Interaction of RNA Polymerase II Fork Loop 2 with Downstream Non-template DNA Regulates Transcription Elongation*1

Maria L. Kireeva ², Céline Domecq ², Benoit Coulombe ³, Zachary F. Burton ³, and Mikhail Kashlev ²

From the ¹NCI-Frederick, National Institutes of Health, Center for Cancer Research, Frederick, Maryland 21702-1201, the ²Gene Transcription and Proteomics Laboratory, Institut de Recherches Cliniques de Montréal and Department of Biochemistry, Université de Montréal, Montréal, Québec, H2W 1R7 Canada, and the ³Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824-1319

Fork loop 2 is a small semiconservative segment of the larger fork domain in the second largest Rpb2 subunit of RNA polymerase II (Pol II). This flexible loop, juxtaposed at the leading edge of transcription bubble, has been proposed to participate in DNA strand separation, translocation along DNA, and NTP loading to Pol II during elongation. Here we show that the Rpb2 mutant carrying a deletion of the flexible part of the loop is not lethal in yeast. The mutation exhibits no defects in DNA melting and translocation in vitro but confers a moderate decrease of the catalytic activity of the enzyme caused by the impaired sequestration of the NTP substrate in the active center prior to catalysis. In the structural model of the Pol II elongation complex, fork loop 2 directly interacts with an unpaired DNA residue in the non-template DNA strand one nucleotide ahead from the active site of Pol II (the i+2 position). We showed that elimination of this putative interaction by replacement of the i+2 residue with an abasic site inhibits Pol II activity to the same degree as the deletion of fork loop 2. This replacement has no detectable effect on the activity of the mutant enzyme. We provide direct evidence that interaction of fork loop 2 with the non-template DNA strand facilitates NTP sequestration through interaction with the adjacent segment of the fork domain involved in the active center of Pol II.

The elongation phase of the RNA polymerase II (Pol II) ² transcription cycle is regulated at the level of promoter-proximal pausing, ³’ RNA processing/polyadenylation, transcription termination, alternative splicing, and epigenetic modulation of histone modifications and DNA methylation (reviewed in Refs. 1 and 2). Understanding the catalytic mechanism of Pol II is essential for revealing the basis of this varied regulation. Conformational changes in the multisubunit RNA polymerases during each NMP addition involve structural rearrangements of several mobile switches and forks in the large Rpb1 and Rpb2 catalytic subunits, which contain several flexible loops adjacent to the nascent transcript and template DNA (3–7). These include the trigger loop, lid, and rudder of the catalytic Rpb1 subunit, and the two flexible loop 1 and loop 2 of the fork region in the Rpb2 subunit. The trigger loop is essential for NTP positioning, catalysis, and the maintenance of transcription fidelity (6, 7); the lid and rudder are involved in the separation of the RNA from the RNA-DNA hybrid and maintenance of the transcription bubble (5). Rpb2 fork 2 appears to be crucial for the interaction of human Pol II with the melted promoter region, initiation, and elongation (8). By contrast, deletion of fork 1 in the β subunit of Pyrococcus furiosus RNA polymerase (RNAP) does not lead to any detectable initiation or elongation defects (9). Deletion of fork loop 2 in the same RNAP impairs transcription, presumably by inhibiting downstream DNA strand separation. A substitution of the conserved fork loop 2 Arg ⁴⁴⁵ in the P. furiosus β subunit with alanine has a phenotype similar to fork loop 2 deletion, suggesting that the interaction of this positively charged residue with the DNA is required for efficient transcription elongation (9). In the majority of the yeast Pol II ternary elongation complex (TEC) structures, fork loop 2 is not resolved. In a few crystals where fork loop 2 is visible, it acquires alternative states: the fully closed state where it interacts with the bridge helix (10) and the opened state turning away from the bridge helix toward the downstream DNA binding channel (11). In the recently published model of the yeast Pol II TEC containing an intact transcription bubble, fork loop 2 interacts with the non-template DNA strand one nucleotide ahead from the active center (12). The apparent flexibility of fork loop 2, its proximity to the active site and its interaction with the bridge helix suggest that, similar to the trigger loop, fork loop 2 could be undergoing conformational changes at each NMP addition cycle.

Due to its location at the leading edge of the transcription bubble, fork loop 2 has been proposed to be crucial for downstream DNA melting (7, 10). Specifically, the two positively charged residues in the loop of Thermus thermophilus RNAP interact with the DNA base pair located immediately downstream from the active site (6). The analyses of the non-template DNA path in the yeast Pol II TEC using fluorescence energy transfer, taken together with the structural information suggest that the DNA strands are separated at Rpb2 Arg ⁵⁵⁰, a functional homologue of Arg ⁴⁴⁵ in the β subunit of P. furiosus RNA polymerase (12). However, the recently characterized yeast Rpb2-R504A substitution did not impair the viability of yeast cells, and did not inhibit transcription on a double-
Fork loop 2 has also been implicated in substrate NTP binding. This region of the β subunit of *Escherichia coli* RNAP and its eukaryotic Rpb2 counterpart appears similar to an NTP binding motif from DNA polymerases (14). Based on this proposed homology, on the apparent allosteric stimulation of *E. coli* RNAP by the incoming NTP substrate (15), and on the properties of the *E. coli* RNAP fork loop 2 deletion mutant (16), fork loop 2 has been suggested to contribute to an allosteric NTP-binding site, distinct from the active site. The Rpb2 fork loop 2 has been suggested to contribute to an allosteric NTP-binding site (14, 16). The pre-steady-state kinetic analyses of formation of two consecutive phosphodiester bonds by human Pol II (17, 18) also suggested the existence of an NTP-binding site, distinct from the active site. The Rpb2 fork loop 2 and the adjacent Rpb2-Arg512 have been proposed to participate in substrate NTP binding and in translocation based on the structure of yeast Pol II (18). Subsequent studies revealed that Rpb2 R512C and R512A mutants are defective in elongation (13, 19, 20); however, this defect may be mediated by interaction of Arg512 with the bridge helix (20). Therefore, a direct role of fork loop 2 in NTP binding and translocation remains to be clearly demonstrated.

