Supporting Information

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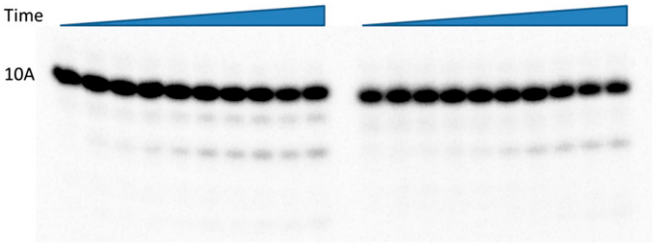

Fig. S1. TFIIS-mediated cleavage of 10A RNA primer with 3′–5′ linkage (*Left*) and 2′–5′ linkage in the end (*Right*). Time points vary from 10 s to 1 h.

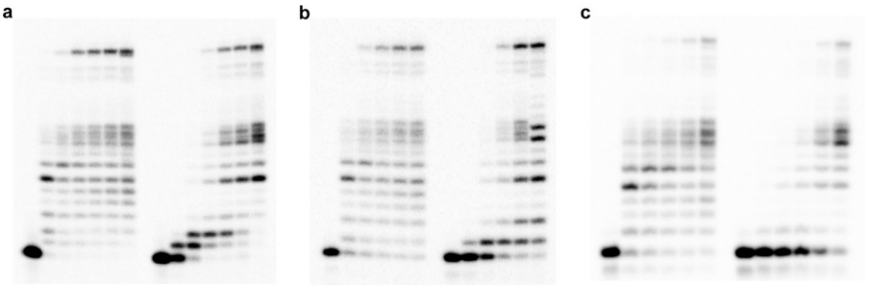

Fig. S2. RNA pol II stalls at all of these three positions. In *A*–*C*, the left section is the elongation through the wild-type template, and the right section is the elongation through the linkage altered template. The concentration of NTP is 25 μM. (*A*) RNA pol II elongation starts from 10A primer. Time points are 0, 30 s, 5 min, 20 min, 1 h, 2 h, and 4 h, from left to right. (*B*) RNA pol II elongation starts from 11A primer. Time points are 0, 30 s, 2 min, 5 min, 20 min, and 1 h, from left to right. (*C*) RNA pol II elongation starts from 12C primer. Time points are 0, 30 s, 2 min, 5 min, 20 min, and 1 h, from left to right.

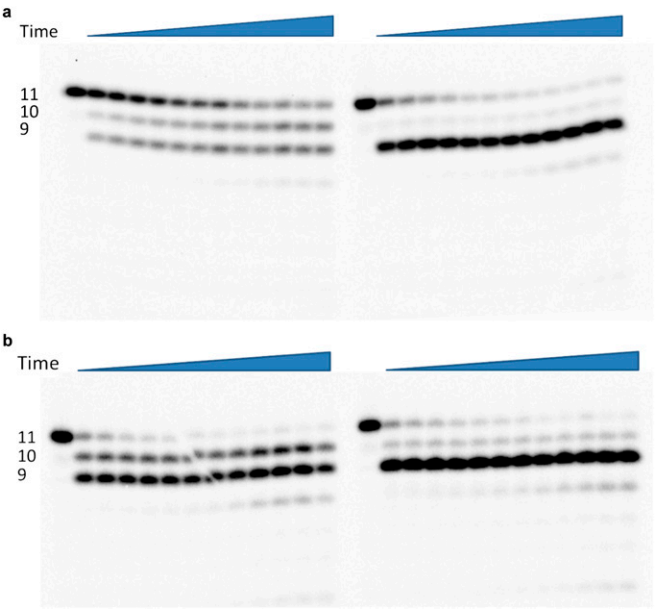

Fig. S3. TFIIS-mediated cleavage of scaffolds 11A (*Left*) and 11U (*Right*) in the template with a 3′–5′ linkage (*A*) or with a 2′–5′ linkage (*B*). Time points vary from 10 s to 1 h.

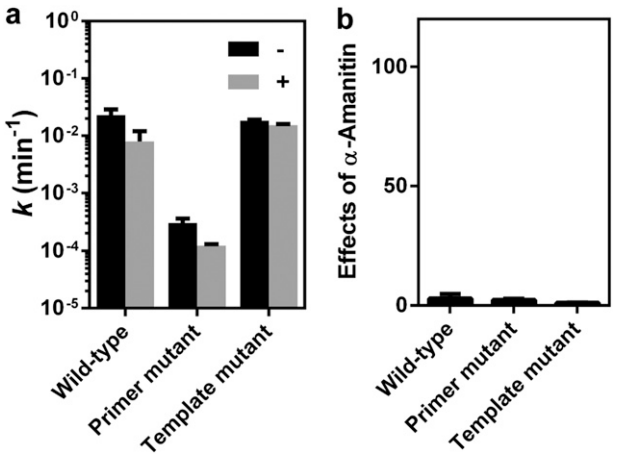

Fig. S4. Effects of α-amanitin during mismatched UTP incorporation. (*A*) Mismatched UTP incorporation rates in the absence (–) and presence (+) of α-amanitin. (*B*) Effects of α-amanitin on UTP incorporation. The effects of α-amanitin refer to folds of the rate changes before and after treatment of α-amanitin.

