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Micrococcal nuclease as a probe for bound and distorted DNA in *lac* transcription and repression complexes

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ABSTRACT

Micrococcal nuclease (MNase) is used to probe the structure of transcription and repression complexes at the *lac* regulatory region *in vitro*. Both the *lac* operator, O_1, and the pseudo-operator, O_3, are found to be protected from MNase digestion by the *lac* repressor on supercoiled DNA, and hypersensitive sites appear on both strands around nucleotide (nt) −26 between O_1 and O_3. This hyperreactive site is coincident with the site of the DNA kink shown previously to form within a loop caused by simultaneous repressor binding to O_1 and O_3. MNase hypersites are also observed both upstream from cAMP receptor protein (CRP) and downstream from bound RNA polymerase in open promoter complexes. In both open and closed complexes the binding of polymerase partially protects the backbone from MNase attack. Catabolite activator protein is shown to be required for both closed and open complex formation. Taken together with previous footprinting data, the results suggest that *lac* transcription complexes involve DNA bent towards a protein core consisting of RNA polymerase and catabolite activator protein.

INTRODUCTION

The structures of several DNA-protein complexes are now known from crystallographic studies. In these cases, a low resolution structure of the bound DNA was inferred from DNA footprinting or probing experiments in solution. When the structure of DNA in the crystal was compared to that inferred from probing studies, the agreement was normally quite good (see 1–3 for example). Since probing studies are rapid and versatile, it is very useful to apply them towards building a solution structure in the many systems where crystallographic data is not yet available.

The ability to build a low resolution structure from such footprinting and probing studies requires a variety of reagents which can sense different aspects of DNA structure. The most widely used probes are the enzyme DNase I (4), which senses interactions in the DNA minor groove (5), and the chemical dimethyl sulfate, which senses close interactions in the DNA major groove (see 6). Many other chemicals have also been used, including ethylnitrosourea (6), ortho-phenanthroline:copper complexes (7), intercalator:EDTA complexes (8), potassium permanganate (9–11), hydroxyl radicals (12) and ultraviolet light (13). The chemical probes have been useful in detecting induced structural perturbations in DNA, in detecting bound proteins that protect DNA from attack, and in interference studies that identify nucleotides whose integrity is critical for protein binding. Their utility in protection studies is somewhat limited by their small size, which often allows them to penetrate DNA-protein complexes to attack the bound DNA; their strong reactivity could also lead to protein modifications that could disturb nucleoprotein complexes. In contrast, enzymes are too large to penetrate complexes easily and react selectively with the DNA.

Although DNase I has been used as a probe for DNA protection by proteins for many years, the extension of footprinting studies to other enzyme probes has been very limited. DNase I is a relatively large molecule which is easily excluded by bound protein from reaching its recognition site, which is in the minor groove of DNA (4). Thus, DNase I footprints generally reveal whether enough protein is near the DNA minor grooves to prevent the entry of the active site of DNase I. DNase I reactivity is also sensitive to changes in the width of the minor groove (5) and thus the DNase cleavage pattern can also be affected by protein-induced changes in DNA structure. It would be very desirable to have available other enzyme probes that sense features of DNA besides those involving the minor groove. For this reason we have explored the possibility of using micrococcal nuclease (MNase) as a footprinting probe of DNA:protein complexes.

MNase is a small protein (M_R = 16,000) which, in contrast to the groove recognition by DNase I, recognizes the phosphodiester backbone of DNA (14,15). It cleaves single-stranded or unpaired regions much more rapidly than double helical regions, presumably because the backbone is more exposed when removed from the constraining environment of a double helix. When guanosines and cytosines are associated with the backbone, cleavage is inhibited (16,17). Thus, MNase is a probing reagent of moderate size that, in principle, could give information about the structure and interactions near the DNA backbone of AT pairs. Thus, it could complement the information about structure and interactions in the minor groove (from DNase I data) and the information obtained from chemical probes. In this paper, we develop MNase as a probe for *lac* transcription complexes and show that in addition to confirming features deduced by previous probing experiments, it reveals interactions not detected previously.

MATERIALS AND METHODS

Materials.

