A Role for the Acidic Trimer Repeat Region of Transcription Factor $\sigma^{54}$ in Setting the Rate and Temperature Dependence of Promoter Melting in Vivo*

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$s^{54}$ is an atypical $\sigma$ factor involved in enhancer-dependent transcription in Escherichia coli. In vivo assays were developed for following the kinetics and thermodynamics of $s^{54}$-dependent melting of the glnAP2 promoter start site. These assays were applied to a series of three $s^{54}$ polymerases containing zero, one, and two copies of the highly acidic trimer repeat region. The results showed that at least one copy of this acid region is required for a complete melting transition at physiological temperature, but is not required for a lower temperature melting transition. The rate of melting the promoter in $\textit{vivo}$ increased with number of copies of this region. Taken together with other observations, the experiments point to a role for the acid region in triggering conformational changes that lead to specific promoter melting. The acid region is required for these changes to occur fully at physiological temperature and influences the rate at which they occur.

Escherichia coli RNA polymerase is a multisubunit enzyme consisting of an exchangeable $\sigma$ subunit and a core enzyme comprised of $\beta, \beta'$, and two $\alpha$ subunits (Burgess et al., 1969). The type of $\sigma$ factor associated with the core enzyme alters the sequence specificity of DNA binding and directs the enzyme to different classes of promoters (for review see Helmann and Chamberlin (1988) and Gross et al., 1989). The major $\sigma$ protein in $\textit{E. coli}$ is $\sigma^{70}$, which produces most of the cellular mRNA. Most minor $\sigma$ proteins have related amino acid sequences and are thus considered to be members of the $\sigma^{70}$ family of proteins (Helmann and Chamberlin, 1988). Although the promoter sequences recognized by different members of this family are different, the overall pathway of transcription initiation appears to be similar (Gross et al., 1992).

There is one minor $\sigma$ factor, $s^{34}$, that is not a member of the $\sigma^{70}$ family of proteins and has a distinct mechanism of transcription initiation. $s^{54}$ associates with the common bacterial core enzyme and changes the sequence specificity of DNA binding in $\textit{E. coli}$ and in a number of other eubacteria (for review see Magasanik (1988); Kustu et al., 1989, 1991, and Collado-Vides et al., 1991). $s^{54}$ was initially found as a consequence of its involvement in mediating nitrogen assimilation but has since been found to mediate diverse metabolic pathways (Kustu et al., 1989). Thus far, $s^{54}$ is the only identified bacterial $\sigma$ protein that appears not to be a member of the $\sigma^{70}$ protein family, as judged by amino acid sequence comparisons (Merrick et al., 1987; Gross et al., 1992). Additionally, the organization of $s^{54}$ promoters differs from that of the $s^{70}$ promoters. The promoter elements recognized by $s^{54}$ holoenzyme are centered at $-12$ and $-24$ in contrast to $-10$ and $-35$ which is common to all other $\sigma$ proteins. The activators of $s^{54}$ transcription generally bind the DNA at some distance from the polymerase. Moreover, these activator binding sites are often enhancer-like in that their precise location and orientation is not critical (Buck et al., 1986; Reitzer and Magasanik, 1986; Collado-Vides et al., 1991; Kustu et al., 1991).

The mechanism of transcription initiation by $s^{54}$ polymerase at the glnAP2 promoter has been studied and found to differ substantially from that of promoters transcribed by $s^{70}$ polymerase. The promoter is bound by the $s^{54}$ polymerase to form a closed complex in a process (Sasse-Dwight and Gralla, 1988; Popham et al., 1989) that does not require the DNA-binding protein, NTRC (NR1). Phosphorylation of NTRC in response to nitrogen deprivation leads to a DNA melting event within the closed complex that is required for transcription initiation (Popham et al., 1989). This event involves looping out of the intervening DNA between the activator and promoter sites, leading to what appears to be protein-protein contacts, and also requires ATP hydrolysis (Su et al., 1990; Weiss et al., 1991). This is very different from the $s^{70}$-type mechanism of initiation and resembles eukaryotic polymerase II transcription in the involvement of remote activators and the requirement for ATP hydrolysis (Gralla, 1991; Wang et al., 1992).

