Site-specific HU binding in the Mu transpososome: conversion of a sequence-independent DNA-binding protein into a chemical nuclease

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HU is a small, basic, sequence-independent DNA-binding protein capable of engineering DNA deformations required for the formation of higher order nucleoprotein structures. One such complex is the Mu Type 1 transpososome, where the ends of Mu are stably synapsed by a tetramer of Mu A and cleaved at their 3' ends. HU is believed to play a critical role in transpososome assembly, which requires the communication of the two Mu ends and the transpositional enhancer. Although footprinting studies have clearly defined the DNA regions bound by Mu A, no protection could be ascribed to the HU protein by DNase I, MPE · Fe(II) or hydroxyl radical methods (Lavoie et al. 1991). To directly probe HU interactions with the transpososome DNA, we have coupled HU to a DNA cleavage reagent, iron-EDTA, and report here the first HU "footprint". HU-nuclease cleavage was detectable at specific sites within an 83-bp spacer DNA separating the left-end-most L1 site from its neighboring L2 site. This HU binding was specific since it could not be competed with 10-fold excess supercoiled DNA. We postulate that HU promotes the formation of a tight DNA bend or loop in this region which facilitates the communication of Mu A monomers during complex assembly. This method may prove generally useful for the localization of sequence-independent DNA-binding proteins on DNA and within higher order nucleoprotein structures.

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Higher order nucleoprotein complexes mediate a variety of DNA metabolic events such as replication, transcription, recombination, and transposition in both eukaryotic and prokaryotic systems (Echols 1986, 1990). The assembly of these structures requires the coordination of multiple protein-protein and protein-DNA interactions and may require the deformation of the DNA helix by melting, wrapping, bending, and/or looping (for review, see Hochschild 1990; Nash 1990; Travers and Klug 1990; Amouyal 1991; Lilley 1991; Harrington 1992; Matthews 1992; Schleif 1992). Such DNA structure alterations are often effected by DNA-binding accessory factors that contribute to the architectural requirements of higher order complexes (for review, see Nash 1990; Boocock et al. 1992; Serrano et al. 1993).

The *Escherichia coli* protein HU typifies a family of small, heat-stable, DNA-binding proteins that stimulate a variety of DNA metabolic reactions (Drlica and Rouvière-Yaniv 1987; Pettijohn 1988; Schmid 1990). HU was originally identified as a stimulatory factor in λ transcription (Rouvière-Yaniv and Gros 1975) and binds DNA in a sequence-independent manner. It has since been shown to positively or negatively affect transcrip-

tion (Drlica and Rouvière-Yaniv 1987), and *oriC* replication (Dixon and Kornberg 1984; Skarstad et al. 1990), and inhibits λ replication (Mensa-Wilmot et al. 1989). Although HU has been shown to bind within the *oriC* preinitiation complex (Funnel et al. 1987) where it promotes DNA melting (Hwang and Kornberg 1992), its site and mechanism of action have not been defined precisely. The *Salmonella typhimurium* phase variation inversion reaction also requires HU. In this system, HU mediates the formation of a tight DNA loop (Haykinson and Johnson 1993; Paull et al. 1993) so that three DNA segments can interact (Heichman and Johnson 1990). In both of these cases, the amount of HU required is much less than that required to coat the DNA; only a few HU dimers (two to five) are required to stimulate *oriC* replication and *hin* inversion (Skarstad et al. 1990; Haykinson and Johnson 1993, respectively).

Cyclization experiments using short DNA lengths have indicated that HU promotes DNA flexibility (Hodges-Garcia et al. 1989; Paull et al. 1993) possibly through DNA-bending; furthermore, HU shows greater affinity for sharply bent, bulged DNA and four-way junctions than for duplex DNA (Pontiggia et al. 1993). HU

has also been shown to wrap DNA, constraining supercoils and forming beaded "nucleosome-like" structures (Rouvière-Yaniv et al. 1979; Broyles and Pettijohn 1986), and to enhance the binding of certain DNA bending proteins (Flashner and Gralla 1988; Yang and Ames 1990; Bonnefoy and Rouvière-Yaniv 1991). By analogy to the integration host factor (IHF) protein (Yang and Nash 1989), a model for HU binding via the minor groove of DNA has been proposed and invokes dramatic bending of the DNA helix (White et al. 1989); however, direct evidence has been lacking.

