The EMBO Journal Vol.17 No.23 pp.7033–7043, 1998

Thermoregulation of *Shigella* and *Escherichia coli* EIEC pathogenicity. A temperature-dependent structural transition of DNA modulates accessibility of *virF* promoter to transcriptional repressor H-NS

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The expression of plasmid-borne *virF* of *Shigella* encoding a transcriptional regulator of the AraC family, is required to initiate a cascade of events resulting in activation of several operons encoding invasion functions. H-NS, one of the main nucleoid-associated proteins, controls the temperature-dependent expression of the virulence genes by repressing the *in vivo* transcription of *virF* only below a critical temperature (~32°C). This temperature-dependent transcriptional regulation has been reproduced *in vitro* and the targets of H-NS on the *virF* promoter were identified as two sites centred around –250 and –1 separated by an intrinsic DNA curvature. H-NS bound cooperatively to these two sites below 32°C, but not at 37°C. DNA supercoiling within the *virF* promoter region did not influence H-NS binding but was necessary for the H-NS-mediated transcriptional repression. Electrophoretic analysis between 4 and 60°C showed that the *virF* promoter fragment, comprising the two H-NS sites, undergoes a specific and temperature-dependent conformational transition at ~32°C. Our results suggest that this modification of the DNA target may modulate a cooperative interaction between H-NS molecules bound at two distant sites in the *virF* promoter region and thus represents the physical basis for the H-NS-dependent thermoregulation of virulence gene expression.

Keywords: curved DNA/enterobacterial pathogenicity/ *in vivo* footprinting/nucleoid/thermoregulation

Introduction

Bacteria entering the human host encounter an increase of their growth temperature to 37°C; this environmental change represents a cue that can trigger virulence gene expression in human pathogens such as *Shigella* species and enteroinvasive *Escherichia coli* (EIEC) (Maurelli and Sansonetti, 1988). These microorganisms cause disease by a similar, complex mechanism of pathogenicity which depends on the expression of chromosomal or plasmid-borne virulence genes (Sansonetti *et al*., 1982; Finlay and Falkow, 1997). The virulence genes carried by pINV are organized in regulons coordinately regulated by a central modulator, VirR (Maurelli and Sansonetti, 1988), corresponding to the major nucleoid protein H-NS (Lammi *et al*., 1984; Spassky *et al*., 1984; Pon *et al*., 1988), which represses their expression at 30°C or at low osmolarity (Tobe *et al*., 1991, 1993; Dagberg and Uhlin, 1992; Porter and Dorman, 1994). In addition to chromosome-encoded H-NS (VirR), two pINV-encoded activators, the proteins VirF and VirB, are involved in the transcriptional control of the invasion genes (Adler *et al*., 1989). In a cascade model, VirF activates transcription of the gene coding for the secondary regulator VirB which, in turn, activates several operons encoding the invasion genes. The activation of *virB* transcription by VirF is highly sensitive to changes in DNA topology and is antagonized at 30°C by H-NS (Tobe *et al*., 1993, 1995). Thus, the cellular level of VirF is crucial for the expression of the invasive phenotype of *Shigella* and *E.coli* EIEC and, while the expression of *virF* is not constitutive at 30°C, it is induced at 37°C (Tobe *et al*., 1991; Colonna *et al*., 1995) and may also be regulated at the post-transcriptional level through tRNA modifications (Durand *et al*., 1994, 1997). Furthermore, *virF* inactivation or hyperexpression results in a complete loss of the invasive phenotype or in the induction of invasiveness at the non-permissive temperature, respectively. More recently, it has been shown that a constitutively higher level of *virF* mRNA and β-galactosidase expressed from a *virF–lacZ* fusion can be found in cells with an *hns*-defective background (Prosseda *et al*., 1998). These data suggested that, as in the case of other virulence-regulating genes such as *cfaD* (Jordi *et al*., 1992), *virB* (Tobe *et al*., 1993) and *papI* (Forsman *et al*., 1992), *virF* expression might also be negatively controlled by H-NS in a temperature-dependent manner. This premise was supported further by data suggesting that this protein may interact with the promoter region of *virF* (Prosseda *et al*., 1998).

