Published in final edited form as:

J Mol Biol. 2008 February 8; 376(1): 153–165.

Threonine 429 of *E. coli* σ^{70} is a key participant in promoter DNA melting by RNA polymerase

Lisa A. Schroeder¹, Mary E. Karpen², and Pieter L. deHaseth^{1,3,4}

¹Center for RNA Molecular Biology, Case Western Reserve University, Cleveland, Ohio 44106

²Chemistry Department, Grand Valley State University, Allendale, Michigan 49401

³Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106-4973

Abstract

Initiation of transcription is an important target for regulation of gene expression. In bacteria, formation of a transcription-competent complex between RNA polymerase and a promoter involves DNA strand separation over a stretch of about 14 base pairs. Aromatic and basic residues in conserved region 2.3 of *E. coli* σ^{70} had been found to participate in this process, but it is still unclear which amino acid residues initiate it. Here we report an essential role for the threonine (T) at position 429 of σ^{70} : its substitution by alanine (T429A) results in the largest decrease in open complex formation yet observed for any single substitution in region 2.3. Promoter recognition itself is not affected by the T429A substitution, thus providing evidence for a role of T429 in the strand separation step. Our data are consistent with a model where the T429 would act as a competitor for the hydrogen bonding stabilizing the highly conserved -11 A-T base pair of the promoter DNA, thus facilitating initiation of strand separation at this particular position in the -10 region. This model suggests an active role for RNA polymerase in disrupting the -11 base pair, rather than just capturing the -11A subsequent to spontaneous unpairing.

Keywords

RNA polymerase; sigma factor; promoter; strand separation

Introduction

Sigma factors constitute a class of bacterial transcription factors which bind to the RNA polymerase (RNAP) “core enzyme”, E, to form “holoenzyme”, $E\sigma^{70}$. In this complex, the sigma factor functions in the sequence-specific recognition of promoter DNA, and plays an essential role in the promoter DNA strand separation step required for initiation of transcription. *E. coli* has 7 different sigma factors, including σ^{70} , which is required for transcription of the housekeeping genes. Four regions of sequence conservation have been recognized, some of which are further divided into sub-regions. Region 2.3, consisting of 18 amino acids, is the DNA melting region of σ^{70} ^{1,2}.

⁴Address all correspondence to this author: Center for RNA Molecular Biology, School of Medicine, Case Western Reserve University, 10900 Euclid Avenue, Cleveland Ohio 44106-4973, Telephone: 216 368 3684, Fax: 216 368 2010 e-mail: pld2@case.edu

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

It is thought that promoter DNA melting is initiated in the -10 region, consensus sequence TATAAT, at the highly conserved A-T basepair which is at position -11 (underlined) in most promoters by a mechanism involving rotation (“flipping”) of the A out of the DNA helix and into a pocket on the RNAP³. Once that has occurred, strand separation would propagate downstream to about position +3, just past the start site of transcription, to form the open complex. This is a complicated process involving conformational changes in the RNAP. The extensive work of Record and his group on open complex formation at the λ P_R promoter has established the occurrence of two intermediates in addition to the first specific (closed) complex⁴⁻⁶. This would lead to the following scheme¹: $\mathbf{R+P \leftrightarrow RP_c \leftrightarrow I_1 \leftrightarrow I_2 \leftrightarrow RP_o}$. Both RP_c and I₁ are sensitive to the competitor, heparin, while I₂ and RP_o are competitor resistant⁴. The promoter region covered by RNAP is smaller in RP_c, extending downstream to about +1, than for I₁, I₂ and RP_o, reaching to about +20⁴.

Much work has been devoted to the study of the mechanism of DNA melting by bacterial RNAP, but to date it is not yet clear how the process is initiated. Several amino acids in region 2.3 of σ^{70} had previously been identified as playing a role in this process^{3,7-10}, but amino acid residues that have been proven to interact with the -11 A-T basepair of promoter DNA during formation of the open complex have yet to be identified. Our recent studies called in question whether the region 2.3 residues Y430 and W433, previously thought to interact with the -11A, are indeed its likely interacting partners¹¹. Some time ago a study by Susskind and colleagues identified a threonine at position 429 of σ^{70} as being uniquely important, as no substitution other than serine was found to result in significant promoter activity in vivo¹². This would be indicative of an essential role for the hydroxyl group that threonine and serine have in common. The experiments reported here demonstrate that the T429A substitution in σ^{70} impedes open complex formation to a greater extent than any other single substitution in σ^{70} . We postulate that T429 may play an essential role in disrupting the -11 A-T base pair as a prelude to flipping the -11A out of the helix.

Results

The T429A substitution greatly impedes stable RNAP-promoter complex formation

In our effort to identify amino acid residues of σ^{70} that are important in the early stages of the RNAP-induced strand separation process, we introduced substitutions at T429 and T432. We chose these amino acids, which are capable of H-bonding, in view of Matlock and Heyduk’s proposal¹³ that amino acid residues of σ^{70} might nucleate promoter DNA melting by competing with the H-bonding stabilizing the -11 A-T base pair. In addition, T429 had previously been found to be of critical importance¹²; see above. Our data indicated that while the T432A substitution had effects that were qualitatively similar to those of Y430A and W433A (e.g. reference¹¹), the T429A substitution was more deleterious than observed for any other single substitution in region 2.3 tested (data not shown).

To explore the DNA target of T429, we used promoter DNAs, bearing abasic sites, as shown in figure 1(a). Abasic sites are useful in order to assess whether a particular amino acid substitution affects either interactions to the missing base, or base pairing involving this base; here they help elucidate the latter (see below). Prior to gel-analysis, the complexes were challenged by the competitor, heparin, to ensure that only stable RNAP-promoter complexes were considered. The results, obtained at room temperature (23-26 °C), reveal the large negative effect of the T429A substitution on the interaction with Short Duplex. A similar negative effect of the T429A substitution is seen with DNA bearing a -11 abasic site in the top (nontemplate) strand, but with this template, complex formation with WT RNAP is reduced as well, when compared to that of the Short Duplex with WT RNAP. These results underscore the importance of both T429 and the -11A for stable complex formation (see figures 1(b) and 1(c)).

If the abasic site is on the template strand (-11 Ab D Bot), a slight increase in the extent of stable complex formation is seen for the WT but a much greater increase for the T429A RNAP. With the -11A already unpaired, due to the absence of its base pairing partner on the template strand, the T429 may no longer be critical. Abasic sites at position -10 also greatly facilitate complex formation, but the differences between the binding of promoters with the abasic sites on the template or the nontemplate strands are not as pronounced. In either case, the weakening of the base pairing due to the abasic sites must be more important than any interactions that might have been disrupted due to the missing bases. Abasic sites at position -12 have smaller effects. The basepair at -12 is not normally melted in the open complex, thus any effects of abasic sites here might be due to their effects on the base pairing at -11¹⁴. The -11 and -10 abasic sites in the template DNA restore complex formation by the RNAP with the T429A substitution to similarly large extents, suggesting that the T429 may function in disrupting either the -11 or the -10 A-T basepairs.

