

Mutations in Sigma Factor That Affect the Temperature Dependence of Transcription from a Promoter, but Not from a Mismatch Bubble in Double-Stranded DNA[†]

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ABSTRACT: Specificity of promoter utilization in bacterial RNA polymerases is imparted by a class of proteins referred to as sigma factors. Conserved region 2.3 of these proteins is thought to play a role in the strand separation process that occurs during the formation of an initiation-competent RNA polymerase–promoter complex. We have used a heterologous system consisting of *Escherichia coli* core RNA polymerase and *Bacillus subtilis* σ^A to probe the effects of amino acid substitutions in region 2.3. In agreement with previous work [Juang & Helmann (1994) *J. Mol. Biol.* 235, 1470–1488] we observe that several amino acid substitutions exacerbate the deleterious effect of low temperature on promoter-dependent initiation. On the other hand, no such enhanced cold sensitivity is found with double-stranded templates that contain short “bubbles” of single-stranded DNA, indicating that the DNA-melting defect imposed by these mutant sigma factors can be suppressed by the use of such bubble templates. These results support the involvement of region 2.3 in the strand separation process that accompanies open complex formation at promoters.

Sigma factors are prokaryotic initiation factors sharing the common properties of binding to the catalytic subassembly of RNA polymerase (referred to as the “core” enzyme) and imparting on the newly formed “holoenzyme” the ability to recognize particular DNA sequences as promoters (Helmann & Chamberlin, 1988; Gross et al., 1992; Lonetto et al., 1992; Helmann, 1994). Sigma factor engages in sequence-specific contacts with the DNA to enable the recognition of different DNA sequences, dependent on the identity of the sigma factor. Significant sequence similarity has been found among sigma factors in regions of putative common function. Commensurate with this sequence conservation, functional hybrid holoenzymes have been reconstituted from sigma factors and core enzymes from different species of bacteria (Achberger & Whiteley, 1980; Shorestein & Losick, 1973) including *Bacillus subtilis* and *Escherichia coli*. One region of sequence identity designated 2.3 has a high proportion of aromatic and positively charged amino acids, which are speculated to function in binding single-stranded DNA (Helmann & Chamberlin, 1988; Juang & Helmann, 1994; Rong & Helmann, 1994).

The formation of a functional (or “open”) RNA polymerase–promoter complex is a multistep process involving DNA strand separation in the promoter region (Leirmo & Record, 1990). It is currently unknown how RNA polymerase disrupts duplex DNA, though destabilization of DNA structure by topological unwinding and localized bending have been invoked as possible mechanisms for the nucleation of the melting process

(Amouyal & Buc, 1987; Heumann et al., 1988; Ayers & deHaseth, 1989). A minimal pathway describing the generation of the open complex by RNA polymerase would be “bind–nucleate–melt” (Leirmo & Record, 1990). The formation of open complexes has been found to be adversely affected by a reduction in temperature (Mangel & Chamberlin, 1974; Kirkegaard et al., 1985; Roe & Record, 1985), an effect that has been ascribed not only to the necessity for strand separation but also to a requisite conformational change in RNA polymerase which would take place at the nucleation step (Roe & Record, 1985; Leirmo & Record, 1990).

In a previous study we had tested the proposed role of aromatic amino acids in region 2.3 in the strand separation process. Several variants (see Figure 1a) of the *B. subtilis* σ^A protein were purified and assayed for their ability to promote open complex formation and transcription initiation at the *B. subtilis* P_{ilv} promoter under a variety of conditions (Juang & Helmann, 1994). The region 2.3 variants fell into three classes: The single class 1 mutant (Y180A) was as effective as wild-type σ^A under all conditions tested. Holoenzymes containing class 2 mutants Y184A, Y189A, W192A, and W193A were cold-sensitive for transcription on linear, but not supercoiled DNA templates. In addition, the Y189A, W192A, and W193A mutants were defective in promoter melting at 40 °C as judged by probing with KMnO₄. Holoenzymes containing class 3 mutants (F178A and F186A) were also impaired for DNA melting, but additionally they were defective for transcription throughout the tested temperature range; this defect was not efficiently suppressed by template supercoiling. Together with *in vivo* studies (Rong & Helmann, 1994), these data suggest that the class 3 mutants harbor defects in addition to DNA melting, perhaps in their ability to bind core RNA polymerase.

