

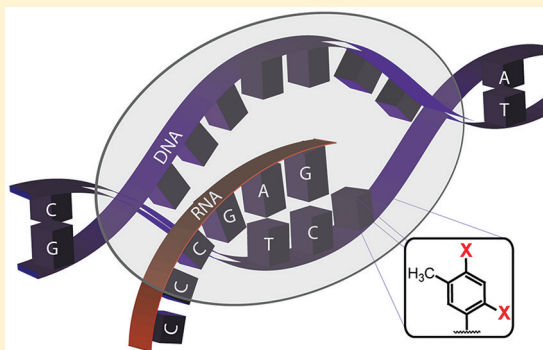
Importance of Steric Effects on the Efficiency and Fidelity of Transcription by T7 RNA Polymerase

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S Supporting Information

ABSTRACT: DNA-dependent RNA polymerases such as T7 RNA polymerase (T7 RNAP) perform the transcription of DNA into mRNA with high efficiency and high fidelity. Although structural studies have provided a detailed account of the molecular basis of transcription, the relative importance of factors like hydrogen bonds and steric effects remains poorly understood. We report herein the first study aimed at systematically probing the importance of steric and electrostatic effects on the efficiency and fidelity of DNA transcription by T7 RNAP. We used synthetic nonpolar analogues of thymine with sizes varying in subangstrom increments to probe the steric requirements of T7 RNAP during the elongation mode of transcription. Enzymatic assays with internal radiolabeling were performed to compare the efficiency of transcription of modified DNA templates with a natural template containing thymine as a reference. Furthermore, we analyzed effects on the fidelity by measuring the composition of RNA transcripts by enzymatic digestion followed by two-dimensional thin layer chromatography separation. Our results demonstrate that hydrogen bonds play an important role in the efficiency of transcription but, interestingly, do not appear to be required for faithful transcription. Steric effects (size and shape variations) are found to be significant both in insertion of a new RNA base and in extension beyond it.



The transcription of DNA is an essential enzymatic process for gene expression and is central to all forms of life. Although studied for decades, it is only more recently that its molecular mechanisms have been revealed.¹ This DNA-templated RNA synthesis is mediated by polymerases that operate with high processivity (~ 100 nucleotides/s)² and high selectivity/fidelity (error rate of $<10^{-5}$),³ thereby averting the appearance of mutagenic or cytotoxic errors.^{4,5} Despite the progress of our qualitative understanding of transcription through structural studies,¹ a more quantitative understanding of the transcription process at a molecular level is still needed.⁶ For instance, biochemical studies have demonstrated that hybridization energy does not fully account for the fidelity observed in DNA replication and transcription, thus suggesting that the confinement within the active site of polymerases affects base pair thermodynamics.^{7–10} Although studies have recently been performed with DNA polymerases to assess the relative importance of hydrogen bonds, sterics, stacking, and solvation during replication (vide infra), the roles of these forces in DNA transcription have not yet been addressed in detail.

We have introduced nonpolar nucleoside analogues for probing the relative importance of electrostatic and steric effects during DNA replication.¹¹ We have previously shown that *Escherichia coli* DNA polymerase I (Klenow fragment) and other A-family DNA polymerases can efficiently and selectively replicate isosteres of thymine (Figure 1) even though they undergo little or no hydrogen bonding.^{12,13} A size-exclusion/

steric matching model that provides a conceptual framework for the role of steric interactions of the base pair within the confined environment of the enzyme's active site has been proposed.^{7,14,15} To test steric effects systematically, we recently reported the design and synthesis of a series of nonpolar nucleoside analogues with sizes varying in subangstrom increments (Figure 1),^{16,17} and we demonstrated that small size variations can dramatically affect the efficiency and fidelity of the enzymatic replication performed by DNA polymerase I in vitro and by cellular enzymes in *E. coli*.^{18,19} Overall, these results demonstrate that the contribution of A-family polymerases to the stability of base pairs is mainly due to steric interactions within the active site.²⁰ Although all DNA polymerases operate by a chemically similar two-metal ion mechanism,²¹ we have shown that these probes can be used to reveal differences in the relative importance of hydrogen bonds and sterics. For instance, hydrogen bonds were found to be important for Y-family error-prone DNA polymerase IV, while steric effects were much less important, suggesting considerable steric flexibility, which may aid in replication beyond damaged bases.¹⁹ In line with the size-exclusion model, we have noted a correlation between the structural flexibility of the active site and the fidelity of the enzyme: rigid active site in high-fidelity

