Nucleotide-Dependent Isomerization of Escherichia coli RNA Polymerase†

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Received April 9, 2004; Revised Manuscript Received July 13, 2004

ABSTRACT: During promoter engagement, RNA polymerase must change conformation or isomerize to its active form. These data show that high concentrations of nucleotides assist this isomerization. When binding to fork junction DNA probes is monitored, isomerization can occur without the need for the DNA that overlaps the transcription start site. When the start site is present, nucleoside triphosphates cause polymerase to change conformation in a way that drives cross-linking to the +1 position on the template strand. Preincubation of transcription complexes with 2 mM initiating nucleotide can drive formation of heparin-resistant complexes under conditions in which isomerization is limiting. It is proposed that complete polymerase isomerization can require nucleotide binding, which can assist formation of the active site that engages the transcription start site.

Before transcription can begin at promoters, RNA polymerase and DNA must undergo a series of conformational changes that have collectively been termed isomerization (*1*−*8*). Both the polymerase and the duplex DNA undergo changes during isomerization. In the process, an initial closed complex is converted to a functional open complex through a series of kinetically defined steps (*3*). The outcome is that polymerase assumes its active structure and is poised to initiate on the single strand DNA that it has created.

Some clues as to the nature of the enzyme changes come from comparing various RNA polymerase structures, and others come from biochemical and biophysical studies (*5*, *9*−*11*). In *Escherichia coli* transcription studies, isomerization is typically assessed by monitoring the resistance of transcription complexes to the inhibitor heparin; free polymerases and those that readily dissociate from closed complexes with DNA are inactivated by heparin (*12*−*14*). Functional open complexes are typically heparin-resistant. Using this method, it has been shown that the DNA sequence of the promoter (*14*) and the temperature strongly influence the isomerization pathway (*12*, *15*). Certain activators also stimulate transcription by facilitating isomerization (*16*−*19*). At ribosomal promoters, binding of the +1 (*20*) or +2 NTP can stimulate transcription (*21*). Since this occurs within a preinitiation complex, it is conceivably an isomerization effect (*20*).

Free RNA polymerase can bind NTPs, but the K_d for binding is higher than for the +1 and +2 NTPs in the polymerase−DNA open complex (*22*, *23*). This raises the possibility that the NTP binding sites are not fully configured and accessible prior to isomerization; in that case, NTP binding could conceivably drive polymerase into the isomerized state.

To test these ideas and to better define what constitutes isomerization, we have initiated studies of RNA polymerase in complexes with fork junction DNA under conditions where its isomerization is incomplete. Fork junction DNA (*24*, *25*) has been cocrystallized with RNA polymerase (*9*). The DNA in such complexes is duplex upstream from position −11 and contains a portion of the downstream nontemplate DNA strand that is essential for tight binding. These probes interact with polymerase in ways that mimic open complex formation (*4*, *9*, *14*, *24*, *26*). Kinetic studies, which detect appropriate intermediate complexes, support this view (*26*). Differences also exist as the probes lack DNA upstream from the −35 element and cannot be transcribed as they lack downstream DNA (*14*, *27*).

The data presented here show that high concentrations of NTPs promote isomerization of polymerase in such complexes, even in cases in which they do not contain a melted template strand containing the transcription start site. Cross-linking studies show that when the downstream template strand is present, even noncognate NTPs can drive polymerase closer to the +1 position on the DNA. In vitro transcription studies show that preincubation with moderate concentrations of cognate NTPs can stimulate formation of heparin-stable complexes.Overall, the data presented here indicate that NTP binding can drive polymerase into an isomerized state that is ready to initiate transcription.

EXPERIMENTAL PROCEDURES

Materials. E. coli holoenzyme and core were purchased from Epicentre. Fork probe deoxyoligonucleotides were purchased from Qiagen. Nucleoside triphosphates (Sigma) were in the Magnesium form.

Electrophoretic Mobility Shift Assay. Promoter probes and electrophoretic mobility shift assay were as described (*18*, *19*) with minor modifications. Heparin is included unless stated to be absent. Briefly, in a 10 uL reaction, 1 nM 5′ ^{32}P phosphorylated-template strand annealed to its complemen-

† This research was supported by National Institutes of Health Grant GM35754 (J.D.G.).