We previously showed that *E. coli* RNA polymerase was able to initiate RNA synthesis at a short region of single-stranded DNA (a bubble) embedded in a double helix, thus bypassing the strand opening step (Tripathi & deHaseth,

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1993; Aiyar et al., 1994). Although both core and holoenzyme can initiate RNA synthesis at the bubble, only holoenzyme forms heparin-resistant complexes. We considered the possibility that the single-stranded DNA binding activity proposed for sigma region 2.3 might be involved in the stabilization against heparin challenge of the complexes formed at the bubble. To test this model, we investigated the ability of wild-type and mutant variants of *B. subtilis* σ^A protein to allow the formation of heparin-resistant, initiation-competent complexes between *E. coli* core RNA polymerase and a mismatch bubble template. This heterologous system enabled us to take advantage of both an existing collection of σ^A variants (Juang & Helmann, 1994) and our previous characterization of the interaction of *E. coli* RNA polymerase and bubble DNA (Tripatara & deHaseth, 1993; Aiyar et al., 1994). We demonstrate that mutations in region 2.3 of σ^A impair the ability of the hybrid polymerases to initiate transcription at low temperature from the λ P_{RM} promoter but not from P_R or the bubble template. These results do not support the idea that interactions between region 2.3 of σ^A and the single-stranded DNA are responsible for stabilizing the holoenzyme-DNA complex against heparin attack. However, the observation that region 2.3 mutations lead to cold-sensitive transcription from the λ P_{RM} promoter, but not from single-stranded bubble DNA, supports the idea that this region is involved in DNA melting.

EXPERIMENTAL PROCEDURES

Materials. *E. coli* RNA polymerase holoenzyme was purified by the methods of Burgess and Jendrisak (1975) and Gonzales et al. (1977), or purchased from Epicentre Technologies (Madison, WI); the latter was also the source of the core enzyme. The mutant sigma factors containing alanine substitutions in region 2.3, as shown in Figure 1, were purified as described (Juang & Helmann, 1994). The bubble template used has been described (Aiyar et al., 1994). Promoter utilization was assayed on restriction fragments containing both the P_R and P_{RM} promoters.

Run-Off Transcription. The reconstitution of holo polymerase from *E. coli* core and *E. coli* σ^{70} or *B. subtilis* σ^A was carried out by mixing 1.5 pmol of core and 13 pmol of sigma factor in a total of 13 μ L buffer (7 mM Tris-HCl, pH 7.9; 0.35 M NaCl; 0.1 mM EDTA; 0.1 mM DTT; 35% glycerol) and incubating for 30 min at 4 $^{\circ}$ C. RNA synthesis was carried out as described (Warne & deHaseth, 1993). The reactions (20 μ L) contained DNA (40 nM bubble oligomer or 25 nM promoter fragment) and RNA polymerase (30 nM of purified holoenzyme or 30 nM of core with or without sigma factor) in 30 mM Tris-HCl (pH 8.2; the pH of each buffer was adjusted at the temperature of its use), 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 10 μ M dithiothreitol, and 45 μ g of bovine serum albumin/mL. After a 10 min preincubation, heparin was added to 50 μ g/ μ L, followed after another 5 min by 5 μ L of an NTP mix, providing final concentrations of 0.2 mM GTP, ATP, and CTP and 2 μ M UTP (containing 15 μ Ci of [α - 32 P]UTP). Elongation was for 15 min at 20 and 30 $^{\circ}$ C, and for 5 min at 37 $^{\circ}$ C.

