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A Novel Assay for RNA Polymerase I Transcription Elongation Sheds Light on the Evolutionary Divergence of Eukaryotic RNA Polymerases

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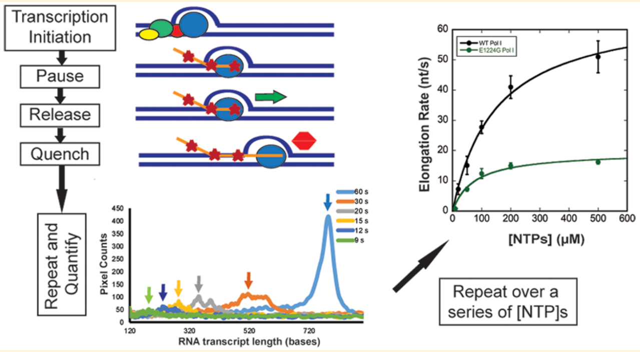
Supporting Information

ABSTRACT: Eukaryotic cells express at least three nuclear RNA polymerases (Pols), each with a unique set of gene targets. Though these enzymes are homologous, there are many differences among the Pols. In this study, a novel assay for Pol I transcription elongation was developed to probe enzymatic differences among the Pols. In *Saccharomyces cerevisiae*, a mutation in the universally conserved hinge region of the trigger loop, E1103G, induces a gain of function in the Pol II elongation rate, whereas the corresponding mutation in Pol I, E1224G, results in a loss of function. The E1103G Pol II mutation stabilizes the closed conformation of the trigger loop, promoting the catalytic step, the putative rate-limiting step for Pol II. In single-nucleotide and multinucleotide addition assays, we observe a decrease in the rate of nucleotide addition and dinucleotide cleavage activity by E1224G Pol I and an increase in the rate of misincorporation. Collectively, these data suggest that Pol I is at least in part rate-limited by the same step as Pol II, the catalytic step.



The three eukaryotic nuclear RNA polymerases (Pols) synthesize different cohorts of RNA species: Pol I synthesizes the majority of the ribosomal RNA (rRNA), Pol II synthesizes the messenger RNA (mRNA), and Pol III synthesizes transfer RNA (tRNA) and the smallest rRNA. The Pols have evolved unique properties to fulfill these roles. It is clear that these Pols are differentially regulated to modulate cellular activities through the use of largely non-overlapping transcription factors.[1] However, little is known regarding the evolutionary divergence of the biochemical properties of the Pols themselves, despite notable structural differences among the three enzymes.[2−4]

There have been extensive efforts to characterize transcription elongation properties of Pol II both *in vivo* and *in vitro* using a vast array of experimental strategies.[5−10] The literature's focus on Pol II has been primarily driven by its critical role in the control of global gene expression. Conversely, few studies have focused on Pol I transcription elongation, despite the emergence of Pol I as a potential chemotherapeutic target.[11−13] Here, for the first time, we devise a new strategy to directly measure the Pol I transcription elongation rate *in vitro* and determine kinetic parameters $k_{\max}$ and $K_{1/2}$ for Pol I multinucleotide addition. We then employ this novel assay to compare these measured parameters to those measured for an interesting Pol I transcription elongation mutant (*rpa190-E1224G*) whose homologous mutation in Pol II (*rpb1-E1103G*) has the opposite phenotype on transcription elongation. This glutamate residue is found in the trigger loop, a universally conserved domain of all multisubunit RNA polymerases.[6,14−16] The trigger loop directly influences the mechanism of transcription elongation.[17] During transcription elongation, the trigger loop adopts at least two unique conformations.[18] The active site is formed when the trigger loop closes to coordinate the incoming nucleotide to the 3′ end of the growing RNA strand.[19] After the nucleotide has been incorporated into the nascent RNA, the trigger loop adopts the open conformation, allowing for pyrophosphate (PP_i) release and translocation down the DNA template.[20−22] Additionally, it has been shown in archaea and bacteria that the trigger loop adopts a number of intermediate conformations during steps that involve proofreading and nucleolytic cleavage, such as pausing, arrest, and backtracking.[16,23−25] A majority of these intermediate conformations are thought to require further movement of the trigger loop away from the active site to allow for nucleolytic cleavage factors [such as GreA and GreB in bacteria, the A12.2 subunit (A12) in Pol I,

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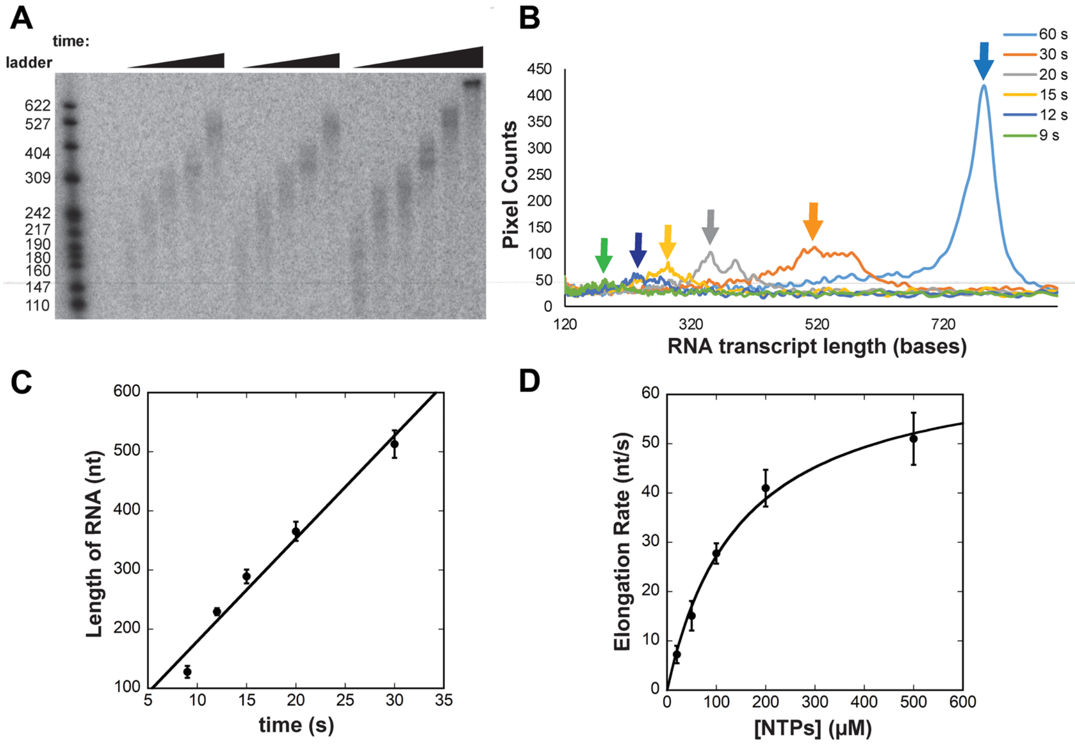

