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Structural Studies of *lac*UV5-RNA Polymerase Interactions *in Vitro*

ETHYLATION INTERFERENCE AND MISSING NUCLEOSIDE ANALYSIS*

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Substantial effort has been made to investigate the interactions that the *Escherichia coli* RNA polymerase makes with promoter DNA during transcription initiation. The *lac*UV5 promoter has been the object of many of these studies, and to date, an incredible wealth of information exists on how RNA polymerase interacts with this promoter. We have sought to expand current knowledge by the use of two chemical interference protocols, phosphate ethylation and missing nucleoside. We have added to existing information with the identification of additional phosphates, for example, at the start site of the template strand that, when ethylated, perturb the binding of RNA polymerase. We have also discovered a number of positions, most remarkably −37 to −34 of the nontemplate strand, where nucleoside loss decreases binding. Finally, we have discovered positions of ethylation and/or nucleoside loss that can stimulate binding. In particular, missing nucleosides and phosphate ethylation near the transcription start site enhance RNA polymerase binding.

In *Escherichia coli*, the binding of RNA polymerase to the *lac*UV5 promoter provides a well studied example of protein-DNA interactions. The binding of RNA polymerase to this promoter has been studied using a wide range of techniques including mutagenesis of the promoter DNA or RNA polymerase, a variety of footprinting techniques, and numerous interference studies (1–12). *lac*UV5 has been best studied because it is a strong promoter that works independently of activator proteins and sequences beyond the core −35 and −10 promoter elements. It also benefits from the fact that the *lac* operon has been a model system for studying both positive and negative transcription regulation for over 40 years. Despite the incredible depth of these studies, it would be of considerable value to determine the effects on RNA polymerase-promoter interaction caused by two types of DNA modification, phosphate ethylation and nucleoside removal.

Ethylation interference can identify critical phosphate backbone contacts for protein-DNA interactions. Phosphate contacts are important to many protein-DNA interactions because the electrostatic interactions provide binding energy and increase the strength of the interaction. Electrostatic interactions have previously been demonstrated to be important for RNA polymerase-promoter interactions. For example, at λp_R, approximately 20 positive ions are displaced from the phosphate backbone of the promoter in order for binding and open complex formation to be achieved (13, 14).

The phosphates are a constant in the four deoxyribonucleotides. The consequence is that the phosphate backbone is relatively uniform regardless of the sequence of bases. Thus, mutation of the DNA sequence is not an option for studying the effects of specific protein-phosphate interactions during binding and open complex formation. Short of protein-DNA cocrystallography, ethylation interference is one of the few techniques available to investigate these fundamental interactions.

Some data are already available concerning the effects of phosphate ethylation on RNA polymerase binding to *lac*UV5 (9, 12). Ethylation interference assays have implicated the phosphates from −39 to −36, −18 to −14, −9, and −8 on the non-template strand and position −32 on the template strand as important for binding of RNA polymerase to *lac*UV5 (9, 12). The phage T7 A1 promoter, which has a perfect −35 hexamer and strong activity in the absence of proteins other than RNA polymerase, displays patterns of ethylation interference (and patterns of G and A methylation protection and interference) remarkably similar to those of the *lac*UV5 promoter (8, 9). These ethylation interference experiments deserve to be revisited, since existing technology allows a more sensitive investigation into the effects of phosphate ethylation on binding and open complex formation at *lac*UV5.

The missing nucleoside experiment can identify important specific contacts to bases in the promoter DNA (15). The missing nucleoside experiment relies on the use of hydroxyl radicals to generate DNA fragments lacking a single nucleoside. The hydroxyl radical treatment results in oxidative degradation of the deoxyribose and leads to destruction of the phosphate backbone and loss of the nucleoside at the position of modification (16). The products are a 5′-phosphoryl group at one end and an equal mix of 3′-phosphoryl and 3′-phosphoglycolic acid at the other end. The modification results in base loss, breakage of the phosphate backbone, and the addition of two negative charges to the backbone (16).

