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A Mechanism of Nucleotide Misincorporation during Transcription due to Template-Strand Misalignment

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Summary

Transcription errors by T7 RNA polymerase (RNAP) may occur as the result of a mechanism in which the template base two positions downstream of the 3′ end of the RNA (the TSn+1 base) is utilized during two consecutive nucleotide-addition cycles. In the first cycle, misalignment of the template strand leads to incorporation of a nucleotide that is complementary to the TSn+1 base. In the second cycle, the template is realigned and the mismatched primer is efficiently extended, resulting in a substitution error. Proper organization of the transcription bubble is required for maintaining the correct register of the DNA template, as the presence of a complementary nontemplate strand opposite the TSn+1 base suppresses template misalignment. Our findings for T7 RNAP are in contrast to related DNA polymerases of the Pol I type, which fail to extend mismatches efficiently and generate predominately deletion errors as a result of template-strand misalignment.

Introduction

Accurate transmission of genetic information is essential to the survival of all organisms. Due to the importance of DNA as the primary genetic repository, most studies of fidelity have focused upon DNA replication, and the accuracy of other processes such as transcription or translation have been less well characterized. In this work, we examined the fidelity of RNA synthesis by the single-subunit DNA-dependent RNAP encoded by bacteriophage T7. T7 RNAP is related to members of a superfamily of nucleotide polymerases that include DNA-directed DNA polymerases (DNAPs) of the Pol I type, mitochondrial RNA polymerase, and reverse transcriptase (RT); a study of mechanisms that affect the accuracy of this enzyme is therefore of general interest.

Previous studies suggested that the accuracy of Pol I DNAPs depends primarily upon the geometry of the active site, which favors the incorporation of substrates that form correct Watson-Crick base pairs and discriminates against the incorporation of non-Watson-Crick base pairs (Kunkel and Bebenek, 2000; Kunkel, 2004; Patel et al., 2001; Doublie and Ellenberger, 1998; Wong et al., 1991; Li and Waksman, 2001). Recent crystallographic results with T7 RNAP and Pol I DNAP have shown that movement of the incoming nucleotide and the template base into the active site requires a nearly identical "open"-to-"closed" conformational change in protein structure (Johnson et al., 2003; Li et al., 1998; Temiakov et al., 2004; Yin and Steitz, 2004) (see Figure 1A). In the open configuration of T7 RNAP, which is in the posttranslocated state and is catalytically inactive, the template-strand base that is to direct the incorporation of the incoming nucleotide (the TSn base) is not located in the active site but is located in an adjacent site (the preinsertion site), where it is poised to interact with the incoming NTP (Tahirov et al., 2002; Temiakov et al., 2004; Yin and Steitz, 2002, 2004). During the transition to the catalytically active closed conformation, the TSn base (and the incoming nucleotide) becomes positioned in the active site (the insertion site) in a manner that is required for the phosphotransferase reaction. Throughout this process the TSn+1 base, which lies immediately downstream of the TSn base, is thought to remain paired to the nontemplate (NT) strand, such that the transfer of the TSn+1 base to the preinsertion site for the next incorporation cycle would require unwinding of the downstream DNA and translocation of the template (Yin and Steitz, 2004). It has been suggested that the open-to-closed conformation change couples translocation to the catalytic cycle, thereby maintaining the correct register of the template strand and preventing frameshift errors (Johnson et al., 2003; Yin and Steitz, 2004).

Consistent with a mechanism for substrate discrimination that involves selective geometry of the active site (the induced-fit mechanism), the majority of residues found to be important for nucleotide selection in Pol I DNAPs are located in or around the insertion site (Bell et al., 1997; Minnick et al., 1999; Suzuki et al., 1997, 2000). However, most mutations that have thus far been found to alter the fidelity of T7 RNAP affect residues around the preinsertion site (Huang et al., 2000). Moreover, the structure of the open conformation of T7 RNAP with incoming substrate revealed that preliminary base pairing of the NTP with the TSn base occurs in the preinsertion site, indicating that T7 RNAP initially selects the correct nucleotide in the open conformation, prior to its transport into the active site (Temiakov et al., 2004). In the structures of the open conformation of Pol I DNAP in the presence of the incoming dNTP, the TSn base is more deeply buried in the preinsertion site, suggesting that steric clash would prevent a similar

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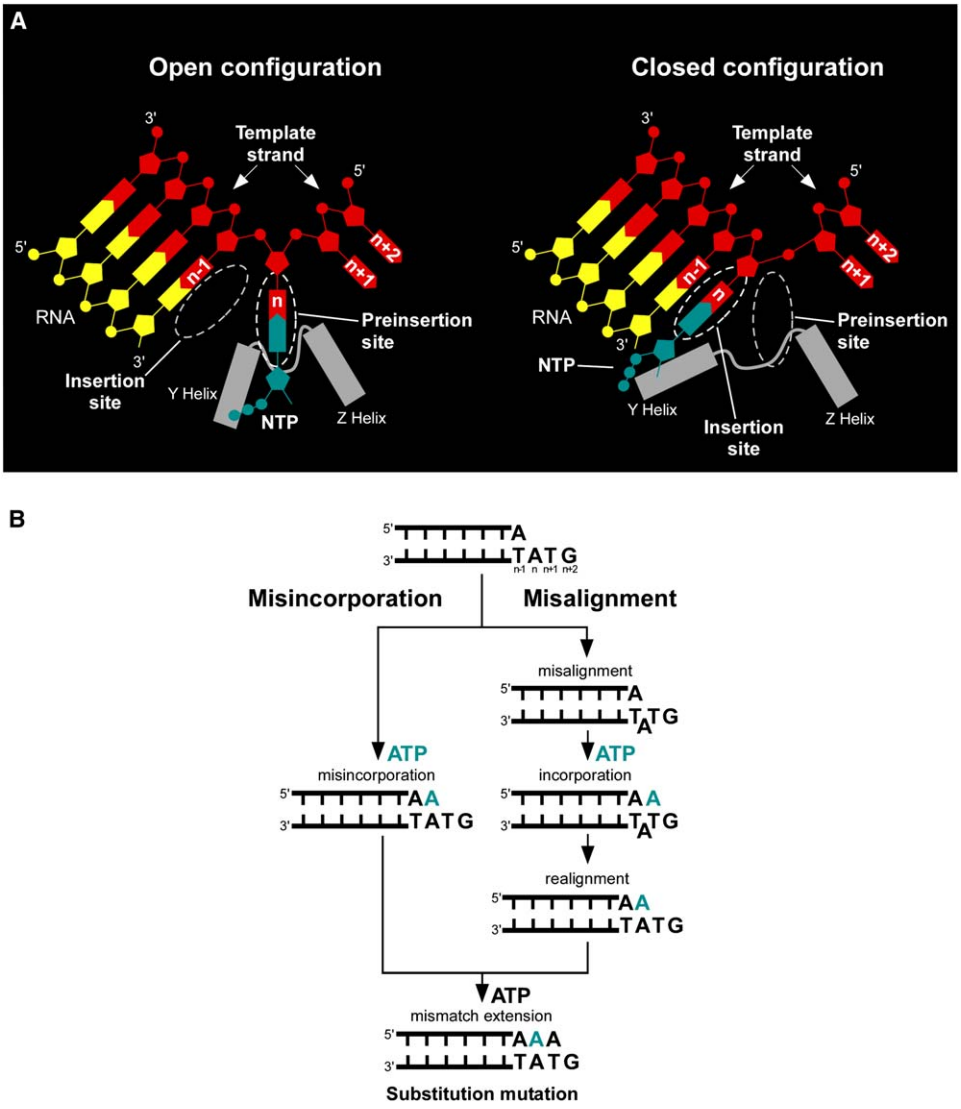

