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High-Resolution Analysis of *Lac* Transcription Complexes inside Cells†

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ABSTRACT: A new primer extension analysis is used to determine the methylation pattern over the *lac* UV5 promoter when dimethyl sulfate is added to growing *Escherichia coli*. The high-resolution analysis reveals altered methylation of 15 bases when the transcription machinery occupies the promoter inside the cell and shows a striking dichotomy in the distribution of methylated bases. Four protected guanosines lie on the side of the helix shown previously to be closely bound by RNA polymerase in vitro [Siebenlist, U., Simpson, R. B., & Gilbert, W. (1980) *Cell (Cambridge, Mass.) 20*, 269–281]. By contrast, the 11 hyperreactive bases lie on the side of the DNA directly opposite from that bound by protein. Those not in the melted region form two distinct "back-side" patches near −35 and −16. We suggest that such hyperreactive patches can be caused by proteins bending the DNA toward themselves to allow a full range of contacts, thus distorting the helix grooves on the "back" side and facilitating attack by the methylating reagent. This leads to a proposal for the formation of transcription complexes in which RNA polymerase interacts with deformed and torsionally stressed DNA.

The characterization of transcription complexes at promoters is a major goal of molecular biology. Much valuable information has come from the study of transcription systems reconstructed in vitro from isolated components [for references to the *lac* system, see Meiklejohn & Gralla (1985)]. One potential problem in this approach is that one can never be certain that all of the important components have been included or that the in vitro conditions reflect the milieu of the cell. In recognition of this potential problem, several recent reports describe methods aimed at studying the state of regulatory DNA in vivo (Becker & Wang, 1984; Nick & Gilbert, 1985). These methods involve isolating DNA from cells containing nicks or modifications introduced in vivo and mapping these relative to nearby restriction cleavage sites.

Recently we described a new method of DNA footprinting in vitro that does not require the close proximity of restriction enzyme cleavage sites or blotting techniques (Gralla, 1985). In addition to yielding especially high-resolution data and being particularly rapid, this technique allows the simultaneous probing of many regions of a DNA sample. DNA samples are probed by primer extension, with the synthetic primer sequences chosen to flank regions of interest. When applied to the interaction of RNA polymerase with supercoiled (i.e., torsionally stressed) *lac* DNA in vitro, this technique confirmed the importance of DNA supercoiling in certain reconstructed systems [see Borowiec & Gralla (1985a,b) for a discussion]. The attacking reagent used in the initial study, DNase I, does not reveal the intimate contacts between protein and DNA as do the chemical probes of DNA developed by Gilbert and colleagues [see Siebenlist et al. (1980)]. We have now adapted the primer extension method to allow the use of these and other probes in vivo or in vitro on supercoiled DNA with single nucleotide resolution.

Materials and Methods

Materials. Escherichia coli proteins RNA polymerase, *lac* repressor, and CRP were all purified in this laboratory [see Meiklejohn & Gralla (1985) for references]. CRP (catabolite activator protein) was a gift of A. Meiklejohn. *Lac* plasmids carried a 207 base pair (bp) fragment containing a *lac* control region inserted into the *Eco*RI site of pAS21 (a modified pBR322 plasmid; Stefano et al., 1980). Commercial sources were used for Klenow fragment of DNA polymerase (BRL) and pBR322 sequencing primers (*Hin*dIII, top strand primer; *Eco*RI, bottom strand primer; Pharmacia P-L Biochemicals), which can be extended through the *lac* insert.

In Vitro Methylation. Lac plasmid DNA (0.5–2.5 μg) was diluted into 100 μL of transcription buffer containing 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 0.2 mM dithiothreitol,

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and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The solution was warmed to 37 °C and dimethyl sulfate added from a freshly diluted 150 mM stock to give a final concentration of 10 mM dimethyl sulfate. Incubation continued for 5 min at 37 °C, and the reaction was quenched by addition of 2 volumes of 3 M ammonium acetate, 1 M β-mercaptoethanol, 20 mM EDTA, and 100 μg of carrier tRNA/mL and precipitated with ethanol. Regulatory proteins were added with slow dilution to the final nominal concentrations of 100 nM *lac* repressor, 50 nM CRP plus 200 μM cAMP, and 25 nM RNA polymerase plus 3.25% glycerol and 100 μg of bovine serum albumin/mL.

For experiments using rifampicin challenge, RNA polymerase was first prebound to *lac* p^s/pAS21 templates. ApA and UTP were added to final concentrations of 500 and 10 μM, respectively, and abortive initiation was allowed for 2.5 min at 37 °C. Rifampicin was added to give a final concentration of 200 μg/mL, the mixture incubated for 2.5 min at 37 °C, and methylation performed as described previously.