H-NS is known to affect, primarily at the transcriptional level, the expression of a fairly large number of genes (reviewed in Atlung and Ingmer, 1997), and although the molecular basis of its regulatory activity probably rests on its preferential interaction with intrinsically curved DNA (Yamada *et al*., 1990, 1991; Tanaka *et al*., 1991), its ability to induce bending of non-curved DNA (Spurio *et al*., 1997) and its ability to induce negative supercoiling (Tupper *et al*., 1994), different mechanisms of repression have been proposed (Higgins *et al*., 1988; Goransson *et al*., 1990; Hulton *et al*., 1990; Owen-Hughes *et al*., 1992; Falconi *et al*., 1993; Ueguchi and Mizuno, 1993; Tupper *et al*., 1994; Barth *et al*., 1995). To explain how H-NS may regulate gene expression in a temperature-dependent manner is even more intriguing. In fact, while induction of gene expression by a temperature increase is

not restricted to the pathogenicity genes, but also concerns the heat shock regulon, the participation of a DNA-binding protein such as H-NS in thermoregulation is a unique characteristic of the virulence genes. Furthermore, unlike the heat response, which entails a temperature increase above the normal growth temperature and which produces a transient expression of the genes belonging to the regulon, temperature-dependent induction of the *vir* regulon in *Shigella* involves a temperature shift within the normal range of growth temperatures and the stable activation of the regulon. Thus, it is clear that temperature control over virulence expression operated by H-NS entails a unique mechanism, clearly different from that responsible for the activation of the heat shock regulon.

To explain the thermoregulation in *Shigella* pathogenicity expression, it is commonly assumed that the temperature control acts via the plasmid-encoded VirF and VirB proteins and involves an H-NS and/or environmentally induced alterations in DNA supercoiling. In fact, it has been reported that H-NS can alter DNA topology constraining negative supercoiling (Tupper *et al*., 1994). Furthermore, it is known that changes in DNA supercoiling can occur in response to the same environmental factors (e.g. temperature, osmolarity and phase of growth) which influence the expression of both *Salmonella* and *Shigella* virulence genes (Galan and Sansonetti, 1996). More specifically, since VirF can bind to *virB* in the absence of a thermal signal, it has been postulated that H-NS antagonizes this transcriptional activator only at low temperature via changes in topology (Tobe *et al*., 1993, 1995). A similar interpretation has been offered for thermoregulation of virulence mediated by histone-like proteins in *Yersinia* (Mikulskis and Cornelis, 1994). However, since the effect of *hns* mutations on the supercoiling of reporter plasmids is not straightforward, being sometimes negligible (Kawula and Orndorff, 1991; Yasuzawa *et al*., 1992) and, in other cases, of opposite sign in different types of bacteria (Dorman *et al*., 1990), alternative explanations have also been formulated. It has been proposed, for instance, that thermoregulation by H-NS could be mediated via the bacterial translational machinery which would produce higher levels of this protein at 30°C compared with 37°C. In turn, this increased concentration of H-NS could cause an increased competition with VirF for binding to *virB* promoter (Tobe *et al*., 1993) or a change in the H-NS quaternary structure favouring its specific (inhibitory) interaction with the upstream region of *virB* (Hromockyj *et al*., 1992). The recent data suggesting that H-NS may also directly control the expression of *virF* in a temperature-dependent manner opens up new perspectives for the elucidation of the mechanism responsible for thermoregulation of pathogenicity in Enterobacteriaceae.

Thus, in the present study we have undertaken the task of establishing whether H-NS indeed plays a role in the temperature-dependent control of *virF* expression and of defining the molecular basis of this thermoregulation. By *in vitro* and *in vivo* footprinting, we have demonstrated that H-NS binds, at 30°C but not at 37°C, to two sites in the upstream region of *virF*, one overlapping the –35 and –10 promoter elements. Furthermore, we have demonstrated that interaction at this site is sufficient for transcriptional repression of *virF* by H-NS but that both sites are necessary for efficient thermoregulation. Analysis at different temperatures of the intrinsic DNA curvature of fragments containing different segments of the *virF* promoter showed that this parameter may cause a structural modification of DNA within the *virF* regulatory region, which controls the accessibility of the DNA target to H-NS.