Figure 1. Models of Base Substitution Errors as a Result of Misalignment or Misincorporation

(A) Organization of the active site of T7 RNAP. In the catalytically inactive open configuration, the template-strand base (TSn) is located in a preinsertion site where it may form incipient hydrogen bonds with the incoming NTP (left). In the closed configuration, the TSn base and the cognate NTP become positioned in the insertion site in a manner that is required for the phosphotransferase reaction (right). This transition involves rotation of the Y and Z α helices (O and O′ helices in the related Pol I DNAPs). Template strand, red; RNA primer, yellow; and incoming NTP, blue.

(B) Mechanisms of substitution errors due to misincorporation or misalignment. In the misincorporation model (left), the +1 NTP (ATP) is misincorporated opposite the TSn base (A), resulting in an A:A mismatch at the 3′ terminus of the RNA:DNA hybrid; this is followed by mismatch extension. In the misalignment model (right), misalignment of the template strand allows incorporation of the +1 NTP (ATP) opposite the TSn+1 base; this is followed by realignment of the primer/template and mismatch extension as above.

(To facilitate comparison between DNAPs and RNAPs, the nomenclature used to identify positions in the nucleic acid components in this work follows the convention used in studies of DNAPs. It should be noted that an alternative nomenclature is also used in RNAP studies, in which the i site is occupied by the 3′ end of the RNA in the posttranslocated state, and i+1 corresponds to the active site, which is occupied by the 3′ end of the RNA in the pretranslocated state and the NTP substrate in the posttranslocated state.)

interaction of the incoming dNTP with the TSn base in this enzyme (Johnson et al., 2003; Kiefer et al., 1998; Li et al., 1998; Temiakov et al., 2004). However, slight rotation of the protein elements in the preinsertion site in Pol I DNAP might expose the TSn base for pairing with the incoming dNTP during the open-to-closed transition, indicating that a similar mechanism of substrate selection might also contribute to the fidelity of Pol I DNAPs (as has been suggested for T4 DNAP [Hariharan et al., 2006]). Structural analysis of DNAPs of the error-prone Y family indicates that factors that may contribute to the low fidelity of these enzymes include limited protein-nucleic acid contacts and a more open substrate binding pocket (Ling et al., 2001; Trincao et al., 2001; Zhou et al., 2001).

A number of assays have been used to estimate the fidelity of nucleotide polymerases in vitro. A previous study of the ability of halted T7 RNAP elongation complexes (ECs) to incorporate incorrect NTPs (Huang et al., 2000) suggested an average misincorporation frequency of 1 in 2×10^4 for this enzyme (which is similar to that of exonuclease deficient Pol I DNAPs [Kunkel,

2004]) and identified a number of mutations around the preinsertion site that affected transcription fidelity (as noted above). A similar error rate for transcripts generated by T7 RNAP in vitro (1 in 3.5×10^4) was determined by an ochre mutation reversion assay (Remington et al., 1998).

In another approach, the fidelity of nucleotide polymerization by DNAPs has been widely examined by observing product extension on primer/template (p/t) assemblies in the presence of correct and incorrect substrate dNTPs (Creighton and Goodman, 1995; Goodman et al., 1993; Kuchta et al., 1988; Kunkel and Bebenek, 2000). Using a similar assay in this work, we found that substitution errors by T7 RNAP may occur either as a result of misincorporation opposite the TSn base or by a mechanism of template-strand misalignment. In the latter case, an NTP that is complementary to the downstream TSn+1 base is added to the 3′ end of the transcript, presumably due to a transient misalignment of the template strand. After incorporation, the template strand is realigned and the mismatched primer is efficiently extended, resulting in a substitution in the RNA product. A similar pattern of substitutions was observed for transcription complexes halted downstream of a promoter on templates having an abasic site or mismatch in the NT strand at the n+1 position, suggesting that misalignment may be relevant in vivo under conditions when lesions or mismatches in the DNA are not repaired prior to transcription.

The observation that misalignment results in substitution errors by T7 RNAP, reported in this study, is in contrast to related Pol I DNAPs, which preferentially generate deletion errors as a result of template-strand realignment (Bebenek and Kunkel, 1990). This may reflect the different consequences of a misincorporation event during transcription versus replication. During DNA replication, the slow extension of a mismatched primer due to a misincorporation event allows time for proofreading mechanisms to correct the mistake. For RNAPs, which are highly processive, a halted transcription complex may be more deleterious to the cell than an occasional RNA substitution error. The ability to extend mismatches more efficiently during transcription may therefore represent an important common feature of RNAPs. In addition, the preference of RNAPs to extend mismatches rather than introduce deletions would tend to preserve the reading frame in the product messenger RNA, resulting in limited amino acid substitutions during translation.

Results

Use of p/t Assemblies to Examine Transcription Fidelity by T7 RNAP

Previous biochemical and structural data demonstrated that highly stable ECs may be formed by incubation of T7 RNAP with synthetic RNA:DNA (p/t) assemblies and that the RNA primer is efficiently extended in the presence of the next (correct) incoming NTP (Temiakov et al., 2002). In this work, we adapted this primer-extension assay to examine transcription errors made by T7 RNAP in the presence of incorrect (noncognate) NTPs, as has been done for DNAPs. The use of a common assay facilitates comparison of T7 RNAP with other members of the Pol I family of nucleotide polymerases.