The *lac* plasmid wt/pAS21 carries a 207 base pair fragment, including the *lac* promoter and operator, which replaces the HindIII to EcoRI fragment of pBR322. Two pBR322 sequencing primers, Hind III (5′-GCAATTTAACTGTGAT-3′) and EcoRI (5′-GTATCACGAGGCCCTT-3′), were synthesized at the UCLA Facility; these read into the *lac* insert from pBR322 sequences. Micrococcal nuclease was purchased from Worthington Biochemical Corp. The *lac* repressor and CRP were purified in this laboratory by A. Meiklejohn and J. Borowiec. RNA polymerase holoenzyme was either purified in this laboratory or purchased commercially from Boehringer Mannheim Biochemicals. Klenow fragment was purchased from Bethesda Research Laboratories.

In vitro treatment with micrococcal nuclease.

Reactions generally contained plasmid DNA at a concentration of 14 μg/ml in buffer A (30 mM Tris-HCl, pH 7.5, 100 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.2 mM dithiothreitol, 33 nM EDTA). *Lac* repressor was added from a freshly diluted 5 μM solution (in buffer A) to give a final concentration of 0.2 μM. Catabolite activator protein was diluted to approximately 1 μM in the presence of 1 mM cAMP and added to a final concentration of 100 nM (with 100 μM cAMP). RNA polymerase was diluted from about 5 μM (1U/μl) to 100 nM into buffer A plus 12.5% glycerol and 200 μg/ml BSA and then added to a final concentration of 40 nM. DNA-repressor and DNA-CRP solution (50 μl) were incubated for 30 min while DNA-RNA polymerase (with or without CRP) was incubated for 10 min at 37°C or 0°C before MNase treatment. The solution was treated with approximately 0.05 unit MNase for 2 min at 37°C or 24 units at 0°C, and then the

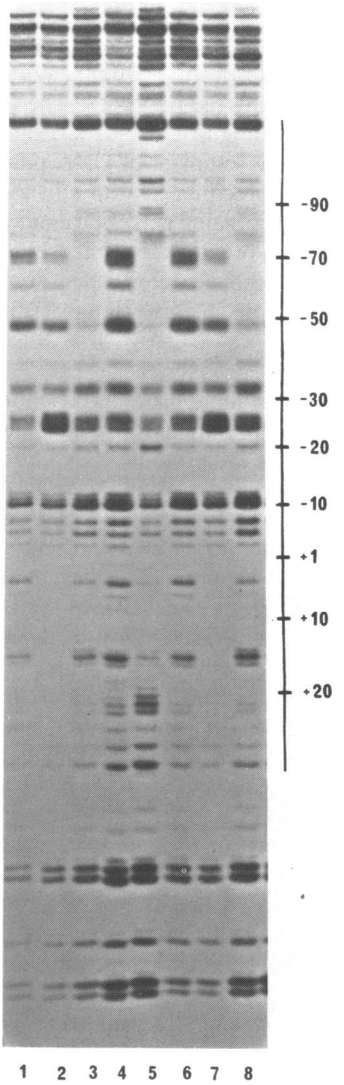

Figure 1. Micrococcal nuclease digestion pattern of the top strand of the *lac* wt DNA in the presence of regulatory proteins using the Hind III primer. Supercoiled DNA was treated with 0.05 unit of micrococcal nuclease following pre-incubation without proteins (lanes 1 and 6), with *lac* repressor (lanes 2 and 7), with CRP-cAMP (lanes 3 and 8), with RNA polymerase alone (lane 4), or with RNA polymerase and CRP-cAMP (lane 5).

reaction was quenched with 25 μl phenol:chloroform (50:50) solution and 10 μl 50 mM EDTA. The mixture was subsequently heated at 80°C for 5 min and extracted with phenol and chloroform. The DNA was then centrifuged through a pre-spun 1-ml Sephadex G-50−80 column, pre-equilibrated in 0.01M Tris pH 7.5 + 1.0 mM EDTA. The samples were brought to 80 μl for primer extension analysis.

Primer extension analysis.
40 μl of each sample was used for primer extension analysis using the alkaline denaturation procedure described previously (18). Briefly, 300,000 cts/min of end-labeled primer was added and followed by NaOH to bring the pH to 11. The DNA was then denatured by heating to 80°C for 2 min and quenching in an ice bath. Primers were hybridized at 45°C for 3 min and extended with Klenow fragment of DNA polymerase at 50−52°C for 10 min. The DNA samples were then precipitated with ethanol and analyzed by 6% (w/v) acrylamide/50% (w/v) urea DNA sequencing gels. Equal amounts of DNA were processed in parallel samples to obtain patterns of equivalent intensity. When variations in intensity occurred, loading differences were accounted for by densitometry, normalizing intensities with respect to reference bands whose intensity is invariant.

