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Drunken-cell footprints: nuclease treatment of ethanol-permeabilized bacteria reveals an initiation-like nucleoprotein complex in stationary phase replication origins

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ABSTRACT

The nucleoprotein complex formed on *oriC*, the *Escherichia coli* replication origin, is dynamic. During the cell cycle, high levels of the initiator DnaA and a bending protein, IHF, bind to *oriC* at the time of initiation of DNA replication, while binding of Fis, another bending protein, is reduced. In order to probe the structure of nucleoprotein complexes at *oriC* in more detail, we have developed an *in situ* footprinting method, termed drunken-cell footprinting, that allows enzymatic DNA modifying reagents access to intracellular nucleoprotein complexes in *E.coli*, after a brief exposure to ethanol. With this method, we observed *in situ* binding of Fis to *oriC* in exponentially growing cells, and binding of IHF to *oriC* in stationary cells, using DNase I and *Bst*NI endonuclease, respectively. Increased binding of DnaA to *oriC* in stationary phase was also noted. Because binding of DnaA and IHF results in unwinding of *oriC in vitro*, P1 endonuclease was used to probe for intracellular unwinding of *oriC*. P1 cleavage sites, localized within the 13mer unwinding region of *oriC*', were dramatically enhanced in stationary phase on wild-type origins, but not on mutant versions of *oriC* unable to unwind. These observations suggest that most *oriC* copies become unwound during stationary phase, forming an initiation-like nucleoprotein complex.

INTRODUCTION

Regulating gene expression, replication and recombination in living cells requires formation of highly specific nucleoprotein complexes. A variety of chemical and enzymatic DNA modifying reagents are available to probe protein–DNA interactions *in vitro*. It is more challenging to study nucleoprotein complexes in living cells, since few of these footprinting reagents cross cell membranes (1). Dimethylsulfate (DMS) does penetrate living cells (2–4). We have previously used this reagent to reveal that nucleoprotein complexes formed at *oriC*, the chromosomal replication origin of *Escherichia coli*, are dynamic in the cell cycle (5). At the time DNA replication initiates, initiator DnaA (6) and bending protein IHF (7) bind to *oriC*, while binding of Fis (8,9), another bending protein, is reduced (5). However, *in vivo* DMS footprinting studies are hampered by its strong base pair preference (2–4). One obvious way to remove this limitation is to destroy the membrane barrier. For example, cells may be gently lysed by chemical or mechanical treatment prior to exposure to the footprinting reagents. While this option has produced DNase I footprints in cell types where gentle lysis is possible (10,11), structural integrity of the cell is completely compromised. Using this method to detect DNA–protein complexes in bacteria is particularly troublesome because treatments sufficiently harsh enough to produce lysis are expected to destroy DNA–protein complexes. In the case of *E.coli*, an intriguing alternative is to permeablize cells to allow entry of enzymatic or chemical nucleases for *in situ* DNA modification. Historically, a variety of chemicals (e.g. toluene, ether and EDTA) were found to permeablize *E.coli* (12), but the state of DNA–protein complexes has not been determined in treated cells.

We were recently attracted by the ethanol fixation procedure used to prepare *E.coli* for flow cytometric analysis of DNA content, as the basis for a suitable permeabilization method for *in situ* footprinting (13). In this report we present a simple and useful variation of this method to allow a wide variety of footprinting reagents access to intracellular DNA–protein complexes in bacteria. We refer to this protocol as drunken-cell footprinting, because bacteria are permeabilized by a brief exposure to ethanol. The method is simple and causes no detectable change in cell morphology or nucleoid conformation after treatment. Chromosomal and extrachromosomal genomes of drunken *E.coli* are susceptible to a variety of enzymatic footprinting reagents including restriction endonucleases, DNase I and P1 nuclease. We used this method to examine the intracellular

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binding of Fis and IHF to the chromosomal origin of replication, *oriC*, as well as the single-stranded state of this replication origin (14,15), in exponential and stationary phase cultures of *E.coli*. As previously reported using DMS *in vivo* footprinting (5), Fis binding was detected only in exponential phase and IHF only in stationary phase using ethanol-treated cells probed with DNase I or the restriction endonuclease *Bst*NI, respectively. In ethanol-treated cells from stationary cultures, a dramatic hypersensitivity to P1 endonuclease cleavage was restricted to the 13mer unwinding region at the left edge of *oriC* (14) at the identical location unwound by DnaA *in vitro*. No P1 hypersensitivity was detected in exponential phase or in stationary cells harboring a mutant plasmid *oriC* defective for unwinding. This result suggests that *oriC* in stationary phase may be locked into a conformation similar to the open complex formed during initiation of DNA replication.

