Scanning Mutagenesis Reveals Roles for Helix N of the Bacteriophage T7 RNA Polymerase Thumb Subdomain in Transcription Complex Stability, Pausing, and Termination*

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Deletions within the thumb subdomain (residues 335–408) of T7 RNA polymerase decrease elongation complex stability and processivity, but the structure of a T7RNAP initial transcription complex containing a 3-nucleotide RNA reveals no interactions between the thumb and the RNA or DNA. Modeling of a longer RNA in this structure, using a T7DNAP-primer-template structure as a guide, suggests that the phosphate ribose backbone of the RNA contacts a stretch of mostly positively charged side chains between residues 385 and 395 of helix N of the thumb. Scanning mutagenesis of this region reveals that alanine substitutions of Arg391, Ser393, and Arg394 destabilize elongation complexes and that substitutions at 393 and 394 increase termination of transcripts 5 or more bases in length. The α-carbons of all 3 of these residues lie on the side of helix N, which faces into the template-binding cleft of the RNA polymerase, and modeling suggests that they can contact the RNA 4–5 bases away from the 3′-end. Alanine substitutions of other residues within 385–395 do not have marked effects on transcription complex stability, but alanine substitutions of Asp388 and Tyr385 reduce pausing and termination at the T7 concatemer junction. Both of these side chains lie on the outer side of helix N, pointing away from the template binding cleft. The thumb subdomain of T7RNAP therefore has roles both in transcription complex stabilization and in pausing and termination at the T7 concatemer junction.

Bacteriophage T7RNA polymerase (RNAP) is the best characterized member of a widespread family of RNAPs that includes many phage RNAPs as well as the eukaryotic mitochondrial RNAPs (1, 2). With the recent description of crystal structures of a T7RNAP-promoter complex (3) and an initial transcription complex (ITC) (4), T7RNAP presents an exceptional system for investigating the structural mechanisms of transcription, particularly because the transcription reaction mediated by the single-subunit T7RNAP is very similar to the transcription reactions mediated by the more complex, mult-subunit RNAPs (5). In particular, the mechanisms of pausing and termination in the T7-like RNAPs and the prokaryotic and eukaryotic RNAPs appear to be similar, because both classes of RNAPs recognize some pause and termination sequences in common (6–8).

An especially intriguing question in the biochemistry of transcription concerns the mechanisms of elongation complex (EC) stability. Such mechanisms must make the EC sufficiently stable to keep the RNAP from releasing the template or RNA while it traverses multiple kilobases of DNA and negotiates obstacles such as DNA-bound proteins. Simultaneously, such stability mechanisms must be responsive to regulatory factors (9, 10) and signals encoded in the template that cause the EC to pause or terminate (11). One of the structural features used to meet the seemingly conflicting requirements of mobility, stability, and responsiveness is an element that, upon RNA binding, undergoes a conformational change that causes the polymerase to clamp onto the template (12–18). Such elements (often dubbed “thumbs” because of their position and the resemblance of polymerases to a cupped right hand) appear to be a ubiquitous feature of polymerase structure and have been shown to be important in stabilizing DNAP-DNA complexes (19, 20). The thumb subdomain of T7RNAP is archetypal. It is an extended structure composed of two α-helices connected by an irregularly structured loop (13). In the apoenzyme, a portion of the thumb that projects furthest from the rest of the polymerase is disordered (14, 15). Upon binding template this region becomes ordered and part of it assumes helical structure (3). A role for the thumb subdomain in stabilizing the EC was revealed by the observation that deletions in the T7RNAP thumb subdomain decrease EC processivity (16) and stability (17, 18).

