hyperbolic [ATP] dependence for the fast or slow phase denoted by x . In this global fitting strategy, $k_{\text{max},x}$ and $K_{1/2,x}$ are global parameters whereas α_{fast} , α_{slow} , and α_1 are local. In contrast to individually fitting each time course to eq 7 to obtain observed rate constant values, as described above, this global fitting strategy enables the [ATP] dependence of the observed rate constants to be extracted from the entire set of time courses in the form of two parameters: $k_{\text{max},x}$ and $K_{1/2,x}$.

The solid lines in Figure 3A-C represent simulations of eq 9 using the values of $k_{\text{max},x}$ and $K_{1/2,x}$ (Table 1) determined from the global WNLLS analysis of the time courses (Figure S1). The broken gray traces in Figure 3A-C represent the 68% confidence intervals about the solid line (see Materials and Methods).

Global fitting of AMP incorporation time courses collected with A12 to eqs 7 and 9 provides estimates of four global parameters describing the A12 single-nucleotide incorporation reaction: $k_{\text{max,fast}}$, $K_{1/2,\text{fast}}$, $k_{\text{max,slow}}$, and $K_{1/2,\text{slow}}$ (see Table 1). Similarly, fitting the AMP incorporation time courses from experiments with WT to eqs 8 and 9 yields three global parameters describing the WT single-nucleotide incorporation reaction: $k_{\text{max,rise}}$, $K_{1/2,\text{rise}}$, and k_{decay} (see Table 1). As shown in Figure S1, the time courses are described well by application of this fitting strategy.

It is clear that the parameters obtained in the analyses described above differ for each polymerase isoform. Of specific interest are the k_{max} values corresponding to the faster of the two observed rate constants governing each (WT- and AA12- catalyzed) nucleotide incorporation reaction. We recently reported that $k_{\text{max,rise}}$ reports on a step that is kinetically coupled to ATP binding. This step is either phosphodiester bond formation or a conformational change.¹⁸ It is noteworthy that $k_{\text{max,fast}} \approx 50 \text{ s}^{-1}$ for A12 and $k_{\text{max,rise}} \approx 200 \text{ s}^{-1}$ for WT are different by a factor of approximately 4 (Table 1). Taken together, the data presented in Figures 1–3 and the values in Table 1 demonstrate that the A12.2 subunit directly influences the polymerization mechanism. This observation raises the question of whether WT- and A12-catalyzed nucleotide incorporation reactions are governed by the same mechanism with different values of the elementary rate constants or whether the two reactions are governed by different mechanisms.

To begin to address this question, we subjected the time courses collected with A12 as a function of [ATP] to global WNLLS analysis using the model given by Scheme 1 and eq 1A-F. Scheme 1, with the exception of removing the step describing cleavage, is identical to the model used to globally fit the WT time courses previously published by us.¹⁸ First, the model assumes that there are two starting populations present before rapid mixing with ATP. These two states are denoted as EC_{10}^* and EC_{10} . Second, the time courses are described by the time-dependent formation of both $(EC11)_1$ and $(EC11)_2$ because the quench-flow experiments are sensitive to both states (see Discussion and Materials and Methods for further description).

Consistent with the analysis described above, fitting the data to Scheme 1 indicates the step that is kinetically coupled to ATP binding for A12 ($k_3 \approx 55 \text{ s}^{-1}$) is roughly 3-fold slower than the equivalent step for WT (i.e., WT $k_3 \approx 180 \text{ s}^{-1}$). Moreover, the step defined by k_3 and k_4 for WT was found to be irreversible, whereas here we find that the A12 data cannot be described well if we assume that $k_4 = 0$. Rather, the data required $k_3 \approx 9 \text{ s}^{-1}$ leading to an internal equilibrium constant for that step of ≈ 6 .

Scheme 1 represents the simplest model that can simultaneously describe all the time courses shown in Figure 4. Simpler versions of Scheme 1 were applied to the data, but all iterations yielded a worse root-mean-square deviation. Although the identities of the intermediates depicted in Scheme 1 are currently not known, comparing the parameter values obtained by fitting the WT and A12 data sets to Scheme 1 provides strong evidence that the A12.2 subunit alters one or more elementary steps of the nucleotide incorporation mechanism. Specifically, the WT nucleotide incorporation reaction is best described by nucleotide binding being immediately followed by a step with a very large equilibrium constant, rendering this step virtually irreversible. This is in sharp contrast to the A12 reaction, which requires that nucleotide binding be directly followed by a reversible step.