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[1] Abbreviations: NTP, nucleoside triphosphate.

10.1021/bi0492814 CCC: $27.50
Published on Web 09/11/2004

tary strand was added to 37.5 nM RNA polymerase final concentration in STA buffer (35 mM Tris-acetate at indicated pH, 8 mM Mg-acetate, 45 mM KCl, and 1 mM β-mercaptoetanol) (*18*, *28*) with 6 ng/uL dIdC. Where indicated, nucleotide was also added to the holoenzyme reaction. Control experiments with added 8−16 mM Mg-acetate showed no substantial effect. Reactions were incubated on ice for 20 min prior to the addition of 50μg/mL heparin for an additional 5 min. The reaction was then run on a 5% PAGE (Minigel system; Bio-Rad) in 1 X TBE buffer. The gel was dried and exposed for quantification using a phosphorimager. The electrophoretic mobility shift assay in Figure 6A used a *p*-azidophenacyl-derivatized promoter probe (*19*) and was conducted in STA buffer with 100 ug/mL acetylated BSA.

Cross-Linking. Cross-linking is conducted as described (*19*) with minor modifications. Template oligodeoxyribonucleotide (Qiagen) was 5′-^{32}P labeled and ammonium acetate precipitated. It was then derivatized with 5.7 mM *p*-azidophenacyl bromide (Sigma) in 57% methanol and 38.5 mM K-phosphate at pH 7. The derivatized DNA was then annealed to its complementary strand. The 5 nM derivatized fork probe is added to 37.5 nM holoenzyme in STA buffer with 100 ug/mL acetylated BSA and 6 ng/uL dIdC in the dark. Where indicated, nucleotide is also added to the reaction. These reactions are incubated on ice for 20 min, and 50μg/mL heparin is then added, when specified, for an additional 5 min on ice prior to UV irradiation on ice (*19*). This is then loaded onto a 6% SDS−PAGE with SDS buffer and run at 200 V at room temperature. Bands are analyzed by a phosphorimager with RNA polymerase subunits as markers.

Sigma 38 in Vitro Transcription. Transcription was conducted essentially as described (*29*) with minor modifications. Briefly, 200 nM *E. coli* sigma 38 (*30*) was combined with 50 nM core in buffer B (50 mM Tris-Cl pH 8, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 100 ug/mL acetylated bovine serum albumin) with 2 mM GTP or CTP (as indicated) and incubated at 37 °C for 10 min. Then 2 nM linearized promoter plasmid (*29*, *31*) was added and incubated for 15 min at 30 °C. A heparin nucleotide mix with 100 μg/mL heparin, 150 μM CTP, 150 μM GTP, 150 μM ATP, and 15 μM [α^{32}P]UTP at 100 nCi/μL was added to the reaction. In control reactions where nucleotide was not used for the preincubation, 2 mM additional GTP or CTP was added with the heparin nucleotide mix. After 6 min at 30 °C, the reaction was stopped by the addition of a urea−dye mix, run on a 6% PAGE with 1 X Tris-buffered EDTA at 21 W for 70 min. Radioactive bands were visualized and quantified by Phosphorimager.

RESULTS

Isomerization Is pH Dependent. The catalytic activities of enzymes change as the pH changes (*32*−*34*), but this has not been investigated systematically for RNA polymerase. In vitro transcription is typically conducted between pH 7.5 and 8.0, presumably because the signal is maximal there. By contrast, crystals of RNA polymerase were obtained at pH 5.8 (*10*). In an initial experiment, we evaluated the isomerization of RNA polymerase-promoter complexes over the pH range from 4.5 to 8. The assay involves taking duplicate samples of polymerase-fork junction DNA complexes and assaying for degree of binding via a standard band shift assay described previously (*14*, *24*). One sample is assayed directly and the other after a brief challenge with heparin. The fraction of complexes that are resistant to heparin is taken to be isomerized (*12*, *14*, *35*). Since the DNA is premelted in all complexes, the process of DNA opening is not driving the isomerization changes.

