A Tunable Ratchet Driving Human RNA Polymerase II Translocation Adjusted by Accurately Templated Nucleoside Triphosphates Loaded at Downstream Sites and by Elongation Factors

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When nucleoside triphosphate (NTP) substrates and α-amanitin are added to a human RNA polymerase II elongation complex simultaneously, the reaction becomes stalled in the core of the bond synthesis mechanism. The mode of stalling is influenced by NTP substrates at the active site and at downstream sites and by transcription factor IF (TFII) and TFII-S. NTP substrates templated at i+2, i+3, and i+4 downstream DNA sites can reverse the previously stable binding of an NTP loaded at the i+1 substrate site. Deoxy-(d)NTPs and NDPs (nucleoside diphosphates) do not substitute for NTPs at the i+2 and i+3 positions (considered together) or the i+4, i+5, and i+6 positions (considered together). The mode of stalling is altered by changing the number of downstream template sites that are accurately occupied by NTPs and by changing NTP concentration. In the presence of the translocation blocker α-amanitin, a steady state condition is established in which RNA polymerase II stably loads an NTP substrate at i+1 and forms a phosphodiester bond but cannot rapidly complete bond synthesis by releasing pyrophosphate. These observations support a role for incoming NTP substrates in stimulating translocation; results appear inconsistent with the secondary pore being the sole route of NTP entry for human RNA polymerase II, and results indicate mechanisms of dynamic error avoidance and error correction during rapid RNA synthesis.

A goal of transient state kinetic analyses is to reveal the internal mechanism of an enzyme reaction by observing synchronized millisecond events on a millisecond time scale. In this work, the mushroom toxin α-amanitin is utilized as a transient state inhibitor, locking human RNA polymerase II in the core of the elongation mechanism. By altering the conditions of stalling and by employing two distinct reaction quenching methods, details of the core mechanism of human RNA polymerase II become apparent.

Recent x-ray crystal structures of Thermus thermophilus RNA polymerase elongation complexes indicate a simple thermal ratchet mechanism for elongation (1, 2), with nucleoside triphosphate (NTP) substrates loading through the secondary pore, a solvent accessible channel, to the active site (designated i+1). Very little space is available in these structures to load NTPs through the main enzyme channel, which holds the DNA duplex and RNA-DNA hybrid. The downstream transcription bubble is closed at the i+2 position by base pairing, so it is difficult to imagine how NTP substrates could interact at i+2 or i+3 template sites. T. thermophilus β Arg^{422} makes a specific contact to the i+1 DNA template phosphate, and appears to provide a specific mechanism for closing the downstream bubble at the i+2 position. This mechanism for closing the downstream bubble is not conserved in human or yeast RNA polymerase II, in which the corresponding residue to β Arg^{422} is Rpb2 Gly^{493} (human) and Rpb2 Gly^{506} (yeast). Furthermore, even if the downstream bubble were open in the T. thermophilus structure, little space is available to flip a dNMP-NTP base pair from the i+2 to the i+1 active site. T. thermophilus structures further indicate that a closed conformation of the “trigger loop- trigger helix” assembly may support the catalytic intermediate, during phosphodiester bond formation.

According to a thermal ratchet mechanism, translocation and pyrophosphate release should be rapid and spontaneous, and neither process can be driven by an incoming, templated NTP substrate. In this report, however, we present data that appear inconsistent with a simple thermal ratchet mechanism for elongation catalyzed by human RNA polymerase II. Furthermore, the data are most consistent with an open configuration of the trigger loop supporting phosphodiester bond addition. Data indicate that the secondary pore is unlikely to be the sole route of NTP entry into human RNA polymerase II. These experiments indicate a ratchet driving forward translocation that is regulated by incoming NTP substrates and transcriptional elongation factors. Elongation catalyzed by human RNA polymerase II, therefore, indicates features that are not apparent in images of the T. thermophilus elongation complex.

Two models have been invoked to describe NTP loading by multisubunit RNA polymerases. The secondary pore NTP loading hypothesis posits that NTPs load only to the i+1 active

The abbreviations used are: NTP, nucleoside triphosphate; TF, transcription factor; d, deoxy.
site and that loading to i+2, i+3, and i+4 downstream sites is impossible (3–9). According to the secondary pore NTP loading hypothesis, pyrophosphate release, and translocation are expected to be rapid and spontaneous, and NTP loading is likely to be rate limiting (4). The NTP-driven translocation hypothesis, by contrast, posits NTP loading at the i+1 and i+2 sites and potential sites further downstream (10–15). The secondary pore is a deep and narrow channel extending from the “funnel” on the surface of the RNA polymerase II molecule to the deeply buried active site (4, 7). The pore appears to be too narrow to exchange two NTPs, so, misloading of an NTP substrate (the usual case according to the secondary pore NTP loading hypothesis), requires release of the incorrect NTP followed by loading of the correct (or another incorrect) NTP (or NDP or other small molecule). In addition to being about 15-Å deep and having a minimum diameter of about 7 Å (NTPs have a minimum diameter of about 6 Å), the secondary pore has significant negative electrostatic potential (4). Because of the negative electrostatics of the pore, Kornberg and co-workers estimated that NTP loading might be rate limiting (20–30 s

Because stable NTP-Mg²⁺ loading can be very rapid, the rate-limiting component of this interval has been interpreted as translocation coupled to pyrophosphate release. As indicated above, the T. thermophilus RNA polymerase elongation complex structures appear most consistent with the secondary pore NTP loading model for bacterial RNA polymerase (1, 2).