MATERIALS AND METHODS

Ethanol-permeabilized bacteria

Cultures of *E.coli* B/r F_{26} *his thyA* (16) transformed with *oriC*-oripBR322 chimeric plasmid pUA5 (17) or pOC170 (18) were grown at 37°C as described (16). pUA5Δ*Bgl*II, used for some experiments, contains a 16 bp *Bgl*II deletion in the leftward 13mer that prohibits *oriC* unwinding (19). To prepare drunken-cells, 1 ml of exponential culture (OD_{450} 0.6) was added to 9 ml of cold 50% ethanol. For overnight stationary cultures (OD_{450} 1.6–1.7), 400 µl was added to 9 ml of cold 70% ethanol to achieve the same degree of permeability, assayed by monitoring DNase I cleavage (described below) of chromosomal DNA on 1% agarose gels. For some experiments requiring non-permeabilized cells, the ethanol was replaced with 9 ml of cold modified phosphate-buffered saline (MPBS) (150 mM NaCl, 4 mM Na_2HPO_4, 2 mM KH_2PO_4). Centrifuged cell pellets were washed once in cold Tris–magnesium (TM) buffer (10 mM Tris, pH 7.4, 10 mM $MgCl_2$) and resuspended in 100 µl cold TM. Samples were warmed to room temperature prior to addition of footprinting reagents.

Footprint analysis

For DMS footprinting in ethanol-treated and untreated cells, 2 µl of 2.65 M DMS (Aldrich) was added to each sample for 1 min at 37°C. Reactions were quenched by diluting each sample with 1 ml ice-cold MPBS. Treated cells were centrifuged and resuspended in 100 µl cold TM buffer. For enzymatic footprints in ethanol-treated cells, either 50 U P1 (Pharmacia Biotech), 45 U *Bst*NI (New England BioLabs) or 2 µl of 1 mg/ml DNase I (Pharmacia Biotech) was added to each sample. DNase I reactions were gently mixed at room temperature for 1 min. P1 and *Bst*NI reactions were incubated at 37°C for 4 min without agitation. Reactions were quenched by adding 5 µl of cold 0.5 M EDTA. All cell samples were then subjected to whole cell lysis as described previously (20). *In vitro* DMS footprints of pUA5/IHF and pUA5/Fis complexes were produced as previously described (5). For DNase I *in vitro* footprinting (100 µl reactions), 1 µl of a 1:100 dilution of DNase I in TM buffer was added along with 4 µl of 0.1 M $MgCl_2$ and 3 µl of 0.1 M $CaCl_2$ for 1 min at 37°C. For *in vitro* *Bst*NI footprints, 1.5 µl of a 7.5 U solution of *Bst*NI in TM buffer was added to samples for 4 min at 37°C. For P1 endonuclease assays of open complex formation, 0.75 µg (300 fmol) of plasmid DNA was added to 40 mM HEPES–KOH, pH 7.6, 8 mM $MgCl_2$, 30% (w/v) glycerol, 320 µg/ml BSA, 5 mM ATP in a 50 µl reaction. P1 endonuclease (1.2 U; Amersham-Pharmacia Biotech, Uppsala, Sweden) in 2 µl of 30 mM potassium acetate, pH 4.8, was incubated with supercoiled DNA for 10 s at 38°C. Proteins were added to reactions at the concentrations indicated in the figures. *In vitro* footprinting reactions were quenched with 100 µl cold stop buffer (3 M ammonium acetate, 1 M β-mercaptoethanol, 250 µg/ml tRNA and 20 mM EDTA), extracted once with an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol and precipitated at –70°C with ethanol. The choice of TM buffer for enzymatic reactions was deliberate due to the presence of endogenous nuclease activities that we observed in some strains of *E.coli* (including B/r F_{26}) that were not detected in TM. Although TM may not be the choice for optimum activity, many DNA modifying enzymes remain active in this buffer and extensive cutting of DNA is not desirable for footprinting. We therefore suggest using TM buffer when possible and careful monitoring of alternative buffer choices for endogenous DNA degradation activity.