However, although deletions in the thumb affect EC stability, no direct interactions between the thumb and either the DNA or RNA are seen in a T7 RNAP ITC structure (4). Because the RNA in this structure is only 3 bases in length, we modeled an extension of the RNA using the structure of the homologous T7DNAP complexed with primer-template (21) as a guide. This modeling suggested that the phosphate-ribose backbone of the RNA 4–5 nucleotides away from the RNA 3′-end could contact a segment of largely positively charged side chains between residues 385 and 395 of helix N of the thumb subdomain. We carried out scanning mutagenesis of this stretch of residues and characterized the effects of the mutations on RNAP activity. Our results reveal two distinct functions for this helix. The side chains of Arg391, Ser393, and Arg394 contribute to TC stability, apparently through interactions with the RNA 4–5 bases away from the 3′-end. The side chains of Tyr385 and Asp388 are not important for TC stability, but mutation of these residues reduces pausing and termination at the T7 concatemer junction, a critical step in T7 phage maturation.
EXPERIMENTAL PROCEDURES

Mutant RNAPs were constructed using the CLONTECH Transformer site-directed mutagenesis kit and the pDPT7 plasmid (22) and followed the manufacturer's instructions. Mutant and wild-type enzymes were expressed and purified as described as described (22). Proteolytically nicked RNAP was prepared by incubating purified T7RNAP with whole *Escherichia coli* cells as described by Muller et al. (23). Transcription reactions were run by mixing RNAPs and templates at twice the desired final concentrations (as specified in figure legends) in 40 mM Tris, pH 8.0, 10 mM NaCl, 10 mM MgCl₂, and 5 mM dithiothreitol. After a 5-min preincubation, reactions were initiated by adding an equal volume of NTPs at twice the desired final concentration (typically 0.5 mM unless otherwise indicated in figure legends) in 40 mM Tris-HCl, pH 8.0, 10 mM NaCl, 10 mM MgCl₂, and 5 mM dithiothreitol. Transcripts were terminated by including 10 μCi/ml of [α-32P]GTP (800 Ci/mM). Reactions were incubated at 37 °C, unless otherwise indicated, and reaction aliquots were taken at the times indicated in individual figures and stopped by addition of an equal volume of 95% formamide, 25 mM EDTA, 0.01% xylene cyanol, and the RNA components were resolved by electrophoresis in 20% polyacrylamide, 1% bis-acrylamide, 6 M urea gels in 1× TBE. Analysis of the resolved transcripts was carried out with a Molecular Dynamics Phosphorimager. RNase T1 treatment of halted ECs was carried out by first forming halted ECs on linearized pPK10 (18) in reactions containing 0.5 mM ATP, 0.5 mM GTP, 0.05 μM [α-32P]UTP and 0.1 mM 3′-dCTP (the +1 to +16 transcript sequence from pPK10 is GGGAGAGGGAGGGAUC). After a 5-min incubation at room temperature to allow formation of the halted, chain-terminated EC, UTP was added to 1 mM to limit labeling of transcripts made following the first round of synthesis. RNase T1 was then added to 0.25 unit/μl, and reaction aliquots were taken at time points indicated in figure legends, terminated by phenol extraction, and mixed with equal volumes of 95% formamide, 25 mM EDTA, 0.01% xylene cyanol, and resolved by electrophoresis on 20% polyacrylamide, 1% bis-acrylamide, 6 M urea gels in 1× TBE.

RESULTS

**Mutant Construction**—Fig. 1A shows the structure of a T7RNAP ITC (4). Superimposed on this structure are the first 4 nucleotides of the primer from a T7DNAP primer-template complex (21). Superposition was carried out by maximizing the alignment of the main chain atoms of the active site asparates of each enzyme (T7 RNAP Asp537/Asp812 and T7DNAP Asp475/Asp585). When this is done the first 3 nucleotides of the primer from the T7 DNAP structure align with the 3 RNA nucleotides in the T7RNAP ITC, and the fourth primer nucleotide approaches a cluster of mostly positively charged side chains (residues 385–395) on helix N of the thumb subdomain. There are two models for the structure of the RNA-DNA hybrid in the T7RNAP TC. A model similar to one previously developed for *E. coli* RNAP and polymerase II (24) proposes that the RNA in the T7RNAP EC separates from the template after forming a 3-base pair hybrid and passes between the thumb and the N-terminal domain as suggested by the green arrow in Fig. 1A (4). Another model proposes that the RNA forms an 8–10-base pair hybrid with the template (25). Although the paths of the RNA in these competing models ultimately diverge, both paths lead the RNA 4–5 nucleotides away from the 3′-end past the 385–395 segment of helix N. We therefore constructed the mutations shown in Fig. 1B and characterized how these mutations affect RNAP function, TC stability, and termination. A deletion mutation (ThDel) that removes residues 359–381 in a part of the thumb subdomain that is disordered in the apoenzyme (14, 15) but that becomes ordered upon binding DNA (3) was similarly characterized, as was an enzyme that was proteolytically cleaved between residues 172 and 173 but in which the separate fragments remain associated (“nicked” RNAP) (23, 26).