The missing nucleoside technique, which has not been used on the *lac*UV5 promoter prior to this study, offers an advantage over the traditional techniques used to study specific contacts (promoter mutation or methylation interference) because it removes structure rather than altering or blocking existing DNA structure. Further, the missing nucleoside experiment is more general than methylation interference, because hydroxyl radicals modify all positions relatively equally.

Other investigators have utilized the missing nucleoside experiment or similar techniques to study the σ^{70}-dependent promoters T7 A1 (17), and *ace* P and *rrnB* P1 (18). At T7 A1, nucleoside removal on the template strand from −34 to −31 and the 2–4 base pairs around −18 of both strands led to

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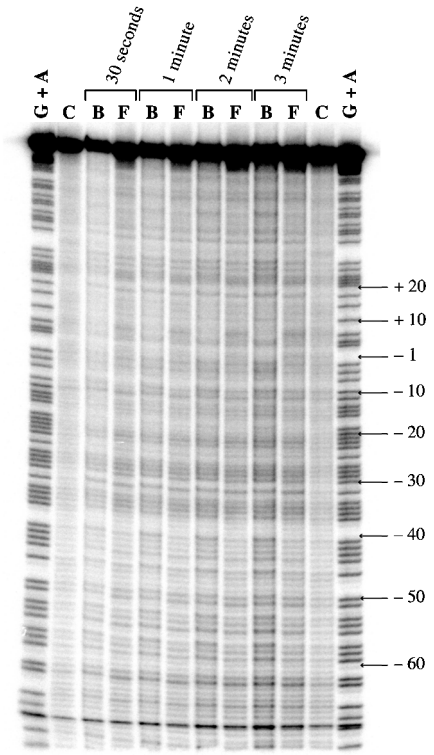

FIG. 1. **Sample ethylation interference gel.** This image is derived from the denaturing gel analysis of the effects of ethylation on the template strand of the *lac*UV5 promoter. It is included as an example of the raw data used for the densitometric analysis of the bound and free DNA. The curves presented in the following *figures* were generated from images such as this one. *G + A* indicates the products of a Maxam-Gilbert G + A sequencing reaction; these serve as markers. *C*, cleavage control of DNA that was ethylated and cleaved in alkali without any exposure to RNA polymerase. *B*, molecules from the bound fraction. *F*, molecules from the free fraction.