Alkaline primer extension and electrophoresis

All footprinting samples were alkaline primer extended as published previously (5). DMS-treated DNA was exposed to piperidine at 90°C for 30 min. A 19mer oligonucleotide primer hybridizing to the top strand of *oriC* at nt 272–290 (5) was end-labeled with ^{32}P and added to 35 µl reactions containing 0.5 µg of piperidine-treated template. After adding 4 µl of 0.01 M NaOH, reactions were incubated at 80°C for 2 min. Cooled samples were then mixed with 5 µl of 0.5 M Tris–HCl, pH 7.2, 0.1 M $MgSO_4$ and 2 mM dithiothreitol and incubated at 56°C for 3 min to hybridize primer with template. Primers were extended with Klenow DNA polymerase I (New England Biolabs) at 50°C for 10 min followed by precipitation with ethanol. Samples were then electrophoresed in 6% polyacrylamide sequencing gels and dried gels were exposed to X-ray film for 1–14 days at room temperature without intensifying screens. Autoradiographs were scanned using a Bio-Rad Model GS-700 Imaging Densitometer and Molecular Analyst v.1.5 software (Bio-Rad) was used to quantify intensities of modified sites. Base positions were determined by comparing the DMS cleavage at G residues to *oriC* sequence and by counting of background bands.

Nucleoid staining and DNA leakage measurements

One milliliter of cell culture (OD_{450} 0.6) was placed in 9 ml of 50% ethanol or 9 ml of MPBS. Cells were washed once in cold TM and resuspended in 1 ml of cold TM. Suspensions were warmed to room temperature, treated with 31 µl of 20 mM propidium iodide (Sigma) and mixed for 1 min at room temperature, then placed on ice. Cells were observed using an Olympus phase contrast microscope model BH-2 with a fluorescence vertical illuminator (Olympus model BH-RFL) at 1000× magnification as described (21). For measurement of DNA leakage, cells (10 ml of OD_{450} 0.3) were labeled for 10 min at 37°C with 100 µl of [^3H]thymidine (Amersham; 1 mCi/ml stock to give 10 µCi/ml final concentration) and chased with cold thymidine for one generation of growth. Cells were permeabilized and treated with DNase I as described above.

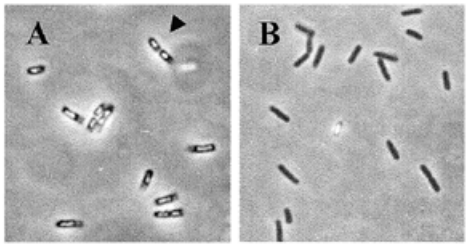

Figure 1. Effect of ethanol treatment on the microscopic appearance of *E.coli* B/r cells and nucleoids. Ethanol-treated (**A**) and untreated cells (**B**) were exposed to propidium iodide and observed under phase contrast microscopy with both UV and visible light as described in Materials and Methods. The arrow in (A) indicates two distinct nucleoids in a cell that has not yet divided. A single dead cell, stained with propidium iodide, is seen in (B).

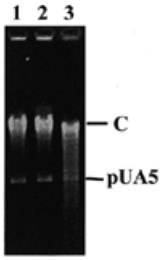

Figure 2. Intracellular chromosomal DNase I digestion in ethanol-treated cells. Samples of *E.coli* B/r/pUA5 were incubated with DNase I, lysed, and whole cell lysates were electrophoresed on agarose gels as described in Materials and Methods. Lane 1, untreated cells; lane 2, ethanol-treated cells with lysis buffer added prior to DNase I incubation; lane 3, ethanol-treated cells with lysis buffer added after DNase I incubation. Position of chromosome (C) and plasmid (pUA5) are marked.

RESULTS

Genomes of ethanol-treated *E.coli* are cut with DNase I *in situ*

Prior to staining *E.coli* for flow cytophotometric measurement of DNA-associated fluorescence, cells are fixed in ethanol (13). Since the integrity of the cells must be maintained for flow cytometry, we chose to use ethanol permeabilization as the basis for an *in situ* enzymatic footprinting method. Exposing growing *E.coli* to 50% ethanol for several minutes had little effect on the cell appearance under the microscope when compared to untreated samples (Fig. 1). Treated cells retained their size and shape, but were no longer able to form colonies. Ethanol-treated cells readily took up propidium iodide and stained nucleoids remained intact and compact in a central intracellular location. To determine if the ethanol treatment caused DNA breakage and leakage that was undetectable by propidium iodide staining, *E.coli* chromosomes were radiolabeled with [^{3}H]thymidine and treated with ethanol as described in Materials and Methods. No significant release of radioactivity was detected in supernatants from pelleted cells (Table 1), indicating that their genomes remained intact. Additionally, our standard DNase I treatment caused only slight release of soluble DNA (<10% of c.p.m.), indicating that our footprints (see below) are generated by a low level of digestion. After centrifugation, ethanol-treated cells remained identical in appearance to untreated cells (data not shown).

