Strand Opening-deficient *Escherichia coli* RNA Polymerase Facilitates Investigation of Closed Complexes with Promoter DNA

**EFFECTS OF DNA SEQUENCE AND TEMPERATURE**

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Formation of the strand-separated, open complex between RNA polymerase and a promoter involves several intermediates, the first being the closed complex in which the DNA is fully base-paired. This normally short lived complex has been difficult to study. We have used a mutant *Escherichia coli* RNA polymerase, deficient in promoter DNA melting, and variants of the polymerase to model the closed complex intermediate at physiologically relevant temperatures. Our results indicate that in the closed complex, RNA polymerase recognizes base pairs as double-stranded DNA even in the region that becomes single-stranded in the open complex. Additionally, a particular base pair in the -35 region engages in an important interaction with the RNA polymerase, and a DNase I-hypersensitive site, pronounced in the promoter DNA of the open complex, was not present. The effect of temperature on closed complex formation was found to be small over the temperature range from 15 to 37 °C. This suggests that low temperature complexes of wild type RNA polymerase and promoter DNA may adequately model the closed complex.

Bacterial RNA polymerase (RNAP) that is able to engage in formation of a functionally competent complex at a promoter consists of the multisubunit core enzyme and the σ^70_0 initiation factor, which imparts on the enzyme the ability to both recognize specific DNA sequence and to melt promoter DNA (1, 2). Formation of a functional complex at a promoter is a multistep process (3) involving several intermediates. Subsequent to formation of the first “closed” RNAP-promoter complex, several rearrangements take place, involving changes in the conformations of both the RNAP and the promoter DNA (3–7), eventually resulting in formation of the transcriptionally competent open complex (RPc) in which strand separation has occurred over about 14 bp of promoter DNA (6–8); see Scheme 1,

\[
\text{R} + \text{P} \leftrightarrow \text{RP}_c \leftrightarrow \text{I}_1 \leftrightarrow \text{I}_2 \leftrightarrow \text{RP}_o
\]

Scheme 1

where RPc is the closed complex; RPc and I1 are both unstable intermediates, I2 is stable, and RPo is the stable final, transcriptionally competent, complex. The latter two complexes have a long half-life, rendering them resistant to heparin, a competitor with promoter DNA for RNAP binding. The rate-limiting step is the I1 to I2 conversion (6, 7). To fully understand the reaction pathway, it is important to know in detail the properties of the intermediate complexes.

Record and co-workers (6, 7) have altered solution conditions and reaction times to favor either I1 or I2. Thus they were able to determine that for the unstable I1 complex, RNAP covers the DNA in the downstream direction up to about +19 (9). In I2, the RNAP covers the DNA over a similarly long stretch as in I1. It has been proposed, although not yet experimentally verified, that in I2 the nucleation of the strand separation process has occurred (6, 7). The closed complex is very short lived at most promoters. Attempts to arrest the reaction pathway (see Scheme 1) at RPc have involved resorting to low temperature incubation (10, 11), working with promoters for which the RPc to I1 step is slow (12, 13) or undetectable (13), and covalently linking the two strands of the DNA so that strand separation is physically blocked (14). These studies found that the region of DNase I protection does not extend beyond the start site of transcription for the closed complex. In subsequent complexes the region of protection reaches to about +19 (10, 12).

Most of the above studies were dependent on particular temperatures or promoter sequences, precluding determinations of effects of promoter sequence at physiological temperature, or of a range of temperatures, on closed complex formation. The studies reported here have employed an RNA polymerase where four amino acid residues (Phe-427, Tyr-430, Trp-433, and Trp-434) in the DNA melting region of the initiation factor σ^70_0 have been substituted by alanine. The resultant “FYWW” variant has been unable to form open complexes with any promoter DNA tested under any condition investigated so far,
including at 25 or 37 °C (15). To determine the equilibrium dissociation constant, $K_d$, of the interaction, we have used DNase I footprinting to monitor the extent of complex formation between FYWW RNAP and promoter DNA as a function of increasing concentration of the RNAP. Our results demonstrate that the closed complex is differentially sensitive to particular substitutions in the promoter sequence and that it is not very temperature-dependent. Enhancement of DNase I cutting by WT but not FYWW RNAP is observed in the spacer DNA of a promoter with an 18 bp spacer DNA separating the −10 and −35 regions.

