In vivo interaction of the Escherichia coli integration host factor with its specific binding sites

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ABSTRACT

The histone-like protein integration host factor (IHF) of Escherichia coli binds to specific binding sites on the chromosome or on mobile genetic elements, and is involved in many cellular processes. We have analyzed the interaction of IHF with five different binding sites in vitro and in vivo using UV laser footprinting, a technique that probes the immediate environment and conformation of a segment of DNA. Using this generally applicable technique we can directly compare the binding modes and interaction strengths of a DNA binding protein in its physiological environment within the cell to measurements performed in vitro. We conclude that the interactions between IHF and its specific binding sites are identical in vitro and in vivo. The footprinting signal is consistent with the model of IHF-binding to DNA proposed by Yang and Nash (1989). The occupancy of binding sites varies with the concentration of IHF in the cell and allows to estimate the concentration of free IHF protein in the cell.

INTRODUCTION

A large number of processes within the living cell depend on specific protein–nucleic acid interactions. Information about the details of such interactions can be derived from the three-dimensional structure of the complex using X-ray crystallography or nuclear magnetic resonance. In the absence of such a structure various footprinting techniques can be employed to probe the interactions. The features revealed by different footprinting techniques vary greatly. Generally one would like to investigate changes of the interactions as a function of different parameters and ideally monitor the interactions in the physiological environment, i.e. within a living cell. The footprinting signal has to report the immediate environment of the bases without perturbing the complex. We use UV laser light as such a non-invasive ‘reagent’ taking advantage of the salient property of light to easily penetrate the living cell thus enabling us to measure protein–DNA interaction under the most relevant conditions.

In the present work we investigate specific protein–DNA interactions involving the integration host factor (IHF) of Escherichia coli. IHF is a heterodimeric protein that belongs to a class of prokaryotic histone-like proteins, together with HU, H-NS and Fis (1,2). IHF is composed of two homologous subunits, α and β, encoded respectively by the two unlinked himA and himD (or hip) genes. IHF, which is not an essential protein, is involved in a variety of cellular processes, such as the site-specific recombination of phage lambda, the transposition of mobile genetic elements, the packaging of viral DNAs, the replication of certain plasmids, and the control of the expression of several operons (for reviews see 1,3,4). IHF binds to sites in DNA (ihf sites) which approximate the consensus YAANNNTTGATW (where W = A or T, and Y = T or C) (5). In this article we will refer to the strand containing this motif as the ‘top’ strand and we call the sequences YAA and TTGAT the consensus trimer and pentamer respectively. IHF’s functionality apparently relies to a large extent on its ability to sharply bend the DNA (by ~130°). IHF is a very abundant protein within the cell and its level varies with growth phase (6). The E.coli genome is estimated to contain a few hundred specific binding sites for IHF (3,5,7) including a class of 70–100 bacterial repetitive elements located at the 3′ end of transcription units called RIBs (8) or RIs (9).

The crystal structure of HU, a homodimeric protein with strong sequence similarity to IHF, has been obtained (10). Based on this high degree of similarity, the assignment of a probable region of interaction with DNA, and footprinting data, Yang and Nash (11) have proposed a model for the IHF–DNA complex. According to the model the β-ribbon ‘arms’ of the dimer contact the minor groove of the binding site. The bound DNA is sharply bent around the IHF dimer. The center of pseudo-symmetry is located just 3′ of the W of the consensus sequence. The consensus sequence therefore constitutes only one half-site and IHF covers a total of ~30 base pairs (bp).

