The EMBO Journal Vol.18 No.19 pp.5434–5444, 1999

The binding motif recognized by HU on both nicked and cruciform DNA

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The heterodimeric HU protein, highly conserved in bacteria and involved in transposition, recombination, DNA repair, etc., shares similarity with histones and HMGs. HU, which binds DNA with low affinity and without sequence specificity, binds strongly and specifically to DNA junctions and DNA containing single-strand breaks. The fine structure of these specific complexes was studied by footprinting and HU chemically converted into nucleases. The positioning of HUαβ on nicked DNA is asymmetrical and specifically oriented: the β-arm binds the area surrounding the break whereas the α-arm lies on the 3′ DNA branch. This positioning necessitates a pronounced bend in the DNA at the discontinuous point, which was estimated by circular permutation assay to be 65°. At junctions, HU is similarly asymmetrically positioned in an identical orientation: the junction point plays the role of the discontinuous point in the nicked DNA. The HU binding motif present in both structures is a pair of inclined DNA helices.

Keywords: DNA containing a nick/DNA junction/DNA structural binding motif/heterodimeric protein/histone-like protein

Introduction

The assembly of the proper architecture of higher-order DNA–protein complexes frequently requires accessory proteins. In *Escherichia coli* several chromosomal proteins, such as LRP, FIS, H-NS, IHF and HU, are good candidates for such a role. The latter two proteins, which belong to the same family, are composed of two non-identical but homologous subunits, α and β, of ~9 kDa (Drlica and Rouviere-Yaniv, 1987; Nash, 1996). Proteins closely related to HU have been identified in virtually every species of bacteria. In most bacteria, however, HU is a dimer of identical subunits, the heterodimeric HU being a characteristic of enterobacteria (Oberto and Rouviere-Yaniv, 1996). In *E.coli*, at the end of the growth phase and during the stationary phase, HU is present mainly as its αβ heterodimeric form (Rouviere-Yaniv and Kjeldgaard, 1979; Claret and Rouviere-Yaniv, 1997). The three-dimensional structure of the homodimeric HU from *Bacillus stearothermophilus*, in the absence of DNA, has been solved by both X-ray crystallography and NMR (Tanaka *et al*., 1984; White *et al*., 1989, 1999; Vis *et al.*, 1995). The two subunits are interwined to form a compact 'body' from which two long β-ribbon 'arms' extend. The three-dimensional structure of IHF complexed to DNA, solved by X-ray crystallography, shows that the folding of IHF is essentially the same as that of HU (White *et al*., 1989, 1999); the 'bodies' of the two proteins can be superimposed with a deviation of 1 Å (Tanaka *et al*., 1984; Rice *et al*., 1996; White *et al*., 1999).

HU plays a pleiotropic role in bacteria. It was first characterized as a histone-like protein in *E.coli* due to its capacity to introduce negative supercoiling into relaxed DNA molecules in the presence of topoisomerase I (Rouviere-Yaniv *et al.*, 1979). More recently, it has been shown that there is cross-talk between the relative quantity of HU in the cell and the activity of topoisomerase I (Bensaid *et al.*, 1996). HU also plays a role in DNA repair (Boubrik and Rouviere-Yaniv, 1995; Li and Waters, 1998), DNA recombination and DNA replication (Bramhill and Kornberg, 1988). The *in vitro* studies of the interaction of HU with DNA have also outlined the role of HU in DNA repair and recombination. Effectively, although HU binds DNA linear fragments with a low affinity and without sequence specificity, it binds four-way DNA junctions and nicked DNA under stringent conditions (200 mM NaCl) and with high affinity (Pontigga *et al.*, 1993; Bonnefoy *et al.*, 1994; Castaing *et al.*, 1995). In fact HU, at least HUαβ and HUα2, binds these two structures with nearly identical binding constants (Pinson *et al.*, 1999). Finally, HU has been shown to participate in Mu transposition (Lavoie and Chaconas, 1993), and in *gal* operon transcriptional regulation (Aki and Adhia, 1997), due to its interaction with supercoiled double-stranded DNA, which stabilizes higher-order DNA–protein complexes built on DNA molecules.

Eukaryotic proteins of the HMG class and histones H1–H5 also bind double-stranded DNA with low affinity and no sequence specificity, but have high affinity to DNA junctions (Bianchi, 1988; Hill and Reeves, 1997). Resolvases, nucleases that recognize DNA junctions without sequence specificity, comprise another class of DNA junction-recognizing proteins (West, 1997; Grainge and Sherratt, 1999). These enzymes bind and cut DNA junctions, but they do not cut DNA containing a nick (Bhattacharyya *et al.*, 1991). Conversely to both resolvases and HMGs, HU specifically binds both structures: one HU dimer binds on DNA containing a nick or a gap (Castaing *et al.*, 1995; Pinson *et al.*, 1999), whilst two HU dimers bind specifically to DNA junctions (Pontiggia *et al.*, 1993; Bonnefoy *et al.*, 1994). We wondered if there might be a common structural motif between these two DNA substrates that are recognized by HU. Therefore, we investigated the complexes formed by

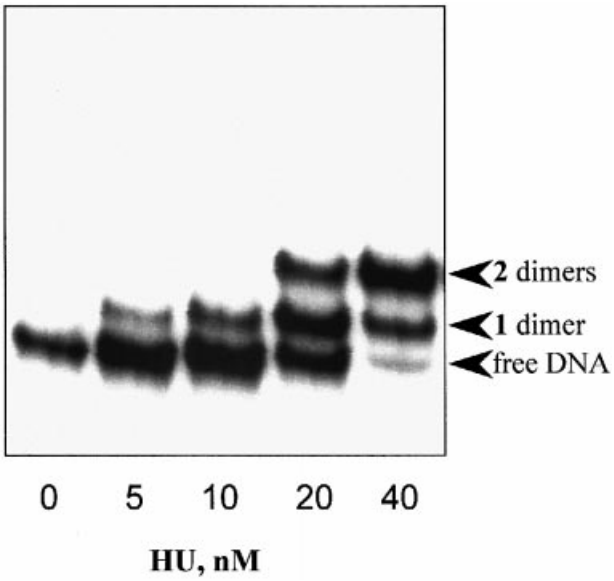


Fig. 1. Separation of the specific complexes DNA junction–HU from non-bound DNA. The ^{32}P-labeled DNA junction and HU (at the indicated concentrations) were mixed in high-salt buffer and the mixture was fractionated on native acrylamide gel buffered with Tris–borate.

the HU protein with both aforementioned DNA substrates in more detail. We show here that HU heterodimers bind these two structures in a specific and similar way. The heterodimer is oriented with respect to either the DNA junction point or the DNA break point. The center of symmetry of the HU$\alpha\beta$ protein lies on the 3′ branch of nicked DNA and its β-arm interacts with the discontinuous point. The same asymmetrical position is found for the HU–junction complex. This asymmetrical positioning reveals an unexpected resemblance between these two distinct DNA structures. HU recognizes, both in the junction DNA and in nicked DNA, a structural motif formed by a pair of helices with the propensity to be inclined. We suggest that this bending at the break point allows contact between the DNA and HU body, thereby rendering the HU–DNA complex more stable.

Results and discussion

Phenanthroline protection analysis

As a model for a DNA junction, we used four synthetic oligonucleotides annealed together to produce a junction with an immobile junction point. The folding of this junction, called Junction 3, in different salt conditions has been well documented by Lilley's group, and in particular its X-shaped configuration has been determined (Duckett *et al.*, 1988, 1990). It has been shown previously that two HU dimers bind specifically to the DNA junction while additional non-specific binding occurs on the branches of the junction when the interaction is studied in low-salt conditions (Bonnefoy *et al.*, 1994; Pinson *et al.*, 1999). This non-specific binding is barely detectable under high-salt conditions (200 mM NaCl), therefore the stringent conditions, which allow the formation of only two specific complexes, were used throughout the present work (Figure 1). To localize the position of HU on this junction, we first used the phenanthroline footprinting technique.