Table 1. Ethanol and DNase I do not cause significant leakage of ^{3}H-labeled DNA from *E.coli*

	%c.p.m. in wash	%c.p.m. in pellet
–Ethanol, –DNase I	1	99
–Ethanol, +DNase I	1	99
+Ethanol, –DNase I	2	98
+Ethanol, +DNase I	4	96

B/r F_{26}/pUA5, grown in the presence of ^{3}H-thymidine, were permeabilized and treated with DNase I as described in Materials and Methods. After treatment, cells were pelleted and the wash was filtered to remove cells. The c.p.m. in pellet value includes both the pellet and any cells removed by filtration.

We next determined whether DNase I cutting of the genome was restricted to the incubation period used with ethanol-permeabilized cells (see Materials and Methods) and not during the lysis and DNA isolation procedure that followed. *Escherichia coli* B/r F_{26}/pUA5 cultures were exposed to ethanol, pelleted, and resuspended in TM buffer as described in Materials and Methods. Drunken-cells were then treated with DNase I in the presence or absence of EDTA and Sarkosyl lysis buffer used to stop cutting and lyse the cells. DNA cutting, measured by gel electrophoresis, was seen only in ethanol-permeabilized cells, but no DNA cutting was observed if EDTA and lysis buffer were added prior to addition of DNase I (Fig. 2).

In vivo DMS footprints are unaffected by ethanol treatment

The results described above indicate that ethanol-permeabilized cells would be useful for enzymatic DNA footprint analysis. However, the effect of ethanol treatment on intracellular DNA–protein complexes was unclear. To address this question, we used DMS *in vivo* footprinting to measure DNA–protein interactions previously studied at *oriC* in exponential and stationary cultures (5). Binding of accessory proteins Fis and IHF is detectable at *oriC* during different growth phases. IHF binds to *oriC* at the time of initiation of chromosome replication during the cell cycle and is undetectable in exponential cultures, where less than 10% of the cells are actively initiating DNA replication at any time (5). Conversely, Fis is bound to *oriC* throughout the majority of the cell cycle and is detectable in samples taken from exponential cultures (5). In stationary phase cultures, IHF levels are increased 5- to 7-fold (22,23) and Fis levels are depleted (24,25). For this reason, Fis binding to *oriC* becomes undetectable while IHF binding is seen in stationary phase (5). To determine if the DNA–protein interactions were still maintained after exposure to ethanol, DMS footprinting was used to measure Fis and IHF binding to *oriC* in treated and untreated cells. Results were compared to *in vitro* DMS footprints obtained for pure *oriC*/Fis or *oriC*/IHF complexes. As shown in Figure 3, no obvious differences in DMS footprints were observed between samples taken from either ethanol-treated (+) or untreated (–) exponential (Fig. 3A, left) or stationary phase (Fig. 3A, right) cultures. When the DNA modification patterns from stationary and exponential