On the basis of this working model we want to validate structural features and examine dynamic features of IHF binding to specific binding sites. Such DNA–protein interactions are most readily studied in vitro. The ionic conditions, the concentrations of components, excluded volume effects and the presence of competing macromolecules within the cell can only be represented approximately in such an experiment. In the present work,
we have therefore undertaken a systematic comparison of the binding characteristics in vitro and in vivo of IHF to five different specific binding sites. We have selected three sites located in RIBs (8), the H1 site of the bacteriophage \( \lambda \) att\( P \) region (12,13) and an \( \text{ihf} \) site found at the 3’ end of the \( \text{ssb} \) gene. We use UV laser footprinting to obtain a structural signal for protein binding to DNA because the method can be applied in an identical fashion in both environments and is very sensitive to the conformation and the immediate environment of the DNA. All sites interact identically with IHF in vitro and in vivo validating the Yang and Nash model [(14) and references therein]. Examination of the interaction of IHF with its binding sites during exponential- or stationary-phases revealed significant changes in the intracellular concentration of IHF.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids**

Strains MC802 and MC803 are IHF+ and IHF- isogenic derivatives (M. Chandler, personal gift) and originated from strains MC240 and MC252 respectively (15) into which the recA deletion from strain JV10289 (16) was introduced by P1 transduction. Plasmids containing the \( \text{uwr} \) RIB (pFBG1), the \( \text{dppA} \) RIB (pFBG14), and the \( \text{gyrB} \) RIB (pFBG30) have been described previously (8). A 182 bp fragment containing the \( \text{ihf} \) site located at the 3’ end of the \( \text{ssb} \) gene (17) was obtained by PCR amplification using as primers the oligonucleotides \( 5’-\text{CAAGTCTCGCCGCAGCAG-3’} \) (from nucleotides 484 to 501) and \( 5’-\text{CCTGTAGATCGTGAGCTC-3’} \) (from nucleotides 665 to 648), and cloned into Bluescript KS+ (Stratagene) resulting in the sequence CTCGAGACTA ATTCCATCTA AGTAGTGAT TCATAGTGGAGCAGAATTC between the two restriction sites XhoI and EcoRI.

**In vitro UV laser irradiation of IHF-DNA complexes**

Prior to incubation with the plasmid containing the \( \text{ihf} \) site, a 5 \( \mu \)M stock of purified IHF was sonicated for 20 s at 0°C in a Branson 2200 ultrasonic bath, in a buffer containing: 50 mM Tris–HCl pH 7.4, 800 mM KCl, 40 mM K\(_2\)HCO\(_3\), 10% glycerol and 2 mg/ml bovine serum albumin. Surpercooled or linearized plasmid (5 nM) carrying the \( \text{ihf} \) site were incubated for 25 min at room temperature in binding buffer (50 mM Tris–HCl pH 7.5, 70 mM KCl, 7 mM MgCl\(_2\), 3 mM CaCl\(_2\), 200 \mu\)g/ml bovine serum albumin, 1 mM EDTA, 1 mM dithiothreitol) in the absence or presence of 50 or 100 nM IHF. After incubation, the binding reactions were divided into 20 \( \mu \)l aliquots and transferred into the inside of the cap of a 500 \( \mu \)l Eppendorf tube.

The samples were irradiated with a single, 5 ns long pulse of 266 nm ultraviolet laser light generated by a Spectra-Physics Quanta-Ray GCR series laser. The energy of one such pulse is ~30 mJ, representing \( 4 \times 10^{10} \) photons, i.e. 67 nmol photons. After irradiation, the samples were completed with MgCl\(_2\) to 10 mM and were precipitated in a dry ice bath by adding 4 vol 100% ethanol. After centrifugation, washing with 70% ethanol and drying, the DNA of each sample was resuspended in 20 \( \mu \)l HE buffer (10 mM HEPES–NaOH pH 7.8; 1 mM EDTA), and was either immediately used for primer extension analysis or stored at -20°C.

