We report the effects of depurination and prenicking at various positions of the phage \( \lambda \) prmu-1Δ265 promoter DNA on the rate of open complex formation. We have found that depurination and prenicking at positions around the −10 region strongly stimulate the rate of open complex formation. Since nicking and depurination are known to destabilize DNA helical structure, our observations indicate that the instability of the −10 region is important for open complex formation. We further infer that (i) the nucleation of DNA melting, which occurs during the isomerization from the closed complex into the open complex, contributes to the rate of open complex formation; (ii) the nucleation of melting occurs around the −10 region; and (iii) the propagation of DNA melting from the nucleation region is not rate-limiting. In addition, we have found that depurination at several positions inhibited open complex formation. We used dimethyl sulfate modification protection studies to show that most of the guanine bases that are among these positions are in contact with RNA polymerase in the open complex.

Open complex formation is the major rate-determining step in the process of transcription initiation with E. coli RNA polymerase (\( \sigma^{70} \)). Many of the previous studies have been based on a model containing two functional steps (1, 2) that include the initial binding of RNA polymerase to the promoter DNA to form the closed complex followed by the isomerization of the closed complex into the open complex. Some studies have also suggested that the isomerization step in the two-step model can be further dissected into two discrete steps: the rate-limiting isomerization step per se and a DNA melting step (3–10). In this extended model, the DNA melting step is argued to be very rapid under normal transcription conditions, and consequently it is normally not rate-limiting. It has been suggested that the isomerization step involves a major conformational change in RNA polymerase, which is thought to be the cause for the nucleation of DNA melting (10, 11).

It is known that the −10 region of the promoters recognized by \( \sigma^{70} \) holoenzyme is thermodynamically less stable than average DNA. The conserved −10 hexamer sequence has a melting free energy that is close to the maximum (least stable) possible based on a calculation using the nearest-neighbor thermodynamic data of Breslauer et al. (12). Therefore, as expected, Margalit et al. (13) have shown that 80% of the up and down mutations in the −10 region correlated qualitatively with the change in the melting free energy. A closer inspection showed that most of the mutations among the exceptions are located outside the −10 (hexamer) region, and consequently are not bona fide exceptions if only the melting free energy of the −10 region is important. There were several exceptions within the −10 region. However, in such cases it can be argued that the effect of the mutation on the specific contact between RNA polymerase and promoter DNA is larger than the effect of the melting free energy change, and consequently obscured this effect.

We have tested whether the structural instability of the −10 region is important for promoter function (presumably in the nucleation of DNA melting) by carrying out depurination and prenicking studies. Both prenicking (14, 15) and depurination (16, 18) are known to destabilize DNA double-helical structure, and prenicking may also increase DNA structural flexibility (19). We found that both defects at positions around the −10 region had strong stimulatory effect on the rate of open complex formation on the prmu-1Δ265 promoter. This suggests that DNA structural instability in the −10 region is important for promoter function, and that DNA melting contributes to the rate of open complex formation. Interestingly, the region displaying the stimulatory effect is much smaller than the melted region detected in the open complex. This is consistent with the hypothesis that DNA melting can be divided into two steps: nucleation, and the subsequent propagation of DNA melting from the nucleation region, which is not rate-limiting. The nucleation region as suggested by these studies is located in a relatively small region around the −10 region. In addition, in both the depurination and prenicking analyses, the isomerization rate constant from the closed complex into the open complex was stimulated by at least 5-fold. This indicates that the nucleation of DNA melting occurs in the isomerization from the closed complex into the open complex.

The stimulatory effect of depurination around the −10 region on open complex formation is clearly due to the involvement of DNA melting this region. For most protein-DNA interactions, depurination is expected to decrease binding due to disruption of essential protein-DNA contacts. Based on this reasoning, depurination has been used in other studies to reveal protein-DNA contact (20). Interestingly, we also found that depurination at some positions had an inhibitory effect on open complex formation, suggesting that these positions are involved in contact with RNA polymerase. We have carried out
DMS<sup>1</sup> modification protection studies of the open complex to confirm that most of the guanine bases among these positions are likely to be involved in contacting RNA polymerase.