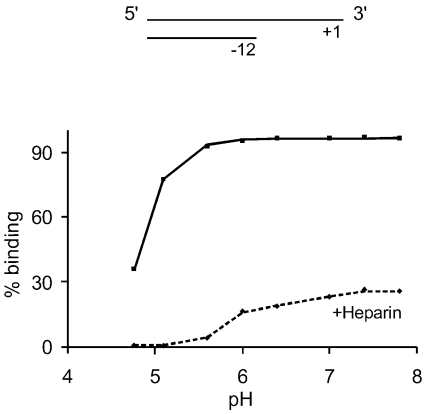

FIGURE 1: pH titration of bound and isomerized holoenzyme on fork junction probes. A schematic of the fork probe is shown at the top with T1/B12 indicating that the nontemplate (top) strand extends to +1 and the template strand extends to −12. The −12 and DNA upstream to −41 is double stranded. All probes are ^{32}P-labeled on the bottom strand. Polymerase binding (solid line) and isomerization (dashed line) on the rrnB P1 fork junction probe are shown (bottom).

The pH titration curves show that only a fraction of the complexes are isomerized, and this changes as a function of pH (Figure 1). At the high pH typically used in vitro, 90% of the complexes are bound, but only 25−30% of these are isomerized (heparin-resistant). The % isomerized is reduced to 10−15% near pH 5.8. As the pH is reduced to 5.5, there is no significant change in binding, but fewer of the complexes resist heparin. At pH 4.8, where a quarter of the DNA can be bound by polymerase, isomerized complexes are nearly undetectable. The data indicate that isomerization is pH dependent. The p*K* for this event is near 6.0. A p*K* near 6 has been observed for other proteins and is sometimes taken to indicate involvement of a histidine (*33*). The source of the low fraction of isomerized complexes even at optimal pH is unknown.

Nucleotides Drive Isomerization. As discussed previously, it is possible that polymerase is not fully isomerized due to the lack of NTPs. The probes used do not contain the template strand near position +1 so NTP binding cannot be templated. The K_d for nontemplated NTPs is expected to be moderately high (*22*) as is that for NTP binding in the absence of magnesium (*36*). Under these conditions, even noncomplementary nucleotides might be effective. We titrated in high concentrations of UTP prior to the addition of fork junction promoter probes and measured both total binding of polymerase to the DNA and fraction of bound polymerase that isomerized and resisted heparin. This was done at a high pH (7.4), where 25−30% of the polymerases are isomerized.

Figure 2 shows that addition of UTP leads to the conversion of preexisting complexes to the isomerized state.

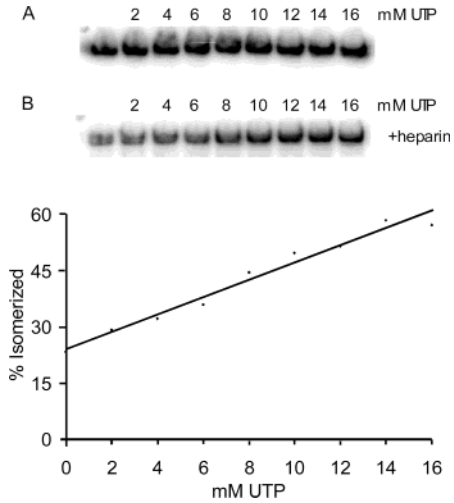

FIGURE 2: UTP stimulates isomerized holoenzyme binding on rrnB P1 T1/B12 fork probes. (A) Binding (without heparin challenge). (B) Heparin resistant binding (isomerization). (bottom) Plot of % isomerized as a function of UTP concentration. Binding was conducted at pH 7.4.