RESULTS

We adapt the primer extension footprinting procedure (18) to use MNase to examine the interaction between the *E. coli* regulatory proteins (the *lac* repressor and the cAMP receptor protein) or RNA polymerase and the *lac* promoter DNA *in vitro*. The *lac* template used is a supercoiled 4540 base-pair plasmid containing the *lac* control region which includes the primary operator (termed O_1) and the upstream pseudo-operator (termed O_3), as well as the *lac* promoter and the binding site for the cAMP receptor protein (CRP). The plasmid does not contain the O_2 operator sequence within the *lac*-Z structural gene (4). In this method, nucleoprotein complexes are formed between the DNA and regulatory proteins and are then cleaved with MNase *in vitro*. The DNA is then purified, denatured and annealed with a ^{32}P-end labeled primer which is extended with the Klenow fragment of DNA polymerase I to the site of cleavage. The extension products are then analyzed on a polyacrylamide DNA sequencing gel to determine the frequency with which MNase cleaved individual positions of the DNA backbone. Comparison with protein-free control reactions allows one to deduce the influence of protein binding on MNase cleavage of the *lac* DNA.

Probing lac repression complexes

We begin by probing the interactions of *lac* repressor with the *lac* DNA template. By using a template containing both the *lac* O_1 and O_3 operators, the ability of MNase to sense two different features of the repression complex can be probed. First, the binding of *lac* repressor to the O_1 and O_3 sites is well characterized by previous probing with other reagents and the sensitivity of MNase to this interaction can be tested. Second, when the DNA is supercoiled, repressor binds simultaneously to O_1 and O_3, causing the looped out DNA between these sites to become structurally distorted (9). This distortion was previously sensed by $KMnO_4$, but not DNase I, and is localized near position −26 in the *lac* promoter. The experiment should also reveal whether MNase can sense this structural distortion.

Figure 1 shows the MNase digestion pattern of the upper strand of the *lac* DNA. Lanes 1 and 6 illustrate the primer extension pattern obtained when MNase is used to treat supercoiled *lac* DNA in the absence of any proteins. The majority of cleavages occur at A and T residues, as expected (sequence not shown). Lanes 2 and 7 of Figure 1 show the digestion pattern of the DNA bound by *lac* repressor. The *lac* O_1 operator is clearly protected from MNase attack, as shown by the strongly diminished cleavage in the region from +24 to −4. Specifically, bands at +24, +22, +21, +15, +7, and +5 are absent in the presence of repressor (compare lanes 6 and 7). This region corresponds exactly with the known *lac* O_1 region. Additionally, in the upper part of the gel, the region from

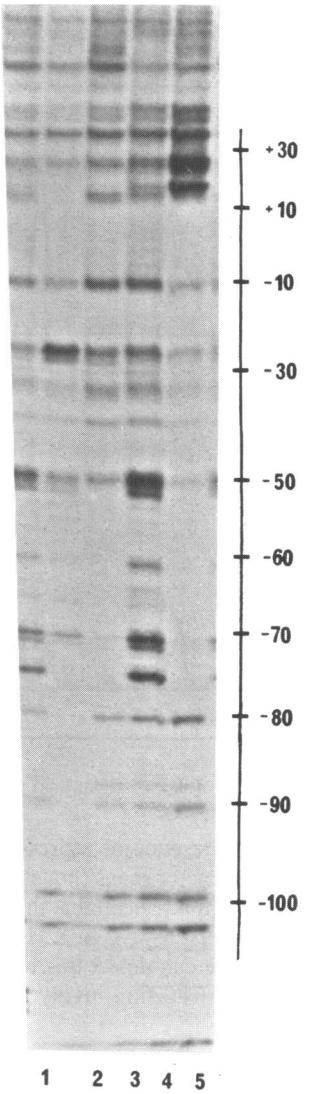

Figure 2. Micrococcal nuclease digestion pattern of the bottom strand of the *lac* wt DNA in the presence of regulatory proteins using the EcoRI primer. Supercoiled DNA was treated with 0.05 unit of micrococcal nuclease following pre-incubation without proteins (lane 1), with *lac* repressor (lane 2), with CRP-cAMP (lane 3), with RNA polymerase alone (lane 4), or with RNA polymerase and CRP-cAMP (lane 5).