Previously, Sasse-Dwight and Gralla (1990) attempted to identify functional domains within $s^{54}$ by assaying the functions of proteins expressed from $s^{54}$ genes containing internal deletions. Two regions containing leucine-rich heptad repeats (Fig. 1), which resemble leucine zippers, were shown to be involved in DNA binding at the $-12$ promoter element. Deletions within a potential helix-turn-helix motif near the carboxyl terminus eliminated all DNA binding, suggesting its involvement in $-24$ promoter element binding. Deletion of a 27-amino acid stretch within a very highly acidic domain (Fig. 1) did not alter DNA binding but significantly reduced the ability of the mutant $s^{54}$ holoenzyme to form open complexes at the start site of transcription. In this report we investigate the role of this acidic region in promoter melting.

Although acidic regions have not previously been implicated in the control of bacterial transcription, they have a well
known involvement in polymerase II-dependent eukaryotic transcription (Ptashne and Gann, 1990). Experiments have suggested an important role of acidity per se, although acidic regions can contain other poorly understood but important elements (Cress and Triezenberg, 1991). In the E. coli, Klebsiella pneumoniae, and Salmonella typhimurium σ44 proteins, amino acids 48-81 form a subregion of a high amino acid homology and negative charge density where (with one exception) every third amino acid is an acidic residue (see Fig. 2a). We have termed this motif the acidic trimer repeats (ATR) region which was deleted in the σ44 mutant, AcDel 9 σ44, studied earlier (Sasse-Dwight and Gralla, 1990). In this report we will study a homologous series of σ44s that differ primarily in that they contain either none, one, or two ATR regions (Fig. 2b). We expect that if the ATR region is a functional module whose role is to primarily provide an acidic domain, then there should be a progression of influence on DNA melting despite the grossly different sizes and possibly altered structures of the proteins. New in vivo assays for the rate and temperature dependence of open complex formation were developed and applied to this series. The results show that at least one ATR region is required for a physiological temperature promoter melting transition and that increasing the number of ATR regions increases the rate at which the promoter can be melted.

**MATERIALS AND METHODS**

*E. coli* Strain, Plasmids, and in Vivo Test Promoter

Mutations were introduced into σ44 through an rpoN gene cloned behind a tac promoter on a pHBlu2-derived plasmid, pTH7, originally supplied by Dr. Boris Magasanik (MIT). These plasmids, described in greater detail below, were used to transform YMC109tk (thi, endA, har, DlacU169, rpoN: Tn10/F' pro lacP' ZU118, Tn5-102), a σ44 minus E. coli host constructed by M. Hsieh and S. Sasse-Dwight in this laboratory. The σ44-dependent test promoter is the chromosomal glnAP2. In the YMC109tk strain there are two chromosomal copies of the glnAP2 promoter. There is the original which controls transcription of the gln ALG operon and a glnAP2-lacZ fusion borne as a λ lysogen (Agn104) (Blackman et al., 1981).

**Plasmids**

Construction of pATuK4—The acidic trimer repeats coding region is closely flanked by a 5′ AluNI restriction site and a 3′ Alul site. The 126-base pair AluNI-Alul fragment was isolated on a 4% NuSieve DNA agarose gel (FMC Bioproducts, Rockland, ME) and inserted into the rpoN gene by partial AluNI digestion of pTH7 (see Fig. 2a). Partial digestion was necessary because pTH7 contains two AluNI sites. AluNI linearized full-length pTH7 was isolated on a 15% agarose gel, purified with GeneClean (Bio 101, La Jolla, CA), and 5′-dephosphorylated with calf intestine alkaline phosphatase (Sigma). It was possible to directionally ligate the 126-base pair AluNI-Alul fragment into the proper pTH7 Alul terminal, since the two AluNI sites are both noncompatible and nonsymmetric. The nonligated AluNI terminal was blunt-ended with T4 DNA polymerase I (which deleted an alanine residue between the two ATR) and subsequently blunt end-ligated to the Alul terminal. This DNA was used to transform the σ44 minus host YMC109tk. The transformants were selected by ampicillin resistance on minimal media agar plates which had arginine as the only nitrogen source (Sasse-Dwight and Gralla, 1990). In order to grow efficiently on this media, transformants had to have a tandem ATR.

