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The *Autographa californica* Nucleopolyhedrovirus IE-1 Protein Complex Has Two Modes of Specific DNA Binding

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Missing contact footprinting with formic acid as a modifying reagent was used to examine specific IE-1 binding contacts to double-stranded oligonucleotides that contained either a consensus *hr* repeat sequence or a sequence from the *pe*38 promoter, which is down regulated by IE-1. The *hr* repeat sequences contain two consensus IE-1 binding motifs (IBMs) flanking a central *Eco*RI site that are oriented in opposite directions with respect to each other. IE-1 was found to contact regions including both IBMs. The bases footprinted in the top strand included the left IBM (IBM-A), whereas bases in the bottom strand were footprinted in a region that included IBM-B and part of IBM-A. When substitution mutations were introduced into either IBM, bases on both strands of the remaining IBM were strongly footprinted. As with the *hr* IBM-mutant constructs, bases footprinted in the *pe*38 promoter construct included both strands of the single IBM.

INTRODUCTION

The genomes of baculoviruses consist of covalently closed, double-stranded DNA molecules ranging in size from 100 to 179 kb (Rohrmann, 1999). A novel feature of these genomes is the presence of homologous regions (*hrs*) located at a number of positions in the genome. *Hrs* have been implicated both as transcriptional enhancers and origins of DNA replication for a number of baculoviruses (Guarino *et al.,* 1986; Guarino and Summers, 1986; Leisy and Rohrmann, 1993; Pearson *et al.,* 1992, 1993; Pearson and Rohrmann, 1995, 1998). In the best characterized baculovirus, *Autographa californica* nucleopolyhedrovirus (AcNPV), *hr* repeat units contain ~70-bp with an imperfect 30-bp palindrome near the center and are repeated two to eight times at each of eight locations around the genome (Kool *et al.,* 1995).

The product of the AcNPV immediate early gene-1 (IE-1) is an important regulator of baculovirus gene expression and has also been demonstrated to be an essential gene for viral DNA replication (Kool *et al.,* 1994; Lu and Miller, 1995). A variety of baculovirus early gene promoter–reporter constructs are activated when they are cotransfected with *ie-1* into uninfected insect cells (Guarino and Dong, 1994; Leisy *et al.,* 1995; Rodems and Friesen, 1993, 1995). This activation is greatly enhanced when the constructs are linked to *hr* sequences. *Hr*-containing DNA has also been shown to bind IE-1 in gel mobility shift assays (Choi and Guarino, 1995; Leisy *et al.,* 1995; Rodems and Friesen, 1995). Deletion analysis of AcNPV IE-1 indicated the presence of separate transcriptional activation and DNA-binding domains (Kovacs *et al.,* 1992; Rodems, *et al.,* 1997; Slack and Blissard, 1997). The requirement for IE-1 in baculovirus DNA replication may result from its function in activating the expression of early genes, some of which are required for viral DNA replication. However, a direct role in origin binding and initiation of the early steps leading to the assembly of a replication complex is also possible.

Gel shift assays and mutational analyses have shown that IE-1 binds to specific sequences within the *hr*-repeat unit (Choi and Guarino, 1995; Leisy *et al.,* 1995; Rodems and Friesen, 1995). IE-1 has also been shown to downregulate certain genes by binding to specific sequences in their promoters (Leisy *et al.,* 1997). Sequence comparison revealed the presence of a common 8-bp sequence motif (5′-ACBYGTAA-3′) in all DNA fragments that have been shown to bind IE-1 (Leisy *et al.,* 1997). The functional importance of this sequence was demonstrated by showing that replacement of three nucleotides within either of the two IBM motifs of an *hr* palindrome abolished enhancer activity in transfection assays (Rodems and Friesen, 1995). Downregulation of transfected *pe*-38 and *ie-2* promoter driven reporter genes was also observed in AcNPV-infected cells and in cells transfected with an IE-1-expressing plasmid. When the single IBM element in these promoters was replaced with a *Bgl*II restriction site, downregulation did not occur (Leisy *et al.,* 1997).

