Transient State Kinetics of Transcription Elongation by T7 RNA Polymerase*1

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The single subunit DNA-dependent RNA polymerase (RNAP) from bacteriophage T7 catalyzes both promoter-dependent transcription initiation and promoter-independent elongation. Using a promoter-free substrate, we have dissected the kinetic pathway of single nucleotide incorporation during elongation. We show that T7 RNAP undergoes a slow conformational change (0.01–0.03 s−1) to form an elongation competent complex with the promoter-free substrate (dissociation constant (Kd) of 96 nM). The complex binds to a correct NTP (Kd of 80 μM) and incorporates the nucleoside monophosphate (NMP) into RNA primer very efficiently (220 s−1 at 25 °C). An overall free energy change (−5.5 kcal/mol) and internal free energy change (−3.7 kcal/mol) of single NMP incorporation was calculated from the measured equilibrium constants. In the presence of inorganic pyrophosphate (PPi), the elongation complex catalyzes the reverse pyrophosphorolysis reaction at a maximum rate of 0.8 s−1 with PPi/Kd of 1.2 mM. Several experiments were designed to investigate the rate-limiting step in the pathway of single nucleotide addition. Acid-quench and pulse-chase kinetics indicated that an isomerization step before chemistry is rate-limiting. The very similar rate constants of sequential incorporation of two nucleotides indicated that the steps after chemistry are fast. Based on available data, we propose that the preinsertion to insertion isomerization of NTP observed in the crystallographic studies of T7 RNAP is a likely candidate for the rate-limiting step. The studies here provide a kinetic framework to investigate structure-function and fidelity of RNA synthesis and to further explore the role of the conformational change in nucleotide selection during RNA synthesis.

The single subunit bacteriophage T7 RNA polymerase (RNAP)2 catalyzes each of the stages of transcription including initiation, elongation, and termination without requiring any accessory proteins that are necessary in multisubunit RNA polymerases (1, 2). Structurally, T7 RNAP is similar to the pol I family of DNA-directed DNA polymerases and reverse transcriptases and shows high sequence homology to mitochondrial RNA polymerases (1). Being a single subunit enzyme, T7 RNAP serves as a model RNAP in understanding the mechanism and regulation of transcription initiation, elongation, and termination.

The mechanism of transcription initiation by T7 RNAP is relatively well understood. The kinetic pathway of initiation has been dissected and the steps of promoter DNA and initiating NTP binding as well as promoter DNA melting have been quantified (3, 4). Recent studies have also provided a more detailed understanding of the transition process from initiation to elongation in T7 RNAP (5–11). The mechanism of transcription elongation catalyzed by T7 RNAP has not been characterized in detail, partly because elongation is an intermediate phase of transcription that begins only after 9–12 nt of RNA is made through promoter-specific initiation (8). In the single subunit T7 RNAP, the transition from initiation to elongation is achieved through major refolding events of the N-terminal domain. The refolding process changes a part of the T7 RNAP structure, that facilitates promoter escape and the channeling of the 5′-end of the RNA into an RNA channel (12, 13). Recent studies have shown that T7 RNAP can assume the refolded elongation structure in the presence of a promoter-free elongation substrate (9, 11, 14, 15). Therefore, T7 RNAP can bypass the initiation phase and catalyze the elongation of RNA in a minimal promoter-free elongation substrate that consists of an RNA/DNA hybrid of 8 bp and a downstream duplex DNA that provides the template for RNA synthesis (16).

The minimal pathway of transcription elongation includes the steps of NTP binding, phosphodiester bond formation (the chemical step), product release, and translocation of RNAP on the DNA for next nucleotide incorporation. High resolution structures of T7 RNAP in the elongation state have provided evidence for conformational changes accompanying NTP binding and PPi release (2, 17). A correct NTP binds to the open state of T7 RNAP in a preinsertion site, where it interacts with the residues along the O helix (2). NTP in the preinsertion site makes base specific contacts with the templating base, but it does not interact with the two metal ions required for the chemical step. NTP binds to the closed state of T7 RNAP in the insertion site, where it interacts with the two metal ions that catalyze the chemical step of phosphodiester bond formation reaction. It has been proposed that correct NTP binding triggers the open to closed conformational change and that this step plays a role in the selection of a correctly base-paired ribonucleotide. Such a conformational change linked to correct
NTP binding has been proposed and kinetically characterized in various other polymerases (18–25).