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1 The abbreviations used are: ATR, acidic trimer repeats; IPTG, isopropyl-1-thio-β-D-galactopyranoside; AT, σ44 protein containing tandem ATR.
and nucleotide sequence analysis. The resultant Δ64 mutant contains the original and a tandem copy of the ATR region separated by 8 amino acids (see Fig. 2b).

Construction of pACDel 9 Δ64 was reported previously (Sasse-Dwight and Gralla, 1990). In this study we have shortened the AcDel 9 Δ64 name to Del 9.

Determination of Δ64 Protein Expression in Vivo—YMC1094k strain cells bearing Δ64-encoded plasmids were diluted 1:100 from saturated overnight cultures and grown to 0.3-0.4 OD600 units in 5 ml of G/gln media (60 mM K2HPO4, 10 mM KH2PO4, 0.46 mM MgSO4, 0.1 mM thiamine, 22.2 mM glucose, 15 mM L-glutamine, 100 μg/ml tetracycline, 100 μg/ml kanamycin, 100 μg/ml ampicillin) in a 37 °C Gyrotory Shaker water bath (New Brunswick Instruments, Edison, NJ). Cells were pelleted at 5000 rpm for 5 min in an SS34 rotor at room temperature (Sorvall, Du Pont Instruments, Wilmington, DE). The cell pellet was then resuspended in 2 ml of fresh SO4-free G/gln media and pelleted again to wash the cells of residual free SO42-. The cells were then resuspended in 2 ml of fresh SO42-free G/gln media, to which was added 2 μl of 0.5 M IPTG and 40 μCi of Na35SO4 (Amersham Corp.), and incubated at 37 °C for 15 min. The cells were harvested by centrifugation and washed in 1 ml of fresh SO42-free G/gln media at 4 °C. The pellet was resuspended in 0.5 ml of cold sonication buffer (0.05 M Tris, 5% glycerol, 2 mM EDTA, 0.1 mM dithiothreitol, 1 mM β-mercaptoethanol, 0.233 M NaCl, 23 μg/ml phenylmethylsulfonyl fluoride, pH 8) and freeze-thawed twice in liquid nitrogen and an ice water bath. To lyse any remaining whole cells, the lysate was sonicated on ice with two 10-s bursts at 4 °C and 10 s intervals mounted on a 100 Sonic Dismembrator set at 35% (Fisher Scientific, Tustin, CA). Cell debris was removed by microcentrifugation at 13,000 × g for 20 min at 4 °C (Eppendorf Brinkmann Instruments, Westbury, NJ). 1 μl of the cell lysates were resolved on an SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) minigel (Bio-Rad). The gel was stained with Coomassie Brilliant Blue R-250, dried, and an autoradiograph was made. Identification of the various Δ64 protein bands were made by comparison with a lysate in which no IPTG was added to induce wild-type Δ64.

In Vivo Dimethyl Sulfate Footprinting

The protocol used was essentially that reported by Sasse-Dwight and Gralla (1988). Rifampicin was used to pretreat these samples in order to trap the Δ64 RNA polymerase at the glnA2 promoter.