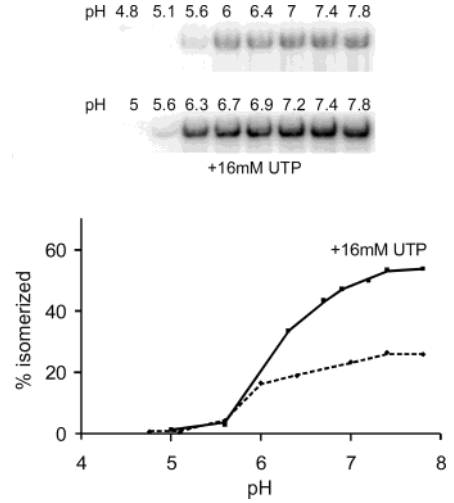

FIGURE 3: pH titration of isomerized holoenzyme binding in the presence of UTP. Heparin resistant holoenzyme binding of rrnB P1 T1/B12 in the absence (top) and presence (middle) of 16 mM UTP at various pHs. Data are analyzed and plotted at the bottom.

Figure 2A shows that the addition of UTP has little effect on the number of complexes that can be formed in the absence of heparin. But in a heparin challenge protocol, more complexes are formed when UTP is added (Figure 2B). At the highest UTP concentration tested, 60% of the complexes are now isomerized (Figure 2B, bottom). This fraction can increase further as the UTP concentration is raised (not shown). The data show that UTP can drive isomerization of RNA polymerase−DNA complexes. Because the DNA is premelted, UTP must be influencing polymerase conformation independent of any DNA melting events.

The pH titration was repeated at the highest UTP concentration. The data show that at every pH tested above the p*K* of 6, UTP increased the fraction of polymerase that is isomerized (Figure 3). We conclude that UTP can bind polymerase and help it to isomerize.

Nucleotide Effect Is Generic. The concentration of UTP needed to isomerize these complexes is nonphysiologically high, as might be expected on a fork junction probe that cannot form a natural open promoter complex. Because the probe does not contain the template sequence near +1, the effect should be generic rather than specific for any particular nucleotide type. Figure 4 shows that ATP also can help in isomerization. Addition of salts alone to mimic those present in nucleotide stocks did not lead to this enhancement nor did addition of 16 mM Mg-acetate (data not shown). The NTPs added were complexed with Mg^{2+}, and so no cation depletion occurred. GTP was the only triphosphate to show weak stimulation, likely because GTP at high concentrations precipitates under conditions of the reaction (see ref *37*).

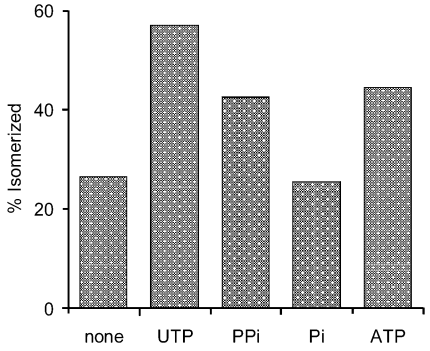

FIGURE 4: Generic stimulation of isomerization. Heparin-resistant binding of rrnB P1 T1/B12 was assayed in the presence of 16 mM indicated compounds at pH 7.4.

The NTP binding sites on RNA polymerase are not fully characterized but would be expected to accommodate the pyrophosphate moiety as occurs for other polymerases (*38*). The data shows that inorganic pyrophosphate can also stimulate formation of heparin-resistant complexes (Figure 4). This effect appears to be specific as inorganic phosphate is ineffective. Nucleoside diphosphate forms of various nucleotides are more effective than monophosphates (data not shown). Overall, the stimulatory effect appears to be related to generic sites within the polymerase detected previously for the binding of nucleotides (*39*, *40*).

Nucleotide Stimulatory Effect Does Not Depend on Unpaired DNA Sequences Near +1. The probes used previoulsy contain an unpaired nontemplate strand, which is a main determinant of binding strength, due to interactions with sigma factor (*9*, *14*, *19*, *24*, *25*). In the open complex, the unpaired template strand from −6 to +1 is believed to bind core polymerase as shown in cross-linking studies (*15*, *41*, *42*). This interaction makes modest contribution to total binding affinity. However, genetic studies suggest that the core may be important for regulation by nucleotide (*43*). Therefore, the nucleotide stimulatory effect was assayed on fork junction probes where the template strand only is unpaired (Figure 5 and schematic of probe in Figure 6, top).