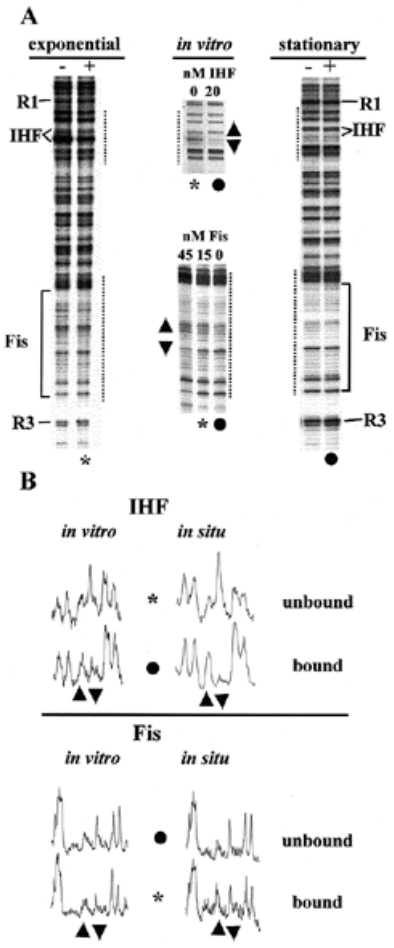

Figure 3. DMS footprints in the presence and absence of Fis and IHF. (**A**) Samples of purified supercoiled *oriC* plasmid (center) or *E.coli* B/r harboring *oriC* plasmids (left and right) were treated with DMS, primer extended and electrophoresed as described in Materials and Methods. Untreated (–) or ethanol-treated (+) samples were obtained from exponential or stationary phase cultures as marked. Up or down arrows indicate enhanced or suppressed DNA modification, respectively. Selected positions of protein binding sites in *oriC* are designated by brackets. Regions chosen for densitometric scans are marked by dashed lines. (**B**) Densitometric scans of selected gel lanes in (A). Scanned lanes are designated with • for lanes corresponding to the exponential pattern or * for lanes corresponding to the stationary pattern.

cultures were compared, differences were detected in IHF and Fis binding sites, as well as DnaA binding sites termed R boxes (26; see sites marked in Fig. 3A). The striking change in DMS sensitivity within the IHF binding region seen in stationary phase cultures (shown in the marked region in Fig. 3A and quantified by densitometric scans in Fig. 3B, upper panel) was nearly identical to the change in DMS *in vitro* footprints obtained when purified IHF protein was added to *oriC* DNA (shown in Fig. 3A, upper center, and scans in Fig. 3B, upper). This result is consistent with the binding of IHF in stationary phase, but not in exponential phase, as previously reported (5). Differences in the Fis binding region of *oriC* in exponential and stationary phase DMS footprint samples were less striking, but comparable to differences seen in the *in vitro* footprints for lower levels of Fis compared to the pattern seen with no protein (Fig. 3A, lower center, and scans in Fig. 3B, lower). As previously reported (5), enhancement/suppression patterns (noted in Fig. 3 by up and down arrows) detected for Fis binding *in vitro* (Fig. 3A, lower center) are most similar to patterns seen in DMS footprints from exponential cultures (Fig. 3A, left). Comparison of the exponential cell footprint with the *in vitro* DMS footprints at different Fis levels suggests that Fis binding to this site within cells is weak since the strong band suppressions seen at only higher Fis levels *in vitro* are not detected *in vivo*.

The DMS footprints obtained from stationary phase also reveal enhanced binding of DnaA to R boxes, as reported previously (5). This binding is detected by increased intensity of the footprints obtained at the weak R3 binding site and stronger R1 (note sites marked in Fig. 3A). Increased binding is also noted at R4 and R2 in stationary phase (data not shown). Filling of R3 is not detected in exponential phase cultures (5,27), but DnaA binds to this site during the cell cycle at the time of initiation of replication coincident with the binding of IHF (5). In stationary phase, enhanced binding of DnaA and IHF to *oriC* suggests that a complex similar to the initiation complex is formed when cells enter stationary phase. If this is the case, unwinding of *oriC* might be detected as an increased sensitivity to P1 endonuclease within the unwound region (see below). Since all differences in DMS patterns between experimental and stationary cultures can be detected in both untreated and ethanol-permeabilized cells, it appears that ethanol treatment used for drunken-cell footprinting has no obvious effect on the intracellular binding of Fis, IHF and DnaA proteins to *oriC*.

DNase I and *Bst*NI footprints of Fis and IHF binding in drunken-cells

To test the drunken-cell method with a footprinting reagent that does not cross the *E.coli* membrane, we used DNase I to examine Fis binding in ethanol-treated cells. DNase I footprints were generated using exponential and stationary phase cell samples and compared to *in vitro* Fis DNase I footprints. As shown in Figure 4, differences were seen in the DNase I cutting pattern caused by Fis binding *in vitro* and between exponential and stationary cells *in situ*. The DNase I pattern with low levels of Fis binding *in vitro* is most similar to the pattern seen in ethanol-treated exponential cells, although the patterns are not completely identical. Enhancement of cutting at bases 233 and 227 and within the 207–210 region along with the strong suppression of cutting at 213 seen in exponential cells are also detectable when Fis is bound *in vitro* (note pattern changes marked by up or down arrows in Fig. 4). This pattern is different to that observed in stationary phase cells, which most closely resembles the *in vitro* DNase cleavage pattern on naked DNA samples. An advantage of using DNase I compared to DMS as an *in situ* footprinting reagent for Fis is the increased number of modified bases detected (Fig. 4C), which enhances the ability to identify occupied binding sites.