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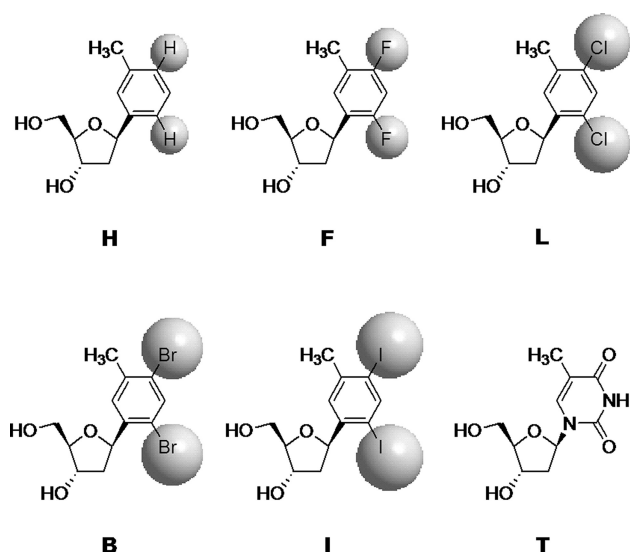


Figure 1. Structures and nomenclature of nonpolar nucleoside analogues of thymidine. The van der Waals spheres of the heteroatoms are colored gray to illustrate the size variations among the series. The interatomic bond lengths vary as follows: 1.08 Å for Ar–H, 1.36 Å for Ar–F, 1.74 Å for Ar–Cl, 1.90 Å for Ar–Br, and 2.10 Å for Ar–I.¹⁸

T7 DNA polymerase I²² and loose active site in low-fidelity Dpo4 polymerase.²³ Here we report the unprecedented use of these nonpolar analogues to probe the roles of steric and electrostatic effects on the efficiency and fidelity of DNA transcription by T7 RNA polymerase.

Unlike most DNA polymerases, RNA polymerases perform a *de novo* templated synthesis.²⁴ The transcription process starts with the enzyme binding a promoter region and unwinding the upstream DNA duplex, thereby opening a “transcription bubble”.²⁵ The initiation step then involves binding and polymerization of individual nucleoside triphosphates (rNTPs) until a sufficiently long transcript (ca. 8–10 nucleotides) has been formed. Because shorter transcripts can easily dissociate from the template, many short products are formed during this abortive cycling. The polymerase then undergoes a major conformational change,^{26–28} which triggers the release of the promoter, and enters its elongation mode that is characterized by a high processivity (~30-fold increase of the rate of formation of the phosphodiester bond in the elongation compared to the initiation phase for T7 RNA polymerase).²⁹ Structural studies have revealed the different steps of the stepwise nucleotide addition process: (1) rNTP binding at a preinsertion site, (2) conformational change of the enzyme, (3) chemical reaction, and (4) translocation. Although there are notable structural differences among DNA-templated RNA polymerases, the overall sequence of events is a common feature. The growing RNA transcript remains within the transcription bubble where it is bound to the DNA template. This DNA–RNA heteroduplex adopts an underwound A conformation, and its stability is an important factor affecting the efficiency of transcription elongation.³⁰

Although structural studies have revealed the molecular basis of DNA transcription, our understanding of this process remains essentially qualitative and the detailed balance of forces that determine the efficiency and the fidelity at the molecular level remains elusive. The present study addresses this by (a) comparing nonpolar thymine template analogues to the polar thymine, thus addressing the importance of electrostatics in the

absence of steric differences, and (b) comparing transcription of variably sized and shape-altered analogues, thus allowing for the evaluation of the role of sterics (size and shape) in the formation of a new RNA–DNA base pair and extension beyond it.