**EXPERIMENTAL PROCEDURES**

**Promoter DNA and RNAP—**The QuikChange kit (Stratagene) was used to carry out site-directed mutagenesis to introduce substitutions into the $P_R$ promoter DNA, contained in the vector pKK232-8. The FYWW σ$^{70}$ was described previously (15). Overexpression was driven by an inducible T7 promoter on a pET11a derivative in BL21(DE3) cells. FYWW σ$^{70}$ purification followed a published procedure (16) with some modifications, the most prominent being induction of expression at a growth temperature of 20 °C, the use of a higher concentration of imidazole than the recommended 50 mM for elution of the nickel-nitrioltriacetic acid column (although 1 mM was used, it is likely a lower concentration would have sufficed), and omission of the ion-exchange chromatography. The purified FYWW σ$^{70}$ (greater than 90% pure) was dialyzed against storage buffer (50 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.1 mM dithiothreitol, 350 mM NaCl, and 10% glycerol). Following concentration by centrifugation through an Amicon ultrafiltration device, the glycerol concentration was adjusted to 50%. The ratio of σ$^{70}$ to core (EPICENTRE Biotechnologies) needed for optimal binding to DNA was measured by titrating varying amounts of FYWW σ$^{70}$ with core. The optimal ratio of FYWW σ$^{70}$ to core RNAP was determined to be 10:1.

**Quantitative DNase I Footprinting—**End labeling of promoter DNA was accomplished by PCR amplification with a template-sequence primer that had been 5'-end-labeled with $^{32}$P and unlabeled nontemplate primer. Binding reactions were routinely carried out at 37 °C in transcription buffer (30 mM Tris-HCl, pH 7.3, 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 10 mM dithiothreitol). For determination of the $K_d$ value at other temperatures, the pH was separately adjusted to 7.3 for each temperature. Labeled promoter DNA fragment (2–4 nM) and RNAP at the desired concentration were incubated for 10 min, and DNase I was then added to an amount determined to be optimal at each temperature investigated (15 °C, 0.04 unit/μl; 25 °C, 0.01 unit/μl; 37 °C, 0.005 unit/μl; and 45 °C, 0.0015 unit/μl). The DNA cleavage reaction was stopped after 30 s by addition of 2 volumes of NH$_4$Ac, followed by addition of glycogen to 150 μg/ml and ethanol precipitation. The pellets were washed with 70% ethanol, taken up in loading solution, boiled for 3 min, and loaded onto 5% polyacrylamide, 7 M urea denaturing gels containing 25% formamide. The gels were run for 2.5–3 h at 1000 V, fixed with 5% acetic acid, 20% methanol, and dried. After a 2–5 day exposure of a PhosphorImaging screen to the dried gel, the image was scanned on a Storm 820 (Amersham Biosciences). Assignments of bands resulting from cuts at −60, −48, −38, +1, and +19 were as in (Ref. 9) and represent a correction to our previous assignments (17). The positions of cuts leading to other bands in the figures were deduced by interpolation of plots of log fragment length versus distance migrated.

Band intensities were obtained by analysis with ImageQuant 5.2. The intensities of bands protected by the RNAP and used to monitor RNAP binding (the group of bands designated as Q in Fig. 2A) were normalized to reference bands outside the footprint (the group of bands designated as N in Fig. 2A), and the degree of saturation ($Y_{app}$) was calculated from Equation 1 (18),

$$Y_{app} = 1 - \left[ \frac{\text{bands inside footprint (complex)}}{\text{bands outside footprint (complex)}} \right] \frac{\text{bands inside footprint (free DNA)}}{\text{bands outside footprint (free DNA)}}$$

$Y_{app}$ was plotted against [RNAP], and the curve was fit in SigmaPlot2001 to the hyperbolic equation $Y_{app} = y_0 + [\text{RNAP}] / (K_d + [\text{RNAP}])$, where $y_0$ equals the $Y_{app}$ in the absence of added RNAP, and to obtain $K_d$, the equilibrium dissociation constant for closed complex formation $K_d = [\text{P}] / [\text{RP}_c]$. Graphic representation of the intensities of the bands in DNase I footprinting gels mapped onto the promoter sequence were obtained with ImageQuant 5.2. Background corrections were carried out by subtraction of the position-dependent intensity in the lane containing undigested DNA. The traces for different lanes were normalized using the first (i.e. most upstream) of the four bands that were used for normalization in the quantitative DNase I footprinting, as indicated above.