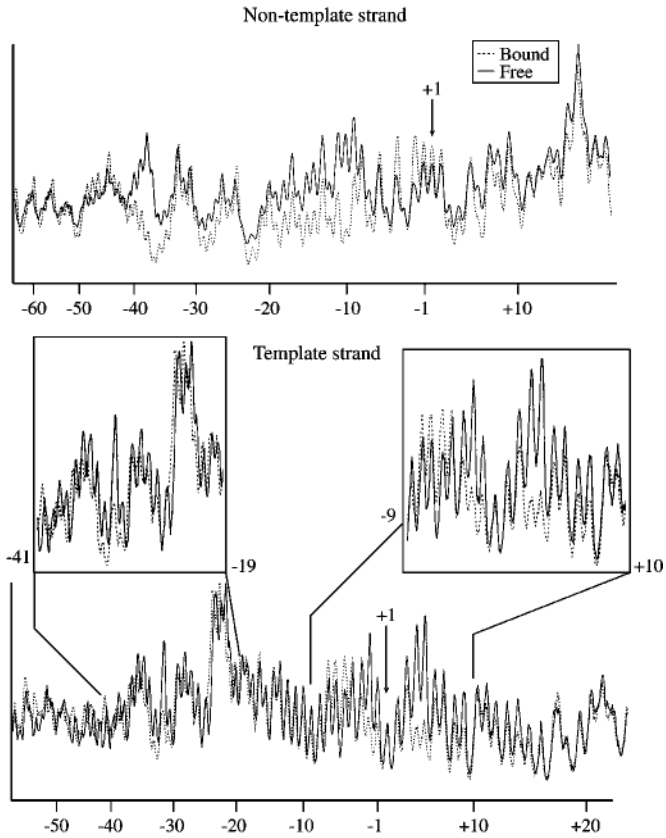

FIG. 2. **Ethylation interference at 30 min.** DNA fragments were modified as described under "Materials and Methods." 0.5–2.5 nM end-labeled, ethylated DNA fragment containing the *lac*UV5 promoter was incubated with RNA polymerase (100 nM) for 30 min at 37 °C and challenged with heparin (20 ng/μl) for 1 min, and then the bound and free DNA molecules were separated and analyzed. The densitometric curves presented were generated using ImageQuant. Data for both strands are presented. *Dashed lines* indicate the cleavage patterns for DNA molecules from the free population. *Solid lines* indicate the cleavage patterns for DNA molecules from the bound population.

decreased affinity of RNA polymerase for the promoter (17). For *ace* P and *rrnB* P1, which have UP elements, enhanced affinity of RNA polymerase for promoter DNA was observed upon base elimination upstream of the −35 hexamer. At all of these σ^{70}-dependent promoters and the phage promoters for T3, T7, and SP6 polymerases, base removal near the melting region (+2 to −14 for T7 A1, −5 to −15 for *ace* P and *rrnB* P1, and +3 to −5 for the phage promoters) enhanced RNA polymerase affinity for the DNA (17–19). At λ*prmup-1*Δ265, prenicking and depurination around the −10 region stimulated the rate of open complex formation (20). Finally, mismatches in the −10 region of λp_R increased the rate of open complex formation (21).

Studies presented in this report identify the critical nucleoside contacts for RNA polymerase binding to *lac*UV5 and extend the available ethylation interference data to new positions. Additionally, the strand specificity of ethylation interference near the start site and the −10 hexamer support current models of initiation that require different roles for each strand. Finally, some bases within the *lac*UV5 promoter, when modified either by ethylation or hydroxyl radicals prior to RNA polymerase incubation, result in increased binding of the protein. This is a novel observation at the *lac*UV5 promoter.

MATERIALS AND METHODS

DNA and Proteins—In all experiments, a DNA fragment extending from +60 to −180 of the *lac*UV5 promoter was end-labeled with ^{32}P. The fragment was 5′-end-labeled with T4 polynucleotide kinase (Promega) or 3′-end-labeled with avian myeloblastosis virus super reverse transcriptase (Molecular Genetics Resources). RNA polymerase was supplied by Epicentre Technologies.

Ethylation Interference Assays—The goal of this protocol was to generate DNA fragments that had one or less ethylated base per molecule. To achieve this end, conditions were adjusted so that approximately 30% or fewer of the molecules were modified (gel analysis indicated that 30% of the molecules were less than full-length). End-labeled DNA fragments were resuspended in 50 mM calcium cacodylate (Sigma) and added to an equal volume of ethanol saturated with ethylnitrosourea (Sigma). This mixture was heated at 50 °C for one hour. The mixture was ethanol precipitated five times followed by a 70% ethanol wash to remove excess ethylnitrosourea. Ethylated DNA was resuspended in water prior to use. Typically 0.5–2.5 nM DNA fragment was incubated with 100 nM RNA polymerase for various times at 37 °C in 100-μl reactions. The binding buffer was 30 mM Tris-HCl, pH 7.9, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 0.2 mM cAMP, and glycerol to 2.5%. At the completion of binding, 20 ng/μl heparin was added, and the complexes were challenged for 1 min at 37 °C. Subsequently, bound DNA molecules were fractionated from free DNA molecules by filter binding or nondenaturing polyacrylamide gel electrophoresis. Fragments were recovered, heated at 90 °C in 0.15 M NaOH to hydrolyze the DNA at the position of ethylation, and bands were resolved with 8 M urea denaturing polyacrylamide gel electrophoresis. Gels were dried and exposed to a storage phosphor screen, and data were analyzed using ImageQuant software (Molecular Dynamics, Inc.). Densitometric traces are provided below. A sample gel is shown in Fig. 1.