Open Complex Formation Rate Assay

The protocol for the open complex formation rate assay was an adaptation of the KMMo footprinting procedure reported by Sasse-Dwight and Gralla (1988, 1990). Cells harboring mutant and wild-type Δ64-encoded plasmids were grown in a shacking water bath at 37 °C to 0.3-0.4 OD600 units in 100 ml G/gln media from a 1:100 dilution of a saturated overnight culture. 100 μl of 0.5 M IPTG was added to the log phase cells and allowed to grow for an additional 60 min. The 100-ml culture was divided into eight 10-ml aliquots and placed back into the 37 °C water bath. Except for the no rifampicin sample, 40 μl of rifampicin (Sigma) at 50 mg/ml methanol was added to each culture at time 0. 0.270 μl of 0.37 M KMnO4 was added to different aliquots at 0.0, 0.16, 0.5, 1.0, 2.0, 4.0, and 6.0 min after rifampicin addition. The KMnO4 was allowed to react with the cells for 2 min. These KMnO4-treated samples were then processed in the same way as the lysate to which no IPTG was added to induce wild-type Δ64.

**RESULTS**

**Mutant Δ64 Proteins Are Expressed to Wild-type Levels—** Previous experiments used plating assays to indicate that mutant forms of Δ64 were expressed (Sasse-Dwight and Gralla, 1990). Now we wished to determine whether the two Δ64 proteins with changes in the ATR region were present at levels comparable with that of the wild-type protein. Total cellular protein was pulse-labeled with 35S]label in cells harboring either the mutants or wild-type rpoN (Δ64) gene. Equal amounts of labeled soluble cell extracts were resolved by SDS-polyacrylamide gel electrophoresis. Δ64 could not be seen by Coomassie staining (not shown) but could be seen by autoradiography (Fig. 3). The Δ64 band is identified by comparison with the lysate to which no IPTG was added to induce wild-type Δ64 (lane 1). As expected, the deletion in the gene increases the mobility of this protein (lane 4) and the insertion decreases the mobility (lane 3). However, the mobility shifts correspond poorly to the predicted molecular weight shifts (approximately 4,400 and 3,000 for Del 9 Δ64), most likely due to the highly charged nature of the ATR region. The autoradiograph in Fig. 3 shows that the two mutants Δ64 s (see above) exhibit approximately the same band intensity as wild-type Δ64 (lane 2).

The Δ64 band intensity is not influenced by the ATR region copy number, since the ATR region does not have any sulfur-containing amino acids. Overall, the experiment shows that all three proteins are produced in approximately equal amounts.

*The Mutant ATΔ64 Directs RNA Polymerase to Bind and Melt the glnA2 Promoter—* Previously, it was shown (Sasse-
Dwight and Gralla, 1990) that AcDel 9 σ44 (called Del 9 in this study), which is missing the ATR region, was capable of directing binding to the glnAP2 promoter in vivo. We used dimethyl sulfate protection footprinting to determine if AT σ44, which has two copies of the ATR region in tandem, also directs RNA polymerase to bind to the glnAP2 promoter. Previous dimethyl sulfate footprinting studies of the glnAP2 promoter (Sasse-Dwight and Gralla, 1988, 1990) have shown that the critical guanines at -13 and -25 are protected by RNA polymerase when a functional σ44 is present in the cell. The essay involves expressing the mutant σ44 in cells lacking the wild-type σ44, trapping the RNA polymerase at the promoter, followed by modifying the DNA with dimethyl sulfate. The sites of attack are then determined by isolating the DNA and cutting it at the methylated sites with piperidine followed by primer extension analysis.

Lanes 1–3 of Fig. 4 show the dimethyl sulfate cleavage pattern using AT, wild-type, and Del 9 σ44 proteins, respectively. The patterns are essentially the same, indicating that the changes in the acid region have not altered the ability to bind the promoter. These three patterns differ from that seen when no σ44 is provided (lane 4). The critical guanines at position -25 is fully protected when any of the three forms of σ44 are present, demonstrating full polymerase occupancy. The -13 position is also protected in all three cases, although the extent of protection is not as strong as reported previously when a less activating medium was used (Sasse-Dwight and Gralla, 1990). We interpret these patterns to mean that the acid region acts as a largely independent module in the sense that even a large insertion or a large deletion does not prevent the remainder of the protein from accomplishing its DNA binding function. The equal binding of mutants and wild-type indicates that there is no significant loss of binding affinity in the mutant transcription complexes. This binding relies on the association of core with σ44 (Popham et al., 1989), and thus the core binding function also appears to be fully intact in these mutants.