In this paper, we have dissected the minimal pathway of single nucleotide incorporation during transcription elongation catalyzed by T7 RNAP using transient state kinetic methods. We have used a synthetic promoter free RNA/DNA elongation substrate composed of an 8-bp RNA-DNA hybrid with a 2-base tail at the 5′-end. The minimal kinetic pathway reported here describes the equilibrium binding affinity of T7 RNAP for the elongation substrate, the time taken by T7 RNAP to assume an elongation-competent state, the binding affinity of correct NTP, the rate of NMP incorporation, and the kinetics of pyrophosphorylase. In addition, we have designed transient state kinetic experiments to investigate the nature of the rate-limiting step in the pathway of single nucleotide incorporation. These studies have measured the rate constants of the elementary steps in the single nucleotide incorporation cycle and therefore provide a kinetic framework for future investigation of structure-function, fidelity, and processive RNA synthesis during the elongation phase of transcription.

**MATERIALS AND METHODS**

Nucleic Acids, T7 RNAP, and Other Reagents—Oligodeoxynucleotides (Fig. 1) were custom synthesized (Integrated DNA Technologies, Coralville, IA) and purified on a 16% polyacrylamide/urea gel. DNA concentration was determined from absorbance at 260 nm and the calculated molar extinction coefficients. RNA (Fig. 1) was purchased PAGED purified and 2′-deprotected/desalted from Dharmacon Research Inc. (Lafayette, CO). RNA was radiolabeled at the 5′-end using [γ-32P]ATP and polynucleotide kinase and purified using a G-50 gel filtration resin (Sigma). Sp-UTPαS (uridine 5′-O-(1-5′-O-(1-thiotriphosphate)) was purchased from Biolog Life Science Institute (Axcor LLC, San Diego, CA).

T7 RNAP was overexpressed in Escherichia coli strain BL21 (26) and purified as described previously (3, 4, 27) with the exception that the CM-Sephadex column was eliminated. Purified enzyme was stored at −80 °C in buffer (20 mM sodium phosphate, pH 7.7, 1 mM Na3-EDTA, and fresh 1 mM DTT) containing 100 mM sodium chloride and 50% (v/v) glycerol. Enzyme concentration was calculated from its absorbance at 280 nm and molar extinction coefficient of 1.4 × 105 M−1 cm−1 (28).

Assembly of the Promoter-free Elongation Substrate—Template DNA, non-template DNA, and 5′-32P-labeled RNA were mixed in 1:1.5:1 ratio in the transcription buffer at a final concentration of 20 μM, heated at 95 °C for 20 min, then stepwise cooled from 75, 55, and 45 °C for 20 min each, 20 °C for another 25 min, and finally to 4 °C for an hour. The transcription buffer consisted of 50 mM Tris acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, and fresh 2 mM dithiothreitol.

Rapid Chemical Quench-Flow Experiments—Pre-steady-state kinetic experiments were conducted at 25 °C using a Model RQF-3 chemical quench-flow apparatus (KinTek Corp., Austin, TX). T7 RNAP and VSR10 (50 mM Tris acetate, 100 mM sodium acetate, 10 mM magnesium acetate, 5 mM DTT) mixture was loaded in one syringe of the quenched-flow instrument and NTP (50 mM Tris acetate, 10 mM magnesium acetate, 5 mM DTT) in the second syringe. The reactions were rapidly mixed and quenched at various times with EDTA (0.2 M final concentration) or 1 N HCl from a third syringe. The HCl-quenched reactions were treated with chloroform and neutralized with 0.25 M Tris base and 1 M NaOH.

**Pyrophosphorolysis**—Pyrophosphorolysis kinetics were measured using VSR11 in the rapid quenched-flow instrument. To determine the Kd of PPi, the reactions were carried out for 0.5 s at various PPi concentrations ranging from 0.05 to 10 mM. Total Mg2+ was kept constant at 20 mM. RNA products (<10 nt were resolved on polyacrylamide sequencing gel and were quantitated and plotted against the respective PPi concentration. The data were fit to a hyperbola (Equation 2) and analyzed as described below.

**Analysis of the Transcription Products**—RNAs were resolved on a 20% polyacrylamide, 1.5% Bis, 7 M urea gel (Bio-Rad sequencing gel apparatus). The gel was exposed to a phosphor screen, scanned on a Typhoon 9410 PhosphorImager instrument (Amersham Biosciences), and quantitated using the ImageQuaNT software (GE Healthcare). The fraction of RNA primer converted to products was determined from the ratio of their respective counts to the total counts, and the concentration of the products was determined by multiplying the fraction with the concentration of the RNA primer. The kinetics were fit to Equation 1 using SigmaPlot software (Jandel Scientific).

\[
Y = y_0 + (1 - A \exp(-k_{obs}t))
\]  
(Eq. 1)

Y is the fraction or molar amount of products, y0 is the y intercept or background, A is the amplitude or the total amount of products formed during the reaction, and kobs is the observed rate constant of product formation.

The observed rate, kobs, plotted as a function of [NTP] was fit to Equation 2.

\[
k_{obs} = \frac{k_{pol}[NTP]}{k_d + [NTP]}
\]  
(Eq. 2)

Kd is the equilibrium dissociation constant of the NTP, and kpol is the maximum rate constant of NMP incorporation.