Missing Nucleoside Experiment—End-labeled DNA fragments were modified at a rate of a single hit per molecule via the Fenton reaction (16). This was verified via gel analysis, as described above. 40 μl of DNA were added to 60 μl of buffer containing 4 mM $(NH_4)_2Fe(SO_4)_2$, 8 mM EDTA, 14 mM sodium ascorbate and 0.01% H_2O_2. The reaction was terminated after 4 min, and the DNA was precipitated, washed with 70% ethanol, and resuspended in water prior to use. Binding and

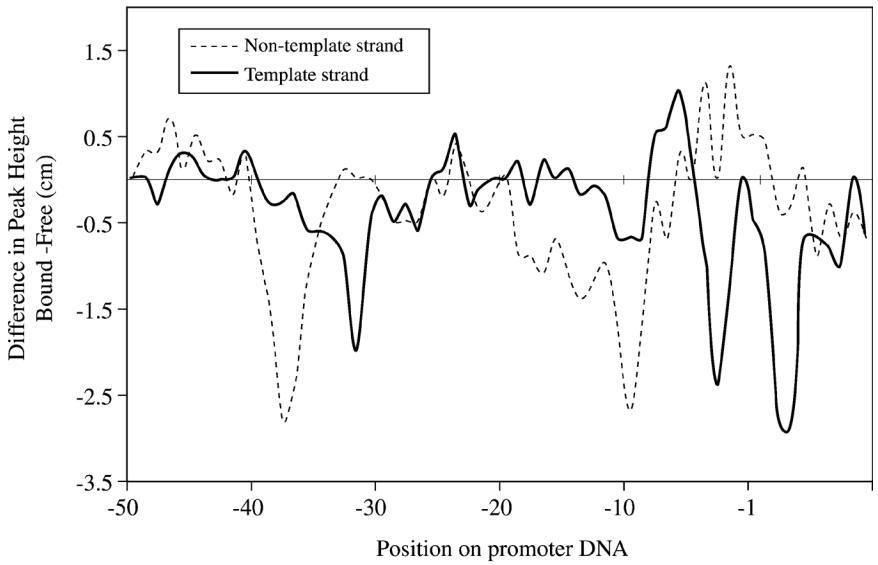

FIG. 3. **Difference plot (bound − free) of ethylation interference at 30 min.** Peak heights were measured, and the differences between the bound peak height and the free peak height for each position for the template and nontemplate strands were calculated. The results were plotted for all positions between −50 and +10. The peak height differences are shown in centimeters on the *y* axis. Values greater than 0 indicate binding enhancement upon ethylation at that position. Values less than 0 indicate binding interference upon ethylation for that base.

analysis were as described above except that alkali treatment was not required for analysis.

RESULTS

Equilibrium Binding of RNA Polymerase to lacUV5—The *lac*UV5 promoter is considered a strong σ^{70}-dependent promoter. It has a perfect −10 hexamer sequence and drives the expression of the *lac* operon independently of CAP. RNA polymerase (the holoenzyme containing σ^{70}) binding to a promoter depends in large part on the match of the promoter core sequence to the consensus for σ^{70} promoters. RNA polymerase binding to *lac*UV5 *in vitro* is, as expected, quite strong. In fact, while the wild-type *lac* promoter gives primarily RNA polymerase complexes with an overlapping promoter-like sequence, P2, *lac*UV5 exhibits binding only at the sequence expected for the biologically active promoter, P1 (7, 22).

We took advantage of these traits of *lac*UV5 to test the effects on binding and open complex formation of phosphate ethylation and loss of nucleosides within the promoter. Equilibrium binding was achieved by incubating RNA polymerase with the modified DNA for 30 min at 37 °C prior to challenging the complexes with heparin to enrich for open complexes and inactivate RNA polymerase molecules not bound in open complexes. Shorter RNA polymerase-DNA incubation periods were also examined.