Figure 1. Novel promoter-dependent *in vitro* transcription assay that allows for the direct measurement of Pol I kinetic parameters. (A) A 15% urea denaturing PAGE gel of three 50 μM WT elongation time course replicates. (B) Replicate 3 WT Pol I elongation time points were quantified via ImageQuant, and the pixel count vs RNA transcript length was plotted for each time point. (C) The pixel positions at the peak pixel count for each time point (color-coordinated arrows in Figure 1B) were then plotted and fit to a line to determine the rate of elongation at 50 μM NTPs. (D) Rates taken from primary plots (Figure 1C) were then plotted vs [NTP], and data were fit to a rectangular hyperbola to produce an elongation rate curve for WT Pol I. Error bars represent the standard deviation about the mean for three independent reactions.

and transcription factor TFIIS for Pol II] to enter the active site after misincorporation.[18,26,27]

In Pol II, it was observed that trigger loop mutant E1103G increases the transcription elongation rate, potentially by stabilizing the closed conformation of the trigger loop, thus favoring the catalytic step, or phosphodiester bond formation.[6] This hypothesis suggests that the catalytic step is rate-limiting for Pol II. We found that multiple mutations in the highly conserved trigger loop region induced opposing phenotypes in Pol I than had previously been described in Pol II.[4] The mutation in Pol I (*rpa190-E1224G* or E1224G Pol I) that is identical to the well described gain-of-function E1103G mutation in Pol II resulted in a decrease in the net transcription elongation rate, not an increase.[4]

This observation raised a critical question: How do identical mutations in these two RNA polymerases result in opposite functional outcomes? To answer this question, it is necessary to define the biochemical consequences of the mutation on Pol I enzymatic activity, to differentiate between two distinct models: (1) Do different steps in transcription elongation limit the activities of Pol I versus Pol II, or (2) have the mechanisms of nucleotide addition diverged between Pols I and II such that an identical mutation has opposing effects on the same biochemical step(s)?

To answer these questions, we deployed our novel biochemical assay to precisely quantify processive multi-nucleotide addition, a previously published single-nucleotide addition assay, as well as misincorporation studies to define the mechanistic consequences of this mutation in the trigger loop hinge of Pol I. We found that overall multinucleotide addition is slowed by the E1224G Pol I mutation and that single-nucleotide addition is also slowed by E1224G Pol I. Furthermore, through model-independent fitting and model-dependent global fitting of nucleotide addition time courses, we determined values for the elementary rate constants for the essential steps in nucleotide addition by E1224G Pol I. Comparing these values to previously published results with wild-type (WT) Pol I, we observe a significantly slower catalytic step for the mutant polymerase. We further support the idea that the rate-limiting step observed in single-nucleotide addition is the catalytic step by executing single-nucleotide addition assays using a slowly hydrolyzing nucleotide analogue of ATP, ATPαS. We observe that the WT enzyme is ~35-fold slower at the rate-limiting step in the presence of ATPαS. This observation is consistent with the rate of nucleotide addition by Pol I in single-nucleotide addition assays being the catalytic step. Furthermore, we note that E1224G causes an increase in the amplitude of misincorporation by Pol I. Collectively, these data suggest that although the major rate-limiting steps for Pol I and II transcription elongation are likely the same, the contribution of the trigger loop to the nucleotide addition cycle is substantially divergent between Pols I and II.

■ RESULTS

Novel Quantitative Analysis of Processive Transcription Elongation by Pol I. In a previous study conducted by our laboratory, genetic and biochemical comparisons suggested that the E1224G mutation in Pol I results in slower transcription elongation.[4] This observation

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was in stark contrast to the well-characterized gain of function phenotype for transcription elongation rate induced by the homologous mutation (E1103G) in Pol II.[6,28,29] The rate of transcription elongation in previous assays for Pol I was inferred from the lag time between the release of synchronized transcription elongation complexes and the appearance of the full-length product. Lag analyses are somewhat error prone due to the limited number of time points during which runoff product accumulation is detected. Additionally, one nucleotide (typically UTP) had to be present at subsaturating concentrations to increase the specific activity of the radiolabeled substrate in the reaction.[4]

To overcome these limitations, we developed a new method for rigorously determining the kinetic parameters of WT and E1224G Pol I (Figure 1). We initiate transcription from the rDNA promoter using purified transcription factors and Pol I, as described previously.[4] Transcription initiation and early elongation are achieved by supplementing the reaction mixtures with ATP, GTP, and UTP. We omit CTP to synchronize transcription elongation complexes at the first encoded C, 56 nucleotides downstream of the transcription start site. After synchronizing transcription elongation complexes, we adjust the concentrations of all three NTPs to the desired final concentration and unleash the halted complexes by addition of CTP to a final concentration equal to that of the other three nucleotides. After a period of time, the reaction is quenched with formamide/EDTA loading dye and samples are analyzed on denaturing polyacrylamide gels (Figure 1A). We use a reference nucleic acid ladder to calculate the position of the polymerase at each time point, based on the migration of the peak of the transcription elongation complexes in comparison to the migration of the ladder (Figure 1B). As in previous assays, we include heparin as a trap, to ensure single-turnover conditions. This assay gives a direct measure of the average rate of the ensemble of polymerases in our reactions (Figure 1B,C).