In Vivo Methylation. *Lac* plasmids were transformed into RecA⁻ *E. coli* strain HB101. Overnight incubations of cells (10 mL) were grown at 37 °C in LB media and diluted 1:100 into 10 mL of LB media the next morning. Methylation experiments were begun when the cellular OD_{660} reached 0.5–1.25. Isopropyl β-D-thiogalactoside (IPTG), when desired, was added to give a final concentration of 500 μM and incubation continued an additional 5 min. When rifampicin was used, 40 μL of a 50 mg/mL rifampicin solution (in methanol) was added to give a final concentration of 200 μg/mL and incubation continued 30 min. At the completion of IPTG and rifampicin treatments, dimethyl sulfate was added from a 10.6 M stock to give a final concentration of 10 mM and incubation continued for 5 min. All cell growth and incubations were at 37 °C with vigorous shaking. At the end of incubation with dimethyl sulfate, cells were rapidly chilled and then collected by centrifugation in an SS34 rotor at 8000 rpm/min for 5 min at 4 °C. The cells were lysed with heat essentially according to Holmes and Quigley (1981), and plasmid DNA was precipitated by using 0.1 volume of 3 M sodium acetate and an equal volume of 2-propanol. The plasmid DNA was brought up in 100 μL of 10 mM Tris-HCl (pH 8.0)/1 mM EDTA (TE) and treated with RNase (0.5 mg/mL for 30 min at 37 °C) and Pronase (1 mg/mL for 20 min at 37 °C). The plasmid DNA was then twice extracted with an equal volume (100 μL) of neutralized, redistilled phenol. The phenol layers were combined and back-extracted with 75 μL of TE. Aqueous layers were combined and extracted with equal volumes of phenol/chloroform/isoamyl alcohol (25:24:1), until the interface was clear, and precipitated with ethanol.

DNA Cleavage, Primer Extension, and Gel Electrophoresis. Plasmid DNA was cleaved with 1 M piperidine following a standard "G-only" DNA sequencing reaction (Maxam & Gilbert, 1980); the reaction was desalted and ethanol precipitated. The primer extension reaction was essentially that of Gralla (1985). Samples were precipitated and electrophoresed on DNA sequencing gels (6% polyacrylamide) according to standard procedures (Maniatis et al., 1982).

RESULTS

The primer extension probing technique is outlined in Figure 1. A DNA modification or cleavage reagent is added to cells or, alternatively, to DNA in vitro. The modified or cleaved DNA is deproteinized, and if necessary, the DNA is broken chemically at those points weakened by modification. Samples of the broken DNA are denatured and annealed separately to ^{32}P end-labeled synthetic oligonucleotide primers (Gralla, 1985). Large fragment of DNA polymerase I (Klenow) then extends the primers until a breakpoint is reached. The primer sequences are chosen to be near a target region, and the extension products mark the breakpoints within this region. A DNA sequencing gel of the radioactive extension products will show bands whose length represents the distance from the 5′ end-labeled terminus of the primer to the cleavage position. If factors were originally associated with the DNA, then the altered accessibility of this region to attack would change the pattern of breakage, resulting in a footprint. Different DNA regions may be probed in parallel using samples of the same DNA preparation by choosing appropriate primers flanking each desired target in the DNA.

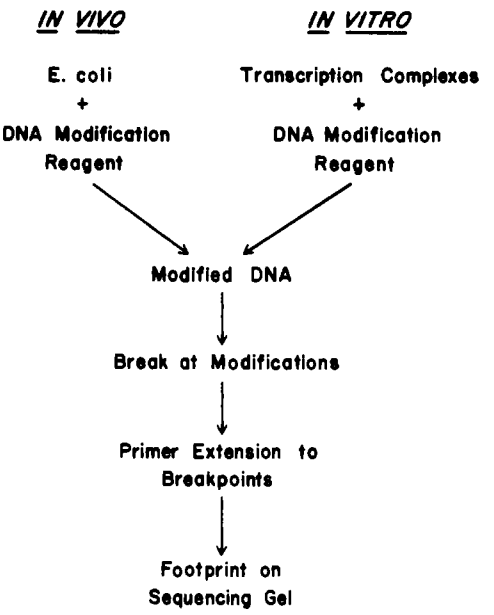

FIGURE 1: Schematic of primer extension protocol. See text for details.

Detection of Protected, Hypersensitive, and Melted Positions in Vitro. To probe intimate contacts between DNA and transcription factors, we use the methylating agent dimethyl sulfate. The in vitro recognition of *lac* DNA was examined by the primer extension procedure to allow comparison with in vivo observations. The promoter chosen was the CRP-independent *lac* p^s promoter, and Figure 2A,B shows the points of dimethyl sulfate attack on this template. Linear plasmid DNA containing the *lac* p^s promoter was methylated and broken at methylated guanosines by using the G-only cleavage of Maxam and Gilbert (1980). The samples were split and probe separately with two flanking primers, chosen to reveal breakpoints on the two DNA strands in the *lac* region. Figure 2A reveals the methylated guanosines on the template ("bottom") strand and Figure 2B those on the nontemplate ("top") strand. The control pattern obtained in lane 1 indicates the cleavage pattern of *lac* DNA methylated in the absence of proteins. The dark bands represent guanosines (marked by dots) in the known sequence and exhibit variations in intensity due to differences in intrinsic reactivity at various positions.