Analysis of Transcription Data. The relative amounts of radioactivity in each band were determined by phosphorimaging of the gels or by densitometry. The intensity of the band corresponding to transcription by just the added core enzyme (typically about 10% of the intensity of the band obtained with core and σ^A on a bubble template, 2% for initiation at a promoter) was subtracted from the bands of the same mobilities in the other lanes. The corrected intensities

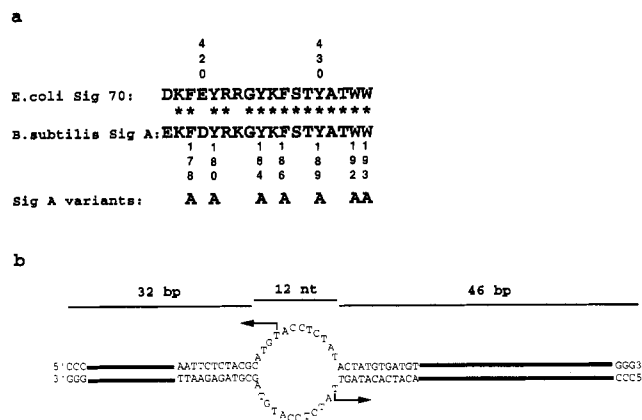


FIGURE 1: (a) The sequences of region 2.3 of wild-type *E. coli* σ^{70} , wild-type *B. subtilis* σ^A , and σ^A variants. (b) The synthetic bubble template. The bubble is asymmetrically positioned on the 90 base pair template, as indicated. The start sites of transcription in the bubble region (Aiyar et al., 1994) are indicated by arrows. Initiation at these sites results in transcripts of 36 and 47 nucleotides in the leftward and rightward directions, respectively.

were then normalized to that obtained for wild-type σ^A . The relative intensities of the 30 °C experiments presented in Figures 5b,c represent normalization to the Y180A rather than the wild-type band in all but one case, due to the lack of active wild-type σ^A at the time. The justification for this deviation derives from the fact that for 31 separate determinations covering two templates and three temperatures, the average intensity of the Y180A transcript (\pm standard deviation) was 1.0 ± 0.4 that of the wild-type σ^A transcript. In addition, the Y180A protein behaves as wild-type both *in vitro* (Juang & Helmann, 1994) and *in vivo* (Rong & Helmann, 1994).

Gel Mobility Shift. RNA polymerase (60 nM holoenzyme or 66 nM core polymerase) and 40 nM DNA (10^6 cpm; end-labeled) were incubated for 10 min in transcription buffer. After addition of heparin to 50 $\mu\text{g}/\text{mL}$ and an additional 5 min of incubation, the complexes of DNA and RNA polymerase were separated from free DNA on 4% [59:1 acrylamide:bis(acrylamide)] nondenaturing gels buffered by $0.5\times$ TBE (Lane et al., 1992).

RESULTS

The collection of amino acid substitutions in region 2.3 of *B. subtilis* σ^A that was used in this study is indicated in Figure 1a; it consists of single alanine substitutions for each of the aromatic amino acids in the region. Also shown is a comparison of the sequences of *B. subtilis* σ^A and *E. coli* σ^{70} in this region; except for three conservative substitutions, the sequences of the two sigma factors are identical. As an initial check for the successful reconstitution of *E. coli* core and *B. subtilis* σ^A , the gel mobility shift experiment shown in Figure 2 was performed. Efficient gel retardation of the bubble DNA fragment was found to require sigma factor: only RNA polymerase holoenzymes reconstituted with *E. coli* σ^{70} or *B. subtilis* σ^A (wild-type or mutant) were able to significantly bind the DNA in a *heparin-resistant* fashion. Interestingly, the complexes containing σ^A consistently migrated with greater mobility than those containing σ^{70} , whether or not the latter had been purified as holoenzyme or reconstituted from purified core polymerase and sigma factor (Figure 2, and unpublished experiments). As neither core (second lane) nor sigma factor (data not shown) by themselves are able to significantly retard the mobility of the bubble DNA, this experiment demonstrates the reconstitution of hybrid holoenzyme from *E. coli* core and