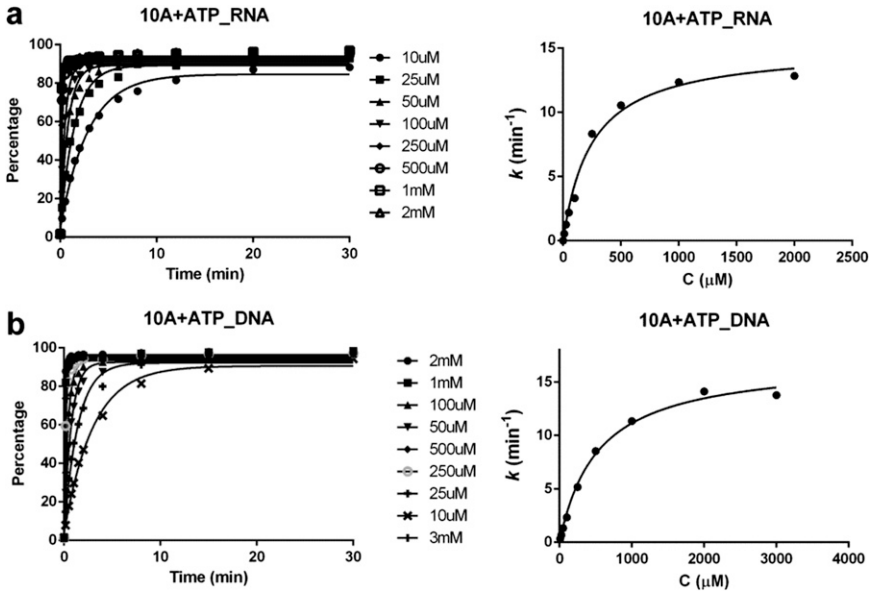

Fig. S5. Representative kinetic fitting curves of nucleotide incorporation in the presence of 2′–5′ linkage alteration. (*A*) Kinetic curves of nucleotide incorporation after the RNA primer with 2′–5′ linkage in the end (see scaffold 10A in Fig. 2*A*). (*B*) Kinetic curves of nucleotide incorporation in the DNA template with 2′–5′ linkage at the addition site (see scaffold 10A in Fig. 3C).

Table S1. Effects of linkage alteration in the DNA template on pol II transcriptional efficiency

Reaction	Linkage	k_{pol}, min^{-1}	K_d, μM	k_{pol}/K_d, $\mu M^{-1}\cdot min^{-1}$	Decrease*
10A + ATP	3′–5′	750 ± 210	90 ± 20	8.3 ± 3.0	~260
	2′–5′	17 ± 1	530 ± 60	0.032 ± 0.004	
11A + CTP	3′–5′	450 ± 20	52 ± 5	8.7 ± 0.9	~1,200
	2′–5′	6.3 ± 0.1	890 ± 70	0.0071 ± 0.0006	
12C + GTP	3′–5′	180 ± 30	78 ± 45	2.3 ± 1.4	~1,000
	2′–5′	3.8 ± 0.1	1,700 ± 100	0.0022 ± 0.0001	

*Decrease = $(k_{pol}/K_d)_{3'\text{-}5'}/(k_{pol}/K_d)_{2'\text{-}5'}$.

Table S2. Effects of linkage alteration in the DNA template on pol II transcriptional fidelity

Checkpoint	Linkage	Reaction	k_{pol}, min^{-1}	$K_{d,app}$, μM	$k_{pol}/K_{d,app}$, $\mu M^{-1}\cdot min^{-1}$	Discrimination*
Nucleotide incorporation	3′–5′	10A + ATP	750 ± 210	90 ± 20	8.3 ± 3.0	$(4.4 \pm 1.8) \times 10^5$
		10A + UTP	0.015 ± 0.003	800 ± 60	$(1.9 \pm 0.4) \times 10^{-5}$	
	2′–5′	10A + ATP	17 ± 1	530 ± 60	0.032 ± 0.004	910 ± 200
		10A + UTP	0.13 ± 0.01	3700 ± 700	$(3.5 \pm 0.7) \times 10^{-5}$	
Nucleotide extension	3′–5′	11A + CTP	450 ± 20	52 ± 5	8.7 ± 0.9	$(1.0 \pm 0.3) \times 10^5$
		11U + CTP	0.26 ± 0.05	3000 ± 700	$(8.7 \pm 2.6) \times 10^{-5}$	
	2′–5′	11A + CTP	6.3 ± 0.1	890 ± 70	0.0071 ± 0.0006	270 ± 60
		11U + CTP	0.021 ± 0.002	810 ± 150	$(2.6 \pm 0.5) \times 10^{-5}$	
Checkpoint	Linkage	Reaction	Cleavage rate (k_{obs}, min^{-1})			Discrimination†
Proofreading	3′–5′	11A	0.6 ± 0.1			12 ± 3
		11U	7.3 ± 1.6			
	2′–5′	11A	9.5 ± 0.8			1.3 ± 0.1
		11U	12 ± 1			

*Discrimination = $(k_{pol}/K_{d,app})_{ATP}/(k_{pol}/K_{d,app})_{UTP}$; $(k_{pol}/K_{d,app})_{11A}/(k_{pol}/K_{d,app})_{11U}$.
†Discrimination = $(k_{obs})_{11A}/(k_{obs})_{11U}$.