E1224G Pol I Is Slower Than WT Pol I in Processive Elongation Studies. We repeated the processive elongation assay for WT and E1224G Pol I over a series of NTP concentrations (Figure 1D). The plot exhibits a hyperbolic behavior and is described by a rectangular hyperbola, which yields a maximum elongation rate (k_{max}) for WT Pol I of (67 ± 4) nucleotides per second, and a substrate concentration at one-half the maximal rate ($K_{1/2}$) of (142 ± 22) μM (Figure 1D). To the best of our knowledge, this is the first characterization of these kinetic parameters for Pol I. Consistent with our previous studies, we found that E1224G Pol I is much slower than WT Pol I, with a k_{max} of (19 ± 2) nucleotides per second and a $K_{1/2}$ of (64 ± 19) μM (Figure 2).

These observations are striking, as it has been described that the E1103G Pol II mutation causes an increase in the Pol II elongation rate.[6,28] The decrease in $K_{1/2}$ observed for the E1224G Pol I enzyme suggests that this mutation causes an increase in Pol I's affinity for NTPs, consistent with a reduced net rate of transcription elongation. Unfortunately, it is difficult to compare the change in affinity for the nucleotide observed to that of E1103G Pol II, as reports of the impact of E1103G Pol II on nucleotide affinity are conflicting.[28,29] Nevertheless, these data quantify key kinetic parameters for Pol I and demonstrate divergent consequences of homologous mutations in Pol II versus Pol I.

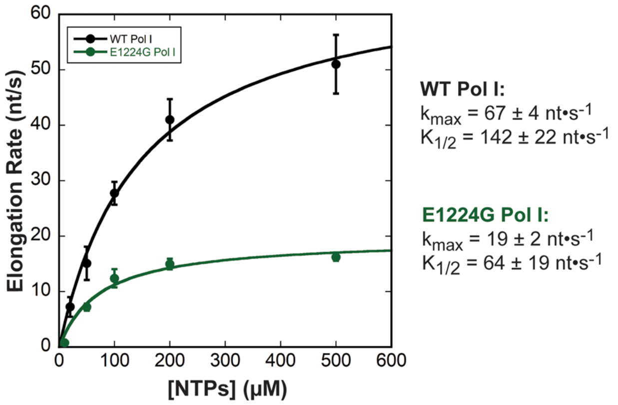

Figure 2. E1224G Pol I is slower than WT Pol I at single-turnover multi-NTP addition and displays a higher affinity ($K_{1/2}$) for NTPs. Promoter-dependent *in vitro* transcription was carried out for E1224G Pol I in the same manner that was used for WT Pol I (Figure 1). WT Pol I is significantly faster than E1224G Pol I. Additionally, E1224G Pol I seems to have a higher affinity ($K_{1/2}$) for nucleotides than WT Pol I.

E1224G Pol I Is Slower Than WT Pol I in Single-Nucleotide Addition Assays. We previously hypothesized that a step other than catalysis is rate-limiting for Pol I, as the E1103G Pol II mutant accelerates Pol II by favoring catalysis, the putative rate-limiting step for Pol II transcription elongation.[28] The data collected in processive elongation studies described above (Figures 1 and 2) reveal a slower overall rate for transcription elongation by E1224G Pol I, which could suggest a potential effect on the catalytic step but does not exclude impedance of other, potentially rate-limiting steps, such as an isomerization or a translocation step. Thus, we included the mutant enzyme in a second experimental strategy that directly monitors single-nucleotide addition and not, necessarily, translocation steps. In this assay, we form elongation complexes on a short template (64 nucleotides) and observe as E1224G Pol I incorporates a single nucleotide into the nascent RNA under rapid mixing conditions (Figure 3A). We then run the samples on a denaturing urea PAGE gel to separate and identify nucleic acid species present at each time point (Figure 3B). We monitor as WT and E1224G Pol I extend a 3′ radiolabeled 10-mer RNA into an 11-mer species over time. At later time points (t > 0.1 s), we observe the accumulation of a CA species, which is produced by the nuclease activity of Pol I. Interestingly, we also note the appearance of a 12-mer species, which was not observed with WT Pol I[30] (Figure 3B).

We fit the data using a model-independent strategy (Figure 3C−E and Figure S1). If E1224G Pol I were rate-limited by a step other than the catalytic step, we would expect to observe no change, or possibly an increase, in the rate of the nucleotide addition step for E1224G Pol I compared to that of the WT. Instead, we observed a significant decrease in the rate of nucleotide addition by E1224G Pol I as compared to WT (Figure 3C and Table S1). These data suggest that the mechanisms of nucleotide addition diverged between Pols I and II such that an identical mutation has opposing effects on the same biochemical step.