We also carried out experiments with Short Duplex and -10 and -11 Abasic Bottom (see figure 1(a)) where the concentrations of RNAP and promoter DNA had been reduced by fivefold, to 10 nM RNAP and 2 nM DNA. All binding percentages were lower as a result (data not shown), indicating that binding was not at saturation, which would have resulted in loss of the ability to fully detect increases in binding affinity. These results confirm that the presence of the abasic site at -11 of the template strand improves binding affinity for T429A RNAP to a much greater extent than that for WT RNAP. A similar difference is observed when comparing the effects of the -10 abasic site in the template strand. The observation that the effect of the template strand abasic site is to preferentially rescue the defect of the T429A substitution is consistent with a role of the T429 in disrupting the -11 and/or -10 A-T base pairing (see discussion).

The T429A substitution does not affect DNA binding by RNAP

The T429A substitution may lead to a defect in stable complex formation due to an impaired ability to bind to promoter DNA in a closed complex under the conditions of the experiments shown in figure 1. To address this possibility, we carried out the EMSA experiments shown in figure 1(d). RNAP and Short Duplex DNA were incubated exactly as for the experiments shown in figure 1(b) and 1(c), but without a heparin challenge, and the mixtures were subjected to electrophoresis on a non-denaturing gel. Comparison of lanes 1 and 5 indicates that under these conditions WT and T429A RNAP show very similar extents of complex formation (99% and 95%, respectively). Additionally, the heparin sensitive complexes are observed to form within 30 seconds of mixing for both WT and T429A (data not shown). In agreement with the results displayed in figure 1(c) (first two bars), it is found that during the 10 minutes of incubation, most of the complexes formed with WT RNAP are heparin resistant (lane 2), but very few formed with the T429A RNAP are (lane 6).

To assess whether the complexes formed in the absence of the heparin are specific, we also used a Short Duplex DNA carrying a randomized sequence instead of the promoter (Randomized in figure 1(a)). It is seen in lanes 3 and 7 of figure 1(d) that the complexes formed with this DNA have diffuse bands in contrast to the sharp bands observed with Short Duplex DNA (lanes 1 and 5). The diffuse bands obtained with the randomized DNA may be due to nonspecific binding of the RNAP at different locations, leading to a range of mobilities of the complexes. As expected, the complexes formed with the randomized DNA and both the WT and T429A RNAP are heparin sensitive. In aggregate, these results indicate that the T429A substitution does not lead to defects in promoter recognition and closed complex formation.

σ^{70} with the T429A substitution can bind core RNAP

We had previously concluded from gel shift analysis of reconstitutions reactions containing both σ^{70} and core enzyme, that the T429A substitution affected the interaction of the σ^{70} with

core enzyme¹⁰. However, inspection of this work revealed anomalies in the gel pattern: while formation of holoenzyme was not detectable, neither was the band representing free core RNAP, casting doubt on our earlier interpretation of the results. The bands on the gel of figure 2(a) demonstrate that the earlier conclusion was incorrect. Upon repeating the experiment, as described in the Materials and Methods, with σ^{70} bearing the T429A as well as four other single substitutions, it is evident that each is able to bind core enzyme equally well as WT σ^{70} to form holoenzyme. The results reveal essentially complete disappearance of the core-band with concomitant appearance of a new band corresponding to the holoenzyme. A duplicate gel (not shown) displayed the same pattern. Addition of a greater excess of σ^{70} over core RNAP in the reconstitution reaction did not rescue the low percentage of heparin-resistant complexes seen with T429A RNAP and Short Duplex DNA (data not shown), providing further indication that under our experimental conditions, the T429A substitution does not impair the σ^{70} -core interaction.

A side chain bearing a hydroxyl group is essential at position 429 of σ^{70}

The results of experiments on the interaction of the Short Duplex DNA template (see first six bars in figure 2(b)) with holoenzyme containing WT σ^{70} and σ^{70} bearing five different substitutions at 429, indicate that with this DNA, the only substitution allowing significant DNA binding activity is T429S. Interestingly, when a fork-junction template is used where the -11A is unpaired (figure 1(a)), all variants showed similar extents of complex formation (next six bars in figure 2(b)), suggesting that the T429 may participate in disrupting the -11 A-T basepairing, so that a substitution for T429 would have no effect if the -11A is already unpaired.

To better differentiate among the effects of the substitutions for T429, we also studied binding to the Long Duplex DNA¹⁵ (see figure 1(a)), a full length promoter, and as such more physiologically relevant. However, with this promoter, the extent of stable complex formation at room temperature is increased such that the difference between WT and T429A RNAP is partially obscured. Therefore, the effects of various substitutions for T429 on heparin-resistant complex formation with Long Duplex DNA were compared at the reduced temperature of 18 °C (see figure 2(c) for a representative gel, and figure 2(d) for a quantitative representation). The serine (i.e. T429S) allowed by far the greatest extent of formation of a stable, heparin-resistant complex among the substitutions tested, consistent with the *in vivo* transcription results from Susskind's group¹², and with the results shown in figure 2(b). Thus the hydroxyl group, shared by T and S, must be essential for formation of a stable complex, while the tyrosine (T429Y) side chain would be too large to properly present its hydroxyl group, and may experience steric hindrance. The cysteine (T429C) showed a low extent of binding, considerably less than the serine, in keeping with the fact that the sulfhydryl group is worse at hydrogen bonding than the hydroxyl. Valine (T429V) is similar in size to threonine, and also has a methyl group, but lacks the hydroxyl group. It allows less promoter DNA binding in a stable complex than serine, but more than the other substitutions tested. Very similar results were also obtained at room temperature with Long Duplex DNA, bearing a 2-AP substitution for the -11A in the nontemplate strand, which is known to mitigate open complex formation^{11,14,16,17}. As a consequence, the 2-AP-substituted promoter also allows good differentiation among RNAP bearing various amino acid substitutions in region 2.3 of σ^{70} , according to their abilities to carry out DNA strand separation.

T429A decreases the rate of stable complex formation with promoter DNA

The mechanism whereby the T429A substitution leads to formation of fewer stable promoter complexes was probed by investigation of the kinetics of the formation and dissociation of stable complexes for WT and T429A RNAP with the Long Duplex at 18 °C. Sample results for the association kinetics obtained by quantification of the stable complex band for each time point in gels such as that shown in figure 3(a), are displayed in figure 3(b) and summarized in

Table 1. The association rate constants for stable complex formation by WT and W433A RNAP are seen to be very similar, and twice as large as that for Y430A RNAP, which in turn is 4-5 times larger than that for T429A RNAP. On the other hand, the amplitudes are all very similar. Eventually, even the T429A RNAP will form open complexes at 80% of the promoters. Experiments carried out at room temperature with Long Duplex DNA bearing the -11 2-AP substitution lead to a similar conclusion (data not shown).