Ethylation Interference Assay—Analysis of the ethylation interference data reveals a pattern that is similar in a number of ways to previous data (9, 12). Fig. 2 shows the effect of ethylation of both the nontemplate and template strands upon RNA polymerase binding. The curves are a sample from a set of experiments. Each experiment was performed at least two times and at various time points. The patterns of interference and stimulation of binding are similar among all experiments. On the nontemplate strand, ethylation of −40 to −35, −29 to −27, −22, −19 to −9, −7, +3, +4, +6, +8, +10, +14, and +15 exhibits interference with binding of RNA polymerase. On the template strand, positions −36 to −31, −29 to −27, −11 to −9, −4 to −2, +1 to +8, and +10 to +14 interfere with binding of RNA polymerase when ethyl groups are present on the phosphates. A significant pattern seen in these data is that ethylation of the nontemplate strand appears more detrimental to binding than ethylation of most positions on the template strand (with notable exceptions at −32, −4 to −2, and +3 to +4). The analysis also indicates that at some positions ethylation can enhance binding. On the nontemplate, these positions are −49 to −47, −45, −24, −6, −4, and −2 to +2; on the template strand they are −24 and −8 to −6. These data are also presented as difference plots in Fig. 3.

Missing Nucleoside Experiment—Analysis of the effect of lost nucleosides on binding is given in Fig. 4. Data for the nontemplate strand indicate that loss of nucleosides from −9 to +4, +18, and +19 increases binding. Loss of the bases from −76 to −72, −70 to −68, −66 to −56, −52 to −40, −37 to −34, −23, −22, −20 to −17, −15, −13 to −10, +6, +7, and +10 to +16 all decrease binding. For the template strand, missing nucleoside data are only available for a 3-min incubation period. Loss of nucleosides from −44 to −42, −33 to −29, −21 to −19, and −14 to +4 increases binding, and loss of bases from −53 to −47, −40, and −39 decreases binding.

We also performed time course experiments (using binding times from 0.5 to 3 min) for both the missing nucleoside and ethylation interference experiments. In general, the data were consistent among the various time points and were representative of the equilibrium results presented in Figs. 2 and 4. Thus, these data are not presented.

DISCUSSION

Enhancement—Perhaps the most interesting result of this work is that the loss of potential contacts, either by removal of various nucleosides or by ethylation of the phosphate backbone, can increase the tendency of the DNA molecules to bind RNA polymerase and form open complexes. This enhancement of binding is present at equilibrium (Figs. 2 and 4) as well as at various time points shortly after the addition of RNA polymerase (data not shown).

Nucleoside loss in the missing base experiment can have four types of effects: 1) the loss of a base that participates in a protein-DNA contact; 2) possible perturbation in the backbone upon the removal of the base and sugar and generation of a gap in the phosphate chain; 3) the generation of two additional negative charges in the phosphate backbone; and 4) loss of interstrand hydrogen bonds (base pairing interactions) and base stacking interactions that could serve to destabilize the local helical structure.

The increase in binding coincident with the loss of bases near the start site has seemingly clear consequences; it may aid in the melting of DNA at the start site upon open complex formation. Other investigators have reported similar effects with both σ^{70}-dependent promoters and promoters for T3, T7, and SP6 bacteriophage polymerases (17–21). In general, the increased affinity of RNA polymerase for the DNA was attributed