Previously, it was proposed (Sasse-Dwight and Gralla, 1990) that the acidic region acted as an essential module used by the bound polymerase to melt the promoter DNA. This was suggested by the diminished melting directed by the bound Del 9 σ44 polymerase. Among the complications in this interpretation is that the deletion is a large perturbation. The AT σ44 also represents a large perturbation of the same region but one that increases rather than decreases the total acidity. To test whether the AT σ44 polymerase could melt the DNA, we used KMnO4 footprinting in vivo.

Fig. 4a shows the assay of KMnO4, hypersensitivity associated with glnAP2 start site melting. Lanes 6 of panels B and C are repetitions of a previous result showing diminished melting in Del 9 compared with wild type (Sasse-Dwight and Gralla, 1990). Lane 6 of panel A shows that start site melting is not diminished using the mutant with tandem copies of the ATR.

The Acidic Trimer Repeat Region Affects the Rate of Open Complex Formation in Vivo—Previous studies on many promoters have focused on the rate at which open complexes form, because this rate may limit transcription. These experiments have been restricted to in vitro conditions due to the lack of a technique to make the measurements in vivo. In order to learn whether the ATR region plays a role in setting this critical rate, we developed a method for measuring this rate in vivo.

The method is an adaptation of the in vivo KMnO4 footprinting protocol (Sasse-Dwight and Gralla, 1988). KMnO4 preferentially oxidizes non-base-paired thymine residues and has been used in vivo to detect open complexes in several contexts (Morett and Buck, 1989; Kassavetis et al., 1990; Wang et al., 1992). At the glnAP2 promoter there is little KMnO4 reactivity at the start site during steady-state transcription. This is because promoter melting is transient. The open complex is rapidly cleared by initiation, and the start site rapidly recloses. However, if the cells are pretreated with rifampicin, KMnO4 detects substantial start site opening at the glnAP2 promoter (Sasse-Dwight and Gralla, 1988). This is because rifampicin binds the β subunit of RNA polymerase.

![Fig. 3. Expression levels of mutant σ44s.](image)

**FIG. 3. Expression levels of mutant σ44s.** The sodium dodecyl sulfate-polyacrylamide gel shows that Del 9 and AT mutant σ44 proteins are expressed to levels similar to wild-type levels. The band pointed out by the arrowhead in lane 2 shows wild-type σ44. The bands marked by the arrowheads in lanes 3 and 4 show AT σ44 and Del 9 σ44, respectively. No IPTG was used to induce wild-type σ44 (lane 1).

![Fig. 4. Dimethyl sulfate footprint of AT, wild-type (Wt), and Del 9 σ44 RNA polymerase at the glnAP2 promoter.](image)

**FIG. 4. Dimethyl sulfate footprint of AT, wild-type (Wt), and Del 9 σ44 RNA polymerase at the glnAP2 promoter.** These data show that the mutant σ44s allow RNA polymerase binding to the glnAP2 promoter. Critical guanines at -13 and -25 are not protected from methylation at the glnAP2 promoter when σ44 is absent (lane 4). Lanes 1 and 3 show that AT σ44 and Del 9 σ44 RNA polymerases bind to the glnAP2 promoter at least as well if not better than wild-type σ44 (lane 2), since the critical guanines are protected at least as well if not better by the mutant proteins.
and prevents the bound polymerase from entering an elongation mode (Sippel and Hartmann, 1968; Wehli et al., 1976; Carpousis and Gralla, 1985). Thus the open complexes can form normally, but the polymerase within them is trapped. Thus the DNA in the open complex remains in the open state where it can be detected by KMnO₄.

In the protocol used here, cells are treated with rifampicin for varying times prior to probing with KMnO₄. This results in the accumulation of varying amounts of trapped open complexes. The samples are treated with KMnO₄, the chromosomal DNA from these samples is isolated, and the oxidized thymines that mark open complexes are detected by primer extension analysis.