The Mutations Leave Catalytic Activity Intact but Reduce Processivity Starting at an RNA Length of ~5 Bases—Fig. 2A shows the transcripts obtained in continuous initiation assays with the mutant enzymes using a template that generates a 59-base run-off transcript from a class III T7 promoter. The figure shows a reaction that was run for 20 min. Reactions run for 5 or 10 min exhibit fewer total transcripts but similar proportions of short and run-off transcripts. The short (2–9 bases) and run-off transcripts are made in large molar excess of the amount of template or RNAP, indicating that the transcripts are being continuously synthesized and released into solution. All of the enzymes are active and synthesize run-off transcripts at levels similar to the wild-type enzyme. However, some of the mutant enzymes show an increase in the proportion of

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**Fig. 1. A**, structure of a T7RNAP ITC (4) (Protein Data Bank code 1QLN). The template strand is green, the nontemplate strand is purple, and the RNA is green. Four nucleotides of DNA from a T7DNAP primer-template complex (Ref. 21; Protein Data Bank code 1TTP) are shown in red. The primer/RNA nucleotides are numbered with respect to the 3′-nucleotide (numbered 1). A previously proposed exit path for the RNA (4) is indicated by the green arrow. The amino acids targeted for mutagenesis are in magenta, and the segment deleted in the ThDel mutant is blue. B, alignment of part of the T7RNAP thumb subdomain sequence with the T3, SP6, and K11 phage RNAP sequences. Positions at which amino acids are conserved in at least three of the sequences are indicated with an asterisk. The element that is disordered in the T7RNAP apoenzyme is indicated along with the location of the deletion in the ThDel mutant. The amino acids targeted for mutagenesis are highlighted in red, and the substitutions made are indicated in cyan, above the T7RNAP sequence.
short transcripts, indicating an increase in termination during initial transcription. Inspection of Fig. 2A reveals that the increases in termination vary as a function of transcript length. S393A, R394A (lanes 11 and 12), and the glycine substituted enzymes (lanes 14–17) show increased termination for RNAs 4 bases in length, whereas the ThDel mutant (lane 2) shows the greatest increases in termination for RNAs 7 bases in length. The increases in termination at 5, 6, 8 or 9 bases also increase the amounts of shorter (2–4 bases) transcripts made because, following release of a transcript, the RNAPs reinitiate. As a consequence, an increase in termination at any point during initial transcription will increase the total number of initiation events and abortive transcripts. However, if the percentage of termination for each RNA is quantified as described in the legend to Fig. 2B, we find that the increases in termination with the mutant enzymes are limited to RNAs 4 bases in length.

**The Mutations Increase Dissociation of 6-mers, but Not 2-mers, from the ITC**—The observed increases in termination during initial transcription could be due to a decrease in the rates of transcript extension and/or to an increase in the rates of transcript dissociation. To determine whether the mutations...
affect transcript dissociation, we measured the steady-state rates of transcript synthesis in reactions in which synthesis was limited to 2-mer (GG) or 6-mer (GGGAGG) production. Presteady-state and steady-state analyses carried out under conditions similar to those used here have shown that steady-state rates of abortive transcript synthesis are limited by the rates of transcript dissociation (27–30), so that destabilization of the complex leads to an increase in the rate of abortive transcript synthesis (30, 31). In the 2-mer reaction the mutants all displayed synthesis rates similar to the w.t. enzyme (Fig. 3, A and C), indicating that the mutations are not increasing 2-mer dissociation. However, when we measured 6-mer synthesis we found that the mutants that displayed increased terminalization during initial transcription also showed increased rates of 6-mer production (Fig. 3, B and D), indicating that these mutations increased the rate of 6-mer dissociation from the complex.