In this work, we directly addressed the role of *Saccharomyces cerevisiae* Pol II fork loop 2 in transcription. We introduced a deletion of the flexible tip of the loop, containing invariant Rpb2 Arg504 and conservative Rpb2 Lys507 residues (Fig. 1A). This part of fork loop 2 occupies variable positions in different crystals of TECs, but the most notable interactions of Arg504 and Lys507 are with the non-template DNA strand and the leading edge of the transcription bubble. Our *in vivo* and *in vitro* data strongly argue that fork loop 2 regulates the catalytic step in the transcription cycle by modulating interactions of the fork region in the Rpb2 subunit with the incoming NTP substrate in the active center of Pol II. Our systematic biochemical analysis, however, provided no evidence supporting a role of fork loop 2 in DNA melting, translocation, and transcriptional fidelity.

**EXPERIMENTAL PROCEDURES**

*Protein Purification* —The wild type Pol II and its variants carrying mutations in the Rpb2 subunit were purified as described (13). TFIIS was purified according to Ref. 21 using an expression construct kindly provided by C. M. Kane.

*Elongation Complex Assembly* —The TECs were assembled on the synthetic nucleic acid scaffolds (22, 23). The reactions were performed in transcription buffer (TB): 20 mM Tris-HCl, pH 7.9, 5 mM MgCl2, 40 mM KCl, 10 mM ZnSO4, 3 mM 2-mercaptoethanol, 20 μg/ml of acetylated bovine serum albumin (BSA). The sequences of the RNA and DNA oligonucleotides used for assembly of the TECs are summarized under supplemental Table S1. In the typical assembly reaction, 5–10 pmol of Pol II was incubated with 15–30 pmol of the preannealed RNA-DNA hybrid in the total volume of 30–50 μl for 10 min at room temperature; 30–100 pmol of non-template DNA strand were subsequently added for another 10 min. The NTP substrates were added as indicated in the figure legends for the last 5 min of incubation with the non-template DNA. The TECs were diluted with 0.5 ml of TB containing 1 M KCl and concentrated using Amicon Ultra (Millipore, Carrigtwohill, Ireland) centrifugal filters with a 100-kDa molecular mass cutoff. The resulting TECs were diluted with TB and concentrated twice.

*Transcription Elongation Assays* —In the bulk elongation and misincorporation assays the reaction was initiated by addition of the NTPs and stopped by addition of the gel-loading buffer. Transcription assays in time intervals shorter than 5 s were performed using the RQF-3 or RQF-4 instruments (Kinetek Corp., Austin, TX). In this case the reactions were stopped by addition of 1 M HCl or 0.5 M EDTA, and the samples were processed as described previously (22). The RNA products were resolved in the 20% acrylamide gels in denaturing conditions. The 32P-labeled RNA products were detected using the PhosphorImager (Typhoon 8600, GE Healthcare). The fluoroscein-labeled RNA products were detected by excitation with a 488-nm laser and read out in the presence of a 520-nm emission filter (Typhoon Trio, GE Healthcare). The quantitative analysis was performed with ImageQuant software. The non-linear regression analyses were done in OriginPro (OriginLab Corp., Northampton, MA).

*Potassium Permanganate Footprinting* —The potassium permanganate footprinting was performed in TB in the absence of BSA and 2-mercaptoethanol essentially as previously described (24). The TECs were assembled with the 5′-end labeled non-template or template DNA strand oligonucleotide. The template DNA strand oligonucleotide was gel-purified after the labeling. The DNA in the TEC was modified by addition of 1 mM KMnO4 for 10 s. The typical reaction volume was 5–10 μl. The modification was stopped by addition of 1 μl of 2-mercaptoethanol. 100 μl of 10% piperidine was added, and the modified DNA was cleaved for 10 min at 90 °C. 10 μl of 3 M sodium acetate and 50 μg of glycogen were added, and the DNA was precipitated with ethanol. The DNA products were resolved in a 10% acrylamide gel in denaturing conditions.

*Exonuclease III (Exo III) Footprinting* (25) —The TECs were assembled with a 5′-end labeled template DNA strand. 15 μl of TB containing 10 units of exonuclease III (New England Biolabs, Bedford, MA) were added to 15 μl of the elongation complex, 7-μl aliquots were taken out at indicated time points, and mixed with the gel loading buffer. The DNA products were resolved in a 10% acrylamide gel in denaturing conditions.

**RESULTS**

*Fork Loop 2 Deletion Does Not Affect Cell Viability* —The mutation in fork loop 2 was designed to remove the entire flexible 4-amino acid segment of the loop, carrying the two conserved positively charged residues constituting the putative NTP binding motif (14) and contacting the downstream DNA. The deleted segment was replaced with a Gly4 linker to minimize disruption of the surrounding protein structure (Fig. 1A). The *rpb2ΔFL2* mutant allele was generated and compared with the *Rpb2* allele, as described previously (13). Briefly, *TRP1*-based plasmid pFL39 (26) carrying a tandem affinity purification-tagged (27) copy of *Rpb2* or *rpb2ΔFL2* under control of an endogenous promoter was introduced into a strain with deletion of the chromosomal copy of *Rpb2*, and carrying a *URA*-based plasmid with a wild-type untagged copy of *Rpb2* under control of a *GAL* promoter (26). The viability of the strain carrying pFL39-*rpb2ΔFL2* on galactose media (Fig. 1B, lanes 1–4)
indicates that the mutation is not dominant lethal. Furthermore, a robust growth of strains carrying pFL39-RPB2 and pFL39-rpb2ΔFL2 on glucose media, taken together with the poor or no growth of the strain carrying an empty vector (lanes 5–8), suggest that deletion of fork loop 2 does not impair the viability and does not substantially affect growth of *S. cerevisiae*. In the *in vivo* tests shown in Fig. 1B, the rpb2ΔFL2 strain is very similar to the RPB2 strain. The rpb2ΔFL2 allele is sufficient to support cell viability, because the URA-based plasmid carrying the wild type RPB2 can be shuttled out from the strain carrying pFL39-rpb2ΔFL2, conferring resistance to 5-fluoroorotic acid (lanes 9–12), without losing viability. Unlike many mutations that strongly affect elongation, the rpb2ΔFL2 deletion is not synthetic with knock-out of the DST1 gene encoding elonga-
tion factor TFIIS (lanes 17–20). Fork loop 2 deletion renders a slightly increased sensitivity to 6-azauracil (lanes 13–16), which may indicate a moderate transcriptional defect.