−75 to about −90 is partially protected (compare lanes 6 and 7). This region is *lac* 0_3, which is known to be only partly bound by *lac* repressor under these conditions. These results show that MNase probing can be used to reveal protein binding and that the *lac* operator backbone is indeed protected by bound repressor, as expected.

The experiment also shows that cleavages at −25, −26, and −27 are much stronger

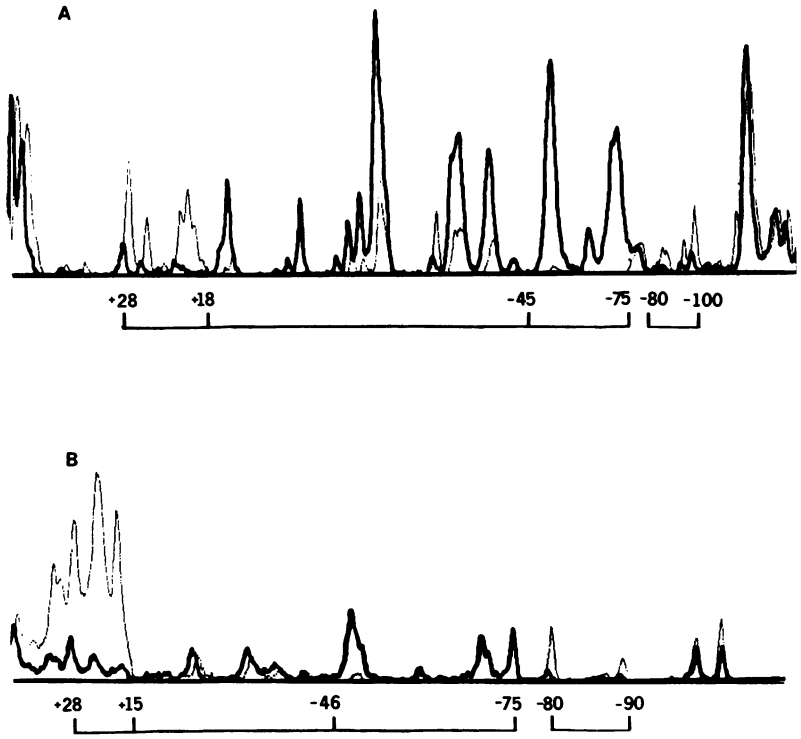

Figure 3. (A) Normalized densitometer scans of lanes 5 (light line) and 6 (heavy line) in Figure 1. (B) Scans of lanes 1 (heavy line) and 5 (light line) in Figure 2.

on this supercoiled template in the presence of repressor than in its absence (best seen by comparing lanes 1 and 2). Densitometer scans (not shown) show that repressor induces a three-fold hyperreactivity of this region. Recall that this is the same region, quite remote from either *lac* operator, that is hypersensitive to $KMnO_4$ attack when a loop is formed between O_1 and O_3. Therefore, MNase can detect this remote DNA distortion induced by repressor binding, implying that the distortion involves increased exposure of the DNA backbone.

These experiments were repeated using a primer which probes interactions on the the other DNA strand (Figure 2). Lane 1 illustrates the primer extension pattern obtained when MNase is used to treat supercoiled DNA in the absence of any proteins. Comparing lane 2 (in the presence of repressor) with lane 1 (no protein) one observes again that the regions around O_1 and O_3, specifically bands at +15, +16, −75, and −80 (also −87 and −89 seen in darker exposures) are protected. The local structural distortion in the loop is also sensed on this strand since nucleotide −26 is hyperreactive in the presence of *lac* repressor. Overall, these results demonstrate that MNase can sense local protein binding to the DNA backbone and also an unusual DNA structure induced in a remote DNA sequence.