The results (Figure 5) show that nucleotide stimulation is still present on this T11/B1 probe. This DNA is duplex from −11 to −41 and contains the template single strand to position +1. This probe does not support a preference for binding the complementary NTP (not shown), which may require a full promoter. Consistent with this, when the template strand sequences downstream from −7 are removed, the stimulation by 16 mM UTP is still present (Figure 5, T11/B7). This same region from −6 to +1 is also not needed on the top strand for stimulation (data not shown). Overall, stimulation by generic nucleotide does not appear to require interactions with either of the strands near +1.

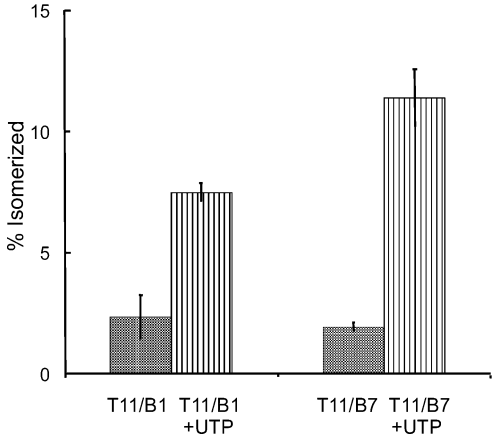

FIGURE 5: Nucleotide effects on fork probes with a bottom single strand. Probes contained the template single strand from −10 to +1 (T11/B1) or to −7 (T11/B7). The effect of UTP on isomerization is shown.

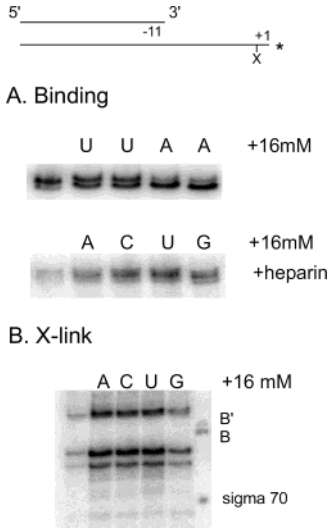

FIGURE 6: Nucleotide directs engagement of the template strand. The radioactive rrnB P1 T11/B1 fork DNA with a *p*-azidophenylacyl cross-linker on the 5′ phosphate of the template +1 nucleotide was used to cross-link to holoenzyme. (A) Binding using this derivatized probe in the presence of 16 mM indicated nucleoside triphosphate (top) and 16 mM indicated nucleoside triphosphate with heparin (bottom; overexposed to reveal detail). **(**B) The samples in panel A (bottom) were cross-linked and run on an SDS gel. Positions of markers are indicated. Binding and cross-linking was conducted at pH 8.

Cross-Linking Shows that Nucleoside Triphosphate Stimulation of Isomerization Leads to Increased Engagement of the Template Strand. In a full open complex, isomerization drives the preinitiation complex to a state where it can initiate RNA synthesis. This requires that polymerase interact with the template strand start site region near +1 (*44*−*46*). Fork junction probes lack DNA upstream from −41 but still bind polymerase in a way that closely mimics open complex formation (*19*, *25*, *26*). Nonetheless, because the polymerase does not need to actually accomplish the DNA melting reaction, it might not easily achieve the final isomerized state. We wished to learn if the stimulation of isomerization on fork junction probes leads polymerase to assume a property centrally involved in functional initiation, the ability to engage the template strand near +1.

The fork junction probe that contains the +1 region on the template strand in single stranded form (as in Figure 6) was modified for this purpose. A UV activatible *p*-azidophenacyl cross-linker was placed on the phosphate upstream of +1 (*19*, *41*) (shown as an × in Figure 6 top). This same DNA strand was radioactively labeled. The goal is to learn if nucleotide-driven isomerization causes the polymerase to cross-link more readily to the +1 transcription start site on the probe at pH 8. A control EMSA experiment shows that the modified probe still binds polymerase (Figure 6A, top). Nucleotide has little effect on the extent of binding, as expected in the absence of heparin. Also as expected, nucleotide addition increases the extent of binding in the presence of heparin due to enzyme isomerization (Figure 6A, bottom).