Figure 2 shows the changes in individual band intensities induced by the binding of the three proteins which recognize *lac* regulatory sequences. The guanosines whose reactivity is altered by binding of repressor (lane 2) within the *lac* primary operator [termed O_1 in Meiklejohn & Gralla (1985)] correspond to those reported previously from experiments using end-labeled restriction fragments (position 17 on the bottom strand in Figure 2A and positions 5, 7, 9, and lesser effects at positions 11 and 12, on the top strand in Figure 2B; Ogata

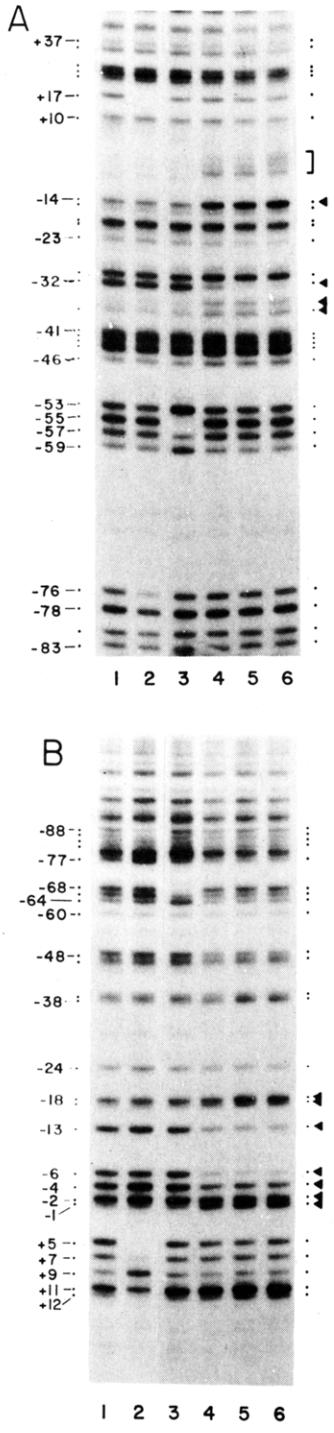

FIGURE 2: Binding of control proteins to the *lac* promoter region in vitro. *Lac* p^s/pAS21 DNA was methylated in the absence of proteins (lane 1) or in the presence of *lac* repressor (lane 2), CRP plus cAMP (lane 3), RNA polymerase (lanes 4, 5), or RNA polymerase in the presence of ApA, UTP, and rifampicin (lane 6). Linear templates were used in lanes 1–4, and supercoiled templates were used in lanes 5 and 6. After methylation and cleavage, primer extension was performed with either *Eco*RI or *Hin*dIII pBR322 sequencing primers to give bottom strand (A) or top strand (B) methylation patterns, respectively. Bands corresponding to G's are indicated by dots on both sides of figure, with various sequence positions listed on left side of figure. Significant changes in methylation pattern caused by RNA polymerase binding are indicated by arrowheads on right side of figure or through the use of a bracket to indicate hypersenswitive region in bottom strand pattern. RNA polymerase induced changes observed are as follows (P, protection; E, enhancement): bottom strand, −14 (E), −32 (P), −35 (adenosine, E), −37 (E) (bottom strand hypersites are observed at positions −4 through −8); top strand, −1 (E), −2 (P), −4 (P), −6 (P), −13 (P), −17 (E), −18 (E).

& Gilbert, 1979). Note also the repressor-induced protection of guanosine at position −76 and slight protection at −78 of the bottom strand and strong enhancement at the top strand guanosine at −77. These changes are within the *lac* pseudooperator which is centered near position −82. Lanes 3 of Figure 2A,B show the guanosine reactivity changes accompanying the binding of CRP–cAMP (−53, −55, −57, −59 on the bottom strand and −64 and −68 on the top strand). These are as deduced previously (Simpson, 1980) using end-labeled restriction fragments.

A comparison of lane 4 with lane 1 in Figure 2A,B shows changes induced by RNA polymerase binding. These differ somewhat from results obtained previously from experiments involving cleavage of end-labeled restriction fragments (Johnsrud, 1978). The changes are distributed on both strands throughout the promoter region and are catalogued in the legend to Figure 2, as well as being marked on the right side of the figure. One observed feature is quite novel and requires explanation. The bracket to the right of Figure 2A marks a region where RNA polymerase induces certain non-guanosine sequences to be methylated and appear as bands on the gel. These constitute a hypersensitive region on the bottom strand extending from −4 to −7 or −8, encompassing the sequence (from −4 toward −8) CACAA. Since the DNA cleavage protocol is designed to break efficiently only at methylated guanosines, the appearance of bands corresponding to methylated adenosines and cytosines is unexpected.

