Supplementary Figure 1: Tgt inhibits transcription by the *E. coli* and *T. thermophilus* RNAPs similarly.

To ascertain that the structural data obtained with the *T. thermophilus* RNAP/Tgt complex would be applicable to other bacterial enzymes, we compared inhibition of the *E. coli* and *T. thermophilus* RNAPs by Tgt (**Supplementary Fig. 1**) using a promoter-specific assay with the λP_R promoter, which is utilized efficiently by both enzymes. Tgt inhibited transcription by the *E. coli* and *T. thermophilus* RNAPs similarly when added either during initiation or elongation: the apparent IC_{50} was only ~ two-fold higher for the *Thermus* enzyme, and the sites at which Tgt induced pausing/arrest during elongation were similar but not identical (**Supplementary Fig. 1b**). Consistent with the report of Matthews and Durbin[1], Tgt did not affect the open complex formation or stability (data not shown).

During elongation, the differences between the enzymes could be due to the differential recognition of the regulatory signals. We have demonstrated that the *B. subtilis* RNAP recognizes only a subset of signals that induce the *E. coli* RNAP to pause, and fails to pause at hairpin-dependent sites[2]. Interestingly, *T. thermophilus* RNAP also did not recognize the *his* pause site during elongation at either 37 ^{o}C (Fig. 1B) or 55 ^{o}C (data not shown). As noted for the inhibition of RNAP III transcription[3], Tgt action was site-specific, and the sites at which Tgt effect was particularly pronounced were mapped to pyrimidine-purine steps on this and other templates (**Supplementary Fig. 1b** and data not shown).

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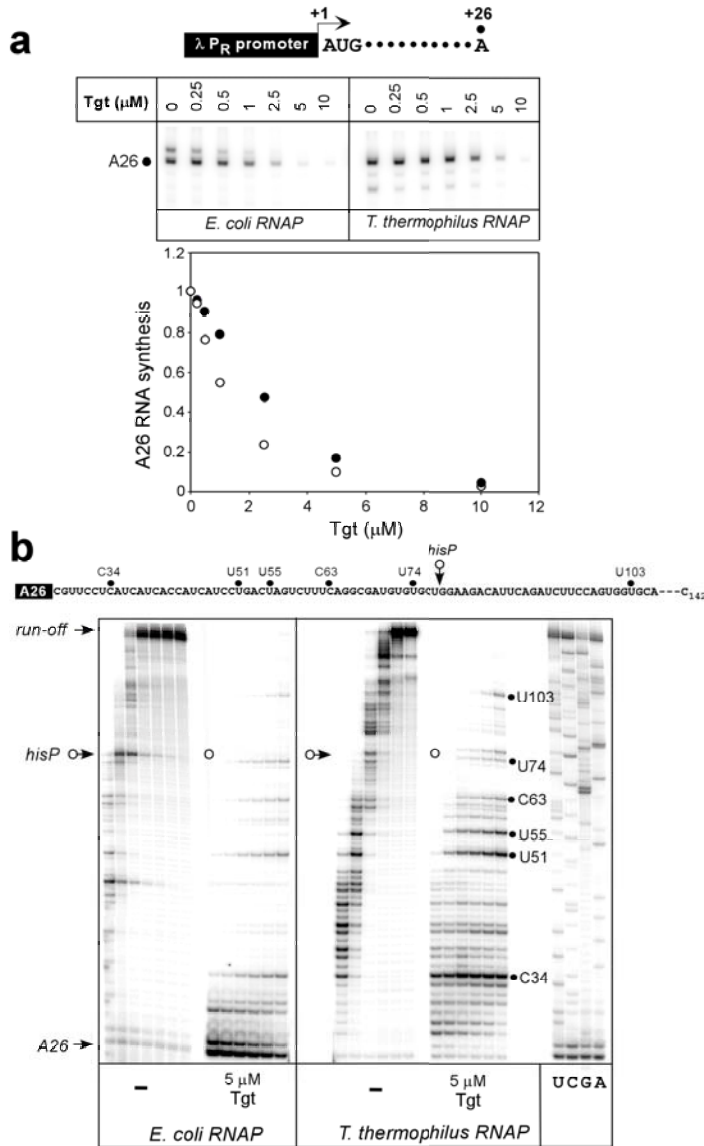