**MATeRIALS AND METHODS**

**DNA Fragment and RNA Polymerase**—The 564-base pair HindIII-EcoRI fragment containing the λ <i>prnpA</i>Δ265 promoter was isolated and labeled as described previously (21). The end-labeled DNA fragment was digested with Hinfl and run on a 5% polyacrylamide gel. The 189-base pair fragment containing the promoter and labeled on either strand at the EcoRI end was eluted from the gel using the Maxam and Gilbert (22) crush and soak procedure.

**E. coli** RNA polymerase holoenzyme (Epo<sup>30</sup>) was isolated according to Burgess and Jendrisak (23) and Lowe et al. (24), and its activity was determined as described by Hawley and McClure (25). The RNA polymerase used in these studies had an activity of 85%. The RNA polymerase concentrations in the text are expressed as active concentrations.

**DNA Prenicking and Depurination**—Random phosphodiester bond cleavages (nicks) were introduced into the labeled 189-base pair fragment DNA at a frequency of about one nick per molecule by treating with DNase I. Following the treatment, the DNA was purified by phenol extraction and ethanol precipitations. The precipitated DNA was dissolved in TE buffer (10 mM TrisCl, pH 7.8, 1 mM EDTA). The DNA samples (with or without 10 μg/ml heparin and 4 μg/ml bovine serum albumin) (21) were treated with piperidine (Fisher) to a final concentration of 2 M dissolved in 1 M piperidine, incubated at 90 °C for 30 min for random nicking, and then cooled slowly to allow renaturation. The DNA was once again purified by ethanol precipitation, and then dissolved in TE buffer.

**Assay for the Effects of Prenicking and Depurination on Open Complex Formation**—Open complex formation on the prenicked or depurinated DNA was assayed using the gel retardation method as described (26, 27) with some modifications. Oligonucleotides were synthesized from 40-mer RNA polymerase and 1 (nextProps) DNA isolated and treated according to Preiss and Grunberg-Manago (26, 27) with several modifications. Open complexes were formed with 40 nM RNA polymerase and 1 nM prenicked or depurinated DNA at 19 °C in standard reaction buffer (30 mM Hepes (adjusted to pH 7.5 with KOH), 200 mM potassium glutamate, 10 mM MgCl<sub>2</sub>, 1 mM dithiotreitol, and 100 μg/ml bovine serum albumin (21)). At time zero, 4 μl of RNA polymerase in reaction buffer was mixed with 16 μl DNA in the same buffer. At various times ranging from 0.5 to 60 min, the reactions were stopped with the addition of 4 μl of 180 μg/ml heparin and 4 μl of 40% glycerol. The samples were immediately loaded into a running 4% polyacrylamide gel (acrylamide to bis-acrylamide ratio of 59:1). The electrophoresis buffer was 10 mM TrisCl (pH 8.0) and 1 mM EDTA. After electrophoresis, the gel was exposed to Kodak X-Omat AR film for about 3 h. The open complex and free DNA bands in the polyacrylamide gel were cut out, and counted in a scintillation counter. The fraction of open complex was observed, and the fraction of open complex formation following 60 min of incubation was about 0.8 (± 0.05). In addition, core enzyme and σ subunit each alone did not bind to the promoter DNA.

**RESULTS**

**Analysis of Open Complex Formation by Gel Retardation Assays**—We analyzed open complex formation on the prenicked, depurinated DNA, as well as untreated DNA using the gel retardation method (27, 28). The result from such a experiment using prenicked template is shown in Fig. 1. We found that on all three templates, a single band corresponding to open complex was observed, and the fraction of open complex formed following 60 min of incubation was about 0.8 (± 0.05).