Thio-Analogue Studies Suggest That Pol I Elongation May Be Rate-Limited by the Catalytic Step. In previous studies, we noted that the kinetic coupling of nucleotide binding to the rate-limiting step is consistent with the rate-limiting step being chemistry in single-nucleotide addition studies.[30] For further confirmation, we conducted single-

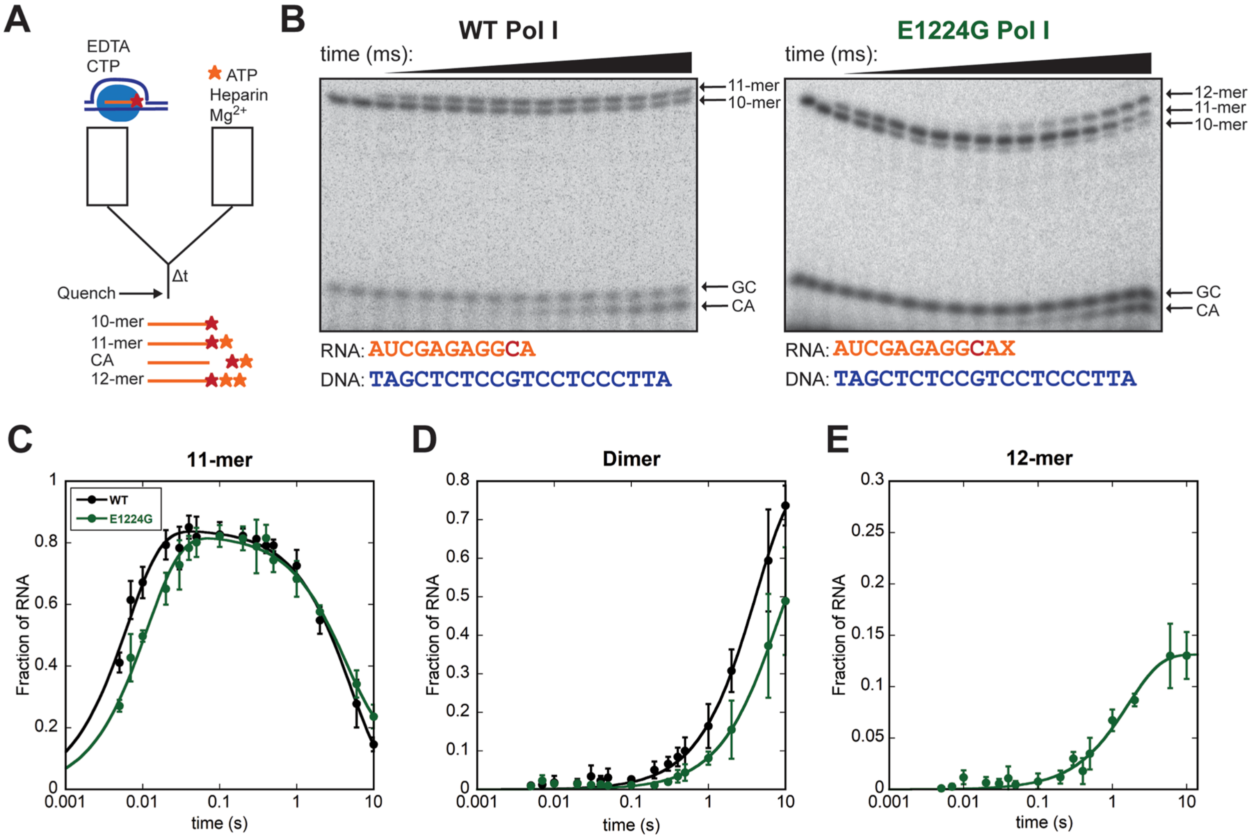

Figure 3. Single-turnover, single-NTP addition assays for E1224G Pol I. (A) Single-turnover, single-NTP addition assay schematic for rapid mixing via chemical quenched flow over a series of time points (0, 0, 0.005, 0.007, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 6, and 10 s from left to right, respectively) used for both WT and E1224G Pol I. (B) The 28% urea denaturing PAGE gels of 1000 μM ATP WT Pol I (left) and E1224G Pol I (right) time courses allow for resolution of the 12-mer, 11-mer, 10-mer, and dinucleotide species CA and GC. Signal intensities for each species were quantified using Image Quant. (C−E) Data for the 11-mer, CA, and 12-mer, respectively, for WT (black) and E1224G (green) Pol I were fit in a model-independent fashion. Data for the 11-mer were fit to the sum of two exponential curves, and data for the CA and 12-mer species were fit to a single-exponential curve. Error bars represent the standard deviation about the mean for three independent reactions. Data were fit using a 95% confidence interval.