Fig. 5A, panel B, shows the application of this assay to wild-type σ₄₃ polymerase at the glnAP2 promoter. The strong bands in each lane are the most strongly oxidized thymines at the start site of the glnAP2 promoter open complex. Prior to rifampicin treatment there is little open complex formation (Sasse-Dwight and Gralla, 1988; this study: Fig. 5a, panel B, lane N). As just discussed, this is because open complexes initiate transcription rapidly and thus are removed almost as soon as they form. Immediately after rifampicin is added, KMnO₄ sensitivity begins to appear, since elongation of open complexes is blocked, leading to their accumulation. The KMnO₄ signal increases with time and apparently plateaus after approximately 1 min. The rate of appearance of the KMnO₄ signal is taken as a measure of the rate at which open complexes form in vivo.

These data were analyzed quantitatively and the result is shown in Fig. 5b (filled square symbols). The $t_{1/2}$ for open complex formation for wild-type σ₄₃ is approximately 0.7 min under these in vivo experimental conditions. The $t_{1/2}$ in vitro has not been measured at this promoter, but other promoters exhibit $t_{1/2}$ that range up to several minutes (McClure, 1985). Thus the assay yields an in vivo result that is within a comparable range.

This experiment was also performed using cells producing the mutant forms of σ₄₃ (Fig. 5a, panels A and C). Qualitatively, the results show that in AT₄, when two ATR regions are present, open complexes accumulate faster than with wild-type σ₄₃, which has one ATR region (compare panels A and B). When the ATR region is missing (Del 9 σ₄₃), open complexes accumulate more slowly, and as noted previously, even with long rifampicin treatment, fewer open complexes accumulate (Fig. 5, panel C).

The results with the mutant proteins were also analyzed quantitatively and are compared to those from wild-type (Fig. 5b). The saturation levels for AT₄ and wt are roughly comparable, although the AVu₄ melting might be slightly higher. In terms of the half-time for open complex formation, the mutant with two ATR regions is 2–3 times faster than wild-type, with a $t_{1/2}$ of approximately 0.25 minutes. The differences are seen primarily at the earliest times which are the only ones that clearly precede the attainment of a plateau.

The mutant without an ATR region behaves differently in this analysis. The KMnO₄ signal reaches a maximum after approximately 1 min and then slowly declines. The results differ from those of the other σ₄₃ proteins in two ways. First, the KMnO₄ signal is always 50–70% less. Second, only in this case does the signal reproducibly decline after reaching a maximum. We do not know the source of this decline for the mutant lacking the ATR region, but one possibility is that the Del 9-mediated open complexes are not stable and are not easily re-formed even with lengthy rifampicin treatment. In any case, the decline at the longer times complicates the quantitative analysis, since it is difficult to determine a $t_{1/2}$ when the saturation level is not known accurately.

In order to compare the Del 9 data to that from the other σ₄₃ proteins, we compared the absolute initial rates in the three cases. This used the first four data points to estimate the initial rate of open complex accumulation. In this analysis the lack of an ATR region causes an approximately 2-fold decrease in the number of open complexes that accumulate in the first minute, before the decline sets in. The presence of an extra ATR region causes an approximately 2.5-fold increase. We conclude that the number of ATR regions present determines at least in part how fast an open complex forms at the glnAP2 promoter in vivo.

At Least One ATR Region Is Required for a Physiological, but Not a Low Temperature Melting Transition in Vivo—The previous experiments have shown that the ATR region influences the rate at which the DNA is melted within the transcription complex. In addition, the lack of an ATR region leads to a lowering of the amount of open complex that can
ultimately be formed. The ability of RNA polymerase to melt a promoter depends on the temperature, presumably reflecting in part the temperature-dependent melting of naked DNA. Such open complex melting curves have been obtained in vitro and have been shown to depend on the promoter DNA sequence (Grimes et al., 1991). In this study, we wished to learn if promoter melting curves could also be influenced by the amino acid sequence of the polymerase, particularly the ATR region implicated in melting. To do this, the KMnO₄ footprinting assay was applied to cells at different temperatures.