In this report, we use missing contact footprinting to examine specific IE-1 binding contacts in a consensus *hr* repeat unit and in a sequence from a promoter that is

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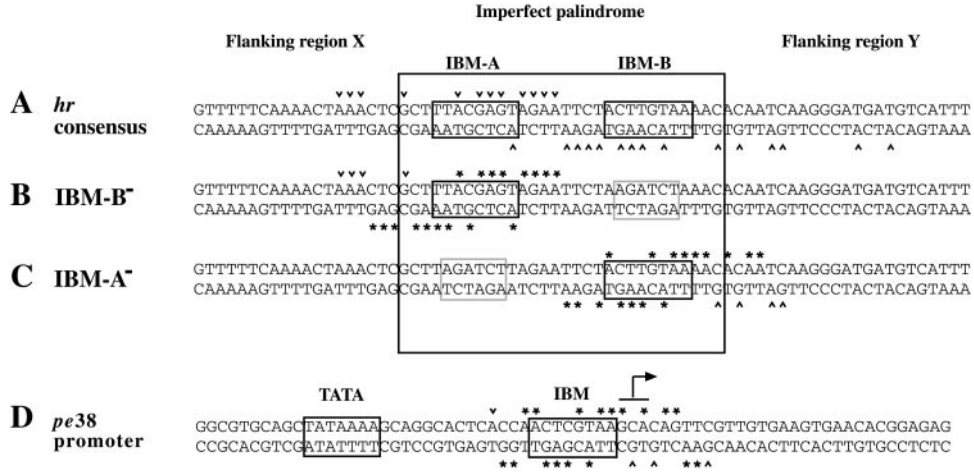

FIG. 1. Sequence of a consensus *hr*-repeat and an IE-1 down-regulated promoter, and summary of missing contact footprinting experiments. (a) Consensus *hr* sequence. The IE-1 binding motifs (IBM-A and IBM-B) are indicated, the 30-bp imperfect palindrome region is boxed, and the flanking regions X and Y are shown. (b and c) *Hr* sequence with a mutation in IBM-B and IBM-A, respectively. *Bgl*II restriction sites were introduced (dashed boxes) to disrupt the IBM motifs. (d) *pe38* promoter sequence. The TATA box and IE-1 binding motif (IBM) are boxed and the position of the *pe*38 mRNA start site is shown. ^, purines that are weakly footprinted; *, purines that are strongly footprinted.

down regulated by IE-1. Our findings suggest that the mode of binding to *hrs,* which contain an inverted pair of IE-1 binding motifs, is distinct from that used for down-regulation of promoters, which contain only a single IE-1 binding motif.

RESULTS

Identification of *hr* sequences that bind IE-1

By comparison of *hr* sequences to promoter sequences that also bind IE-1, we previously defined a putative IE-1 binding motif (IBM), ACBYGTAA, which is conserved among these sequences (Leisy *et al.,* 1997). Each *hr* repeat unit contains an inverted pair of these motifs, IBM-A and IBM-B, flanking a central *Eco*RI site that lies at the exact center of an imperfect palindrome (Fig. 1a). The promoters that are downregulated by IE-1 contain an IBM sequence just upstream of the mRNA start site (Fig. 1d). To define nucleotides that physically interact with IE-1, we conducted missing contact footprinting studies using double-stranded oligonucleotide constructs containing a consensus *hr* sequence derived from comparison of the complete set of *hr* repeated sequences (Kool *et al.,* 1995). In these assays the double-stranded oligonucleotides, end-labeled on one strand with ^{32}P, were subjected to limited depurination by treatment with formic acid. The treated DNA was then mixed with IE-1-containing cell extracts, and DNA–protein complexes (shifted DNA) were separated from free DNA on a nondenaturing polyacrylamide gel. Super-shift experiments using anti-IE-1 antibodies have demonstrated that IE-1 is a component of the protein complex bound to the DNA (Leisy *et al.,* 1995, 1997, Rasmussen *et al.,* 1996). *In vitro* synthesized IE-1 has also been shown to bind to *hr*-containing DNA, suggesting that the binding of IE-1 is direct and not mediated by other proteins in the transfected cell extract (Rodems and Friesen, 1995). DNA isolated from the complex was treated with piperidine to cleave the DNA at the positions of missing bases and then compared on a sequencing gel with similarly treated control DNA that had not been reacted with IE-1 and with unshifted DNA. Missing or underrepresented bands in lanes containing shifted DNA, commonly referred to as footprints, indicate the positions of important contact points for IE-1 binding. In control experiments using extracts from pBluescript transfected cells, only very minor amounts of shifted DNA were observed. These shifted bands migrated faster than the IE-1-DNA complexes (data not shown). We were unable to obtain sufficient material from these bands for footprint analysis. By examining IE-1 footprints on sequences that had been labeled either on the top or bottom strands of the consensus *hr*-repeat (Fig. 2), we found that the region footprinted in the top strand lies to the left of the palindrome center and extends at least 6 bases into flanking region X (Fig. 1a). On the bottom strand, the footprint covered a larger area, extending upstream into IBM-A and extending downstream $\geq$17 nt into the adjoining flanking region Y (Fig. 1a). There are also faint bands visible in Fig. 2B that correspond to positions occupied by pyrimidines. These pyrimidines also appear to be footprinted in the region near IBM-B.