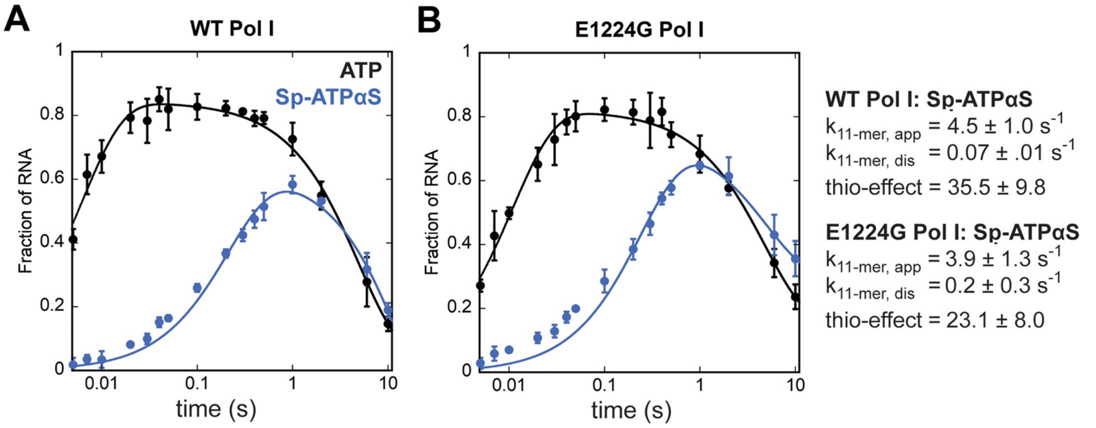

Figure 4. Sp-ATPαS studies suggest that WT Pol I may be rate-limited by the catalytic step in single-nucleotide addition experiments. Single-turnover, single-NTP addition assays were carried out with nucleotide analogue Sp-ATPαS for (A) WT and (B) E1224G Pol I via rapid mixing in a chemical quenched flow. Rate constants were calculated via model-independent fitting (lines) of data (circles) for WT and E1224G Pol I in the presence of 1 mM ATP (black) or 1 mM Sp-ATPαS (green). Error bars represent the standard deviation about the mean for three independent reactions. Data were fit using a 95% confidence interval.

nucleotide addition assays with a slowly hydrolyzable nucleotide analogue of ATP, Sp-ATPαS (the active isomer of ATPαS), with both WT and E1224G Pol I (Figure 4). It is accepted for Sp-ATPαS studies that if the observed single-turnover rate constant is decreased by >10-fold, then the observed rate constant (or rate-limiting step) of the reaction pathway is consistent with the catalytic step.[31−34] We note a substantial decrease in both WT and E1224G Pol I nucleotide addition rate constants at saturating concentrations of Sp-ATPαS (35- and 23-fold, respectively) (Figure 4). Importantly,

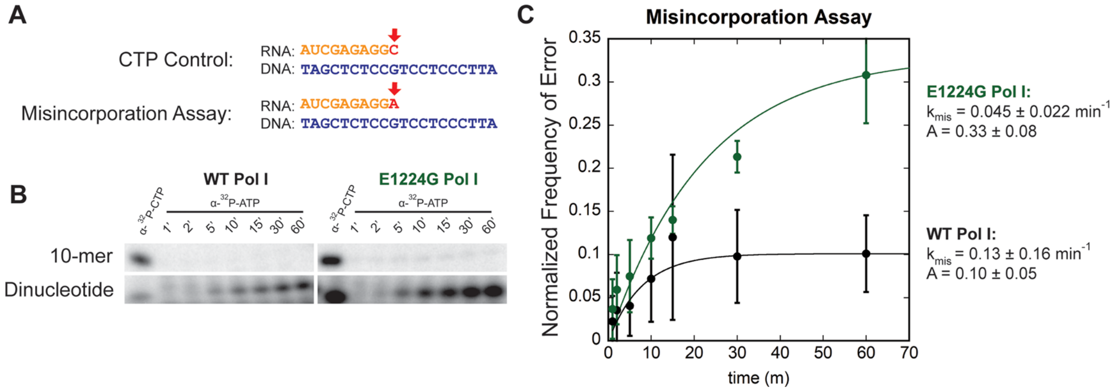

Figure 5. Misincorporation assay that shows a greater amplitude of E1224G Pol I misincorporation. (A) Schematic of the misincorporation assay strategy, where ECs are radiolabeled with a noncognate nucleotide, [α-^{32}P]ATP. (B) For each time course, ECs radiolabeled with CTP for 10 min were used as a normalization control for the active elongation complex. Time points were taken discontinuously for both the WT (left) and the mutant (right) polymerase and run on a 28% denaturing urea gel. (C) The normalized fraction of misincorporation was calculated by normalizing to both the intensity of the radioactive signal and the active elongation complex (total signal of the CTP control lane). Averages of three independent reactions are shown for WT and E1224G Pol I (circles), and error bars represent the standard deviation about the mean. Data were fit to a single-exponential curve using a 95% confidence interval.

while this evidence points to the chemical step being rate-limiting, these data alone do not allow us to conclude that catalysis is the only step that could be rate-limiting. Previous analyses of thio-analogue studies concluded that a >10-fold change in the observed rate-limited step could be consistent with either chemistry or steric effects caused by the slight change in shape and charge of the thio-analogues.[35]

E1224G Is Slower at Dinucleotide Production Than WT Pol I. For Pol I, there are two possible steps that can occur after incorporation of a nucleotide. (1) The polymerase can undergo a second cycle of nucleotide incorporation, or (2) it can backtrack one nucleotide and cleave the nascent RNA to produce a dinucleotide species. Through model-independent and model-dependent fitting of the single-nucleotide addition data, we observed an overall decrease in rate constants describing dinucleotide production, or nucleolytic cleavage, for E1224G Pol I as compared to WT (Figure 3C,D, and Table S1). These data support the hypothesis that the E1224G Pol I mutation may be somehow obstructing the nucleolytic cleavage activity of the Pol I A12 subunit.