This region is known to be specifically melted by RNA polymerase on *lac* DNA (Siebenlist et al., 1980; Kirkegaard et al., 1983; Carpousis & Gralla, 1985); thus, the observed hypersensitive site represents methylation of melted DNA. Recent experiments of Inoue and Cech (1985) provide a possible explanation for why the primer extension footprinting protocol shows bands corresponding to this melted DNA region. Those investigators found that primer extension using reverse transcriptase on methylated RNA templates was specifically blocked by adenosines and cytosines methylated at Watson–Crick base pairing nitrogens and used this observation to map unpaired regions in RNA. Apparently, this elongation blockage occurs under our primer extension conditions as well. Thus, melted adenosines and cytosines can appear as hypermethylated sites in the protocol if they are susceptible to dimethyl sulfate attack at their Watson–Crick hydrogen bonding positions. It is worth noting that, in vitro, no unpaired adenosines or cytosines are induced by CRP or repressor, confirming that these proteins do not detectably melt DNA.

The introduction of DNA supercoiling to the *lac* p^s promoter has been shown to have significant effects on the rate at which RNA polymerase can form active transcription complexes with the promoter (Borowiec & Gralla, 1985a). We wished to examine if DNA supercoiling caused any significant changes on RNA polymerase–promoter contacts as probed via dimethyl sulfate. In contrast to the action of DNase I, dimethyl sulfate does not initially cleave the DNA strands so that any changes which require the constant application of superhelical energy may be revealed by the dimethyl sulfate probe.

Lane 5 of Figure 2A shows one significant change upon the introduction of supercoiling. A guanosine within the conserved −35 promoter element (−32 on the bottom strand) is seen to have very strong polymerase protection. This residue was partially protected on linear DNA (compare lane 4 with lane 1). DNA supercoiling appears to strengthen the polymerase–DNA interaction in the −35 region, firmly excluding attack by dimethyl sulfate at position −32. The effect of DNA

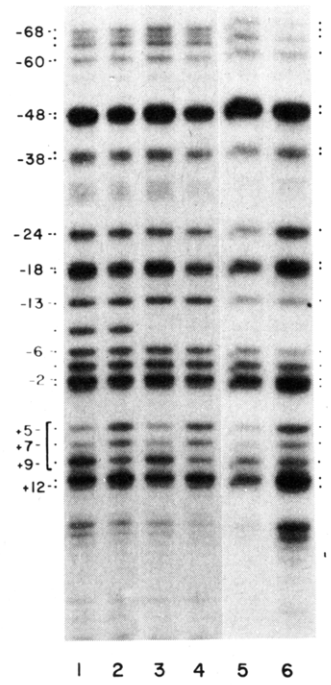

FIGURE 3: *Lac* repressor binding in vivo. Methylation was performed on uninduced (lanes 1, 3, and 5) or induced (lanes 2, 4, and 6) cultures of *E. coli* containing *lac*/pAS21 plasmids. The following *lac* promoters were examined: L157 (lanes 1 and 2), L157:p^s (lanes 3 and 4), and L8:UV5 (lanes 5 and 6). DNA was isolated as given under Materials and Methods and probed with top strand primer. Guanosines are indicated as in Figure 2. The bracket shown on the left side of the figure indicates changes observed upon induction of cultures.

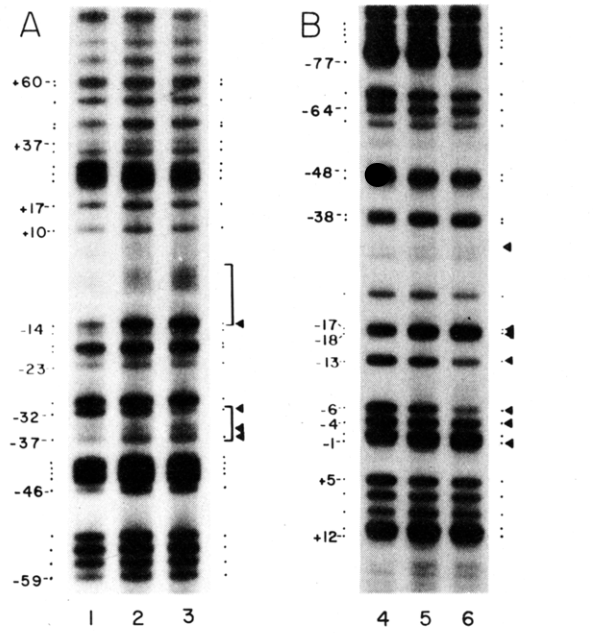

FIGURE 4: Interactions at the promoter in vivo. Control L8:UV5/pAS21 DNA methylated in vitro is shown in lanes 1 and 4. Induced cultures of *E. coli* transformed with L8:UV5/pAS21 were methylated in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of 200 μg of rifampicin/mL. DNA was isolated as given under Materials and Methods and probed with bottom strand (A) or top strand (B) primers. Guanosines are indicated as in Figure 2. Brackets to the right of panel A indicate two regions of RNA polymerase induced changes in the methylation pattern (see text for details). Individual bases at which the most significant changes were observed are indicated by arrowheads to the right of each panel.