Footprinting of *hr* sequences containing mutations in IE-1 binding motifs

Hr sequences containing mutations in one of the IE-1 binding motif (IBM) sequences often retain the ability to bind IE-1 (Guarino and Dong, 1994; Leisy *et al.,* 1995;

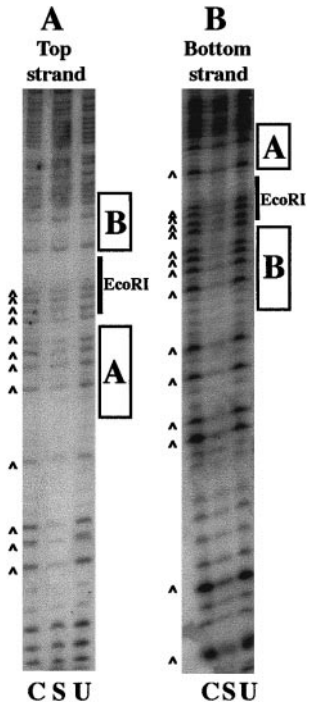

FIG. 2. Missing contact footprinting of the consensus *hr* repeat unit. Seventy-two base oligonucleotides corresponding to the top and bottom strands of the consensus *hr* repeat unit were annealed, treated with formic acid, and reacted with IE-1-containing cell extract. IE-1 bound DNA was separated from unbound DNA by electrophoresis on a native acrylamide gel. The bands corresponding to bound and unbound DNA were excised, eluted from the gel, treated with piperidine, and electrophoresed on a DNA sequencing gel. (A) Footprint of the top strand. (B) Footprint of the bottom strand. Below each panel the lanes are marked: (C) control DNA that was not reacted with IE-1 extract, (S) DNA from the shifted band, and (U) unshifted DNA. ^, purines that are weakly footprinted.

Rodems and Friesen, 1995). The footprints that we observed with the intact *hr* sequence are quite weak. This may reflect the fact that a modification that prevents the interaction of IE-1 with one IBM does not prevent the interaction of IE-1 with the other IBM and therefore does not prevent formation of a shifted complex (see below). Mutational studies have shown that IE-1 binds to an *hr* half-palindrome with lower affinity compared to a complete *hr* palindrome (Rodems and Friesen, 1995). This may account for the weak footprinting that we observed.

To observe the footprinting patterns when only one IBM is present, we performed missing-contact footprinting assays on constructs containing mutations in IBM-A or IBM-B (Fig. 3). In each case, a *Bgl*II sequence was used to replace the central 6 bp of the 8-bp IBM sequence. With constructs containing an IBM-B$^-$ mutation, the region footprinted on the top strand was similar to the region footprinted in the consensus *hr* repeat unit, except that the footprint was more pronounced (Fig.3A). However, we now observed a strong footprint on the bottom strand of IBM-A as well (Fig. 3B). The results with the IBM-A$^-$ mutant construct were similar to those with the IBM-B$^-$ mutant (Figs. 3C and 3D). The very strong footprint that was observed on the top strand in the region spanning IBM-B of the IBM-A$^-$ mutant was unexpected because this region did not appear to be even weakly footprinted in the intact *hr* consensus sequence (Fig. 2A). These results are summarized in Figs. 1b and 1c.