1. The abbreviation used is: DMS, dimethyl sulfate.
DNA are labeled in lanes 3 and 7 of the gel. The bands corresponding to open complexes and free DNA are labeled B and F, respectively.

Fig. 1. The binding of RNA polymerase holoenzyme, core enzyme, and σ subunit to prenicked DNA. The binding reactions were performed at 19°C in standard reaction buffer containing: 30 mM Hepes (pH 7.5), 200 mM potassium glutamate, 10 mM MgCl₂, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin. 40 nM RNA polymerase holoenzyme, core, or σ subunit was incubated with 1 nM prenicked DNA in a volume of 20 μl for 0.5 or 60 min. The reactions were stopped by the addition of 4 μl of heparin (final 30 μg/ml) and 4 μl of glycerol (final 6%). The samples were analyzed by electrophoresis on a 4% polyacrylamide gel. The figure shows a photograph of the resulting autoradiogram. The proteins added to the reactions were: lane 1, none; lanes 2 and 3, holoenzyme; lanes 4 and 5, core enzyme; and lanes 6 and 7, σ subunit. The reaction times were 0.5 min for lanes 1, 2, 4, and 6; and 60 min for lanes 3, 5, and 7. The bands corresponding to open complexes and free DNA are labeled B and F, respectively.

phoresis on a sequencing gel. The resulting autoradiograms were quantified by densitometry scanning. In these autoradiograms, each band represented a population of DNA molecules that carried a defect (nick or apurinic site) at a certain position. Consequently, the integration value of a band in a lane for the open complex DNA sample and the integration value of the corresponding band in the lane for the free DNA sample at the same time point were, respectively, proportional to the amounts of DNA in the open complex and free DNA forms. Based on this reasoning, we calculated the fraction of open complex formation (F_{RPo}) for each band or group of bands at each time point using each pair of the integration values. The rate of open complex formation (k_{obs}) for each band was then determined by a plot of ln(1 - F_{RPo}) versus time. The magnitude of the stimulatory or inhibitory effect of DNA defect at each position was calculated by comparing the k_{obs} for each band with the overall rate of open complex formation for the whole promoter region, which is referred to as the average rate of open complex formation (k_{av}). In the sections below, we will first describe the results from the prenicking studies, and the results from depurination studies will follow.

Position-dependent Effects of Prenicking on Open Complex Formation—Fig. 2 shows representative portions of the autoradiogram of the sequencing gel from a experiment with prenicked DNA. As shown in the figure, several bands corresponding to positions around the −10 region appeared in the lane for the open complex DNA sample and disappeared in the corresponding lane for the free DNA sample at a very early time point (0.5 min), which indicates a stimulatory effect of prenicking at these positions on open complex formation. In the contrary, prenicking at several positions (e.g. −36 on top strand) has an inhibitory effect, which is more obvious at the 60-min time point. Therefore, prenicking at certain positions can either stimulate or inhibit open complex formation.

To determine the magnitude of the stimulatory or inhibitory effect of prenicking at each position, we determined the fraction of open complex formed (F_{RPo}) and rate of open complex formation (k_{obs}) for each band or group of bands as described above. The average rate of open complex formation (k_{av}) was determined by quantifying the whole region from −45 to +45. Fig. 3A shows the plots used to determine k_{av} based on data from both strands. We found that the value of k_{av} derived is comparable to the k_{obs} determined in the gel retardation assay. This indicates that prenicking in the region from −45 to +45 did not significantly change the overall rate of open complex formation.