The NTP-driven translocation hypothesis requires NTP loading to downstream template sites, and, therefore, posits an unpaired downstream bubble. Based on available x-ray crystal structure data, however, the extent of opening of the downstream bubble remains controversial (1, 2, 6, 17–19). All available yeast RNA polymerase II elongation complex structures are open in the downstream region. Some of these structures are open to the i+5 or i+6 downstream positions (18), and base pairs that have the capacity to form in the downstream region are not observed to form (6, 18, 19). A T. thermophilus RNA polymerase elongation complex was observed to close in the downstream region (closed at i+2), although a key residue for closure, β R422 (corresponding to Saccharomyces cerevisiae Rpb2 G506), is not conserved in RNA polymerase II (2). If RNA polymerase II has the capacity to close the downstream bubble, therefore, RNA polymerase II uses different contacts for closure than T. thermophilus RNA polymerase. Some chemical reactivity studies support a model of variable downstream template opening for multisubunit RNA polymerases (20, 21), and functional studies indicate that the downstream bubble of human RNA polymerase II can interact with NTP substrates (12).

According to the NTP-driven translocation model, an NTP loads to the active site by transfer as a dNMP-NTP base pair from the i+2 to the i+1 site. NTP-driven translocation appears to be coupled to pyrophosphate release, linking initiation of each new bond addition cycle to the end of the previous cycle. According to this model, incorrect NTPs rarely load to the active site because NTPs are not present on the DNA template prior to active site loading, enhancing fidelity. In this model, the secondary pore is the route of pyrophosphate release and may be a route for expulsion of NTP substrates rejected at the active site. Furthermore, NTP-driven translocation can be utilized as a means to dislodge an NTP from the active site in response to a translocation block, indicating a role for downstream NTPs in dynamic error avoidance and error correction (10, 12). Normally, a translocation block signals a transcription error.

In this report, we show templated effects of i+2, i+3, and i+4 NTPs on the fate of an NTP substrate loaded at i+1. This result demonstrates the major tenet of the NTP-driven translocation model: that NTPs load to downstream template sites while the active site is occupied. It appears from these studies that translocation pressure can be tuned by limiting the number of downstream sites that are accurately filled by NTPs and by altering the concentrations of accurately templated downstream NTPs. Elongation factors TFII F and TFIIS also appear to adjust translocation pressure. We present a model for a tunable ratchet driving translocation that responds to accurately templated downstream NTPs and elongation factors, and that couples NTP-driven translocation to NTP substrate tightening at the i+1 active site.

EXPERIMENTAL PROCEDURES

Isomerization reversal experiments and quantification of data were done essentially as described (12). Briefly, human C40 (40 nucleotide RNA ending in 3′-biotinylatedCMP) RNA polymerase II elongation complexes were formed by accurate initiation from the adenovirus major late promoter, using an ApC dinucleotide primer. The transcribed sequence was modified (Fig. 1) to allow C40 synthesis without substantial overrun. 5′-biotinylated DNA templates were immobilized on streptavidin beads. All reactions were done at 25°C. C40 complexes were radiolabeled with [α-32P]CTP. C40 complexes were extended to A43 by addition of 100 μM ATP for 30 s (s). During the 30-s incubation, A43 complexes were transferred into the left sample port of the KinTek RQF-3 Rapid Chemical Quench Flow instrument and then extended by computer-controlled rapid mixing. Reactions were quenched with EDTA or HCl, as indicated in individual protocols.
To reactions in which TFIIS was added during the ATP running start, 20 μM CTP and UTP were added during the preincubation to maintain the C40 stall position. In this protocol, CTP and UTP are diluted to a working concentration of 5 μM by two equal volume mixings. When TFIIS was not present during the preincubation, CTP and UTP were also omitted (except as specified in individual protocols). To compensate for inconsistencies in sample recovery or gel loading, transcripts were quantified as a ratio of relevant bands within each gel lane. Gel lanes were analyzed independently for percent of signal present in A43 plus all longer transcripts. The Molecular Dynamics Phosphorimager was calibrated and found to give a nearly linear response over a range of at least 20,000-fold 32P exposure above a detectable background. A linear response over a 100-fold exposure range is sufficient to detect all relevant transcripts.

Molecular images were prepared using the graphics program Visual Molecular Dynamics (22). Structures were modified (i+2 and i+3 NTPs placed by modeling, DNA strands extended, non-template DNA placed), as described previously (10).

RESULTS

Robust Isomerization Reversal—In Fig. 1, we show an isomerization reversal experiment modeled on those of Gong et al. (12). Interestingly, the reaction is observed to advance and then retreat, particularly when accurately templated downstream NTPs are present (Fig. 1A). In the absence of accurately templated downstream NTPs, reversal is diminished and transient (Fig. 1B). Phosphorimager quantification of the experiment is shown in Fig. 1C, with the data represented on two time scales (1 and 0.2 s (s)). Normally, enzymatic reactions do not advance and then retreat. So how can such an unusual observation be understood?

"Isomerization reversal" refers to reversal of stable NTP loading to the i+1 active site of RNA polymerase II in response to: 1) a translocation block (α-amanitin); and 2) accurately templated downstream NTPs. Neither α-amanitin nor downstream NTPs induce strong reversal by themselves (supplemental materials, Fig. S1).