The HU protein is required for an early step in Mu DNA transposition *in vitro* (Craigie et al. 1985; Craigie and Mizuuchi 1987; Surette et al. 1987; refer to Fig. 1)

Figure 1. Mu *in vitro* strand cleavage reaction. A supercoiled mini-Mu donor plasmid containing the Mu ends in their natural orientation and the transpositional enhancer is converted to stable higher order nucleoprotein complexes or transpososomes in the presence of the Mu A and *E. coli* HU proteins as well as divalent metal ions (for review, see Mizuuchi 1992; Haniford and Chaconas 1992). IHF, a second *E. coli* factor, stimulates the reaction through the enhancer located ~1 kb from the Mu left end and lowers the HU requirement (Surette and Chaconas 1989). Although the transpososomes contain both Mu A and HU, the Mu A protein is responsible for maintaining the structural and functional integrity of the complexes (Lavoie and Chaconas 1990) through multiple protein-protein and protein-DNA interactions that constrain the ends of Mu. In Mg^{2+} , nicks are introduced at the 3' ends of Mu, yielding supercoiled Mu and relaxed vector domains to form a type 1 complex, also known as a cleaved donor complex (CDC) (Surette et al. 1987; Craigie and Mizuuchi 1987, respectively). In Ca^{2+} , the Mu ends are stably synapsed but uncut to yield a stable synaptic complex (SSC), which we call a type 0 transpososome; cleavage occurs rapidly upon addition of Mg^{2+} (Mizuuchi et al. 1992). The generation of these 3' hydroxyl groups precedes the strand transfer step (Craigie and Mizuuchi 1987; Surette et al. 1987).

and *in vivo* (Kano et al. 1989; Huisman et al. 1989). This initial step requires contact between three separate DNA elements: the two Mu ends, and the transpositional enhancer (Leung et al. 1989; Mizuuchi and Mizuuchi 1989; Surette et al. 1989; Allison and Chaconas 1992; Surette and Chaconas 1992). This results in the oligomerization of Mu A to a tetrameric unit (Lavoie et al. 1991; Baker and Mizuuchi 1992; Baker et al. 1993) that stably and noncovalently binds the ends of Mu to form the type 1 transpososome (Craigie and Mizuuchi 1987; Surette et al. 1987). Although both Mu A and HU have been localized to the transpososome core in >90% of complexes (Lavoie and Chaconas 1990), the *in vitro* role of HU is limited to the appropriate synapsis of the left and right Mu ends; HU is not required for strand cleavage (Mizuuchi et al. 1992) or subsequent strand transfer (Lavoie and Chaconas 1990). Moreover, HU does not contribute to maintaining the structural integrity of the formed transpososome complexes, as it can be completely removed by a 0.5 M NaCl wash leaving only Mu A (Lavoie and Chaconas 1990).

Interestingly, HU affinity for the transpososome core is much greater than for supercoiled DNA and is independent of DNA sequence. We have sought to understand the nature of this high affinity binding of HU. Previously, in an attempt to locate HU within the transpososome, we have footprinted type 1 complexes in the presence or absence of HU and found no nuclease protection that could be ascribed to the HU protein (Lavoie et al. 1991). We report the first site-specific HU-binding site and discuss its functional significance.

Results

Conversion of HU into a chemical nuclease

We attempted unsuccessfully to localize the HU-binding site in the transpososome by using a variety of DNA footprinting reagents including DNase I, micrococcal nuclease, methidiumpropyl-EDTA · Fe(II) [MPE · Fe(II)] and hydroxyl radicals (data not shown). We then adopted the alternative approach of chemical modification of the HU protein with an EDTA · Fe(II) complex capable of directly cleaving the sugar phosphate backbone via hydroxyl radical chemistry (see Fig. 2). This approach presents several advantages. The iron-EDTA complex can be stably introduced into the native protein (Ermácora et al. 1992) and does not promote spontaneous cleavage of the DNA (Ebright et al. 1992; Mazzarelli et al. 1993). This permits assembly of higher order nucleoprotein structures that require supercoiled substrates. Upon the addition of a reducing agent, DNA cleavage ensues rapidly, is highly localized, and shows little or no sequence specificity (Ebright et al. 1992; Mazzarelli et al. 1993).

The [EDTA-2-aminoethyl]-2-pyridyl disulfide · iron [EPD · Fe(II)] reagent has been used to cleave DNA within ~30 Å of the protein derivatization site (Ebright et al. 1992; Mazzarelli et al. 1993). It reacts with sulfhydryl residues and becomes coupled to proteins through a disulfide bond (Ebright et al. 1992; Ermácora et al. 1992;

Figure 2. Conversion of HU to a chemical nuclease. The HU protein was thiolated by reaction of 2-iminothiolane (Traut's reagent) with primary amino groups. EPD·Fe(III) could then be coupled to the modified HU by a disulfide exchange reaction. EDTA·Fe(III) was reduced by ascorbate to EDTA·Fe(II), the active species in the Fenton reaction (1896) in that iron(II) reduces hydrogen peroxide to give hydroxyl radicals that mediate DNA cleavage (see Tullius et al. 1987).