EXPERIMENTAL PROCEDURES

Synthesis of Nonpolar Nucleoside Analogues. Compounds H, F, and L were synthesized as previously described.¹⁷ Compounds B and I were prepared according to modified procedures (Supporting Information). The synthesis of compound MeL is reported in the Supporting Information. The corresponding DMT-protected and phosphoramidate derivatives were prepared as previously described.¹⁶

Oligonucleotide Syntheses. DNA oligonucleotides were synthesized on an Applied Biosystems 394 synthesizer using standard α -cyanoethylphosphoramidite chemistry. DNA oligonucleotides were synthesized in DMT-off mode, deprotected in concentrated NH_4OH at 55 °C overnight, purified by preparative 20% denaturing polyacrylamide gel electrophoresis, isolated by the crush and soak method, characterized by MALDI-TOF mass spectrometry (Supporting Information), and quantitated by absorbance at 260 nm. Molar extinction coefficients were calculated by the nearest neighbor method as previously described.^{16,18}

In Vitro Transcription. One microliter of a 1 μM solution of promoter (final concentration of 100 nM), 1 μL of a 1 μM solution of DNA template (final concentration of 100 nM), and 1 μL of Tris–HCl with NaCl (10 mM each) were annealed by being heated to 95 °C over 3.5 min and then slowly cooled to 4 °C over 60 min. One microliter each of 200 μM GTP, CTP, and UTP (final concentration of 20 μM) were added, followed by 1 μL of [α -³²P]ATP [10 μCi (Perkin-Elmer)]. One microliter of 10 \times Takara buffer [400 mM Tris–HCl (pH 8.0), 80 mM MgCl_2 , and 20 mM spermidine] and 1 μL of 50 mM DTT were added. After a quick centrifugation of the mixture, 1 μL (50 units³¹) of T7 RNAP (Takara) was added, and the mixture was incubated at 37 °C. The reaction was quenched by addition of 10 μL of gel loading buffer (10 M urea in 1 \times TBE), and then the mixture was heated at 75 °C for 3 min and subjected to denaturing gel electrophoresis (20% polyacrylamide, 7 M urea, and 1 \times TBE). Quantification was performed on a PhosphorImager (Molecular Dynamics) with ImageQuant.

Transcript Digestion and Two-Dimensional (2D) TLC Analyses. Ribonuclease I (RNase I) was used to digest RNA transcripts and yield a mixture of 3'-monophosphate nucleosides. Because the phosphorus atoms of phosphodiester remain linked to the 3'-hydroxyl group, the 3'-radiolabeled nucleoside monophosphates are those that were connected to the 5'-end of radiolabeled ATP in the RNA transcript. Therefore, we excised band N' + 1 (see Figure 3) because the radiolabeled phosphorus atom present at the 3'-end (A) of the transcript will be transferred to the 3'-monophosphate nucleoside Y, thus making the site of interest for the determination of the transcription fidelity visible by radioimaging.

The band of interest was located by exposure to film (Kodak X-OmatARS). It was then excised, and the product was eluted with 200 μL of water for 8 h. One microliter of 3 M sodium acetate, 1 μL of ~1 mM tRNA (Sigma Aldrich), and 1.2 mL of ethanol were then added to the solution which was then kept overnight in the freezer. After a centrifugation, the supernatant solution was removed. Sixteen microliters of water, 2 μL of 10 \times

TNE buffer, and 1 μ L of RNase I [10 units (Epicentre Biotechnologies)] were successively added, and the mixture was incubated at 37 °C for 3–4 h. The reaction mixture was then directly loaded onto TLC plates (HPTLC plates, 100 mm \times 100 mm, Merck, Darmstadt, Germany). Elution was performed with the following mixtures: first dimension, 66/1/33 isobutyric acid/ammonia/H₂O; second dimension, 100 (v)/60 (w)/2 (v) sodium phosphate (0.1 M, pH 6.8)/ammonium sulfate/*n*-propanol. Quantification was performed on a PhosphorImager (Molecular Dynamics) with ImageQuant.

RESULTS

Templates Containing Analogues. We prepared template DNAs containing nonpolar isostere F or native T to evaluate the importance of hydrogen bonding in the synthesis of a new base pair at a position 13 nucleotides downstream from the transcription start site (Figure 2). We also synthesized

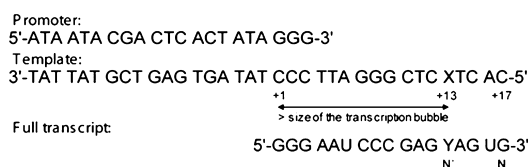


Figure 2. Sequences of the DNA promoter top strand, DNA template, and RNA full transcript. X denotes the positions where nonpolar analogues (H, F, L, B, and I) were placed. Y, at position N' of the RNA transcript, denotes the nucleoside placed opposite X during the enzymatic transcription by T7 RNAP.

templates with variably sized thymine analogues H, F, L, B, and I (Figure 1) to measure the role of steric effects in this process. Modified DNAs were characterized by mass spectrometry (see Supporting Information).