To further interrogate the differences between the mechanisms of WT- and A12-catalyzed nucleotide incorporation, we measured the [ATP] dependence of the apparent activation energies governing each polymerase-catalyzed reaction. Panels A and B of Figure 5 display Arrhenius plots of k_{rise} and k_{fast} , i.e., the fast phase rate constants for WT and A12, respectively. Each plot displays the natural log of the observed rate constant measured at 1 mM and 50 μM ATP as a function of the inverse temperature (circles). The solid lines represent weighted fits to the linearized Arrhenius equation (eq 6). Figure 5C and Table 1 display the apparent activation energies (E_a) obtained from the fits in panels A and B of Figure 5.

The data in Figure 5 and Table 1 indicate that at saturating ATP (1 mM), WT- and A12-catalyzed nucleotide incorporation are governed by different energetics. Specifically, the presence of the A12.2 subunit confers a roughly 60% increase in barrier height. In addition, with the change from 1 mM to 50 μM ATP, the WT-catalyzed reaction experiences a significant drop in activation energy as compared to that of the A12-catalyzed reaction in which there is no observed drop. These data clearly reveal that A12.2 fundamentally alters the energetics of Pol I-catalyzed nucleotide incorporation. Together, all of these data

demonstrate the central role of A12.2 in Pol I nucleotide incorporation activity and suggest critical evaluation of analogous factors in other polymerase systems.

DISCUSSION

Here we have used transient-state kinetic techniques to characterize the nucleotide incorporation properties of a Pol I isoform lacking the A12.2 subunit. Consistent with previous studies, we found that A12 lacks nucleolytic activity but retains robust polymerization activity.^{14,21} However, the details of nucleotide incorporation differ dramatically between WT and A12.

From Figure 1, it is apparent that A12 and WT Pol I give rise to very different nucleotide incorporation time courses. Qualitatively, the difference in A12 and WT nucleotide incorporation time courses is most apparent in A12's lack of nucleolytic activity. This nucleolytic activity is responsible for the dramatic rise and fall of the extension product observed in WT Pol I-catalyzed nucleotide incorporation time courses.¹⁸ More surprisingly, we observed substantial differences in nucleotide incorporation kinetics between the A12 variant and WT polymerase.

WT and A12 AMP Incorporation Mechanisms.

We recently reported a characterization of WT Pol I nucleotide incorporation and nuclease kinetics.¹⁸ In that study, we described nucleotide incorporation and nucleolytic activity with a single simple model and, in doing so, provided estimates of elementary rate constant values governing polymerization and nucleolytic cleavage. Here we have found that time courses collected with A12 can be described well by the same model with the step describing nascent RNA cleavage removed. However, the parameter values obtained for the mutant enzyme differ substantially from those of the WT enzyme. From these observations, it is tempting to conclude that the two isoforms are following the same elementary kinetic mechanism and the magnitudes of the rate constants for the same elementary steps are altered by the A12.2 subunit. However, it is possible that the same model fortuitously describes single-nucleotide incorporation time courses from each polymerase isoform, and the observed steps for A12 are different from those observed for WT. Experiments are currently underway to determine if phosphodiester bond formation, for example, is kinetically coupled to ATP binding and if pyrophosphate release represents one of the rate-limiting steps.

In using Scheme 1 to fit WT single-nucleotide incorporation time courses, we assigned the final two 11-mer states [denoted in this work as $(EC_{11})_1$ and $(EC_{11})_2$] as a postincorporation EC in the pyrophosphate-bound and pyrophosphate-free form, respectively. We assigned a lower bound on the rate constant governing the release of pyrophosphate from WT Pol under the assumption that pyrophosphate must be released from the active site prior to dinucleotide production. In the case of A12-catalyzed single-nucleotide incorporation, our assay is not sensitive to any steps that follow pyrophosphate release, and therefore, we are unable to place bounds on the rate constant governing pyrophosphate release. While a slow step connecting $(EC_{11})_1$ and $(EC_{11})_2$ in Scheme 1 is required to adequately describe the A12 time courses, it is not likely that this step is rate-limiting in processive elongation. This is

because A12 cells are viable and the cells would not survive if transcription were limited to approximately two to three nucleotides per second.

Multiple explanations could account for the slow step. For example, in the case of the WT enzyme, if the next nucleotide encoded in the template is not present then the enzyme would back up and cleave off two nucleotides. The A12.2 subunit is required for this cleavage activity. However, in the absence of A12.2 and the next correct nucleotide, the $\sim 3 \text{ s}^{-1}$ observed rate constant may represent backing up and waiting for cleavage that will not occur because of the absence of A12.2. Thus, the enzyme slowly isomerizes into a “dead-end” complex that is not likely to occur if the next nucleotide were present to be incorporated. This so-called “dead-end” complex likely does not exist for WT but does exist for the A12 variant. Single-turnover multinucleotide addition experiments are underway to test this possibility. Heterogeneity in the enzyme preparation is an alternative possibility that is difficult to rule out with these ensemble approaches. Thus, single-molecule techniques will be imperative for testing this possibility.