Probing CRP-cAMP interaction with DNA

Previous experiments have shown that when CRP-cAMP binds its *lac* recognition site, a tight complex is formed which induces a bend within the bound DNA (19,20). We probed

this complex to determine which features will be sensed by MNase. Lanes 3 and 8 of Figure 1 show the digestion pattern of the complex on the top strand. Comparing these with the control lanes, 1 or 6, the region from −48 to −75 is strongly protected by CRP. Specifically, bands at −49, −50, −61, −69, −70, −71, and −72 are protected. Moreover, the downstream protection around −50 is somewhat weaker than the upstream protection and nucleotide −54 is slightly hypersensitive (more visible in longer exposures; not shown). On the bottom strand (Figure 2), the results also show that the region from −50 to −75, specifically bands at −62, −71, and −75, are protected in the presence of CRP (Figure 2, compare lane 3 to lane 1), while bands around −50 are only partially protected. These results are quite consistent with previous studies, including the observation that CRP binding is stronger at the upstream half-site than at the downstream half-site. The only indication of a DNA bend, however, is the weak hyperreactivity near nucleotide −54 on the top strand. Apparently, the bend does not lead to exposure of a protein-free backbone that can be attacked strongly by MNase.

Probing open transcription complexes

Next, we probed *lac* open transcription complexes involving bound RNA polymerase and CRP-cAMP. Probing of the top strand of open promoter complexes (lane 5 of Figure 1) shows that only a few bands outside the CRP site (−50 to −70) are strongly protected. Instead the entire promoter region is partially protected (compare lanes 5 and 6). These observations are confirmed by the densitometer scan shown in Figure 3A. This figure superimposes scans with (light line) and without (dark line) protein. The peaks at the edges, corresponding to non-promoter DNA, are of similar intensity in the presence and absence of protein. Strong protection is observed within and downstream of the CRP site (−45 to −75). Most of the bands underneath the RNA polymerase are about half protected (−45 to +18). Experiments using a bottom strand probe show somewhat stronger, but still incomplete, protection (compare lanes 1 and 5 in Figure 2 and see scan in Figure 3B). Overall, the open complexes are somewhat more sensitive to MNase attack than DNase I attack, in which the region is very strongly protected (4). This could be because MNase is a smaller protein that can approach the bound DNA more closely. Alternatively, the backbone, sensed by MNase, may be less closely contacted by polymerase than the minor groove, sensed by DNase I. These experiments also confirm that crp-cAMP is required for specific open complex formation (compare lane 4 with lane 5).

The most unusual features of the MNase cleavage pattern are the strong hyperreactive cleavages that accompany open complex formation. First, on the top strand, comparison of lane 5 of Figure 1 with lane 6 shows RNA polymerase-induced hyperreactivity of residues +20, +21, and +22. Less striking, but clearly visible hyperreactivity also occurs in the region upstream from the CRP site (lane 5 versus lane 6 of Figure 1). These observations are confirmed by the densitometric analysis shown in Figure 3A. The two hyperreactive regions are seen here in the +18 to +28 and −80 to −100 segments. Hyperreactivity is also visible on the bottom strand (Figure 2), which is confirmed by the densitometer scans (Figure 3B; −80 to −90 and downstream of +15), although the lower resolution of the data makes quantification unreliable. We conclude that there are two regions where open complex formation induces a structure that is hyperreactive to MNase attack. These regions bracket the transcription complex, that is, they appear just upstream of the CRP-protected region and just downstream of the polymerase protected region. They indicate that *lac* transcription complexes alter the structure of DNA at the periphery of the tightly bound region.