No decay of the “stable” complexes containing either WT or T429A RNAP was detectable over a time scale of 60 minutes (data not shown), precluding determinations of a dissociation rate constant. Because the complexes are so stable, it is quite possible that effects of the T429A substitution on the stability of the RNAP-promoter complexes may have remained undetected. Complexes of RNAP with Short Duplex DNA are considerably less stable¹¹, but that template could not be used here with T429A for either the association or the dissociation experiments, in view of the very low extents of binding obtained (e.g. see figure 1(c)). Thus it is difficult to compare the current results with those obtained previously for complexes of RNAP containing substitutions at Y430 and W433¹¹ with Short Duplex. With the latter DNA, no effects of the Y430 and W433 substitutions were observed on the association kinetics, but the complex half life was found to be a factor of two less than that observed with WT RNAP.

The T429A substitution slows, but does not prevent, promoter DNA melting

To directly determine the effect of the T429A substitution in σ^{70} on RNAP-dependent promoter DNA melting, we carried out KMnO₄ probing experiments to monitor DNA strand opening. The T's in single stranded regions of DNA are oxidized by KMnO₄, thus rendering the DNA sensitive to piperidine-induced cleavage. In figure 4 the results are shown of such an experiment with Long Duplex DNA at 18 °C. The samples where the promoter DNA was incubated with WT RNAP for 10 or 60 min prior to probing with KMnO₄ showed prominent bands (figure 4 (a)), indicative of extensive strand separation. The data have been quantified and are shown in figure 4(b) as percentage of the extent of melting seen with WT RNAP after a 60 min incubation with the promoter DNA. With T429A RNAP, strand separation is slow, consistent with the observed kinetics (figure 3, above), but at 60 min a significant extent of melting could be observed. For Y430 and W433 RNAP, the extents of DNA melting are very similar to those seen for WT RNAP (data not shown). For reactions containing 50 nM RNAP and 10 nM promoter DNA incubated for 10 min, it is expected that most of the DNA will be bound by RNAP containing WT, T429A, Y430A, and W433A σ^{70} (see figure 1(d)), so that the results described here reflect a pronounced defect in the ability of the T429A RNAP to melt promoter DNA. Consistent with expectations, the non-melting FYWW RNAP^{18,19} did not show any detectable melting of -11 2-AP-substituted Long Duplex DNA, even after an hour of incubation at room temperature and a concentration of 200 nM, which would result in most of the FYWW RNAP being bound by the promoter DNA¹⁹ (data not shown).

The T429A substitution exerts its effects at a step after closed complex formation

In order to try and pinpoint at what step in the kinetic scheme the consequences of the T429A substitution manifest themselves we carried out DNase I footprinting on the Extended Long Duplex, a promoter with the sequence of the Long Duplex but in the context of a longer DNA fragment, necessary to obtain good footprinting patterns (see figure 1(a) for the DNA sequences and figures 5(a) and (b) for the footprinting). In order to be able to capture the closed complex, we used short incubation times (0.5 min) and low temperature (9 °C). Incubation of the Extended Long Duplex for 0.5 min with WT RNAP (or Y430A and W433A RNAP (data not shown)) or for 10 min with T429A RNAP led to a long, heparin resistant DNase I footprint, stretching from about -44 to +25, characteristic of an open complex. However a 0.5 min incubation of Extended Long Duplex with T429A RNAP resulted mainly in a shorter, heparin-

sensitive footprint, extending from approximately -44 to about +5, characteristic of a closed complex.

This assignment is further supported by probing the above complexes with KMnO_4 in order to directly monitor RNAP-induced strand opening. Indeed, KMnO_4 probing of the complexes exhibiting a long footprint indicated that strand separation had occurred, as deduced by DNA strand breakage at the +2/-1, -6 and -10 T's in the nontemplate strand (see figure 5(c)). However, much fainter bands can be seen for the complexes with the short footprint, formed after a 0.5 min incubation of the Extended Long Duplex with T429A RNAP. From the band intensities, it can be calculated that these conditions lead to the melting of approximately 26% of the DNA as compared to the WT RNAP, in agreement with the 26% estimated from EMSA analysis of heparin-challenged binding reactions carried out under the same conditions as the DNase I footprinting (data not shown). The two prominent bands downstream of the presumed closed complex boundary are each about 50% protected from DNase I cleavage (see figure 5 (b)). Some of this protection is due to the open complexes formed at 26% of the promoters during the 0.5 min incubation with T429A RNAP. Some or all of the remaining 20-30% protection may be due to I_1 , consistent with our observation that there likely is both heparin resistant and-sensitive protection of the above two bands (data not shown). For this strong promoter, the I_1 to I_2 conversion is expected to be very fast, thus such accumulation of I_1 would be indicative of an effect of the T429A substitution on the I_1 to I_2 conversion as well. Technical reasons (heparin-sensitivity of the DNase I cleavage reaction itself) precluded more precise quantification of the extent of heparin-sensitive protection of the DNA.

We also footprinted the complexes formed by T429A RNAP and the well-studied λP_R promoter DNA; see figures 6(a) (gel) and 6(b) (traces). Closed complexes show a short footprint at this promoter¹⁰ as well, and also at a variant of the P_R promoter bearing a G-12T substitution¹⁹, while for I_1 , I_2 and RP_o complexes, a footprint is observed that is extended in the downstream direction^{4,5}. With WT RNAP, after a 10 minute incubation at room temperature, essentially all the DNA is protected over a region extending to +20 (lane 2) in a complex that survives a heparin challenge (lane 3), consistent with an open complex. On the P_R promoter, weaker than the Extended Long Duplex, after a 10 minute incubation of the DNA and T429A RNAP, a short (lane 4) and heparin sensitive (lane 5) footprint is observed, characteristic of the closed complex. The traces of the gel shown in figure 6(a) are presented in figure 6(b), which clearly displays the differences between the long and the short footprints. The results are in good agreement with those obtained before¹⁹, not only with respect to the footprints for WT RNAP, but also in comparing the short footprints for T429A RNAP with that seen earlier for FYWW RNAP. Thus the T429A substitution causes an accumulation of RP_c , consistent with a defect of T429A RNAP in carrying out the RP_c to I_1 step. In contrast to the results obtained for the Extended Long Duplex, there is little additional protection of DNA downstream of the presumed closed complex boundary for the T429A RNAP, obviating the need for invoking effects of the T429A substitution at steps beyond the RP_c to I_1 transition.