In vitro transcription assays. Enzymatic transcription reactions were performed with T7 RNAP on a 35-mer DNA template featuring a) the consensus double-stranded T7 RNAP 18-mer promoter, and b) a 17-mer containing the nonpolar nucleoside analogues at position 13 (Figure 2). The modification was placed 13 nucleotides downstream of the initiation position to ensure that the enzyme was in elongation mode once it reached that position. The context of the modified nucleobase (X) was 5'-TXC, which should be noted because context can exert an influence on RNA polymerase fidelity.^{32,33}

Enzymatic transcription reactions were monitored by internal radioisotopic labeling with [α -³²P]ATP. Because T7 RNAP does not exhibit intrinsic proofreading,³³ we applied the standard model³⁴ used in running start reactions with DNA polymerases to quantify the efficiency of transcription that is defined by the ratio of the rate constant for polymerization (k_{pol}) and the rate constant for pausing (k_{off}). The efficiency of transcription at site m can thus be calculated as follows:

$$\left(\frac{k_{\text{pol}}}{k_{\text{off}}} \right)_m = \frac{\sum_{n=1}^{\infty} I_n}{I_m} \quad (1)$$

where I_m is the intensity of the band corresponding to the stalled product at position m and I_n is the intensity of each elongated transcript. In this study, transcripts up to 18-mer were taken into account as longer transcripts were seen to be minor byproducts most probably arising from RNA-templated RNA synthesis.³⁵ As defined, the efficiency of transcription

represents the ability of the polymerase to pass a site m and produce extended transcripts.

Transcription Efficiency. We first assessed the overall transcription efficiency by comparing the amount of full transcript produced with the different templates. A gel electrophoretic analysis of the enzymatic reactions with different DNA templates is shown in Figure 3. Full-length

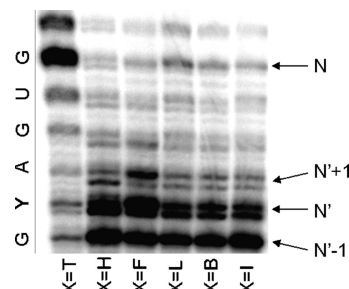


Figure 3. Enzymatic transcription with different DNA templates (X = T, H, F, L, B, and I). N denotes the band corresponding to the full transcript, whereas N' denotes the band corresponding to the transcript stalled at the position of the artificial modification X. The transcript sequence is noted on the left. The reaction time was 30 min.

transcript (N) is clearly visible for the thymine template and for the most efficient of the analogues. We noted that several bands appear doubled both for natural and unnatural templates. A composition analysis was performed on the two N' + 1 bands excised separately and showed no significant differences in composition at this position (see Supporting Information). In the subsequent data analyses, we therefore considered the ensemble of two bands together, because the apparent inhomogeneity occurred at an earlier site in the transcript.

In comparison to that of the natural thymidine-containing template, the transcription of DNA templates containing nonpolar analogues gave markedly lower yields of the full transcript (5–10% relative to the natural template). To gain a more detailed understanding of the origin of this lower efficiency, we determined the efficiency of insertion and elongation with eq 1. The results are represented in Figure 4.

Upon comparison of the natural template T with its nonpolar isostere F, the results show that the efficiencies of both insertion and extension are considerably decreased (by ~ 1 order of magnitude). Comparison among the series of nonpolar analogues allows probing of the effect of small size variations on the efficiency of transcription, and interesting effects were observed. First, the smallest and largest substrates, H and I, respectively, display the poorest efficiency of insertion, significantly lower than that of analogue F [Student's t test between H and F, $\text{Prob} > |t| = 0.0538$ ($p = 0.0715$); Student's t test between F and I, $\text{Prob} > |t| = 0.0528$ ($p = 0.0613$)]. Second, a small preference for F over L and B is observed. The relationship between the efficiency of transcription and the size of the analogues thus follows a bell-shaped trend similar to previous observations with DNA polymerase I,¹⁸ except that the maximum here lies at F, while previous DNA polymerases showed a slightly stronger preference (L). In contrast, the efficiencies of elongation show little dependence on the size of the analogues, thereby suggesting that steric constraints are lower further into the enzyme active site.