The reaction design is shown at the top of Fig. 1. Human RNA polymerase II is used to synthesize C40 elongation complexes (a 40-nucleotide RNA ending in 3'5'-CMP). The encoded RNA sequence downstream of C40 is 40CAAAGCCUUU49. Complexes are combined with elongation factor TFIIF, which stimulates forward translocation. On the bench top, 100 μM ATP and elongation factor TFIIS, which stimulates RNA dinucleotide cleavage and re-start, are added for 30 s to advance the RNA to A43. The combination of TFIIF and TFIIS promotes forward translocation (TFIIF) and increases the fraction of elongation complexes on the active synthesis pathway (TFIIS). During the

The gels shown in A and B. Two time scales (1 and 0.2 s) are shown. G44 + % indicates G44 plus all longer transcripts, indicated as percent of total (A43 and longer transcripts). Synthesis of the G44 bond is strongly and stably reversed in response to both α-amanitin and accurately templated downstream NTPs, using the EDTA quench procedure.
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30-s stall at A43, the elongation complex preparation is transferred to the sample port of the KinTek RQF-3 rapid chemical quench flow instrument. Using the rapid mixing device, the sample is combined with 2.5 mM GTP, 2.5 mM CTP, 2.5 mM UTP, and 1 mM α-amanitin for various times (0.002 to 1 s) before quenching with 0.5 mM EDTA (Fig. 1A). The G44 signal is highest at the 0.002 and 0.005-s time points and then decreases. Despite the abundance of CTP substrate, a very small amount of C45 is detected, starting at 0.05 s. Little C45 is synthesized, and no longer transcripts are detected above background, because α-amanitin is added to the reaction, and α-amanitin blocks translocation (11, 12, 23). α-amanitin appears to allow synthesis of G44 primarily from those elongation complexes that were initially post-translocated at the A43 stall position. After G44 synthesis, C45 synthesis requires translocation, so little C45 and no longer transcripts are observed, indicating that α-amanitin effectively blocks translocation. While GTP for G44 synthesis occupies the i+1 active site, CTP can potentially occupy the i+2 and i+3 downstream template sites, and UTP can potentially occupy the i+4, i+5, and i+6 downstream sites, depending on the availability of downstream DNA for specific NTP interactions.

This experiment demonstrates 69% transient (0.2 s) and 58% sustained (1 s) isomerization reversal with added CTP and UTP, calculated as 100% — ((G44 + % (0.2 or 1 s))/(G44 + % (burst; 0.002 or 0.005 s)) × 100%). G44+ indicates G44 plus all longer transcripts (in this case, C45). We observe greater isomerization reversal (IR = 69% or 58% in this experiment) than observed in similar experiments using the same template in Gong et al. (12) (IR = 25%) because we used 1 mM α-amanitin in this experiment and 0.5 mM α-amanitin in comparable experiments in Gong et al. (12) (supplemental Fig. S2). Also, UTP, which stimulates reversal, was omitted in the experiments of Gong et al. (12).

When 2.5 mM CTP and UTP are substituted with 5 mM ATP (an NTP that is not accurately templated at adjacent downstream sites), isomerization reversal is significantly reduced and is overcome with longer times of incubation (IR = 25% at 0.2 s and IR = 0% at 1 s). Because the extent of reversal with negative control samples varies with the time of EDTA quench addition, reversal appears to be primarily determined by the activity of GTP- and/or ATP-Mg2+. Because of prior addition, all reactions in Fig. 1 contain 5 μM CTP and UTP (added to maintain the C40 stall position in the presence of the RNA cleavage factor TFIIHS). 5 μM CTP and UTP may be sufficient to contribute weakly to reversal. We suggest that NTPs that are not accurately templated may interact weakly at downstream sites and promote transient reversal. When accurately templated downstream NTPs are limiting, GTP-Mg2+ becomes stabilized in the i+1 active site and becomes increasingly resistant to reversal with the passage of time.

The observation of reversal with control samples is of interest, because it shows isomerization reversal in response to a translocation block. Furthermore, a mechanism for exchange of the i+1 GTP substrate is indicated that responds to free pools of NTPs. The data indicate that free NTP-Mg2+ is necessary to displace the i+1 GTP substrate, because little or no reversal is detected with the earliest times of EDTA addition (0.002–0.005 s), a treatment that eliminates free NTP-Mg2+ but not GTP-Mg2+ tightened in the active site. Isomerization reversal, therefore, 1) requires free NTP-Mg2+, 2) is strongly stimulated by accurately templated downstream NTP-Mg2+, and 3) appears to be a more dramatic phenomenon than can be fully scored using the current reaction design because of rapid GTP-Mg2+ re-loading after release, prior to EDTA addition.

Because the experiment in Fig. 1 indicates the activity of accurately templated CTP and possibly ATP at downstream sites, i.e. i+2, i+3, and i+4, on the fate of the GTP loaded and initially tightened in the active site (i+1), this experiment indicates NTP loading at downstream sites while the active site is occupied. EDTA quenching is expected to chelate Mg2+ from downstream CTP- and UTP-Mg2+ very rapidly. The i+1 GTP-Mg2+, however, can be protected within the active site from EDTA quenching, and can proceed to form the G44 phosphodiester bond after EDTA addition. We posit that, because of NTP-driven translocation, CTP and UTP strain against the α-amanitin translocation block, resulting in expulsion of a large fraction of the i+1 GTP-Mg2+, which had previously been fated for G44 bond synthesis. Thus, RNA polymerase II could utilize incoming NTPs in dynamic error avoidance and error correction, to relieve a translocation block (12). Essentially, RNA polymerase II appears to react to the α-amanitin translocation block by weakening binding of the i+1 GTP in the active site. Because GTP is the accurately templated substrate for G44 synthesis, RNA polymerase II cannot easily reject GTP, limiting the extent of isomerization reversal. As a result, only 69 or 58% reversal is achieved.