**In vivo UV laser irradiation of living E.coli cells**

Cultures (5 ml) of strains MC802 (IHF+) and MC803 (IHF-), transformed with the plasmid carrying the \( \text{ihf} \) site, were grown in LB medium (18) to an OD\(_{600}\) of 1 or to saturation. The cells were washed in minimal M9 medium, and resuspended in M9 minimal medium to an OD\(_{600}\) between 1 and 1.5. Sixteen samples of the bacterial suspensions (50 \( \mu \)l) were irradiated as described above, pooled to yield a total volume of 800 \( \mu \)l and immediately frozen in a dry ice bath. The plasmid DNA was extracted using the alkaline lysis protocol of Maniatis (18) with proportionally adjusted volumes. The plasmid DNA was resuspended in 40 \( \mu \)l of HE buffer containing 20 \( \mu \)g/ml RNase I. Half of each plasmid DNA preparation, i.e. 20 \( \mu \)l, was used for primer extension analysis.

**Primer extension reactions**

One microliter of a solution of 0.5 \( \mu \)M primer (radioactively labeled at the 5’ end) was added to 20 \( \mu \)l samples of irradiated plasmid. The samples were denatured by boiling for 3 min and chilled on ice for 5 min. After addition of 2.5 \( \mu \)l of 10x HMD buffer (0.5 M HEPES pH 7.5, 0.1 M MgSO\(_4\), 2 mM dithiothreitol) the samples were incubated for 3 min at 50°C (primer annealing) and chilled again for 5 min on ice. We added 2 \( \mu \)l of a mix containing 10 mM Tris–HCl pH 7.5, 1 mM dithiothreitol, 50 \( \mu \)M EDTA, 25% glycerol, 2.5 mM of each deoxyribonucleotide, 0.3 \( \mu \)M T7 DNA polymerase (Pharmacia), and incubated the reaction for 8–12 min at 37°C. The samples were precipitated with ethanol and analysed by denaturing polyacrylamide gel electrophoresis (18) on an 8% sequencing gel.

**Footprinting using T7 RNA polymerase**

In vitro and in vivo irradiated plasmid DNA was linearized and transcription from the T7 promoter located in the pSK- vector was performed using T7 RNA polymerase (Pharmacia). The conditions used were similar to those described by Hün and Johnston (19). Briefly, half of the plasmid sample resuspended in 5 \( \mu \)l of H\(_2\)O was transcribed in a volume of 10 \( \mu \)l containing 40 mM Tris–HCl pH 7.5, 800 mM MgCl\(_2\), 2 mM spermidine, 10 mM DTT, 125 \( \mu \)M of each ATP, CTP, GTP and UTP; 5 \( \mu \)Ci \([\alpha-32P]UTP\); 10 U RNase inhibitor and 25 U T7 RNA polymerase. The optimal concentration of nucleoside triphosphates is 500 \( \mu \)M, but a reduction to 125 \( \mu \)M, which is near the \( K_m \), does not lead to adverse effects for transcription elongation (19). To determine precisely the location of termination sites in the template, we generated a reference RNA ladder by adding to the transcription reaction 30 \( \mu \)M 3’ dATP as RNA chain terminator [RNA products ending with a 3’ deoxyribose migrate as if they were one-half nucleotide shorter (19)].

**RESULTS**

**UV laser irradiation and primer extension**

The principle of signal detection by UV laser footprinting is illustrated in Figure 1. The DNA sample, either purified DNA or whole cells, is irradiated with a short pulse of UV laser light either in the presence or in the absence of the DNA-binding protein (in vivo: using a strain that is wild-type or carries a deletion of the
The primer ensuing photoreactions ensures a dimer of photons delivered to the sample in a single pulse is superior to the number of absorbing molecules. The high intensity laser radiation ensures a very short (5 ns) time of exposure to the radiation. The ensuing photoreactions take place on the microsecond time scale (20), i.e. fast enough to preclude rearrangements of the complex. The signal obtained therefore represents a true equilibrium (or steady state) situation and is not significantly influenced by the measurement procedure.