Mazzarelli et al. 1993]. Because HU is devoid of cysteines, 2-iminothiolane (Traut's reagent) was used to add sulfhydryl groups at primary amines (Traut et al. 1973; Jue et al. 1978). A mock-derivatized HU control was treated with the EPD·Fe(III) in the absence of 2-iminothiolane. Residues essential for DNA binding were protected from modification by performing the reaction with HU bound to DNA cellulose (Shellman and Pettijohn 1991). The EPD-derivatized HU protein made in this way was fully active in the generation of type 1 complexes (data not shown).

Site-specific HU binding in the transpososome

The HU–chemical nuclease was used to generate type 0 or type 1 complexes; DNA cleavage was induced by the addition of sodium ascorbate and hydrogen peroxide. After the labeling of individual strands at each Mu end, a standard sequencing gel was used to resolve the HU–nuclease cleavage products. To our surprise, cleavage was detected at only one of the two Mu ends, the left end, shown in Figure 3. Furthermore, strand scission was restricted to the 83-bp sequence between the L1 and L2 Mu A-binding sites and was seen in three main clusters. Cleavage was dependent on the presence of complex, HU–nuclease and reducing agent (Fig. 3A, cf. lanes 1–3 with lane 4 and lanes 7 and 8 with lane 9). An additional control confirmed that the signal stemmed from the derivatized HU and that its DNA-binding properties had not been grossly altered; derivatized HU was removed from the DNA by a 0.5 M NaCl treatment (Fig. 3B, lane 6 vs. 7). The efficient removal of HU in 0.5 M NaCl indicates further that cleavage was HU dependent and did not result from transfer of the EPD·iron from HU to the A protein via disulfide exchange.

The stability of binding of the derivatized HU in the complex was assessed by a competition experiment. The

addition of 10× excess competitor DNA, either supercoiled and devoid of Mu A-binding sites or linear Mu right end, did not significantly diminish the signal (Fig. 3A, cf. lane 4 with lanes 5 and 6; Fig. 3B, cf. lane 4 with 5).

The type 0 complex has been proposed to reflect an earlier intermediate in the strand cleavage reaction where the ends of Mu are correctly synapsed but uncut and requires HU for assembly (Mizuuchi et al. 1992). We compared the HU–nuclease cleavage pattern on the continuous strand in both type 1 and type 0 complexes and found no difference (see Fig. 3A); thus, type 0 complexes were used to probe cleavages on the nicked strand (see Fig. 3B). As on the continuous strand, the primary cleavages map to the spacer region between L1 and L2; however, faint cleavage products were also detectable on the nicked strand, near the Mu–host junction (arrow). These bands are specific to the complex but could sometimes be observed in the presence of mock-treated HU or in the absence of reducing agent and H₂O₂. We postulate that the sugar–phosphate backbone is strained in these positions, possibly as a prelude to cleavage at the terminal nucleotide of Mu.

HU–nuclease cleavages cluster in regions of dyad symmetry

The cleavages from the HU–nuclease were mapped to nucleotide resolution (Fig. 4) and cluster around two elements of dyad symmetry, termed dyads 1 and 2. The cleavages also varied in relative intensity, the strongest mapping to dyad 2, a perfect 12-bp self-complementary element. Because HU has been shown recently to bind to four-way junctions (cruciforms and Holliday structures) (Pontiggia et al. 1993), we assessed the importance of self-complementary sequences in dyad 2, that contains the strongest HU–nuclease cleavage sites on both

Figure 3. HU-nuclease cleavage at the Mu left end. Type 1 or type 0 transpososomes were generated using HU-nuclease or mock-derivatized HU as described in Materials and methods. (SC) Supercoiled mini-Mu DNA incubated in the absence of Mu A (no complex formation). Hydroxyl radical DNA cleavage was induced by the addition of sodium ascorbate and H_2O_2 either directly or following the addition of supercoiled nonspecific (+) or linear right end (+R) competitor DNA. Mu A-binding sites are represented by brackets. (→) The junction between the Mu and vector sequences. The continuous and nicked strands are shown in A and B, respectively. Markers are Maxam-Gilbert G-specific reactions of the appropriate strands.

strands (Fig. 4). We constructed mutants that destroy intrastrand base-pairing ability without changing the GC content. The HU requirement for type 1 complex formation was unaffected in the mutants (Fig. 5A), and the nuclease cleavage patterns were largely unchanged (Fig. 5B). Subtle changes in the ratios of cleavage were detectable on the nicked strand, however, especially for the triple base pair mutant (pBL3M2). These may reflect a low level sequence specificity or local structural alteration that affects hydroxyl radical cleavage. Thus, HU action in the Mu strand cleavage reaction does not seem to require cruciform extrusion or binding.