Comparison of *hr* and *pe38* promoter footprints

Because IE-1 is known to downregulate the transcription of selected genes by binding to promoter regions containing single IBMs (Leisy *et al.,* 1997), it is possible that the footprint patterns that we observed involving both strands of a single IBM represent a mode of IE-1 binding used for negative transcriptional regulation that is distinct from the mode of binding normally used within *hr* repeat units. To test this hypothesis, we performed missing-contact footprinting on double-stranded oligonucleotides containing sequences from the *pe-38* promoter (Fig. 4) and compared the footprint patterns with those obtained with the wild-type and mutant *hr* repeat units. We found that the top strand was strongly footprinted, extending ≥4 bases upstream and 6 bases downstream from the IBM motif (Fig. 4A). On the bottom strand, the footprinted region extended ≥3 bases upstream and 9

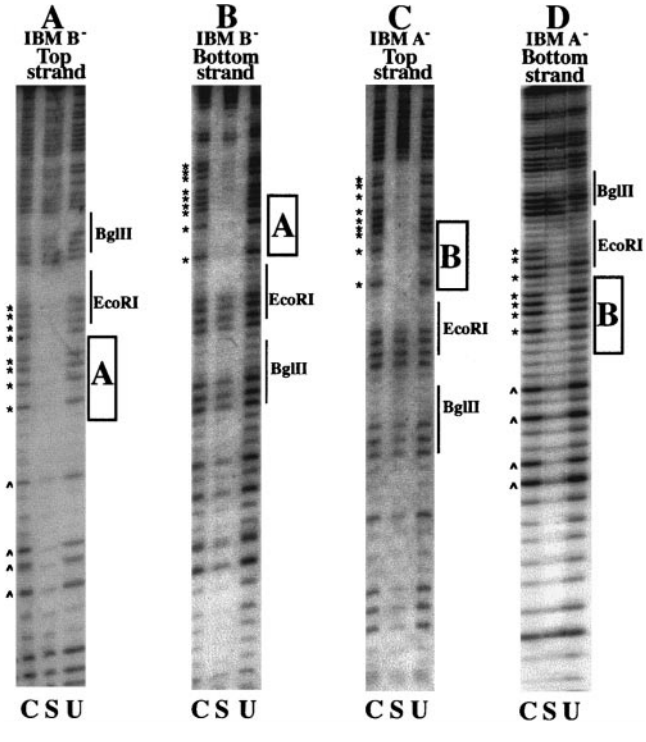

FIG. 3. Missing contact footprinting of mutant *hr* repeat units. Seventy-two base oligonucleotides corresponding to the top and bottom strands of the consensus *hr* repeat unit with substitution mutations in IBM-B (A and B) or IBM-A (C and D) were treated as described in the Fig. 2 legend. (A and C) Footprint of the top strand. (B and D) Footprint of the bottom strand. Lanes are labeled as in Fig. 2. ^, purines that are weakly footprinted; *, purines that are strongly footprinted.

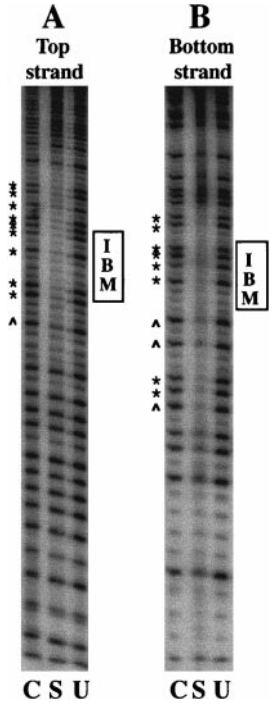

FIG. 4. Missing contact footprinting of the *pe*38 promoter region. Seventy base oligonucleotides corresponding to the top and bottom strands of the *pe*38 promoter region were treated as described in the Fig. 2 legend. (A) Footprint of the top strand. (B) Footprint of the bottom strand. Lanes are labeled as in Fig. 2. ^, purines that are weakly footprinted; *, purines that are strongly footprinted.