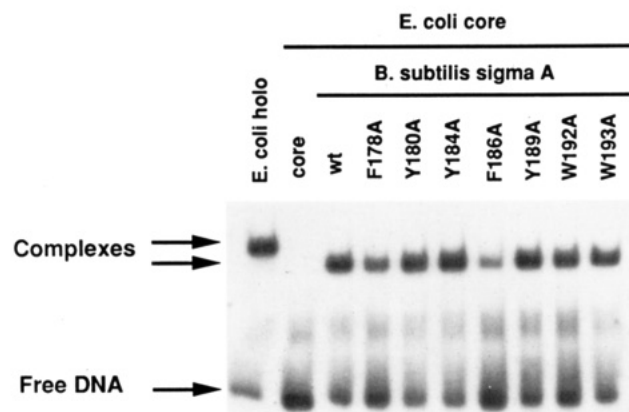


FIGURE 2: Gel electrophoretic analysis of the interaction of core polymerase and purified and reconstituted holoenzymes with bubble template. The first and second lanes contained purified *E. coli* holoenzyme and core enzyme, respectively; all other lanes contained *E. coli* core polymerase and added *B. subtilis* sigma factor as indicated above the lanes. Labeled bubble template and RNA polymerase were incubated and then challenged with 50 μ g/mL heparin for 5 min prior to loading onto the nondenaturing polyacrylamide gel. The positions of the free DNA and its complexes with RNA polymerase are indicated.

B. subtilis sigma factor. In this as well as other similar experiments (data not shown), holoenzyme containing σ^A variants with substitutions at positions 178 and 186 retained less DNA than enzymes reconstituted with the other sigma factor variants. While caution needs to be exerted in the interpretation of such single-concentration mobility shift experiments, the results are consistent with those of other experiments showing reduced activity for these two class 3 sigma variants both *in vitro* [see below and Juang and Helmann (1994)] and *in vivo* (Rong & Helmann, 1994).

We next determined the ability of the various hybrid RNA polymerase holoenzymes to bind to the bubble and synthesize RNA as a function of temperature. RNA polymerase and bubble DNA were preincubated at several temperatures to allow complex formation, heparin was added to inactivate free RNA polymerase, and RNA synthesis was initiated by the addition of nucleoside triphosphates. Under these conditions the amount of RNA synthesized is a measure of the formation of heparin-resistant initiation complexes. Autoradiographs for representative transcription experiments at 20, 30, and 37 $^{\circ}$ C are shown in Figure 3 for the bubble template and in Figure 4 for the promoter fragment. At all three temperatures, and with both templates, transcription is dependent upon the presence of sigma factor. As had been previously observed (Aiyar et al., 1994), the bubble template yields transcription products of 36 and 47 nucleotides, representing initiation of RNA synthesis in divergent directions at the two edges of the bubble. For unknown reasons some day-to-day variability in the intensity of the 36 mer band is observed, as is exemplified by the particular experiment shown in Figure 3a, where the amount of this product is small compared to that of the 47 mer. With the *B. subtilis* sigma factors an additional product (labeled 18 mer due to its comigration with the marker of that length) is observed; this product has not been further characterized.

Formation of transcription-competent RNA polymerase-promoter complexes is adversely affected by lowering the temperature, and in the single-round transcription assays carried out with the bubble template a small effect of temperature was observed as well. The amount of bubble RNA synthesized by holoenzyme reconstituted with either σ^{70} or σ^A decreased by a factor of 2 when the experiment was