A helical projection of the HU-nuclease cleavages between L1 and L2 is depicted in Figure 6. The arrows represent cleavage sites and cluster on one face of the helix across minor grooves. This pattern is consistent

with the spacer DNA being wrapped around the HU protein, possibly to mediate formation of a tight loop or bend between the L1 and L2 Mu A-binding sites.

Discussion

HU interacts stably with the L1–L2 spacer DNA

Using the Mu in vitro strand-cleavage reaction, we report here the first direct demonstration of a specific HU-binding site in a higher order nucleoprotein structure. By employing an HU-chemical nuclease, we have shown that HU binds to the left end of Mu, within an 83-bp pair intervening DNA segment separating the left end-most Mu A-binding site L1 from the L2 and L3 sites. The cleavages are specific, span three to four minor grooves,

Figure 4. Nucleotide localization of HU-nuclease cleavages. Mu left end sequence. Upper- and lowercase letters denote Mu and vector DNA, respectively. Bases are numbered 1 from the first T of Mu; the Mu A-binding sites are underlined. Relative intensities of the HU-nuclease cleavages were derived as described in Materials and methods and are represented by the histograms above (continuous strand) and below (nicked strand) the sequence; these are not directly comparable between strands. The elements of dyad symmetry are denoted by brackets showing intrastrand base-pairing potential. Mutations generated in dyad 2 are shown. Restriction sites used for labelling are indicated: *Bam*HI and *Sty*I were used for the nicked and continuous strands, respectively.

and cluster within two regions of dyad symmetry, with the most frequent cleavages mapping to dyad 2, a 12-bp element. HU is known to have a much higher affinity for bent DNA structures, such as four-way junctions (cruciforms and Holliday structures), than for duplex DNA, and its binding is mediated through structure rather than sequence recognition (Pontiggia et al. 1993). Although the inverted repeats between L1 and L2 are very short and not likely to form stable cruciforms, we investigated further the requirement for cruciform formation for HU action by two approaches. In the first approach, we treated HU-containing and -depleted type 1 complexes with P1 nuclease to probe for cruciform loops or destabilized regions. Previously, P1 cleavage of the single-stranded DNA regions of cruciforms was shown to be unaffected by the presence of HU (Pontiggia et al. 1993). We did not detect any P1 cleavage in the L1–L2 region in the presence or absence of HU, suggesting that stable cruciforms were not extruded in the type 1 transpososome (data not shown). In the second approach, we disrupted the base-pairing potential for cruciform extrusion at two or three positions in dyad 2, where the major HU-nuclease cleavages are found. We investigated the effect of these changes both on the HU requirement in the type 1 complex formation and on the binding of HU to the L1–L2 spacer. The HU concentration dependence in the strand-cleavage reaction was unaffected in the mutants. Furthermore, HU-nuclease experiments using the mutants revealed that HU binding between L1 and L2 was similar to wild type and mapped to both dyads. Taken together, the above data indicate that cruciform formation is unlikely to be involved in HU binding and function at the L1–L2 spacer.

HU has been assumed to promote a variety of site-specific DNA metabolic events by its ability to affect DNA flexibility through very short-lived, sequence-independent DNA interactions. We were thus surprised at the discrete cleavage pattern generated by the HU-nuclease and from our inability to displace HU from its binding site(s) in the type 0 and Type 1 complexes with

10× excess DNA. This unusual stability of HU binding was also observed by electron microscopy and was shown by control experiments not to be an artifact of chemical modification of the HU (data not shown). Why, then, is HU binding in the Mu transpososome so tight? It is tempting to speculate that on duplex DNA, HU binding is transient because it requires a structural distortion of the DNA such as bending or wrapping. These conformational perturbations are energetically costly and are usually short lived, resulting in release of HU protein as the DNA rapidly returns to its normal structure. If, however, the altered DNA structure induced by HU is stabilized by the formation of a higher order nucleoprotein complex involving Mu A, the HU can then remain stably bound to its preferred DNA conformation for long periods of time. The nature of this proposed altered structure is discussed below.

Finally, it is worth noting that for reasons not yet understood, HU stably bound to a specific region of DNA can escape detection by a variety of footprinting reagents (Lavoie et al. 1991; Mizuuchi et al. 1991; Hwang and Kornberg 1992; data not shown); further investigation of the role of HU in other systems may benefit from the approach used here.