Stable T7 RNAP ECs were assembled on a nucleic acid scaffold in which a ^{32}P-labeled 7 nt RNA primer was annealed to a template DNA strand having an unpaired downstream region. The ability of the RNAP to extend the primer after addition of substrate was then determined by gel electrophoresis and autoradiography (Figure 2). Consistent with previous results, T7 RNAP efficiently extended the RNA primer by 1 nt in the presence of the correct substrate (e.g., UTP for the p/t used in Figure 2A, lane 2). Further extension of the 8 nt product due to misincorporation was also observed during the extended incubation time used in these assays (upper bands in lane 2). Incubation with the noncognate nucleotides CTP and GTP resulted in much less-efficient extension of the primer to 8 nt and beyond (lanes 4 and 5) due to misincorporation events.

Misalignment of the Template Strand as a Potential Mechanism for Transcription Errors

Unexpectedly, incubation with ATP (which is complementary to the downstream TSn+1 base on the p/t used in Figure 2A and is therefore referred to as the +1 NTP) resulted in efficient extension by 2 nt, with little detectable extension by 1 nt (Figure 2A, lane 3). Two mechanisms that might account for this result are presented in Figure 1B.

In the misincorporation model, incorporation of AMP opposite the TSn base (A) would result in the formation of an A:A mismatch at the 3′ terminus of the RNA primer (Figure 1B, left). Efficient extension of the mismatch by correct incorporation of AMP opposite the next base (TSn+1, T) would then result in rapid conversion of the 8 nt product to a final size of 9 nt, with little detectable accumulation of the intermediate product (i.e., the rate of mismatch extension is greater than that of misincorporation). Although this mechanism can account for the failure to observe the intermediate product, it does not account for the preferential extension of the primer only in the presence of the +1 NTP versus other noncognate NTPs (because misincorporation would have to occur in all cases as the initial, rate-limiting step).

In an alternative model, it is proposed that T7 RNAP misaligns the template strand by rotating the TSn base out of the active site, allowing the formation of an A:T base pair at +1 (Figure 1B, right). After incorporation of AMP, the template strand would be realigned and the mismatched 3′ terminus would be extended as above. This mechanism would account both for the preferential extension of the primer in the presence of the +1 NTP (due to the opportunity to base pair with the TSn+1 base) as well as the failure to detect an intermediate 8 nt product (due to rapid extension of the mismatched primer following realignment).

To differentiate between the misincorporation and misalignment models, we examined primer extension in different sequence contexts (Figures 2B–2D). In each case, the primer was more efficiently extended by 2 nt in the presence of the +1 NTP than by 1 nt in the presence of other noncognate NTPs (compare lane 3 to lanes 4 and 5 for Figures 2A–2D), demonstrating the importance of an opportunity to form a base pair with the TSn+1 base. This was observed regardless of the nature of the mismatch that would occur opposite the TSn base

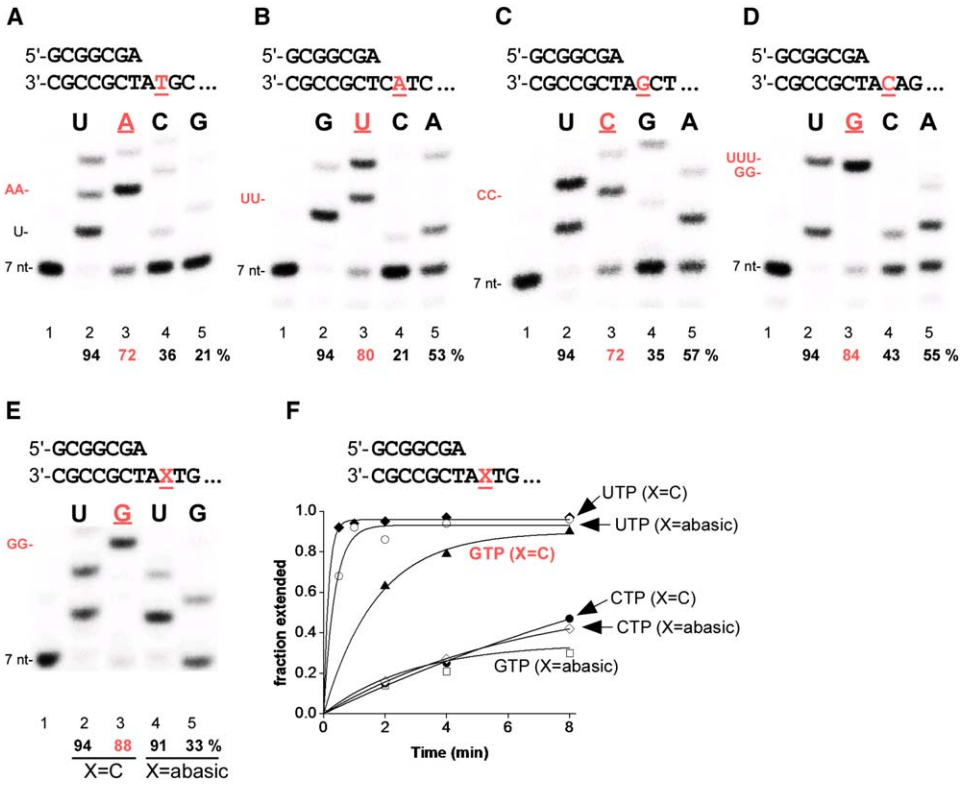

Figure 2. Extension of p/t in the Presence of Correct and Incorrect Nucleotides

Elongation complexes were assembled on p/t assemblies having an unlabeled template DNA strand (bottom sequence) and a 5′-labeled RNA primer (top sequence). After addition of the substrate indicated and incubation for 10–25 min, reactions were terminated and the RNA products were resolved by gel electrophoresis. Where indicated, the template strand had an abasic site (X) at the TSn+1 position. The percentage of primer extended at the conclusion of each reaction is indicated below the lane numbers in (A)–(E); rates of extension versus time of incubation are shown in (F). p/t assemblies were as follows: R7/RP107, R7/RP174, R7/RP106, RP/TS01, R7/TS40, and R7/TSA40 ([A]–[F], respectively).

(e.g., purine:purine, pyrimidine:pyrimidine, and purine:pyrimidine mismatches).

Interestingly, for the p/t used in Figure 2D, we observed extension by 1 nt in the presence of the correct substrate (UTP) followed by efficient extension for an additional 2 nt, to give a product of 10 nt (upper band in lane 2). On this p/t, UTP is complementary to both the TSn and TSn+2 bases. Thus, after the primer is extended 1 nt by correct incorporation opposite TSn (lower band in lane 2 of Figure 2D), incorporation of UMP opposite TSn+2 during the next two nucleotide-addition cycles as a result of misalignment and subsequent mismatch extension results in further extension by 2 nt (upper band in lane 2 of Figure 2D).