The geometry of the HU arms, and the charge distribution combined with DNA footprinting and structural data from IHF, suggested that the arms might wrap around the minor groove of the DNA (White *et al.*, 1989, 1999; Yang and Nash, 1989; Rice *et al.*, 1996). To establish this and to investigate further the interaction of HU with DNA junctions, we used the phenanthroline–Cu complex, an efficient chemical nuclease that cleaves the phosphodiester backbone of DNA after its intercalation into the minor groove. This compound has been shown to be sensitive to local conformational changes in the DNA induced by protein binding (Spasky and Sigman, 1985). The difficulty in applying this technique to HU was that all the previous attempts to localize HU on duplex DNA had failed (Lavoie and Chaconas, 1993; J.Rouviere-Yaniv and others, unpublished data). Therefore, no clear footprint for this protein bound to naked DNA existed. To overcome this problem, we tried to stabilize the HU–DNA contacts during footprinting. It has been shown by Garner and Revzin (1981) that the 'cage-effect' of the gel matrix maintains the integrity of the nucleoprotein complex during electrophoresis. The HU–DNA complexes were therefore treated with phenanthroline within the native gel subsequent to separation of the bound and free DNA (Papavassiliou, 1994). Following phenanthroline treatment, the four DNA strands of the junction were analyzed on sequencing gels. To reveal those DNA sites modified by HU binding, the cleavage patterns of bound DNA (lanes 'b') were compared with free DNA (lanes 'fr') (Figure 2A). Our results show clearly that HU modifies the phenanthroline cleavage, decreasing (→) or increasing (●—) the cleavage patterns, proving that HU does indeed interact with the minor groove.

These modified DNA sites were mapped relative to the DNA sequence (Figure 2B). Junction branches are named according to their 5′ strands. Branches R and H were clearly less protected by HU than their symmetrical counterparts, branches B and X. This finding was unexpected and not easily explained since we have two HU dimers in the complex analyzed (Figure 1). Therefore, we decided to study in parallel the specific binding of HU to another DNA structure. A DNA containing a single-strand break with which HU forms a single specific complex (Castaing *et al.*, 1995; Pinson *et al.*, 1999) was selected. To facilitate the comparison with the junction, we used a nicked molecule made from branches B and X of the junction substrate (Figure 2B). The binding of HU on this nicked molecule was probed by phenanthroline, again directly in the gel (Figure 2C). Residues whose phenanthroline sensitivity is altered upon HU binding were mapped and compared with the pattern observed with DNA junction (Figure 2B). HU clearly interacts with the minor groove in both DNA structures. A marked similarity in the footprinting patterns of the DNA branches B and X is observed in both structures (Figure 2B). The protected region for both DNAs is large, ~20 bp. This extent of protection can be explained by bending of the DNA molecules upon HU binding, as will be discussed below.

The resemblance observed between the protected sites on the corresponding B and X branches of both substrates raised two points. First, we wondered whether the preferential binding to this half of the junction was due to

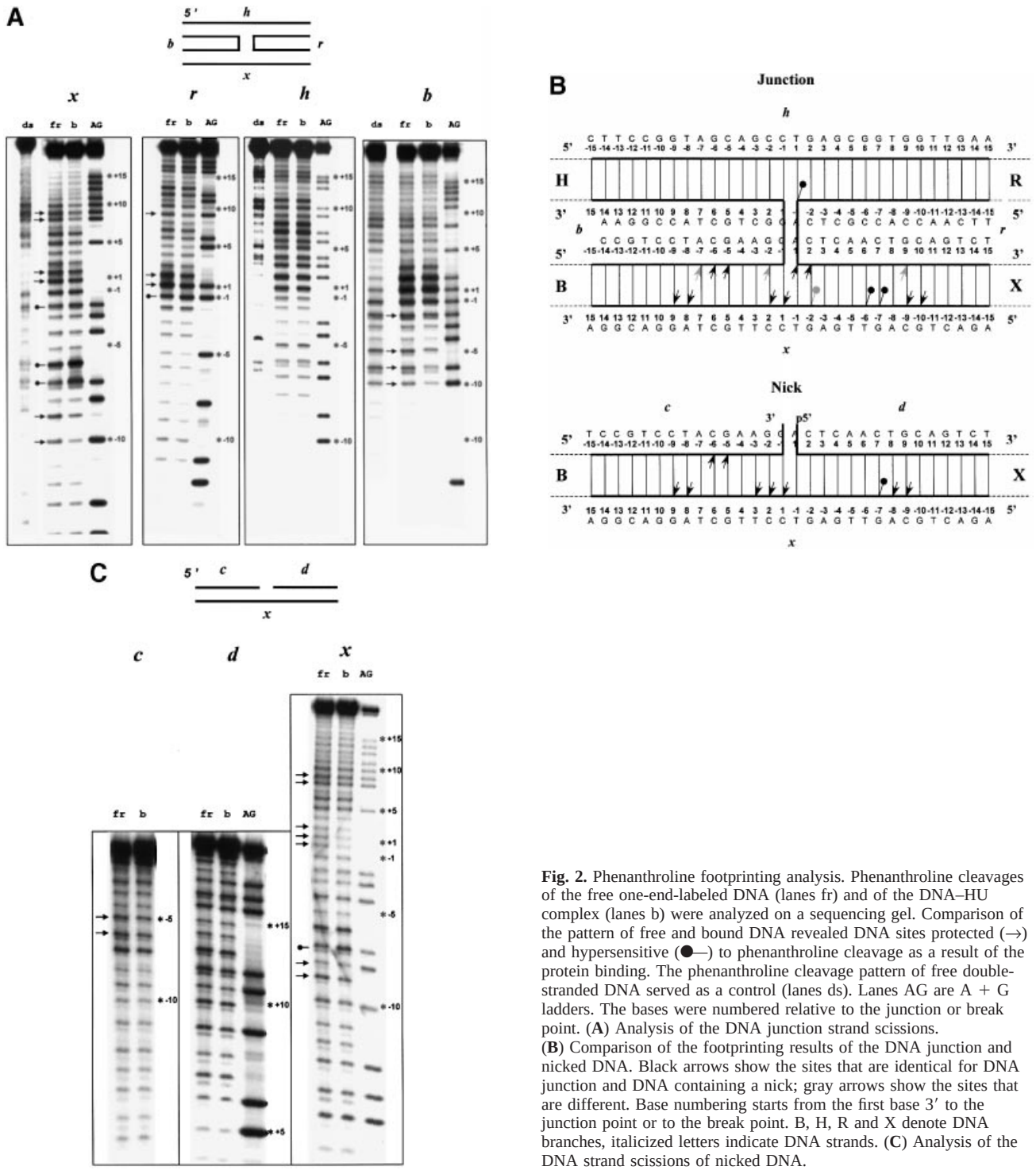


Fig. 2. Phenanthroline footprinting analysis. Phenanthroline cleavages of the free one-end-labeled DNA (lanes fr) and of the DNA–HU complex (lanes b) were analyzed on a sequencing gel. Comparison of the pattern of free and bound DNA revealed DNA sites protected (→) and hypersensitive (●—) to phenanthroline cleavage as a result of the protein binding. The phenanthroline cleavage pattern of free double-stranded DNA served as a control (lanes ds). Lanes AG are A + G ladders. The bases were numbered relative to the junction or break point. (**A**) Analysis of the DNA junction strand scissions. (**B**) Comparison of the footprinting results of the DNA junction and nicked DNA. Black arrows show the sites that are identical for DNA junction and DNA containing a nick; gray arrows show the sites that are different. Base numbering starts from the first base 3′ to the junction point or to the break point. B, H, R and X denote DNA branches, italicized letters indicate DNA strands. (**C**) Analysis of the DNA strand scissions of nicked DNA.

a sequence preference. To explore this possibility, we constructed a nicked molecule with the R and H branches of Junction 3. We recall that the cleavage pattern of these R–H branches of the junction was practically unmodified in the presence of HU (Figure 2B), but HU protected this R–H nicked structure as it had protected the B–X nicked structure, although to a lower extent (data not shown). This might reflect a slight preference of HU for the sequences contained in the B and X branches. The sensitivity of this technique is probably not sufficient to obtain clear protection of the R and H branches, for which HU has less affinity when they form part of the DNA junction.