**KmnO$_4$ Probing—**Complexes of RNAP and end-labeled promoter DNA were formed as indicated above, except that the buffer contained 1 mM MgCl$_2$ instead of 10 mM. The KmnO$_4$ probing protocol was exactly as described (19). Separation of the fragments generated from the unmodified DNA was accomplished by running the samples on a 10% sequencing gel. Analysis of the bands was by the use of ImageQuant 5.2 software.

**RESULTS**

The Variant of the $P_R$ Promoter Used in This Work—The reference promoter for our studies is $P_R - 12T$, shown in Fig. 1A. It is a derivative of the $P_R$ promoter of bacteriophage λ, with the G-12T substitution in the −10 region, which gives the −10 region the consensus sequence TATAAT. The $P_R - 12T$ promoter has just one nonconsensus base (the start site-proximal T on the nontemplate strand of the −35) among both the −10 and −35 regions. The G-12T substitution was introduced in $P_R - 35$ regions. The G-12T substitution in the PR promoter of bacteriophage λ, with the G-12T substitution in the −10 region, which gives the −10 region the consensus sequence TATAAT. The FYWW σ$^{70}$ variant (abbreviated as FYWW RNAP), so as to counteract a binding defect resulting from the quadruple substitution in σ$^{70}$ (15). A 2-fold decrease in the $K_d$ (see Table 1) demonstrates that the G-12T substitution indeed improves closed complex formation. To be able to carry out

3 L. Tsujikawa and P. L. deHaseth, unpublished data.
FYWW RNAP Is Greatly Defective in Formation of an Open Complex with Even a Consensus Promoter—In prior experiments with the WT PR promoter, we had shown that the FYW RNAP (alanine substitutions in α70 at 427, 430, and 433 but not at 434) formed readily detectable closed complexes with the WT PR promoter, including the signature short footprint extending to just +1 in downstream direction, although eventually some strand separation did occur. The open complex, formed with WT RNAP, was seen to extend to about +19 for both PR− and PR+ (9). We carried out an experiment to evaluate whether the FYWW RNAP would be suitable for studies on closed complex formation. For this we used one of our strongest promoters, PR− − 12T − 30A. The DNase I footprints in Fig. 1B, lanes 12 and 13, is essentially upstream of the start site. This is commensurate with weaker binding and formation of a closed complex. In the presence of heparin, the protection afforded by FYWW RNAP is essentially abolished (Fig. 1B, compare lanes 13 (FYWW RNAP), lane 12 (FYWW RNAP with heparin challenge), and lane 2 (free DNA)).

To directly compare the extents of melting by FYW and FYWW RNAP, we probed complexes of RNAP and the consensus PR− − 12T − 30A promoter with KMnO4 both in the presence and absence of heparin. The results are shown in Fig. 1B, lanes 1–6. It is seen that after a 10-min incubation at 37 °C of 200 nM FYW RNAP with PR− − 12T − 30A, the extent of strand separation is essentially the same as that for 60 nM WT RNAP, which saturates the promoter (Fig. 1B, compare lanes 1 and 2 with 3 and 4). However, much fainter bands (about 2% of those obtained with WT RNAP) are seen for the FYWW RNAP (Fig. 1B, lanes 5 and 6). A similar result was obtained for FYWW RNAP at the PR− − 12T promoter (data not shown). As at 200 nM FYWW RNAP only about 75% of the promoter DNA is occupied, full occupancy would have resulted in about 3% promoter melting by FYWW RNAP. This small extent of open complex formation should not significantly affect the quantitative interpretation of our binding experiments. A similar result concerning the relative abilities of FYW and FYWW RNAP to form open complexes was obtained with the Lac UV5 promoter (15).

Based on the outcome of the above studies, the FYWW RNAP was chosen for our subsequent experiments.