Chelation of Mg2+ at early times (0.002 and 0.005 s) results in robust G44 synthesis, because of rapid inactivation of CTP- and UTP-Mg2+. Chelation of Mg2+ at a later time (i.e. 0.02 s) results in dramatically reduced G44 synthesis, apparently because of the activities of CTP and UTP acting at downstream template positions. In supplemental materials (Fig. S3), a similar isomerization reversal experiment is shown in which CTP and UTP are replaced by CTP and ATP. Because substituting UTP with ATP slightly reduces isomerization reversal, it appears that i+4 UTP contributes to reversal.

An important feature of the isomerization reversal experiment is that α-amanitin is used on a millisecond time scale as a transient state inhibitor. In this reaction design, α-amanitin is added together with accurately templated substrate and downstream NTPs, and α-amanitin exerts its effects within milliseconds. RNA polymerases catalyze an elongation step on about a 5–50 ms time scale, underscoring the need for millisecond time resolution in analysis of single bond additions (11–15). In isomerization reversal experiments, conditions for forward synthesis and inhibition by α-amanitin are established together, and what results is a glimpse of the internal mechanism of RNA synthesis, as the reaction is driven forward by substrate and blocked at the translocation step by inhibitor.

NTPs Occupy Template At Least to i+4—I In previous work, accurate NTP interactions were detected at the i+2 and i+3 downstream sites, while the i+1 substrate site was occupied (12). Here, we report templated interactions of NTPs at least to the i+4 position, while the i+1 site is occupied. The assays shown in Fig. 2 differ from those shown in Fig. 1 in that TFIIHS...
was omitted, reducing the magnitude of the G44 transcription signal, but, perhaps, increasing the translocation force generated by TFIIF (in the absence of TFIIIS). Furthermore, α-amanitin was added at 0.5 mM, instead of 1 mM. Because we wished to test many conditions and many time points, we needed to reduce the cost incurred from adding the higher concentration of α-amanitin. Also, by slightly slowing the rate of α-amanitin binding by limiting concentration, some elongation complexes may be able to further advance through the bond addition mechanism before inhibition is fully established. One goal of the transient state inhibition experiment is to reveal the inner mechanism of RNA polymerase II elongation.

Fig. 2 shows isomerization reversal experiments with addition of different downstream NTPs and analogues to test the requirements for occupying the i+2 and i+3 CTP sites (considered together) and the i+4, i+5, and i+6 UTP sites (considered together), while the i+1 active site is occupied by GTP. Precisely how many downstream DNA template sites are bound by NTP substrates has not been determined, so we wondered whether the i+4 site is accurately occupied. For this experiment, concentrations of downstream NTPs were reduced 5-fold compared with Fig. 1, from 2.5 to 0.5 mM, close to physiological levels, to show that physiological concentrations of downstream NTPs are sufficient to induce isomerization reversal. Reducing the concentration of CTP (i+2 and i+3) in the reversal experiment is also expected to accentuate any effects of UTP (i+4, i+5, and i+6). Because TFIIIS was omitted from these reactions, there was no need to include traces of CTP and UTP to maintain the C40 stall position, before the addition of substrate NTPs, so CTP and UTP were not added to these reactions during the preincubation.

Depending on the combinations of NTPs added, reversal curves separate into 3 classes (Fig. 2). In every case, 2.5 mM GTP is included as the substrate for G44 synthesis because this concentration allows very rapid binding of GTP at i+1. α-amanitin is added at 0.5 mM. Downstream NTPs are added at 0.5 mM. Reactions are stopped by addition of EDTA. The combination of GTP, CTP, and UTP induces the most robust reversal (IR = 50% at 0.2 s). Omission of CTP results in almost no sustained reversal, because the i+2 and i+3 sites must be accurately occupied by CTP to strongly stimulate reversal. Reactions lacking CTP, therefore, result in limited, transient reversal, and serve as controls for the importance of downstream, accurately templated NTPs.

GTP, UTP, ATP, and dCTP will not substitute for CTP, at the i+2 and i+3 positions (IR = 8–10% at 0.2 s) (Fig. 2). CDP added in place of CTP also does not stimulate robust reversal, although the curve does not precisely overlay with the other control curves lacking CTP (IR = 14% at 0.2 s; green stars; the curve has a reduced burst height). The slight, transient reversal observed in control samples lacking CTP can be attributed to effects of non-templated NTP-Mg2+ and α-amanitin. Probably, inaccurately templated NTPs contribute to reversal in control experiments, because different times of EDTA addition result in different responses, indicating that the mechanism for reversal involves EDTA chelating Mg2+ from NTP-Mg2+. It is not possible to exclude non-templated NTPs from the reaction, because i+1 substrate GTP must be added for G44 bond synthesis.

Surprisingly, UTP, which is accurately templated at the i+4, i+5, and i+6 downstream sites, stimulates isomerization reversal at G44 (Fig. 2). In the presence of CTP, which is required for reversal, withholding UTP from the reaction reduces reversal. This result indicates that NTPs can simultaneously and accurately occupy the i+1 (GTP; active site), i+2 (CTP), i+3 (CTP), and i+4 (UTP) sites, at a minimum. Whether NTPs can interact at the i+5 (UTP) and i+6 (UTP) sites has not been determined. Because dTTP and dUTP (2′-dideoxy UTP) do not stimulate reversal (in the presence of CTP), dTTP and dUTP cannot substitute for UTP at the i+4, i+5, and i+6 positions (considered together). GTP, ATP, CTP, and UDP also fail to substitute for UTP in the reversal experiment.