Discussion

We have established the exquisite importance of the σ^{70} T429 residue to formation of a stable, strand separated, RNAP-promoter complex by demonstrating that the T429A substitution is highly deleterious to this process. Several lines of evidence are consistent with T429 being involved in promoter DNA strand separation. First, as is shown by the results presented in figure 1(d), the T429A substitution does not affect closed complex formation, indicating that subsequent step(s) involved in the nucleation of promoter DNA melting are affected. Second, the effects of the T429A substitution are mitigated if the -11A (or -10T) is already unpaired, as is the case for both Fork DNA and for promoter DNA with an abasic site in the template strand, but not (or to a much smaller extent) in the nontemplate strand (see figures 1(c) and 2

(b)). That the level of abasic promoter DNA binding is not completely restored to that seen with WT RNAP points to T429's likely involvement in interactions other than that necessary for strand separation of the -11 A-T basepair (see below). Third, the T429A substitution slows the kinetics of formation of the stable promoter complex (see figures 3 and 4). Finally, by DNase I footprinting (figures 5(a) and (b), and 6(a) and (b)) we were able to detect an accumulation of the closed complex. Our data are consistent with an effect of the T429A substitution on the RP_c to I_1 step. However, as pointed out above (see Results and figure 5(b)), for the Extended Long Duplex we cannot conclusively rule out accumulation of an I_1 -like intermediate and an (additional) effect of the T429A substitution on the I_1 to I_2 conversion. No such additional inhibition needs to be invoked for the P_R promoter.

Proposed mechanism of action for T429

The evidence presented above strongly supports a role for T429 in the nucleation of strand separation of promoter DNA. A large body of data indicates that initiation of DNA melting occurs at the -11 A-T base pair^{3,11,13,14,16,20-22}. Taken together, this would suggest that an important target for T429 is the A-T base pair at -11. However, the data in figure 1(c) indicate that the effects of abasic sites at -10 in either strand of promoter DNA are large as well. There are several possible explanations for this observation. The abasic sites at -10 may facilitate initiation of DNA melting at this position, and/or exert an indirect effect by decreasing the stability of the -11 base pair. However, the possibility that T429 also interacts with the -10 base pair cannot be ruled out. Interestingly, the data displayed in figures 4(a) and 5(c) show that WT RNAP protects the -10T to a greater extent from $KMnO_4$ oxidation (relative to the -6T) than T429A RNAP, supporting the proximity of T429 and -10T during at least some phase of open complex formation. Thus, all four bases participating in base pairing at -11 and -10 need to be considered as possible targets of T429.

In order to determine the feasibility of basepair disruption through H-bonding to the hydroxyl of T429, we focused on the area surrounding the T429, using the 6.5Å structure obtained by Darst and co-workers²³. This structure is a complex of E. coli holoenzyme with a fork-junction DNA template similar to the Fork DNA used in this work, which would likely represent an open complex. Ideally, we would use a closed complex or other intermediate complex structure for modeling, but these have not yet been solved. Using the coordinates of this "open" complex structure, we have constructed the model presented in figure 7 (see Materials and Methods), which shows that the bases at -11 and -10 of the nontemplate strand are within 9 Å of T429. As T429 exerts its effect at an early stage during open complex formation (figures 5 and 6), it is likely that a subsequent conformational change may have occurred during this process, which would have moved the T429 out of H-bonding distance (3 Å) from the -11 (and/or -10) base pairs, its likely targets. Residues Y430 and W433, also shown to be in close proximity to the -10 region (figure 7), play important, albeit not critical roles in open complex formation⁸⁻¹⁰.

Support for conformational changes during open complex formation is derived from modeling of the template strand downstream of -12 as forming a B-helix with the nontemplate strand, to emulate a closed complex (model not shown). This strand would clash with amino acids in region 2.3 of σ^{70} , including the T429, and thus this region of σ^{70} must have been re-positioned subsequent to strand separation and insertion of the template strand into the tunnel leading to the active site of the RNAP. Additional support comes from the observation that Q437 and T440, both of which have been shown to interact with the -12T base²⁴, are also outside of H-bonding range to this base. Thus, conformational changes on the pathway from the closed to the open complex likely positioned the amino acid and base interacting partners so they could no longer interact. In conclusion, the modeling demonstrates not only that T429 is on the surface of the sigma subunit and able to contact the nontemplate strand, but also that only a small

adjustment in distance would suffice for the T429 to be able to interact with the -11 or -10 base region.

We propose that T429 helps initiate strand separation at either position by using its hydroxyl group to compete for hydrogen bonding between the bases at -11 and/or -10, thereby weakening the base pairing between these bases. This would facilitate capture of the base(s) by the RNAP to initiate the strand separation process, a concept that was proposed earlier by Heyduk and coworkers¹³. Any disruption of base pairing at both -11 and -10 by T429 is likely to be sequential, with the -11 base pair melted first. The exact target of T429 may not be easily elucidated, as it is likely that each DNA base is contacted by multiple amino acid residues and vice versa²⁵, forming a network of interactions.

Comparison of T429A RNAP with a σ^{70} -substituted RNAP that forms stable closed complexes

In previously published work^{18,19}, we have characterized another RNAP bearing substitutions in region 2.3 of σ^{70} . With four aromatic amino acids in region 2.3 substituted by alanine (F427, Y430, W433 and W434) this form of σ^{70} was designated FYWW. While the footprints of T429A (50 nM RNAP, 0.5 min incubation) and FYWW (150 nM RNAP, 10 min incubation) RNAP on the Extended Long Duplex at 9 °C are similar (data not shown), there are fundamental differences in the behavior of these two enzymes. We have not been able to detect a significant amount of open complex formation by FYWW RNAP under any conditions assayed so far. However the T429A RNAP characterized here exhibits slow kinetics, but has been found to form open complexes on almost all Extended Long Duplex promoters in the solution in 10 min (see figure 5). This distinction likely reflects a fundamental difference in the mechanisms by which the substituted amino acid residues participate in strand separation. While T429 would accelerate the initiation of strand separation, the T429A RNAP would retain the ability to carry out the subsequent steps (involving capture of the -11A) once the -11A has spontaneously rotated out of the DNA helix. On the other hand, the four substitutions which are substituted in the FYWW σ^{70} are proposed to form the -11A binding pocket on the RNAP. This mutated RNAP would then be deficient in holding on to the -11A, rendering it unable to form open complexes regardless of the strength of the promoter or the time of incubation. The model presented here is compatible with an active role for RNAP in effecting promoter DNA strand separation, which may also require DNA bending to further weaken the base pairing at -11²⁶. The alternative would be a passive role where RNAP would merely capture the -11A during a spontaneous excursion out of the DNA helix.