Irradiation is carried out with 266 nm light, roughly at the absorption maximum of nucleic acid bases. The resulting photoreactions include covalent DNA–protein cross-links (21,22), reactions of the excited bases with solvent molecules (23,24) and intra-strand reactions of the bases [the most prominent reaction of this type is the formation of pyrimidine dimers (25)]. The modification of a base may be detected by primer extension using T7 DNA polymerase. Polymerization of the DNA stops at the nucleotide opposite (or just before) the modified base (26,27). A comparison between samples containing or missing the DNA binding protein identifies the positions where the immediate environment of the DNA has changed due to the presence of amino acid side chains, exclusion of solvent molecules, or change of the DNA conformation, i.e. constitutes a footprint.

**Figure 2. UV laser footprint of IHF at the dppA binding site.** The T7 DNA polymerase synthesizes the top strand, thus revealing the modifications induced on the bottom strand. A sequencing reaction using the same primer is shown on the side. The position of the pentamer and trimer consensus elements of the *ihf* site is indicated by brackets on the side of the gel. Samples in lanes 1–4 were not irradiated. Primer extension was performed using *in vitro* samples (lanes 1 and 2 and 5–8) or *in vivo* samples (lanes 3, 4, 9 and 10). The *in vitro* reactions contained 2.5 nM (lanes 5 and 7) or 5 nM (lanes 6 and 8) of DNA. The arrowhead indicates the position of the major signal (located on the central G residue of the consensus pentamer) that appears upon binding of IHF. The arrow marks the position of a band (C in the TACGAAA sequence) in lanes 5–10 that can be used as an internal reference. The asterisk indicates a band (T in the TCCG sequence) in lanes 5–10 that is weaker *in vivo* than *in vitro* irrespective of the presence or absence of IHF (lanes 9 and 10 versus lanes 5–8).

**Characterization of IHF-induced footprinting signals *in vitro***

We selected different *ihf* sites located in RIBs (gyrB, dppA, uvrD), in λ *attP* (HI site) and at the 3' end of *ssb* gene to study the IHF–DNA interaction by UV laser footprinting. By gel retardation, it has been shown that these binding sites are saturated at 100 mM IHF (8 and data not shown). The primer extension profiles obtained after irradiation of the dppA RIB and gyrB RIB are presented in Figures 2 and 3, respectively.

One determinant for the appearance of a footprint is the ability of the polymerase used in the primer extension to recognize the damaged base and arrest elongation. Certain sequences constitute a natural block to elongation even without prior exposure to UV radiation, as can be seen in lanes 1–4 of Figure 2. These elongation arrests (or pauses) constitute an inevitable "background" of the
primer extension reaction but do not interfere with the footprinting signal since we only consider changes in the elongation pattern upon protein binding.

A second factor determining the appearance of a UV laser footprint is the probability with which a nucleic acid base can undergo a particular photoabortion after excitation with 266 nm light. The predominant reaction, with a quantum yield of $-4 \times 10^{-2}$ (20), is the formation of thymine dimers (more generally pyrimidine dimers). This can clearly be seen in the bottom part of Figure 2. The template strand for the primer extension reaction consists almost exclusively of thymine and cytosine residues (corresponding to adenine and guanine in the sequencing reaction) in this region. Many strong elongation arrests are observed in the samples that have been irradiated (lanes 5–10) compared with samples that have not been exposed to UV radiation (lanes 1–4). However it is also evident from Figure 2 that photoreactions are not limited to any particular sequence. The precise chemical nature of the reaction that constitute this large spectrum of photoreactivity remains mostly uncharacterized but each reaction provides nevertheless a potential footprinting signal.

Since the footprint is determined by changes in band intensities we have to compare lanes that contain the same amount of total material loaded. It is difficult to achieve, especially for in vivo footprinting reactions where the yield of the plasmid preparation is variable. We therefore limit our analysis to the predominant signals that can easily be detected and use signals located outside the ihf site as an internal reference within each gel lane. For a quantitative analysis several reference bands outside the protein binding site have to be used to normalize the signal in order to ensure that protein binding does not perturb a larger region of the DNA (see Discussion).