These results demonstrate the high selectivity of downstream NTP binding. Binding is accurately templated, but the capacity for accurate base-pairing to template is not sufficient. NTPs cannot be substituted with NDPs or dNTPs, even when these analogues have comparable base-pairing specificity. This result demonstrates selectivity of NTP interactions at downstream sites, consistent with the high fidelity of RNA synthesis catalyzed by multisubunit RNA polymerases.

In Fig. 2, we demonstrate strong suppressive effects of UTP on synthesis of G44, in the presence of CTP and α-amanitin. As predicted by the NTP-driven translocation hypothesis, UTP also suppresses synthesis of C45 and C46 in the presence of the
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UDP fail to replace UTP in reversal reactions. Once again, this result is most consistent with the NTP-driven translocation model. This result does not appear consistent with the secondary pore NTP loading hypothesis.

The Core Mechanism of RNA Synthesis—To gain more insight into the reversal experiment, we compared EDTA quenched reactions to reactions quenched with HCl. In addition, to gain insight into the effects of accurately templated downstream NTPs, the concentrations of CTP and UTP were varied (Fig. 4). Stopping the reaction with acid shows the timing of synthesis of the phosphodiester bond (12, 14, 15, 24, 25). Bond synthesis, however, may remain reversible until pyrophosphate is released. If the phosphodiester bond has been formed, however, the bond cannot reverse after acid addition. If the bond has not yet formed, the bond cannot be formed after HCl addition. By contrast, after EDTA quench addition, two outcomes are possible: 1) phosphodiester bond formation; and 2) phosphodiester bond reversal. Because an NTP-Mg$^{2+}$ can be sequestered from EDTA chelation within the i+1 active site, the NTP-Mg$^{2+}$ can progress to synthesize a phosphodiester bond after EDTA addition. Alternatively, the NTP-Mg$^{2+}$ can potentially be destabilized in the active site after EDTA addition, as in the isomerization reversal experiment.

An ongoing issue in RNA synthesis is the dynamics of the pyrophosphate release step and its link to translocation. Based on transient state kinetic analyses of human RNA polymerase II elongation, the Burton laboratory posits that pyrophosphate release is coupled to NTP-driven translocation, explaining the surprisingly high NTP dependence of the rate-limiting step during elongation that corresponds to both translocation and pyrophosphate release (12, 14, 15). By contrast, the secondary pore NTP loading hypothesis seems to require that pyrophosphate release and translocation be rapid, spontaneous and NTP-independent. Without these features in a secondary pore NTP loading model, RNA polymerase would pause after synthesis of every bond, independent of substrate NTP concentration. According to estimates of the Kornberg laboratory, for NTPs to enter the active site through the secondary pore by diffusion, NTP loading would likely be rate limiting for RNA synthesis (20–30 nucleotides s$^{-1}$) (4). So far as we can discern from rapid kinetic studies, NTP loading is not rate limiting for RNA synthesis (>1450 ± 330 s$^{-1}$), and pyrophosphate release is not rapid and spontaneous (about 30 s$^{-1}$ and coupled to the NTP-driven translocation step) (14).

In the presence of $\alpha$-amanitin, RNA polymerase is stalled at the core of the bond addition mechanism, and the position of stalling is dependent on accurately templated downstream NTPs (Fig. 4). Stalling is indicated by the failure of the HCl quench (phosphodiester bond formation) and EDTA quench (stable GTP-Mg$^{2+}$ loading) curves to converge. With addition of GTP, CTP, and UTP to the reaction, a long-lived steady state is established in which the HCl quench curve rises above the EDTA quench curve, but does not rise to the level of control samples (gray symbols) lacking CTP and UTP (Fig. 4, A–D). Higher concentrations of CTP and UTP appear to develop increased translocation strain on the elongation complex, and therefore increase the persistent separation of the HCl and EDTA quench curves (compare Fig. 4, A–C). Long-lived eleva-

alpha-amanitin translocation block (Fig. 3). To provide a fair comparison between those samples that contain CTP and UTP to those samples that contain CTP but lack UTP, the synthesis of C45 and C46 was normalized, respectively, to synthesis of the previous G44 and C45 bonds. Only those samples containing CTP are considered, because these are the only samples that can synthesize C45 and C46. Remarkably, inclusion of UTP in the reaction strongly suppresses C45 synthesis and (within detection) abolishes C46 synthesis. For C45 synthesis, UTP is accurately templated at the i+3, i+4, and i+5 downstream positions. For C46 synthesis, UTP is accurately templated at the i+2, i+3, and i+4 positions. ATP, GTP, CTP, dUTP, TTP, and dTTP fail to replace UTP in reversal reactions. Once again, this result is most consistent with the NTP-driven translocation model. This result does not appear consistent with the secondary pore NTP loading hypothesis.

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The bond reversal reaction requires endogenous pyrophosphorolysis, providing further evidence of phosphodiester bond reversal in response to addition of EDTA. According to our interpretation of isomerization reversal, G44.PPi transcripts, which score as G44 signal requires reversal of phosphodiester bond synthesis, in this case, after addition of the EDTA quench. The bond reversal reaction requires endogenous pyrophosphate, once again indicating the difficulty in releasing pyrophosphate and also indicating coupling of pyrophosphate release to NTP-driven translocation, a step that is effectively blocked by α-amanitin. Addition of 10 mM exogenous pyrophosphate has no effect on isomerization reversal reactions (data not shown), and pyrophosphate was not an added component of these reactions. Only very small amounts of pyrophosphate are generated from elongation reactions, so exogenous pyrophosphorolysis does not contribute to reversal.