Supplementary Figure 1 Tgt inhibits transcription by the *E. coli* and *T. thermophilus* RNAPs. (**a**) Effect on halted complex formation on the phage λ P_R promoter template pIA226[4]. Open complexes were formed by the *E. coli* (○) and *T. thermophilus* (●) RNAPs (at 37 and 55 °C, respectively), and then incubated with increasing concentrations of Tgt and the substrates (ApU, GTP, [α-^{32}P]GTP, ATP and UTP) for 15 min at 37 °C. Samples were quenched and separated on a 10% denaturing gel. The relative amounts of synthesized A26 RNA products are plotted below the gel panel. (**b**) Effect on RNA chain elongation. Halted A26 TECs were formed on pIA226 by the *E. coli* and *T. thermophilus* RNAPs during a 15-min incubation at 37 and 55 °C, respectively, incubated with Tgt (5 μM) for 2 min at 37 °C, and challenged with NTPs (25 μM each) and rifampicin (at 25 μg/ml). Aliquots were withdrawn at 5, 10, 20, 40, 90, and 180 s, quenched with the STOP buffer, and separated on a 10% denaturing gel. Positions of the halted A26 TEC and the run-off transcript are indicated with arrows, the *his* pause RNA is indicated by an open circle. *T. thermophilus* RNAP does not recognize the *hisP* and other hairpin-dependent pause sites. Positions of the Tgt-induced pause sites (mapped using chain-termination reactions with 3'-O-Me NTPs) that are common to both bacterial enzymes are shown with bullets.

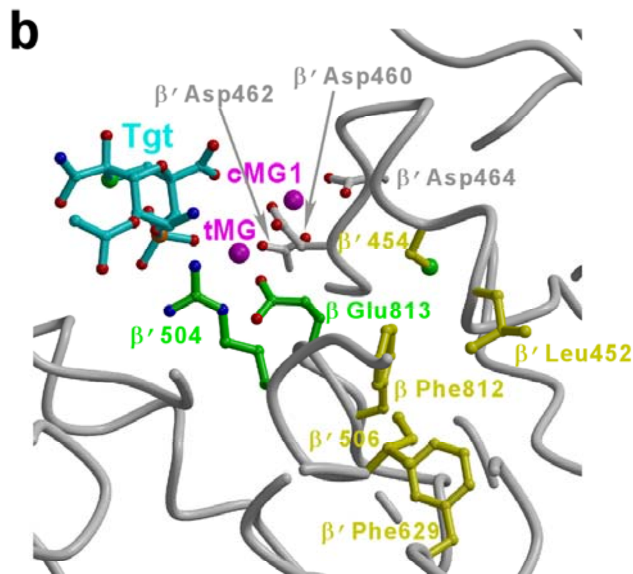
Supplementary Figure 2: Comparison with the eukaryotic enzymes

While eukaryotic pol III RNAP is sensitive to Tgt, pol II requires significantly higher Tgt concentrations for inhibition[1]. The simplest explanation is that pol II RNAPs carry amino acid substitutions that disrupt Tgt binding by either a loss-of-contact or steric hindrance mechanism. However, among the Tgt-binding residues (**Fig. 2**) no substitutions are unique for the pol II enzymes, making this hypothesis unlikely. Alternatively, given the concerted mode of the Tgt binding, one can easily envision how such binding interface would be compromised by mutations that either displace the residues directly involved in Tgt binding or destroy their supporting structural framework. One apparent candidate that may play such a role is a hydrophobic core region adjacent to the Tgt-binding site (in particular, to the β'Gln504 and βGlu813 residues crucial for the Tgt function; **Supplementary Fig. 1**).