Effects of Abasic Sites in the Template Strand

To account for the preferential incorporation of the +1 NTP versus other noncognate substrates, the misalignment model proposes the formation of a base pair between the +1 NTP and the TSn+1 base. We therefore asked whether introducing an abasic site at the TSn+1 position would suppress this effect (Figure 2E). As compared to a control template having C at TSn+1, which resulted in a high rate of primer extension by 2 nt in the presence of GTP (lane 3), addition of the same substrate to a p/t template having an abasic site at TSn+1 resulted in inefficient extension, and by only 1 nt (lane 5). The abasic site at TSn+1 had little or no effect on the rate of incorporation of the correct substrate (UTP) or that of another incorrect substrate (CTP; Figure 2F). Significantly, the rate of incorporation by GTP on the abasic template (leading to extension by 1 nt) was nearly identical to that of another noncognate substrate (CTP) on this template or on the control template (Figure 2F), indicating that when the opportunity for base pairing at +1 is removed, incorporation of GMP is the result of misincorporation opposite the TSn base (as is the case for CMP). The use of a template having an abasic site at the TSn position resulted in a slight stimulation in incorporation of the +1 NTP (data not shown), suggesting that removal of the TSn base might facilitate misalignment by reducing steric constraints within the active site.

Evidence for a Single NTP Binding Site

Recent observations with multisubunit RNAPs have suggested that binding of the +1 NTP in these enzymes might occur in a secondary site, without altering the register of the TSn base (Abbondanzieri et al., 2005; Foster et al., 2001; Gong et al., 2005; Westover et al., 2004), and that binding of this nucleotide might activate the complex, resulting in higher rates of correct incorporation opposite the TSn base (Foster et al., 2001). Conceivably, binding of the +1 NTP and activation of the transcription complex by this mechanism might stimulate misincorporation opposite the TSn base in T7 RNAP, which could also result in preferential extension of the primer in the presence of the +1 NTP.

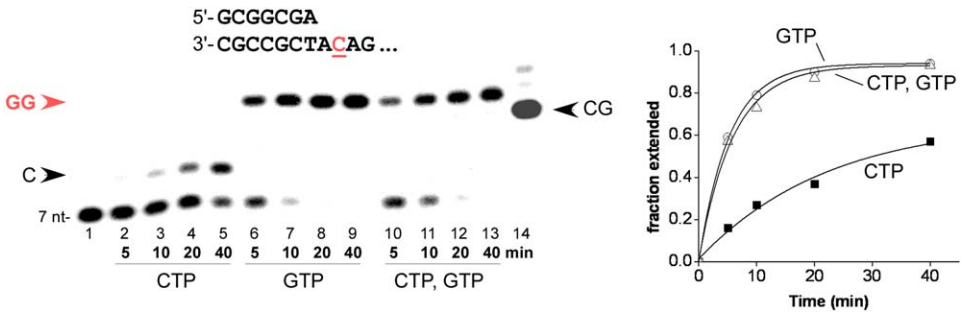

Figure 3. Evidence for a Single NTP Binding Site

Elongation complexes were assembled on the p/t indicated (R7/TS01), and substrates were added as follows: CTP (lanes 2–5), GTP (lanes 6–9), or CTP and GTP together (lanes 10–13). Samples were withdrawn at the times indicated and resolved by gel electrophoresis; the percent extension of the primer versus time of incubation is plotted to the right. The CG reference band (lane 14) was generated on a p/t, which allows for correct extension of the primer by 2 nt in the presence of CTP and GTP.

To explore this, we compared the rates of primer extension due to incorporation of a different noncognate nucleotide in the presence or absence of the +1 NTP (Figure 3). As before, we observed a slow extension of the primer by 1 nt in the presence of the noncognate substrate CTP alone (lanes 2–5) and rapid extension by 2 nt in the presence of the +1 NTP alone (GTP; lanes 6–9). In the presence of GTP and CTP together, the primer was rapidly extended by 2 nt to yield a product that is characteristic of extension by GG (the different mobilities of products extended by GG or CG in this gel system readily allow a discrimination as to which nucleotides are incorporated; compare lanes 13 and 14). Significantly, no extension by CG was observed under these conditions, indicating that rapid extension by 2 nt in the presence of both substrates is due to the sequential incorporation of two GMP residues and not due to an enhanced misincorporation rate for CMP followed by efficient mismatch extension.

Efficient Mismatch Extension by T7 RNAP

In the experiments above, there is little evidence for the formation of an intermediate product (extension by 1 nt) in the presence of the +1 NTP (Figure 3, lanes 6–9) indicating that the efficiency of mismatch extension by T7 RNAP is relatively high and is more rapid than misincorporation. To examine this, we compared the rate of extension of primers having a mismatch at the 3′ terminus to extension of primers having a correctly paired 3′ terminus (Figure 4A; UTP). In the same experiment, we also examined the rate of extension of a correctly paired primer due to misincorporation (in the presence of CTP) or due to incorporation of the +1 NTP (GTP). As predicted by the model, we found that the rate of extension of the mismatched primer in the presence of the correct NTP is very rapid (comparable to the extension of a correctly paired primer within the resolution of this experiment) and is much faster than extension due to misincorporation. Significantly, extension by the +1 NTP (which involves misalignment followed by mismatch extension) is also more rapid than misincorporation, again indicating that the pathway that leads to extension by 2 nt in the presence of the +1 NTP does not involve an initial misincorporation event (which would be rate limiting).

Comparison of Error Mechanisms of Pol I DNAP versus T7 RNAP

The efficient extension of a mismatched primer by T7 RNAP is in contrast to results of previous studies with Pol I DNAPs, in which it has been reported that the presence of a mismatched base within the p/t reduces the rate of extension by factors of 100 to one million (Echols and Goodman, 1991; Goodman et al., 1993; Kunkel and Bebenek, 2000). The slow rate of extension of a mismatched primer has important implications for fidelity and error correction by DNAPs, as it affects the balance between extension and editing due to exonuclease activity and other repair mechanisms (T7 RNAP is not known to have an editing/exonuclease function [Huang et al., 2000]).

To facilitate comparison between the two systems, we examined primer extension by T7 RNAP and the Klenow fragment (KF) of DNAP in the same sequence context (Figure 4B). In contrast to the results obtained for T7 RNAP (right panel), no preferential incorporation of the +1 dNTP was observed for KF (compare left panel, lanes 7–11 for incorporation of the +1 dNMP [dGMP] versus lanes 12–16 for incorporation of another incorrect dNMP [dCMP]). Moreover, the mismatched primer resulting from misincorporation of the +1 dNMP was poorly extended by KF, resulting in primer extension by only 1 nt (lanes 7–11). Thus, consistent with previous reports, the opportunity to form a base pair between the +1 dNTP and the TSn+1 base does not stimulate the misincorporation rate for Pol I DNAP nor is there a high rate of mismatch extension (Bebenek and Kunkel, 1990; Kuchta et al., 1988; Kunkel and Bebenek, 2000; Wong et al., 1991).