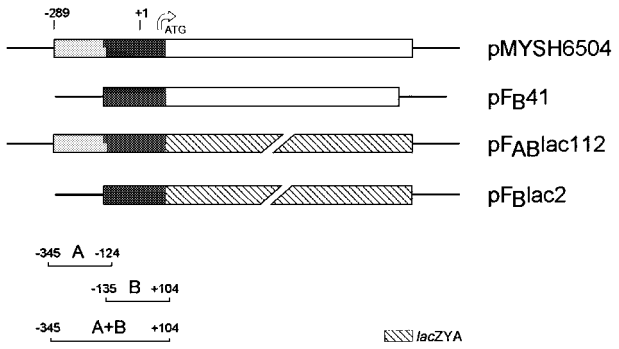

Fig. 1. Schematic representation of the constructs used in this study. Plasmid pMYSH6504 is a pBR322 derivative containing the entire *Shigella flexneri virF* gene including the promoter and an extended upstream region (from –289), while pF_B41 is derived from pMYSH6504 which contains a shorter *virF* promoter region (from –135). pF_{AB}lac112 and pF_Blac2 were obtained by fusing fragments A + B (–345 to + 104) and B (–135 to +104) of *virF*, respectively, to the promoterless *lacZ* gene in pMC1403 (see Materials and methods).

Results

Temperature- and H-NS-dependent activity of the virF promoter in vivo

As mentioned above, previous data had shown that the *in vivo* expression of *virF* present on a recombinant plasmid is influenced by temperature and pH in wild-type but not in *hns*-defective *E.coli* strains. Furthermore, purified H-NS was found to interact with the *virF* promoter, at least as judged by electrophoretic gel-shift experiments (Prosseda *et al*., 1998). The affinity of H-NS for the *virF* promoter was estimated to be similar to that displayed for *virB*, whose interaction with H-NS is well documented (Tobe *et al*., 1993) and was attributed to an intrinsic curvature predicted in this DNA. These results suggested that *virF* expression is under the control of H-NS which represses transcription by binding to its promoter at low pH and low temperature (Prosseda *et al*., 1998).

To confirm this hypothesis and, more importantly, to elucidate the molecular basis of a possible H-NS-dependent thermoregulation of *virF* expression, we sought to characterize, both *in vivo* and *in vitro*, the nature, conditions and functional consequences of the interaction between H-NS and *virF*. To this end, the constructs and the DNA fragments schematically presented in Figure 1 were prepared and transformed into *E.coli* cells (either wild-type or carrying two different *hns* alleles). As seen in Table I, the level of β-galactosidase activity expressed *in vivo* from the *virF–lac*Z fusion carried by pF_{AB}lac112 (Figure 1) was found to be 4–5 times lower at 30°C compared with 37°C in the two wild-type strains (MC4100 and HN4122), while it was essentially the same at 30 and 37°C in the two *hns* null alleles.

When pF_{AB}lac112 was replaced by pF_Blac2, which carries a shorter upstream fragment of *virF* (Figure 1), repression of *lacZ* at 30°C (compared with its expression