**The Mutant Enzymes Exhibit Fast Rates of Promoter Escape and Slow Rates of Halted EC Dissociation**—The previous observations indicate that some of the thumb subdomain mutations destabilize ITCs that contain RNAs >4 bases in length. To determine whether these mutations would also destabilize ECs, we measured the rates of transcript synthesis in reactions in which transcript extension was halted during elongation by omitting an NTP. We used a template (pPK5) whose initially transcribed sequence is GGGAGGGGAGACU (18), so that a 14-mer is made when only GTP and ATP are present. We first determined, for all of the mutant enzymes, whether the rates of 14-mer synthesis in these reactions would be limited by the rate of halted EC turnover or by the rate of promoter escape. Fig. 4 shows a representative experiment measuring the rate of promoter escape for the w.t. and 5 mutants. On the promoter used here the RNAP pauses when the RNA reaches 6 nucleotides in length (43). As measured from the decrease in the amount of 6-mer and the appearance of the 14-mer, the w.t. enzyme (Fig. 4, A, lanes 1–5, and B) clears this promoter and forms the halted complex at a rate of ~0.6 min⁻¹ at room temperature. With the exception of S393G (Fig. 4, A, lanes 21–25, and B), the mutant enzyme rate of promoter escape is similar to that of the w.t. enzyme. We found that all of the mutants, with the exception of S393G and S393A (not shown), displayed rates of promoter escape similar to that of the w.t. enzyme. The escape rates for S393G and S393A were 2–3-fold slower. Therefore, in these reactions the synthesis of one 14-mer/template occurs rapidly. However, following this initial burst, the synthesis of additional 14-mer is much slower (Fig. 4). We therefore conclude that following the initial burst the rate of synthesis of the 14-mer (for all of the mutants) is limited by the rate at which the halted EC dissociates.

**Thumb Subdomain Mutations Increase the Dissociation Rates of ECs Halted on Supercoiled Templates**—We measured halted EC turnover in reactions in which halted complexes were formed on pPK5. The +1 to +16 sequence of the transcript from this template is GGGAGGGGAGACU. By omitting CTP and including 3′-dCTP in reactions with pPK5, we could form halted ECs containing a 15-nucleotide RNA that could not be extended because it lacked a 3′-OH. This made it possible to measure the stability of the EC in either the presence or absence of UTP so that we could assess the effects of NTP binding on complex stability. It has been found that EC stability is affected by template supercoiling (17, 18), so we measured EC stability on both linear and supercoiled templates. Representative data for the four different reaction conditions (supercoiled or linear template; ±1 mM UTP) are shown in Fig. 5A for the w.t. RNAP and for four modified RNAPs that exhibit distinctive behavior. The w.t. enzyme and the D388A mutant are seen to form stable complexes that turn over at a rate of <0.01 min⁻¹ under all four conditions. The nicked enzyme forms a less stable complex; without UTP present the nicked EC turns over at a rate of 0.1–0.2 min⁻¹ on both the linear and supercoiled templates. Addition of 1 mM UTP stabilizes the nicked complex and reduces turnover to <0.01 min⁻¹. Because GTP and ATP are present at 0.5 mM in these experiments, it is clear that this stabilizing effect requires an NTP that is complementary to the template base immediately downstream of the RNA 3′-end (with a template whose initial transcribed sequence is GGGAGGGGAGACU stabilization of complexes halted by 3′-dUMP incorporation required CTP addition; data not shown). The complex formed by R391G is stable on the linear template, but on the supercoiled template it turns over at a rate similar to that of the nicked complex. Addition of UTP stabilizes the R391G complex. Data for all the enzymes are summarized in panels B–D of Fig. 5.