As previously shown using DMS, the intracellular footprint for Fis binding at its *oriC* site was not as striking as that obtained using high levels of purified Fis protein *in vitro*. Although it is not clear why *in vitro* footprints are not fully identical to *in vivo*/*in situ* footprints obtained with DMS and DNase I, respectively, the consistent difference obtained with both intracellular footprinting reagents suggests that binding of

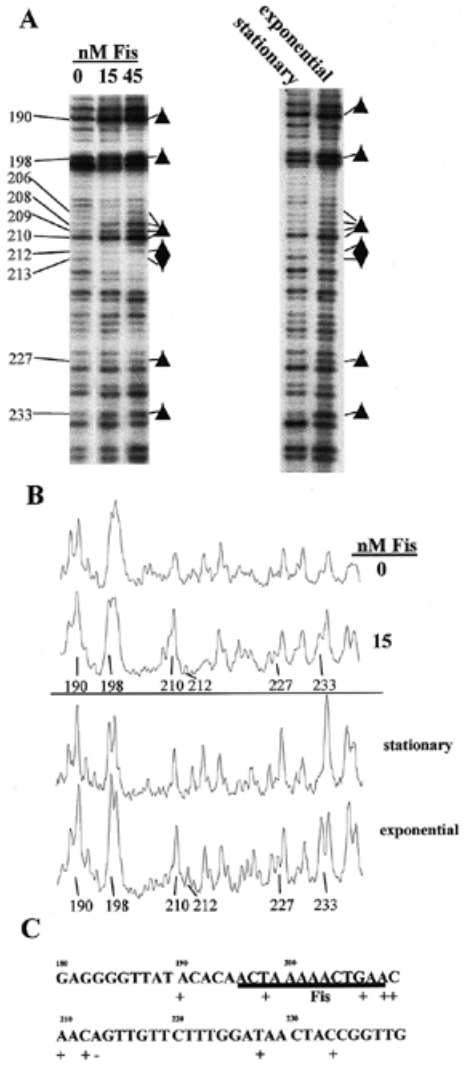

Figure 4. DNase I footprints in the presence and absence of Fis. (**A**) Samples of purified supercoiled *oriC* plasmid (left) or ethanol-treated *E.coli* B/r harboring *oriC* plasmids (right) were treated with DNase I, primer extended, and electrophoresed as described in Materials and Methods. Ethanol-treated samples were obtained from exponential or stationary phase cultures. Up or down arrows indicate enhanced or suppressed cutting, respectively. Selected nucleotide positions are designated. (**B**) Densitometric scans of selected gel lanes in (A). (**C**) *oriC* nucleotide sequence around the Fis binding site. Nucleotide positions of enhanced or suppressed cleavage with respect to the underlined Fis consensus binding site are marked with a + or –, respectively.