Fig. 3B shows several plots used to determine the k_{obs} for single bands or small groups of bands. The plots are representative of three types of bands each displaying a k_{obs} very different (larger or smaller) or similar to k_{av}. Three fitted lines representing each type of the bands are shown: the fitted line in the middle was derived from the data for the whole region from −45 to +45 on the top strand, and corresponded to a different k_{obs} (defined as k_{av}) value of 7.8 ± 0.5 × 10^{-4} s^{-1}; the lower line was fitted to the data for a small group of bands, −7 to −9, and corresponds to a k_{obs} of 1.4 ± 0.3 × 10^{-3} s^{-1}, which is approximately 20-fold higher than k_{av}; the upper line was fitted to the data for the −35 band and corresponds to a k_{obs} of 3.4 ± 0.4 × 10^{-4} s^{-1}, which is less than half k_{av}. For the bands that displayed rapid kinetics, the rate constants could not be accurately determined; the values reported here are lower estimates. On the other hand, for the bands with slow kinetics, open complex formation might not have reached a final value at 60 min. Consequently, we are not certain whether open complex formation for these bands would reach the same F_{max} that we have used to normalize F_{RPo} in determining the k_{obs}.

In our analysis, the effects on both the rate and F_{max} would contribute to the apparent changes in k_{obs}. Consequently, the magnitudes of inhibition on k_{obs} would be slightly overestimated in those cases where smaller F_{max} would be reached.

The effects of prenicking at different positions on the rate of open complex formation are summarized in Fig. 4. The magnitudes of stimulation and inhibition are expressed as R (= k_{obs}/k_{av}) and 1/R, respectively, i.e. in terms of how many fold the rate of open complex formation was stimulated or inhibited. It is clear from the figure that prenicking at most positions within the promoter did not significantly affect the rate of open complex formation. However, prenicking at positions around the −10 region (−12 to −1 on the top strand, and −12 to −4 on the bottom strand) strongly stimulated open complex formation. The magnitude of stimulation is up to 20-fold. The k_{obs} values for the these positions were up to 8 times larger than the isomerization rate constant k_r (1.8 × 10^{-3} s^{-1}) measured on the unmodified template using abortive initiation assays (15), i.e. prenicking at these positions stimulated the isomerization rate constant by at least 8-fold (see “Discussion”). Significant stimulation was not observed outside this region.

Fig. 4 also shows that prenicking at several positions had a small inhibitory effect on open complex formation. Inhibition of >50% was observed at positions −17, −18, −19, −25, −35, −36, and −37 on the top strand, and at positions +3 to +5 on the bottom strand.

We considered the possibility that the complexes formed on some of the nicked DNA molecules might not be open complexes. To show this conjecture to be false, we carried out the following experiments. First, following open complex formation with prenicked DNA, transcription reaction was carried out with the addition of all four nucleoside triphosphates together with heparin. The complexes remained following transcription and the free DNA were then separated by gel retardation.
Fig. 2. The effects of prenicking on open complex formation. The gel retardation assay of the prenicked DNA was described under “Materials and Methods.” DNA from the bound (B) and free (F) bands of nine different samples with incubation times ranging from 0.5 to 60 min, and the free band of a control (with no RNA polymerase added) in the retardation gel was isolated and analyzed by electrophoresis on a sequencing gel. The figure shows part of the resulting autoradiogram for the top strand (A) and the bottom strand (B). In each panel, lane 1 was derived from the free band of the control; lanes 2, 4, and 6 were from the bound bands of samples that were incubated for 0.5, 6, and 60 min, respectively; lanes 3, 5, and 7 were from the free bands of the same samples. The identity of the bands relative to DNA sequence positions is based on alignment of the bands generated from DNase I digestion with the bands generated from Maxam and Gilbert sequencing reactions (21).

method. The results showed that the amount of complex detected in the polyacrylamide gel decreased by about 60% after transcription, and importantly, a similar result was obtained with unnicked DNA. This indicates that there is no general deficiency for open complexes formed on prenicked DNA to transcribe. To further show that the complexes formed on all of the DNA species (i.e., DNA molecules nicked at different positions) were equally capable of transcription and consequently are open complexes, DNA from both the complex and free DNA bands was isolated and analyzed on a sequencing gel. Quantification analysis of the resulting autoradiogram shows that the intensities of all the bands in the lane for the stable complex DNA sample decreased by a similar extent after transcription. Therefore, the complexes formed on templates with nicks at different positions are indeed open complexes.