**Fork Loop 2 Deletion Enhances Transcription Pausing by Decreasing the Rate of NMP Incorporation**—The Pol II core enzyme variants carrying the wild-type Rpb2 (WT Pol II) and Rpb2/H9004FL2 subunit (dFL Pol II) were purified and characterized using a single round elongation assay as previously described (13). The mutation only moderately decreased the rate of accumulation of the runoff product at both low (10 μM) and high (200 μM and 1 mM) NTP concentrations (Fig. 2A and supplemental Fig. S1). The overall inhibition was about 2-fold and it appeared to be sequence-specific. The mutation did not affect the escape from the A20 stall site or pausing in the next U21 site at low NTP concentration (supplemental Fig. S1, lanes 1–7, compare the upper and lower panels), but pausing at the U27 position was noticeably enhanced in the mutant (Fig. 2A). The mutation also increased the duration of pausing at A26, U33, and G41 positions of the template (supplemental Fig. S1). The mutation had the most prominent effect on slowing down escape from the U27 pause (compare lanes 2–4 and 7–9 in Fig. 2A).

**FIGURE 2.** Fork loop 2 deletion slows down elongation and enhances transcription pausing and arrest of Pol II. TEC9 was assembled using RNA9, TDS76, and NDS79. A, bulk elongation with 200 μM NTP. The positions of the RNA in the starting TEC20, the runoff product (56 nucleotides), and the U21 and U27 pausing/arrest sites are indicated at left. B, intermediates of pausing and arrest by Pol II backtracking. C, irreversible transcription arrest of the stalled TEC27. D, products of the TFIIS-induced transcript cleavage in the TEC27. The scans of the gel lanes, normalized to the total amount of the radioactivity in the lane, are shown in C and D. E, bond formation rate determined for the WT and dFL Pol II. Transcription was performed by addition of 1 mM ATP and CTP to TEC9; the reaction was stopped by addition of 1 M HCl. The time course of NMP incorporation and the observed rates for the WT Pol II are shown by black symbols, for the dFL Pol II, by gray symbols.
Pausing and arrest at the U27 site was previously shown to involve backtracking of Pol II (28). U27 pausing involved reversible backtracking at a short distance, whereas arrest was associated with long distance irreversible backtracking (28). Next, we analyzed whether parameters of backtracking at the U27 site were affected by the mutation. As previously reported (29), an entry of Pol II into the backtracked pause requires time. Therefore, the pause efficiency increases with a decrease of NTP concentration (supplemental Fig. S1, lanes 9–18), suggesting that the probability of entering the backtracked state increases with the decrease of the CMP incorporation rate during the TEC27 → TEC28 transition (k_2, Fig. 2B). The efficiency of backtracked pausing is determined by the ratio between k_a and the rate of entering the backtracked state (k_{-2}, Fig. 2B). dFL Pol II could have a decreased k_a and/or an increased k_{-1} at the U27 site. On the other hand, a distinction between backtracked pausing and irreversible arrest is determined by the propensity of Pol II to enter the short distance (k_{+1}) versus the long distance backtracking (k_{+2}), and on the corresponding rate of the return to the active state by reversal of backtracking (k_{-1} and k_{-2}, respectively). It is assumed that the k_{-1} is much higher than k_{-2} due to the higher activation barrier for Pol II to recover from the long distance rather than from the short distance backtracking. Note that despite enhanced pausing at the U27 site, the deletion mutant was arrested to the same degree as the WT enzyme, which argued that k_{+2} and k_{-2} were not affected by the mutation. To further distinguish between the effects of the mutation on k_a, k_{+1}/k_{-1}, and k_{+2}/k_{-2}, we stalled Pol II at the U27 site for 10 min by NTP deprivation to provide a sufficient time for development of the backtracked states. Subsequently, the stalled TEC27 was incubated with 1 mM NTPs for 90 s to chase all the active and reversibly backtracked complexes to the end of the template and to isolate and quantify the irreversibly arrested fraction (Fig. 2C). This assay revealed that the mutation did not strongly affect the efficiency of irreversible U27 arrest, which was 75 and 80% for the WT and dFL Pol II, respectively. This result indicated that long distance backtracking (k_{+2}/k_{-2}) was not altered by the mutation.