We first characterized the UV laser footprint for IHF under precisely controlled conditions in vitro. In Figure 2 we show UV laser footprints of the dppA site using different amounts of DNA in the primer extension reaction (2.5 nM in lanes 5 and 7; 5 nM in lanes 6 and 8). The relative footprinting pattern within a lane is independent of DNA concentration and lanes can be compared by reference to a band located outside the IHF binding site, e.g. the band marked with an arrow in Figure 2.

In the absence of IHF, the primer extension profile reveals signals induced by the modification of irradiated DNA (Fig. 2, lanes 5 and 6; Fig. 3, lanes 2 and 6). A strong band appears when the samples are irradiated in the presence of IHF (marked with an arrowhead in Figs 2 and 3). This strong signal corresponds to the central G on the top strand of the pentamer consensus sequence TTGAT (Fig. 4). In Figure 2 the strong band at the central guanine residue of dppA RIB is less apparent in lane 7 containing a lower amount of DNA than in lanes 8 and 9. However when the comparison is made using a reference band (indicated by an arrow), the signal is evident even in the underloaded lane 7 (compare lanes 5 and 7).

This predominant band, specific for the IHF–DNA complex, is located close to the pseudo-dyad axis of the complex in the model of Granston and Nash (14). The gyrB RIB has an identical sequence around the G-residue (Fig. 4) on the bottom strand, just on the other side of the pseudo-dyad axis. In Figure 3 we also

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{example_image.png}
\caption{Figure 3. UV laser footprints of IHF on the gyrB site obtained by primer extension on the irradiated plasmid DNA. The T7 DNA polymerase is synthesizing the top strand in lanes 1–4 and the bottom strand in lanes 5–8. The plasmid was irradiated in vitro in the absence (lanes 2 and 6) or in the presence (lanes 1 and 5) of 100 nM IHF. The in vivo footprint was performed in an IHF–strain (lanes 4 and 8) or in an isogenic IHF+ strain (lanes 3 and 7). The brackets along the gels indicate the position of the consensus trimer and pentamer elements. The arrowheads indicate the position of the major IHF-specific signal (G within the consensus pentamer for the top strand, and G in the sequence ATGAAA for the bottom strand). The asterisk marks the position of a band on the bottom strand that is only present in vivo (lanes 7 and 8 versus 5 and 6).
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{example_image.png}
\caption{Figure 4. Summary of the major UV laser footprinting signals for five ihf sites. The trimer and pentamer elements of the ihf site are written in bold type face for each site. Signs above the sequence of the top strand or underneath the sequence of the bottom strand indicate major variations of the footprinting pattern produced by the polymerase when synthesizing this strand. These variations therefore reflect chemical modifications on the opposite strand. A '+' sign indicates that the corresponding band is stronger in the presence of IHF. A '-' sign indicates a weakening of the intensity of the corresponding band in the presence of IHF. In vivo data are marked closer to the written sequence than the in vitro data.
\end{figure}
show the UV laser footprint for the bottom strand of gyrB RIB: one predominant signal is found on the G-residue within the sequence ATGAAA in the presence of IHF (Fig. 3, lane 5; band marked with an arrowhead). The signals on both strands of the gyrB RIB therefore occur within the same sequence context and correspond to positions where the β-ribbon arms of IHF are predicted to contact the DNA (see Discussion).

**In vivo footprinting of IHF on RIBs**

The footprinting experiment was repeated using whole cells containing pFBG14 (dppA RIB) or pFBG30 (gyrB RIB). After irradiation the plasmid was extracted and primer extension performed as for the *in vitro* experiment. A comparison of the reactivity pattern of DNA extracted from a wild-type strain and an IHF deletion mutant reveals the footprint of IHF-binding on the dppA and gyrB binding sites (compare lanes 9 and 10 in Fig. 2 and lanes 3 and 4 or lanes 7 and 8 in Fig. 3). A strong specific signal (marked with an arrowhead in Figs 2 and 3) is present on the plasmids extracted from an IHF⁺ strain and absent in the corresponding IHF⁻ strain. Three features characterize the footprint: (i) the changes of band intensities upon binding of IHF are exactly the same as those observed in the equivalent *in vitro* experiments; (ii) the photo-reactivity patterns of the final IHF-DNA complexes are identical *in vitro* and *in vivo*; (iii) the overall UV laser footprints performed in cells lacking IHF are very similar, but not identical (see below), to those obtained from the same plasmid *in vitro* in the absence of IHF (compare for example lanes 5 and 10 in Fig. 2).