Each of the samples from this heparin challenge protocol was irradiated with UV light to allow the functional group at the +1 position to cross-link to RNA polymerase. These five samples, a control without any nucleotide and 4 with different NTPs, were loaded on an SDS−PAGE gel. The free DNA runs off the gel under these conditions, but radioactive template strands that are cross-linked to protein can be detected by autoradiography (*19*). The autoradiograph shows that each of the nucleotides enhances cross-linking of polymerase to the probe at +1 on the DNA template strand (GTP stimulation is low, as stated previously, due to precipitatation (*37*)). The bands are located near markers for uncrosslinked β and β' subunits. A cross-linker placed on the same position was previously shown to cross-link to β and β' subunits in the absence of nucleotide (*41*). The multiple bands likely represent cross-links to multiple locations on these subunits.

The key point is that nucleotide has caused the polymerase to engage the template strand +1 position more closely, as demonstrated by the higher level of cross-linking. This is part of the activation of enzyme isomerization and not related to DNA melting because the DNA is premelted. We infer that NTPs favor conformational changes in the polymerase that direct it to engage the point on the template at which transcription will begin.

Moderate Concentration of the +1 NTP Stimulates Open Complex Formation in an Isomerization-Limited System. These data imply that NTPs drive isomerization and thus systems that isomerize poorly may be stimulated by the presence of NTPs. This was tested using transcription of sigma 38-dependent promoters, where sigma mutants that are defective in isomerization have been identified (*30*, *31*). The system in general was set up to limit isomerization by linearizing the template to remove DNA supercoiling and transcribing at 30 °C. In this assay, sigma 38 polymerase-promoter complexes were preincubated with 2 mM +1 initiating nucleotide prior to the addition of a heparin. A nucleotide mix containing all four NTPs was then added to allow one round of transcription from the open complexes formed. A comparison reaction (without preincubation) received 4 NTPs so that the final concentrations of all nucleotides were identical in both reactions during elongation.

Figure 7 shows that preincubation with initiating NTPs prior to heparin challenge increases transcription (see lanes marked +). The effect is greatest when the isomerization-defective mutant of sigma 38 is used. The NTP used in the

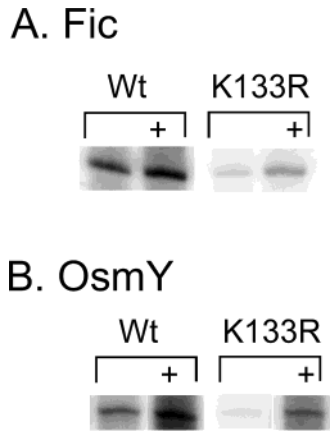

FIGURE 7: Initiating NTP can stimulate transcription in an isomerization-limited context. Heparin-resistant complexes were formed either in the presence of the initiating NTP (+signs) or with the 2 mM MgCl2 and 8 mM NaCl (each left lane) contained in the NTP solution. The K133R isomeriation mutant of sigma 38 was used as indicated. (A) Use of 2 mM CTP (*55*, *56*) at the fic consensus promoter led to a 2-fold stimulation in transcription for wild-type and 4-fold stimulation for K133R. (B) Use of 2 mM GTP (*57*) at the osmY promoter led to a 3-fold stimulation in transcription for wild-type and a greater than 5-fold stimulation for K133R.

preincubation was only 2 mM, indicating that the NTP effect can be observed at much lower concentrations in the context of a true transcription complex.

DISCUSSION

These experiments have shown that nucleotides can stimulate the isomerization of RNA polymerase, which is reflected in an increase in the fraction of heparin-resistant complexes. In the absence of nucleotides, the polymerase bound to fork junction DNA is not fully isomerized. Nucleotides bind RNA polymerase in these complexes and alter its conformation to isomerize it. The isomerized polymerase can readily engage the template strand at the transcription start site. These enzyme conformational changes do not require the presence of the template strand but also occur when the template strand is present. They can be driven by nucleotides that bind free RNA polymerase, although the K_m for these effects is higher (K_m not determined) than expected for free RNA polymerase (*22*). When in vitro transcription is conducted under conditions that limit isomerization, only a moderate concentration of the +1 NTP is required to stimulate functional complex formation. The implications of these observations for how RNA polymerase isomerizes to its functional state are discussed next.