The differences for T7 RNAP and DNAP in their ability to extend a mismatch are likely to account for the different outcomes of the two systems as a result of a misincorporation event in the presence of the +1 NTP. Whereas for T7 RNAP the outcome is predominately a base substitution error (this work), previous studies indicate that for KF the predominant result is a deletion error (Bebenek and Kunkel, 1990). The pathways that are thought to lead to these alternate outcomes are shown in Figure 4C. In the case of T7 RNAP (right panel), misalignment promotes the preferential incorporation of the +1 NTP as the initial step in the process. Subsequent

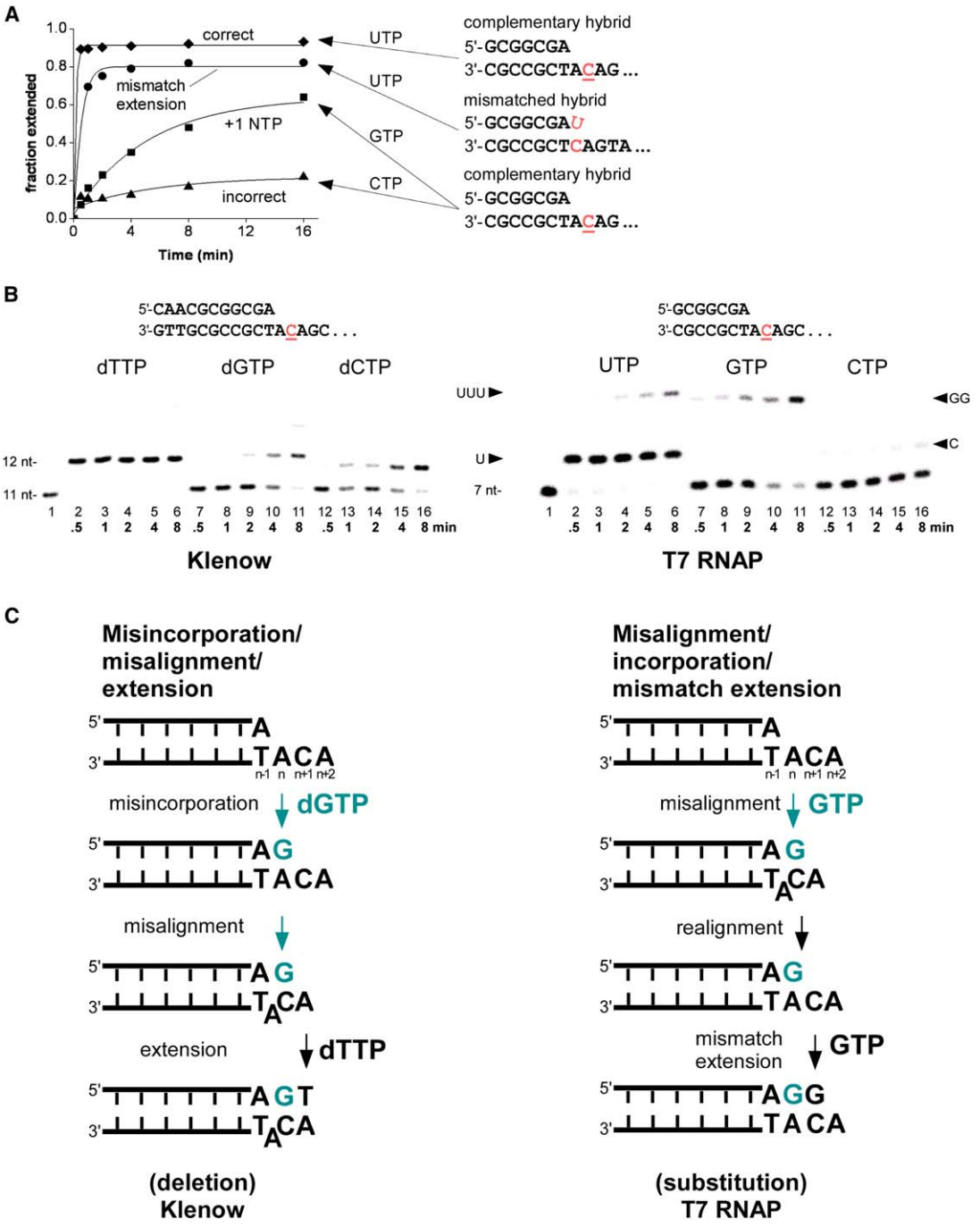

Figure 4. Mismatch Extension and Comparison of Mechanisms of Misalignment Errors by T7 RNAP and Pol I DNAPs

(A) T7 RNAP ECs were assembled on p/t having either a fully complementary primer (complementary hybrid, R7/TS01) or a primer with a mismatch at the 3′ terminus (mismatched hybrid, R8/RP108), as indicated. Substrates were then added, and extension of the primer in the presence of the correct (UTP), incorrect (CTP), or +1 NTP (GTP) was determined versus time of incubation.

(B) Comparison of primer extension by KF DNAP (left) and T7 RNAP (right). The sequences of the p/t assemblies used are shown at the top of each panel. Samples were withdrawn after addition of the indicated substrate and resolved by gel electrophoresis. p/t assemblies: RP180/RP181 (left) and R7/TS01 (right).

(C) Models for substitution or deletion errors as a result of template-strand misalignment.

In the misincorporation/misalignment/extension model (left, Klenow), the initial event is a misincorporation of dGMP opposite the TSn base (A). Misalignment of the 3′ end of the primer then allows for correct base pairing with the TSn+1 base and correct extension by dTTP, resulting in a deletion. In the misalignment/incorporation/mismatch extension model (right, T7 RNAP), the initial event is the incorporation of GMP opposite the TSn+1 base (C) as a result of misalignment. This is followed by template realignment and efficient mismatch extension, resulting in a substitution error.