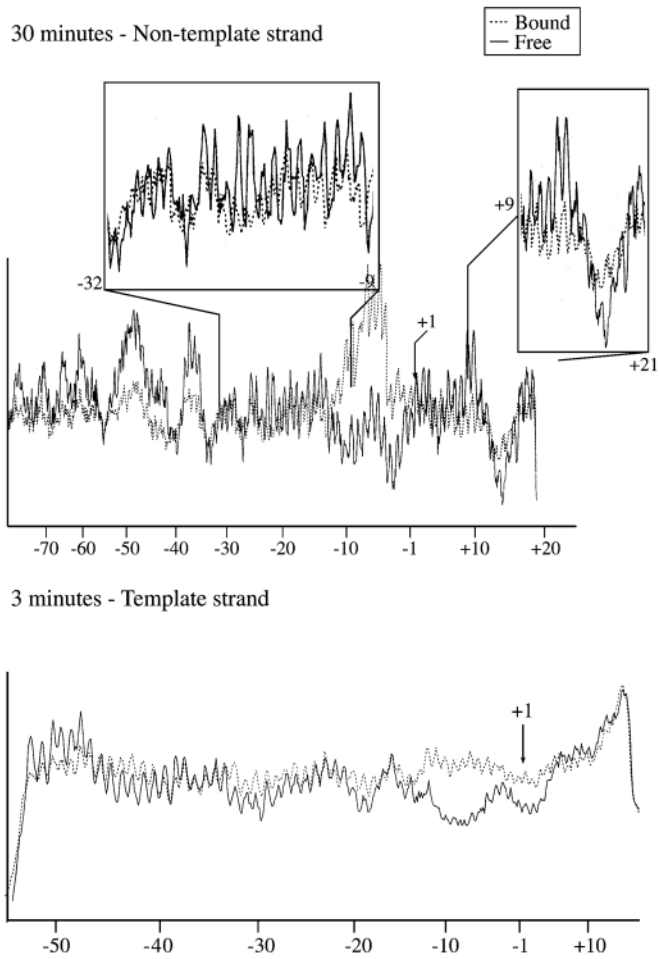

FIG. 4. **Missing nucleoside data at 30 and 3 min.** DNA fragments were modified as described under "Materials and Methods." 0.5–2.5 nM end-labeled, hydroxyl radical-modified DNA fragment containing the *lac*UV5 promoter was incubated with RNA polymerase (100 nM) at 37 °C and challenged with heparin (20 ng/μl) for 1 min, and then the bound and free DNA molecules were separated and analyzed. The densitometric curves presented were generated using ImageQuant. Data are shown for the nontemplate strand for a 30-min incubation and the template strand for a 3-min incubation. *Dashed lines* indicate the cleavage patterns for DNA molecules from the free population. *Solid lines* indicate the cleavage patterns for DNA molecules from the bound population.

to enhanced flexibility and/or disruption of the helix in this region, leading to stabilization of the RNA polymerase-promoter DNA complex and nucleation of DNA melting. Enhanced flexibility and/or helix disruption could contribute to the enhanced binding of RNA polymerase to the *lac*UV5 promoter upon nucleoside loss between −10 and +5. This model is supported by the lack of strand bias; base removal has similar effects for either strand between −10 and +5.

It is not difficult to imagine that the loss of one partner in a base pair interaction in the melting region might decrease the energy input required to separate the DNA strands at the start site. Further, the generation of two additional negative charges in the phosphate backbone could promote strand separation and enhance melting. All of these mechanisms would make the formation of an open complex with that molecule more thermodynamically favorable than for a molecule unmodified in the melting region.

An interesting observation in regard to this stimulation is apparent in a similar analysis of the wild-type *lac* promoter. Although the wild-type *lac* promoter shows some increase in binding with the loss of nucleosides near the P1 start site (like *lac*UV5), the major stimulation of binding occurs with the loss of nucleosides between −26 and −19 (data not shown). The P2 start site of *lac* is at −22. This suggests that destabilization of the P1 melting region will not significantly alter the balance between P2 and P1 distribution for RNA polymerase for the wild-type *lac* promoter sequence. In other words, the destabilization resulting from lost nucleosides does not appear to compensate for a suboptimal promoter sequence. Thus, it appears that nucleoside loss at the melted region and coincident stimulation of open complex formation is a general property of promoters and is not a response to a specific structural component of some σ^{70} and phage promoters.

Phosphate ethylation at the start site can also lead to enhanced open complex formation. Contrary to the missing nucleoside results, the enhancement that results from ethylation is strand-specific; nontemplate strand ethylation is stimulatory, while template strand ethylation is strongly inhibitory (discussed below).