In this experiment, cell cultures are grown to mid-late log phase at 37 °C. σ⁴ protein is induced with IPTG, and samples are removed and equilibrated at several lower temperatures for 30 min. Each sample is then pretreated with rifampicin, and promoter opening is assessed with the standard KMnO₄ procedure.

Results obtained for wild-type σ⁴ at four different temperatures are shown in Fig. 6a, lanes 5–8. As the temperature is lowered from 37 to 23 °C, there is a modest decrease in the KMnO₄ signal (compare lanes 7 and 8). However, when the temperature is lowered a few more degrees to 16 °C, there is a large reduction in the KMnO₄ signal (lane 6). A large reduction in signal is also seen as the temperature is lowered further to 0 °C (lane 5). The KMnO₄-sensitive bands were excised from the gel and counted. Fig. 6b shows the data plotted as a conventional DNA melting curve.

The results (Fig. 6b, filled squares) resemble known melting curves obtained with naked DNA and with polymerase-dependent open complexes in vitro (Grimes et al., 1991). The apparent melting temperature is approximately 15 °C. This value is between the values of 9 and 25 °C determined by permanganate probing of two other promoters in vitro (Grimes et al., 1991). All of these values are much lower than the melting temperature of naked DNA since these are enzyme catalyzed reactions. This experiment has not been done in vitro for the glnAP2 promoter, so it is not yet possible to compare in vitro with in vivo results at the same promoter.

This experiment was also performed with the two σ⁴ mutants, and the results are shown in Fig. 6a, lanes 1–4 (AT) and 9–12 (Del 9). The KMnO₄ signals were quantified and are plotted on Fig. 6b. The melting curves for AT and wild-type forms of σ⁴ are indistinguishable. However, for Del 9, the melting behavior is similar only at low temperatures. In fact, at 0 and 16 °C, all three proteins seem to catalyze identical extents of melting. However, above 16 °C the melting transition that is common to wild type and AT is simply absent for Del 9. This transition is nonlinear and is reminiscent of the cooperative melting behavior of DNA. We infer that σ⁴ needs at least one copy of the ATR region to facilitate full promoter melting at physiological temperatures. Promoter melting at temperatures below 16 °C is partial and occurs independently of the ATR region.

**DISCUSSION**

In these experiments, the acidic trimer repeats of σ⁴ seem to act as a required melting module. Neither a duplication nor a deletion of this region hinders the other critical functions of σ⁴, namely binding to the −12 DNA promoter element, binding to the −24 promoter element, and by inference, the binding to core polymerase. Thus large changes in this region are tolerated with respect to those functions. On the other hand, both the deletion and duplication of the ATR region strongly influence promoter melting, although in opposite ways, as discussed further below.

This influence on promoter melting was assayed by developing and applying new methods for measuring the rate and temperature dependence of open complex formation in vivo. These are adaptations of the standard in vivo KMnO₄ footprinting assay. This allows for the first time an assessment of the kinetics and thermodynamics of open complex assembly in vivo. The rate of open complex formation at the glnAP2 promoter was found to be slightly less than 1 min in activating media. Although this measurement has not been made at the glnAP2 promoter in vitro, it has been made at many other bacterial promoters in vitro, and those measurements generally gave t₁/₂ of up to a few minutes. The in vitro rates can vary with solution conditions such as the concentration and nature of ions and the concentrations of macromolecules (McClure, 1985). It will be informative to study the same promoters in vivo and in vitro to assess the relevance of the variety of solution conditions used for in vitro transcription. Similarly, it will be useful to apply the simple melting curve procedure used here to compare with the melting behavior in solution. Although rifampicin is postulated not to affect steps prior to chain initiation (McClure and Cech, 1978), minor effects on the rate of open complex formation cannot be excluded. If precise rate differences are important to determine, one should compare the in vivo rate to the in vitro rates, both in the absence and presence of rifampicin.