Global fitting of the sequential addition of two nucleotide kinetics was fit using MATLAB (MathWorks Inc) (gfit, open-source software for global analysis of experimental data, also available from the authors).

**Nitrocellulose-DEAE Double Filter Binding Assay**—The binding affinity of T7 RNAP for the elongation substrate, VSR10, was determined by the nitrocellulose-DEAE filter binding assay (29, 30). DEAE and nitrocellulose membranes (Schleicher & Schuell) were treated with 0.5 M NaOH for 5 min, washed thoroughly with doubly distilled water, then equilibrated in the transcription buffer for 12–24 h before use. Radiolabeled VSR10 (0.3 μM) and T7 RNAP (0–2 μM) were preincubated for 30 min, filtered through a bilayer of nitrocellulose and DEAE membranes under vacuum, and washed with the transcription buffer. The filters were dried and exposed to phosphor screens. The counts on the nitrocellulose membrane gave a measure of the amount of T7RNAP-VSR10 complex, and that on the DEAE provided the amount of free VSR10. The fraction bound (Fb) was calculated as the ratio of counts on nitrocellu-
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The kinetic analysis of T7 RNAP binding to the promoter-free elongation substrate was performed using a filter binding assay (see “Materials and Methods”). In this assay, radio-labeled VSR10 was incubated with varying amounts of T7 RNAP for 30 min and the mixture was filtered through nitrocellulose-DEAE membranes. T7 RNAP-VSR10 complex bound to the nitrocellulose membrane and free VSR10 bound to the DEAE membrane was determined at each T7 RNAP concentration. Fig. 2 shows the hyperbolic increase in the concentration of VSR10-T7 RNAP complex as a function of increasing T7 RNAP concentration. The data were fit to a quadratic equation (Equation 3) that provided an equilibrium dissociation constant, $K_d$, of 96 ± 18 nM for the VSR10-T7 RNAP complex and an amplitude of 0.2 μM.

Slow Binding of T7 RNAP to the Promoter-free Elongation Substrate—The kinetics of T7 RNAP binding to VSR10 were investigated by following the elongation of 10-mer RNA to 11-mer under defined mixing conditions. In setup one, T7 RNAP was preincubated with radio-labeled VSR10 for at least 30 min before initiating the reaction by mixing with UTP. In setup two, T7 RNAP was not preincubated with VSR10, and the elongation substrate was added with UTP to initiate the reaction. The elongation of 10-mer to 11-mer in setup one under the preincubated conditions occurred at a rapid rate constant of 123 ± 19 s⁻¹ and amplitude of 0.8 (Fig. 3A). On the other hand, the elongation of 10-mer to 11-mer in setup two under the non-preincubated conditions was extremely slow and occurred with an observed rate constant of 0.01 ± 0.0007 s⁻¹, which is >4000 times slower than the rate of UMP incorporation. The slow rate constant of single nucleotide incorporation remained unchanged with higher concentrations of T7 RNAP (Fig. 3B). Therefore, the bimolecular rate constant of T7 RNAP binding to the promoter-free substrate does not appear to be the slow step. The results indicate that a slow conformational change prior to or subsequent to binding must limit the observed rate of correct UMP incorporation in the VSR10 substrate under the non-preincubated conditions.

The Refolding of T7 RNAP Is a Slow Step—The N-terminal domain of T7 RNAP undergo major refolding events to assume the elongation competent conformation (9, 11). It is therefore likely that the refolding of T7 RNAP is a slow step that limits the rate constant of UMP addition in the above experiments under the non-preincubated conditions. The kinetics of T7 RNAP refolding from its initiation conformation to the elongation conformation can be probed by limited trypsin digestion (8). The 170-180 loop of T7 RNAP is susceptible to trypsin cleavage in the initiation conformation. Therefore, the full-length 98-kDa T7 RNAP-promoter complex is cleaved into 80- and 20-kDa fragments during limited trypsin digestion (Fig. 3C, lane 3) (the 20-kDa fragment is further digested to 15- and 5-kDa fragments and not shown). When T7 RNAP assumes the elongation conformation, the 170-180 loop is refolded into subdomain H, which forms part of the RNA channel, and hence the lysines in this loop are protected from trypsin cleavage. In
the elongation conformation, however, T7 RNAP does not interact with the promoter; hence, the lysine in the 98–100 loop becomes susceptible to trypsin. Therefore, T7 RNAP bound to the promoter-free elongation substrate VSR10 is cleaved into 88- and 10-kDa fragments (Fig. 3C, lane 4). Appearance of the 88 kDa and disappearance of the 80 kDa are therefore signatures of initiation to elongation conformational changes.