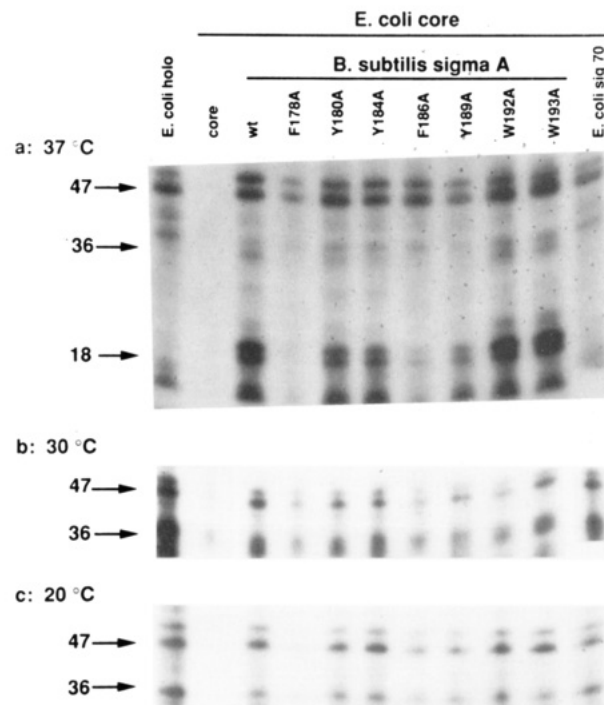


FIGURE 3: Transcription of bubble template with various forms of RNA polymerase. The RNA polymerases were exactly as in Figure 2, except for the additional last lane containing RNA polymerase reconstituted from *E. coli* core polymerase and sigma factor. The bubble template (Figure 1b) was incubated with RNA polymerase at the indicated temperatures followed by a heparin challenge; then RNA synthesis was initiated by addition of NTPs. The products were separated on a denaturing polyacrylamide gel and visualized by autoradiography. The sizes of individual RNAs are indicated. Due to differences in the exposure times between the experiments done at different temperatures, cross-temperature comparisons of intensities are not meaningful.

carried out at 20 $^{\circ}$ C as compared to 37 $^{\circ}$ C. The data displayed in Figure 5 provide a quantitative display of temperature sensitivities above and beyond that characteristic of σ^A , to which the data have been normalized in order to facilitate the comparison of sigma factor mutants at each temperature. In Figure 5a, the relative amounts of 47 mer (chosen in view of some variability in the amount of the 36 mer; see above) RNA synthesis from the bubble template at each temperature (averaged from at least three experiments) are shown. While there are differences in the activities of the various reconstituted RNA polymerases (sigma factors bearing substitutions at positions 178 and 186 being least active at all three temperatures tested; compare to the results of Figure 2), the relative activities are temperature-independent within the error of the experiment. This temperature independence is still observed when the preincubation is reduced from 10 min to 30 s (data not shown). Therefore, even the mutant sigma factors allow the relatively rapid formation of heparin-resistant complexes on this bubble template at the temperatures tested.

The effects of temperature on promoter utilization had been determined for the homologous system containing *B. subtilis* core and sigma factor (Juang & Helmann, 1994). In order to directly compare the effects of temperature on bubble template and promoter utilization by the hybrid RNA polymerases, we measured promoter-driven RNA synthesis under the same conditions. As promoters utilized by *B. subtilis* σ^A and *E. coli* σ^{70} are very similar, we used as template a fragment carrying the P_R and P_{RM} promoters of *E. coli* bacteriophage λ and, as above, performed single-round transcription assays. With promoter DNA, reducing the

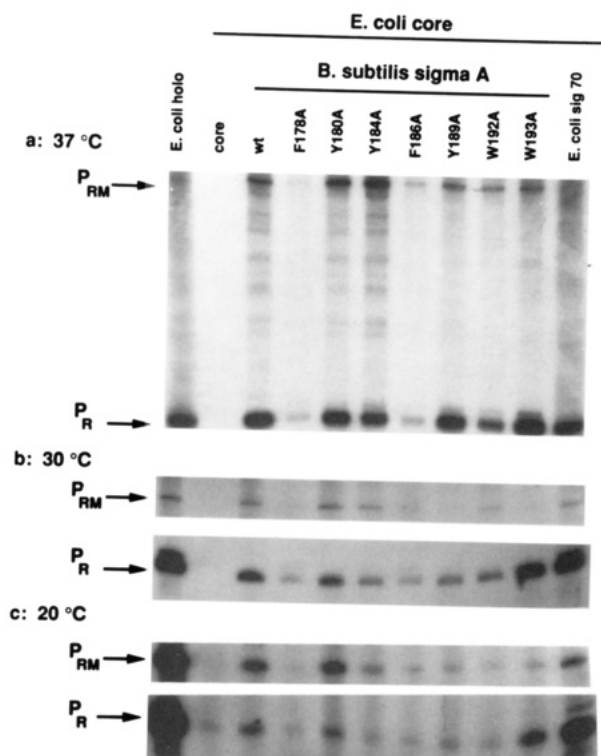


FIGURE 4: Transcription from the P_R and P_{RM} promoters with various forms of RNA polymerase. The RNA polymerases were exactly as in Figure 3. A DNA restriction fragment bearing the P_R and P_{RM} promoters was incubated with the RNA polymerases at the indicated temperatures followed by a heparin challenge; then RNA synthesis was initiated by addition of NTPs. The products were separated on a denaturing polyacrylamide gel and visualized by autoradiography. The bands corresponding to initiation at each of the two promoters are indicated. Due to differences in the exposure times between the experiments done at different temperatures, cross-temperature comparisons of intensities are not meaningful.