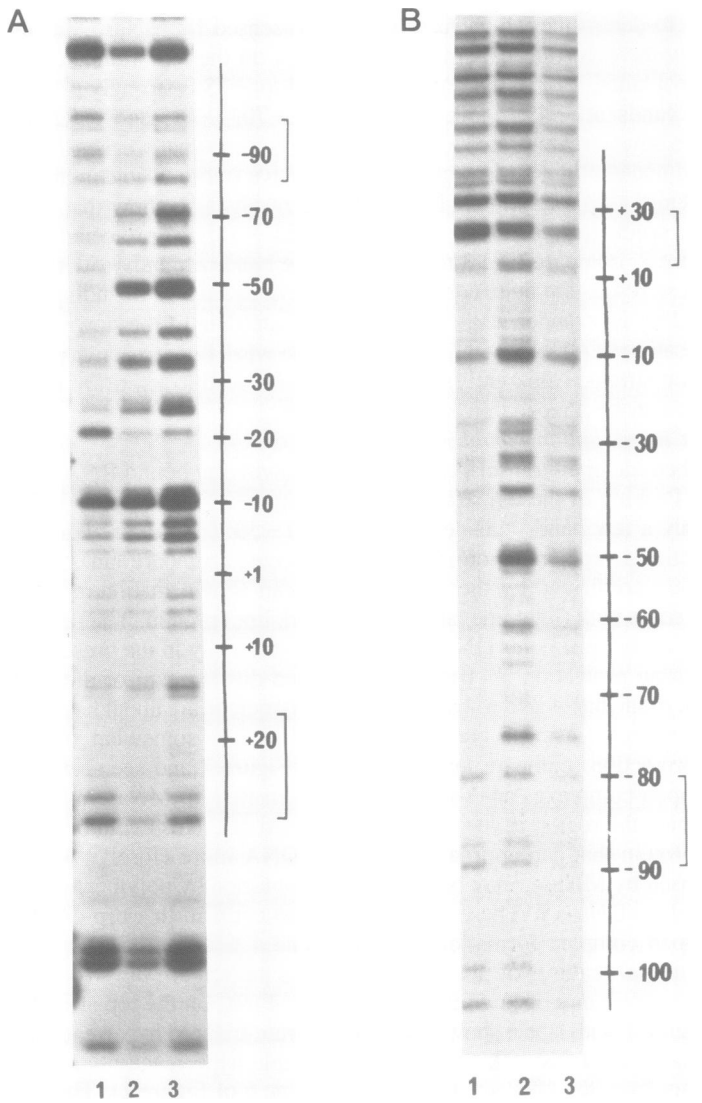

Figure 4. Micrococcal nuclease digestion pattern at 0°C corresponding to closed complex conditions. (A) Digestion pattern of the top strand. Supercoiled DNA was treated with 24 units of micrococcal nuclease following preincubation without proteins (lane 3), with RNA polymerase (lane 2), or with RNA polymerase and CRP-cAMP (lane 1). (B) Digestion pattern of the bottom strand. Lane 1, DNA with RNA polymerase and CRP-cAMP; lane 2, DNA alone; lane 3, DNA with RNA polymerase. The brackets denote the region hypersensitive to attack in the open complexes shown in Figures 1 and 2.

Probing closed transcription complexes

Next, we used MNase to probe *lac* transcription complexes formed at low temperature where strand opening is inhibited. It is believed that the *lac* promoter can still be recognized under these conditions, but the complexes formed are relatively unstable closed complexes

(see 21,22). It has been proposed that one role of catabolite activator protein is to stabilize and properly position RNA polymerase in these complexes (23,24). The experiment shown in Figure 4 probes these complexes and illustrates directly the role of CRP in their formation.

Comparison of lanes 2 and 3 in Figure 4A reveals the changes in MNase cleavage pattern when RNA polymerase alone is incubated with DNA under closed complex conditions. No specific protection of the promoter region (−40 to +20) is observed, although there may be some generalized protection accompanying the addition of RNA polymerase (lane 2, in which all bands are lighter). By contrast, when CRP and RNA polymerase are added together (lane 1), a specific protection pattern is observed compared to digestion of naked DNA (lane 3). This protection begins upstream of the CRP binding site (beyond −70) and continues well beyond its downstream border to at least position −25 within the RNA polymerase interaction site. This extended protection caused by the addition of CRP is clearer when the other DNA strand is probed (Figure 4B). When CRP and RNA polymerase are added together (lane 1), bands from −75 to −10 and to a lesser extent to +15 are protected compared to the pattern observed when no proteins are present (lane 2). Addition of polymerase alone does not lead to specific protection of the promoter (lane 3). We conclude that the presence of CRP is necessary for formation, at 0°C, of a specifically positioned complex at the *lac* promoter. MNase may be particularly useful in probing such complexes since it detects interactions with the DNA backbone which could occur prior to the intimate contacts within the DNA grooves; recall that other probes such as dimethyl sulfate and DNase I detect groove contacts.

When the MNase attack patterns of these low temperature complexes (Figure 4) are compared to those of open complexes (Figures 1 and 2) certain differences can be noted. The most obvious difference is that the MNase hypersites that border the protected regions in open complexes (Figure 1, lane 5, upstream of −70 and downstream of +20; Figure 2, lane 5, downstream of +15) are either absent or barely discernable in closed complexes (Figure 4A bracketed region in lane 1 compared to lane 3; Figure 4B, bracketed region in lane 1 compared to lane 2). Within the promoter, open complex protection may be slightly stronger, especially in the +1 to +20 region. Thus, among the changes occurring during formation of open complexes, one should include changed interactions at the border of the transcription complex and stronger interactions inside it.