Having HCl quench curves rise above EDTA quench curves provides further evidence of phosphodiester bond reversal in response to addition of EDTA. According to our interpretation of isomerization reversal, G44.PPi transcripts, which score as G44 transcripts by HCl quenching, revert to A43, in response to EDTA addition. Because G44 transcripts are detected by HCl quenching and lost after EDTA quenching, G44 transcripts are not lost in the quantification by extension to C45 (or beyond) and, thus, escaping detection. Bond reversal in response to addition of three independent experiments done on different days. Some error bars are obscured by the graph symbols.

| NTPs | 0, 0.25, 0.5, and 2.5 mM | A, 2.5 mM CTP and UTP. The gray line indicates the negative control experiment with 0 mM CTP and UTP (also shown for reference in B and C). B, 0.5 mM CTP and UTP. Error bars indicate standard deviation of three independent experiments done on different days. Some error bars are obscured by the graph symbols. C, 0.25 mM CTP and UTP. D, 0 mM CTP and UTP. A–C, in which downstream NTPs are present, the HCl quench curve rises above the EDTA quench curve. In D, in which adjacent downstream NTPs are absent, the EDTA quench curve rises above the HCl quench curve.

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EDTA addition indicates that chelation of CTP- and UTP-Mg\(^{2+}\) may inhibit accurately templated NTP-driven translocation, which is required to maintain formation of the G44 bond. Furthermore, accurately templated NTP-driven translocation appears to be coupled to phosphodiester bond synthesis and pyrophosphate release. Comparing Fig. 4, A–C shows that reducing CTP- and UTP-Mg\(^{2+}\) concentration reduces separation of the HCl and EDTA quench curves, indicating that lower concentrations of incoming NTPs may generate reduced translocation pressure against the α-amanitin translocation block.

The experiment shown in Fig. 4, in which HCl quench and EDTA quench curves fail to converge within 1 s, was done in the presence of TFII F and the absence of TFII S. In previously published experiments (12), which were done in the presence of both TFII F and TFII S, HCl quench and EDTA quench curves converged within 0.2–0.4 s when accurately templated downstream NTPs were present. By contrast, in the absence of accurately templated downstream NTPs, convergence required about 2 s. Slow convergence of HCl and EDTA quench curves was explained because accurately templated NTP-driven translocation was necessary for bond completion or bond reversal, particularly in the presence of a translocation block. Comparing the results in Fig. 4 with previous results indicates that TFII S will interact with elongation complexes during active synthesis and help in some way to cause either bond completion (pyrophosphate release) or isomerization reversal (or both).

To confirm the previous result, we did the experiment shown in Fig. 5, in which we compare the convergence of HCl and EDTA quench curves in the presence and absence of TFII S. As expected, TFII S causes rapid convergence of the HCl quench and EDTA quench curves (within about 0.1 s) (Fig. 5). In Fig. 5, reactions that include TFII S are identical to those that lack TFII S, except that, in this case, TFII S was added with GTP, CTP, UTP, and α-amanitin. If TFII S is added to the reaction during the ATP pulse, the results are similar to those previously observed in Gong et al. (12) (supplemental materials, Fig. S2).

Convergence of HCl quench and EDTA quench curves, in the presence of TFII S, demonstrates a previously unknown function of TFII S. TFII S interacts with stalled elongation complexes that have loaded an NTP into the active site and are engaged in forming a phosphodiester bond. TFII S affects isomerization reversal, and reversal is an apparent fidelity mechanism, involving rejection of the substrate NTP in response to a translocation block. It was previously unknown that TFII S could interact with actively transcribing complexes. Furthermore, because TFII S contributes to convergence of HCl and EDTA quench curves, separation of HCl and EDTA quench curves observed in Figs. 4 and 5 is not due to an artifact of quantification. The observation of phosphodiester bond reversal in Gong et al. (12) is confirmed, indicating that accurately templated NTP-driven translocation is coupled to isomerization reversal, bond formation, and pyrophosphate release.

**DISCUSSION**

**NTP-driven Translocation**—In the presence of a translocation block (α-amanitin), we demonstrate robust effects of NTPs that are accurately templated at adjacent downstream positions (i+2, i+3, and i+4, at a minimum) on the fate of a substrate NTP loaded and initially tightened in the i+1 active site of human RNA polymerase II. Neither improperly templated NTPs, nor accurately templated NDPs, nor accurately templated dNTPs show these effects, revealing chemical selectivity at multiple downstream positions. Because these results demonstrate simultaneous NTP occupancy of the active site and downstream sites, these results support the NTP-driven translocation model and appear inconsistent with the secondary pore being the sole route of NTP loading for human RNA polymerase II. Based on available structures (6, 17–19, 26), it does not appear that NTPs can access the i+2, i+3, and i+4 sites through the secondary pore.

Recent x-ray crystal structures of *T. thermophilus* RNA polymerase elongation complexes indicate that NTP-driven translocation may not be general for all multi-subunit RNA polymerases. Because *T. thermophilus* structures have a closed downstream bubble (closed at i+2), this raises the question of whether bacterial RNA polymerase can bind an NTP substrate at i+2. It should be noted that merely because *T. thermophilus* has a closed downstream bubble in some elongation complex structures, this does not show that the downstream bubble is always closed. For instance, allosteric effects of substrate NTPs with *Escherichia coli* RNA polymerase may indicate interactions of an NTP substrate at or near the i+2 site (27, 28). Single molecule studies of elongation catalyzed by *E. coli* RNA polymerase are also most consistent with NTP substrates binding at or near i+2 (29).