supercoiling on the binding of CRP and repressor to the *lac* control region was also examined. No changes in the binding of these proteins to the CRP site or O_1 operator were seen, although DNA supercoiling caused a significant increase in the affinity of the *lac* repressor for the pseudooperator (not shown). This latter phenomenon is currently being investigated more thoroughly.

In Vivo Recognition of the Lac Promoter–Operator. Next, having established the high-resolution map in vitro, we applied the primer extension technique to *lac* regulatory DNA carried on plasmids inside growing *E. coli*. A small bacterial culture was divided in half, and one sample received the artificial *lac* inducer IPTG. Dimethyl sulfate was added to both cultures, and a few minutes later, the cells were harvested and lysed. A crude plasmid DNA fraction was isolated from the supernatants, and this DNA was broken by using the G-only cleavage reaction of Maxam and Gilbert (1980). It was then denatured and probed for breakpoints using primer extension. As an initial control, the weak L157 *lac* promoter variant was used under repression conditions to minimize promoter occupancy by RNA polymerase (L157 has a point substitution at −32 which weaknes the promoter substantially; Maquat & Reznikoff, 1978). The top strand primer was used initially since it should most easily reveal a repression complex by probing the characteristic triplet of guanosines contacted by repressor in vitro (see Figure 2B, lanes 1 and 2).

Figure 3 shows the cleavage pattern obtained by using L157 DNA isolated from uninduced (lane 1) and induced (lane 2) cells. The two patterns may be compared band-by-band and are identical except for the triplet of strong guanosine changes at positions 5, 7, and 9 within the *lac* operator (bracketed to the left of the figure). Comparison with the *lac* repressor interaction patterns in vitro (Figure 2) confirms that the uninduced culture has repressor bound to operator and the induced culture does not [see also Becker & Wang (1984) and Nick & Gilbert (1985)]. The experiment was repeated with the slightly stronger L157:p^s *lac* promoter (Ackerson & Gralla, 1983) with a similar result (compare lanes 3 and 4; a guanosine is replaced in the −10 region of the UV5 and p^s promoters, and this results in a missing band). The strength of these interactions is somewhat surprising since the operators are contained on plasmids with a copy number comparable to or greater than the number of *lac* repressor molecules inside cells. This high sensitivity in detecting *lac* operator occupancy suggests that this method will be useful in testing proposals which relate the occupancy of DNA sites in other operons to transcription.

Finally, the above experiment was repeated with cells transformed with the very strong promoter variant *lac* UV5 in order to learn whether RNA polymerase can now occupy the promoter (Figure 3, lanes 5 and 6). Again, the operator triplet changes after *lac* induction from that of repressor bound to repressor free (compare lane 5 with lane 6). Now, however, the induced pattern differs from that of the weak L157 promoter in regions away from the operator and begins to resemble more closely the polymerase-bound DNA in vitro (compare with Figure 2, lanes 4 and 5).

The altered reactivity of bases associated with the induced *lac* UV5 promoter was investigated at high resolution on both strands in the experiments shown in Figure 4. Each half of the figure compares the guanosine reactivity on naked DNA in vitro (lanes 1 and 4) with the reactivity of induced promoter DNA inside cells (lanes 2 and 5). Also shown is the pattern observed when cells not only were induced but also were treated with the drug rifampicin (lanes 3 and 6). Although rifampicin blocks transcription initiation (but not abortive initiation), it does not disrupt open promoter complex in vitro (Sippel & Hartmann, 1968; Carpousis & Gralla, 1985). This is confirmed by a control shown in Figure 2, lane 6, in which

RNA polymerase–DNA complexes formed in vitro were challenged with rifampicin and nucleoside triphosphates to allow abortive initiation prior to primer–extension footprinting. The pattern observed was identical with that from unchallenged open promoter complexes. Therefore, the use of rifampicin in vivo might be expected to simply increase occupancy of the promoter in the cell.

The data in Figure 4 reveal protected and hyperreactive bases and melted DNA caused by trapping RNA polymerase at the *lac* UV5 promoter inside *E. coli*. First, consider the result on the template strand. Comparison of lane 1 with lane 3 reveals two principal regions in which polymerase has caused altered methylation. These are bracketed to the right of the figure and include the two major conserved sequence elements in *E. coli* promoters; the −4 to −14 region includes the −10 promoter element, and the −32 to −37 region includes the −35 promoter element. The former region includes the series of hyperreactive adenosines and cytosines which marked melted promoter DNA in vitro. Additionally, it includes a hyperactive guanosine at position −14. The −35 region includes a strongly protected guanosine at −32 and somewhat enhanced reactivity at guanosine −37 and adenosine −35. It is interesting that the protection at −32 is strengthened by rifampicin treatment; recall that this is the same protection which was strengthened by DNA supercoiling in vitro.