DISCUSSION

The development of micrococcal nuclease as a probe for DNA-protein interactions has extended our knowledge of the structure of *lac* transcription and repression complexes. The enzyme is used to detect DNA regions protected from attack due to bound protein in open and closed complexes and regions of unusual DNA structure induced by protein binding. Some of these interactions were not detected during previous probing with numerous reagents, indicating that MNase can be a very useful reagent in complementing data obtained by other methods.

Structural features of lac open promoter complexes

One unexpected result of these experiments is the appearance of regions of MNase hyperreactivity at the upstream and downstream borders of the *lac* transcription complex. These regions are not within the DNA bound tightly by protein, as defined by previous DNase I footprints, and confirmed by these MNase footprints. That is, they are upstream of the CRP binding site and downstream from +17, which is the last downstream position strongly protected by RNA polymerase.

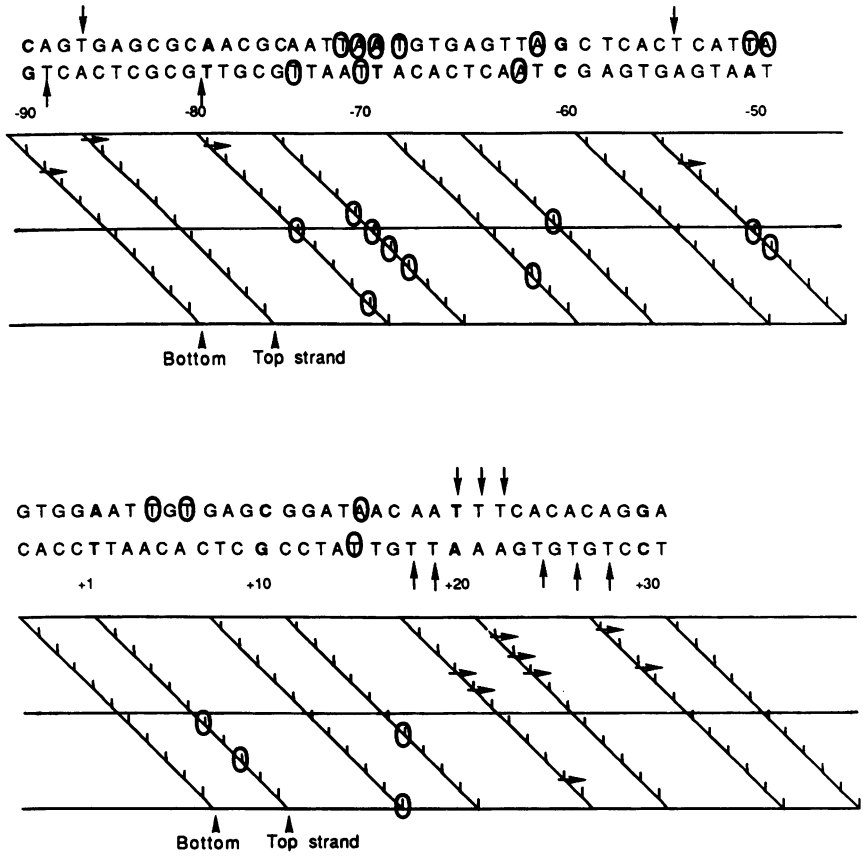

Figure 5. Helix maps of interactions in the −50 to −90 and +1 to +30 regions in the open complex at the *lac* promoter. The DNA helix (10.5 base pairs per turn) is shown in planar representation such that the helix has cut along a single edge, unfolded and drawn flattened. Diagonal lines represent the sugar-phosphate backbone with tick marks being phosphates. Strong protections are shown by ovals and enhancements are shown by arrows. The changes are shown on the phosphate backbone because micrococcal nuclease binds to one strand of DNA and cleaves phosphodiester bonds. The two regions shown are in register assuming 10.5 base pairs per turn in the intervening DNA.