bases downstream (Fig. 4B). These findings were very similar to what we observed with the IBM-A$^-$ and IBM-B$^-$-mutant *hr* sequences (Figs. 1b–1d), suggesting that the manner in which IE-1 binds to DNA containing a single IBM motif is distinct from the manner in which it binds to the *hr* palindromes. The distinct modes of binding to the *hr* and *pe*38 promoter constructs that we have characterized may be related to the different functional roles of IE-1 when bound to these regions.

DISCUSSION

In this report we have characterized two modes of binding of IE-1 to sequences containing IE-1 binding motif (IBM) sequences. By missing-contact footprinting, using formic acid as the modifying agent, we found that the nucleotides important for binding an intact consensus *hr* sequence lie to the left of the palindrome center on the top strand. On the bottom strand, the footprint is most prominent in the region to the right of the palindrome center. Kremer and Knebel-Morsdorf (1998), using phenanthroline-copper footprinting assays of an *hr*4a repeat sequence detected an extensive footprint on the bottom strand that spanned both IBM sequences but did not detect footprints on the top strand of this region. The disparity between their results and ours may reflect differences in the sensitivities of each assay or could be an indication that IE-1 uses different modes of binding to different *hr* sequences, depending on variation in sequence.

The observations we report regarding the binding of IE-1 to *hr* palindromes are consistent with a model for IE-1 binding proposed by Friesen (1997) in which one subunit of an IE-1 dimer interacts with a recognition sequences in one half of the palindrome and the second subunit interacts with a recognition sequence in the other half. Our data show that the bases contacted on each strand by IE-1 extend over a region greater than one turn of the DNA helix. This indicates that IE-1 may wrap itself around the DNA to contact these continuous stretches of bases. Alternatively, IE-1 may unwind the DNA to allow simultaneous contact with long stretches of bases. The latter possibility seems unlikely since IE-1 does not contain an NTP binding motif characteristic of DNA helicases (Lu *et al.,* 1997) and therefore would not be expected to have a DNA unwinding capability. Yet another possibility is that different conformations of the DNA occur during a transition from an initial IE-1:DNA binding complex to a stable complex, such as a transition from a linear to a cruciform structure, and different sets of bases are contacted at various stages of this transition. Under this scenario, the bases footprinted in the missing contact footprint assay would represent not only those bases contacted in the stable structure, but those contacts required for initial binding and transition, as well.

Our data are also consistent with previous reports in which it has been demonstrated that a variety of different palindrome mutations, including deletion of *hr* half- palindromes, and alteration of the spacing between *hr* half-palindromes results in constructs that still retain the ability to bind IE-1. However, *hr* repeat elements with these mutations are inactive in transient replication assays and have lost their capacity to enhance expression when linked to early gene promoters (Guarino and Dong, 1994; Kremer and Knebel-Morsdorf, 1998; Leisy *et al.,* 1995; Rodems and Friesen, 1993, 1995). It is likely that in these inactive constructs, IE-1 is binding to a single IBM as we observed in Figs. 3 and 4 for the mutant *hr* sequences and the inhibitory consensus sequence. These data suggest that with this mode of binding the conformation of the IE-1-DNA complex is not conducive to the initiation of DNA replication or for transcriptional enhancement. In addition, when a single IE-1 binding motif is located close to an mRNA start site, IE-1 binds both strands of DNA and may inhibit initiation of transcription by physically blocking the interaction of the RNA polymerase complex with the DNA.