Position-dependent Effects of Depurination on Open Complex Formation—Representative portions of the sequencing autoradiograms resulting from the depurination studies are shown in Fig. 5. The average rate of open complex formation, $k_{av}$, was determined by quantifying the region from -45 to +50 as a whole, and corresponded to a value of $k_{av} = 7.0 \pm 0.6 \times 10^{-4}$ s$^{-1}$. Again, this value is very similar to that determined in gel retardation assay, suggesting that partial depurination in this region does not have an overall effect on the rate of open complex formation.

As shown for prenicking, even though depurination at most positions did not have a strong effect on open complex formation, at some positions it had a strong stimulatory effect, and at yet some other positions an inhibitory effect was observed. For example, the -35 band had a $k_{obs}$ of $3.1 \pm 0.2 \times 10^{-4}$ s$^{-1}$, which is less than half $k_{av}$, whereas the -9 band had a $k_{obs}$ of $7.0 \pm 4.7 \times 10^{-4}$ s$^{-1}$, which is about 10 times larger than $k_{av}$. As discussed for prenicking studies, the rate of open complex formation may be overestimated for the bands with slow kinetics, while for bands showing rapid kinetics, the rate constants determined are probably lower estimates.

Fig. 6 summarizes the magnitude of the stimulatory or inhibitory effect of depurination at each position of the promoter DNA on the rate of open complex formation. As shown in the figure, depurination at all positions from -10 to -4 on the top strand and from -12 to -6 on the bottom strand had a major stimulatory effect on the rate of open complex formation. The magnitudes of the stimulation range from 8- to about 15-fold. The $k_{obs}$ values for these positions were up to 6 times larger than the isomerization rate constant $k_f$, i.e., depurination at these positions stimulated the isomerization rate constant by at least 6-fold as discussed for prenicking. Depurination at positions -2 and -3 resulted in some stimulation, but it was of much lower magnitude (2-3-fold). In addition, 2-4-fold stimulation was also observed at positions +19, +20, and +26 on the bottom strand.

Those positions where depurination inhibited open complex formation can also be identified in Fig. 6. The positions showing more than 50% inhibition on $k_{obs}$ were +3 to +5, -13, -14, -16, -17, -30, -32, and -33 on the top strand, and -28, -35, and -36 on the bottom strand. These positions might be in contact with RNA polymerase during open complex formation (see “Discussion”).

DMS Modification Protection at Guanine Bases—We carried out DMS modification protection studies to reveal the RNA polymerase-promoter contacts on the guanine bases in the open complex. As shown in Fig. 7, the guanines at positions -14 and -16 on the top strand, and at positions -3 and -31 on the bottom strand were the only bases protected from DMS modification. The guanines at positions -2, -10, -17, and -33 on the top strand were enhanced. These results will be compared with those from the depurination analysis in terms of RNA polymerase-promoter interaction (see “Discussion”).

DISCUSSION

We have found that both prenicking and depurination around the -10 region strongly stimulated the rate of open complex formation. This indicates that: (i) the structural instability of the -10 region is important for promoter function; (ii) the process of destabilizing a small region of promoter DNA around the -10 region, which we refer to as the nucleation of...
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DNA melting, contributes to the rate of open complex formation; (iii) the nucleation of DNA melting may normally occur in the region where prenicking and depurination stimulated open complex formation (if so, then this region is smaller than the entire DNA melting region); (iv) the propagation of DNA melting from the nucleation site(s) is not rate-limiting.