T7 RNAP was incubated with VSR10 for different time intervals ranging from 5 s to several minutes, and then treated with trypsin very briefly (estimated 5 s) before analysis by SDS-PAGE. Fig. 3C shows the time course of the change in the proteolysis pattern indicating a conformational change in T7 RNAP upon binding to the promoter-free elongation substrate. It is clear from the results that the initiation to elongation conformational change is slow and occurs at a rate constant (0.01 s−1) (Fig. 3D) that is very similar to the observed rate constant of UMP incorporation in setup two under the non-preincubated conditions (see above). These results support the idea that the refolding of T7 RNAP from its initiation conformation to the elongation competent conformation is a slow step that limits the incorporation of nucleotide into the RNA primer in the promoter-free substrate.

$k_{pol}$ and $K_d$ of Correct Nucleotide Incorporation—The kinetics of correct UMP addition were measured under single turnover conditions (1 μM VSR10 and 2 μM T7 RNAP) with the goal of determining the equilibrium dissociation constant ($K_d$) and the intrinsic rate constant of UMP addition ($k_{pol}$). Promoter-free substrate was preincubated with T7 RNAP for at least 15 min before initiating the reaction with UTP in a rapid chemical quenched-flow instrument. After various time intervals from 2 ms to 20 s, 1 N HCl was added to quench the reactions. The kinetics of 10-mer elongation to 11-mer were measured at various [UTP]. The RNAs were resolved on a denaturing polyacrylamide sequencing gel (Fig. 4A), and the time course of 11-mer RNA formation was fit to a single exponential equation (Equation 1) to obtain the rate constant of UMP addition at each UTP concentration. The observed rate constants were plotted against [UTP] (5–250 μM) and the concentration dependence was fit to a hyperbolic equation (Equation 2), which provided UTP $K_d$ of 76 ± 33 μM and $k_{pol}$ of 222 ± 40 s−1 (Fig. 4B).

Pulse-Chase Experiments—Structural studies of T7 RNAP indicate that NTP binds via a minimal two-step mechanism involving a conformational change following NTP binding (2,
17). Pulse-chase and pulse-quench experiments were designed to investigate whether the isomerized complex after NTP binding accumulates during the reaction, that is whether the chemical step or the conformational change upon NTP binding was the rate-limiting step. In the pulse-quench conditions, the acid quenches all the enzyme-bound species immediately, whereas in the pulse-chase conditions, the reactions are chased with excess cold UTP, and this allows for the enzyme-bound species to be converted to products during the chase time. If chemistry is rate-limiting, then the isomerized enzyme-bound species will accumulate and consequently depending on the mechanism either a faster rate or higher amplitude of UMP addition will be observed in the pulse-chased conditions relative to the pulse-quenched conditions.

In the pulse-quenched experiment, a solution of T7 RNAP-VSR10 complex was rapidly mixed with [α-32P]UTP, and after predetermined reaction times quenched with 1 M HCl (Fig. 5A). In the pulse-chase experiment, T7 RNAP-VSR10 complex was rapidly mixed with [α-32P]UTP, and after predetermined reaction times the complex was mixed with excess of unlabeled UTP for 10 s, sufficient time for chasing intermediates, before acid quenching. The resulting 11-mer radiolabeled RNA was resolved from the free [α-32P]UTP on a denaturing polyacrylamide gel (Fig. 5B), and its molar amount was plotted against the reaction time. The kinetics of 11-mer formation under pulse-chase or pulse-quench conditions (Fig. 5C) were identical (35 s−1). In both cases, the amplitude was close to 60%. The
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![Graph of UTP and UTP incorporation over time](Image)

**FIGURE 6. Kinetics of reaction with UTP and UTPαS.** T7RNAP (2 μM) was preincubated with VSR10dsES (1 μM) and reacted with 50 μM UTP (○) or 50 μM UTPαS (□). The reactions were quenched with 1 n HCl after predetermined time intervals and analyzed by denaturing sequencing gel. The formation of 11-mer as a function of time was fit to a single exponential (Equation 1) with a rate constant of 90 ± 11 s⁻¹ for UTP and for UTPαS of 125 ± 14 s⁻¹.

experiments were repeated several times and overlapping kinetics of UMP incorporation was observed under pulse-chase and pulse-quench conditions. The experimental results indicate that an isomerized ternary complex such as a closed T7 RNAP-VSR10-UTP ternary complex does not accumulate during the reaction. We therefore conclude that the chemical step must be faster than the conformational change step accompanying the NTP binding step.