The presence of two forms of binding complicated the footprinting analysis of the *hr* palindrome consensus sequences because inhibition of binding on one strand due to a missing contact would lead to the alternative form of binding on the same molecule in which IE-1

TABLE 1

Oligonucleotides Used in Footprinting Assays

A) *hr* length: 72 bases
5′-GTTTTTCAAAACTAAACTCGCTTTACGAGTAGAATTCTACTTGTAAAACACAATCAAGGGATGATGTCATTT-3′
B) *hr*.rev length: 72 bases
5′-AAATGACATCATCCCTTGATTGTGTTTTACAAGTAGAATTCTACTCGTAAAGCGAGTTTAGTTTTGAAAAAC-3′
C) *hr*A$^-$ length: 72 bases
5′-GTTTTTCAAAACTAAACTCGCTTAGATCTTAGAATTCTACTTGTAAAACACAATCAAGGGATGATGTCATTT-3′
D) *hr*A$^-$.rev length: 72 bases
5′-AAATGACATCATCCCTTGATTGTGTTTTACAAGTAGAATTCTAAGATCTAAGCGAGTTTAGTTTTGAAAAAC-3′
E) *hr*B$^-$ length: 72 bases
5′-GTTTTTCAAAACTAAACTCGCTTTACGAGTAGAATTCTAAGATCTAAACACAATCAAGGGATGATGTCATTT-3′
F) *hr*B$^-$.rev length: 72 bases
5′-AAATGACATCATCCCTTGATTGTGTTTAGATCTTAGAATTCTACTCGTAAAGCGAGTTTAGTTTTGAAAAAC-3′
G) *pe*38prom length: 70 bases
5′-GGCGTGCAGCTATAAAAGCAGGCACTCACCAACTCGTAAGCACAGTTCGTTGTGAAGTGAACACGGAGAG-3′
H) *pe*38prom.rev length: 70 bases
5′-CTCTCCGTGTTCACTTCACAACGAACTGTGCTTACGAGTTGGTGAGTGCCTGCTTTTATAGCTGCACGCC-3′

Note. A) and B), top and bottom strand, respectively, of a consensus *hr* direct repeat sequence. C and D), top and bottom strand of a consensus *hr* direct repeat sequence containing a *Bgl*II site substitution in IBM-A. E) and F), top and bottom strand of a consensus *hr* direct repeat sequence containing a *Bgl*II site substitution in IBM-B. G) and H), top and bottom strands of the *pe*38 promoter sequence. *Bgl*II site substitution mutations are underlined.

would bind both the upper and lower strands of the other half palindrome. This would result in formation of a DNA–protein complex and slower migration of the DNA in a gel-shift assay. These complexes will contain molecules with depurinated bases in one of the binding regions, which, upon cleavage, will form bands on a sequencing gel in a region representative of the contact region. It is likely that the only reason footprints are observed at all with intact palindromes is because IE-1 has a lower affinity for palindrome half-sites than it does for intact *hr* palindromes. Therefore the band intensities in regions corresponding to the IBM sites would be expected to be reduced compared to those from regions outside of these binding sites, as we have observed.

MATERIALS AND METHODS

Preparation of whole cell extracts

Whole-cell extract preparation and gel shift assays were done as previously described with minor modifications (Leisy *et al.,* 1995). Briefly, 1.5×10^7 Sf9 cells were plated onto a 150-mm tissue culture plate, allowed to attach overnight, and then transfected with either 20 μg of ple-1 (Leisy *et al.,* 1995) or pBKS$^-$ (Stratagene, Inc.) plasmid DNA using cellfectin reagent according to manufacturer's recommendations (Invitrogen Corp.). After a 5-h transfection period, the transfection mixture was removed, and 14 ml TNM-FH (Summers and Smith, 1987) was added to the cells. At 24 h posttransfection, the cells were scraped, transferred to 15 ml conical tubes, and centrifuged at 1300 RPM for 3 min on a Beckman GS-6R tabletop centrifuge, and the supernatant was removed. The cells were resuspended in 14 ml phosphate buffered saline (PBS) that had been used to rinse the plate and then recentrifuged. The cells were again resuspended in PBS and recentrifuged. The cells were then resuspended in 1 ml PBS and transferred to a 1.7 ml eppendorf tube. The cells were centrifuged in a variable speed microfuge for 4 min at 3000 RPM, the supernatant was removed, and the cells were recentrifuged for 1 min, and as much PBS was removed as possible without disrupting the cell pellet. The cell pellet was resuspended in 250 μl of whole-cell extraction buffer [10 mM HEPES (pH 7.2), 0.4 M NaCl, 0.1 mM EGTA, 1 mM EDTA, 20% glycerol, 0.5 mM phenylmethlsulfonyl fluoride] and incubated on ice for 10 min. The cells were pelleted at 3000 RPM in a microfuge at 4°, the supernatant was removed, divided into 20 μl aliquots and immediately frozen in liquid nitrogen. The whole-cell extracts were stored at −80° until needed.