temperature from 37 to 20 °C resulted in reductions of 30-fold and 2-fold for P_R transcription, and 20-fold and 4-fold for P_{RM} transcription with wild-type σ^A and σ^{70} , respectively (data not shown). Due to the 10 min preincubation time between RNA polymerase and DNA prior to the addition of heparin and the start of RNA synthesis, these numbers have only qualitative significance. If open complex formation is fast relative to the 10 min incubation time even at 20 °C, this would result in a small or no apparent effect of temperature while the true effect might be much greater. Alternatively, the RNA polymerase containing σ^A may form predominantly heparin-sensitive complexes at 20 °C. Indeed, the *B. subtilis* σ^A holoenzyme forms heparin-sensitive complexes at most promoters investigated, even at high temperature (Dobinson & Spiegelman, 1987; Whipple & Sonenshein, 1992; Rojo et al., 1993).

The relative amount of RNA synthesized from the P_{RM} promoter was reproducibly temperature-dependent for several of the σ^A mutants (Figures 4 and 5c). The σ^A proteins containing the Y184A substitution were the most cold-sensitive, with moderate effects displayed by the F186A (note the higher average activity at 37 °C for this variant in Figure 5c than in the experiment of Figure 4a), Y189A, W192A, and W193A mutants. The P_{RM} values were obtained by monitoring the prominent paused transcript, which had been observed by us (Hershberger & deHaseth, 1991) and others (Hwang et al., 1988). However, two potential complications might affect the interpretation of the temperature dependence of this P_{RM} signal. The pause yielding the band that is quantified is more prominent at lower temperatures (Kuzmin and

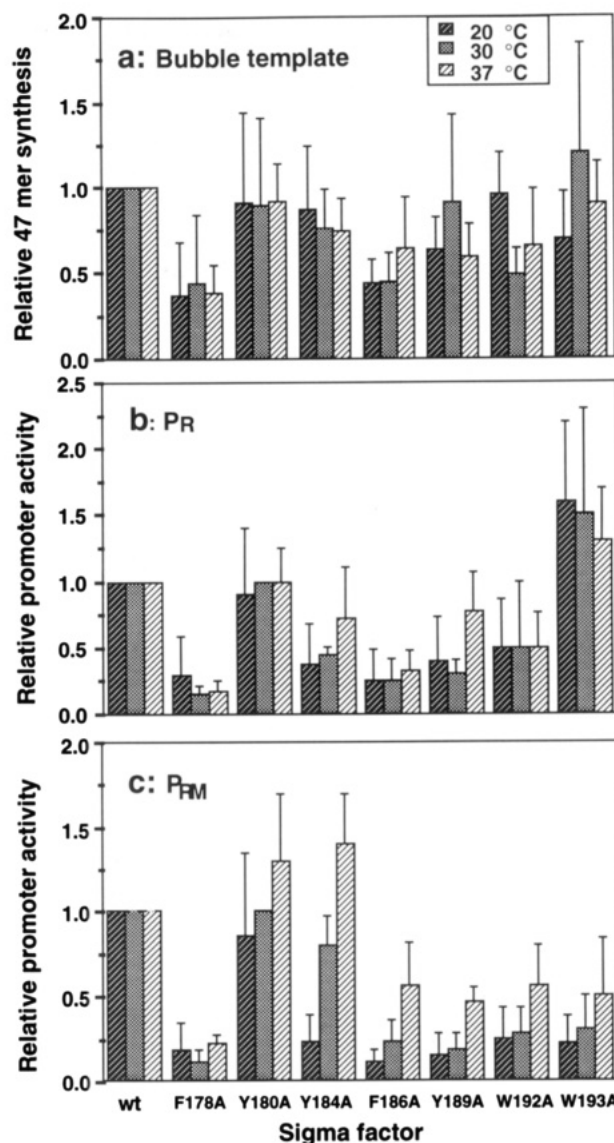


FIGURE 5: RNA synthesis obtained with the various sigma factors relative to that with wild-type σ^A , as a function of DNA template and temperature. The values are averages of 3–5 independent experiments with standard deviations as given by the error bars. The temperatures for all three panels are as indicated in the inset of panel a. The transcripts were as indicated in the panels. The products used for quantitation were as follows (see Experimental Procedures): (a) bubble transcript: 47 mer; (b) P_R transcript: full-length run-off product; (c) P_{RM} transcript: paused product.