The above conclusion was supported by the direct measurement of the backtracking distance in the WT and mutant TEC27. The transcript in TEC27 was labeled at the 3’ end by incubating the purified unlabeled TEC26 with α-[32P]UTP. After removal of the non-incorporated NTPs, TEC27 was treated with TFIIS, an elongation factor that promotes transcript cleavage by the Pol II active site. The length of the 3’ RNA cleavage products is indicative of the distance that Pol II has backtracked (see Ref. 30 and the scheme of the TFIIS-induced RNA cleavage in Fig. 2D). As expected, cleavage generated the short (2 nucleotides) and long (10–11 nucleotides) 3’-end products corresponding to the short distance backtracked pauses and the long distance arrest (Fig. 2D, the black trace, and supplemental Fig. S2, lanes 2–4 and 9–11). The irreversibly arrested fraction of TEC27 was isolated by addition of NTPs to TEC27: in the presence of NTPs the active and reversibly arrested fractions of TEC27 extended the RNA to form the runoff product, but the arrested fraction remained intact. TFIIS treatment of the arrested fraction of TEC27 produced exclusively the 10–11-nt cleavage products (Fig. 2D, the red trace, and supplemental Fig. S2, lanes 5–7 and 12–14), confirming that the arrested TEC27 underwent long distance backtracking. Importantly, the yield and pattern of the cleavage products in the stalled and chased TEC27 were very similar for the WT and dFL Pol II strongly indicating that parameters of the irreversible (k_{+2}/k_{-2}) and reversible (k_{+1}/k_{-1}) backtracking were not affected by the mutation. Thus, fork loop 2 deletion appeared to increase the efficiency of the U27 pause indirectly, by slowing down incorporation of the next cognate CMP (decreasing the k_a). The additional analyses of NMP incorporation by the mutant enzyme in several sequence contexts not associated with Pol II backtracking (Fig. 2E, Table 1, and supplemental Fig. S3) revealed that fork loop 2 deletion caused a ~2-fold decrease of the NMP incorporation rate at these sites regardless of the nature of the incoming substrate (purine or pyrimidine). This reduction appeared to be comparable with the effect of the mutation on pausing in the bulk transcription assay (Fig. 2A).

It has been proposed that fork loop 2 constitutes part of a NTP-binding and pre-selection site in the primary channel of Pol II, thus contributing to transcription fidelity (14, 17). To test this hypothesis, we determined the apparent maximum incorporation rate (k_{pol}) for the complementary (CMP) and non-complementary (UMP) NMPs in TEC9 that was used as a standard complex in our published assay for mis-incorporation by Pol II (25). We also determined the apparent K_{diss} for CTP and UTP for the WT and dFL TECs (Table 2 and supplemental Fig. S4). In agreement with the individual NMP incorporation assay, the k_{pol} of CMP incorporation was decreased in the mutant compared with the WT Pol II. The affinity for the correct substrate appeared slightly lower in the dFL Pol II. The k_{pol} for the mismatched substrate was also decreased in the mutant. Importantly, the changes in the apparent K_{diss} and k_{pol} parameters rendered by fork loop 2 deletion did not result in a significant change of the apparent transcription fidelity compared with WT Pol II. The effects of the fork loop 2 deletion on the
correct NMP incorporation, misincorporation, and transcription fidelity argued against a direct role in substrate NTP binding and substrate preselection.

**Fork Loop 2 Does Not Participate in Downstream DNA Melting during Transcript Elongation**—The structural analysis of the TECs formed by Pol II and *T. thermophilis* RNA polymerase suggested that fork loop 2 may play a role in melting the downstream DNA duplex (6, 7, 10). We tested this hypothesis with the specially designed TEC carrying a run of three thymidine residues (dT) in the non-template DNA strand at the leading edge of the transcription bubble and one dT base at the upstream end of the bubble (Fig. 3A). The degree of DNA melting in a series of TECs approaching the dT run was determined by sensitivity of dT residues to modification by potassium permanganate (Fig. 3A). We used the standard nomenclature for the *i* and the *i+1* sites corresponding to the 3'-end RNA-binding site and the empty active center in the post-translocated TEC (31). The *i+2* site is located 1 bp downstream from the active center in the primary DNA-binding channel of the post-translocated enzyme (31). Lane 2 shows that in TEC9 carrying dT residues in the *i-9, i+3, i+4*, and *i+5* positions, the dT*_{i+2}* residue was strongly modified indicating its full opening. In contrast, all three downstream dT residues were modified to a much lesser extent (lane 2, and the top gel scan), indicating that the downstream DNA beyond the *i+2* register was predominantly double-stranded. This low sensitivity was further decreased to the background level after chase of TEC9 with 4 NTPs prior to addition of permanganate (Fig. 3A, lane 3). Accordingly, TEC9 → TEC10 → TEC11 transition by incubation with CTP followed by ATP led to the enhanced modification of the three downstream dT residues and a corresponding decrease of the upstream dT*_{i-9}* modification. Thus, this assay depicted the forward progression of transcription bubble into the dT run (compare lanes 2 and 4, in the top and middle plots). Importantly, modification of the dT*_{i+2}* in TEC9 was substantially weaker than that of the dT*_{i-9}*. The dT*_{i+3}* signal was also weaker in TEC9 than in TEC10 where the corresponding residue moved to 1–nt distance from the active center to become the dT*_{i+2}*. This result argued that the extent of DNA melting in TEC9 was primarily limited to the 1–bp distance downstream from the active center, whereas the residues located further downstream remained base paired. The moderate sensitivity of the dT*_{i+3}* residue in TEC9 and the dT*_{i+2}* residue in TEC10 to permanganate indicated the existence of the equilibrium between the hybridized and non-hybridized states of the *i+2* and *i+3* bases (lanes 2 and 4). Finally, the DNA residue located immediately downstream from the active center became completely single-stranded after entering the *i+1* position in TEC11 as indicated by its hypersensitivity to permanganate (lane 5). These data are in agreement with the previously reported extent of the downstream DNA melting in *E. coli* RNAP and *S. cerevisiae* Pol II TECs (32). The lack of any difference in the modification pattern between the WT and dFL Pol II in all TECs tested unambiguously demonstrated that the fork loop 2 does not play a role in downstream DNA melting (compare lanes 2–5 and 6–9; also the black and red trace in the top, middle, and bottom plots).