We then monitored the initial phase of transcription and determined the transcription efficiency of insertion at low levels

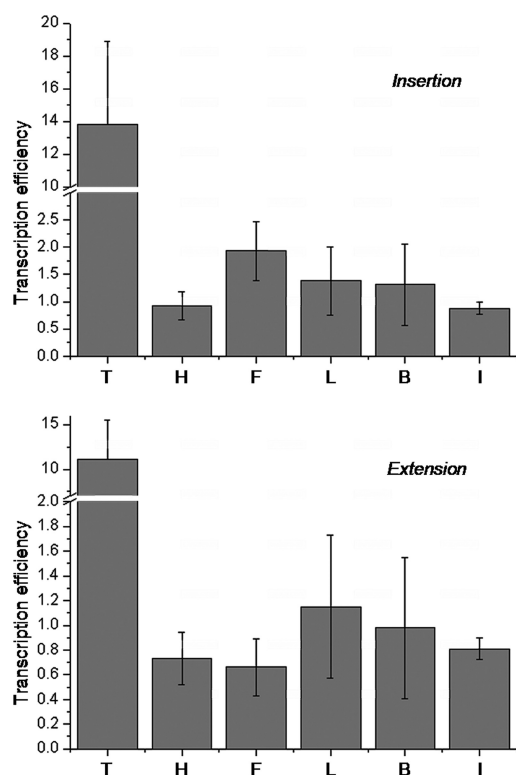


Figure 4. Comparison of the transcription efficiencies of insertion (top) and extension (bottom) for varied DNA templates ($X = T, H, F, L, B,$ and I). Data were obtained from eq 1 in which $m = N' - 1$ (insertion) or N' (extension). The reaction time was 30 min (three replicates). Error bars represent one standard deviation.

of product conversion (i.e., at short reaction times) (Figure 5 and Supporting Information).

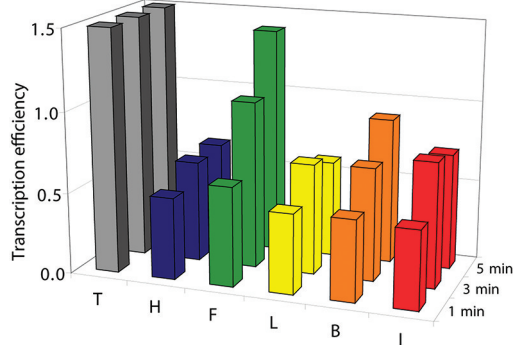


Figure 5. Comparison of the transcription efficiencies of insertion between different DNA templates ($X = T, H, F, L, B,$ and I) during the initial phase of transcription at low levels of product conversion. Data obtained from eq 1 where $m = N' - 1$. Bars for T are truncated, actual values being 10.1 at 1 min, 9.6 at 3 min, and 10.0 at 5 min. The reaction time is noted at the right (1, 3, and 5 min).

Overall, this experiment confirms the ~ 10 -fold reduction in the efficiency of transcription when thymine is replaced with a nonpolar analogue. However, the results clearly show variations among the series of analogues, F being processed much better (~ 2 times) than the other size variants, thereby confirming that the correlation between the efficiency of insertion and the size of the nonpolar analogues follows a curve centered on F, which is the same size as thymine.

In addition to nucleobase size, another relevant steric property is shape. To assess how the shape of thymine, with its substitution at positions 2 and 4, and the lack thereof at the 3 position affect activity, we prepared a previously unknown nonpolar nucleoside analogue having a distorted shape. Compound Me_L features an extra methyl group protruding at the 3 position of the phenyl ring (Figure 6), disrupting the

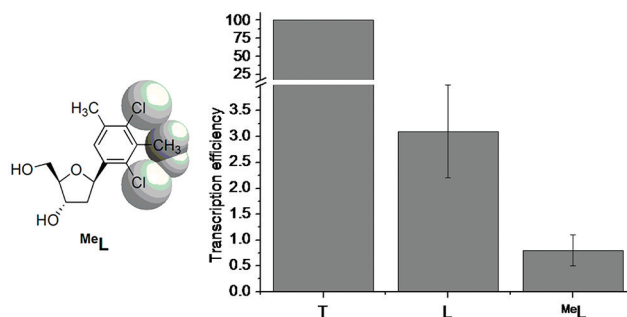


Figure 6. Structure of shape-modified nonpolar nucleoside analogue Me_L with van der Waals spheres colored gray (left) and full transcript yields of enzymatic transcription reactions with different DNA templates ($X = T, L,$ and Me_L) (right). Normalized yields (template T, 100). The reaction time was 30 min (three replicates). Error bars represent one standard deviation.

thymine-like shape markedly.³⁶ It is a nonpolar mimic of N-3-methylated thymine, a common form of DNA damage.^{37–40} We tested the effect of this shape modification by comparing enzymatic transcription reactions with DNA templates L and Me_L (Figure 6 and Supporting Information).