Instability of the −10 Region Is Important for Open Complex Formation—Based on the results from promoter mutations, it is clear that base pair identity in the −10 region is important for RNA polymerase-promoter DNA recognition during open complex formation. Our observation that destabilization of the −10 region by prenicking and depurination strongly stimulated open complex formation provides strong evidence that the instability of DNA double-helix structure in this region is also important for promoter function. The −10 region of E. coli promoters is less stable than average DNA (13). In addition, it has been shown that all promoters have at least two of the three highly conserved base pairs, −1T, −1A, and −7T (1).

Therefore, the −10 region of the promoter has two functions. First, the base pair identities in this region are important for RNA polymerase recognition; second, the sequence of this region seems to be optimized for DNA melting (presumably at the step of nucleation) during open complex formation.

That depurination and prenicking can destabilize DNA double-helix structure has been shown in several studies. The effects of an abasic site include significant reduction of $T_m$ of oligonucleotides (16), and a free energy loss of 6.5 kcal/mol (17, 18). NMR studies have shown that a nick in an oligonucleotide destabilized the DNA with changes in enthalpy and entropy that roughly corresponded to the loss of a single base pair (14). Much stronger effects were observed when a nick was introduced into a dumbbell-shaped, double-hairpin molecule based on thermodynamic studies (15).

Mechanism of DNA Melting—The observation that the rate of open complex formation can be significantly stimulated by destabilizing a specific region of promoter DNA indicates that destabilization of this region may normally contribute to the rate of open complex formation. We refer to this destabilization of a small region in promoter DNA during open complex formation as the nucleation of DNA melting. Consequently, the region where stimulation was observed is taken as a rough estimate of the nucleation region. Based on our results, the nucleation process has the following characteristics:

First, the nucleation is at least part of the isomerization step from the closed complex to the open complex. This suggestion is supported by our observation that depurination and prenicking stimulated the rate constant, $k_r$, for the isomerization step by as much as 8-fold. The actual stimulatory effect on $k_r$ must be many times larger than what was observed because helical defects in the −10 region are expected to have two opposite effects: one is the stimulatory effect caused by destabilization of the DNA double-helix structure, and the other is the inhibitory effect caused by the elimination of the interactions between RNA polymerase and the promoter DNA at the depurinated positions.

Roberts and Roberts (29) have identified the non-template strand as being responsible for the sequence-specific interaction of RNA polymerase in the −10 region of λ P_R. The lack of stimulation by depurination at the −11 position observed here is consistent with their proposal. Part of the stimulation they observed on heteroduplex templates with mismatched base pairs is likely due to the effect of helical defects described here.

Second, the nucleation occurs in a relatively small region that overlaps the −10 region. It is known that upon open complex formation, the region from the −10 hexamer sequence to the transcription start site becomes single stranded (7, 30–32). Based on KMnO₄ analysis, we have estimated that the minimum size of the DNA melting region on the prrnp-Δ265 promoter is 13 base pairs extending from −11 to +2. However, only at positions in the −10 region and the several base pairs downstream from it (from −12 to −4) did prenicking and depurination stimulate open complex formation. Therefore, the

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2 X.-Y. Li and W. R. McClure, unpublished results.
The nucleation region (where stimulation was observed) is smaller than the DNA melting region. Immediately downstream from the assigned nucleation region is a d(G-C) dinucleotide, which is thermodynamically very stable. The finding that depurination at these positions did not have a strong effect on open complex formation strengthens the idea that the nucleation occurs in a discrete region that does not extend to this position. Additional evidence for a stepwise process in promoter DNA melting comes from the protection studies of Chen and Helman (3), and from the characterization of an RNA polymerase mutant that melted promoter DNA in discrete steps (6).

The results and interpretation of Werel et al. (33) appear to argue for a larger region involved in the putative nucleation function. Their use of the T7 A1 promoter and pretreatment with hydroxyl radical do not allow a detailed or direct comparison with our results. Moreover, Werel et al. did not measure rates of association to the gapped templates they prepared. Instead, overall “affinities” were scored after a long dialysis step against TE buffer, and consequently they might have followed an effect on dissociation of preformed open complex. Probably for this reason, the stimulation observed for gaps in the melting region was modest ranging from about 2-fold to
5-fold at 22 °C and up to 10-fold at 4 °C. Nevertheless, it appears likely that a similar effect of DNA helical defects is responsible for our results and those reported by Werel et al.