deHaseth, unpublished). In addition, open complex formation at the P_{RM} promoter is affected by the presence of the nearby P_R promoter in a temperature-dependent fashion (Hershberger et al., 1993). As the results displayed in Figure 5c involve the comparison of relative temperature sensitivities, and the above two effects are not likely to be dependent on the identity of the sigma factor, the interpretation of our results should not be affected. To specifically determine whether the presence of the nearby P_R promoter affected the results, we investigated the temperature dependence of initiation at P_{RM} on a template where P_R had been inactivated by mutation. The results were essentially the same as those shown in Figures 4 and 5c (data not shown).

Our findings are similar to the pattern of cold sensitivity observed when the corresponding *B. subtilis* holoenzymes were used to transcribe the *B. subtilis* P_{IIV} promoter (Juang & Helmann, 1994). In contrast with the results for the P_{RM} promoter, none of the substitutions affected the relative

temperature dependence of transcription from the P_R promoter by more than a factor of 2 (Figure 5b), suggesting that initiation from this promoter is not limited by DNA melting under these conditions [see also Roe et al. (1985)]. The data summarized in Figure 5b,c show that the F178A variant is the only one which even at 37 °C is poorly active in initiating transcription from both P_R and P_{RM} , in agreement with the results shown in Figures 2 and 3.

DISCUSSION

We have demonstrated that several amino acid substitutions in region 2.3 of *B. subtilis* σ^A (most prominently Y184A) introduce a low-temperature barrier to productive complex formation at the P_{RM} promoter but not at a mismatch bubble or the P_R promoter. The simplest interpretation of this pattern of temperature sensitivity is that these substitutions impair a step which is rate limiting in the former case but not the latter ones; such a step could be open complex formation. The molecular mechanism by which RNA polymerase mediates site-specific DNA melting is presently unclear, but appears to be facilitated by localized untwisting and bending of the DNA duplex (Amouyal & Buc, 1987; Heumann et al., 1988; Ayers et al., 1989). It has been proposed that region 2.3 might participate in the melting step of open complex formation (Juang & Helmann, 1994; Helmann, 1994). Region 2.3 contains several conserved aromatic amino acids similar to those which in single-stranded nucleic acid binding proteins are thought to stack with exposed bases. There is good genetic and biochemical evidence that the adjacent region 2.4 determines recognition of part of the -10 consensus element (Walburger et al., 1990; Siegle et al., 1989; Dombroski et al., 1992), which would position region 2.3 close to the stretch of DNA that is melted in an open complex (Daniels et al., 1990). A direct physical contact between sigma factor and the melted DNA is strongly supported by the observation of a photochemical cross-link between σ^{70} and the -3 position of an open complex (Simpson, 1979). Whether the conserved amino acids of region 2.3 directly stabilize the melted DNA of the open complex is not established, but the consequences of amino acid changes in this region of sigma are consistent with that model (Juang & Helmann, 1994; Rong & Helmann, 1994)).