**Effects of the Mutations on Termination at Class I and Class II Terminators**—Because some of the thumb mutants affect EC stability they might be expected to affect termination. T7RNAP terminates specifically at two types of terminators (32–34). The class I terminator is similar to classic E. coli RNAP intrinsic terminators and is comprised of a sequence that can form a stable hairpin in the RNA immediately upstream of a run of
uracils (32). Class II terminators are comprised of the sequence AUCUGUU followed by a run of uracils (33, 34). In T7 DNA this sequence is found at the concatemer junction (CJ), and termination or pausing by T7RNAP at the CJ is essential for phage development (35). Fig. 6A shows that none of the mutations had marked effects on class I termination; the percentage termination for all of the enzymes reproducibly fell in the 63–70% range. Fig. 6B shows termination at a class II terminator. As reported previously, the nicked enzyme fails to recognize the class II terminator (34, 35). The ThDel and Y385A mutants also show almost complete bypass of the class II terminator (3–4% and 7–8% termination for ThDel and Y385A, respectively), whereas the D388A mutation exhibits a substantial reduction in termination (19% termination). The percentage of class II termination for the w.t. enzyme and the other mutants reproducibly fell between 54 and 73%.

Class II terminators act as both pause and termination signals. Sequences that contain the class II CAUCUGUU recognition element (the CJ sequence) but that lack a run of uracils act as pause sites (33). To determine whether the thumb mutations affect pausing at such a site, we measured the rate of appearance of run-off and paused/terminated transcripts in continuous initiation assays with the w.t., Y385A, nicked, and ThDel enzymes (Fig. 7). In the case of the w.t. enzyme, the pause at the class II site delays the appearance of significant amounts of run-off transcripts until ~80 s after initiation of the reaction. However, in the reactions with nicked, ThDel, and Y385A enzymes, run-off transcript is detected 20 s after initia-
EC stability and termination are due to a similar change in the RNA-RNAP interaction, we treated thumb mutant ECs with RNase T1. Fig. 8 shows representative data for the w.t. complex (lanes 1–4), the nicked complex (lanes 5–8), and five thumb mutants (lanes 9–28). The reduced protection of the upstream region of the RNA in the nicked complex is apparent; T1 treatment of the nicked complex results in the rapid degradation of the RNA to 8 nucleotides in length, whereas in the reaction with the w.t. complex the 8-mer is generated more slowly and 11–15-mers accumulate. Quantitation reveals that the ThDel EC also shows loss of protection of the upstream region of the RNA. To determine whether the effects of the thumb mutations on EC stability and termination are due to a similar change in the RNA-RNAP interaction, we treated thumb mutant ECs with RNase T1. Fig. 8 shows representative data for the w.t. complex (lanes 1–4), the nicked complex (lanes 5–8), and five thumb mutants (lanes 9–28). The reduced protection of the upstream region of the RNA in the nicked complex is apparent; T1 treatment of the nicked complex results in the rapid degradation of the RNA to 8 nucleotides in length, whereas in the reaction with the w.t. complex the 8-mer is generated more slowly and 11–15-mers accumulate. Quantitation reveals that the ThDel EC also shows loss of protection of the upstream region of the RNA, but to a more modest extent than the nicked enzyme. With the four point mutants, the T1 digestion patterns are similar to that seen with the w.t. complex, and all of the other thumb mutant complexes also showed patterns of T1 sensitivity like the w.t. complex (not shown). Thus, the reduced EC stability and termination for the thumb mutants cannot be attributed to the kind of large change in the RNA-RNAP interaction that is seen with the nicked enzyme.