Characterization of the Interaction of FYWW RNAP with Promoter DNA—In Fig. 2A, a footprinting gel for titration of PR− − 12T DNA with the FYWW RNAP is shown. From a quantification (see “Experimental Procedures”) of the [FYWW RNAP] dependence of the protection of the DNA between −38 and +1, the data shown in Fig. 2B were obtained. Using a hyperbolic fit, a value for the equilibrium dissociation constant, Kd, of 85 nM was obtained. Three independent titrations yielded an average value for Kd of 87 ± 7 nM. The WT and PR+ promoter has been extensively studied (6, 9, 20, 21), but because of its transient nature, little information is available concerning the closed complex. The Kd for the I1 complex (90–150 nM (6, 20)) is quantitatively DNase I footprinting studies (18), all promoters shown in Table 1 were 5′-end-labeled on the template strand by using a 5′-labeled downstream primer in the PCR amplification of the promoters.

TABLE 1

| DNA template | Sequence* | Kd (nM) |
|--------------|-----------|---------|
| Consensus sequences | TGTGACA | TATAAT |
| λ PR− − 12T | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 87 ± 7 |
| λ PR− WT | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 190 ± 16 |
| λ PR− − 12T − 5C | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 80 ± 14 |
| λ PR− − 12T − 7C | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 253 ± 40 |
| λ PR− − 12T − 11C | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 149 ± 15 |
| λ PR− − 12T − 15T | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 79 ± 6 |
| λ PR− − 12T SM | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 49 ± 9 |
| λ PR− − 12T S16 | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 102 ± 16 |
| λ PR− − 12T S18 | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 154 ± 26 |
| λ PR− − 12T − 30A | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | 63 ± 7 |
| λ PR− − 12T − 35C | TTATTTTATATTCTCCTGGCCTTCCTGATAAAGG | >265* |

* = 35 (left) and −10 (right) regions are indicated in boldface. Changes with respect to the PR− − 12T promoter are underlined.

* Kd values were determined as described in the legend to Fig. 2 and under “Experimental Procedures.”

* Estimated lower limit is based on the following experimental values: 235, 472, and 609 nM.

FIGURE 1. Characterization of complexes formed between FYW or FYWW RNAP and the PR− − 12T−30A promoter at 37 °C. A, sequence of the promoter. The promoter has a −12T, whereas WT PR has a G at this position. For all experiments the radiolabel is α32P-phosphate (black dot) at the 5′ end of the template strand. B, comparison of complexes of the PR− − 12T−30A promoter with the FYW and FYWW RNAP by KMnO4 probing (lanes 1–6) and DNase I footprinting (lanes 7–13). Lanes 1 and 2, WT RNAP; lanes 3 and 4, FYW RNAP; lanes 5 and 6, FYWW RNAP. Samples loaded in lanes 1, 3, and 5 were subjected to a heparin challenge (100 μg/ml for 10 min) prior to loading. Lanes 7, DNA only; lanes 8 and 9, WT RNAP; lanes 10 and 11, FYW RNAP; lanes 12 and 13, FYWW RNAP. Samples loaded in lanes 8, 10, and 12 were subjected to a heparin challenge (100 μg/ml for 10 min) prior to loading onto the gel. The extents of the “long” and “short” footprints (reflecting the RP− and RP+ complexes, respectively) are shown next to the gel. Assignments of bands resulting from cuts at −48, −38, +1, and +19 were as in Ref. 9.
within a factor of 2 of our 190 nM obtained with the PR promoter (see Table 1) in a similar buffer, but this comparison may not be warranted because in forming I₁, likely conformational changes in the RNAP take place at the expense of the binding free energy (6, 7). The $K_d$ value obtained here for PR is similar to that determined for the interaction of WT RNAP with the much weaker
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Effects of Alterations in the Spacer DNA Separating the −10 and −35 Regions and Further Upstream—Two of these substitutions (T-35C and T-30A) are in the −35 region, known to be involved in important contacts between σ70 and promoter DNA. Two alterations affect the length of the spacer DNA separating the −10 and −35 regions, either increasing (P_R − 12T S18) or decreasing it (P_R − 12T S16) with respect to the optimal separation of 17 bp in the WT P_R promoter. The −12T SM is a block substitution in the spacer DNA consisting of 7 bp of A + T-rich DNA, previously found to greatly increase in vitro transcription from the lacUV5 promoter (31). This block substitution in the spacer DNA was found to reduce the K_d by a factor of 1.8 (see Table 1), indicating some effect on closed complex formation. Two changes (P_R − 12T − 30A and P_R − 12T S16) were considered to have no significant effect, and two (P_R − 12T − 35C and P_R − 12T S18) led to an increase in the K_d. Thus the deletion of a base pair in the spacer DNA (P_R − 12T S16) did not have a significant effect on closed complex formation, but the insertion of a base pair did, increasing the K_d by a factor of 1.8 for P_R − 12T S18.