The pattern of *in vivo* samples were obtained after irradiation of *exponential* (dppA RIB in Fig. 2 and gyrB RIB in Fig. 5) or stationary-phase (gyrB RIB in Fig. 3) cultures. We observe an identical change in band intensity upon binding of IHF for the *in vitro* footprint and the corresponding *in vivo* experiment using a stationary phase culture (Fig. 3). Since all *in vitro* experiments have been carried out at saturating concentrations of IHF, we conclude that the *ihf* site in gyrB RIB is saturated with IHF *in vivo* when the cells were in stationary phase (gyrB RIB in Fig. 3). However, both dppA and gyrB RIBs were not completely occupied by IHF (smaller change of band intensity upon IHF binding) when the *ihf* sites were probed during exponential growth (Figs 2 and 5 respectively and see Discussion).

**Comparison of five *ihf* sites**

A summary of the footprinting patterns of five different *ihf* sites *in vitro* and *in vivo* is shown in Figure 4. Only the most prominent signals are shown. Two situations have to be considered when comparing the *in vivo* and *in vitro* footprints: similarities or differences in the footprinting pattern in the absence of IHF indicate the degree of resemblance of the *in vitro* and *in vivo* environment of the DNA (e.g. additional proteins binding *in vivo*); the changes of photoreactivity upon addition of IHF characterize the interaction of IHF with its binding sites.

The overall reactivity pattern of the DNA in the absence of IHF is very similar *in vitro* and *in vivo* (compare corresponding lanes in Figs 2 and 3) indicating that the DNA is in a comparable environment and conformation. Small differences between the *in vitro* and *in vivo* experiments may be due to additional proteins binding *in vivo* (see Discussion). Two examples of such bands located outside the *ihf* site marked with an asterisk in Figures 2 and 3. In Figure 2 a band below the *ihf* site is absent in the *in vivo* experiment (lanes 9 and 10) but present under all condition *in vitro* (lanes 5–8). Another band in the bottom strand of the gyrB just above the IHF-binding site can be detected *in vivo* but is absent *in vitro* (Fig. 3, lanes 5–8).

Within the *ihf* site a band is visible on the cytosine of the consensus trimer CAA in the top strand of the gyrB site *in vivo* (Fig. 3, lane 4). This band disappear upon binding of IHF (Fig. 3, lane 3) and is absent under all conditions *in vitro* (Fig. 3, lanes 1 and 2). This example illustrates that the environment of the *ihf* site is dominated by the bound IHF protein, leading to an identical footprinting signal *in vitro* and *in vivo* in the presence of IHF, despite the different starting environment of the site.
Nature of footprinting signal

A predominant photoreaction upon irradiation with UV light is the formation of pyrimidine dimers. Another photoreaction with a high quantum yield is the formation of covalent cross-links between the protein and the DNA (20,27). However, the efficiency of this reaction varies greatly from protein to protein. The possibility of forming cross-links between IHF and its site was tested upon irradiation of complexes between IHF and double-stranded 35mer or 40mer oligonucleotides corresponding to λ attP H1 or to the ihf site located in gyrB RIB. By denaturing SDS polyacrylamide gel electrophoresis, no covalent cross-links (detection limit ~0.1% of the input DNA) could be detected (data not shown).