E1224G Pol I Is More "Error" Prone Than WT. In the single-nucleotide addition assays described here (Figure 3), we observed a significantly greater amplitude of misincorporation by the mutant polymerase compared to that of the WT, as noted by the appearance of a 12-mer species (Figure 3E). We observed that at its peak the 12-mer species represents 18% of the total RNA population. In comparison, in previous studies performed by our lab there was minimal (<3%) misincorporation observed by WT Pol I (Figure 3B and ref 30). Interestingly, an increase in the rate of misincorporation was also observed in previous studies of E1103G Pol II (though in Pol II the increase in the rate of misincorporation is more substantial).[28] As we observe a decrease in the rate of transcription, coupled with an increase in the rate of misincorporation, these data highlight the fact that Pol I may not be sensitive to the same proportional relationship or "balance" between the transcriptional dynamics of misincorporation and speed of transcription as observed in other RNA polymerases.[28]

To test whether the observed increase in the rate of misincorporation could be the result of a contamination of the cognate nucleotide (GTP) in our reactions, we employed a misincorporation assay in which we used the same oligonucleotide templates and RNA primer as in Figure 2, incubated with noncognate [α-^{32}P]ATP instead of [α-^{32}P]CTP (Figure 5A). In this strategy, the extended RNA is visible only if the polymerase incorporates the radiolabeled nucleotide. If the nucleotide is added, the polymerase can then either cleave the misincorporated RNA, producing a dinucleotide species (a GA dimer), incorporate the next nucleotide, or pause. We note that at all time points we do not see any accumulation of the 10-mer. Instead, we observe accumulation of only the GA dinucleotide cleavage product; thus, the cleavage step must be significantly faster than misincorporation. We compare the GA signal to the total signal of a negative control reaction (elongation complex incubated with cognate [α-^{32}P]CTP) (1) to ensure that we can account for the relative abundance of the active elongation complex between WT and E1224G Pol I and (2) to compare the migration of the dinucleotide species during gel electrophoresis. It is worth noting that the dinucleotides GA and GC migrate at slightly different rates during gel electrophoresis, so these species can be uniquely identified (Figure 5B). This assay reveals the relative misincorporation rate of E1224G and WT Pol I by comparing the accumulation of the signal for the GA dimer to that of the GC dimer over time. We observe a greater overall amplitude (3-fold) of misincorporation for the mutant over WT Pol I (Figure 5B,C). These data suggest that the accumulation of the 12-mer observed with the mutant polymerase in single-nucleotide addition assays is in fact due to misincorporation and not due to contaminated reaction substrates. In addition, we note that the rate of misincorporation is faster for WT Pol I (Figure 5C). It is reasonable to conclude that this increase in the rate of misincorporation by WT Pol I compared to that of the mutant reflects the faster rates of nucleotide addition and dinucleotide cleavage by the WT enzyme.

Model-Dependent Analysis of Single-Nucleotide Addition for E1224G Pol I. To further investigate which step(s) in single-nucleotide addition limits the reaction, the single-nucleotide addition assay was carried out over a series of NTP concentrations, and the data sets were globally fit to the reaction scheme in Figure 6A. This scheme is similar to that

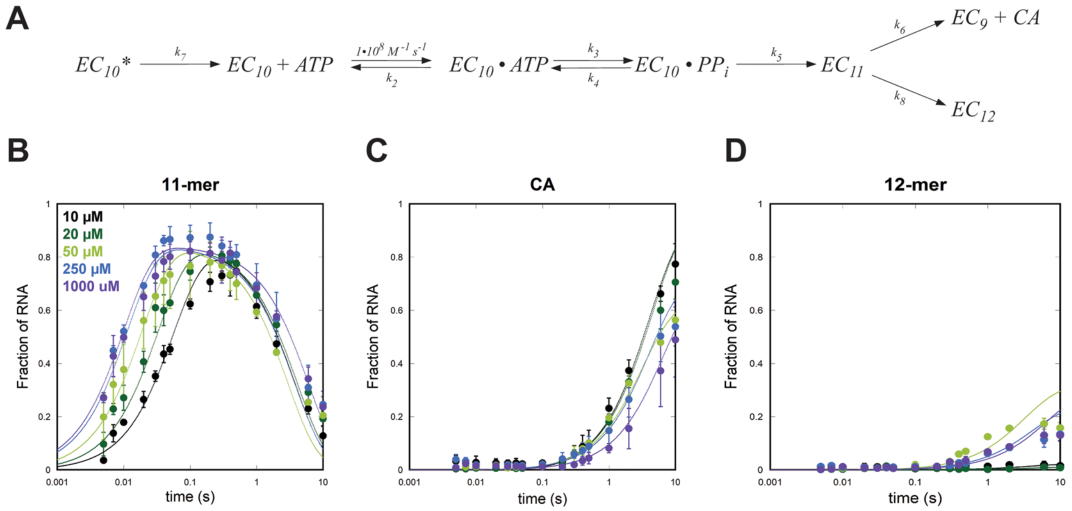

Figure 6. Global fits of E1224G Pol I transcription kinetics. (A) Simplest model that best describes data collected for E1224G Pol I single-nucleotide addition assays. (B–D) Averages of three independent reactions are given for each species fit to the scheme in panel A in MatLab (11-mer, CA, and 12-mer, respectively) for each ATP concentration (circles). Error bars represent the standard deviation about the mean for three reactions. Lines represent data gobally fit in MatLab to the scheme in panel A.

previously developed for WT Pol I[30] with the addition of a step to describe the formation of the 12-mer species (k_8) subsequent to the pyrophosphate release step (k_5) (Figure 6A). We found that E1224G Pol I was slower than WT Pol I at the phosphodiester bond formation step (k_3) (WT k_3 = 180 s^{-1}, and E1224G Pol I k_3 = 103 s^{-1}) (Figure 6A and Table 1).[30] This observation, consistent with our processive elongation data (Figure 2) and our model-independent fitting (Figure 3C), suggests that this mutation inhibits the catalytic step by Pol I, in direct opposition to results observed previously with Pol II.[28] It is also worth noting that the ATP dissociation rate constant, k_2, is slower for E1224G Pol I than for WT Pol I. This reduced "off rate" for ATP is in agreement with the observation in processive elongation assays that the mutant polymerase displays a lower $K_{1/2}$ for substrate NTPs (Figure 2). These data indicate two distinct possibilities. (1) The E1224G Pol I mutation stabilizes the closed conformation of the trigger loop, but this stabilization somehow disfavors the catalytic step, pointing to major mechanistic differences between Pols I and II. (2) The E1224G Pol I mutation does not stabilize the closed conformation of the trigger loop, highlighting the possibility of key conformational differences in the active sites of Pols I and II.