HU-dependent loop formation between L1 and L2

The binding of HU between L1 and L2 is consistent with the formation of a tight DNA loop that would allow distant Mu A monomers to interact. In the simplest case, communication between these two sites would involve the extrusion of the ~80 bp of intervening DNA. The ~80-bp spacer is predicted to be too small to allow efficient looping back of the DNA on itself, as judged by the inability of DNA fragments <126 bp to cyclize (Shore et al. 1981). HU has been shown to induce DNA flexure (Hodges-Garcia et al. 1989) and allow cyclization of fragments as small as 78 bp (Paull et al. 1993). Moreover, the HU-nuclease cleavage pattern is restricted to one face of the DNA helix, suggesting that the looped out DNA is

Figure 5. HU requirement and nuclease cleavage in dyad 2 mutants. (A) The strand-cleavage reaction as a function of HU concentration was analyzed for wild-type mini-Mu (WT) and the two dyad 2 mutants. Reactions were performed and quantitated as described in Materials and methods. (B) Type 0 complexes containing HU-nuclease were generated using wild-type and dyad 2 mutants as described in Fig. 4. (+) Addition of sodium ascorbate and hydrogen peroxide. Dyads are indicated by brackets. The sequence is numbered from the terminal base pair of the Mu left end.

sharply bent or wrapped around an HU core, which may prove to be a general feature of tight, HU-dependent loops.

A similar function for HU has been shown in the *hin* system, where HU is required to mediate interactions between the enhancer and the left *hix* site when the enhancer is moved to <104 bp from *hixL* (Haykinson and Johnson 1993) and appropriately phased (Paull et al.

1993). The nature of the intervening DNA is not important for the reaction (Paull et al. 1993) but is looped out of the complex (Heichman and Johnson 1990). Also, the HU requirement for efficient inversion can be circumvented by moving the enhancer farther from the *hixL* site, indicating that HU activity in *hin* recombination is primarily limited to loop formation (Haykinson and Johnson 1993). Experiments of this type are complicated in the Mu system because addition of DNA to the L1–L2 region (plus 200, 320, or 440 bp) renders the substrate inactive in transpososome formation under our standard reaction conditions (data not shown). Alternatively, HU may induce a precise geometrical configuration of the Mu left end required for interaction with the Mu right end and/or with the transpositional enhancer, as formation of the transpososome involves a myriad of protein–protein and protein–DNA contacts (Allison and Chaconas 1992, and unpubl.).

The question of HU stoichiometry is also worthy of comment. Haykinson and Johnson (1993) have shown that significant inversion stimulation (50%) occurs in the presence of approximately three HU dimers per DNA substrate. Similarly, significant stimulation (50%) of the Mu in vitro strand-cleavage reaction requires ~10 HU dimers per DNA substrate. Thus, modulation of DNA flexibility in site-specific recombination reactions does not require coating of the DNA with HU but, rather, relies on a small number of HU molecules acting at a precise location.

Interpretation of the HU footprint derived with the HU-nuclease

The HU-nuclease used in these studies was generated by randomized derivatization of solvent-accessible primary amino groups in native HU while bound to DNA. The total number of primary amino groups per HU dimer is 22; the actual number modified in the HU-nuclease is unknown, although at least 30% of our HU-nuclease preparations showed altered migration on SDS-polyacrylamide gels in the absence of 2-mercaptoethanol as compared with unmodified HU (data not shown). The observed cleavage pattern from HU-nuclease is therefore expected to reflect derivatization with EPD · Fe(II) at a variety of HU sites. Coupling of the reagent to a unique site in the DNA-binding portion of the CAP and $\gamma\delta$ resolvase proteins yielded single (CAP) or multiple ($\gamma\delta$ res) DNA cleavages indicative of the protein–DNA interface (Ebright et al. 1992; Mazzarelli et al. 1993, respectively). The single or multiple cleavages observed presumably reflect the positioning and rotational freedom of the 14 Å EPD arm in DNA-bound protein. Because hydroxyl radicals are diffusible to ~17 Å (Dervan 1991), we reasoned that EPD · Fe(II) modification of HU outside its DNA-binding domain would also result in DNA cleavage up to a distance of ~30 Å from the modification site. On the basis of this information, three interpretations of our data are possible. First, EPD · Fe(II) coupled in or close to the DNA-binding domain of HU would result in a DNA-cleavage pattern indicative of HU dimer positions on

Figure 6. Helical representation of HU cleavages. Arrows are roughly proportional to cleavage intensity. Weaker cleavages shown by dots in Fig. 4 are not shown. Elements of dyad symmetry are shown in brackets.