The second point concerns the junction itself. In the absence of HU, on the free DNA, phenanthroline attack analysis revealed hypersensitive sites at +2 and +1, mainly on the *r* and *b* strands of the DNA junction (Figure 2A, lanes 'fr'). When a plain, double-stranded DNA is used, this hypersensitivity was not observed (lanes 'ds'); therefore, this hypersensitivity seems to be structure specific. This point is strengthened by the fact that the hyper-reactivity is much more marked on the discontinuous

strands than on continuous strands (*r* and *b* strands versus *x* and *h* strands). We conclude that the intercalation of phenanthroline between base pairs of the adjacent branches of the junction is facilitated compared with its intercalation between double-helix base pairs. To check if this was a characteristic specific to Junction 3, the same experiment was performed with a different junction. A similar hypersensitivity was observed on a junction whose sequence was described by Pontigga *et al.* (1993). Moreover, there is no equivalent of these hypersensitive sites in the nicked structure, which again tends to suggest that this effect is due to the structure of the junction, which is absent in the nicked molecule. The interesting point is that the binding of HU decreases this effect: the hypersensitivity to phenanthroline of the stacked bases of the junction point is decreased when HU binds to the junction (Figure 2A, lanes 'b'). This could certainly suggest a structural change of the junction upon HU binding, which will be discussed below.

HU chemically converted into a nuclease

Using HU protein chemically converted into a nuclease and two different *E.coli* systems, Chaconnas' group (Lavoie *et al.*, 1996) and Adhya's group (Aki and Adhya, 1997) have shown that within high-order protein–DNA complexes, the heterodimeric HU is oriented and precisely positioned with respect both to the DNA and to the other proteins of the complex. Affinity cleavage mapping by chemically converting HU into a nuclease has given information about the position of the reactive groups of the protein on the DNA (Lavoie and Chaconas, 1993). Further analysis of the specific cleavages sites of 16 HU mutants modified with (EDTA-2-aminoethyl)-2-pyridyl disulfide-complex (EPD-Fe) permitted the determination of the position of HU on the Mu transpososome (Lavoie *et al.*, 1996). We wondered if the heterodimeric HU, when bound specifically to structures such as junction or nicked DNA, could also be oriented specifically with respect, this time, to the discontinuous points of either the junction or the nicked structure. We used the same tool to approach this problem.

To determine the position of HUαβ on our two DNA substrates, we used a series of modified HU heterodimers composed of a mutated complementary subunit, in addition to a wild-type subunit, coupled to an EPD-Fe moiety. These HU nucleases, a gift from G.Chaconas, were respectively HUβ-78, HUβ-74 and HUα-43, named according to their mutated residue. The iron, Fe(III), chelated with EDTA, is reduced by ascorbate to EDTA-Fe(II) which, in turn, reduces hydrogen peroxide, creating DNA strand scission in the vicinity of the site of the modification (Tullius *et al.*, 1987). The positions of the specific cleavage of these derivatized HU are well documented for Mu transpososome DNA (Lavoie *et al.*, 1996). The single mutations and the chemical modifications do not noticeably perturb the DNA binding properties of HU; in fact, modified HUs were indistinguishable from the wild-type HU protein in terms of DNA binding and stimulation of transpososome formation (Lavoie *et al.*, 1996). By gel-retardation assay, we verified that these modified HUs bound specifically to both the junction and nicked DNA sequences (data not shown).

No cleavage was observed when derivatized HU was bound to DNA in the absence of reducing agents, while in their presence, HU nucleases cleaved the DNA junction with low efficiency (not shown). Therefore, we again changed the reaction conditions to make the cleavage more specific, as we had for the phenanthroline footprinting. HU–DNA complexes were treated with reducing agents, directly in the gel, subsequent to their separation from both unbound HU nuclease and unbound DNA. By eliminating the diffusion of the protein, this procedure enables the correct molar ratio between HU and DNA (2:1 for junction DNA and 1:1 for nicked DNA) to be maintained during the experiment. The positions of scission by HU derivatives were determined for junction (Figure 3A) and for nicked DNA (Figure 3B). Some strand scission took place in the presence of reducing agents, even in the absence of derivatized HU (lanes '–'). These faint bands can be seen in Figure 3A, lanes '–' of the *r* and *b* strands. The intensity of these bands was taken as background, and subtracted from the cleavage pattern obtained with derivatized HU (lanes '+'). Cleavages where the intensity noticeably and reproducibly exceeds the background are taken as specific. These specific cleavage sites (marked as o in Figure 3A and B) were mapped on the DNA sequences of both substrates. The cleavage patterns, on DNA junction, of HUβ-78 are given in Figure 3A, those of HUβ-74 gave similar results (data not shown). The cleavage patterns of the three HU nucleases (β-78, β-74 and α-43) on strand *x* are given in Figure 3B. In Figure 3C, we compare the cleavage patterns of nicked DNA (bottom) and of junction DNA (top). Again, a high similarity is revealed on the B and X arms, as it was for the phenanthroline protection (Figure 2B). Clearly, branches B and X of both structures are cleaved in a way that is asymmetrical relative to the break or to the junction point. Branches R and H of the junction were cleaved ~5-fold less than their symmetrical counterparts. Reduced cleavage of these branches R and H of the junction by HU nuclease is similar to the reduced protection of the same branches from phenanthroline attack. Again, this may be explained as a HU preference for the sequences of branches B and X of the junction. However, the greater sensitivity of HU-nuclease assay reveals some cleavage sites on these branches not observed with phenanthroline.

Position of HUαβ on nicked DNA

To dock HU on the uniformly bent B-DNA in the MU transpososome by the interpretation of their specific cleavage data, Chaconas' group used as the initial complex the NMR structure of HU (Vis *et al.*, 1995; Lavoie *et al.*, 1996). The distances between the Cα atoms of the modified residue and the C1′ of the observed cleavage sites were found to be 10–22 Å, which corresponds to the maximal reach of EPD (14 Å spacer, 10 Å diffusibility). These values were similar to those (7–16 Å) obtained for the Cro and CAP proteins using a similar technique (Ebright *et al.*, 1992). To establish the position of HU dimer on the nicked DNA, we took as an initial complex the structure of IHF with nicked DNA which has been described by Rice *et al.* (1996). The body of HU from *Bacillus subtilis* (Tanaka *et al.*, 1984; White *et al.*, 1999) and that of IHF (Rice *et al.*, 1996) can be superimposed with a root-mean-square deviation (r.m.s.d.) of 1.0 Å. The