**DISCUSSION**

A Role for Helix N of the T7RNAP Thumb Subdomain in Stabilizing the TC—The effects of alanine substitutions at Arg391, Ser393, and Arg394 indicate that these side chains are involved in stabilizing the TC. Their contributions to complex stability become important once the RNA reaches a length of 4–5 bases (Fig. 2). This supports modeling that places the phosphate-ribose backbone of the RNA 4–5 bases away from the 3′-end close to the 385–395 segment of the thumb (Fig. 1A). As shown in Fig. 9 the different contributions of the side chains within this segment to complex stability can be interpreted in terms of their positions on helix N. The side chains of Ser393 and Arg394 face toward the RNA and into the large template binding cleft of the polymerase. The side chain of Arg391 faces away from the cleft, but its α-carbon is on the same side of helix N as that of Arg394, so Arg391 could swing into position to interact with the RNA. The S393A and the R394A mutations destabilize both ITCs (Fig. 2) and ECs (Fig. 5), whereas the R391A mutation destabilizes ECs (Fig. 5) but does not markedly increase termination during initial transcription (Fig. 2). Arg391 may only move into position to interact with the RNA following the isomerization that accompanies promoter release. Side chains 385–389, 392, and 395 either point away from the RNA and template binding cleft or are too far from the RNA, as indicated by modeling, to contact it (Fig. 9). Consistent with this, alanine substitutions at these positions do not have large effects on TC stability (Figs. 2, 3, and 5).

Glycine Substitutions Are More Disruptive than Alanine Substitutions—Upon binding template, part of helix N that is disordered in the apoenzyme assumes a helical conformation (3, 14, 15), indicating both that this part of the thumb subdomain is sensitively poised between helical and nonhelical conformations and that its secondary structure is important for function. To test this we introduced glycines at positions 391–394. Glycine is helix destabilizing (36), so if the secondary structure of helix N is both important for function and sensitive to perturbation, the glycine substitutions should disrupt its activity. We found that glycine substitutions had larger effects on termination during initial transcription and on EC stability than did alanine substitutions (Figs. 2 and 5). In fact, although Lys392 points away from the RNA and the template binding cleft and a K392A substitution does not affect TC stability, a K392G substitution markedly destabilizes the EC and increases termination during initial transcription. Similarly, although an R391A substitution has little effect on initial transcription, an R391G substitution has a large effect.

The Effects of the Mutations on EC Stability Are Influenced by Template Topology—A partial deletion of the thumb subdomain results in an EC, which is exceptionally unstable on supercoiled, but not linear, templates (Fig. 5). It has been suggested that this is because an extended RNA-DNA hybrid forms on supercoiled templates and destabilizes the EC (17, 18). The point mutations in the thumb show a DNA topology-dependent effect on EC stability similar to the thumb deletion. The effects of the thumb mutations on EC stability may be contrasted with the effect of nicking the N-terminal domain, which similarly destabilizes ECs on supercoiled or linear templates (Fig. 5 and Refs. 17 and 18). The decreased stability, processivity, and RNA displacement activity of the nicked EC has been proposed to be due to disruption of an interaction between the single-stranded RNA and an RNA binding site on the N-terminal domain (18, 23, 26, 31). The loss of protection from RNase T1 in the RNA 8–14 bases away from the 3′-end in the nicked EC supports this hypothesis, and the lack of an effect on protection from RNase T1 with the thumb point mutants (Fig. 8) reinforces the conclusion that the thumb and N-terminal domain stabilize the TC through distinct mechanisms.

**The Thumb Deletion and the Point Mutations Affect Initial Transcription Differently**—Although the effects of the thumb deletion and the point mutations on EC stability and RNA interactions are similar (and distinct from the effects of nicking), the thumb deletion and the point mutants have distinct

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**Fig. 7.** Run-off transcription of EcoRV-cut pDL68 (33) for the indicated times (s). RNAP and template concentrations were as in Fig. 2. The sequence of the pause/termination site in pDL68 is CAUCUGUU.
effects on initial transcription. Point mutations between 391 and 394 increase termination for RNAs 4 bases in length, but the largest increases in termination for the ThDel enzyme are for 8- and 9-base RNAs (Fig. 2). The small effect of the thumb deletion on termination for shorter RNAs is consistent with modeling (Fig. 9), which indicates that the amino acids within the deleted segment are too far away to contact RNAs 4–6 nucleotides in length. The effect of the deletion on the stability of TCs with longer (>7 bases) RNAs indicates either that the deleted portion of the thumb makes interactions with the RNA 8 or more bases away from the 3’-end or that the destabilizing effect of the deletion is only manifest once the RNA reaches a length of ~8 bases and the RNAP releases the promoter.