DNA; thus, in the Mu transpososome, one to four HU dimers would bind in the minor grooves where cleavage is observed (Fig. 6), on one face of the helix. Such an arrangement would require the tight packing of HU dimers observed at high concentration on oligonucleotides (Bonnefoy and Rouvière-Yaniv 1991) and might reflect the formation of higher order HU structures able to wrap DNA (Rouvière-Yaniv et al. 1979; Broyles and Pettijohn 1986).

Second, EPD·Fe(II) coupled primarily to the non-DNA-binding portion of HU would yield a pattern similar to that from a classical footprint. In this case, some regions of the DNA not in actual contact with HU would be expected to be close enough to the derivatized sites of the protein to experience cleavage. Thus, all of the observed cleavages could result from one HU dimer, located in the “protected” minor groove (bp 70–80) central to the nicks (see Fig. 6). If the DNA were to wrap or sharply bend around the HU dimer, the DNA backbone would thus be brought into proximity of the EPD·Fe(II) moieties, and one face of the DNA helix would be preferentially cut, yielding the very large pattern observed. Such a scenario is reminiscent of how the IHF protein is believed to interact with its target site, protecting some 45 bp from nuclease digestion (Craig and Nash 1984; Yang and Nash 1989) by bending the DNA around itself (see Yang and Nash 1989; Nash 1990). Considering the structural (Drlica and Rouvière-Yaniv 1987) and functional (Surette and Chaconas 1989; Skarstad et al. 1990; Goodman et al. 1992) similarities between HU and IHF, it is tempting to speculate that HU could bind DNA in this way, albeit without the sequence recognition element. Generation of an IHF–nuclease, using the same chemistry as used for HU, has allowed us to probe the IHF-binding site found in the Mu transpositional enhancer. An equally broad footprint spanning ~50 bp and restricted to one side of the helix was observed with the IHF–nuclease (data not shown). Because IHF binds its recognition sequence as a dimer (Yang and Nash 1989), the extended cleavage pattern is consistent with our second interpretation. Although further experiments will be required to unequivocally assign HU–nuclease cleavages as originating from modified residues within and outside of the DNA-binding domain, we infer from the IHF–nuclease result that a dimer of HU–nuclease could account for the large pattern observed.

A third possibility that we consider unlikely is that HU modified in the non-DNA-binding portion cleaves DNA in *trans*. In this case, modification of HU in a region far from the DNA-binding domain could result in cleavage of a second DNA helix that is not bound by HU but lies juxtaposed in the type 1 complex. This option

does not seem viable because in the absence of the L1–L2 spacer region no HU–nuclease cleavage was detected in the corresponding (and unoccupied) DNA region (data not shown).

In summary, we have chemically coupled the *E. coli* protein HU with an iron–EDTA-based, DNA cleavage reagent and used it to probe higher order complexes involved in Mu DNA transposition. The HU–nuclease has provided the first site-specific footprint of a sequence-independent DNA-binding protein. Surprisingly, stable HU binding was observed at the Mu left end. HU–nuclease cleavages were mapped to an 83-bp spacer between the L1 and L2 Mu A-binding sites and lead us to postulate that HU binding induces a tight DNA bend or loop required for complex assembly. Finally, this approach may prove valuable for other proteins refractory to standard footprinting methods.

Materials and methods

Reagents and enzymes

Chemicals for protein modification were obtained from the following sources: *bis*(sulfosuccinimidyl)suberate (BS³) and 2-iminothiolane (Traut's reagent) were from Pierce; Sepharose 6B and DNA–cellulose were purchased from Pharmacia. Hydroxylapatite, Chelex 100 (100–200 mesh) was from Bio-Rad. Promega restriction enzymes and BRL Mo-MLV reverse transcriptase and low melting-point agarose were used. The EPD·Fe(III) was a generous gift from Dr. Mario Ermácora and Dr. Robert Fox (Yale University, New Haven, CT).

DNA substrates and proteins

The mini-Mu donor substrates used in this work have been described elsewhere, pGG215 (Surette et al. 1987), pBL03 (Lavoie et al. 1991), or were derived from pBL03. Briefly, these DNAs contain ~1.7 kb from each of the Mu ends cloned into pBR322 (pGG215) or the filled-in *Hind*III site of pUC19 (pBL03). pBL3M1 and pBL3M2 are site-directed mutants containing GC → CG or GCT → CGA changes, respectively, beginning at nucleotide 81 from the left end of Mu.