**Isomerization Reversal and the Trigger Loop Hypothesis**—In x-ray crystal structures, multiple placements of the trigger loop (Rpb1 1081 to 1093, *S. cerevisiae* residues) have been observed,
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including “open” and “closed” conformations (1, 2, 18). The trigger loop appears to close over the i+1 NTP substrate as part of an induced fit mechanism, tightening the catalytic intermediate to promote catalysis and ensure transcriptional fidelity. The trigger loop, however, is not required for phosphodiester bond formation by multisubunit RNA polymerases. A radical deletion of the E. coli RNA polymerase trigger loop is slow for elongation, but it remains active (30). A four amino acid substitution mutant designed to block trigger loop dynamics is also very slow in elongation (2). Based on the T. thermophilus elongation complex structures, E. coli β′ Met932, Arg933, and His936 are predicted to be key residues to support elongation and to contribute to transcriptional fidelity. Although mutant proteins in these residues were tested for pausing half-life, activities in elongation compared with wild-type RNA polymerase were not reported (30). E. coli β′ T934M, found in this critical region, is rapid in elongation compared with wild type (31). Mutations in the trigger loop increase the duration of pauses at an RNA hairpin-dependent pause site from the histidine operon, demonstrating that the trigger loop has an important role in regulating transcriptional pausing (30).

Trigger loop closing, however, appears inconsistent with α-amanitin (18, 23) and with TFIIIS (17, 32) binding to the active elongation complex. Because the data presented in this report indicate that α-amanitin and TFIIIS affect NTP-Mg2⁺ isomerization and pyrophosphate release, it appears that α-amanitin and TFIIIS can interact with human RNA polymerase II during catalysis. Results in the human RNA polymerase II system, therefore, are most understandable if an open trigger loop conformation supports catalysis. At this time, we do not have a clear explanation for this apparent discrepancy with the T. thermophilus elongation complex structure. Yeast RNA polymerase II elongation complexes with closed trigger loop conformations do not appear to be precise catalytic intermediates because of a bend in the bridge α-helix and distortions in base pairs at the i and i+1 positions (2, 18). To obtain x-ray crystal structures that approach a catalytic intermediate requires blocking NMP incorporation, so non-incorporated NTP analogues or a 3′-deoxy chain terminator at the RNA 3′-end are used. Structures with a closed trigger loop conformation, therefore, could represent a response to the block to NMP incorporation. Critical mutations in the trigger loop are so far characterized most completely for transcriptional pausing, rather than for catalysis or transcriptional fidelity, so it is not yet clear whether a closed trigger loop conformation is normally utilized for bond synthesis (30).

A Tunable Ratchet Driving Translocation—To explain the effects of α-amanitin, TFIIIF, TFIIIS, and downstream NTPs in adjusting translocation pressure, we propose a model for a tunable ratchet driving translocation (Fig. 6). Fig. 6A shows a schematic of a tunable translocation ratchet, with the bridge α-helix forming its core. Fig. 6B shows a structural image of the bridge α-helix and a proposed conformational switch (the “funnel”; S. cerevisiae Rpb1 673 to 763), proposed to help roll the ratchet domain forward and back. A primary feature of the tunable ratchet model is that it posits conformational coupling between the RNA polymerase II active site (i+1) and the NTP-driven translocation mechanism, which involves templated NTP binding to downstream sites (i+2, i+3, and i+4). In this model, the bridge α-helix forms the center of a cylindrical domain that rolls forward to translocate the DNA and then slides back relative to DNA, during each bond addition step.

Tightening of the i+1 NTP substrate is posited to place strain on the DNA template stimulating forward translocation. A right angle bend in the template DNA over the bridge α-helix is suggested to link catalysis to translocation. The bridge α-helix has been described as a ratchet driving forward translocation (3, 6). We posit that the bridge α-helix is at the core of a domain that rolls forward, stimulating forward translocation. Tightening of the i+1 NTP is posited to increase contact between the triphosphate and basic residues Rpb2 Arg1020 and Rpb1 Lys752 (residue numbers are for S. cerevisiae). Located on the highly conserved 750-GSKG-753 loop, which is linked to the “funnel” (Rpb1 663 to 763), Rpb1 Lys752 is posited to be a major sensor of the state of the catalytic cycle (18). As catalysis progresses, a rocking motion is developed in the funnel that stimulates rolling of the rolling ratchet domain. Movement of accurately templated downstream NTPs contributes to translocation force. NTP-driven translocation is thought to be coupled to pyrophosphate release, so bond completion would be coupled to accurate NTP driven translocation for the next bond addition. As the next NTP is transferred into the RNA polymerase II active site, the bridge α-helix domain rolls back, sliding relative to the DNA template (Fig. 6A).

Kornberg and co-workers (6, 18) suggested that the bridge α-helix might cycle between bending and straightening to drive forward translocation. Nudler and co-workers (3) suggested a similar dual ratchet mechanism for elongation, involving conformational changes of the bridge α-helix and associated “G” or “trigger” loop. We favor a mechanism in which the bridge α-helix lies at the core of a larger domain that rolls (relative to template) more than bends to drive forward translocation. Rolling of the bridge α-helix forward causes Rpb1 Tyr836 to clash with template DNA, helping to induce a change in the location of the 90-degree bend in the DNA template strand over the bridge (Fig. 6A). Changing the position of the bend in the DNA is suggested to be an important part of a switching mechanism to alter contacts between basic groups (Rpb1 Arg1020, Lys830, Lys132, Arg137, Arg139, Arg1386, and Rpb2 Arg1129) and DNA phosphates (Fig. 6B, lower panel). Altering the DNA bend point is posited to cause first releasing and then re-setting phosphate contacts to the next downstream position, supporting stepped translocation of the DNA.