a

	β		β′					
Eco	809	G Y N F E D S I	450	H P L V C A A Y	501	V P S Q D V V L G	626	Y T G F A Y A
Tth	681	G Y N F E D A I	729	H P L V C E A F	780	K P S R D I I L G	936	Y Y G F T F S
Ath	665	G Y N F E D A V	479	H P L V C K G F	530	V P T Q D M L I G	34*	T L G F Q Q A
Sc III	763	G Y D I E D A L	501	N E C V C T P Y	552	A A T Q D F I T G	699	K L C A R F L
Sc II	831	G Y N Q E D S M	471	N L S V T S P Y	522	G I V Q D T L C G	651	K V V N F W L

b



Supplementary Figure 2. Hydrophobic core adjacent to the Tgt binding site in bacterial RNAP. (**a**) Sequence alignment shows the RNAP regions flanking the conserved hydrophobic core in bacterial, chloroplast, and eukaryotic pol III RNAPs (yellow boxes) sensitive to Tgt and their polar substitutions in the Tgt-insensitive pol II (red boxes). Two Tgt-binding residues that might be affected by alterations of the hydrophobic core are highlighted by green boxes. The same species as in **Figure 2** were used for comparison. (**b**) Three-dimensional drawing of the Tgt-binding site shows the two Tgt-binding residues (green balls-and-sticks) in the vicinity to the hydrophobic residues making a local hydrophobic core that might be essential for formation of the Tgt binding site (yellow balls-and-sticks).

While this region is conserved among the bacterial and pol III enzymes, in pol II its conformation is defined by the hydrogen bonds made by the polar side chains substituting the hydrophobic residues observed in bacterial RNAP (**Supplementary Fig. 2a**). This may result in a reduced flexibility of the Tgt-binding site in pol II, thus presumably hindering the induced fit of the inhibitor to RNAP and disturbing this concerted mode of Tgt recognition.

Finally, resistance of pol II to tagetitoxin can arise from different catalytic properties of the enzyme itself (see below). Indeed, marked differences in transcription elongation and termination exist between pol II and pol III; some of these differences were attributed by Chedin and colleagues to the pol III subunit Rpc11, which apparently combines properties of pol II subunit Rpb9 and its transiently associated elongation factor TFIIS[2]. Considering profound differences in sequence and architecture of pol II and pol III, we presently cannot offer a simple explanation for their differential sensitivity to tagetitoxin.

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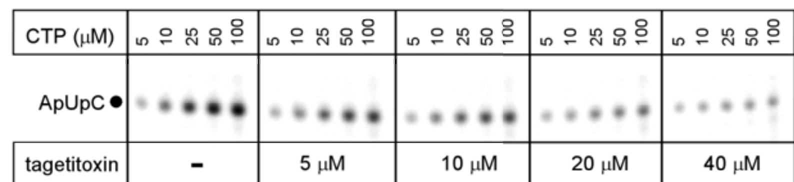
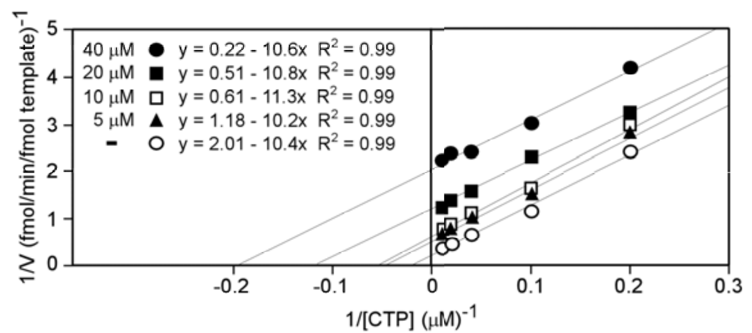
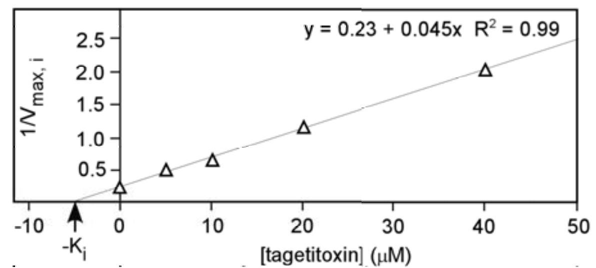
Supplementary Figure 3: Tgt acts as an uncompetitive inhibitor.

Mathews and Durbin[1] suggested that Tgt acts as an uncompetitive inhibitor of transcription at the λ P_R promoter. However, under certain conditions, mixed inhibition would yield the pattern similar to the uncompetitive inhibition[2], whereas the location of the Tgt-binding site next to the catalytic center suggests that a partial competition with the incoming substrate could be possible. To evaluate this possibility, we performed steady-state transcription initiation assays on T7A1 promoter and monitored synthesis of the ApUpC RNA from ApU and CTP substrates (**Supplementary Fig. 3**).