The experiments revealed that the shape-modified analogue Me_L is processed less efficiently by T7 RNAP and leads to significantly more stalling. The full transcript yield is 4 times lower with Me_L than with L, thereby demonstrating that a thymine-like shape makes a significant contribution to the overall efficiency of base pair formation (presumably opposite adenine, vide infra). More detailed analysis revealed that both the efficiency of insertion and extension are adversely affected when L is replaced with Me_L (see the Supporting Information), which is consistent with the notion that the base pair being synthesized must fit into an active site that normally surrounds a canonical pair.

Transcription Fidelity. To determine the fidelity of the enzymatic transcription of the thymine analogues, we analyzed the composition of transcripts by performing an enzymatic digestion followed by 2D TLC separation and quantification by radioimaging (Figure 7 and Supporting Information). This

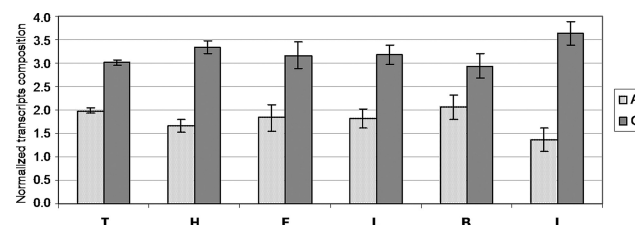


Figure 7. Normalized composition of transcripts $N' + 1$ determined by enzymatic digestion followed by 2D TLC separation and quantification by radioimaging. Error bars represent one standard deviation. Minimum of three replicates.

established method has been previously used in similar contexts.^{41,42}

When $X = T$, the enzymatic digestion of transcript $N' + 1$ should yield a ratio of three radiolabeled GMP mononucleotides to two radiolabeled AMP molecules (see Experimental Procedures for analysis). Any deviation from this composition should therefore reflect a decreased fidelity. As shown in Figure 7, the composition analysis of transcript $N' + 1$ formed from the enzymatic transcription of the natural DNA template where $X = T$ indeed gives the expected ratio of three GMP mononucleotides to two AMP molecules. When the enzymatic transcription was conducted with the modified DNA template containing the nonpolar isostere F, the results showed a similar composition of transcript, thereby indicating a comparable fidelity of transcription. Interestingly, significant differences were observed among the series of nonpolar analogues (Figure 7). The smallest (H) and largest (I) analogues gave the largest deviation from the ideal fidelity one expects if they were recognized as thymine analogues, showing that they are processed with a significantly lower fidelity [the theoretical limit for the case of a 0% fidelity opposite site X being a value of 1.25 for A (Figure 7)].

DISCUSSION

Effects on the Efficiency of Insertion. Our results clearly show an overall reduction in the efficiency of nucleotide insertion by T7 RNAP when hydrogen bonding groups are missing in the templating base. This can be due to either an inhibition of the required conformational change of the enzyme or a misalignment of the rNTP within the active site of T7 RNAP. Patel et al. have recently suggested, on the basis of kinetics studies, that the induced fit conformational change is the rate-determining step of transcription of DNA by T7 RNAP.²⁹ It is therefore reasonable to surmise that the variations in transcription efficiency observed here are due to perturbation of the rates or populations of open versus closed conformations of the enzyme. Crystal structures of the complexes with a complementary rNTP in the preinsertion⁴³ and insertion⁴⁴ sites have shown that the template base and the incoming rNTP adopt more favorable orientations and are in closer contacts, through hydrogen bonds, in the insertion site than in the preinsertion site. Furthermore, previous studies with DNA Pol I have shown that hydrogen bonds play a significant (although not essential) role in stabilizing the closed ternary complex.⁴⁵ Because DNA Pol I and T7 RNAP share considerable homology,^{46,47} it is plausible that the same is true for T7 RNAP. Overall, these data suggest that hydrogen bonds are important for triggering the conformational change in T7 RNAP and that the effect is greater for this RNA polymerase than for A-family DNA polymerases. Specifically, which hydrogen bonds play the most important role is discussed below.