Although we have shown here that the nucleation region includes the whole −10 RNA polymerase-recognition region and several additional base pairs as well, in other cases the recognition region and the nucleation region may be separable. For example, the −10 region of the promoters recognized by σ^{2} holoenzyme can be divided into two segments, CCCC and ATt(−10)Aa (lowercase letters indicate weak conservation). It has been shown that the guanine residues of the first segment are all in contact with RNA polymerase (34). By analogy to the promoters recognized by σ^{70} holoenzymes, the second segment but not the first would be melted in the open complex. Therefore, it seems that the first thermodynamically stable segment would be responsible for RNA polymerase recognition, while the second segment may have to do with the melting of DNA although it may also be involved in RNA polymerase recognition and binding. Thus, in this case the nucleation region may not overlap the recognition region completely. Similarly, the consensus sequence of the promoters recognized by the T7 RNA polymerase is about 20 base pairs long, but less than half of the sequence is melted in the open complex. The DNA melting region is centered around a TATA sequence. This sequence is similar to the −10 region of the promoters recognized by the T7 RNA polymerase and consequently may be the nucleation site of DNA melting. Interestingly, Jorgensen et al. (35) have found that depurination in the DNA melting region of the T7 f10 promoter also enhanced the binding efficiency of T7 RNA polymerase. Although the sequences recognized by different σ factors have diverged during evolution, they may have maintained small segments that are suitable for DNA melting.

Third, the observation that only prenicking and depurination in a small region stimulates open complex formation suggests that propagation of DNA melting is not rate-limiting. If DNA melting in the region downstream from the nucleation region were rate-limiting, destabilization of the downstream region by prenicking or depurination should also significantly increase the rate of open complex formation.

It has been suggested that the isomerization from the closed complex into the open complex involves two discrete steps: the isomerization per se and DNA melting (4, 8–10). DNA melting is not rate-limiting at higher temperatures, but may become rate-limiting at lower temperatures. We have argued above that the propagation of DNA melting from the nucleation region is not rate-limiting even though our studies were carried out at 19 °C. This is consistent with the following findings. The intermediate complex preceding DNA melting did not accumulate, even at a lower temperature (15 °C); the apparent activation energy for open complex formation in the temperature range of 15 °C to 25 °C is only about 20 kcal/mol, which is similar to the activation energy observed on the λPR promoter.

FIG. 6. Summary of the effects of depurination on open complex formation. The autoradiograms resulting from the depurination analysis as shown in Fig. 5 were quantified using scanning densitometry. The rate of open complex formation (k_{obs}) for each band was determined and compared with k_{av}. The magnitudes of the stimulatory (R) or inhibitory (−1/R) effect of depurination at various positions on the rate of open complex formation, calculated as described in Fig. 4 legend, are shown. The open columns are for purines on the top strand, and the solid columns are for purines on the bottom strand.
at higher temperatures (10). Therefore, the stimulation by depurination and prenicking on the rate of open complex formation reported here was at the isomerization-nucleation step rather than the DNA unstacking step. Our suggestion that nucleation contributes to the rate of isomerization and that the properties of structural stability in the −10 region is important in this process is complementary to the suggestion of Roe et al. (10) that a major conformational change in RNA polymerase may occur in the isomerization step.

Destabilizing the double-helix structure of the −10 region might facilitate open complex formation by decreasing the activation energy for the nucleation. If this is true, the observed magnitude of stimulation (up to 20-fold) resulting from prenicking and depurination would correspond to a change in activation energy of about 1.7 kcal/mol. This is only a small portion of the total activation energy for open complex formation, which is 20 kcal/mol for this promoter.