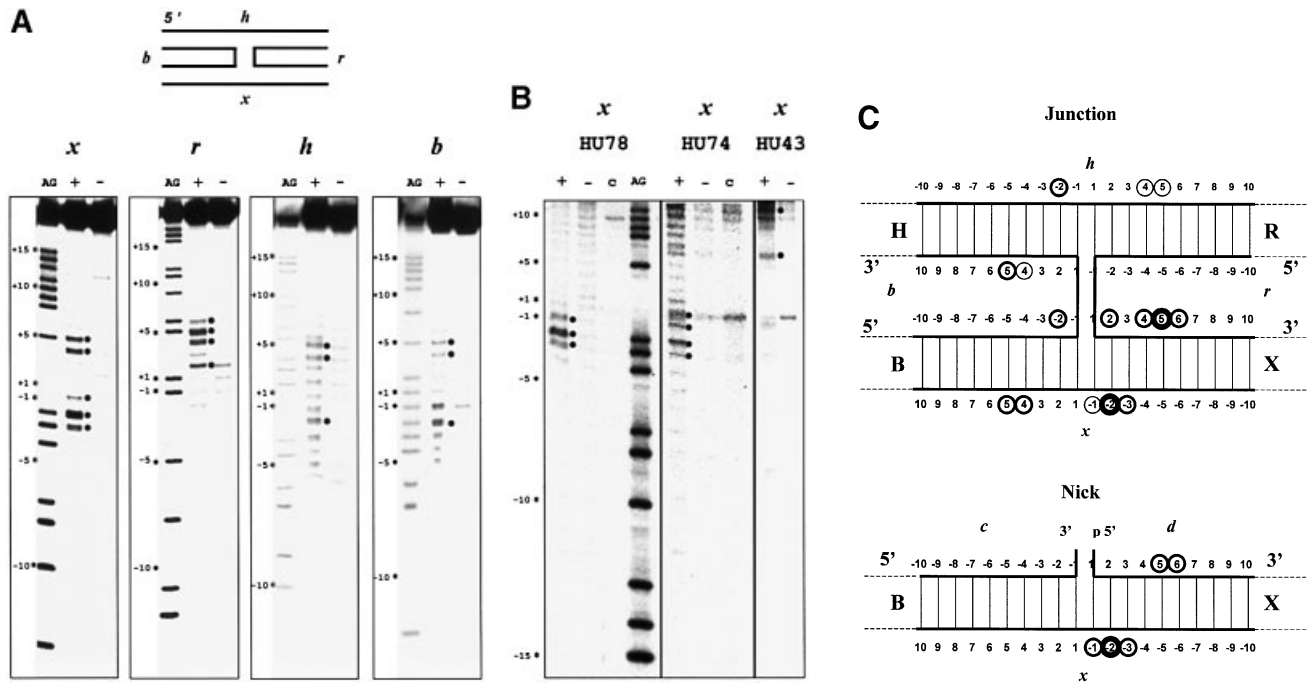


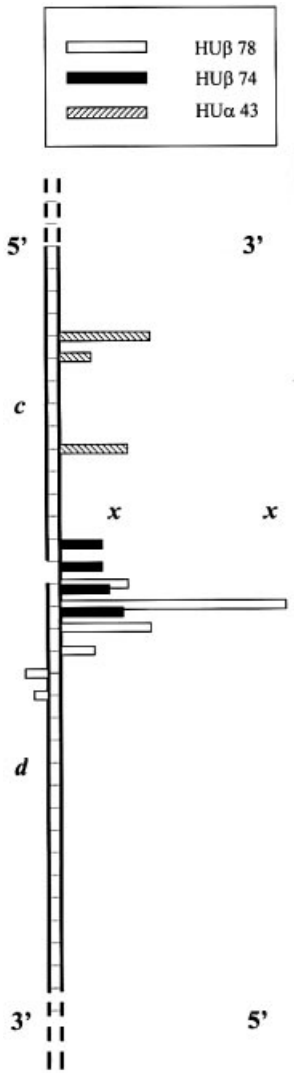
Fig. 3. HU nuclease DNA cleavage pattern. (**A**) DNA junction cleavage by HUβ-78 coupled with EPD-Fe. DNA–protein complexes were treated with reducing agents to initiate a specific cleavage within the native gel, subsequent to their gel-separation from free protein and DNA (lanes +). Free DNA treated in the same manner served as a control (lanes –). AG is A + G sequencing ladder. Points indicate the positions of specific cleavage by derivatized HU, base numbers are indicated. (**B**) DNA cleavage of strand *x* of DNA containing a nick with HUβ-78, HUβ-74 and HUα-43 (lanes HU78, HU74 and HU43, respectively) coupled with EPD-Fe. Protein–DNA complexes were treated with reducing agents within native gels (lanes +). Free DNA treated in a similar manner served as a background control (lanes –), an additional control is protein–DNA complexes not treated with reducing agents (lanes c). (**C**) Comparison of the pattern of the specific cleavage caused by HUβ-78 coupled with EPD-Fe on a DNA junction and on nicked DNA. Sites of specific cleavage of HUβ-78 coupled with EPD-Fe are circled; the thickness of the circle corresponds to the yield of the cleavage.

distances between HU residues that were modified in our experiments and their homologues in IHF are <1 Å. Therefore, we substituted HU for IHF and measured the distances between the modified HU residues (78, 74 and 43) and the C1′ atoms of the corresponding cleavage sites (Figure 4, left). The distances reported in Figure 4 were calculated for two possibilities; in the first, HUβ replaced IHFβ on nick ('β–nick'), and in the second, HU-α was on nick ('α–nick'). The complex obtained with HU β-arm on nick, 'β–nick', fits the specific cleavage data (white area where distances do not exceed 24 Å). For HUβ-78, the Cα–C1′ distances were found to be 12–14 Å, for HUβ-74, 12–15 Å and for HUα-43, 17–18 Å. The opposite substitution, when HU α-arm is on nick, does not fit our cleavage data. For HUβ-74 and HUα-43 cleavages, the distances to the corresponding DNA sites (respectively 30 and 37 Å) are not in a range compatible with the cleavage obtained (dark area in Figure 4). Clearly our cleavage data fits only when HUβ replaces IHFβ and lies on the nick.

The next step was to position and to orient HUαβ with respect to the DNA break point. For this, we calculated the Cα–C1′ distances for all the potential positions and orientations of HU on the nicked molecule. These parameters (see Material and methods) were again calculated for either HU β-arm or HU α-arm on nick, for all the possible positions of HU relative to the break point (Figure 4, right). When the Cα–C1′ distances exceeded 24 Å, the position of HU is considered to be incompatible with the cleavage data. There are a few permissive areas for cleavage (Figure 4, in white), and distances that are incompatible with the cleavage data (in dark). This exercise enabled us to position the HU center of symmetry between 2 and 6 bp on the right 3′ branch of the nicked DNA (Figure 5). The best fit for the cleavage data was obtained when the center of symmetry of HU is 3.5 bp on the 3′ branch of the nicked DNA (r.m.d. 14 Å). For the complexes localized between 2 and 6 bp to the break point, the average distance is 14–17 Å, a reasonable fit.

The structure of the IHF complexed with nicked DNA shows that the protein introduces two large kinks into the DNA where the proline at the tip of each arm intercalates between base pairs (Rice *et al.*, 1996). This proline is conserved in every known member of the HU–IHF family (Oberto *et al.*, 1994). The conformation of the turn at the tip of the arms is similar in the structure of both proteins (Vis *et al.*, 1995; Rice *et al.*, 1996). Therefore, we wanted to know if the nicked DNA complexed with HU was in a straight conformation (Figure 5A) or was kinked (Figure 5B). To resolve this point, we calculated the Cα–C1′ distances using a straight B-DNA conformation (the lanes 'β, st' for 'beta-on-nick, straight DNA' in Figure 4). A straight conformation for nicked DNA is clearly incompatible with the HUα-43 specific cleavage data (>28 Å), whereas a kinked conformation would decrease the distance to the cleavage site to 18 Å. Thus, nicked DNA is kinked upon HU binding between the left branch of the DNA and the DNA surrounded by the HU arms (Figure 5B).