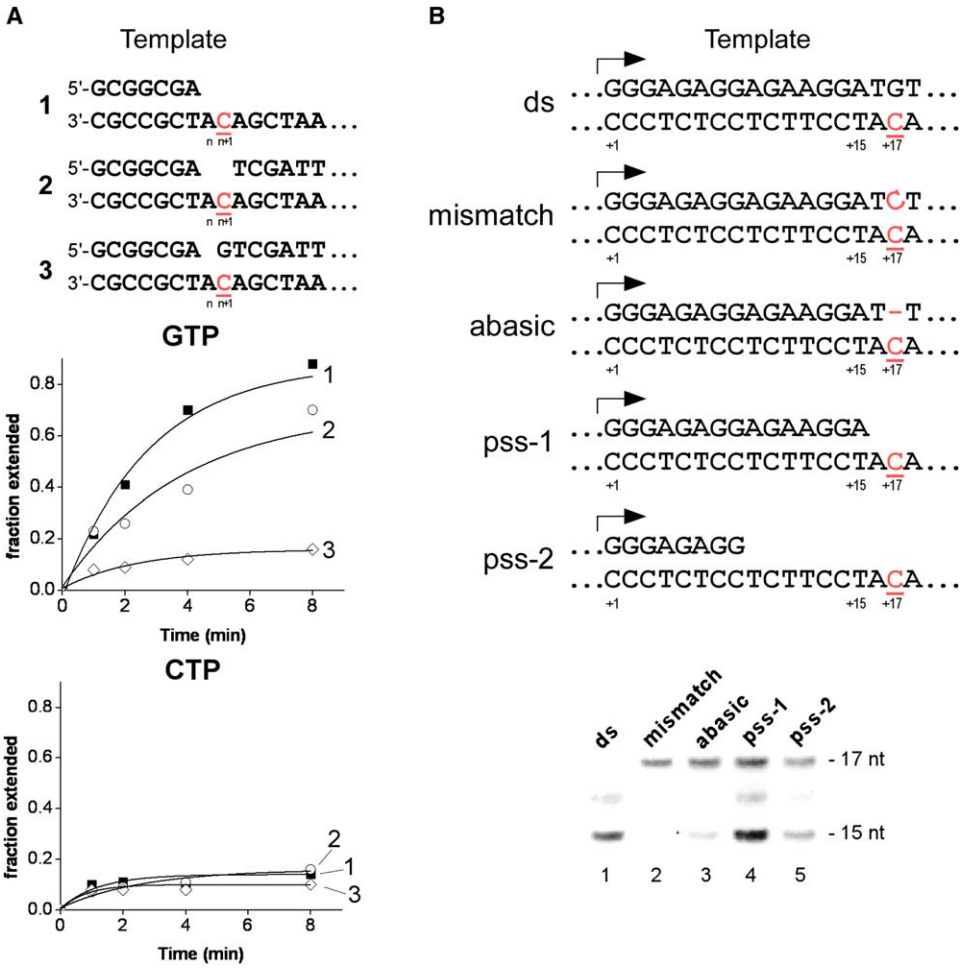

Figure 5. Effects of Template Topology on Misalignment

(A) Effects of a complementary NT strand. Rates of primer extension were determined on p/t assemblies lacking a complementary downstream NT strand (template 1) or on assemblies having a complementary NT strand commencing at +2 (template 2) or +1 (template 3) in the presence of the +1 NTP (GTP; top) or a different noncognate NTP (CTP; bottom). p/t assemblies: 1, R7/TS01; 2, R7/TS01/NT02; and 3, R7/TS01/NT03.

(B) Effects of mismatches and abasic sites in the NT strand. T7 RNAP was incubated with promoter templates having the sequences indicated in the presence of GTP and (^{32}P) α-ATP (which allows for correct extension up to 15 nt downstream of the start site) for 30 min, and the products were resolved by gel electrophoresis. Note that for complexes halted at +15, GTP is the +1 NTP. Extension by an additional 2 nt (to +17) was readily apparent on templates lacking a complementary NT strand base at the +1 position. Templates: ds, RP146/RP147; mismatch, RP147/RP157; abasic, RP147/RP160; pss-1, RP147/RP148; and pss-2, RP147/RP159.

realignment and efficient mismatch extension then "seals" this substitution into the nascent transcript. In the case of KF (left panel), the initial event is misincorporation of the +1 dNTP (apparently not as a result of template-strand misalignment, for there is no preferential incorporation of the +1 nucleotide; see Figure 4B, above). However, because a mismatched primer is poorly extended by DNAP, the situation is thought to be resolved by misalignment of the template strand, allowing correct pairing of the 3′ end of the primer with the next downstream base and subsequent extension, resulting in a deletion error (Figure 4C, left; Bebenek and Kunkel, 1990; Kunkel and Bebenek, 2000; Kunkel, 2004).

Effects of Changes in the Organization of the Transcription Bubble on Frameshift Fidelity

The p/t assemblies used in the experiments above are similar to those used to explore the fidelity of DNAPs (and were specifically chosen for this reason to allow comparisons between various systems). However, they do not have all of the features that are thought to be present in a transcription elongation complex. In particular, they lack a complementary NT DNA strand downstream of the active site. We therefore examined primer extension on p/t assemblies that included (1) no downstream NT strand (template 1), (2) a complementary downstream NT strand beginning at the n+2 position (template 2); or (3) a downstream NT strand beginning at the n+1 position (template 3) (Figure 5A). Although the rate of extension in the presence of a noncognate substrate (CTP) was the same on all templates (bottom panel), the rate of extension by the +1 NTP (GTP) was slightly reduced on template 2 and dramatically reduced on template 3 (top panel). The latter template requires melting of the duplex DNA at the n+1 position to allow base pairing with the +1 nucleotide (GTP). In this case, the rate of extension by the +1 NTP was comparable to misincorporation in the presence of CTP, indicating

that the presence of a complementary NT base at the n+1 position suppresses misalignment. In separate experiments, we found that the presence of a complementary strand does not suppress extension of a mismatched primer (data not shown).

We next asked whether ECs halted downstream of a promoter on a double-stranded (ds) template would generate similar misalignment errors and whether the frequency of these errors might be affected by lesions or mismatches in the NT strand (Figure 5B). Here, the sequence of each template was designed to allow the formation of an EC halted 15 nt downstream of the promoter (EC15) in the presence of GTP and ATP alone (due to lack of the next incoming nucleotide, UTP). Importantly, each template included a cytidine in the template strand at the TSn+1 position (+17), allowing detection of misalignment errors due to incorporation of GMP (GTP is present in the reaction used to generate EC15).