Since the signal is obtained by primer extension, the photoreaction has taken place in the opposite, i.e. template strand. Most of the strongest signals that we obtain can be interpreted as pyrimidine dimers (see Discussion). The sequence on the template strand opposite the major signal in the pentamer consensus sequence of the top strand is 5'-TTC-3' (gyrB, H1, dppA and uvrD sites in Fig. 4); the signal may therefore correspond to an elongation arrest of the DNA polymerase caused by the formation of a pyrimidine dimer on the template strand. The absence of this signal at the same location in the ssb site reinforces this hypothesis as no pyrimidine dimer formation is possible on the template strand.

Extensions using T7 RNA polymerase

The detection of bases modified by UV irradiation-induced photoreactions relies on the capacity of T7 DNA polymerase to pause elongation in front of or across a modified nucleotide. This property has been documented in the case of pyrimidine dimer formation but it is possible that other base modifications are tolerated by T7 DNA polymerase. The spectrum of UV modified bases recognized might be specific for the particular DNA polymerases used for primer extension. We have further tested the influence of this parameter by using a different polymerase, T7 RNA polymerase.

The primer extension reaction is no longer necessary. Instead we used the promoter for T7 RNA polymerase located on the cloning vector. Detection of modified bases is achieved by transcription from this promoter toward the ihf site in the presence of a radioactive nucleotide labeled in the α-position. Such an RNA UV laser footprint is shown in Figure 5 for the top strand of the gyrB site. As for the reactions using T7 DNA polymerase we observe natural termination (or pause) sites of the RNA polymerase using a non-irradiated DNA as template (lanes 1–3). An example of such a strong intrinsic elongation arrest is the band marked with an asterisk in Figure 5. The pattern obtained with the irradiated sample in the absence of IHF reveals elongation stops caused by irradiation (lane 4). The RNA polymerase arrests elongation in front of pyrimidine dimers (as does the DNA polymerase). This can clearly be seen for the stretch of four thymines (A in the sequencing reaction) just below the ihf site. In presence of IHF, one predominant signal consisting of a doublet is detected, again located near the pseudo-dyad axis of the IHF binding site (Fig. 5, lane 5). As for DNA polymerase there is a precise correspondence between the in vitro and in vivo UV laser footprints (Fig. 5, compare lanes 4 and 5 with lanes 6 and 7).

DISCUSSION

We have exploited the property of UV light to induce photoreactions in the DNA that are very sensitive to the immediate environment of the bases. Binding of a protein perturbs this environment in several ways: new chemical groups (amino acid side chains) are brought into the vicinity of the DNA bases (possibly leading to new photoreactions, i.e. cross-links between the DNA and the protein); potentially reactive solvent molecules are excluded from the vicinity of the DNA (thus preventing their reaction with the DNA); and the conformation of the DNA may be changed (bending of the DNA, for example, will reorient the planes of consecutive bases thus increasing or decreasing the probability of an intramolecular photoreaction such as the formation of a pyrimidine dimer). Contrary to chemical footprinting techniques that necessitate long incubation times (relative to the time scale of conformational rearrangements, i.e. typically milliseconds) UV laser footprinting does not perturb the complex under investigation because the signal is acquired within several microseconds (20), i.e. before the complex can rearrange. This footprinting technique should be very generally applicable to the study of protein–DNA interactions since it is applicable in vitro and under the most relevant conditions, i.e. within the living cell. In the present study we have concentrated on easily detectable, large changes in the photoreactivity of bases upon binding of IHF to different specific binding sites in vitro and in vivo. However a quantitative analysis of primer extension patterns shows a great variety of more subtle changes. A detailed study of RNA polymerase–promoter interactions using this technique is currently underway in our laboratory.