The main feature of the complex between HU and



Modified residue	78			74			43		
Subunit in nick	β	α		β	α	β, st	β	α	β, st
+14	41	40		45	49		28	42	
+13	37	37		41	48		26	39	
+12	33	33		36	44		22	35	
+11	28	28		32	40		→ 17	31	
+10	24	24		29	35		12	28	
+9	23	21		28	31		10	28	30
+8	24	21		29	30		11	31	28
+7	26	23		29	32	35	14	34	29
+6	28	26		29	34	32	→ 18	37	28
+5	29	28		27	37	30	22	39	30
+4	28	28		24	38	26	23	38	30
+3	24	26		20	36	22	23	35	29
+2	22	25	→	16	35	17	24	34	27
+1	18	22	→	12	31	13	24	30	23
-1	→ 14	17	→	12	25	12	20	26	20
-2	→ 12	12	→	15	20	15	18	24	18
-3	→ 14	11		19	15	19	19	24	19
-4	17	13		23	13	23	23	26	23
-5	21	18		26	15	26	28	29	28
-6	23	21		27	17	27	32	31	32
-7	23	23		27	20	27	34	30	34
-8	24	24		27	22	26	36	28	36
-9	24	25		27	24	25	36	25	35
-10	20	23		25	25	25	34	20	35
-11	17	21		23	25	27	31	16	36
-12	15	19		23	24	32	28	11	37
-13	16	18		26	22	36	26	9	40
-14	19	19		30	21	41	27	11	45
-15	23	22		34	23	44	30	14	49
-16	27	27		37	29	46	34	17	54
-17	30	31		40	33		37	19	
-18	32	33		41	37		39	20	
-19	33	36		42	41		40	22	
-20	33	36		41	42		39	21	
-21	35	37		43	44		38	23	

Fig. 4. Localization of HU nucleases cleavage on DNA containing a nick and the distances between the Cα atom of the modified residues and the C1′ of the X strand bases. The height of the stripes corresponds to the yield of cleavage. Distances were calculated on the basis of IHF–nicked DNA X-ray structure (Rice *et al.*, 1996), where the IHF subunit in the region of the DNA break was substituted with HUβ (lane β) or with HUα (lane α). Lane 'β, st' represents the distances in the complexes where IHFβ subunit is substituted with HUβ, and kinked DNA with straight B-DNA. Arrows indicate the sites of specific cleavage.

nicked DNA is that the center of symmetry of the HU dimer is lying on the right-hand DNA branch, 3′ from the break point (Figure 5B). The asymmetry of this location may reflect a preferential binding of the HU β-arm when this arm runs antiparallel to the broken strand and the proline 63 is intercalated correctly between the bases. This model is also in agreement with the phenanthroline footprinting data (Figure 2B). The protection of bases 9–5 in branch B further proves that the DNA is curved upon HU binding, whereas protection of bases 1–3 in branch B, and protection of bases 7–9 in branch X, are perfectly explained by the interaction of the HU arms with the DNA minor groove.

HU introduces a 65° kink into nicked DNA at the break point

To explain our cleavage data obtained upon HU binding nicked DNA, we postulated that, similarly to IHF which kinks DNA upon binding a nicked molecule (Rice *et al.*, 1996), HU must also bend the DNA, since a straight conformation of the DNA molecule is incompatible with the cleavage data (Figure 4). A kinked conformation with an angle of at least 35° between the 5′ and 3′ branches of the DNA must be introduced to make the distance between Cα of HUα-43 and the DNA cleavage site <24 Å. How to demonstrate this putative DNA bending upon HU binding was not obvious, since 'real' circular permutation assays are not easy to perform with proteins that do not recognize a specific sequence on DNA.

A first piece of evidence that HU curves nicked DNA is shown in Figure 6A. In this experiment, we compared the mobility of HU complexed either with a 40 bp double-stranded DNA or with a 40 bp nicked DNA. The nicked DNA complex migrates 10% more quickly in the polyacrylamide gel than the double-stranded DNA complex (compare bands C1 and C1′), while the mobility of both free DNAs is the same. Bending of the nicked DNA makes the HU complex more compact than the complex with the double-stranded DNA, therefore leading to a faster migration. The effect of bending in this experiment

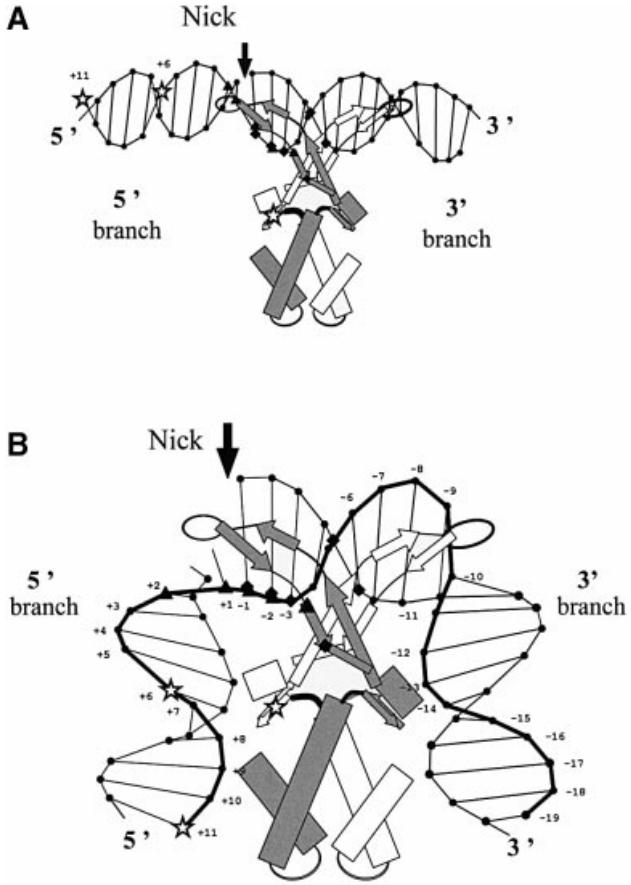


Fig. 5. Model of HU–nicked DNA complex based on IHF complex with nicked DNA where the IHF subunit is substituted with the HU subunit. The β-subunit is shaded, the α-subunit is in white. Positions of the modified residues and corresponding sites of the DNA-specific cleavage are indicated as follows: diamonds, HUβ-78; triangle, HUβ-74; stars, HUα-43. Bases of the continuous, *x*, strand are numbered. (**A**) Straight B-DNA (Arnott and Hukins, 1973) was fitted to the kinked DNA. (**B**) The configuration of the kinked DNA was taken from Rice *et al.* (1996) with its fit to the protein.

is opposite to that observed in general for circular permutation assays when a DNA of 200–500 bp is used (Crothers and Drak, 1992).

Another approach was the circularization of a DNA fragment by DNA ligase in the presence of HU, previously performed by Pettijohn's group (Hodges-Garcia *et al.*, 1989). This experiment certainly showed that HU bends DNA, but does not allow direct measurement of the angle of bending, possible only by the circular permutation technique. To overcome this problem, we explored the possibility of exchanging the sequence-specific binding site of the conventional technique by a structure-specific binding site. For this, we used a single-strand break as a 'protein binding site' in a circular permutation assay. Comparison of the gel mobility of HU complexes with the same DNA fragment carrying a DNA break either in the middle or at one extremity (Figure 6B) shows that the ratio of their respective mobilities is 0.87, estimated by the method of Thompson and Landy (1988) to correspond to an angle of 65° upon HU binding. Therefore, these data demonstrate that the specific binding of one dimer of HU to nicked DNA introduces a curvature of 65° between the two double helices of the DNA at the break point.