The application of these methods to the ATR region of σ⁴ confirmed its importance in melting the promoter during transcription. The melting curves showed that at least one ATR region is required for a physiological temperature melting transition in the promoter. A lower temperature transition was not affected by the deletion of the ATR region. Although the full ATR region has a net negative charge of −12, its partial deletion leaves intact an adjacent region with an approximate net negative charge of −25. Perhaps it is this residual acidity that allows the low temperature melting to

![Fig. 6. a, promoter DNA temperature-dependent melting. In vivo KMnO₄ probing of glnAP2 open complex levels mediated by AT (lanes 1–4), wild-type (WT) (lanes 5–8), and Del 9 (lanes 9–12) σ⁴'s at 0, 16, 23, and 37 °C. b, GlnAP2 temperature-dependent melting curve. The open complexes from a were quantified and plotted as % melting versus temperature.](image-url)
occur. This question cannot be simply answered by deleting the residual acidity, since it overlaps with other important functional domains of σ^44. A more sophisticated mutational analysis will be required to test this hypothesis.

The addition of ATR region modules had a progressive influence on the rate at which open complexes formed. In the absence of an ATR region, melting is slow. The melting temperature experiment shows that this corresponds to the low temperature melting transition. With a single ATR region the rate increases and the additional physiological temperature melting occurs. With tandem ATR regions the rate increases further, but no additional melting transition is introduced. The extra ATR region also does not detectably lower the apparent melting temperature. However, it does increase the rate of open complex formation, implying that it lowers the activation barrier that slows DNA melting. The extra ATR could provide extra electrostatic energy to do this. It could also perturb the conformation of the ATσ^44 holoenzyme to more closely resemble the enzyme in the open complex state.

The ATR region seems to act as a module, presumably electrostatic. However, although the primary chemical characteristic of the ATR region is acidity, it is not presently known which feature of this region is critical for melting the DNA. The hypothesis that acidity is the critical feature for DNA melting is supported by the lack of homology in this region in eight sequenced σ^44s (GenBank, University of Wisconsin). The sequence of this region is not generally conserved, and the size can be up to 21 amino acids larger than the E. coli region. Perhaps this helps explain why our insertion and deletion constructs are so well tolerated. Despite the lack of length and sequence conservation, the acidic character of the region is conserved, suggesting a functional role for the acidity.

How does the acidic region assist in promoter melting? Acidic regions are known to be important for activation of certain genes by eukaryotic RNA polymerase II. Experiments in those systems have led to a number of proposals, including suggestions that acidic regions bind basal transcription factors. Experiments with the ATR region demonstrate that when the Del 9 mutant (where the ATR region is deleted) retains the defect in melting. This defect occurs in cells both expressing and lacking NTRC. This would suggest that the ATR region is involved in the recruitment of this protein. The ATR region is also unlikely to be involved in core polymerase binding. This is because σ^44 is required along with the core RNA polymerase for glnAP2 promoter recognition (Popham et al., 1989), and promoter recognition is still intact in the mutant with no ATR region. Thus the acidic trimer repeat region of σ^44 is unlikely to function by recruiting either of the other protein components of the glnAP2 transcription apparatus.

An alternative role of the acidic region would be to promote conformational changes leading to melting within the assembled closed transcription complex. Sasse-Dwight and Gralla (1990) suggested that this could occur by the negatively charged acidic region of σ^44 being driven by NTRC-phosphate and ATP hydrolysis toward the negatively charged DNA. This could create an energetically unfavorable electrostatic environment which would be relieved by the DNA duplex melting. The separated strands would be delivered to single-strand binding sites, possibly in the core enzyme where the template strand is read during transcription. Alternatively, the acidic region could approach another region within the transcription complex and trigger conformational changes, possibly again driven by electrostatic repulsion. These models are consistent with the results reported here. The acidity of the ATR region would induce the observed physiological temperature melting transition, and as observed, an extra copy of the ATR region would provide the additional negative charge to cause this melting to occur faster.

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