Oligonucleotides, end-labeling reactions, and annealing

The sequence of the oligonucleotides used in this study are shown in Table 1. The oligonucleotides were prepared at the Center for Gene Research, Oregon State University and subsequently purified by electrophoresis on nondenaturing polyacrylamide gels with recovery by the "crush and soak" method (Sambrook *et al.,* 1989). One strand (10 pmole) of each pair of complementary oligonucleotides (either the top or the bottom strand) was end-labeled with γ-[^{32}P]ATP (New England Nuclear) using T4 polynucleotide kinase (New England Biolabs) and methods recommended by the supplier. The end-labeled oligonucleotide was purified on a 1 ml G25-spin column (Pharmacia) then mixed with threefold excess of the unlabeled complementary oligonucleotide. To anneal the

strands, 0.1 vol of 10× annealing buffer was added (final concentration = 20 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl), the reaction was heated to 90°C, and then was slow cooled to room temperature. The DNA was then ethanol precipitated and resuspended in H_20.

Formic acid footprinting

Missing contact footprinting using formic acid as the modifying agent was performed essentially as described by Cao and Revzin (1993).

Modification of DNA with formic acid. The ^{32}P-end-labeled, annealed oligonucleotides were resuspended in 23 μl TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA); 0.5 μl 10 μg/μl *Escherichia coli* tRNA was added, and then 77 μl formic acid was added. The reaction was incubated for 5 min at room temperature, and then the DNA was precipitated by the addition of 10 μl 3 M NaOAc, pH 5.3, and 250 μl 95% ethanol. The modified DNA was allowed to precipitate at −80° for 30 min and then pelleted by microcentrifugation for 30 min. The pellet was rinsed with 70% ethanol, resuspended in 250 μl 0.3 M NaOAc, pH 5.3, and reprecipitated by adding 750 μl 95% ethanol and incubating at −80° for 30 min. The modified DNA was then microcentrifuged, rinsed with 70% ethanol, dried under vacuum, and resuspended in a small volume of H_20.

Preparative gel shifts. Formic-acid-treated DNA ($2–3 \times 10^6$ cpm) was mixed with 20 μl whole-cell extract in a 50 μl reaction containing 10 mM Tris (pH 7.9), 100 mM NaCl, 1 mM dithiothreitol, 20% glycerol, and 5 μg dIdC (Sigma). After 20 min at 4°, the DNA–protein complexes were resolved by electrophoresis at 200 V for 3–5 h on 4.2% 19:1 acrylamide:bis gels containing 3% glycerol, 8.0 mM Tris–HCl (pH 7.9), 6 mM sodium acetate, 1 mM EDTA. Electrophoresis was performed in a 4° room. After exposure of the gels to X-ray film (Sterling), they were positioned over the film and sections of the gel corresponding to the shifted and unshifted bands were excised and the DNA was eluted by the crush and soak method (Sambrook *et al.,* 1989).

Cleavage reactions. The DNA was cleaved at the positions where formic acid modification had occurred by resuspension in 100 μl 10% piperidine and heating to 90°C for 30 min. The DNA was ethanol precipitated twice, with 70% ethanol washes following each precipitation. Approximately 15,000 cpm (Cherenkov) of each sample were resolved on a urea-polyacrylamide gel. The gel was fixed in 10% acetic acid, 10% methanol for 25 min and then transferred to Whatmann 3MM paper and dried with a gel dryer. The dried gels were then exposed to a phosphoimager screen overnight and scanned.

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