This conclusion was further confirmed by permanganate footprinting of the opposite DNA strand in a series of TECs containing several dT residues in the template strand at positions similar to those probed in the non-template strand (Fig. 3B). The template dT*_{i+1}* residue in the active center of TEC8 appeared fully unpaired and ready to hybridize with the incoming cognate ATP. The dT*_{i+2}* site became protected after the TEC8 → TEC9 transition indicating incorporation of an AMP opposite the dT*_{i+1}* (lanes 1 and 2). Note, that dT*_{i+2}* and dT*_{i+3}* residues remained double-stranded in TEC9, but they became completely (*i+1*) and partially (*i+2*) opened in TEC10 (lane 3). We ruled out the possibility that partial protection of the dT*_{i+2}* site in TEC9 was caused by the protein as opposed to DNA base pairing. Indeed, introduction of an abasic site to the non-template strand (dT*_{i+2}*) opposite the complementary dT*_{i+2}* residue in TEC9 made the formerly fully accessible to modification (Fig. 3B, compare lanes 2 and 8). This result directly demonstrated an existence of DNA base pairing at the *i+2* position located only 1 nucleotide downstream from the active center of Pol II. The intermediate sensitivity to permanganate of the *i+2* thymidine in both the template and non-template strand indicated the presence of an equilibrium between the opened and hybridized states. Once again, the dFL Pol II showed no difference from the WT enzyme in the degree of the DNA melting probed on the template DNA strand (compare lanes 1–3 with 4–6, and 7–9 with 10–12). Taken together, these data strongly suggest that the fork loop 2 is not involved in downstream DNA melting during elongation.

**Fork Loop 2 Does Not Promote Forward Translocation of Pol II**—Due to its location at the front boundary of the transcription bubble, fork loop 2 was proposed to participate in forward translocation of Pol II (33, 34). The post-translocated TEC originates from the pre-translocated TEC by a 1-base pair forward movement of Pol II along DNA and RNA. Translocation results in transfer of the 3’-end of the RNA from the *i+1* site (the active center) to the *i* site. This thermally driven motion is fully reversible causing a rapid shift of Pol II between pre-translocated and post-translocated states separated by a 1–bp distance on DNA (35). Exonuclease III (Exo III) has been broadly employed to probe the translocation equilibrium by time-resolved footprinting of the transient pre-translocated and post-translocated boundaries in the stalled Pol II (25). Exo III footprinting was performed with the same series of TECs as described in the legend to Fig. 3B. The data of Fig. 4 demonstrated that the dFL Pol II did not exhibit any translocation defects compared with the WT enzyme for all three TECs that we tested (compare the left side upper and lower panels). In fact, we detected a slight

---

**TABLE 2**

Transcription fidelity of WT and dFL Pol II variants

The *k*incorporation and *K*incorporation parameters were obtained by hyperbolic fits of the observed incorporation rates (*k*incorporation) plotted versus the substrate concentration (see supplemental Fig. S4 for details).

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>dFL</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>k</em>incorporation (CTP), s⁻¹</td>
<td>61 ± 2.6</td>
<td>37.6 ± 2.4</td>
</tr>
<tr>
<td><em>K</em>incorporation (CTP), μM</td>
<td>63 ± 7.1</td>
<td>110 ± 16</td>
</tr>
<tr>
<td><em>k</em>incorporation/Kincorporation (CTP), μM⁻¹ s⁻¹</td>
<td>0.97 ± 0.15</td>
<td>0.33 ± 0.07</td>
</tr>
<tr>
<td><em>k</em>incorporation (UTP), × 10⁻⁶ s⁻¹</td>
<td>7.8 ± 0.39</td>
<td>2.4 ± 0.19</td>
</tr>
<tr>
<td><em>K</em>incorporation (UTP), μM</td>
<td>1400 ± 150</td>
<td>1600 ± 250</td>
</tr>
<tr>
<td><em>k</em>incorporation (UTP), × 10⁻⁶, μM⁻¹ s⁻¹</td>
<td>5.5 ± 0.92</td>
<td>1.5 ± 0.36</td>
</tr>
<tr>
<td>Fidelity, × 10²</td>
<td>1.8 ± 0.57</td>
<td>2.2 ± 0.98</td>
</tr>
</tbody>
</table>
**FIGURE 3.** Fork loop 2 deletion does not affect the extent of downstream DNA melting. The arrows on the gels indicate the base in the i+1 position for each elongation complex. **A**, potassium permanganate footprinting of the non-template DNA strand. The TECs were assembled with RNA7, TDS50, and NDS50 in the presence of ATP and GTP. C10 and A11 were obtained from TEC9 by addition of CTP or CTP and ATP, respectively. The top, middle, and bottom plots represent the scans of lanes 2 and 6, 4 and 8, and 5 and 9, respectively. The signal for the dFL Pol II (red trace) has been normalized to the signal in the WT Pol II (black trace). The signal intensities can be directly compared between the three plots. Scheme on the left summarizes the bubble dynamics during TEC9 to TEC11 progression. The single-stranded thymines are shown in red. The gray arrow indicates the RNA. **B**, potassium permanganate footprinting of the template DNA strand. The elongation complexes were assembled with RNA7FL, TDS45, and NDS45 in the presence of GTP (TEC8) or GTP and ATP (TEC9). TEC10 was obtained from TEC9 by addition of 5 μM CTP. The other symbols are the same as in panel **A**.
stimulation of the forward translocation in the dFL Pol II (compare lanes 7–10 in the left side upper and lower panels).

In contrast to the result obtained with the dFL mutant, an induced melting of the downstream DNA does promote forward translocation of Pol II and this effect is independent on fork loop 2. DNA melting was artificially induced by introduction of the abasic sites into the non-template DNA strand downstream from the active site. In TEC8, the translocation equilibrium was shifted toward the post-translocated state on the template carrying the two abasic sites at positions as compared with the non-modified template (compare lanes 1–4 and 21–24 in Fig. 4). However, in TEC9, the downstream abasic sites, moved to the positions, did not have a significant impact on translocation (compare lanes 5–8 and 25–28). This result suggested that DNA base pairing ahead of the active center (in the position) hinders forward translocation, but the magnitude of the effect depends on sequence context. These effects on translocation were fully preserved in dFL Pol II confirming that fork loop 2 does not promote, and might even slightly restrict, forward translocation. However, this role is independent from the extent of downstream DNA melting.