In the presence of a fully complementary NT strand (ds template), we observed inefficient extension beyond 15 nt, and only by 1 nt (lane 1), presumably due to a low level of misincorporation. However, in the absence of a complementary NT strand (templates pss-1 and pss-2), we observed efficient extension by 2 nt by the halted ECs (lanes 4 and 5). Strikingly (but consistent with the results above), efficient extension by 2 nt was also observed on templates having a mismatch or an abasic site in the NT strand at the +1 position (mismatch and abasic templates, lanes 2 and 3, respectively). As abasic sites and mismatches are the result of mutagenesis or DNA replication errors, these findings may have relevance to transcription fidelity in vivo under circumstances in which these alterations remain unrepaired prior to transcription.

Discussion

In this work, we present evidence for a mechanism of nucleotide misincorporation during transcription by T7 RNAP that involves template-strand misalignment. We find that T7 RNAP efficiently extends an RNA primer by 2 nt in the presence of an NTP complementary to the TSn+1 base (the +1 NTP). Addition of other noncognate nucleotides results in a lower efficiency of misincorporation and extension by only 1 nt. Use of a template having an abasic site at the TSn+1 position eliminates preferential extension by the +1 nucleotide, supporting a mechanism of misalignment in which base pairing between the +1 NTP and the TSn+1 base is required. In the proposed misalignment model, the TSn base is "flipped out" of the helical axis in order to allow positioning of the TSn+1 base in the preinsertion site (Figure 1B, right). After incorporation of the +1 substrate, the template is realigned, and the resulting 3′ terminal-mismatched primer is efficiently extended, resulting in a substitution in the RNA product.

Structural and kinetic studies of DNAP p/t assemblies involving a variety of mismatches and misalignments indicate that there is considerable flexibility in protein structure and in the organization of nucleic acids that allow DNAP to accommodate deviations from normal p/t structure (Garcia-Diaz et al., 2006; Johnson and Beese, 2004; Ling et al., 2001, 2004; Tippin et al., 2004; Zang et al., 2005). Furthermore, a recent study with a flipped-out base in the human DNA polymerase of the X family demonstrates how rotation and displacement of a single base can be accomplished without significantly perturbing the configuration of the enzyme or the double-helical structure on either side of the lesion (Garcia-Diaz et al., 2006). Modeling based on available T7 RNAP structures (Temiakov et al., 2004; Yin and Steitz, 2004) suggests that the EC may readily accommodate a flipped-out TSn base in the open configuration, without significant alterations of protein or RNA:DNA hybrid structures (Figure 6). The opportunity for the incoming NTP to base pair with the TSn+1 base in the preinsertion site in this configuration may help to stabilize a misaligned intermediate, and interactions between the flipped-out TSn base with adjacent protein side chains and the sugar moiety of the TSn+2 nucleotide might further contribute to the stability of the complex. In the closed configuration, however, modeling suggests that some rearrangements of the RNAP and/or of the RNA:DNA hybrid would be needed to allow stacking interactions between the TSn+1 and TSn-1 bases that are likely to be required for catalysis. Though the details of such alterations are difficult to predict (and therefore to model), we speculate that similar rearrangements might also allow accommodation of a mismatch at the 3′ end of the primer after substrate incorporation and realignment of the TSn base. Information as to the actual organization of the complex during this mode of misincorporation will need to await biochemical and structural studies.

In contrast to Pol I DNAP, T7 RNAP appears to be able to extend a primer with a mismatch at its 3′ terminus more efficiently. This disparity is likely to account for the different outcome of the two systems to a misincorporation event, which results predominately in base substitution errors for T7 RNAP in the presence of the +1 NTP and deletion errors for KF. The dissimilar abilities of T7 RNAP and DNAP to extend a mismatched primer are likely to reflect the different roles of these enzymes in transmission of genetic information. For DNAPs, the results of a misincorporation event are of serious consequence and should be avoided. Thus, mismatch extension is strongly discriminated against, thereby favoring error correction by exonuclease activity or other mismatch-editing mechanisms (Kunkel and Bebenek, 2000; Kunkel, 2004). In the case of a highly processive RNAP, an occasional misincorporation event is likely to be of less consequence to the cell than the formation of a stalled transcription complex, which has been shown to impede head-on passage of a replication fork, potentially leading to genomic instability (Elias-Arnanz and Salas, 1999; Mirkin et al., 2006). Taken together, these observations underscore the importance of avoiding transcription arrest in maintaining normal DNA metabolism processes. This property may therefore be common to all RNAPs. Previous work with *E. coli* RNAP has shown a higher rate of mismatch extension than misincorporation for this enzyme as well (Erie et al., 1993). Moreover, using a similar p/t extension assay to study the fidelity of bacterial RNAPs and yeast Pol II, we have recently observed an identical pattern of misincorporation events in the presence of the +1 NTP, suggesting that this phenomenon may be universal for all RNAPs (Kashkina et al., 2006 [this issue of *Molecular Cell*]).