Two hypersensitive sites are seen with promoters that have 17-bp spacer DNAs (near the top of Figs. 1B and 2A) and 16 bp spacer DNAs (data not shown) as follows: at −38 and −48, meaning that cleavage of the phosphodiester bonds between −38 and −37 and between −48 and −47 is enhanced in the complex of FYWW RNAP and promoter DNA, compared with the free DNA. An interesting feature of the footprint for the complex between WT RNAP and the P_R − 12T S18 promoter is that it includes another hypersensitive site at −27, reflecting enhanced cleavage between −27 and −26 in the complex as compared with the free DNA (see Fig. 3, A and B). Hypersensitivity at about −27 is commonly observed with promoters that have a spacer DNA greater than 17 bp, such as lacUV5 (for a review see Ref. 32). Remarkably, no hypersensitivity is observed at −27 (or at −48) for complexes formed with P_R − 12T S18 and FYWW RNAP, as most clearly shown in Fig. 3B. Quantitative data pertinent to hypersensitive sites in open and closed complexes for promoters P_R − 12T S18, P_R − 12T, and P_R − 12T S16 are shown in Table 2. Here values of 1 indicate cleavage to the same extent in the free DNA as in RNAP-bound DNA, greater than 1 indicate enhancement, and smaller than 1 indicate protection. Cleavage at −38 was enhanced in complexes formed both with WT RNAP and with FYWW RNAP (~4- and ~3-fold, respectively). The slight difference between the two values may just reflect the fact that nearly 100% of the promoter DNA has bound WT RNAP (as deduced from the absence of many of the bands visible in the footprint of the free DNA), but for FYWW at 200 nM the occupancy is only about 60% based on the K_d of 154 nM for this promoter (see Table 1). The intensity of cleavage at −48 (see Fig. 3) was independent of spacer length, and modestly enhanced by WT but not by FYWW RNAP. Cleavage at −27 is only hypersensitive for P_R − 12T S18 with WT RNAP, and the enhancement is much greater than that for the cleavage reaction at −48. Interestingly, FYWW RNAP did not have an effect on cleavage at −27.
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![Graph A](image1)

![Graph B](image2)

**TABLE 2**

Quantification of band intensities at positions \(-27, -38, \text{ and } -48\) as a function of spacer length

| DNA        | RNAP  | Fold enhancement<sup>a</sup> |
|------------|-------|------------------------------|
|            |       | \(-48^{c}\) | \(-38^{c}\) | \(-27^{c}\) |
| \(\lambda P_r - 12T\) | WT    | 1.3 | 4.3 | <1<sup>d</sup> |
|            | FYWW  | 0.8 | 2.8 | <1<sup>d</sup> |
| \(\lambda P_r - 12T S16\) | WT    | 2.2 | 4.1 | <1<sup>d</sup> |
|            | FYWW  | 1.0 | 2.6 | <1<sup>d</sup> |
| \(\lambda P_r - 12T S18\) | WT    | 1.8 | 3.8 | 3.5 |
|            | FYWW  | 1.1 | 2.8 | 1.3 |

<sup>a</sup> Fold enhancement was compared with the same band in free DNA.
<sup>b</sup> Band locations are as indicated in Figs. 2 and 3; for simplicity, no numbering change was used to reflect the different spacer lengths.
<sup>c</sup> Fold enhancement was determined at concentrations of WT RNAP of 60 or 200 nM, both much higher than the value of the \(K_d\) at 5 nM and at a concentration of 200 nM of FYWW RNAP.
<sup>d</sup> <1 is to indicate that for promoters with 16 and 17 bp spacers, only protection is seen for cutting at \(-27\).