Breaking phosphate contacts results in a reverse roll of the ratchet domain, causing the DNA template to slide relative to the bridge α-helix. In this way, the bridge α-helix is at the core of a molecular ratchet driving forward translocation. We propose that larger motions of RNA polymerase II may support the ratchet roll of the bridge domain. For instance, the funnel may rock toward the bridge α-helix, to enhance the forward roll, and then rock back during template sliding, during the reverse ratchet roll (Fig. 6). Because the funnel includes Rpb1 Lys752, which is poised to interact with the i+1 NTP triphosphate, this establishes a connection that is mediated through the funnel structure between the i+1 active site and translocation driven by accurately templated NTPs at i+2, i+3, and i+4. A func-
**FIGURE 6.** A model for a tunable ratchet driving translocation by human RNA polymerase II. A, bridge α-helix (yellow) is depicted as forming the core of a cylindrical domain (light blue) that rolls forward and then slides back, relative to the DNA template (green; phosphates indicated as yellow dots) during each translocation step. The “funnel” (pink) connects active site and rolling ratchet functions. Rpb2 R1020 and Rpb1 Lys752 are proposed to be sensors of progression of each bond addition. RNA and NTP substrates are red. Mg<sup>2+</sup> atoms are green dots. Tilting of the funnel (pink), gray lines and letters indicate rotation of the ratchet domain. Rpb1 Tyr<sup>836</sup> (S. cerevisiae residue number) is purple. Tyr<sup>836</sup> is posited to be important in switching the bend point in the DNA template strand as it passes over the bridge α-helix. a, relaxed elongation complex; b, isomerized (tightened) elongation complex; c, complex after phosphodiester bond formation; d and e, maximally strained elongation complexes, in which NTP-driven translocation is coupled to pyrophosphate release; and f, relaxed elongation complex. α-amanitin (αA) primarily blocks the dominant translocation step, but limited translocation is suggested to be associated with multiple steps in the mechanism. B, structural model of the rolling ratchet mechanism. Top panels, three views of the bridge α-helix (Rpb1 810 to 845) (yellow) and C-terminal cap (Rpb1 846 to 868) (yellow) and the funnel region (Rpb1 526 to 763) (mauve) are shown in relation to the transcription bubble. The proposed cylindrical ratchet domain location is indicated by white circles (left and right images) and a white rectangle (center image). Bottom panel, a detail of the right angle bend in the DNA template as it passes over the bridge α-helix is shown with basic residue contacts to phosphates. NTP substrates are shown at the i+1 (orange), i+2 (pink), and i+3 (lime) positions. The i+2 and i+3 NTPs were placed by modeling. Mg<sup>2+</sup> atoms are green; the DNA template strand is green; the non-template DNA strand is white; RNA is red; phosphorous atoms are indicated as tan spheres; basic amino acids (Rpb1 Lys<sup>332</sup>, Lys<sup>336</sup>, Lys<sup>386</sup>, Arg<sup>337</sup>, Arg<sup>339</sup>, Arg<sup>386</sup>, Arg<sup>1389</sup>, and Rpb2 Arg<sup>1129</sup>) are blue; Rpb1 Tyr<sup>836</sup> is purple; phenylalanine residues found at the N-terminal end of the bridge α-helix (Rpb1 813 to 815) are white. DNA missing from available structures was placed by modeling (10). Multiple crystal structures were used to generate this figure (6, 19).
translocated elongation complexes has not been observed in millisecond phase kinetic studies, although a rapidly reversible thermal ratchet and spontaneous pyrophosphate release are predicted features of the secondary pore NTP loading model and the dual ratchet model. In this report, we show that human RNA polymerase II does not release pyrophosphate spontaneously. Isomerization reversal experiments indicate that pyrophosphate release is linked to translocation, rendering pyrophosphate release coupled to accurate NTP-driven translocation a potential fidelity check point in each step of RNA synthesis. In the presence of the translocation blocker α-amanitin, the reaction can be halted after phosphodiester bond synthesis but prior to pyrophosphate release. Accurately templated downstream NTPs seem to drive forward translocation, because they appear to adjust the set point of the ratchet. These results support the NTP-driven translocation mechanism. No model requiring either rapidly reversible translocation or secondary pore NTP loading appears consistent with these data.

The primary issue in considering NTP-driven translocation is not whether NTPs enter the structure through the secondary pore, as has been generally assumed, or through the main enzyme channel, as appears to be the case for human RNA polymerase II. The essential issue is the fidelity of RNA synthesis. NTP-driven translocation induced by accurately templated downstream NTPs is a high fidelity mechanism, because it provides a mechanism for pre-sorting and repeated confirmation of accurately loaded NTP substrates. For human RNA polymerase II, if NTPs are retained in the queue, NTP substrates are confirmed versus template at the i+4, i+3, i+2, and i+1 sites, prior to NMP incorporation at i+1. Remarkably, although necessary, the capacity for accurate base-pairing is not sufficient for retention in the queue. Because they fail to support isomerization reversal, very close NTP analogues such as dNTPs and NDPs appear to be rejected downstream of the active site, even when they are accurately templated. In addition to preventing inaccurate loading of NTPs to the active site, accurately templated downstream NTPs appear to generate translocation force that is used either: 1) to complete synthesis of correct bonds; or 2) to prevent and reverse transcription errors in progress. In this manner, accurately templated NTP-driven translocation appears to be coupled to fidelity mechanisms.

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