Supplementary Figure 3. Tgt acts as an uncompetitive inhibitor. (**a**) Steady-state abortive initiation assays were performed on T7A1 promoter template with the wild-type *E. coli* RNAP in the presence of increasing concentrations of Tgt. Samples were analyzed on a 15% denaturing gel. (**b**) Double reciprocal plots of inverse reaction velocity as a function of inverse substrate (CTP) concentration; linear fits are shown. (**c**) Intercept of the double reciprocal plot as a function of Tgt concentration. From these data, K_i for Tgt was 5 µM. Linear regression analysis was performed with Lotus 1-2-3 (IBM).

Double reciprocal plots (1/V vs 1/S) generated in the presence of different concentration of Tgt were nearly perfectly parallel. Moreover, the intercept replot as a function of inhibitor concentration was linear. This pattern is consistent with the uncompetitive but not with mixed inhibition pattern, as the latter would yield the hyperbolic replot[2]. Thus, in agreement with Mathews and Durbin and in support of the molecular modeling (**Fig. 5**), we conclude that Tgt acts as an uncompetitive inhibitor of RNAP, and does not compete with the incoming substrate NTP.

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Supplementary Table 1. Plasmids and oligonucleotides used in this work.

Name	Description	Source or note
pIA171	T7 A1 promoter transcription template	[1]
pIA226	λ P_R promoter - A26 - *his* pause transcription template	[2]
pIA299	P_{T7} –*rpoA*-*rpoB* -*rpoC*	[3]
pIA423	P_{T7} –*rpoA*-*rpoB* -*rpoC* [intein-CBP]	[3]
pIA434	P_{T7} –*rpoA*-[His_6]*rpoB* ^{QP513}-*rpoC* [intein-CBP] cloned between *Nco*I and *Sda*I sites of pIA423	this work
pIA509	P_{T7} –*rpoA*-*rpoB*-*rpoC*: co-expresses wild-type *E. coli* α, β, and β' core RNAP	this work
pIA530	P_{T7} –*rpoA*-*rpoB* -*rpoC* ^{N458D}[His_6]	[4]
pIA545	P_{trc}-[His_6]*rpoB*^{MfeI561}: silent *Mfe*I site introduced by site-directed mutagenesis at *rpoB* codon 561 in pIA160	[5]
pIA611	P_{trc}-*rpoC* ^{R731A}[His_6]; site-directed mutagenesis in pRL662	this work
pIA614	P_{trc}-*rpoC*^{Q504A}[His_6]; site-directed mutagenesis in pRL662	this work
pIA618	P_{T7} –*rpoA*-*rpoB* -*rpoC* ^{R731A}[His_6]: *Bsm*I-*Hin*dIII fragment from pIA611 cloned into pIA299	this work
pIA620	P_{trc}-[His_6]*rpoB*^{E813A}; site-directed mutagenesis in pIA545	this work
pIA621	P_{trc}-*rpoC*^{Q504A}[His_6]; *Sna*BI-*Sph*I site from pIA614 recloned into pRL662	this work
pIA622	P_{trc}-[His_6]*rpoB*^{S1105A}; site-directed mutagenesis in pIA545	[5]
pIA623	P_{T7} –*rpoA*-[His_6]*rpoB*^{S1105A} –*rpoC*: *Nco*I – *Sbf*I fragment from pIA622 cloned into pIA509	this work
pIA624	P_{T7} –*rpoA*-[His_6]*rpoB* ^{E813A} -*rpoC*: *Nco*I – *Sbf*I fragment from pIA620 cloned into pIA509	this work
pIA664	P_{trc}-*rpoB*^{R678A} [His_6]; site-directed mutagenesis in pIA545	this work
pRL662	P_{trc} -*rpoC*-[His_6]	[6]

TRANSCRIPTION TEMPLATES	
abortive initiation	PCR of pIA171 with 14 and 18 (ApUpC synthesis)
transcript cleavage and elongation	PCR of pIA226 with 17 and 18

OLIGONUCLEOTIDES FOR TEMPLATES		
14	GGAGAGACAACTTAAAGAGA	T7A1 upstream
17	CGTTAAATCTATCACCGCAAGGG	λP_R upstream
18	GGAAGATGATCTTCCGGGGGCTTTC	T7A1 and λP_R downstream

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