Conclusion

Our data demonstrate that T429 is a key player in the nucleation of promoter DNA melting. The hydroxyl group of the threonine was found to be critical, providing support for a model where it would play a role in disrupting base pairing of the -11 and/or -10 base pairs of promoter DNA to facilitate flipping of the -11A out of the helix. Thus RNAP would play an active role in nucleating the strand separation step that results in formation of the open RNAP-promoter complex.

Materials and Methods

Materials

Oligodeoxynucleotides were synthesized by Invitrogen or Integrated DNA Technologies. (γ -³³P) ATP was purchased from Perkin Elmer, DNA modifying enzymes from either New England Biolabs or Roche and *E. coli* RNAP core from EpiCenter. All chemicals were from Sigma, Fisher, or Amresco.

Protein purification, characterization, and reconstitution

E. coli σ factors were purified using a non-denaturing protocol from Zhi and Jin²⁷ with minor modifications, as described¹¹. Holoenzyme was reconstituted from purified RNAP core (400 nM) and WT or substituted σ^{70} by incubation on ice for 1 hour. The amount of σ^{70} added (5-15-fold excess over the core enzyme, depending on the particular preparation) was determined in separate assays to give maximal formation of stable complexes with promoter DNA, radiolabeled and annealed as previously described¹¹.

Gel shift assays for holoenzyme formation

Reconstituted RNAP, 7.5 μ l, was added to 2.5 μ l of 4 x native dye solution (125 mM Tris-HCl, pH 6.8, 40% glycerol, 0.01% Bromophenol Blue). All 10 μ l were then loaded onto a Tris-HCl 4-20% PAGE Readygel from BioRad (catalog # 161-1105) and run at low voltage in native running buffer (25 mM Tris, 170 mM glycine, 0.1% SDS; pH 8.3). The gel was then rinsed with ddH₂O and stained for 1 hour with Gelcode® Blue from Pierce. Documentation of the gel was performed using a Chemi Genius Bio Imaging System from Syngene.

Electrophoretic mobility shift assay (EMSA)

These binding assays were carried out as previously described¹¹ in Fork binding buffer (FBB: 30 mM Hepes, pH 7.5, 1 mM DTT, 0.1 mg/ml BSA, 100 mM NaCl, 0.1 mM EDTA, pH 8, 1% glycerol). Generally, 10 nM annealed DNA, and 50 nM of RNAP holoenzyme were incubated at room temperature (23-26°C) for 10 minutes (or 5 minutes where indicated). To assay for formation of stable complexes, reactions were challenged with 200 μ g/ml of heparin for an additional 10 minutes (or 5 minutes where indicated); for reactions without heparin challenge, 1 μ l of ddH₂O was added and incubation was continued for another 10 minutes. Four percent non-denaturing gels were loaded and run at room temperature as described, and after drying, the gel was analyzed by PhosphorImaging (Molecular Dynamics) using ImageQuant 5.2 software to quantify the radiolabeled free and RNAP-bound DNA. For EMSA experiments performed at 18 or 9°C, an Echo Therm chilling/heating plate from Torrey Pines Scientific was used to maintain temperature, and gels were run at the same temperature in a Protean II xi cell electrophoresis unit (BioRad). Here and elsewhere, error was determined from half of the spread of the values (two experiments), or the standard deviation (three or more experiments).

Obtaining association rate constants by EMSA

Experiments for determining k_{obs} were performed essentially as described¹¹. Briefly, the binding reaction was initiated by addition of reconstituted RNAP (50 nM final concentration) to 10 nM annealed, radiolabeled DNA in 10 μ l 1 x FBB at 18°C. After the desired incubation times, 1 μ l of 2 mg/ml heparin (final of 200 μ g/ml) was added for 30 seconds to dissociate non-specific and heparin sensitive complexes. Addition of dye and loading is as described above for EMSA experiments run at 18°C. Fractions of DNA bound were determined and plotted as a function of time and fit using Kaleidagraph version 3.52 to the following single exponential equation: $y = y_{max} * (1 - \exp(-k_{obs} * t))$ where y_{max} is the amplitude and k_{obs} is the pseudo first-order rate constant for the association of RNAP and promoter DNA to form a heparin resistant complex. Experiments for determining the k_{off} of heparin resistant complexes were performed in 30 μ l reaction mixtures containing the same components as for the association kinetics. After a ten minute incubation at 18°C, complexes were challenged with 200 μ g/ml of heparin and time points taken thereafter. Addition of dye and gel loading is as described above.

DNase I footprinting

End-labeling of the 237 basepair λ PR fragment was as described¹⁹. The DNA (~3 nM), ³³P labeled at the 5' end of the template strand, was incubated with 100 nM reconstituted RNAP

at room temperature in transcription buffer (30 mM Tris-HCl, pH 7.3, 20 mM KCl, 10 mM MgCl₂, 0.1 mM EDTA, 50 µg/ml bovine serum albumin, 10 mM dithiothreitol). The final volume of the reactions was 20 µl. After 10 minutes of incubation, DNase I was added to a final concentration of 0.01 U/µl and incubation at room temperature was allowed to proceed for 30 seconds. For reactions requiring a heparin challenge, 1 µl of 2 mg/ml heparin was added and incubation proceeded for 1 minute before adding the DNase I. Reactions were stopped by addition of 2 volumes of 4M NH₄Ac, followed by ethanol precipitation in the presence of 80 µg/ml of glycogen. Pellets were washed with 70% ethanol, resuspended in formamide dye¹¹ and loaded onto a 6% sequencing gel containing 25% formamide. Gels were fixed with 5% acetic acid and 20% methanol, dried, and exposed for 2 days to a PhosphorImaging screen before scanning (Molecular Dynamics).

The Extended Long Duplex promoter DNA was derived from cloning the 65 bp annealed Long Duplex promoter into a pCR-Blunt II-TOPO vector (Invitrogen). End-labeling of the 242 basepair Extended Long Duplex was performed by PCR amplification using a nontemplate-sequence primer that was 5'-end-labeled with ³³P and an unlabeled template-sequence primer. DNase I Footprinting experiments using the Extended Long Duplex promoter were performed similarly, except that the FBB used for the EMSA experiments was additionally supplemented with 5 mM MgCl₂ (final concentration in the reactions), RNAP concentration was 50 nM, and reactions were incubated at 9°C for either 0.5 or 10 minutes. The volume and incubation time of the DNase I reaction was the same, but the DNase I was to 0.06 U/µl instead in view of the lower temperature.

DNase I footprinting patterns were analyzed using ImageQuant 5.2. To compare different reactions, the quantified traces for the different lanes were normalized to the same peak (a band chosen that is outside of the footprinted region) using Microsoft Excel. To correct for the non-alignment of peaks in different lanes due to uneven running of the gel (only for the graph in figure 6(b)) the bands just upstream of the -48 peak for the No RNAP lane were shifted to the right, and those for T429A RNAP to the left to properly align the peaks. The traces shown in figure 5(b) did not require modification of this type.