The Mu A and *E. coli* IHF proteins were purified as described previously (Lavoie et al. 1991; Nash et al. 1987, respectively) with the addition of a heparin–Sepharose column as the final step in IHF purification (Surette and Chaconas 1989). Mu A was quantitated using an extinction coefficient of ϵ_{280} 1 mg/ml = 1.83 (Kuo et al. 1991), which indicated that our previous quantitation by Bradford assay (Surette et al. 1987; Lavoie et al. 1991) overestimates Mu A by a factor of 2.3. IHF was quantitated by TCA Lowry with bovine serum albumin standards as described (Surette and Chaconas 1989).

E. coli HU was overexpressed from a strain kindly provided by Li Huang and Roger McMacken (The Johns Hopkins University, Baltimore, MD). Briefly, 300 ml of cells was grown in LB + 50 μ g/ml ampicillin at 30°C to an A_{595} of 1.7–2.0. Two hundred

milliliters of the same growth medium prewarmed to 42°C was added, and the culture was induced for 25 min at 42°C, 250 rpm. After centrifugation (6000g, 4°C) the pellet was rinsed in 25 mM HEPES–KOH at pH 7.6, 1 mM DTT, and 0.1 mM EDTA and resuspended in 10 ml of this buffer, transferred to an Oakridge tube, and frozen in liquid nitrogen (LN₂). After slowly thawing the cells in 10°C water, 0.25 volume of 4 M KCl and 0.01 volume of 25 mg/ml of egg lysozyme were added and incubated for 30 min on ice. The cell suspension was then refrozen in LN₂, allowed to thaw slowly as before, and the freeze–thaw cycle was repeated once more. Following the centrifugation of the lysate (65,000 g for 1 hr, 4°C), the supernatant was dialyzed against 2 × 2 liters of buffer A [40 mM imidazole–HCl at pH 6.75, 1 mM EDTA] using a tubing of MWCO 3500 for all HU dialysis steps. The dialysate was made 20% (wt/vol) in glycerol and loaded onto a pre-equilibrated P11 column (6 ml). After washing the column with 0.5 volume equilibration buffer, HU was eluted using a 50-ml linear gradient of buffer A plus 20% glycerol from 0–0.7 M KCl collecting ~1.5-ml fractions. HU-containing fractions were identified in strand–cleavage assays, pooled (the nuclease peak following HU elution was not included), and dialysed against 2 liters of buffer A overnight. After addition of glycerol to 20% (wt/vol), the dialysate was loaded onto a 0.4-gram hydroxylapatite column pre-equilibrated in the same buffer. The column was then washed with 2–3 ml of buffer B (50 mM PIPES at pH 6.7, 20% glycerol) and eluted with a 15-ml gradient: buffer B → 0.3 M NaH₂PO₄ at pH 6.8, 20% glycerol, in 0.5-ml fractions. HU activity was assayed as before, and active fractions were pooled and dialyzed overnight against 1 liter of buffer C [25 mM HEPES–NaOH at pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 5% glycerol]. Approximately 5 mg of HU in 5 ml of buffer C was recovered. To further purify our HU from low-level contaminants, 40% of the dialysate (2 mg) was then loaded onto a 0.4-gram heparin–Sepharose 6B column, preswollen, and equilibrated in buffer C. A 0.1–1.3 M NaCl gradient was used to elute HU, which was recovered in ~1 M NaCl. HU was quantitated by TCA Lowry, as described previously (Surette and Chaconas 1989) and contained <0.5 ng of IHF/μg of HU as judged by Western blots.

In vitro reactions

Type 1 and type 0 *in vitro* reactions were carried out as described previously (Surette and Chaconas 1989, Mizuuchi et al. 1992, Surette and Chaconas 1992, respectively). Typically 25- to 100-μl reactions containing a 10-fold increase in the standard protein and DNA concentrations were run for 5 min at 30°C (e.g., 150 μg/ml of mini-Mu, 30 μg/ml of Mu A, 37.5 μg/ml of HU, and 1 μg/ml of IHF). This apparent decrease in Mu A concentration reflects the change in quantitation method (absorbance vs. Bradford assay) rather than an actual change in the amount of protein used. All complexes were formed in 25 mM HEPES–NaOH at pH 7.8 (to avoid interference of Tris with amine-specific reagents) in the presence of 100–140 mM monovalent cation. Type 1 and type 0 reactions required 10 mM MgCl₂ or CaCl₂, respectively. Reactions were monitored by agarose gel electrophoresis, and the extent of reaction was quantified by whole band analysis of fluorescence using a CCD camera and Gel Print Tool Box software from Biophotonics Corp. (Ann Arbor, MI).