**Kinetics of UTPαS Incorporation**—The single turnover kinetics of UMPαS addition was measured to investigate the nature of the rate-limiting step in the pathway of correct nucleotide addition (18, 31). T7 RNAP was preincubated with VSR10 for 30 min and mixed with 50 μM UTP or 50 μM UTPαS (S₁ isomer) for various predetermined reaction times before quenching with 1 n HCl. The single turnover kinetics of UMP or UMPαS addition (Fig. 6) were fit to a single exponential equation (Equation 1) with rate constants of 88 ± 11 s⁻¹ and 125 ± 14 s⁻¹ for UTP and UTPαS, respectively, and amplitudes close to 70%. The ratio of rate constants k_{UMP}/k_{UMPαS}, 88/125 = 0.7, indicates that the thio-UMP is incorporated as efficiently as the normal nucleotide substrate, suggesting that the chemical step is not rate-limiting.

**Kinetics of Sequential Addition of Two Nucleotides**—The kinetics of two nucleotides was measured to investigate whether any of the steps after phosphodiester bond formation such as PPᵢ release and/or translocation of T7 RNAP are significantly slower. The kinetics of 10-mer elongation to 11-mer and 12-mer was measured in a rapid quench-flow instrument by mixing T7 RNAP-VSR10 with a mixture of UTP and CTP. Quantitation of the RNA (Fig. 7A) show the time dependent disappearance of the 10-mer, formation and decay of the 11-mer intermediate, and the formation of the final 12-mer product with lag kinetics (Fig. 7B). We observed about 40% conversion of 10-mer to 12-mer in this experiment. This amplitude appears to be variable; therefore, we assumed that only 40% of the T7 RNAP-VSR10 complex was productive in the sequential model used to fit the kinetic data. Global fitting of the kinetics to a sequential nucleotide addtion model (see "Materials and Methods") provided rate constants of 11-mer (120 s⁻¹) and 12-mer (100 s⁻¹) formation. From the observation that the rate constant of 11-mer formation is very close to that of 12-mer formation, we conclude that the steps after the incorporation of the first nucleotide such as PPᵢ release/translocation are not significantly slow to limit the addition of the next nucleotide.

**Pyrophosphorolysis**—Pyrophosphorolysis is the exact reverse reaction of nucleotide incorporation during which the product PPᵢ bound to the RNAP active site reacts with the 3’-base of the RNA to generate rNTP and an RNA shortened by one nucleotide. VSR11 substrate with 11-mer RNA that contains a U-nucleotide at the 3’-end was used to measure the exact reverse of the single nucleotide incorporation kinetics described above. T7 RNAP was preincubated with 5’-32P-labeled VSR11 and rapidly mixed with PPᵢ, for predetermined reaction times before quenching with EDTA. The 11-mer RNA was converted to 10-mer, and at longer times the 10-mer was further converted to shorter RNAs. The RNA products were resolved on a sequencing polyacrylamide gel, and 10-mer and shorter products were quantitated (Fig. 8A and B). The kinetics at 5 mM PPᵢ were fit to a single exponential (Equation 1) with a rate constant of 0.6 ± 0.1 s⁻¹. To determine the K_d of PPᵢ, the pyrophosphorolysis reactions were measured at different concentrations of PPᵢ, and the fraction of RNA products shorter than the 11-mer starting substrate was quantified and plotted against PPᵢ concentration from 0.05 to 10 mM. The PPᵢ dependence was fit to a hyperbola (Equation 2) and provided PPᵢ K_d of 1.3 ± 0.4 mM (Fig. 8C). From the PPᵢ K_d and the rate constant of pyrophos-
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**DISCUSSION**

We have dissected the minimal pathway of single nucleotide addition by T7 RNAP in the elongation phase of transcription, which describes all the steps from substrate binding to product release (Scheme 1). A synthetic RNA/DNA hybrid substrate was used to investigate the elementary steps using transient-state kinetic methods. The elongation substrate contained a promoter-free sequence and a 10-mer RNA that formed an 8-bp RNA-DNA hybrid and 2-base ssRNA tail at the 5'-end. In addition, we introduced a single-stranded DNA gap of 2-nt based on studies that showed that a nicked substrate was not efficient at catalyzing elongation (16). Several studies in the literature have shown that the promoter-free elongation substrate forms a competent complex with T7 RNAP and the RNA is properly channeled during elongation (9, 11, 14–16, 32).