Overall, it is clear that the A12.2 subunit fundamentally influences the elementary kinetic mechanism. Whether this is through altering elementary rate constant(s) for the same step(s) or making a different step rate-limiting remains to be determined.

Nucleotide Incorporation Activation Energies.

As we and others have discussed, observed rate constants are functions of the underlying elementary rate constants that govern a given reaction.^{18,22,23} The form of an observed rate constant function is dictated by the reaction mechanism. With this in mind, it is clear that activation energies obtained from the temperature dependence of observed rate constants represent a convolution of the energetics of the underlying elementary steps and should be viewed as apparent activation energies.²⁴ Here we have compared the apparent activation energies of the fast phases (k_{fast} or k_{rise}) of the A12 and WT nucleotide incorporation time courses at two different ATP substrate concentrations. As we have determined previously, the fast phase of the nucleotide incorporation reaction is reporting on a step that is kinetically coupled to ATP binding. This step could be either phosphodiester bond formation or a conformational change that immediately follows ATP binding.¹⁸

Upon measuring the apparent activation energies of the fast phase of the A12 and WT time courses at saturating (1 mM) and subsaturating (50 μM) ATP substrate concentrations, we found that the apparent activation energies of nucleotide incorporation differ between the two polymerase isoforms over the temperature range examined. The data in Figure 5 reveal that at saturating ATP, WT-catalyzed nucleotide incorporation is governed by an energetic barrier roughly 60% higher than that of the A12-catalyzed reaction. Interestingly, as [ATP] is decreased, the apparent activation energy of WT-catalyzed nucleotide incorporation decreases.

It is not clear whether A12 and WT Pol I follow the same nucleotide incorporation mechanism with different barrier heights governing the elementary steps of the pathway or whether the two polymerase isoforms have fundamentally different nucleotide incorporation

mechanisms. What is clear is that the A12.2 subunit is an integral component of Pol I-catalyzed nucleotide incorporation.

CONCLUSIONS

All cellular multisubunit RNA polymerases are known to possess cognate cleavage factors.^{1,2} In addition, it appears that these factors act via a conserved mechanism involving binding the RNA polymerase secondary channel. Regulation of RNA polymerase activity through the secondary channel has emerged as a common theme.^{25–28} Multiple factors that bind the secondary channel to regulate transcription initiation, pausing, and termination have been identified.²⁶ Our data demonstrate that the assignment of a secondary channel binding factor into a given category, i.e., “cleavage factor”, must be more nuanced as all of these factors are likely to exert different effects on the nucleotide incorporation properties of the polymerase.

Given the degree of conservation between cellular multi-subunit RNA polymerases, we hypothesize that TFIIS and C11 exert similar effects on the Pol II- and Pol III-catalyzed nucleotide incorporation reaction as we observe here for the interaction between A12.2 and Pol I. Structural studies demonstrated a large amount of reorganization of Pol II upon TFIIS association,²⁹ consistent with potential dramatic effects on polymerization activity. Indeed, previous kinetic studies by the Burton lab suggest that TFIIS can impact human Pol II elongation efficiency, particularly in the presence of another transcription factor, TFIIF. However, that study suggested that TFIIS alone does not increase the rate of nucleotide addition.¹¹ Rather, Burton and colleagues found the dominant effect of TFIIS to be on transcription efficiency or pausing. Further investigation of the kinetic effects of TFIIS on Pol II nucleotide addition kinetics seems warranted. The degree to which these “cleavage factors” stably associate with their cognate enzymes and influence RNA synthesis remains an open and interesting area of investigation.

Given these considerations, it seems likely that many secondary channel binders are capable of directly modulating steps of the nucleotide incorporation cycle. The potential impact of this idea is substantial. Many bacteria express multiple factors that occupy the secondary channel of the RNA polymerase. If each of these factors has a different effect on the nucleotide incorporation parameters of the enzyme, then there will be cohorts of different RNA polymerases with elongation properties potentially tailored to their respective gene targets or nutritional conditions. Such elongation-targeted “regulons” would represent a novel control mechanism in bacteria. Similar effects could also be possible in eukaryotes, because TFIIS has been identified in association with RNA polymerase III-transcribed genes.¹⁵ Together, all of these data and hypothetical considerations indicate that cleavage factors, and possibly all secondary channel binding factors, must be characterized individually with regard to their effects on the nucleotide incorporation cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of single-AMP incorporation reactions catalyzed by WT and $\Delta A12$. (A) Denaturing PAGE separation of product and reactant RNA species from a $\Delta A12$ -catalyzed single-AMP incorporation reaction performed at $50 \mu\text{M}$ substrate ATP using the quenched-flow method. (B) Same as panel A except for WT polymerase. (C) Quantification of the extension product from triplicate measurements of $\Delta A12$ -catalyzed single-AMP incorporation reactions performed at $50 \mu\text{M}$ substrate ATP. Circles represent the average of three independent measurements, and error bars represent one standard deviation about the average. (D) Same as panels C except for WT polymerase.