Fork Loop 2 Stimulates Phosphodiester Bond Formation and NTP Sequestration by Interacting with an Unpaired Non-template DNA Base—From our biochemical analyses, a moderate stimulation of NMP incorporation was the only positive function assigned to fork loop 2 (Fig. 2, and Tables 1 and 2). A clue to understanding this stimulation came from an experiment addressing the role of downstream non-template DNA in the Pol II catalysis. We compared the rates for NMP incorporation by WT and dFL Pol II on two template variants: the fully double-stranded DNA and a template carrying an abasic site in the non-template strand expanding the transcription bubble by 1 nucleotide downstream from the active center. This expansion inhibited the catalytic activity of Pol II similarly to the effect of the fork loop 2 deletion (Fig. 5A, black bars). Importantly, this effect was not observed in dFL Pol II, for which the elongation activity was already 2-fold lower than that of the WT enzyme on this template (Fig. 5A, red bars). A similar trend was observed for the TEC9 formed by WT and dFL Pol II on a paired and an abasic template (Fig. 5B, black and red bars). Apparently, elimination of the base (compare panels B and A, respectively) had the same inhibitory effect on Pol II catalysis as deletion of the fork loop 2 and the inhibition required the presence of the intact loop. Most remarkably, an introduction of the mismatch in place of the abasic sites did not inhibit the activity of WT Pol II (Fig. 5C).

Next, we addressed if the abasic site in the downstream DNA affects phosphodiester bond formation per se, or whether it inhibits earlier steps in the nucleotide addition cycle, such as the sequestration of the substrate NTP in the active site. We used the previously developed assay for NTP sequestration in the active site (22, 25). The single nucleotide addition by Pol II observed in the experimental setup when the catalytic reaction is rapidly quenched with EDTA appears significantly faster than the same NMP incorporation quenched using HCl (36).
This difference was observed in the WT and dFL Pol II (Fig. S5). The substrate addition quenched with EDTA is analogous to a pulse-chase reaction and allows detection of the substrate NTP stably sequestered in the Pol II active center, thus providing the information about the isomerization of the active center, which precedes the bond formation (25). In a typical EDTA-quenched reaction, the NMP incorporation time course appears profoundly biphasic (supplemental Fig. S5). We used the amplitude of the fast fraction as the quantitative measure of the substrate NTP sequestration and observed that WT Pol II sequestered the incoming NTP more efficiently than the dFL Pol II on the fully double-stranded DNA template, but not on the i+2 abasic template (Fig. 5D). Qualitatively, the time courses of NTP addition obtained for WT and dFL Pol II in the acid quench and EDTA quench setups were clearly different on the fully double-stranded DNA template and were indistinguishable on the template carrying abasic sites in the downstream DNA (supplemental Fig. S6).

Taken together, the analyses of bond formation (HCl quench) and NTP sequestration (EDTA quench) suggested that the optimal catalytic activity and NTP sequestration by Pol II primarily depends on the interaction of fork loop 2 with the unpaired i+2 residue in the non-template DNA strand. Based on the results of permanganate footprinting (Fig. 3), the i+2 residue appeared to be a mixture of the opened and paired states indicating that fork loop 2 transiently interacts with the opened state to stimulate bond formation. This conclusion was consistent with the location of the fork loop 2 in the structural model of the Pol II TEC containing an intact transcription bubble (12). In this structure, fork loop 2 was in direct contact with the α-phosphate of the unpaired i+2 residue in the transcription bubble (Fig. 7A).

Fork Loop 2 Interaction with the Downstream DNA Affects Conformation of the Rpb2 Fork Residues Adjacent to the Active Site—We further explored the mechanism by which the putative interaction of fork loop 2 with the i+2 non-template base regulates transcription using site-directed mutagenesis of the extended fork region. It has been previously proposed that fork loop 2 may affect the conformation of the extended fork domain in the Rpb2 subunit (11). Specifically, a short segment of the fork, containing Rpb2 Glu529, Glu530, and Gln531 residues (subdomain I), constitutes part of the active center potentially donating the side chain of Glu529 for the i+1 NTP alignment (11).

Indeed, the rpb2-ES29Q mutation has prominent effects on the NMP incorporation rate ($k_{inc}$) and NTP sequestration by Pol II on a fully double-stranded DNA template. NMP incorporation in the mutant is slowed more than 4-fold (supplemental Fig. S7, panels A and C). At the same time, NTP sequestration appears to be more efficient in the slow Rpb2 ES29Q mutant than in the WT Pol II (supplemental Fig. S7B). Significantly, opposite effects of the Rpb2 ES29Q mutation on NTP sequestration and on bond formation clearly demonstrate that stable loading of the NTP to the active site is not sufficient for rapid phosphodiester bond formation. Evidently, Rpb2 Glu529 plays a dual role in transcription elongation. The enhanced NTP sequestration and the apparent increase in the affinity to the substrate NTP (supplemental Fig. S7, panels B and D) rendered by the ES29Q substitution suggest that Rpb2 Glu529 restricts NTP loading to the active site. At the same time, the decreased bond formation rate indicates that the same residue promotes later step(s) in the NMP incorporation, presumably, positioning the NTP in the active site. The central role of Rpb2 Glu529 in transcription elongation is further confirmed by the less severe phenotype imposed by mutations in the residues flanking...
Glu529. Indeed, the double Rpb2 P528D,G530A substitution, which might misorient Glu529, is similar to WT Pol II in the bulk elongation assay, only slightly decreases the bond formation rate and has a mild defect in NTP sequestration [supplemental Fig. S7].