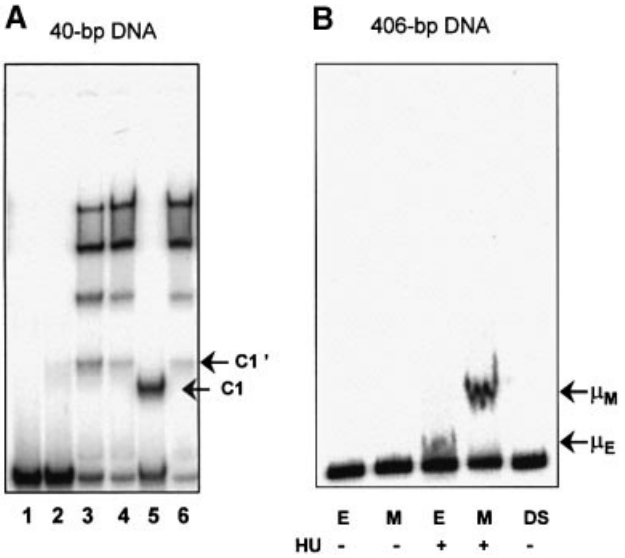


Fig. 6. (**A**) Gel mobility of the complexes of HU with 40-bp nicked DNA (C1) and double-stranded DNA (C1′) of the same sequences. Labeled double-stranded DNA was mixed with HU protein in buffer containing 10 mM NaCl. In lanes 1–4 and 6, the concentration of HU is 0, 20, 40, 80 and 100 nM, respectively. Lane 5, labeled nicked DNA with 10 nM of HU. (**B**) Circular permutation assay. Gel mobilities of the HU complexes with 406-bp DNA containing a nick in the middle (M) and at the end (E) were compared.

Positioning of HUαβ on junction DNA

The location of the specific cleavage by HUβ-78 nuclease observed on DNA junction (Figure 3A) was mapped on its sequence (Figure 3C). This figure shows that the cleavage pattern on junction DNA was very similar to that obtained for nicked DNA, in the same way as the phenanthroline protection patterns were similar for these two DNA structures (Figure 2B). In consequence, the positioning of the HU dimer on the junction DNA can be considered in the same way as for nicked DNA. The binding of the HU dimer to the B and X branches of the junction is superposable to its binding to a B–X nicked molecule. Therefore, the center of symmetry of HU is located on the X branch, 3′ from the junction point (Figure 7A). In the proposed model, the HU β-arm (in dark) interacts with the DNA in the region of the junction point, exactly as was found for nicked DNA. In a symmetrical manner, the cleavage sites of the H branch of the junction (although the yield of cleavage was lower) can be interpreted as the binding of an HU dimer to the H and R branches of the junction, similar again to the binding of the H–R nicked molecule. The center of symmetry of HU will lie 3′ from the junction point; the *h* strand plays the role of the continuous strand.

This is certainly the most logical and simple model in agreement with the localization of the major cleavage sites, identical on the two substrates (Figure 7A). In this configuration, strands *x* and *h* play the role of the continuous strands, which may reflect the preferential folding of Junction 3 used here (Duckett *et al.*, 1988). However, the cleavage pattern is more complicated than this. In addition to these identical cleavage sites, present on both structures, there are six cleavage sites found only on DNA junction. They constitute 20% of the total cleavage of HUβ-78. Their locations are almost strictly symmetrical with those of the first class. To explain these

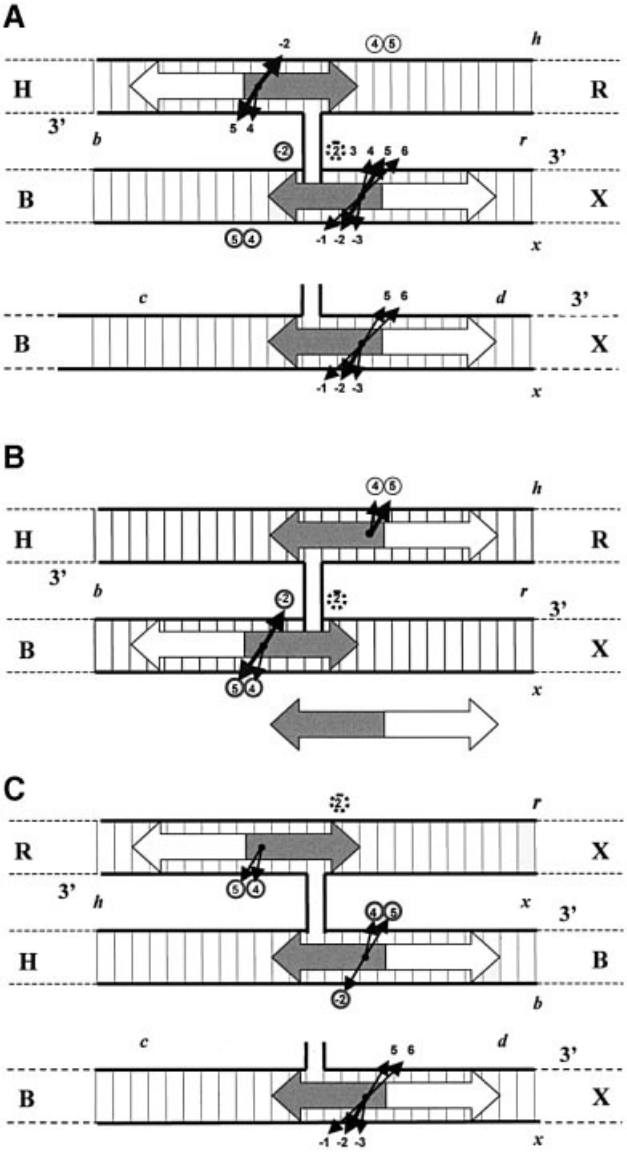


Fig. 7. Localization of HU dimer and HUβ-78 nuclease cleavage on the DNA junction and on the DNA containing a nick. The position of the HU β-arm is in gray, the position of the HU α-arm is in white. Arrows indicate the cleavage sites present on both DNA substrates; cleavage sites that are different are circled. (**A**) HU positioning on the nicked DNA (bottom) and HU positioning on DNA junction corresponding to the cleavage similarity. (**B**) Hypothetical symmetrical position of HU on DNA junction that could have explained HU cleavage sites being symmetrical to ones on nicked DNA (this hypothesis is rejected by the data). (**C**) Position of HU on DNA junction with the branch pairing opposite to that shown in (A). This configuration explains the junction cleavage sites that are symmetrical to the ones on nicked DNA (this hypothesis is accepted by the data).

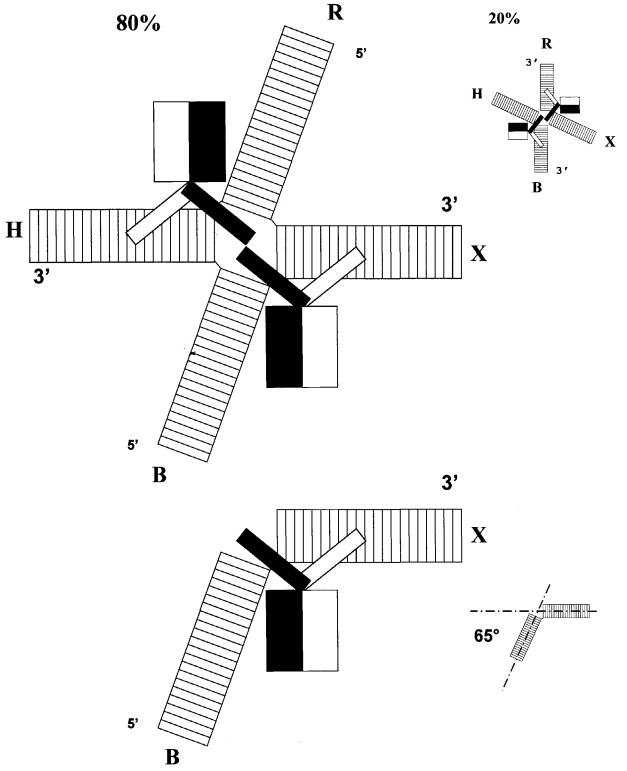


Fig. 8. Schematic representation of the similar positioning of HU on the two DNA substrates. The β-subunit is dark and the α-subunit is white. As shown in this work: (i) the β-subunit arm interacts with the discontinuous point of both the DNA structures; (ii) the HU center of symmetry lies on the right 3′ branch of the DNA 2–6 bp away from the discontinuous point; (iii) DNA is kinked at the discontinuous point. (**A**) Preferential position of HU on the DNA junction; (**B**) alternative configuration of the DNA junction and HU. (**C**) Positioning of HU on the DNA containing a nick.