Numerous changes can also be seen in the nontemplate strand by comparing lane 4 with lane 6. These include strong guanosine protections at −6 and −13 within the same −4 to −14 region which showed altered reactivities on the other strand. Weaker guanosine protection within this region is observed at position −4. Hyperreactive residues are also observed at guanosines −1, −17, and −18 and at adenosine −33; the latter change was not caused by RNA polymerase binding in vitro. Although there are minor differences in the reactivity pattern caused by polymerase binding in vivo and in vitro, particularly in the −35 region, the major changes are in identical positions. However, as discussed below, these differ somewhat from previous results in a way that leads to a new interpretation of the molecular basis for the altered pattern of dimethyl sulfate attack. Moreover, the in vivo experiments do not require the difficult taks of isolating the components and optimizing their interactions in vitro. We hope that the technique will allow high-resolution probing of DNA binding sites for proteins which have not yet been purified. This can be accomplished with primers designed to be complementary to sequences flanking these DNA sites.

Discussion

Interaction Map at the Promoter in Vivo. Figure 5A displays altered base reactivities to dimethyl sulfate attack caused by occupancy of the *lac* UV5 promoter inside the cell. The changes observed include 4 guanosines protected from attack and 11 bases with enhanced reactivity. Some of these correspond to changes seen in preliminary experiments by Nick and Gilbert (1985). The high-resolution picture is made possible, in part, by the primer extension protocol which does not rely on blotting or the close proximity of restriction sites and is therefore limited principally by the resolution of DNA sequencing gels.

Five of the hyperreactive residues lie on the template strand between −1 and −8 in a region known to be made hyperactive by RNA polymerase melting of *lac* DNA in vitro (see Results). Thus, we conclude that these seven base pairs from the minimum region of melted promoter DNA inside the cell. Not all residues on both strands of this region are hyperreactive, most likely because the opposite strand bases are held tightly

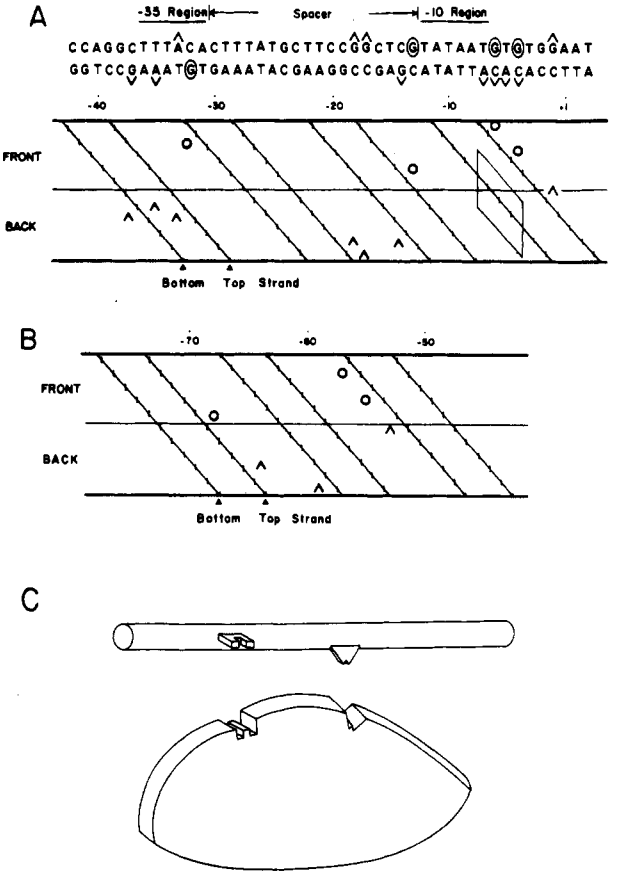

FIGURE 5: Helix maps of RNA polymerase and CRP interactions with *lac* promoter DNA and proposed model for initial recognition of the promoter by RNA polymerase. The DNA helix (10.5 base pairs, turn) is shown in a planar representation such that the helix has been cut along a single edge, unfolded, and drawn flattened. Diagonal lines represent sugar–phosphate backbone with tick marks being phosphates. (A) Helix map of RNA polymerase/promoter interactions in vivo (from Figure 4). The sequence of the *lac* L8:UV5 promoter is shown in register with the helix map; enhancements of methylation are shown by carets, and protections are shown by ovals or circles. The changes in guanosine methylation are shown in the major groove, and adenosine changes are shown in the minor groove (Siebenlist et al., 1980). A diamond enclosing bottom strand sequences −4 to −7 indicates that these hypermethylations may occur by attack from either side of the DNA. (B) Helix map of CRP site interactions in vitro (from Figure 2). Protections and enhancements are as indicated for panel A. (C) Cartoon illustrating why the DNA must be distorted as part of promoter recognition by RNA polymerase [modified from Stefano & Gralla (1982b)]. The DNA is proposed to bend and twist to allow simultaneous contact of the −10 and −35 elements to their recognition sites on the enzyme. This would allow formation of an intermediate "stressed" complex (not shown). This stress could be partly relieved by DNA melting to form the open complex. The hyperreactive methylation patches may then represent the residual more modest bending which remains in the open promoter complex.