**TABLE 3**

Temperature dependence of \(K_d\) for complexes between FYWW RNAP and \(P_r - 12T\) promoter DNA

| Temperature | \(K_d\) \(\text{nM}\) |
|-------------|-----------------|
| 15          | 112 ± 11<sup>b</sup> |
| 25          | 127 ± 4<sup>c</sup> |
| 37          | 87 ± 7<sup>c</sup> |
| 45          | 270 ± 44<sup>c</sup> |

<sup>a</sup> \(K_d\) values were determined as described in the legend to Fig. 2 and under “Experimental Procedures.”
<sup>b</sup> Data are average ± S.D.
<sup>c</sup> Data are the average of two determinations; error indicated is 1⁄2 the spread in the values.

not affect cleavage at \(-27\) for the \(P_r - 12T S18\) promoter, although it afforded protection at this site for the \(P_r - 12T S16\) and \(P_r - 12T\) promoters. We conclude that the intensity increases in the bands because of cleavage at \(-27\) and \(-48\) are specific to formation of the open complex with WT RNAP.

Of the two substitutions in the \(-35\) region, T-30A had essentially no effect, whereas T-35C increased the \(K_d\) beyond our ability to reliably quantify the extent of the change. We estimate that for the latter substitution, our data are compatible with an increase in \(K_d\) to at least 265 nM. The effect of the T-35C substitution indicates that the binding assay employed here is capable of detecting large increases in \(K_d\) (i.e., decreases in affinity) in comparison to the \(K_d\) for the \(P_r - 12T\) promoter. The same is true at the other end of the range. For WT RNAP, which essentially completely protects promoter DNA from approximately \(-38\) to \(+10\), we determined a \(K_d\) of 5 nM. Thus our failure to observe substantial effects on \(K_d\) for several promoter substitu-
stranded DNA. At some weak promoters, RNAP is known to form a closed complex in the absence of an activator (12, 13). Li and McClure (12) took advantage of the slow conversion to the open complex at the phage $\lambda P_{\text{KM}}$ promoter, to kinetically enrich for the closed complex. Their detailed study included both the determination of the binding constant for closed complex formation at 19 °C and the demonstration that this complex has a short footprint and is heparin-sensitive. Perhaps the most reliable method for determining the binding constant for the RNAP and promoter in the closed complex has been to monitor the kinetics of open complex formation as a function of [RNAP] (34). Although the assumptions that underlie the interpretation of the results were found to be justified (e.g. see Ref. 12), this method is technically demanding and not suited for structural studies.

The complexes formed between FYWW RNAP and the $P_r$-derived promoters studied here exhibit the short DNase I footprint seen in prior work, as well as the sensitivity to heparin (Figs. 1 and 2). This is consistent with the complex formed between promoter DNA and the FYWW RNAP being arrested at the closed complex (RP, in Scheme 1). Note that $I_1$, although heparin-sensitive and not opened, does not have a short DNase I footprint (7). Additionally, it was found that formation of the FYWW RNAP-promoter complex is facilitated by CRP protein under the conditions where CRP has been known to facilitate initial binding of RNAP to the promoter (15). Thus, despite a binding defect (see “Results”), the FYWW RNAP is a valuable tool for the determination of relative effects of particular substitutions in promoter DNA, and of temperature, on closed complex formation. Such studies would have been difficult to accomplish by other methods of arresting progression of the reaction between RNAP and a full-length promoter at the closed complex.

The Effects of Substitutions at $-15$ and Downstream—The T-7C substitution increased $K_d$ about 3-fold, but this would still be a small effect compared with its 20-fold reduction in RNA synthesis in vivo (30). Still, recognition of this T in double-stranded DNA is surprising, as it is in the region that becomes single-stranded in the open complex, and RNAP can recognize it as such (35). The near 2-fold effect of the A-11C substitution is unexpected for the same reason, and because two of the substitutions (Trp-434 and Trp-433) that impede the ability of FYWW RNAP to carry DNA strand separation may also function in recognition of the upstream bases of the $-10$ region. Thus, perhaps the effects of not only the T-12G but also the A-11C substitutions should be considered lower level estimators. On the other hand, our own recent results have raised the possibility that Trp-433 may not in fact recognize the $-11A$. Relatively small effects of substitutions in the $-10$ region on closed complex formation have also been observed for short constructs where the DNA strands were chemically linked at $-5$ (14). We also observed that neither the G-15T, which generates the extended $-10$ TGTATAAT region, nor the G-5C substitutions (which interferes with a $\sigma^{70}$ contact) had much effect on the $K_d$. It appears that the large effects of substitutions at these two positions observed on RNA synthesis in vivo and open complex formation (27–29) in vitro must be due to effects on steps beyond closed complex formation.