KMnO₄ probing

Reactions contained 10 nM annealed Long Duplex DNA (5' end-labeled at the nontemplate strand) in Tris Binding Buffer (TBB: 40 mM Tris-HCl, pH 8, 40 mM KCl, 1 mM MgCl₂) and were started by the addition of RNAP to 50 nM (final volume 20 µl). Incubation was carried out at 18°C for 1, 10, or 60 minutes, then KMnO₄ was added to a final concentration of 2 mM. After 30 seconds, 5 µl of stop solution containing 1.5 M NaOAc, pH 8, 4 mg/ml glycogen, and 300 mM β-mercaptoethanol was added and reactions were placed on ice. Further work-up was exactly as described, except that an 8% sequencing gel was used instead of a 10% gel¹¹. To compare extents of RNAP-induced melting of the Long Duplex DNA, the -1 and +2 T bands were used to quantify the strand opening. The total radioactivity of these bands in each lane was divided by that of full length DNA, and the values were then normalized to that obtained for WT RNAP after a 60 minute incubation.

For reactions performed at 9°C, the same TBB was used as for the previous KMnO₄ experiment, except that the MgCl₂ concentration was 5 mM instead of 1 mM in order to make more direct comparisons to the DNase I footprinting experiments. Here, about 3 nM of nontemplate strand ³³P-labeled 242 bp Extended Long Duplex DNA and 50 nM RNAP was incubated at 9°C for 0.5 or 10 minutes. Heparin was added to a final concentration of 100 µg/ml and incubated for 1 minute before adding 1 µl of 63 mM KMnO₄ (final concentration of 3 mM due to the lower temperature of the experiment). Additional work-up is as described for the protocol above, except that a 6% sequencing gel was used for loading the reactions. To quantify strand-opening, all four melted bands were used (-10T, -6T, -1T, +2T).

Structural modeling

The structure presented in figure 7 is based on that determined by Darst and co-workers²³ for the complex of holoenzyme with a fork junction DNA template (double stranded to -12, single stranded from -11 to -7 of the nontemplate strand). Starting with the backbone coordinates of PDB file 1L9Z²³, we built and minimized the sigma subunit residues 252-257 (T. aquaticus numbering) and the nontemplate strand bases in the -12 to -7 region, using CHARMM version 30 with the version 27 parameter set²⁸. The figures were made using VMD 1.8.2²⁹. To emulate the double stranded DNA characteristic of a closed complex, using the program 3DNA³⁰, an eight base pair canonical B-DNA double helix was modeled in as the template DNA, complementary to the -7 to -14 promoter sequence, GCTATACT²³. The -8 to -11 nontemplate backbone of the B-DNA model was superimposed upon the backbone of the 1L9Z crystal structure. The base positions were similar to those found with CHARMM.

Acknowledgements

We thank Dr. Mark Caprara for critically reading a version of this manuscript and Victoria Cook for generously supplying the non-melting FYWW RNAP used for some unpublished experiments. This work was supported by NIH grant GM 31808 to PLdH.

References

1. deHaseth PL, Zupancic M, Record MT Jr. RNA polymerase-promoter interactions: the comings and goings of RNA polymerase. *J. Bacteriol* 1998;180:3019–3025. [PubMed: 9620948]
2. Gross, C.; Chan, C.; Dombroski, A.; Gruber, T.; Sharp, M.; Tupy, J.; Young, BA. Cold Spring Harbor Symposia on Quantitative Biology. 63. Cold Spring Harbor Laboratory Press; Plainview, NY: 1998. The functional and regulatory roles of sigma factors in transcription; p. 141-155.
3. Helmann JD, deHaseth PL. Protein-nucleic acid interactions during open complex formation investigated by systematic alteration of the protein and DNA binding partners. *Biochemistry* 1999;37:5959–5967. [PubMed: 10320321]
4. Craig ML, Tsodikov OV, McQuade KL, Schlax PE Jr, Capp MW, Saecker RM, Record MT Jr. DNA footprints of the two kinetically significant intermediates in formation of an RNA polymerase-promoter open complex: Evidence that interactions with the start site and downstream DNA induce sequential conformational changes in polymerase and DNA. *J. Mol. Biol* 1998;283:741–756. [PubMed: 9790837]
5. Saecker RM, Tsodikov OV, McQuade KL, Schlax PE, Capp MW, Record MT Jr. Kinetic studies and structural models of the association of *E. coli* σ^{70} RNA polymerase with the λP_R promoter: Large scale conformational changes in forming the kinetically significant intermediates. *J. Mol. Biol* 2002;319:649–671. [PubMed: 12054861]
6. Davis CA, Bingman CA, Landick R, Record MT Jr, Saecker RM. Real-time footprinting of DNA in the first kinetically significant intermediate in open complex formation by *Escherichia coli* RNA polymerase. *Proc. Natl. Acad. Sci. USA* 2007;104:7833–7838. [PubMed: 17470797]
7. Juang Y-L, Helmann JD. A promoter melting region in the primary sigma factor of *Bacillus subtilis*: Identification of functionally important aromatic amino acids. *J. Mol. Biol* 1994;235:1470–1488. [PubMed: 8107087]
8. Fenton MS, Lee HJ, Gralla JD. *Escherichia coli* promoter opening and -10 recognition: mutational analysis of σ^{70} . *EMBO Journal* 2000;19:1130–1137. [PubMed: 10698953]
9. Tomsic M, Tsujikawa L, Panaghie G, Wang Y, Azok J, deHaseth PL. Different roles for basic and aromatic amino acids in conserved region 2 of *Escherichia coli* σ^{70} in the nucleation and maintenance of the single-stranded DNA bubble in open RNA polymerase-promoter complexes. *J. Biol. Chem* 2001;276:31891–31896. [PubMed: 11443133]
10. Panaghie G, Aiyar SE, Bobb KL, Hayward RS, deHaseth PL. Aromatic amino acids in region 2.3 of *Escherichia coli* sigma 70 participate collectively in the formation of an RNA polymerase-promoter open complex. *J. Mol. Biol* 2000;299:1217–1230. [PubMed: 10873447]
11. Schroeder LA, Choi A-J, deHaseth PL. The -11A of promoter DNA and two conserved amino acids in the melting region of σ^{70} both directly affect the rate limiting step in formation of the stable RNA