E. coli RNA polymerase holoenzyme binds and initiates poorly at the P_{RM} promoter, and very well at P_R . During the course of investigating the interaction of the various holoenzymes with a restriction fragment containing these two *E. coli* promoters, it became clear that at 37 °C hybrid enzymes containing the *B. subtilis* σ^A utilized the P_{RM} promoter much more efficiently than the *E. coli* holoenzyme with the σ^{70} . This result is consistent with the report of Henkin and Sonenshein (1987), in which they described the isolation of promoter mutations that led to improved function with *B. subtilis* holoenzymes. Two regions implicated by these workers as important for recognition by *B. subtilis* RNA polymerase might be relevant for the interpretation of our results: an RTRTGN (R = purine; N = any base) sequence immediately upstream and a stretch of A's downstream of the -10 region. The P_{RM} promoter shows 3 matches to the RTRTG and has 2 A's downstream of the -10 region, while the P_R promoters has 2 matches upstream of the -10 and none downstream. Thus our results suggest that sigma factor itself could be responsible for the interaction with the extended -10 region of the promoter established by Henkin and Sonenshein (1987). In some previous studies with *E. coli* core enzyme reconstituted with *B. subtilis* σ^A , it was noted that the resulting holoenzyme

had properties characteristic of *B. subtilis* RNA polymerase (Achberger & Whiteley, 1980), while in others it appeared that the hybrid enzyme more closely resembled the *E. coli* holoenzyme (Davison et al., 1980; Shorenstein & Losick, 1973). Since these assays used complex templates with many promoter sites, it is difficult to compare these early studies with our current work.

We have observed cold-sensitive transcription from the λ P_{RM} promoter by five of the reconstituted holoenzymes (F184A, F186A, F189A, W192A, and W193A). The most dramatic effect was observed with the Y184A σ^A . At 20 °C, reconstituted holoenzyme containing Y184A σ^A is reduced 5-fold in its ability to form heparin-resistant complexes at this promoter as judged by single-cycle transcription assays. Interestingly, studies with the *B. subtilis* holoenzyme suggest that Y184A is actually the least impaired among the five mutants displaying cold sensitivity. Of the five, only Y184A can support cell growth in single copy (Rong & Helmann, 1994) and allow efficient melting at the P_{ilv} promoter *in vitro* (Juang & Helmann, 1994). Nevertheless, the Y184A holoenzyme is cold sensitive for P_{ilv} transcription in multiple-cycle assays, and this cold sensitivity is suppressed by template supercoiling, consistent with a melting defect at low temperature (Juang & Helmann, 1994). In addition, a *B. subtilis* strain containing the Y184A sigma mutant is cold sensitive for growth (50% increase in generation time at 18 °C). The cold-sensitive transcription observed at P_{RM} with the Y189A, W192A, and W193A σ^A variants is also consistent with the effects observed on the P_{ilv} promoter. In each case, holoenzymes containing these mutant proteins were cold sensitive for P_{ilv} transcription and defective in open complex formation at 40 °C (Juang & Helmann, 1994). In addition, it was previously noted that substitutions in region 2.3 of σ^A affected promoter recognition (Juang & Helmann, 1994). That result is confirmed here: At all three temperatures investigated, polymerase containing σ^A with the W193A substitution gives 50% more synthesis of the P_R product than that containing wild-type σ^A or any of the other variant sigma factors.

No temperature dependence of functional complex formation on the bubble template was observed in single-round transcription experiments, following a challenge with heparin. On this template, the DNA is maintained in a single-stranded state by virtue of the extended mismatch between the template and nontemplate strands, and any strand separation function of the polymerase is bypassed. Indeed, Aiyar et al. (1994) have shown that, in the absence of heparin, core polymerase by itself will initiate RNA synthesis from the region of mismatch. They proposed that in this case sigma protein blocked access of heparin to a target site on RNA polymerase. Since the ability to form heparin-resistant complexes is not impaired by amino acid substitutions in region 2.3 which affect the formation of open complexes, we suggest that these amino acids are not essential for heparin resistance.

The data presented in this paper are consistent with the notion that region 2.3 facilitates the formation of initiation-competent open complexes at natural promoter sites. In order to rigorously exclude other possible interpretations of the data (for example, that the mutants investigated affect closed complex formation), more extensive studies of the effects of the substitutions on the kinetics of open complex formation are required. Such an analysis will be carried out with a homologous system consisting of *E. coli* core polymerase and sigma factor, in which alanine substitutions have been introduced at the positions indicated in Figure 1a. Our working model proposes that region 2.3 provides favorable interactions

with the displaced nontemplate strand to help stabilize the strand-separated intermediate. This interaction is predicted to become important at lower temperatures when open complex formation is limiting for RNA synthesis.

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