How Do NTPs Drive RNA Polymerase Isomerization? Nucleotides have long been known to bind free RNA polymerase with a moderately high K_d and to stabilize it against heat inactivation (*22*, *47*). However, the number, specificity, and tightness of binding of nucleotides to free polymerase are unsettled issues. Similar K_d values were observed with or without magnesium (*36*), and from two to nine sites were reported in these early studies (*36*, *48*). The current study with fork junction probes shows that a high binding constant is associated with isomerization on fork junction probes. The value of the binding constant for nucleotide is uncertain, as it was not assayed directly and the value could be altered by the presence of DNA probes. When cognate NTPs are used in isomerization-limited transcription experiments, lower concentrations are effective (Figure 7). In aggregate, these considerations show that free polymerase, polymerase bound to fork junction probes, and polymerase in transcription complexes can bind nucleotide and that the binding can change the properties of the enzyme.

The K_m for the initiating nucleotide in the context of the full transcription complex where the polymerase is normally fully isomerized and the template strand is engaged is thought to be 80 μM (*23*), which is lower than the K_m for free polymerase and fork junction complexes. We suspect that much of this lowering is due to enzyme conformational changes because NTPs are bound largely through interactions of site-bound metals with the phosphates (*38*), implying that the contribution from base-template interactions may be minor. That is, free polymerase may include conformers in which the NTP binding sites are not optimally formed. Overall, isomerization is the series of changes associated with the conversion of the inactive free enzyme to the form that can easily bind and condense the +1 and +2 NTPs. In the case of DNA polymerases, which bind NTPs similarly, nucleotides are known to induce isomerization by bringing the enzyme fingers closer to the DNA (*38*).

The molecular nature of the conformational changes that accompany isomerization are not known. The various structures of RNA polymerase solved in recent years (*5*, *9*, *10*) certainly include isomerization intermediates, but it is premature to identify the precise changes induced by NTPs, although a model was presented recently for the case of the ribosomal promoters (*21*). Definitive identification of the fully isomerized form may require cocrystallization with initiating NTPs.

Isomerization during Transcription Initiation. Promoter engagement by bacterial RNA polymerase has long been separated into DNA binding and complex isomerization phases (*1*−*5*, *49*). The latter phase includes the opening of the DNA to allow the template strand to be read. More recently, use of premelted fork junction probes has allowed polymerase isomerization to be studied independent of the need to melt the DNA (*14*, *24*). Such studies have demonstrated that promoter elements can function by driving enzyme isomerization independent of driving DNA melting (*14*, *19*). Transcription rates can be limited at the isomerization step (*14*, *50*, *51*).

The current data suggest that promoters that isomerize only with difficulty might be preferentially subject to stimulation (*52*, *53*) by NTPs. In a preliminary test, sigma 38 functional complex formation under isomerization-limited conditions was promoted by the initiating NTP (Figure 7). An extreme case may be the ribosomal promoters that isomerize very poorly (*20*) and where regulation by initiating NTP concentration has been demonstrated (*20*). Recently, we have found that transcription from these promoters is stimulated by the +2 (*21*) as well as the +1 NTP (*20*), which suggests that filling the 2 NTP binding sites likely assist rate-limiting isomerization steps at these promoters. The natural process of isomerization might be best studied in the presence of NTPs for isomerization-limited promoters (as in Figure 7), although early studies showed that the very strong early T7 promoters are not strongly responsive to nucleotide (*54*). The specific stimulatory effect of −10 region promoter DNA sequence on the isomerization step (*14*) may be to induce the conformation of polymerase that readily binds and

condenses the cognate nucleotides. The negative effect of sequences associated with ribosomal promoters could be to induce a form of polymerase that resists isomerization and thus build-in a requirement for high concentrations of nucleotides (*21*).

ACKNOWLEDGMENT

We thank Dr. Konstantin Severinov for critically reading an early draft of this manuscript. We also thank Dr. Shun J. Lee for purified sigma 38 proteins and plasmids.

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BI0492814