**Slow Kinetics of Elongation Complex Formation**—By using a promoter-free elongation substrate, we are able to bypass the initiation phase and measure the pre-steady-state kinetics of single nucleotide incorporation during the elongation phase. The first step in the kinetic pathway is the binding of T7 RNAP to the promoter-free elongation substrate. Our studies showed that at equilibrium, T7 RNAP formed a tight complex with the elongation substrate with a $K_d$ of 96 nM. During the course of our kinetic measurements, we observed that the elongation kinetics were slow when T7 RNAP was not preincubated with the promoter-free elongation substrate. T7 RNAP concentration-dependent studies indicated that a step before or after T7 RNAP-elongation substrate complex formation is slow. Limited proteolysis studies confirmed that the initiation to elongation conformational refolding of T7 RNAP is the slow step that limits the observed rate of elongation. T7 RNAP free in solution exists largely in the initiation conformation (33). This is consistent with the observation that T7 RNAP binds to a promoter DNA fragment at close to diffusion-limited rate constants (27). To bind the promoter-free elongation substrate, T7 RNAP has to undergo refolding of the N-terminal domain (9, 11). Our studies indicated that T7 RNAP refolding when initiated by the addition of a promoter-free substrate is a very slow process. Our measurements indicated that protein refolding rate constant (0.01–0.03 s$^{-1}$) is 8000 times slower than the NTP addition rate constant (Scheme 1). The promoter-free elongation substrate, T7 RNAP (400 nM) and VSR10 (100 nM) were preincubated and reacted with limiting UTP (200 nM) and varying amounts of PPi (0.5–5 mM) for 5–30 s. The 10- and 11-mer RNAs were resolved on a denaturing sequencing gel and quantitated at each PPi concentration. The reaction reached equilibrium within 5 s as expected. The ratio of 10-mer to 11-mer remained constant from 5 to 30 s (supplemental Fig. 1). An overall equilibrium constant of $7.5 \times 10^4$ was calculated from the average amounts of 10-mer and 11-mer and from the relationship: $K_{eq} = [11\text{-mer}] [\text{PPi}]_{\text{free}}/[10\text{-mer}] [\text{NTP}]_{\text{free}}$. From this value of the overall equilibrium constant, the internal equilibrium constant $K_2$ for UMP incorporation was calculated as 500.

**overall and Internal Equilibrium Constants of Single Nucleotide Addition**—The minimal pathway of single nucleotide incorporation consists of the steps of NTP binding (represented by the equilibrium constant $K_1$), chemical step ($K_2$), and PPi release ($K_3$). The overall equilibrium constant of single nucleotide incorporation $= K_1 K_2 K_3$. The experiments described above have provided the values of $K_1$ ($1/K_1$ of UTP) and $K_3$ ($K_3$ of PPi). Knowing the overall equilibrium constant therefore provides the value of the internal equilibrium constant $K_2$, which includes the steps of chemistry and conformational changes before and after chemistry. To measure the overall equilibrium constant of 10-mer to 11-mer conversion in the

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**FIGURE 8. Single-turnover pyrophosphorolysis kinetics during transcription elongation.** A, T7 RNAP (2 µM) was preincubated with VSR11 (1 µM) and rapidly mixed with 5 mM PPi in the quenched-flow instrument. After predetermined time intervals (lanes 0–10 represent times, 0, 0.008, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 20, and 30 s) the reactions were quenched with 0.3 M EDTA and run on a denaturing gel. B, RNA products (<11-mer) were quantitated and plotted versus time, and the kinetics was fit to a single exponential equation (Equation 1) to yield a rate constant of 0.6 ± 0.1 s$^{-1}$. C, pyrophosphorolysis reactions were repeated at various concentrations of PPi (0.05–10 mM). The pyrophosphorolysis products at 0.5 s were plotted against total PPi concentrations, and the data were fit to a hyperbola (Equation 3) with a $K_d$ of 1.3 ± 0.4 mM.
elaboration transition (34) showed a 10-fold faster conformational change relative to WT T7 RNAP (supplemental Fig. 2). Future studies of this mutant should provide additional insights into the refolding process.

**Kinetic Pathway of Single Nucleotide Incorporation during Transcription Elongation**—The minimal pathway of nucleotide incorporation starting from free T7 RNAP consists of the steps outlined in Scheme 1. As discussed above, T7 RNAP forms an elongation competent complex with the promoter-free substrate at a slow rate. The correct UTP binds to T7 RNAP-VSR10 to form a ternary complex with a \( k_d \) of 80 \( \mu \)M. The incorporation of UMP into the RNA primer occurs at a maximum rate constant of 220 s\(^{-1}\). The efficiency of correct UTP incorporation, \( k_{pol}/k_d \) by T7 RNAP is therefore \( 3 \times 10^6 \text{M}^{-1}\text{s}^{-1} \), which is comparable with the values of DNA polymerases (18, 19, 22). Comparison of the NTP \( K_d \) values of polymerases indicates that DNA polymerases (18, 19, 22) and reverse transcriptases (25, 35) bind correct NTPs with higher affinities (\( K_d \) values around 2–20 \( \mu \)M) relative to T7 RNAP. Interestingly, RNA polymerases such as *E. coli* RNAP (24), human RNAP II (21), and poliovirus RNA-dependent RNAP (36) bind correct NTPs with a relatively weak affinity, similar to that of T7 RNAP. A possible rationale for the higher \( K_d \) values of RNA polymerases versus DNA polymerases is the higher concentrations of rNTPs in the cell relative to dNTPs.