We reasoned that if fork loop 2 affects conformation of Glu529, activity of the E529Q mutant might be independent from the downstream non-template base, similar to the activity of Pol II carrying the fork loop 2 deletion. First, the extent of NTP sequestration on the fully double-stranded DNA template and the template carrying abasic sites in the downstream DNA has been compared for the WT Pol II and Rpb2 mutant variants (Fig. 6A and supplemental Fig. S7). NTP sequestration by Rpb2 E529Q Pol II was only slightly lower on the abasic template compared with the fully double-stranded template (Fig. 6A). At the same time, NTP sequestration by the Rpb2 P528D,G530A double mutant was affected by the abasic site to the same extent as NTP sequestration by the WT Pol II. Therefore, interaction of the downstream non-template DNA base with fork loop 2 appears to regulate NTP sequestration primarily via Rpb2 Glu529.

Next, the apparent maximum polymerization rate (kpol) was determined on the fully double-stranded and the abasic templates. Unlike the observation for NTP sequestration, the NMP incorporation rate by the Rpb2 E529Q mutant was as sensitive to the non-template base presence as the bond formation rate of WT Pol II. The double Rpb2 P528D,G530A substitution apparently impaired allosteric regulation of NMP incorporation by the downstream DNA, similar to the fork loop 2 deletion (Fig. 6B). The distinct effects of the mutations in the fork region on the sensitivity of NTP sequestration and apparent maximum NMP incorporation rate to the downstream DNA structure further suggest that sequestration and bond formation may be regulated independently by distinct allosteric pathways. Thus, the effect of the fork loop 2 deletion on transcription elongation is likely to be multipartite and may affect several steps of the nucleotide addition cycle.

The properties of the Rpb2 fork mutants suggest that the allosteric signal from the fork loop 2 interaction with the downstream DNA base is coupled to the active center through the fork domain (Fig. 7A, bottom panel). This interaction is likely to be mediated by a network of contacts between fork loop 2 (subdomain II) and subdomain I containing the Glu529 residue (Fig. 7A). Interestingly, this interaction between the fork loop 2 and subdomain I is disrupted in several crystals of the yeast Pol II due to rotation of fork loop 2 away from subdomain I (10, 37).

**DISCUSSION**

Fork loop 2 in the Rpb2 subunit represents a small flexible element in the catalytic cleft of RNA polymerase II (Figs. 1A and 7A). This loop constitutes one of the three subdomains (I, II, and III) of the larger fork domain (Rpb2 residues 466–546, Fig. 7A, shown in cyan) forming multiple contacts with the RNA-DNA hybrid, active center, and downstream DNA (7, 10). Fork loop 2 forms part of the fork spreading away from the active center on the top of the bridge helix toward the front end DNA duplex (Fig. 7A). In the crystal structures of TECs by T. thermophilus RNAP the loop is juxtaposed against the i + 2 base pair of the downstream DNA (4, 5). Due to its particular location, several groups have proposed that the fork loop participates in DNA melting and forward translocation of the enzyme (5–7, 10). The main and unexpected finding of this work is that fork loop 2 is not essential for DNA melting or translocation. Instead, we found that it plays a subtle stimulatory role promoting sequestration of an incoming NTP in the active center and increasing the rate of phosphodiester bond formation. Interestingly, fork loop 2 function appears to depend on its interaction with the downstream non-template DNA. Consistent with this finding, in the structural model of the “complete” elongation complex by the yeast Pol II carrying the entire transcription bubble (12), fork loop 2 is flipped almost 180 degrees from its conformation observed in the elongation complex with partially downstream DNA and interacts with the unpaired i + 2 residue of the non-template DNA strand (Fig. 7A). A similar positioning of fork loop 2 was observed in several crystals of Pol II (10, 38) (Fig. 1A, bottom panel).

Our results strongly argue that interaction of fork loop 2 with the downstream non-template DNA affects the crystallization of the entire fork domain extending to the Pol II active site (Fig. 7A, top panel, colored in cyan). A short segment of the fork,
Molecular mechanism for stimulation of elongation by fork loop 2

A positioning of the fork domain relative to the active center and the downstream DNA in the structure of yeast TEC containing the intact modeled transcription bubble. Three subdomains (I, II, and III) of the intact fork domain are marked and location of Rpb2 Glu529 and the deleted Arg504 to Gly530 residues of the fork loop 2 are shown as space-filling models. Subdomains I and II form extensive interaction with each other; the tip of the fork loop 2 is rotated to contact the non-hybridized i+2 residue in the non-template DNA strand (shown in red). The i+3 and i+4 residues are engaged in the downstream DNA duplex (shown in green and gray, respectively). The i+1 NTP substrate superimposed from the TEC structure (PDB code 2H2E) is shown in magenta. B, scheme depicting a model for effects on NTP sequestration generated by fork loop 2 interaction with the non-template DNA downstream from the active site may serve as an anchor decreasing the intrinsic thermal fluctuations of both DNA strands at the leading edge of the transcription bubble and thus promoting the DNA template i+1 residue loading to the active center. It has been previously shown that the i+1 template residue in the post-translocated elongation complex can occupy a broad range of positions, including one properly aligned with the i+1 site to one that is completely flipped out of the i+1 site and positioned on the top of the bridge helix (39). By interacting with the opened i+2 residue in the non-template strand, fork loop 2 may decrease these fluctuations either directly or indirectly by preventing a transient base pairing of the i+2 non-template residue to its counterpart in the template strand. Such a pairing may stabilize the inactive flipped out configuration of the i+1 template base by stacking interactions with the i+2 base pair. Transcription inhibitor α-amanitin was previously shown to stabilize the flipped out state of the i+1 base (39). We rationalized that if the deletion of fork loop 2 increases the intrinsic mobility of the i+1 base, fork loop 2 deletion might counteract the catalytic inhibition by α-amanitin. The data of supplemental Fig. S8, however, showed that the WT and the dFL Pol II had essentially identical sensitivity to the drug arguing against the role of fork loop 2 in counteracting the intrinsic flexibility of the template DNA.