by polymerase and are thus made inaccessible to attack [see Carpousis & Gralla (1985) for a discussion].

When all of the 15 changes observed are displayed on a helix map of DNA (Siebenlist et al., 1980), a striking dichotomy is observed. That is, the four protected positions define one "face" of the promoter helix, and the hyperreactive residues define the opposite face (Figure 5A). The protected residues all lie within the helical face shown by ethylation–interference experiments to interact with RNA polymerase during the process of open complex formation in vitro (Siebenlist et al., 1980), and presumably defines the final helical face to which the RNA polymerase is bound. However, the hyperreactive residues occur in three distinct patches which encompass the

back side where protein should be absent. One, as just discussed, corresponds to the melted region, and the other two are centered near −35 and −16. Since these hyperreactive pathces define a helical face which is opposite to the face used during binding, the proposal that enhancements result from dimethyl sulfate collecting at hydrophobic patches at the enzyme–DNA interface (Johnsrud, 1978; Siebenlist et al., 1980) seems inadequate to explain the observation. Instead, we seek an alternative molecular explanation for increased susceptibility to attack by dimethyl sulfate.

One potential clue to understanding the hyperreactivity phenomenon may be seen by inspecting the altered dimethyl sulfate reactivity due to binding CRP protein to the promoter in vitro (Siebenlist et al., 1980; Simpson, 1980; Figure 2 above). When CRP binds to this site, it closely approaches one side of the helix (Siebenlist et al., 1980) and causes the bound DNA to bend slightly (Kolb et al., 1983; Wu & Crothers, 1984). Figure 5B reveals that the CRP–DNA complex also shows the dichotomy of protected residues on the bound face of DNA and hyperreactivity on the back face. In this case, we suggest that the hyperreactivity is a consequence of the DNA bending slightly to contact CRP closely, thus opening the back of the helix somewhat and allowing dimethyl sulfate increased access to the reactive edges of the bases in the major groove.

Therefore, we propose that patches of hyperreactivity to dimethyl sulfate can mark distortions in the helix which facilitate entry of the attacking reagent and interpret the in vivo RNA polymerase interactions in this manner. Thus, we infer that as part of RNA polymerase binding, the DNA bends somewhat near −35 and −16 to contact the enzyme more closely. This would open the back side of the DNA to facilitate dimethyl sulfate attack, resulting in the two observed patches of hyperreactivity. Helix distortions involving untwisting in this region during promoter binding have previously been proposed as essential to promoter recognition in vitro (Stefano & Gralla, 1982b; Borowiec & Gralla, 1985a).

This leads to a proposal for the molecular pathway of formation of the open promoter complex. RNA polymerase initially cannot simultaneously interact with both −10 and −35 promoter elements (Figure 5C; Stefano & Gralla, 1982b). Promoter DNA must bend and untwist to allow the polymerase more complete contact with both consensus elements. Though the changes in the DNA helix now allow closer RNA polymerase–DNA contacts, the DNA becomes distorted or "stressed" in the process. This stress can be partly relieved in either of two ways: (1) by dissociation of this stressed complex to the closed complex or free components or (2) by melting the DNA in the −10 region to form the open complex. The hypersensitive sites observed may be due either to a small amount of residual stressed complex or to residual stress in the open complex itself.

The existence of this stressed promoter complex is consistent with very recent studies of the pathway of open complex formation. The open complex of the *lac* UV5 promoter has been found to be in equilibrium with a second complex (Straney & Crothers, 1985; Spassky et al., 1985). Kinetic studies on the *lac* UV5 promoter have led independently to the proposal that there is an unmelted intermediate between the "closed" and open complex (Buc & McClure, 1985). Since opening the intermediate complex is fast at 37 °C (Buc & McClure, 1985), energy in a polymerase–promoter complex must be used to overcome the substantial barrier involved in unwinding 17 base pairs of DNA (Gamper & Hearst, 1982). We suggest that this energy is provided by the torsional and bending energy associated with a stressed intermediate complex, which is transiently stabilized by extensive contacts between the enzyme and the −10 and −35 regions of the promoter.