Effects of Substitutions in the Spacer DNA Separating the $-10$ and $-35$ Regions and Further Upstream—The greatest increase in binding affinity (i.e. lowest $K_d$) we have observed for the interaction of our $P_r$ promoter variants with the FYWW RNAP was the result of a block substitution in the spacer DNA. The substitution we introduced was previously found to increase the extent of open complex formation at another promoter by a factor of 50 (31). The relatively modest effect on closed complex formation observed here would suggest that the main effect is on a later step on the pathway to open complex formation (see Scheme 1). Previously it was observed that the effects of insertion and deletion of a base pair into 17 bp long spacer DNA were considerable (about 10-fold both in vivo and in vitro (5, 36–38)), but here too only modest effects on closed complex formation were detected. With $P_r - 12T$ 18S, which has a longer spacer DNA, an additional DNase I band because of cutting at $-27$ is detected in the footprint of the open complex, the result of WT RNAP-induced hypersensitivity to DNase I in the spacer DNA. Hypersensitivity at about $-27$ is common for open complexes at promoters that have a spacer longer than 17 bp (32) and likely the result of a local distortion introduced while accommodating the longer DNA between the $-10$ and $-35$ regions. No hypersensitivity or protection is observed with the FYWW RNAP, indicating that the RNAP-induced distortion happens subsequent to formation of the closed complex. It is envisaged that the conformational changes undergone by the RNAP during the process of open complex formation would alter its “grip” on the DNA in the $-10$ and $-35$ regions to introduce the distortion. A 1 bp deletion in the spacer DNA leads to a smaller increase in $K_d$ than the 1 bp insertion. This is consistent with RNAP-dependent unwinding of the spacer DNA to properly align the $-10$ and $-35$ regions (38). A difference between the closed complex studied here and the low temperature complex formed at the lacUV5 promoter which has an 18 bp spacer also is that the latter does show the hypersensitivity at about $-27$ (10) despite its lack of strand separation.

Of the two substitutions in the $-35$ region, one is at a more highly conserved base than the other. The T-30A introduces the consensus sequence at a moderately conserved position (54% of promoters (39)), with modest effects, consistent with its effect on RNA synthesis in vivo (30). The other substitution introduces a nonconsensus bp (C) at the upstream edge of the $-35$ region, a position where 69% of promoters have a T (39). This substitution is very deleterious to both closed complex formation (see Table 1) and to RNA synthesis in vivo (30). Our own prior work had shown a greater than 10-fold effect of the G-33C substitution on initial binding (40), also at a well conserved base pair (61% of analyzed promoters have a $-33G$) in the $-35$ region. In aggregate, these results are consistent with interactions of RNAP and the $-35$ region being the most important contributors to the stability of the closed complex.

Effects of Temperature—Our finding that the $K_d$ for the interaction between the FYWW RNAP and the $P_r - 12T$ promoter changes by less than a factor of 2 between 15 and 37 °C, is important; it validates the common practice of using the low

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4 Schroeder, L. A., Choi, A., and deHaseth, P. L. (2007) Nucleic Acids Res., in press.
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temperature complex between WT RNAP and a promoter as a model for the closed complex (e.g., see Refs. 10 and 11). The precipitous increase in $K_d$ between 37 and 45 °C was unexpected, as we had previously demonstrated that for WT E. coli RNAP, as measured by KMnO₄ probing, promoter strand separation was optimal at 45 °C (19). The possibility that the FYWW RNAP may be more temperature-sensitive than WT RNAP is supported by our finding that the $K_d$ for open complex formation at 45 °C with WT RNAP is 5 nM (data not shown), the same value as was determined at 37 °C. We have not compared the stabilities of the WT and FYWW σ⁷⁰, directly, but in view of the multiple substitutions in the FYWW σ⁷⁰, it seems plausible that this variant would indeed be more temperature-sensitive than WT σ⁷⁰.

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