*NTP Binding Strongly Stabilizes the EC—*NTP binding reduces the turnover rates of the w.t. and mutant ECs to below detectable levels (Fig. 5). Similar observations were made by Mentesanas et al. (18) and have also been observed in human immunodeficiency virus, and type 1 reverse transcriptase (37). In addition to the strong stabilization against dissociation, we have also found that NTP binding restricts the lateral mobility (sliding) of the EC on the template (38). EC stabilization caused by NTP binding may be due directly to template-NTP-RNAP interactions, or it may reflect an NTP induced isomerization from an “open” to a “closed” conformation in the RNAP. Such an isomerization has been detected kinetically (39) and proteolytically (40) in DNAPs homologous to T7RNAP and has been observed directly in crystal structures (21, 41). The latter studies revealed that this isomerization causes the fingers domain of the polymerase to close around the template strand. Presumably, such an NTP-induced isomerization could significantly enhance EC stability.

The Functional Importance of Different Side Chains between Positions 385 and 395 Is Consistent with Their Conservation—Three amino acids (Tyr385, Arg391, and Ser393) within 385–395 are well conserved among the phage RNAPs, and at another two positions (Asp388 and Arg394) there is conservation of charge (Fig. 1B). As judged from the effects of alanine substitutions, Arg391, Ser393, and Arg394 make the most important contributions to TC stability. To evaluate the importance of charge at positions 391 and 394, we made lysine substitutions at both positions. Relative to the alanine substituted enzymes, both the R391K and R394K mutants form more stable ECs (Fig. 5), indicating that charge is a functionally important feature of the side chains at these positions.

A Role for Helix N in Pausing/Termination at the Concatemer Junction—Mutation of the other two well conserved side chains (Tyr385 and Asp388), which both project from the outer surface of helix N (Figs. 1 and 9), does not affect TC stability but does reduce pausing and termination at the T7 DNA CJ. Proteolytic nicking of the N-terminal domain and deletion of part of the thumb that is close to Tyr385 (Fig. 9) also reduce pausing and termination at the CJ. The mechanism of termination at the CJ is not well understood, so it is difficult to speculate on how these alterations in RNAP structure affect response to the CJ. It is, however, likely that nicking of the N-terminal domain and the thumb mutations work through distinct mechanisms, because nicking leads to a large change in protection from RNase digestion (38), as well as to reduced EC stability (17, 18) and processivity (23, 26), and a defect in RNA displacement (38). The Y385A and D388A mutations have none

![Fig. 9. Structure of a T7RNAP TC.](image-url)
of these effects. The effect of nicking on CJ recognition may, therefore, be a consequence of a gross alteration in the RNA-RNAP interaction, whereas the effects of the Y385A and D388A mutations are limited to CJ pausing and termination. It is possible that the Tyr<sup>385</sup> and Asp<sup>388</sup> side chains directly recognize the CJ in the RNA. In one model for the RNA path in the T7RNAP EC, the RNA passes through a cleft formed by the thumb and N-terminal domain (4). The RNA, after emerging from this cleft, could contact Tyr<sup>385</sup>/Asp<sup>388</sup> on the outer surface of helix N (Fig. 1). The distance between the RNA 3'-end and the RNA in contact with Tyr<sup>385</sup>/Asp<sup>388</sup> would then be ~9 nucleotides, which is consistent with the position at which termination occurs relative to the CJ. However, a recent cross-linking analysis proposes a distinct RNA path in which the RNA 8–10 nucleotides away from the 3'-end lies near the RNAP promoter specificity loop (25). In this case, a direct role for Tyr<sup>385</sup> and Asp<sup>388</sup> in recognition of the CJ appears unlikely, although it cannot be ruled out because it is not known whether the RNA path in a normal EC differs from that in a complex despite their conformational changes important for re-

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