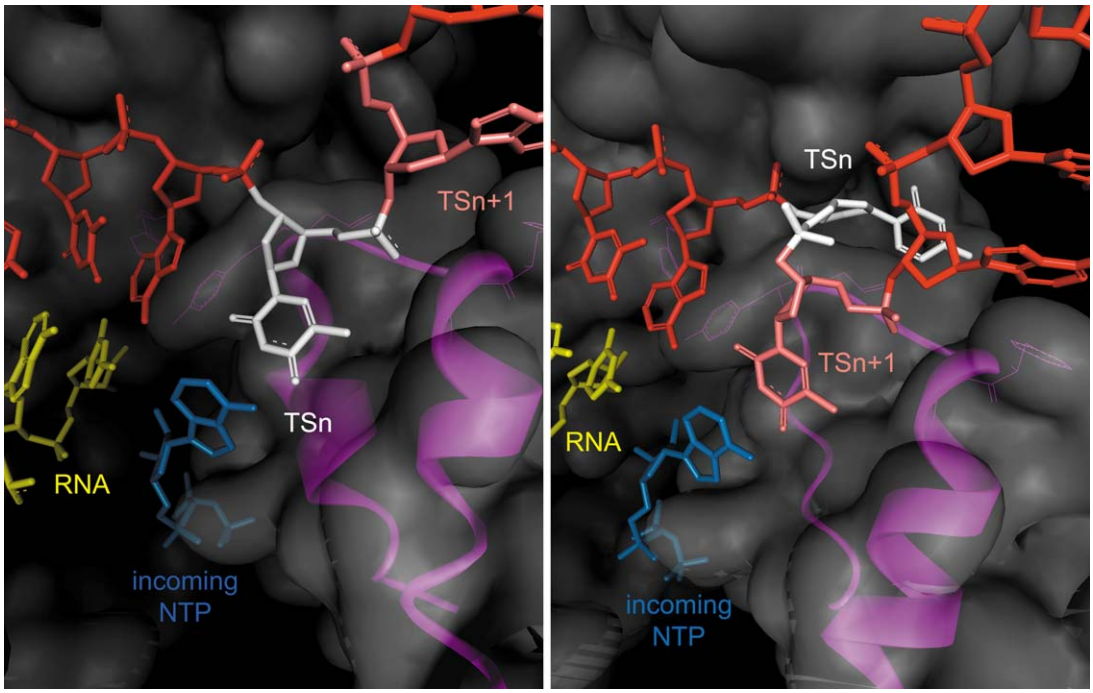

Figure 6. Model of an EC with a Misaligned Base

Comparison of the structure of the T7 RNAP EC observed in the open configuration in the presence of the correct incoming NTP (left; Temiakov et al., 2004) and a model of a misaligned EC in which the TSn base is rotated out of the preinsertion site, allowing the TSn+1 base to pair with the incoming substrate (right). The protein is shown as a transparent surface; DNA template strand (TS) is red, RNA primer yellow, incoming NTP blue, TSn base white, TSn+1 base light red, and Y and Z helices (residues 624 to 658) and side chains of residues implicated in fidelity (Y639, G640, F644, G645, and H784) (Huang et al., 2000) are in pink.

Another related difference between T7 RNAP and Pol I DNAP is that T7 RNAP shows preferential incorporation of the +1 NTP, presumably due to misalignment of the template strand and the opportunity to form a base pair between the +1 NTP and the TSn+1 base. As shown in Figure 4B, and consistent with previous reports in the literature (Bebenek and Kunkel, 1990; Kuchta et al., 1988; Wong et al., 1991), we did not observe a preferential incorporation of the +1 dNMP by KF. This suggests that there may be less flexibility in Pol I DNAP with regard to template-strand misalignment and/or a decreased opportunity to form a base pair between the incoming substrate and the TSn+1 base. In this regard, it may be worth noting that in the available structures of Pol I DNAP the TSn base is more deeply buried in the preinsertion site and is less available for base pairing with the incoming substrate than the comparable TSn base in the T7 RNAP EC is (Johnson et al., 2003; Kiefer et al., 1998; Li et al., 1998; Temiakov et al., 2004).

Although the Pol I DNAPs do not appear to show preferential incorporation of the +1 nucleotide due to template-strand misalignment, such a mechanism has been observed for DNAPs of the X, Y, B, and C families, where it is referred to as dNTP-stabilized misalignment (Bloom et al., 1997; Efrati et al., 1997; Kobayashi et al., 2002; Kokoska et al., 2002; Kroeger et al., 2006; Ling et al., 2001, 2004; Tippin et al., 2004; Wolfle et al., 2003; Zang et al., 2005). In this case, however, deletion errors are still the predominant outcome, perhaps due again to inefficient mismatch extension by these enzymes.

We found that the organization of the transcription bubble, and in particular, the presence of a complementary downstream NT strand, is important to the fidelity of transcription. Thus, the presence of a complementary downstream NT strand commencing at +1 suppresses primer extension in the presence of the +1 nucleotide but has no effect on the incorporation of other noncognate substrates or upon extension of a mismatched primer. Importantly, we found that misalignment errors may also be generated on templates in which transcription is initiated from a promoter and that the presence of the downstream NT strand on these templates suppresses misalignment (see Figure 5B). The presence of a noncomplementary base (mismatch) or an abasic site in the NT strand at +1 greatly stimulates misalignment errors on these templates. These results indicate that misalignment may be relevant to transcription fidelity in vivo where mismatches and lesions go unrepaired by DNA and transcription-coupled repair mechanisms.

In conclusion, we report here the first observation of substitution errors generated by a template-misalignment mechanism during transcription. Several types of substrate-template misalignments have been previously reported in DNA replication (Kunkel, 1990, 2004; Kunkel and Bebenek, 2000) and translation (Choi et al., 2003; Prufer et al., 1992; Stahl et al., 2002), indicating that misalignment presents a common challenge to the accurate transmission of genetic information.

Experimental Procedures

Extension of p/t by T7 RNAP

Purification of T7 RNAP and assembly of p/t scaffolds were carried out as previously described (Temiakov et al., 2002, 2003). Synthetic

RNA and DNA oligomers were obtained from Dharmacon and Integrated DNA Technologies (IDT), respectively; sequences are provided in the Supplemental Data available with this article online. Primers were labeled at the 5′ end with γ-(^{32}P)-ATP by polynucleotide kinase (New England Biolabs). p/t assemblies were formed by annealing equimolar concentrations of synthetic DNA oligonucleotides and complementary radiolabeled RNA for ~7 min at 70°C, followed by slow-cooling to room temperature (23°C –25°C). ECs were assembled by incubating T7 RNAP with a preannealed p/t assembly at final concentrations of 2 and 1 μM, respectively, in transcription buffer (TB: 20 mM Tris HCl [pH 7.9], 8 mM $MgCl_2$, 0.1 mM EDTA, and 5 mM β-mercaptoethanol) for ~10 min at room temperature. Primer extension was carried out by subsequent addition of 50 μM cognate or noncognate NTPs for 10–30 min at 37°C (with the exception of the experiment shown in Figure 4A, which was carried out at room temperature). Reactions were terminated by the addition of stop buffer (50 mM EDTA in 90% formamide), and the products were resolved by electrophoresis in 20% polyacrylamide gels containing 6 M urea and quantified by using ImageQuant analysis (GE Health). The percentage of primer extension was determined as the ratio of the intensity of the extension product(s) versus the sum of the intensities of the product(s) plus the initial unextended primer.

Extension of p/t by KF

p/t assemblies were constructed as above with the exception of the use of a synthetic deoxyribonucleotide oligomer as a primer (oligomers RP180 and RP181; see Supplemental Data). Ten units of exonuclease-deficient KF (New England Biolabs) was incubated with the p/t indicated at a final concentration of 1 μM for ~10 min in 1× TB at room temperature followed by the addition of 50 μM dNTP substrate for the indicated time at 37°C. Reactions were terminated, and the products were resolved as above.

Extension of RNA on Promoter Templates

The formation of T7 RNAP-halted elongation complexes was carried out as previously described (Mentesana et al., 2000). Briefly, dsDNA templates were assembled as above (for p/t assemblies). Sequences of oligomers are provided in the Supplemental Data. Halted T7 RNAP ECs were formed by incubating 20 nM of T7 RNAP with 50 nM of the preannealed DNA template indicated in the presence of 500 μM GTP, 50 μM ATP, and α-(P^{32})-ATP in 1× transcription buffer for 30 min at room temperature. RNA products were resolved and quantitated as above.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.molecule.org/cgi/content/full/24/2/245/DC1/.

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