The introduction of negative DNA supercoiling into the DNA template was shown to lead to strengthened interactions between RNA polymerase and the *lac* p^s promoter (Figure 2) and also to cause a very large (40-fold) increase in the rate of open complex formation at this same promoter (Borowiec & Gralla, 1985a). Topological considerations indicate that processes which cause the DNA to become unwound are energetically favored by negative DNA supercoiling. The primary mode by which DNA supercoiling increases the open complex rate would therefore be expected to lie in stimulating the melting of the DNA helix. However, we have proposed previously that supercoiling also affects the ability of polymerase to recognize unmelted promoter DNA, based on the observation that high superhelicity slows the rate of open complex formation (Borowiec & Gralla, 1985a). Thus, supercoiling may induce DNA conformational changes that strongly influence the ability of the DNA to be recognized and form a metastable stressed complex with RNA polymerase.

The specifics of the hypothetical model of open complex formation lead to a strong prediction. The helical distortions proposed here should be sensed by other DNA-modifying reagents which have limited access to bases buried in the helical grooves and should lead to the appearance of hyperreactive sites at the proposed distortions. More generally, the use of a number of DNA probes in combination with the primer extension protocol appears to be a powerful technique to describe the state of regulatory DNA inside the cell.

Registry No. RNA polymerase, 9014-24-8.

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A 10S Particle Released from Deoxyribonuclease-Sensitive Regions of HeLa Cell Nuclei Contains the 86-Kilodalton–70-Kilodalton Protein Complex†

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ABSTRACT: Digestion of HeLa cell nuclei with micrococcal nuclease or deoxyribonuclease I (DNase I) released the 86-kilodalton–70-kilodalton (kDa) protein complex in particles sedimenting at approximately 10 S in sucrose density gradients. Immunoaffinity-purified ^{32}P-labeled complexes contained 86- and 70-kDa polypeptides with phosphorylated serine residues and DNA fragments, of which the largest was 110 base pairs long. Digestion of nick-translated nuclei with micrococcal nuclease released ^{32}P-labeled 10S particles that were immunoaffinity-purified; they contained labeled 110-base-pair DNA fragments. The micrococcal nuclease digests were analyzed by two-dimensional electrophoresis, which separated nucleosomes in the first dimension and the associated proteins in the second. Western blots of the separated proteins showed that the 86-kDa–70-kDa complex was associated with the mono-, di-, and trinucleosomes. A more extensive electrophoretic separation revealed that the 10S particle from nick-translated nuclei migrated with a subfraction of the mononucleosomes that lacked H1 histones. These results suggest that the 10S particle which contains the 86-kDa–70-kDa complex is associated with an unfolded nucleosome that is present in DNase I sensitive regions.

Recently, monoclonal antibodies were developed in our laboratory that recognized a polypeptide of M_r 86 000 and a p*I* 6.0 (Yaneva et al., 1985a). With these antibodies, the antigen was immunoaffinity-purified and shown to be in a complex of two polypeptides of M_r 86 000 and M_r 70 000. The release of this complex by treatment of nuclei with DNase I and the reduction of nucleolar immunofluorescence by treatment with the DNase I suggested that this antigen was a DNA-binding nonhistone protein (Yaneva et al., 1985a). Preliminary immunofluorescence studies with synchronized HeLa cells showed cell cycle variations in the pattern of distribution of the antigen within the nucleus and nucleolus (Zweig et al., 1984). Reeves (1985) has recently identified a similar antigen localized to the nucleus with monoclonal antibodies to HeLa nuclei. This antigen is a DNA-binding, nonhistone protein that is recognized by a number of sera from patients with autoimmune diseases (systemic lupus erythematosus, mixed connective tissue diseases, and scleroderma). An antigen that binds selectively to DNA and consists of 80- and 70-kDa[1] polypeptides was also found by Mimori et al. (1985), who reported that it was recognized by autoimmune sera from patients with rheumatic disorders. It is possible these studies all deal with similar antigens.

This study was done to learn whether the 86-kDa–70-kDa complex was in a particle and if it relates to nucleosomal structures. Although DNA and histones form the basic nucleosome structure (McGhee & Felsenfeld, 1980; Kornberg & Klug, 1981), not much is yet clear about the presence of nonhistone proteins. It has been suggested that nonhistone proteins maintain a special unfolded conformation of the nucleosome to permit transcription and replication (Weisbrod, 1982; Levinger & Varshavsky, 1982); a consequence of this altered conformation is increased DNase I sensitivity of these regions of the chromatin (Weisbrod & Groudine, 1980; Weintraub, 1985). It has been shown (Gazit et al., 1980) that proteins extracted from the cell nucleus with 0.35 M NaCl, particularly HMG 14 and 17, confer DNase I sensitivity (Weisbrod & Groudine, 1980; Weisbrod, 1982a,b). A role

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[1] Abbreviations: kDa, kilodalton(s); Tris, tris(hydroxymethyl)-aminomethane; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; ELISA, enzyme-linked immunoabsorbent assay; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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