- polymerase-promoter complex, but they do not necessarily interact. *Nucleic Acids Res* 2007;35:4141–4153. [PubMed: 17567604]
12. Waldburger C, Susskind MM. Probing the informational content of *Escherichia coli* sigma 70 region 2.3 by combinatorial cassette mutagenesis. *J. Mol. Biol* 1994;235:1489–1500. [PubMed: 8107088]
 13. Matlock DL, Heyduk T. Sequence determinants for the recognition of the fork junction DNA containing the -10 region of promoter DNA by *E. coli* RNA polymerase. *Biochemistry* 2000;39:12274–12283. [PubMed: 11015206]
 14. Heyduk E, Kuznedelov K, Severinov K, Heyduk T. A consensus adenine at position -11 of the nontemplate strand of bacterial promoter is important for nucleation of promoter melting. *J. Biol. Chem* 2006;281:12362–12369. [PubMed: 16531399]
 15. Schroeder LA, deHaseth PL. Mechanistic differences in promoter DNA melting by *Thermus aquaticus* and *Escherichia coli* RNA polymerases. *J. Biol. Chem* 2005;280:17422–17429. [PubMed: 15731103]
 16. Lim HM, Lee HJ, Roy S, Adhya S. A “master” in base unpairing during isomerization of a promoter upon RNA polymerase binding. *Proc. Natl. Acad. Sci (USA)* 2001;98:14849–14852. [PubMed: 11734629]
 17. Tsujikawa L, Strainic MG, Watrob H, Barkley MD, deHaseth PL. RNA polymerase alters the mobility of an A-residue crucial to polymerase-induced melting of promoter DNA. *Biochemistry* 2002;41:15334–15341. [PubMed: 12484772]
 18. Sun L, Dove S, Panaghie G, deHaseth PL, Hochschild A. An RNA polymerase mutant deficient in DNA melting facilitates study of activation mechanism: Application to an artificial activator of transcription. *J. Mol. Biol* 2004;343:1171–1182. [PubMed: 15491604]
 19. Cook VM, deHaseth PL. Strand opening-deficient *E. coli* RNA polymerase facilitates investigation of closed complexes with promoter DNA: effects of DNA sequence and temperature. *J. Biol. Chem* 2007;282:21319–21326. [PubMed: 17507375]
 20. Fenton MS, Gralla JD. Function of the bacterial TATAAT -10 element as single-stranded DNA during RNA polymerase isomerization. *Proc. Natl. Acad. Sci. USA* 2001;98:9020–9025. [PubMed: 11470900]
 21. Fenton MS, Gralla JD. Effect of DNA bases and backbone on σ^{70} holoenzyme binding and isomerization using fork junction probes. *Nucleic Acids Research* 2003;31:2745–2750. [PubMed: 12771200]
 22. Lee HJ, Lim HM, Adhya S. An unsubstituted C2 hydrogen of Adenine is critical and sufficient at the -11 position of a promoter to signal base pair deformation. *J. Biol. Chem* 2004;279:16899–16902. [PubMed: 14990576]
 23. Murakami KS, Masuda S, Campbell EA, Muzzin O, Darst SA. Structural basis of transcription initiation: An RNA polymerase holoenzyme-DNA complex. *Science* 2002;296:1285–1290. [PubMed: 12016307]
 24. Waldburger C, Gardella T, Wong R, Susskind MM. Changes in conserved region 2 of *Escherichia coli* sigma 70 affecting promoter recognition. *J. Mol. Biol* 1990;215:267–276. [PubMed: 2213883]
 25. Jen-Jacobson L. Protein-DNA recognition complexes: conservation of structure and binding energy in the transition state. *Biopolymers* 1997;44:153–180. [PubMed: 9354759]
 26. deHaseth PL, Nilsen TW. When a part is as good as the whole. *Science* 2004;303:1307–1308. [PubMed: 14988541]
 27. Zhi H, Jin DJ. Purification of highly-active and soluble *Escherichia coli* σ^{70} polypeptide overproduced at low temperature. *Methods in Enzymology* 2003;370:174–180. [PubMed: 14712643]
 28. Brooks BR, Bruccoleri RE, Olafson BD, States DJ, Swaminathan S, Karplus M. CHARMM: A program for macromolecular energy, minimization, and dynamics calculations. *J. Comp. Chem* 1983;4:187–217.
 29. Humphrey W, Dalke A, Schulten K. VMD - Visual Molecular Dynamics. *Graphics* 1996;14:33–38.
 30. Lu X-J, Olson WK. 3DNA: a software package for the analysis, rebuilding and visualization of three-dimensional nucleic acid structures. *Nucleic Acids. Res* 2003;31:5108–5121. [PubMed: 12930962]

(a)

Short Duplex

5' CGCACGGTGT **TTGACA** TTTATCCCTTGCGG **TGGTATAAT** TAACGTA **A** 3' -35 -15 -11 +1
 3' GCGTGCCACA **AACTGT** AAATAGGGAACGCC **ACCATATTA** ATTGCAT **T** 5'

- -10, -11, -12 Abasic Top and Bottom derived from Short Duplex

Fork

5' ————— **TTGACA** ————— **TGGTATAAT** ————— **A** 3'
 3' ————— **AACTGT** ————— **ACCA** 5'

Randomized

5' ————— **CAATTC** ————— **CAGAGATTC** ————— **A** 3'
 3' ————— **GTTAAG** ————— **GTCTCTAAG** ————— **T** 5'

- Long Duplex = Short Duplex with a +2 to +20 extension = 65 bp
- Extended Long Duplex = promoter DNA from -138 to +104 containing the 65 bp Long Duplex = 242 bp

 λ P_R

5' ACCGTGCGTG **TTGACT** ATTTTACCTCTGGC **GGTGATAAT** GGTTCGA 3'
 3' TGGCACGCAC **AACTGA** TAAAATGGAGACCG **CCACTATTACCA** ACGT 5'

- Fragment used contains promoter DNA from -135 to +102 = 237 bp

(b)

(c)

(d)

Figure 1.

The T429A substitution is highly deleterious to open, but not closed complex formation. (a). DNA templates used in this work. The consensus -35, extended -10, and -10 promoter elements are shown in bold, as is the transcription start site. The promoter in the Randomized DNA has been inactivated by base substitutions. (b). The effects of T429A substitution in σ^{70} on the ability of holoenzymes to form heparin-resistant complexes with the DNA templates indicated at room temperature: EMSA gel. Only the regions of the gel containing the free and RNAP-bound DNA are shown; Ab = Abasic. (c). Quantification of extents of binding determined by EMSA gels such as that shown in (b). D = Duplex; Bot = Bottom strand. (d). RNAP was incubated for 10 min at room temperature with either Short Duplex DNA, or Randomized DNA. The mixtures were analyzed by EMSA both with and without prior heparin challenge. Only the regions of the gel containing the free and RNAP-bound DNA are shown.

(a)

(b)

Figure 2.