Most DNA polymerases bind dNTPs by a two-step mechanism, which includes a rapid NTP binding step followed by an isomerization step prior to chemistry (18, 22, 25, 36, 37). This induced fit mechanism of correct NTP binding has been proposed to play a role in assuring high fidelity in polymerases (18, 23, 38–40). Structural and recent fluorescence studies indicate that the NTP-induced isomerization step represents the movement of the O helix in polymerases (23, 41, 42). Structures of T7 RNAP show a correct NTP bound in two different conformational states providing direct evidence for isomerized ternary complexes (2, 17). In the open conformation of T7 RNAP, a correct NTP is bound to a preinsertion site along the O helix making base specific contacts with the templating base without interacting with the two metal ions required for chemistry. In the closed conformation of T7 RNAP, the NTP is bound in the insertion site, where it makes Watson–Crick interactions with the template base. The phosphates of the NTP are coordinated to the two metal ions in the insertion site and hence the ternary complex represents the structure of the isomerized complex ready to undergo chemistry. It has been proposed that the NTP-induced isomerization step must play a role in selecting a correctly base-paired nucleotide and in discriminating ribo- versus deoxyribonucleotide during RNA synthesis.

To determine whether the 220 s\(^{-1}\) rate constant of UMP incorporation into the primer in the elongation substrate represents the rate of isomerization or the rate of the chemical step, we investigated the kinetics of UTP\(\alpha\)S incorporation, UMP addition under pulse-quench and pulse-chase conditions, and the sequential addition of two nucleotides. The pulse-chase experiments failed to detect the accumulation of any isomerized ternary complex before the chemical step. These results indicated that the isomerization step is slow, and the chemical step of NMP incorporation is fast. To investigate whether the steps after the chemical step such as PPi release/translocation are slow, we measured the rate constant of the second nucleotide addition in a reaction where two NTPs were added sequentially. We found that the rate constants of first and second nucleotide incorporation in the sequential reaction with UTP and CTP were similar, which indicated that the steps after the chemical step are not slow in a single nucleotide addition cycle. The results support a mechanism in which an isomerization step accompanying NTP binding before the chemical step is rate-limiting. Based on available structural data on T7 RNAP, we propose an interesting possibility that the preinsertion to insertion conformational change of NTP observed in structural studies of T7 RNAP is the rate-limiting step. Further studies are needed to investigate this proposal, for which methods will have to be developed to directly assay the isomerization step to measure its intrinsic rate constant.

After NMP is added to the primer, T7 RNAP releases the PPi and translocates to allow the next nucleotide addition. Translocation is one of the least understood steps in the mechanism of polymerases. Two models, power-stroke and Brownian ratchet, have been proposed to explain translocation. The power-stroke mechanism postulates that the PPi release drives translocation (17), whereas the Brownian ratchet model postulates that NTP binding biases the translocated state (43). The present studies do not provide any additional insights to distinguish between the two models. In the presence of PPi, T7 RNAP catalyzes the reverse reaction of pyrophosphorolysis, and detailed experiments provided PPi \( K_d \) of 1.2 mM and maximum rate constant of the reverse reaction equal to 0.8 s\(^{-1}\). Our measurement of the overall equilibrium constant for single UMP
addition indicated that UMP addition is accompanied by a free energy change of \(-5.5\) kcal/mol. From the overall equilibrium constant and the \(K_v\) values of UTP and PP\(_i\), an internal equilibrium constant of 500 was calculated for UMP addition. The internal equilibrium constant includes the chemical step and any other conformational changes before and after. Nucleotide addition is therefore more reversible on the RNAP active site as noted for DNA polymerases (18). In the VSR10 substrate used in these studies, T7 RNAP does not have to unwind the downstream DNA to add UMP to the primer because the template base is single stranded. It will be interesting to see how the free energies of the reaction will change when T7 RNAP needs to melt the downstream template DNA.

In conclusion, these detailed studies of RNA synthesis by T7 RNAP in the elongation mode have revealed the minimal pathway of a single ribonucleotide addition. There are many steps that need to be characterized in more detail such as the isomerization step, the PP\(_i\) release kinetics, and translocation kinetics. These will require the development of direct assays that can probe the dynamics of conformational changes and translocation. The reported studies, however, provide a minimal kinetic framework to investigate structure-function and fidelity of RNA synthesis.