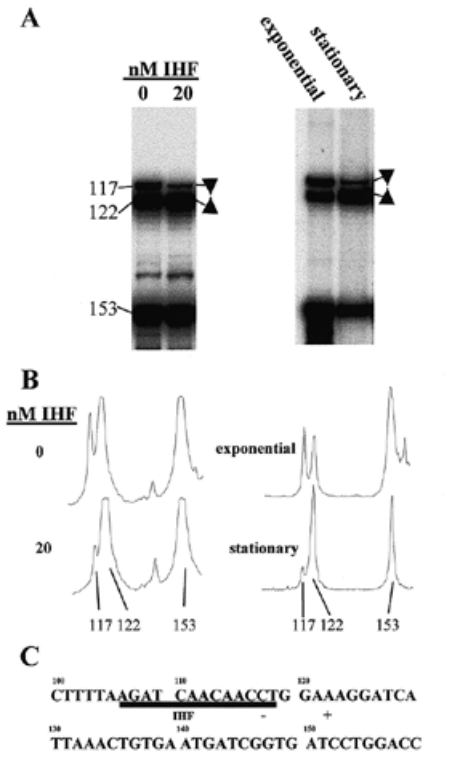

Figure 5. *Bst*NI restriction in the presence and absence of IHF. (**A**) Samples of purified supercoiled *oriC* plasmid (left) or ethanol-treated *E.coli* B/r harboring *oriC* plasmids (right) were treated with *Bst*NI endonuclease, primer extended and electrophoresed as described in Materials and Methods. Ethanol-treated samples were obtained from exponential or stationary phase cultures. Up or down arrows indicate enhanced or suppressed cutting, respectively. Selected nucleotide positions are designated. (**B**) Densitometric scans of gel lanes in (A). (**C**) *oriC* nucleotide sequence around the IHF binding site. Nucleotide positions of enhanced or suppressed cleavage with respect to the underlined IHF consensus binding site are marked with a + or –, respectively.

Fis to *oriC in vivo* is relatively weak. Although it remains to be determined why this is the case, the strength of Fis binding at *oriC* may reflect the dynamic nature of the intracellular complex.

In order to demonstrate the flexibility of the method, we chose a different enzyme to examine the binding of IHF to DNA. The nucleotide sequence encompassing the binding site of IHF was used to select a restriction endonuclease whose accessibility should be affected by the presence of IHF. *Bst*NI was chosen to detect IHF binding due to the proximity of its recognition site ($CC^{A}/_{T}GG$) to the *oriC* consensus sequence for IHF binding (28) (Fig. 5C). Assays were performed in TM buffer at 37°C since these conditions were determined (by DMS footprinting) not to disturb IHF binding to *oriC*. As shown in Figure 5, the pattern of cutting produced by *Bst*NI on naked DNA *in vitro* and in exponential drunken-cells was identical in the *oriC* IHF binding region although under these buffer and temperature conditions the recognition site stringency was relaxed (Fig. 5A). For example, an additional cut was seen within the degenerate recognition site AAAGG at position 122, just to the left of the IHF consensus recognition site (Fig. 5C). In the presence of IHF *in vitro* and in the stationary phase drunken-cell sample, a clear suppression of *Bst*NI restriction at position 117 and enhanced restriction at 122 was observed, indicative of IHF binding (Fig. 5A and scans in Fig. 5B). Despite relatively few DNA modification sites, *Bst*NI appeared to be a useful probe for the detection of IHF binding to its *oriC* site *in vitro* and *in situ*. The wide range of available restriction endonucleases dramatically increases the number of footprinting probes for DNA–protein analysis using the drunken-cell method.

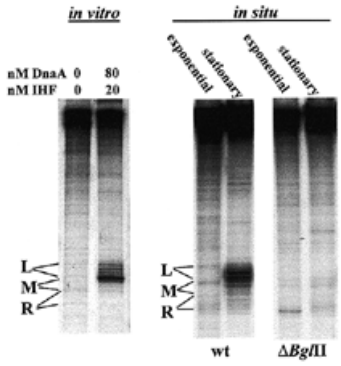

Figure 6. P1 endonuclease cutting in the *oriC* 13mer region *in vitro* and *in situ*. Samples of purified supercoiled *oriC* plasmid (*in vitro*) or ethanol-treated *E.coli* B/r harboring *oriC* plasmids (*in situ*) were treated with P1 endonuclease, primer extended and electrophoresed as described in Materials and Methods. *In situ* samples were obtained from exponential or stationary phase cultures harboring wild-type (wt) or unwinding mutant (Δ*Bgl*II) *oriC* plasmids. The concentration of purified protein associated with *in vitro* samples is indicated. The positions of P1 cut sites are designated in the leftward (L), middle (M) or rightward (R) set of repeated 13mer sequences in the unwinding region of *oriC*.