Taken together, this study presents a pathway for detailed characterization of nucleotide addition, nascent RNA cleavage, and misincorporation by Pol I in the ensemble. Using these methods, we defined the mechanistic consequences of an evolutionarily conserved mutation in the trigger loop of Pol I.

■ DISCUSSION

Potential Mechanism for the Decreased Rate of Transcription with the E1224G Pol I Mutant. As shown in single-nucleotide addition assays, it is most likely the decrease in k_3 in Figure 6A (the catalytic step or the conversion of bound ATP to RNA_{11} and bound PP_i) of E1224G Pol I that causes the observed decrease in the rate of transcription elongation (Figure 6, Figure S2, and Table 1). This was surprising, as it suggests that the two homologous mutations in Pol I and Pol II (E1103G and E1224G) cause distinct phenotypes but likely affect the same rate-limiting step in transcription. We previously used chimeric Pol II complexes that carried the trigger loop of Pol I to demonstrate that the E-to-G mutation in the hinge region remained a gain-of-function allele irrespective of the trigger loop sequence.[4] This suggests that the conformational changes that are caused by the E1224G mutation in Pol I are likely the same as or similar to those induced by the mutation in Pol II. However, due to differences in the local protein environment or other differences in the interactions between the trigger loops and the active sites of Pols I and II, this mutation has a different mechanistic effect on the rate of phosphodiester bond formation for the two enzymes.

Possible Implications of the A12 Subunit on Trigger Loop Function and Pol I Transcription Elongation. A previous study utilizing optical tweezers observed that transcription elongations by Pols I and II are distinct and pointed to the differences between the Pol's non-essential subunits to explain this observation.[36] It is worth noting that one large structural difference between the active sites of Pol I and II is the persistent presence of the A12 "nucleolytic cleavage" subunit in the Pol I active site, which is absent from Pol II.[17,26,37] Instead, Pol II recruits TFIIS for transient nucleolytic activity to rescue transcription from misincorporation and pausing.[38] The Pol I A12 subunit is composed of a C-terminal domain that is homologous to the Pol II "cleavage factor" TFIIS and an N-terminal domain that is homologous to Pol II Rpb9. Additionally, A12 in its entirety is homologous to the C11 subunit of Pol III. It is possible that the coordination of the A12 subunit with the trigger loop is responsible for the large difference in functionality observed between the E1103G Pol II and E1224G Pol I mutations. This is especially intriguing in light of our observation of a decrease in the rate of nucleolytic cleavage by E1224G Pol I (Figures 3D and 6C). It is also worth noting that in previous studies we have shown the combination of the E1224G Pol I mutation and deletion of *RPA12* is synthetically lethal, and it has likewise been shown that the E1103G mutation in Pol II is synthetically lethal with deletion of *RPB9*.[4,8,39] Rpb9 lacks the proofreading activity of A12, but it is critical for transcriptional fidelity *in vivo*, possibly responsible for the structural coordination of TFIIS to the Pol

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II active site.[39] It has also been shown in force-assisted studies that A12 and TFIIS are crucial for backtracking recovery for Pol I and Pol II, respectively.[36] Collectively, these observations suggest direct but different roles for Pol I A12 and Pol II Rpb9 in nucleotide addition. Perhaps interactions between A12/Rpb9 and the trigger loop/active center govern the divergence in phenotypes observed in mutations in the trigger loops of Pols I and II.

Table 1. The E1224G Pol I Mutation Decreases the Rate of Nucleotide Addition and Increases the Amplitude of Misincorporation[a]

	WT	lower bound	upper bound	E1224G	lower bound	upper bound
k_1[b] (M^{-1} s^{-1})	1 × 10^8	–	–	1 × 10^8	–	–
k_2 (s^{-1})	9800	8100	12100	4731	3230	6290
k_3 (s^{-1})	180	160	220	102	85	131
k_4[b] (s^{-1})	≈0	–	–	≈0	–	–
k_5 (s^{-1})	>300	>300	–	>52	>52	–
k_6[c] (s^{-1})	0.42	0.40	0.44	0.22	–	–
k_7 (s^{-1})	0.04	0.01	0.07	0.08	0.00	0.41
k_8[c] (s^{-1})	–	–	–	0.003	–	–
$[EC_{10}]/[EC_{total}]$	0.77	0.74	0.79	0.85	0.79	0.91

[a]Parameters were globally fit for species from single-NTP nucleotide addition assays for E1224G Pol I (Figure 3D,E) and from previous characterizations of WT Pol I.[30] Lower and upper bounds were calculated by grid searching using 68% confidence intervals about the mean. [b]Parameters fixed in global analysis. [c]Average minima for locally fit parameters.