Figure 2.

ATP substrate titration of WT- and $\Delta A12$ -catalyzed single-AMP incorporation reactions. (A) Time courses of the extension product from $\Delta A12$ -catalyzed single-AMP incorporation reactions performed at 10 μM , 20 μM , 50 μM , 100 μM , 300 μM , 500 μM , 1 mM, and 2 mM (green, dark blue, red, gray, orange, black, light blue, and brown, respectively). Circles represent the average of three independent measurements, and error bars represent one standard deviation about the average. Lines represent WNLLS fits of the data to eq 7. (B) Same as panel A except for WT polymerase and lines represent WNLLS fits of the data to eq 8.

Figure 3.

Observed rate constants and amplitudes from WT- and A12-catalyzed single-AMP incorporation reactions. (A) k_{fast} , obtained from the fits displayed in Figure 2A (circles) plotted as a function of [ATP]. Error bars represent the 68% confidence interval on each fitted value. The solid line represents an evaluation of eq 9 using the parameter values obtained from the global WNLLS fit displayed in Figure S1. The broken lines represent the 68% confidence interval of the global fit displayed in Figure S1. (B) k_{slow} , obtained from the fits displayed in Figure 2A (circles) plotted as a function of [ATP]. The error bars and lines were calculated as described for panel A. (C) k_{rise} , obtained from the fits displayed in Figure 2B (circles) plotted as a function of [ATP]. The error bars and lines were calculated as described for panel A. (D) Amplitude parameters obtained from the fits displayed in Figure 2 plotted as a function of [ATP]. Green circles, blue circles, and red squares represent the fitted amplitude values corresponding to A12 k_{fast} , A12 k_{slow} , and WT k_{rise} , respectively. The error bars represent the 68% confidence interval on the fitted parameter value.

Figure 4.

Global WNLLS analysis of data in Figure 2A using Scheme 1. In Scheme 1, EC_{10} denotes the elongation complex bound to the radiolabeled 10-nucleotide RNA in two isoforms, one poised for immediate elongation, EC_{10} , upon nucleotide binding and one that must proceed through a slow conformational change, EC^*_{10} . EC_{10} and EC^*_{10} are in equilibrium before rapid mixing with ATP. The fraction of total EC population in the EC^*_{10} state is given by global parameter x . $EC_{10}\cdot ATP$ represents the elongation complex bound by ATP, and $(EC_{11})_1$ and $(EC_{11})_2$ represent the elongation complex with the RNA extended by one nucleotide. The time courses were described by the summation of the time-dependent functions describing the formation of $(EC_{11})_1$ and $(EC_{11})_2$ scaled by a single amplitude term, a . Solid lines represent the global best fit based on the values of the rate constants given in Scheme 1, where $x = 0.486 \pm [0.08, 0.1]$ and $a = 0.86 \pm 0.03$.

Figure 5.

Temperature dependence of WT- and A12-catalyzed single-AMP incorporation kinetics. (A) Arrhenius plot of k_{fast} values obtained from fitting A12-catalyzed single-AMP incorporation time courses collected at 1 mM and 50 μ M ATP to eq 7 (black and gray circles, respectively). Error bars represent the 68% confidence interval on the fitted parameter value. Solid lines represent weighted least-squares fits to eq 6(B). Same as panel A, except WT data were fit to eq 8 to obtain k_{rise} . (C) Activation energies obtained from the fits displayed in panels A and B. WT values are colored black and A12 values gray. Error bars represent the 68% confidence interval of the fitted parameter value.

Scheme 1

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Table 1^a

polymerase	$k_{\text{max, rise/fast}}$ (s^{-1})	$k_{1/2, \text{rise/fast}}$ (μM)	$k_{\text{decay}}/k_{\text{max, slow}}$ (s^{-1})	$K_{1/2, \text{slow}} (\mu\text{M})$	E_a^b (kcal mol^{-1}) (1 mM ATP)	E_a^b (kcal mol^{-1}) (50 μM ATP)
WT	$197 \pm [22, 27]$	$96 \pm [17, 21]$	$0.34 \pm [0.05, 0.06]$	not applicable	38 ± 6	26 ± 3
A12	$50 \pm [10, 20]$	$43 \pm [36, 74]$	$3.7 \pm [0.5, 0.6]$	$21 \pm [10, 22]$	24.05 ± 0.04	21 ± 11

^aErrors are the $\pm 68\%$ confidence interval from the fit.

^b E_a values correspond to k_{rise} and k_{fast} .