Despite convincing evidence supporting the idea about the long-range interactions in the fork domain, our data cannot entirely exclude the third possibility that fork loop 2 interaction with DNA directly affects its contacts with the bridge helix, a long α-helix involved in interaction with front DNA, i+1 template base, Pol II translocation and catalysis (40). Notably, Rpb2 Arg512, the residue adjacent to fork loop 2, plays a pivotal role in transcription elongation. Rpb2 R512C and R512A substitutions impose profound transcription elongation defects, slowing down phosphodiester bond formation (13, 19). Molecular dynamics simulations suggest that the movement of Rpb2 Arg512 is conformationally coupled with the bridge helix bending and thus may affect the active site conformation, hydration, and consequently, the phosphodiester bond formation rate (20). Deletion of fork loop 2 characterized in this work might affect the conformation of Arg512 of the extended fork loop 2 thus impairing the NTP sequestration in the active site.

The extent of DNA melting and the mechanism of maintenance of the downstream edge of the transcription bubble has been an open question in the field. The permanganate footprinting of the DNA in several consecutive E. coli TECs containing Rpb2 Pro528, Glu529, Gly530, and Gln531 residues (subdomain I), constitutes a part of the active center potentially donating the side chain of Glu529 for interaction with the i+1 NTP (Fig. 7A). Unlike the fork loop 2 deletion, the E529Q mutation promoted NTP sequestration. However, the E529Q substitution essentially eliminated inhibition of NTP sequestration by the i+2 abasic site, which was similar to the effect of the fork loop 2 deletion. Furthermore, the double PS28D,G530A mutation exhibits similarity to the fork loop 2 deletion by rendering the NMP incorporation insensitive to the downstream abasic site (Fig. 6). Taken together, these observations strongly suggest that the allosteric signal from fork loop 2 interaction with the downstream non-template DNA is communicated to the active center through the fork domain (Fig. 7A). Fig. 7B illustrates how the closed conformation of the active center is supported by a concerted action of the four elements including fork subdomains I and II, the closed trigger loop, and the bridge helix. If this network of interactions is not established due to the fork loop 2 deletion or an abasic site in the i+2 position, the active center isomerization becomes more reversible increasing the probability of the NTP substrate release before bond formation (Fig. 7B, right panel). The Rpb2 Glu529 residue appears to play a key role in the regulation of the active site isomerization and catalysis (Fig. 6).
revealed a highly variable extent of downstream DNA melting, ranging from fully double-stranded downstream DNA to the unwinding of 2–3 bases downstream from the RNA 3′ end (41). It is likely that the apparent high variability of the downstream edge of the transcription bubble could be caused by RNAP backtracking (42). The crystallographic (5–7, 10) and biochemical (32) analyses of DNA base pairing in the elongation complexes that were not prone to backtrack have been performed on nucleic acid scaffolds lacking the full-length non-template DNA. Therefore, the results of these studies do not provide information about the native transcription bubble boundaries. The permanganate footprinting and translocation assays performed in this work using the elongation complexes assembled with the fully complementary template and non-template DNA strands demonstrated that: (i) the i + I template DNA base is fully unpaired in the active elongation complexes; (ii) the i + 2 DNA base is partially unpaired; and (iii) the artificial melting of the downstream DNA supports the post-translocated state of the elongation complex, but does not always increase its catalytic activity. We conclude that downstream DNA melting is not necessarily rate-limiting in transcription elongation, and that contact of the Rpb2 fork loop 2 with the downstream DNA is not required for DNA melting.

The downstream DNA in the elongation complex is located in the “jaw” domain formed by Rpb1, -2, -5, and -9 subunits of bacterial RNAP (3). Several reports have shown that the downstream DNA plays a significant role in transcription pausing and termination indicating a communication between the downstream DNA and the active center of the enzyme (43–45). Our finding that the downstream non-template DNA supports the optimal catalytic conformation of the Pol II active site suggests that the protein interactions with DNA ahead of the active center impose multiple allosteric and long-range effects on NTP binding and catalysis. This work revealed one of these signals wired to the active center from the downstream DNA through fork loop 2 and fork subdomain I of the Rpb2 subunit. The other candidate is the Rpb1 1371–1382 amino acids region, which is located in close proximity to the i+2 to i+5 region of the non-template DNA strand (7, 10). Its homologue in the E. coli Thermus elongation complex directly interacts with the same region in the non-template DNA (6). As opposed to the stimulatory contact mediated by fork loop 2, some of these contacts may have inhibitory effects on bond formation. Indeed, deletion of part of the jaw domain of the β' subunit of E. coli RNAP (43) and point mutations in the bridge helix of Methanocaldococcus jannaschii RNAP facing the downstream DNA have been shown to increase the catalytic activity of the enzyme (37). Thus, the downstream DNA interactions are likely to be employed for the positive and negative regulation of transcription elongation. The full scope of these effects on transcription remains to be established.

REFERENCES

Rpb2 Fork Loop 2 in Transcription Elongation

Interaction of RNA Polymerase II Fork Loop 2 with Downstream Non-template DNA Regulates Transcription Elongation
Maria L. Kireeva, Céline Domecq, Benoit Coulombe, Zachary F. Burton and Mikhail Kashlev

doi: 10.1074/jbc.M111.260844 originally published online July 5, 2011

Access the most updated version of this article at doi: 10.1074/jbc.M111.260844

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2011/07/05/M111.260844.DC1

This article cites 45 references, 20 of which can be accessed free at http://www.jbc.org/content/286/35/30898.full.html#ref-list-1