The strongest signal common to all ihf sites investigated is an increase in band intensity of the guanine residue in the pentamer sequence 5′-TTGAA-3′. This signal is not due to a covalent cross-link between IHF and the DNA but most likely corresponds to the formation of a pyrimidine dimer on the opposite strand (the template for the primer extension) 5′-T*TCAA-3′ or 5′-TT*CAA-3′. The formation of this pyrimidine dimer is impossible in the ssb binding site (the sequence does not contain two adjacent pyrimidines in this region) and the corresponding signal on the central guanine is absent. Only the T*C dimer can form at the H1 site of lambda. It is therefore likely that a T*C dimer forms in all cases and that the T7 DNA polymerase arrests elongation just across the first nucleotide of the dimer as has been observed for E.coli DNA polymerase I and the Klenow fragment (26). The detection of a doublet of transcription arrest using T7 RNA polymerase is consistent with this interpretation: elongation would stop in front of the pyrimidine dimer or on the first nucleotide of the dimer depending on enzyme and NTP concentration as was observed for DNA polymerase I (26).

The assumption that the signal on the central guanine is due to the formation of a pyrimidine dimer leads to a straightforward structural interpretation. Although the precise structural perturbation caused by a pyrimidine dimer remains controversial (28) most models predict a small unwinding of the DNA helix and a bend towards the major groove at the site of the pyrimidine dimer (29–31). Experiments using nucleosomes (32,33) or looped DNA (34) confirm the theoretical predictions and show that the formation of thymine dimers is enhanced at positions where the major groove is compressed. On the basis of the model for the IHF–DNA complex proposed by Nash and co-workers (11,14) this would correspond to an out-of-plane bend of the DNA away
from the beta-ribbon arms that bind in the minor groove of the DNA. Insertion of the beta-ribbons would widen the minor groove and compress the major groove located on the opposite side of the DNA helix. The center of pseudo 2-fold symmetry of the complex is located just 5' of the WATCAANNNTTR consensus sequence (14) and the predicted deformation should be symmetrical about this point. Indeed, the only site (gyrB) that does possess an identical symmetry related GAA sequence on the opposite strand also shows increased photoreactivity at this position upon IHF binding (Fig. 4). We note however that the sequence of gyrB is almost symmetrical (8/9 match to consensus in the orientation shown and 7/9 match to consensus in the opposite orientation) and IHF may well bind in both orientations to this site. The observed binding signal would thus constitute a population average of the two binding modes.

The UV laser footprints are very similar in vitro and in vivo. Even though the initial environments of the DNA in the absence of IHF are different, the final reactivity pattern (within the ihf site) in the presence of IHF is identical in both situations. This strongly suggests that IHF interacts identically with its binding sites in vitro and in vivo.

Additional proteins, solvent molecules, etc. are very likely to be bound to the DNA in vivo. Protein binding as well as other factors will lead to differences between the in vivo and in vitro reactivity patterns (some of them are marked by asterisks in Figures 2, 3 and 5). Proteins bound within the IHF binding site will be displaced by IHF (e.g. the band within the consensus trimer in Fig. 3), whereas signals outside the ihf sites will be indifferent to the presence or absence of IHF. If a protein modifies the organization of a larger region of DNA UV laser footprinting could reveal signals outside the binding site that are sensitive to protein binding. This does not seem to be the case for the ihf sites studied.

The predominant signal for binding of IHF to the gyrB site is the band at the central guanine residue. The increase in intensity of this band upon addition of IHF (at saturating concentration) in vitro is identical to the difference in band intensity in vivo when comparing cells in stationary-phase that express or lack IHF (Fig. 3 and data not shown). We conclude therefore that the binding site is saturated in vivo in stationary phase. However, when an identical experiment is performed with cells from an exponentially growing culture the intensity of the same signal is ~2-fold lower (data not shown). These results correlate IHF abundance and the level of in vivo occupancy of a binding site and confirm the measurements of Ditto and collaborators (6) who showed by Western immunoblot analysis an increase of IHF concentration from exponential- to late-stationary-phase cultures. Quantitative titerations of several ihf sites spanning a large range of binding affinities are underway to determine the value of the effective concentration of free IHF in vivo under different growth conditions.

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