We have only a few clues regarding the nature of these structural changes that accompany open promoter complex formation. They have not been detected in previous studies using DNase I, dimethyl sulfate or $KMnO_4$, all of which sense DNA groove geometry; by contrast, MNase probes DNA backbone exposure. In the case of the *lac* repression loop, MNase detected a previously identified distortion in a protein-free DNA region constrained within a bent DNA loop stabilized at its base by repressor interacting with remote operators. Thus, by analogy, we suspect that the open complex hyperreactivity reflects sharp bends in the promoter at both borders of the transcription complex. The bends should involve increased exposure of the DNA backbone, facilitating MNase attack.

The helix maps of these hyperreactive sites in border regions, kept in register assuming 10.5 base pairs per turn, are shown in Figure 5. The sites in the −50 to −90 and +1

to +30 regions, where the backbone is exposed to MNase attack, are all on the outside or unprotected face of the bound DNA. In this display, those residues that are strongly protected by bound CRP and RNA polymerase are indicated by circles and are seen to all lie on one face of the DNA helix. These backbone protections lie on the same helix face containing major groove positions protected by dimethyl sulfate attack in open complexes (6). The MNase hyperreactive sites lie on the opposite helix face from the protections, implying that the backbone on this face is perturbed in a manner that leads to its greater exposure. These observations are analogous to those made using dimethyl sulfate, in which case the dichotomy was interpreted as promoter DNA bending to make contact with the curved surface of the polymerase (25). Included in this array are the strong hypersites outside the protected region which likely represent polymerase induced bends in the DNA at the borders of the transcription complex. Thus, the transcription complex seems superficially to resemble nucleoprotein structures such as nucleosomes and replication complexes in which the DNA is bent around the surface of a protein core (26).

Closed complexes at the lac promoter.

MNase also proved to be a useful probe of *lac* promoter complexes formed at 0°C and the results were relevant to the important issue of the mechanism of transcription activation by CRP protein. Specific complexes were not detectable in the absence of CRP, but were readily detectable in its presence. Thus, the probing experiments suggest that at least one role of CRP is to stabilize RNA polymerase in closed promoter complexes, as also inferred from previous kinetic transcription experiments (23).

Although *lac* closed complexes had not been footprinted previously, these MNase results can be compared with DNase I probing of other closed complexes. Such experiments were reported on the *lac* UV5 promoter, which differs from *lac* wild-type in that DNA sequence changes allow transcription in the absence of CRP protein. Those experiments (22) suggested that the *lac* UV5 closed complex was specifically positioned with interspersed DNase protections and hyperreactive sites occupying opposite faces of the DNA. The above MNase probing of the wild-type promoter does not show the interspersed hypersites, which could be due to the use of MNase to probe backbone conformation or to the presence of CRP in the wild-type experiments. The MNase hypersites that are observed are associated primarily with open complexes except for one at −21 which is also present in closed complexes. The open complex-specific hypersites occur just beyond the borders of the strongly protected region; they are, however, also on the unprotected face of the DNA (Figure 5). Taking these data together with other probing results (6,25), it appears that *lac* promoter recognition occurs by bound CRP and DNA sequence elements attracting polymerase to one side of the DNA, followed by a series of complex conformational changes that include DNA bending towards the protein core.

Micrococcal nuclease as a structural probe

Previously, micrococcal nuclease has been used primarily as a probe of chromatin structure (27). It cleaves chromatin preferentially in linker regions and may also have reduced access to regions bound by non-histone proteins (11). These previous uses of MNase involved detecting double strand DNA breaks. Since MNase cuts only one DNA strand at a time, previous studies detected those cleavages where initial MNase cleavage is followed by a second independent cleavage to the opposite strand; DNA nicking is invisible using the previous detection procedures. By contrast, the primer extension method used here detects sites of nicking, which allows the use of MNase as a footprinting reagent for nucleoprotein complexes. Footprinting using end-labelled DNA rather than by primer extension should give equivalent information.

The results of footprinting with MNase show that the enzyme is useful for detecting bound proteins as well as for detecting structural perturbations induced in DNA. The protected regions are roughly equivalent to those observed previously in DNase I footprinting, indicating that proteins are closely associated with the DNA backbone as well as the DNA grooves. The proteins detected include RNA polymerase, CRP and *lac* repressor. In addition, the reagent detects a DNA distortion accompanying repression loop formation, and a structural distortion bordering the *lac* transcription complex. DNase I did not detect either induced structural perturbation, suggesting that micrococcal nuclease will prove to be a versatile reagent complementing those currently available.

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