Inhibition—The data that demonstrate an interference with binding fit fairly well with published work as described above. The key difference is that this work expands the extent of positions that are critical for their phosphate contacts and defines the regions where loss of nucleosides alter binding of RNA polymerase at *lac*UV5.

In terms of missing nucleosides, we expected to find that loss of the conserved positions would be the most severely detrimental to binding. However, none of the bases from −12 to −7 were strongly identified by this analysis. *lac*UV5 is a strong promoter, and its −10 hexamer is a perfect match to the consensus. Perhaps this promoter has so many optimal contacts that loss of a single one in any molecule is insufficient to perturb the binding in this assay. It is also possible that the decreased binding energy due to a lost −10 region contact is compensated by facilitated DNA melting of the start site. Alternatively, no specific contacts to the −10 hexamer are absolutely required for binding and open complex formation. This seems unlikely on the basis of 30 years of work with promoter recognition by RNA polymerase, in particular some recent work that implicates specific contacts between σ^{70} and the nontemplate strand at positions −11 and −12 (20, 21, 23, 24). In fact, Fig. 4 shows that nucleoside loss on the nontemplate strand between −13 and −10 results in a modest decrease in RNA polymerase binding consistent with a role in σ^{70} binding.

For the −35 hexamer, encompassing −36 to −31 at *lac*UV5, positions upstream of −32 appear necessary for binding. This is in line with the work done on T7 A1 (17). Further, numerous positions upstream of −37 extending to beyond −50 as well as nucleosides downstream of +6 (most notably on the nontemplate strand) give evidence of inhibiting binding upon removal. Obviously, RNA polymerase must make contacts beyond the hexamers and spacer DNA, and it appears that the nontemplate strand is more important to these protein-DNA interactions than the template strand.

Regarding the positions of ethylation that interfere with binding, all previously identified positions were again found to be critical to binding (9, 12). On the template strand, the peak of interference around −32 has been more clearly defined to extend from −36 to −31. On the nontemplate strand, the positions of ethylation that have negative effects on binding have been expanded to include sequences from −50 to +15. This pattern of interference indicates that RNA polymerase makes close contact with the DNA backbone over the entire span of the hydroxyl radical and DNase I footprints at *lac*UV5.

Ethylation has relatively similar inhibitory effects on binding for each strand with two notable exceptions: the upstream DNA from −20 to −8 and the start site DNA. Modification of

the nontemplate strand from −20 to −8 leads to significant inhibition of open complex formation (Fig. 3). Template strand ethylation for the same positions has very little effect. However, template strand ethylation at the start site has a large negative effect on open complex formation, while ethylation of the nontemplate strand at the start site is modestly stimulatory. These two examples of strand specificity of ethylation effects support a model of RNA polymerase-promoter interaction requiring close protein-DNA associations between the σ subunit and the nontemplate strand upstream of the start site and between the template strand near +1 and the active site of RNA polymerase (20, 21, 23, 24).

Conclusions—A number of the observations from this work offer new information for the *lac*UV5 promoter. Most striking, nucleoside loss on either strand between −10 and +5 increases the likelihood of RNA polymerase forming an open complex on that molecule. These results indicates that individual bases at the start site do not play a major informational role in the process of RNA polymerase recognition and binding to promoters. This observation appears to be a general phenomenon of RNA polymerase-promoter DNA interactions.

Both nucleoside loss and ethylation of certain positions of the nontemplate strand −10 hexamer region result in inhibition of open complex formation. These results support the role of the nontemplate strand in protein-DNA interactions with the σ^{70} subunit.

Ethylation of the template strand, but not the nontemplate strand, near the start site leads to significantly decreased open complex formation. This result supports the role of template strand DNA in an interaction with the active site of RNA polymerase.

Finally, we have extended the scope of positions that are subject to ethylation interference of binding. The pattern spans a range similar to the coverage of DNA by RNA polymerase in various footprinting assays.

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