symmetrical cleavages, specific to the junction, two modes of HU positioning on the junction could be considered (Figure 7B and C). In the first, HU has no fixed position. The symmetry in the cleavage sites observed on the B and X branches, versus the zero point, is explained by the alternative positioning of HU on the B or X branches (Figure 7B). However, in that case, HU will be positioned on the B branch, 5′ to the zero point, instead of the 'real' positioning on the X branch, 3′ to the zero point, which we have demonstrated to occur. Obviously, the B and the X branches are not, *sensu stricto*, symmetrical within the junction. In a spacial configuration it is the H branch that is symmetrical to the X branch. Additionally, the β-arm is found in this case (Figure 7B) to run parallel to the exchanging strand, whereas it is antiparallel in the nicked structure as well as in the junction (Figure 7A). Therefore, this alternative positioning must certainly be disregarded.

The last possibility for the positioning of HU on the junction is that a fraction of the junctions have an alternative pairing of the four branches for HU binding (Figure 7C). This configuration differs from the 'classical' one of Junction 3 (Figure 7A). Strands *b* and *r* play the role of the continuous strands, whereas strands *x* and *h* are the exchanging ones (Figure 7C). This alternative configuration explains the symmetrical cleavage pattern observed. These two configurations of Junction 3 bound to HU, presented in Figure 8, co-exist only upon HU binding and certainly indicate an effect of HU on the junction structure. The HU-induced configuration is, in our experiments, less frequent (yield 20% of the total cleavage) than that of the usual configuration of Junction 3. In this latter configuration, the HU β-subunit interacts with the junction point. The HU center of symmetry juxtaposed 2–6 bp from the junction point on the 3′ side of the R branch instead of the 3′ side of the X branch as it is in the majority of cases (80% of the junctions) and as it is in the nicked DNA (Figure 8). In both configurations

of the junction, two HU dimers are juxtaposed on the opposite junction branches and may interact with each other via their β-arms, separated by ~6 Å (Figure 8). However, only the β-arms can contact each other; the interaction between either the two bodies, or the α-arms of the facing dimers, is not possible according to the data. This finding is in agreement with the apparent lack of cooperativity in the binding of the first and second HU dimers to the DNA junction (Pinson *et al.*, 1999). Effectively, this asymmetrical positioning gives HU dimers the possibility of binding the junction without interfering with each other.

Concluding remarks

IHF, like the HMG proteins and the TATA box binding protein, contacts the minor groove of DNA. It was a safe assumption that HU might also bind to the minor groove. The phenanthroline protection experiments demonstrated that HU contacts the minor groove of the DNA in the junction as well as in the nicked structure, but more unexpected, the comparison of the patterns of the DNA sites that undergo changes upon HU binding revealed a strong resemblance between these two DNA structures. As regards phenanthroline protection, a junction can be considered as two nicked molecules for HU binding (Figure 2B). This assertion is confirmed by the cleavage patterns of the HU nuclease, where again a junction can be considered as two nicked molecules (Figures 3C and 7), the break point in the nicked DNA being equivalent to the junction point in both cases.

The position and orientation of the HU heterodimer on the DNA containing a nick (Figure 5) show that the HU β-arm reveals a selective binding to the strand break with an orientation of the arm antiparallel to the broken strand (direction 3′ to 5′) and that the center of symmetry of the HU dimer lies on the 3′ branch of the DNA (the branch containing the 3′ end of the broken strand). Finally, we demonstrated by different approaches that HU binding to the break point introduces a kink in the region of the nick. First, the cleavage data by HU nuclease do not support a straight DNA configuration (Figures 4 and 5). Second, the extent of protection by phenanthroline (~20 bp) observed on the B and X branches of the junction and on the nicked molecule (Figure 2B) is too large to be compatible with a straight configuration. More precisely, by a circular permutation assay modified for a DNA binding protein recognizing a structure instead of a sequence, we could estimate the bending at the break to be ~65°. This DNA bending permits, or induces, an interaction between the 5′ branch of the DNA molecule and the HU body. The implication of the HU body in the interaction with the nick may explain why the binding with a nicked molecule is at least 100-fold stronger than that with plain double-stranded DNA (Castaing *et al.*, 1995; Pinson *et al.*, 1999).

The position and orientation of the heterodimeric HU on the junction molecule are comparable to those on the nicked structure (Figures 7 and 8). For HU binding, a DNA junction can be considered as two DNA molecules containing a nicked motif. However, this assembly of four DNA strands is not a plain connection of two nicked molecules; the structures are different. The connection of four DNA branches leads to the the folding of the junction, a complexed structure (Duckett *et al.*, 1988). The rotation of the branches is constrained by the joining of the four branches and the tension created by this connection may introduce some fluctuations in the DNA fine structure. This could explain the existence of the several strong phenanthroline-hypersensitive sites that were observed with free junction DNA, but are absent with both nicked and double-stranded DNA, as discussed above. The differences between the two structures could justify why junction-resolving enzymes cleave DNA junction and leave nicked DNA intact (Bhattacharyya *et al.*, 1991). We show here that HU binds both DNA junction and nicked DNA in an identical manner. The capability of the HU arm to bind the region of discontinuity in both structures and the capability of the DNA branches to bend easily upon HU binding, render the HU complexes with both DNAs very similar. Therefore, it is possible to conclude that the DNA junction is equal to two nicked DNAs in respect of HU binding. We cannot, however, exclude an additive role of HU on the junction which certainly could play a role in establishing two possible configurations of the junction. An alternative pairing of the junction branches is the only possible interpretation for the symmetrical cleavage pattern obtained with the junction but not with nicked DNA. Finally, the phenanthroline footprinting data (Figure 2B) support the idea that branches B–X as well as branches R–H of the junction are also inclined upon HU binding.

HU and IHF dimers have similar three-dimensional structures. We show here that the HU position on the nicked DNA is very similar to the position of IHF on its specific DNA binding site, which has a nick in its middle. The orientation preference of IHF protein to nicked DNA (Rice *et al.*, 1996) was perhaps linked to the sequence specificity of the IHF protein. Clearly, the position of IHF in the crystal structure could be caused by the position of the binding site or by the break point, or by both. Crystals are perhaps obtained when the break point is correctly positioned with regard to the IHF-specific sequence. For HU protein it is not an issue: it binds DNA without sequence specificity and selects structural features of DNA for binding (Churchill and Travers, 1991; Pinson *et al.*, 1999). Another difference between IHF and HU binding to DNA concerns the DNA bending. While IHF introduces two kinks of 80° into nicked DNA (Rice *et al.*, 1996), we show here that HU introduces only one kink at the DNA break point, which allows a contact between the 5′ branch of DNA and the HU body. Furthermore, it should be recalled that even if HU can, sometimes at least, replace IHF *in vitro* (Segall *et al.*, 1994), *in vivo* overproduction of IHF cannot compensate for the absence of HU in *hupAB* mutants (Boubrik *et al.*, 1991) and that HUαβ, and this form only, is required for the optimal survival of *E.coli* (Claret and Rouviere-Yaniv, 1997). It is possible that certain residues in the DNA-interacting arms of IHF require specific base pairs while HU can accommodate any base sequences and for specific binding requires only two branches of DNA with a propensity to be inclined. It is, therefore, possible to predict that HU should be able to bind specifically to a multitude of structures found in the cell that are not ‘plain duplex DNA’, such as incomplete junction, DNA strand invasion, etc.