Potential Impacts of the Pol III Homologue E1070G on Transcription Elongation. The Pol II E1103G mutation has been well-described in the literature as a gain of function mutation that increases the polymerase's affinity for the substrate and transcription rate and decreases transcriptional fidelity. Additionally, substitutions of amino acids near the conserved glutamate residue also result in an increase in elongation rate in bacteria and archaeal polymerases.[14−16] In Pol III, the mutation homologous to E1103G Pol II is *rpc160-E1070G*. This mutation has not been extensively characterized in the literature; however, one recent study showed that E1070G Pol III decreases transcription fidelity, similar to the effect of E1103G Pol II and E1224G Pol I, and therefore results in termination defects.[40] In that study, the impact of E1070G on the Pol III elongation rate was not directly tested; however, the observed termination defect served as an *in vivo* indicator of increased transcription elongation rate. Future studies that aim to characterize the effect of this mutation *in vitro* to determine the mechanistic consequences of the mutation on Pol III will be particularly interesting, especially because Pol III also carries a nucleolytic cleavage subunit (C11).[26,27]

Catalysis Is Likely the Rate-Limiting Step for Pol I Transcription Elongation. The rate-limiting step for Pol II transcription has been somewhat controversial; however, it is generally accepted that catalysis is rate-limiting, or at least partially rate-limiting, for Pol II transcription elongation.[28,29] Therefore, mutations that favor the closed conformation of the trigger loop, which favors nucleotide addition, result in a gain of function.[6,29] We have observed that the nucleotide addition step is also rate-limiting for Pol I transcription elongation. In this study, we show that multi-NTP addition is slower in E1224G Pol I than in the WT. Additionally, we show that for E1224G Pol I single-nucleotide addition assays, the rate of the catalytic, nucleotide addition step (k_3) is also decreased compared to that of WT Pol I (Table 1 and Figure 6A). This decrease is not as dramatic as that seen with multi-NTP addition (∼3-fold vs ∼1.5-fold), but this may be partially dependent on the identity of the nucleotide. It is worth noting that our single-nucleotide addition assay is likely insensitive to translocation steps and backtracking, and thus, the mutation may induce additional defects not apparent in this particular assay. Despite the lack of information about translocation steps for Pol I, it is clear that E1224G Pol I has a significant, negative impact on elementary rates that govern nucleotide addition by Pol I.

Understanding the mechanisms that govern RNA polymerase function is critical for understanding the fundamental steps in gene expression. Here, we show that Pol I activity is sensitive to the chemical step in nucleotide addition, and this observation is similar to previous observations for Pol II. However, mutations in universally conserved domains within the enzymes have opposing effects on that step. These findings provide insight into the evolution of unique features within the eukaryotic RNA polymerases, potentially revealing useful vulnerabilities of each enzyme. To fully understand the degree to which nucleotide addition mechanisms have diverged among RNA polymerases, each enzyme must be characterized extensively using the array of assays described here and as described in related works from other groups.[23,28,29,40]

■ MATERIALS AND METHODS

Yeast Strains, Media, and Growth Conditions. Yeast strains were identical to those described previously.[4]

Protein Purification. E1224G Pol I, WT Pol I, and initiation factors (TBP, CF, and Rrn3) were purified as previously described.[4] Proteins were stored in storage buffer (elution buffer with 40% glycerol) at −80 °C.

Multinucleotide *in Vitro* Transcription. The transcription elongation assays were performed as previously described,[41] with some key differences. For all transcription reactions, during the labeling reaction, the UTP concentration was kept to 10 μM to favor the incorporation of ^{32}P-labeled UTP. Prior to the release of synchronized transcription elongation complexes at the 56th nucleotide downstream of the transcription start site, UTP levels were adjusted to the concentration of all other substrates for the given NTP concentration. Additionally, the elongation reactions were quenched at time points prior to the polymerase reaching the end of the template. This allowed for direct measurement of the peak intensity of the population of polymerases while they were in active elongation complexes. The peak signal intensity for each time point was plotted against a radiolabeled ladder, and this was used to calculate the average position of the ensemble of polymerases at a given time point. These data were quantified in ImageQuant; error analysis was performed in Scientist 3.0, and data were plotted in Kaleidagraph.

Single-Nucleotide *in Vitro* Transcription. Single-nucleotide addition assays for E1224G Pol I were performed over a series of ATP concentrations as previously described.[30] A detailed description of experimental and analytical methodology can be found in the Supplemental Methods.

Sp-ATPαS Single-Nucleotide *in Vitro* Transcription. Sp-ATPαS assays were performed as described above for single-nucleotide addition assays but were carried out with 1 mM Sp-ATPαS (BioLog Life Science Institute A040-05) instead of ATP.

Misincorporation Assay. Elongation complexes were set up as previously described for single-nucleotide addition assays as described previously in Single-Nucleotide in Vitro Transcription methods section; however, labeling was done with noncognate [α-^{32}P]ATP instead of [α-^{32}P]CTP. Misincorporation was analyzed by measuring the intensity of the signal of the GA dimer over a series of time points and normalized to the radioactive signal and the relative amount of the active elongation complex (total signal, 10-mer + GC signal, from elongation complexes radiolabeled for 10 min with [α-^{32}P]CTP).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b01256.

Tables S1 and S2, Figures S1 and S2, and Supplemental Methods (PDF)

Accession Codes

UniProt accession IDs. RNA polymerase I subunits: Rpa190, P10964; Rpa135, P22138; Rpa49, Q01080; Rpa43, P46669; Rpc40, P07703; Rpa34, P47006; Rpb5, P20434; Rpb6, P20435; Rpc19, P28000; Rpb8, P20436; Rpa12, P32529; Rpb10, P22139; Rpb12, P40422. Transcription initiation factors for RNA Pol I: Rrn6, P32786; Rrn7, P40992; Rrn11, Q04712; TBP, P13393; Rrn3, P36070.

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Author Contributions

C.E.S. conceptualized and designed research and methodology, did formal analysis, performed investigations, wrote the original draft of the manuscript, and provided funding. Z.M.I. designed software, did formal analysis, executed data curation, and wrote the manuscript. A.L.L. conceptualized and designed research and methodology, provided supervision, and wrote the manuscript. D.A.S. conceptualized and designed research and methodology, provided supervision, wrote the manuscript, and provided funding.

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Notes

The authors declare no competing financial interest.

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