P1 endonuclease detects *oriC* unwinding in drunken-cells

Another application of the footprinting method described here is the ability to detect intracellular DNA conformational changes such as bends and single-stranded regions using enzymes that specifically detect these changes. In the case of *E.coli oriC*, a P1 endonuclease assay was developed to detect unwinding in the 13mer region produced by the initiator protein DnaA *in vitro* (14). Using the same assay, we wondered if it might be possible to detect *oriC* unwinding in ethanol-treated cells. We observed that *in vitro* (in the presence of DnaA and IHF) and in drunken stationary cells, the unwinding of *oriC* was detected by positioning of P1 cut sites in the same region of the left and middle 13mers (Fig. 6). Significant P1 sensitivity in the 13mers was not detected *in vitro* in the absence of DnaA/IHF or in exponential phase. Cutting was also absent in exponential or stationary cultures harboring plasmids carrying *oriC* unable to unwind due to a 16 bp deletion mutation (Fig. 6) (19). We showed previously that in stationary phase, DnaA was loaded at its R sites (see Fig. 3, right) and IHF was bound (Figs 3 and 5). These conditions should lead to open complex formation (unwinding of *oriC*). Enhanced P1 cutting of *oriC* in stationary phase samples is consistent with *oriC* unwinding in this growth phase. $KMnO_4$ sensitivity in *oriC* also correlates with the presence of single-stranded DNA (15) and enhanced $KMnO_4$ sensitivity was detected in stationary cells (data not shown).

DISCUSSION

Although a wide variety of DNA modifying reagents are available for detection of DNA–protein interactions by footprinting *in vitro* (29), detection of these interactions in cells is significantly limited due to the dearth of DNA modifying reagents that enter cells. As analysis of DNA replication, recombination and gene expression proceeds, there will be an increased need for intracellular studies of DNA–regulatory protein interactions. The drunken-cell method reported here expands footprinting analysis in *E.coli* by allowing DNA modifying reagents that cannot cross membrane barriers to enter cells. By expanding the number of different DNA modifying enzymes and chemicals that can be used for intracellular footprinting, it becomes easier to validate the presence of regulatory proteins at particular binding sites using multiple footprinting reagents. Although it will be necessary to test each case individually, we show that brief exposure of *E.coli* to ethanol produced a permeabilized bacterium, but did not destroy the interaction of two well-studied DNA binding proteins, Fis and IHF, with their known binding sites in *oriC*. Fis and IHF are members of a dynamic complex that is assembled and disassembled on *oriC* during the cell cycle (5) and the application of drunken-cell footprinting to the study of these complexes should provide additional insight into the molecular nature of this bacterial growth control mechanism.

The detection of *oriC* unwinding presented here indicates that ethanol treatment did not drastically alter the conformational state of supercoiled DNA. We believe that the drunken-cell footprinting method is suitable for measurement of the onset and duration of *oriC* unwinding during the cell cycle in synchronized *E.coli* (30) and study of the intracellular *oriC*–protein complex that produces the DNA conformational change. The observed enhancement of P1 cutting in stationary phase suggests that *oriC* is unwound in stationary phase. It is well established that binding of initiator DnaA to supercoiled *oriC* produces an open complex characterized by single-stranded regions in the 13mer repeats as a prerequisite for assembly of new replication forks (14). It was assumed that unwinding of *oriC* would be a transient condition during the cell cycle (5) and might be detrimental for cell survival if maintained for extended periods of time. Based on findings reported here, this is not the case. Unwinding in *oriC* was stable and persistent for at least 12 h of stationary phase in our experiments. More studies are required to determine the kinetics of *oriC* unwinding as cells enter and leave stationary phase.

Since cells entering stationary phase are likely to continue to accumulate DnaA initiator protein as growth is slowing (31), unwinding of *oriC* may be a normal outcome, although continued replication initiation is not (32). It is not yet clear whether the inability to synthesize sufficient amounts of DnaA protein (or another factor) prohibits new rounds of chromosome replication or if a stationary phase protein prohibits new rounds (33). The question also remains whether it is advantageous for *E.coli* to lock its origin of replication into the unwound state in stationary phase. There are two obvious advantages. First, when cells are again able to proceed through the cell cycle once more, their replication origins are ready to fire and should respond rapidly to the proper initiation signal. Second, the unwound origin might be the very best substrate for whatever mechanism resets the origin and starts the assembly of new initiation complexes. It remains to be determined if either situation is a feature of bacterial cell growth control.

There may be some DNA–protein interactions or conformational changes that will not survive ethanol treatment, and there are also DNA modifying reagents that may not be useable in ethanol-permeabilized cells. We do not yet know the limitations of this method, particularly with regard to the compatibility of some buffer conditions. The fact that drunken *E.coli* cells are

not viable at the time they are exposed to the DNA modifying reagent is a significant difference from the DMS (34) or UV light (4) footprinting methods. We recommend use of these *in vivo* reagents in conjunction with drunken-cell footprinting to evaluate the nature of DNA–protein contacts under physiological conditions of interest.

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