**RNA Polymerase-Promoter Interactions**—We have found that depurination at several positions had an inhibitory effect on the rate of open complex formation, presumably by disrupting protein-DNA interactions. Five of these positions are located in the −35 region of the promoter, and are expected, considering the importance of base identities of this region in promoter function. Interestingly, removal of −31G by depurination did not have much effect on open complex formation even though this base was protected in the open complex from DMS modification on this promoter, as well as several other promoters (36, 37). A possible explanation is that the protection resulted from an indirect effect rather than a specific contact to the N7 group of −31G by RNA polymerase.

Two of the bases (−14G and −16G), whose removal by depurination showed inhibitory effects on open complex formation, were also protected from DMS modification in the open complex. This suggests that these two bases may have contacts with RNA polymerase during open complex formation, in agreement with the results of Michin and Busby (38) on the gal P1 promoter. The removal of −33G and −17G by depurination also significantly inhibited open complex formation, indicating that these bases are in direct interaction with RNA polymerase in the DMS protection experiment, however, we observed enhancement at these positions. A possible explanation that is consistent with both results is that RNA polymerase makes contact with the O6 group of these two bases so that their N7 groups are still available for DMS methylation in the open complex. This type of protein-DNA contact at the O6 group of a guanine residue has been shown to exist in the interactions of λ repressor (39) and phage 434 repressor (40) with their respective operator site. Interestingly, the positions −14, −15, and −17 showed weak conservation in the compilation of the sequences of known promoters; and mutations have been isolated at positions −14, −15, and −16 on several promoters (41). This evidence and our results suggest that the contacts of RNA polymerase at the positions in the region from −14 to −17 may be important for open complex formation on this and other promoters.

We have also observed an inhibitory effect upon depurination at two other guanine positions (−3 and +5) where DMS modification protection or enhancement in the open complex was not observed. It is possible that, as mentioned above, RNA polymerase might make contact with the O6 group but not the N7 group of these guanine residues so that DMS modification, which occurs at N7 of guanine bases, would not be blocked.

**Prenicking Also Inhibited Open Complex Formation**—We found that prenicking at several positions inhibited open complex formation slightly. A possible explanation is that prenicking at these positions altered DNA structural properties such as flexibility. It has been suggested (42–45) that there might be a DNA rotational change, or the formation of other DNA structural stress, during open complex formation. The DNA structural stress was argued to facilitate DNA melting. Therefore, prenicking might have inhibited open complex formation by eliminating the DNA stress. An example showing that a nick can relieve DNA torsional stress and consequently alter protein-DNA binding affinity came from a study by Koudelka et al. (19). It was shown that a nick in the middle of the operator DNA increased the phase 434 repressor binding, presumably by relieving a structural stress resulting from DNA bending caused by the repressor binding. An alternative explanation for our observations is that prenicking might have caused disruption of RNA polymerase-promoter interactions by altering DNA structure around the nick. It has been shown that nicks can cause slight distortion in DNA structure (14). This explanation is consistent with the observation that the positions showing inhibitory effects are within or close to protected regions in the hydroxyl radical footprint of the open complex.

**Conclusion**—Our finding that depurination or prenicking in the −10 region greatly stimulated open complex formation suggests that the intrinsic instability of this region is important for promoter activity. Our results also suggest that the nucleation of DNA melting, i.e., the destabilization of a small region of DNA, contributes to the rate of open complex formation. Nucleation may occur around the −10 region. Moreover, we have also shown that depurination at some positions had an inhibitory effect on open complex formation, indicating that these positions are important for open complex formation on this promoter. About half of these positions were found to be in the −35 region, which is consistent with the importance of base pair identity in this region for promoter function. Therefore, our results support the following model: both regions of the promoter are important in the direct interactions with RNA polymerase, whereas, the DNA melting free energy around the −10 region but not that of the −35 is important for open complex formation.

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