Materials and methods

Construction of 5′-labeled DNA probes

The 40 nucleotide synthetic oligonucleotides used for the construction of DNA Junction 3 (Duckett *et al.*, 1988) are (from 5′ to 3′):
x: AGTCTAGACT GCAGTTGAGT CCTTGCTAGG ACGGATCCCT
r: AGGAATTCAA CCACCGCTCA ACTCAACTGC AGTCTAGACT
b: AGGGATCCGT CCTAGCAAGG GGCTGCTACC GGAAGCTTCT
h: AGAAGCTTCC GGTAGCAGCC TGAGCGGTGG TTGAATTCCT.
DNA containing a nick was constructed from oligonucleotides *x* and:
c: ACTCAACTGCAGTCTAGACT
d: pAGGGATCCGTCCTAGCAAGG, 5′ phosphorylated.

Other DNAs were constructed from the complementary stands of the same oligonucleotides. DNAs were constructed by annealing the appropriate oligonucleotides, one being 5′-labeled with T4 polynucleotide kinase and [^{32}P]ATP, and gel-purified. Annealing reactions were carried out by incubating the oligonucleotides (300 nM) for 3 min at 80°C in 20 mM Tris–HCl pH 8.0, 400 mM NaCl, 0.2 mM EDTA and then allowing them to cool slowly.

HU protein and gel electrophoretic separation of the HU–DNA complexes

HUαβ was prepared from *E.coli* strain JRY1 by a protocol that permits the purification of the protein without nucleases associated with HU and without the denaturation–renaturation step (O.Pelligrini, manuscript in preparation). HU heterodimers containing EPD-Fe coupled to HU mutant subunit (HUβ-S78C, HUβ-Q74C and HUα-A43C) were a generous gift from G.Chaconas. Varying amounts of HU protein were incubated with 5′-^{32}P-labeled DNAs (1–3 nM) for 15 min in the cold room in 16 ml of the binding buffer, 20 mM Tris–HCl pH 8, 200 mM NaCl, 0.05 mg/ml bovine serum albumin, 7% glycerol. Samples were loaded on 8 or 10% polyacrylamide gels (29:1) and electrophoresed at 4°C in 90 mM Tris–borate pH 8.6.

Phenanthroline footprinting

1,10-Phenanthroline–copper footprinting of DNA–HU complexes was performed essentially as described by Papavassiliou (1994). The DNA–protein complexes were gel-separated from unbound DNA. Gels were then soaked in the buffer, 10 mM Tris–HCl pH 8.0, 100 mM NaCl, for 10 min at room temperature, treated with freshly prepared phenanthroline–copper solution ($CuSO_4$ final concentration 0.045 mM, phenanthroline 0.2 mM) for 2 min and 3-mercaptopropionic acid was added to 5.8 mM. The strand scission reaction was quenched by adding 2,9 dimethyl-1,10-phenanthroline to 2.8 mM after 12 min. Gel slices corresponding to bound and free DNA were excised after exposure with X-ray film. DNA was then eluted from the gel by diffusion and ethanol precipitated. The samples of bound and free DNA were dissolved, with equal radioactivity per milliliter, in 7 M urea, heated to 100°C for 2 min, and electrophoresed in 15% TBE–urea sequencing gels with an A + G marker. Quantification was performed using a Molecular Dynamics PhosphorImager (400 S).

HU nuclease cleavage reaction

The standard chemical reactions of the Fe strand scissions were used (Lavoie *et al.*, 1996; Tullius *et al.*, 1987). HU coupled with EPD-Fe was mixed with DNA in binding buffer and DNA–protein complex was gel-separated from non-bound DNA, as described above but without EDTA. Gels were soaked twice in buffer (10 mM Tris–HCl pH 8.0, 100 mM NaCl) for 10 min at room temperature, the strand scission reaction was then initiated by adding sodium ascorbate to 5 mM and hydrogen peroxide, 0.02%, then quenched after 10 min by adding 0.1 M thiourea and 20 mM EDTA. For strand scission analysis, DNA was treated as for phenanthroline footprinting.

Protein–DNA distance measurements

To move DNA containing a nick along HU and to calculate the resulting distances between Cα atoms of HU and C1′ atoms of the sites of specific cleavage, we used the distances from the initial complex between nicked DNA and IHF protein (Rice *et al.*, 1996), where IHF is substituted with HU (Tanaka *et al.*, 1984; White *et al.*, 1999). HU sliding along the DNA was modeled by the change in the numbering of the DNA bases: one base step to the right on the DNA corresponds to an increase of 1 to the DNA base numbers, while distances to the C1′ of the bases remain unchanged. This allowed calculation of the distances between Cα atoms of modified HU residues and all the C1′ atoms in the moved complex, except for those bases located next to the break. We also used the straight B-DNA to measure the Cα–C1′ distance. The B-DNA was fitted to the nicked DNA within the initial complex. Distances to the straight DNA C1′ atoms led us to estimate the distances to the C1′ of kinked DNA in the region of the DNA break. Positions of HU where distances between the Cα of the modified residue and the corresponding site of specific cleavage exceed 24 Å were estimated as incompatible with the cleavage data (Figure 4). The distances were determined on the basis of the modeling of the structure of heterodimeric HU of *E.coli* (S.W.White, personal communication), based on the recently obtained X-ray data of the homodimeric *B.stearothermophilus* HU (White *et al.*, 1999). Protein–DNA distances and conclusions are identical for the two HU molecules. The method used to calculate the distances is sufficiently precise to estimate the distances between the protein residues and DNA in all possible orientations, and to position the protein on DNA.

Circular permutation assay

To construct the DNA containing a single-strand break in its middle, we used a 406 bp PCR product of pBR322 made with BOT (5′-CTAGCTTCCCGGCAAC) and TOP (5′-ACGCTCAGTGGAACG) oligonucleotides, digested with the nicking restriction endonuclease N.*Bst*NBI (NEB) at a position 203 from the end. The same fragment containing a single-strand break at position 14 was constructed by PCR of the double-stranded 406 bp fragment with only one primer END (5′-pAATTAATAGACTGGA, 5′-phosphorylated). The product was purified on agarose gel and annealed with 5′-labeled oligonucleotide BOT. Band-shift assay for 406 bp DNA containing a positioned strand break was performed as described above, but binding buffer contained 400 mM NaCl, and the gel contained 5% acrylamide (30:1) and 4× TBE (360 mM Tris–borate, 1 mM EDTA) to reduce the non-specific binding of HU to the continuous branches of DNA.

Acknowledgements

We are greatly indebted to George Chaconas for the gift of the three modified HU proteins used in this work, to Stephen White for communicating unpublished data concerning the coordinates of heterodimeric HU of *E.coli* and to Olivier Pellegrini for kindly providing the HU proteins. This work was supported by the CNRS (UPR 9073) and grants from l'Association de la Recherche contre le Cancer (ARC), l'INTAS (93-1028) and EDF. D.K. was the recipient of postdoctoral fellowships from successively EMBO, ARC (Association de la Recherche sur le cancer) and FRM (Fondation de la Recherche Medicale); A.B. received several short fellowships from INTAS.

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Received May 10, 1999; revised and accepted August 6, 1999