Our observation that varying templating base size in very small gradations affects efficiency significantly suggests that the steric accommodation of the incoming NTP within the enzyme active site also contributes to the transcription efficiency, most probably by affecting the positioning of reactive groups.⁴³ This conclusion is in line with biochemical studies that have shown that in T7 RNAP mutations that facilitate the accommodation of incorrect base pairs in the catalytically competent closed conformation increase the rate of misincorporation.^{33,43} The observation that a template base (^{Me}L) having a shape improper for pairing with any natural base gives a lower efficiency of

insertion also supports the idea that steric effects do play a role in transcription efficiency.

Effects on the Efficiency of Extension. Our results show that the extension step with T7 RNA polymerase depends strongly on hydrogen bonding groups in the templating base. Hydrogen bonds involving nucleobases in polymerase active sites can play a role in two ways: via H-bonds between the bases, and between the bases and the enzyme in the minor groove. Hirao et al.⁴¹ and Romesberg et al.⁴² have shown that T7 RNAP is capable of efficiently incorporating and extending synthetic base pairs lacking hydrogen bonds between them. This is in contrast with our present observation, where transcription efficiency is apparently lower. However, the compounds used in those prior studies all feature polar moieties pointing toward the minor groove, while our compounds do not. This suggests that the most important electrostatic effects are not hydrogen bonds between the bases but rather those directly with the enzyme. Biochemical studies have previously shown that the H784A mutation results in an enhanced rate of extension of misincorporations.³³ In addition, examination of recent structural data reveals likely polar contacts between H784 and the minor groove edge of the 3'-end of the RNA transcript (distance of 2.8 Å between N3 of H784 and N3 of the terminal adenine in the RNA transcript).⁴⁴ Our results suggest that moving a nonpolar base opposite such a polar group is destabilizing at the transition state of extension, possibly by desolvation of the polar group. Similar effects have been seen with DNA polymerases previously.⁴⁸ Our results suggest that electrostatic interactions between H784 and the minor groove edge of the base pair are important for the extension.⁴⁹ Overall, the results suggest that H784 plays a crucial role as a checkpoint and that it senses newly formed base pairs through polar contacts in the minor groove of the 3'-end of the RNA transcript.

Effects on Fidelity. The results obtained here show similar values of fidelity when thymine is replaced with synthetic nonpolar analogues, thereby showing that the enzyme recognizes these substrates as if they were thymine templates. This result demonstrates that in T7 RNAP, Watson–Crick hydrogen bonds are not essential for the determination of the fidelity of transcription. This is consistent with the results of Hirao et al.⁴¹ and Romesberg et al.,⁴² who have shown substantial fidelity in novel nonpolar base pairs. Biochemical studies have shown that enzyme mutations that stabilize the catalytically active closed complex increase the rate of misincorporation.³³ This gives support to a screening and selection of the appropriate rNTP that takes place when the enzyme is in the catalytically incompetent open state, the induced fit conformational change from the open to the closed state being thus powered by the energy of binding of the rNTP to the active site opposite the templating DNA nucleobase.⁴³ Because T7 RNAP does not display intrinsic proofreading,³³ the fidelity of the transcription is determined by the selectivity of recognition of the correct rNTP in the preinsertion complex. Our results indicate that fidelity is retained in the absence of hydrogen bonds between the partners. Small size variations, tested with the series of nonpolar analogues varying in size in subangstrom increments, indicate that the preinsertion site is somewhat plastic and can accommodate the smallest size variations reasonably well. However, larger size variations, as seen with analogues H and I, significantly reduced the fidelity of the transcription, thereby indicating the presence of significant steric gating.

Overall, the results show the utility of size-varied nonpolar nucleobase analogues for analyzing mechanisms in a class of polymerases distinct from DNA polymerases. Future work will apply these analogues to the mechanistic study of enzymes involved in eukaryotic transcription.

■ ASSOCIATED CONTENT

● Supporting Information

Experimental details about the synthesis and characterization of compounds **B**, **I**, and **M^eL** and additional data on the enzymatic transcription assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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■ ABBREVIATIONS

TLC, thin layer chromatography; NTP, nucleoside triphosphate; DTT, dithiothreitol; TBE, Tris-Borate-EDTA.

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