HU derivatization and DNA cleavage

Chemical modification of basic residues of HU has been shown to occur on DNA–cellulose (Shellman and Pettijohn 1991). To

preserve HU activity in stimulating transpososome formation, 20 μg of HU protein (~23 nmoles of primary amino groups) was prebound to a 50-μl DNA cellulose column (made from a cutoff 200-μl capillary tube) in 50 mM HEPES–NaOH at pH 7.8 and 50 mM NaCl (buffer D). This buffer was made from Chelex 100-treated stocks and deoxygenated by bubbling nitrogen (using a gas dispersion tube) through a prewarmed (90°C) solution for 1 hr in a cold room. The column was rinsed with 3 volumes of buffer D and 1.2 column volumes of freshly made reaction cocktail consisting of 0.4 mM 2-iminothiolane and 0.2 mM EPD · Fe(III) in buffer D was flushed into the column. Nitrogen gas was flushed into the top of the column before sealing, and the reaction proceeded for 4 hr at room temperature. Mock-derivatized HU columns had the 2-iminothiolane omitted from the reaction cocktail. To terminate the reaction, the column was washed with 5 volumes of buffer D; no HU eluted in these 50 μl fractions. HU was eluted by increasing the salt to 0.5 M NaCl (buffer D plus 0.5 M NaCl) and was detected by immunoblotting in the first 2 column volumes (100 μl final) to yield a final concentration of ~200 μg/ml. This HU was used directly in type 1 assays and was found to nick supercoiled DNA only in the presence of a reducing agent; 1 mM sodium ascorbate + 0.03% H₂O₂, 10 mM or 20 mM sodium ascorbate were equally effective in promoting nicking. As expected, the extent of nicking was proportional to the concentration of HU; under the conditions used to generate type 1 complex, >90% of the DNA was nicked in the presence of ascorbate. Also, using suboptimal amounts of the HU–nuclease, the nicking reaction was found to be complete after 2 min; longer incubations had little effect. The mock-derivatized HU was ineffective in promoting DNA nicking in the presence or absence of ascorbate.

HU–nuclease cleavage in the transpososome

Fivefold concentrated reactions were assembled on ice using pBL03; type 1 complexes were generated in 10 mM MgCl₂ and type 0 in 10 mM CaCl₂. To reduce background (HU–nuclease-independent nicking), all buffer stocks were treated with Chelex 100 before use. Following a 5-min incubation at 30°C, the reactions were stopped by immediate removal to ice for 2–3 min. Reaction efficiency was >90% conversion of input DNA to complex form. For experiments using competitor DNA, a 10× excess of supercoiled non-Mu DNA (pSD7–Sac or KP91 for the continuous or nicked strands, respectively) or PCR-generated Mu right end fragment containing the R1, R2, and R3 Mu A-binding sites was added after the initial 30°C incubation. To remove HU from the transpososome core, 0.5 M NaCl was added to complexes at the same time as competitor DNA in the presence of the protein cross-linker BS³, which inactivated HU in solution, and incubated for 10 min at room temperature. HU–nuclease cleavage was then induced by the addition of freshly prepared sodium ascorbate and hydrogen peroxide to 1 mM and 0.03% final, respectively. Hydroxyl radical cleavage was allowed to proceed for 2–5 min and quenched by the addition of thiourea and EDTA to 100 mM and 20 mM, respectively, as described previously (Lavoie et al. 1991). The samples were deproteinized by a phenol–chloroform (1:1) extraction followed by gel filtration spin column chromatography (50 mM HEPES–NaOH at pH 7.8, 100 mM NaCl). MgCl₂ was added to the DNA containing void volume (to 10 mM), and StyI or BamHI (continuous and nicked strands, respectively) was added. 3′-Labeling used Mo-MLV reverse transcriptase and [α³²P]dCTP (6000 Ci/mole) supplemented by cold dATP, dGTP, and dTTP for labeling at the BamHI site. After a 30-min incubation at 37°C, unincorporated label was removed by spin column chromatography as before. Segregation of the label was accomplished by a

second restriction digest; the *SspI* and *MluI* enzymes were used (continuous and nicked strands, respectively) and the DNA was subjected to electrophoresis through a 1.2% low melting-point agarose gel. The left end of Mu was isolated from the gel by phenol extraction and ethanol precipitation (Selden and Chory 1987) and redissolved in 3–5 μ l of water to which 1 volume of formamide loading dye was added. Samples were heated to 95°C for 3 min, loaded onto a 6.6% standard sequencing gel, and run at constant current (36 mA, 50°C). Finished gels were dried and autoradiographed without an intensifying screen. Densitometry was performed on the X-ray film as described previously (Lavoie et al. 1991). Maxam–Gilbert G reactions were performed as described (Maxam and Gilbert 1980) using linear DNA labeled analogously to the samples.

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