Comparison of the effects of various substitutions for residue 429 of σ^{70} on formation of holoenzyme and stable complexes at promoters. **(a).** Determination of the ability of the σ^{70} substituted at position 429 to interact with RNAP core enzyme. Bands due to holoenzyme (holo) and free σ^{70} are indicated. The lane labeled WT Holoenzyme contained RNAP that was purified as the holoenzyme as opposed to holo RNAP reconstituted from sigma factor and core RNAP. **(b).** Effects of T429 mutant RNAP on the interaction with Short Duplex and Fork DNA as determined by EMSA analysis of heparin-challenged binding reactions carried out at room temperature. For each template, the data were normalized to that obtained with the WT RNAP. **(c).** Representative EMSA gel for determining the effects of various substitutions for the T429 on formation of heparin-stable complexes between RNAP and the Long Duplex DNA at 18°C. RNAP and DNA were incubated for 5 min at 18 °C, followed by heparin challenge for 5 min. The EMSA gel was run at 18 °C as well. Only the regions of the gel containing the free and RNAP-bound DNA are shown. **(d).** Quantification of the gels such as that shown in (c), above. Values were normalized to that obtained with the WT RNAP.

(a) **T429A RNAP – Long Duplex, 18°C**

Figure 3.

The T429A substitution in σ^{70} slows the kinetics of stable complex formation between RNAP and Long Duplex promoter DNA at 18°C. **(a).** A representative EMSA gel showing the increased formation of heparin-stable complexes of T429A and Long Duplex DNA over time at 18°C. The samples were incubated individually and run on the gel for different amounts of time, causing the observed differences in migration of free DNA and complexes. **(b).** A plot of the fraction of stable complexes versus time for WT and T429A RNAP. The circles represent the data points, and the lines the single exponential fits to the points. The average parameters for the fits to duplicate sets of data are shown in Table 1.

(b)

Figure 4.

KMnO₄ probing of T429A RNAP and Long Duplex DNA complexes reveals slow melting of the promoter compared to WT RNAP at 18°C. **(a)**. Representative 8% KMnO₄ footprinting gel. The time is the length of incubation of radio-labeled DNA and RNAP before KMnO₄ is added. F.L. stands for full-length DNA at 65 bases. Bands representing oxidation of the -10, -7, -6, -1, and +2 T's are marked. The nontemplate strand of the Long Duplex DNA was 5' end-labeled with ³³P. **(b)**. Quantification of the data from the gels such as that shown in (a). Values are expressed as the % of melting of the -1 and +2 T bands divided by the band for the full-length DNA (see Materials and Methods), and then normalized to the WT RNAP value after a 60 minute incubation. The numbers after the RNAP on the x-axis represent time of incubation in minutes.

(b)

Figure 5.

The T429A substitution exerts its effects at a step beyond formation of the closed complex. **(a).** DNase I footprinting of RNAP complexes at the Extended Long Duplex promoter. Image of the radioactive bands of single stranded DNA fragments on a 6% denaturing gel. The 5' end of the nontemplate strand is radio-labeled. The area of the footprint representing the closed and open complexes are marked. Band positions (within one-to-two base accuracy) were identified by DNA sequencing (Sequenase Version 2.0 PCR Product Sequencing Kit, USB). The band marked with an asterisk was used for normalization in figure 5(b). The numbers after the RNAP represent time (in minutes) of incubation of RNAP and promoter DNA prior to addition of heparin, where indicated, and DNase I. **(b).** Scan of individual lanes of the gel shown above as Figure 5(a). The scans shown (all without heparin addition) represent lane 1 (No RNAP), lane 2 (WT RNAP, 0.5 minutes of incubation), and lane 4 (T429A RNAP, 0.5 minutes of incubation). The asterisk is the band used for the peak normalization of the No

RNA polymerase (RNAP) reaction. Band positions and the closed and open complex boundaries are assigned as represented in the gel for figure 5(a). **(c).** RNAP-induced strand opening at the Extended Long Duplex promoter. KMnO_4 modification of thymine residues that become single-stranded (band positions denoted on the right side of the gel) are compared in the presence of heparin without RNAP (Lane 1), with a 0.5 minute incubation with WT RNAP (lane 2) and T429A RNAP (lane 3), and a 10 minute incubation with T429A RNAP (lane 4). The % of promoter DNA melting (determined as described in the Materials and Methods), normalized to that for the WT RNAP reaction incubated for 0.5 minutes, is shown below the lane for each reaction with RNAP.

(b)

Figure 6.

DNase I footprinting on the λP_R promoter reveals that a significant fraction of T429A RNAP-DNA complexes for this promoter are closed complexes. **(a)**. A 6% denaturing gel of the bands due to cutting by DNase I in the presence of RNAP and the 237 bp λP_R promoter DNA. The 5' end of the template strand is labeled with ^{33}P . The short footprint representing closed complex formation is labeled as RP_C , and the long footprint is labeled as RP_O . Bands representing DNA of a certain length are marked, and the asterisk denotes the band used for normalization in figure 6(b). **(b)**. Scan of individual lanes of the gel represented above as figure 6(a). The scans shown (all without heparin addition and 10 minutes of incubation) correspond to lane 1 (No RNAP), lane 2 (WT RNAP), and lane 4 (T429A RNAP) of figure 6(a). The asterisk is the band used for the peak normalization of the No RNAP reaction. Band positions and the closed and open complex boundaries are assigned as represented in the gel for figure 6(a). As described in the Materials and Methods section, the traces for No RNAP and T429A

RNAP were adjusted in this scan in order to properly align the peaks due to slight uneven running of the lanes of the gel shown in figure 6(a).

Figure 7.

T429 is in the vicinity of the -10 region. A model of the -10/-11 binding pocket in the RNAP holoenzyme open complex (adapted from PDB 1L9Z²³) showing that in the open complex, T429 is positioned in the proximity of the -10 region of the nontemplate strand. A portion of the σ^{70} is rendered in cyan. The side chains of T429 (252), Y430 (253) and W433 (256) are shown (the *T. aquaticus* numbering is given in parentheses). The nontemplate DNA strand is shown in dark blue, with the -11 base in yellow and the -10 base in green.

Table I

The effects of alanine substitutions for various RNAP on the kinetics of stable complex formation with the Long Duplex promoter at 18 °C.

RNAP ^a	A ^b	k _{obs} (min ⁻¹) ^c
WT	0.90 ± 0.0	0.40 ± 0.01
T429A	0.80 ± 0.01	0.05 ± 0.00
Y430A	0.88 ± 0.01	0.23 ± 0.01
W433A	0.91 ± 0.01	0.39 ± 0.03

^a In all cases the concentration of the RNAP was 50 nM and that of the DNA, 10 nM.

^b A is the amplitude of the reaction in fraction of total DNA in the stable complex.

^c k_{obs} is the pseudo first order rate constant.