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REFERENCES

1. Jeruzalmi, D., and Steitz, T. A. (1998) EMBO J. 17, 4101–4113
2. Temiakov, D., Patlan, V., Anikin, M., McAllister, W. T., Yokoyama, S., and Vasylyev, D. G. (2004) Cell 116, 381–391
3. Jia, Y., and Patel, S. S. (1997) J. Biol. Chem. 272, 30147–30153
4. Jia, Y., and Patel, S. S. (1997) Biochemistry 36, 4223–4232
5. Guo, Q., Nayak, D., Brieza, L. G., and Sousa, R. (2005) J. Mol. Biol. 353, 256–270
6. Ma, K., Temiakov, D., Anikin, M., and McAllister, W. T. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 17612–17617
7. Ma, K., Temiakov, D., Jiang, M., Anikin, M., and McAllister, W. T. (2002) J. Biol. Chem. 277, 43206–43215
8. Bandwar, R. P., Tang, G. Q., and Patel, S. S. (2006) J. Mol. Biol. 360, 466–483
9. Yin, Y. W., and Steitz, T. A. (2002) Science 298, 1387–1395
10. Steitz, T. A. (2004) Curr. Opin. Struct. Biol. 14, 4–9
11. Tahirov, T. H., Temiakov, D., Anikin, M., Patlan, V., McAllister, W. T., Vasylyev, D. G., and Yokoyama, S. (2002) Nature 420, 43–50
12. Huang, J., and Sousa, R. (2000) J. Mol. Biol. 303, 347–358
13. Temiakov, D., Mentesana, P. E., Ma, K., Musteau, A., Borukhov, S., and McAllister, W. T. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 14109–14114
14. Daube, S., and von Hippel, P. H. (1992) Science 258, 1320–1324
15. Daube, S., and von Hippel, P. H. (1994) Biochemistry 33, 340–347
16. Temiakov, D., Anikin, M., and McAllister, W. T. (2002) J. Biol. Chem. 277, 47035–47043
17. Yin, Y. W., and Steitz, T. A. (2004) Cell 116, 393–404
18. Patel, S. S., Wong, I., and Johnson, K. A. (1991) Biochemistry 30, 511–525
19. Benkovic, S. J., and Cameron, C. E. (1995) Methods Enzymol. 262, 257–269
20. Burton, Z. F., Feig, M., Gong, X. Q., Zhang, C., Nedialkow, Y. A., and Xiong, Y. (2005) Biochem. Cell Biol. 83, 486–496
21. Nedialkow, Y. A., Gong, X. Q., Hovde, S. L., Yamaguchi, Y., Handa, H., Geiger, J. H., Yan, H., and Burton, Z. F. (2003) J. Biol. Chem. 278, 18303–18312
22. Washington, M. T., Prakash, L., and Prakash, S. (2001) Cell 107, 917–927
23. Double, S., Sawaya, M. R., and Ellenberger, T. (1999) Structure (Camb.) 7, R31–R35
24. Foster, J. E., Holmes, S. F., and Erie, D. A. (2001) Cell 106, 243–252
25. Hsieh, J. C., Zinnem, S., and Modrich, P. (1993) J. Biol. Chem. 268, 24607–24613
26. Davanloo, P., Rosenberg, A. H., Dunn, J. J., and Studier, F. W. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2035–2039
27. Jia, Y., Kumar, A., and Patel, S. S. (1996) J. Biol. Chem. 271, 30451–30458
28. King, G. C., Martin, C. T., Pham, T. T., and Coleman, J. E. (1986) Biochemistry 25, 36–40
29. Wong, I., and Lohman, T. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5428–5432
30. Hingorani, M. M., and Patel, S. S. (1993) Biochemistry 32, 12478–12487
31. Kuchta, R. D., Cowart, M., Allen, D., and Benkovic, S. J. (1988) Biochem. Soc. Trans. 16, 947–949
32. Datta, K., Johnson, N. P., and von Hippel, P. H. (2006) J. Mol. Biol. 360, 800–813
33. Sousa, R., Chung, J. Y., Rose, J. P., and Wang, B. C. (1993) Nature 364, 593–599
34. Guillerez, J., Lopez, P. J., Proux, F., Launay, H., and Dreyfus, M. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 5958–5963
35. Pandey, M., Patel, S., and Gabriel, A. (2004) J. Biol. Chem. 279, 47840–47848
36. Arnold, J. J., and Cameron, C. E. (2004) Biochemistry 43, 5126–5137
37. Dahlberg, M. E., and Benkovic, S. J. (1991) Biochemistry 30, 4835–4843
38. Double, S., and Ellenberger, T. (1998) Curr. Opin. Struct. Biol. 8, 704–712
39. Wong, I., Patel, S. S., and Johnson, K. A. (1991) Biochemistry 30, 526–537
40. Tsai, Y. C., and Johnson, K. A. (2006) Biochemistry 45, 9675–9687
41. Li, Y., Korolev, S., and Waksman, G. (1998) EMBO J. 17, 7514–7525
42. Johnson, S. J., Taylor, J. S., and Beese, L. S. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 3895–3900
43. Guo, Q., and Sousa, R. (2006) J. Mol. Biol. 358, 241–254