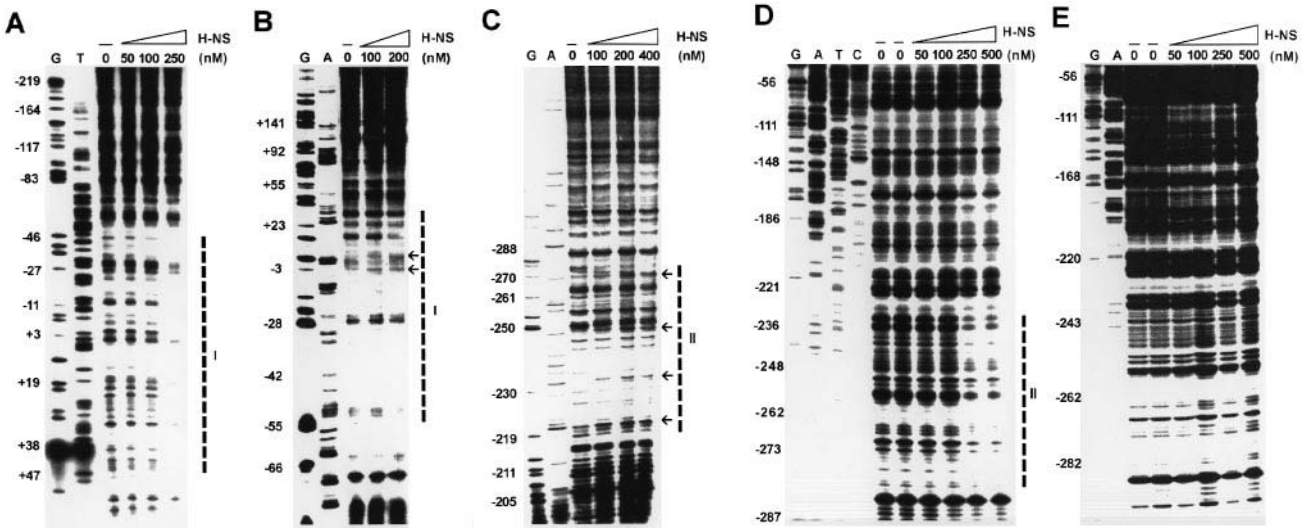

Fig. 2. DNase I fooprinting of the *virF* promoter region by H-NS. Plasmid pMYSH6504 was incubated at 28°C (**A**–**D**) or at 37°C (**E**) with or without the indicated concentrations of H-NS expressed as nM dimer; the samples were processed as described in Materials and methods using BX8 (A), F322 (B), F321(C) and FO1 (D and E) as primers. Lanes G, A, T and C represent *Taq*I polymerase sequencing reactions using the same primers. The H-NS-protected sites are indicated with vertical broken lines and labelled I and II, the sites hypersensitive to DNase I are indicated by horizontal arrows.

Table I. Temperature-dependent expression of *virF–lacZ* fusions

Strains/plasmids	β-Galactosidase activity[a]		
	Expressed at 30°C	37°C	Expression ratio (37/30°C)
MC4100 (wt)/pF_{AB}lac112	833 (±15%)	4180 (±10%)	5.02
HN4104 (*hns118*)/pF_{AB}lac112	7537 (±8%)	5781 (±9%)	0.77
HN4122 (wt)/pF_{AB}lac112	542 (±18%)	2162 (±7%)	3.99
HN4124 (*hns2*)/pF_{AB}lac112	2005 (±1%)	1970 (±1%)	0.98
HN4122 (wt)/pF_{B}lac2	1051 (±9%)	1703 (±10%)	1.62
HN4124 (*hns2*)/pF_{B}lac2	1929 (±6%)	1918 (±4%)	0.99

[a]Units of β-galactosidase are calculated according to Miller (1992). The results represent the average of at least four independent experiments, and standard deviation values expressed as a percentage are reported in parentheses. The plasmid copy number, determined as previously described (Prosseda *et al*., 1998), does not exhibit a significant difference between wild-type and *hns* mutants strains.

at 37°C) was <2-fold and, again, seen only in the wild-type *hns* background (Table I). These data confirmed and extended the previous observation that *in vivo* H-NS may control *virF* expression in a temperature-dependent manner (Prosseda *et al*., 1998).

Binding of H-NS to the virF promoter region is temperature dependent

The above result prompted us to map the binding sites of H-NS on the *virF* promoter by performing *in vitro* [DNase I and dimethyl sulfate (DMS)] and *in vivo* (DMS) footprinting experiments at different temperatures in the presence and absence of H-NS. DNase I footprinting carried out on supercoiled plasmid (pMYSH6504) DNA containing the whole *virF* gene (Figure 1) revealed the existence of two fairly extended sites (sites I and II) which were protected preferentially by H-NS when the DNA target and the purified protein were incubated at 28°C. Although the precise borders of these sites are not very sharp, it is relevant that there is a long (160 bp) non-protected fragment between site I and site II. Site I spans approximately from –46 to +46 on one filament and from –54 to +33 on the other (Figure 2A and B) and site II from –278 to –234 on one filament and –274 to –218 on the other (Figure 2C and D). Within both sites, a few DNase I-hypersensitive diester bonds (indicated by the arrows in Figure 2B and C) are visible. In contrast to the results obtained at 28°C (Figure 2A–D), very little or no protection by H-NS was observed when identical footprinting experiments were carried out at 37°C; in fact, within the limits of accuracy of this type of analysis, we cannot detect any significant difference in the DNase I digestion patterns obtained at 37°C in the absence and presence of up to 500 nM H-NS in the regions of H-NS site I (not shown) and site II (Figure 2E). These results indicate that the interaction of H-NS with these two DNA targets is strongly affected by a moderate (10°C) temperature variation.

The interaction of H-NS with the *virF* promoter region was also investigated *in vivo* analysing the DMS modification on plasmid pMYSH6504 (Figure 1) in *E.coli* YK4122 (wt) and YK4124 (*hns*2) at 37 and 28°C. As seen in Figure 3A and B, there is a substantial difference between the *in vivo* footprinting patterns obtained at 37°C and at 30°C in the strain containing wild-type H-NS; two stretches of DNA with several bases, mostly G residues whose positions are indicated as C on the complementary strand, are substantially less accessible to DMS at the lower

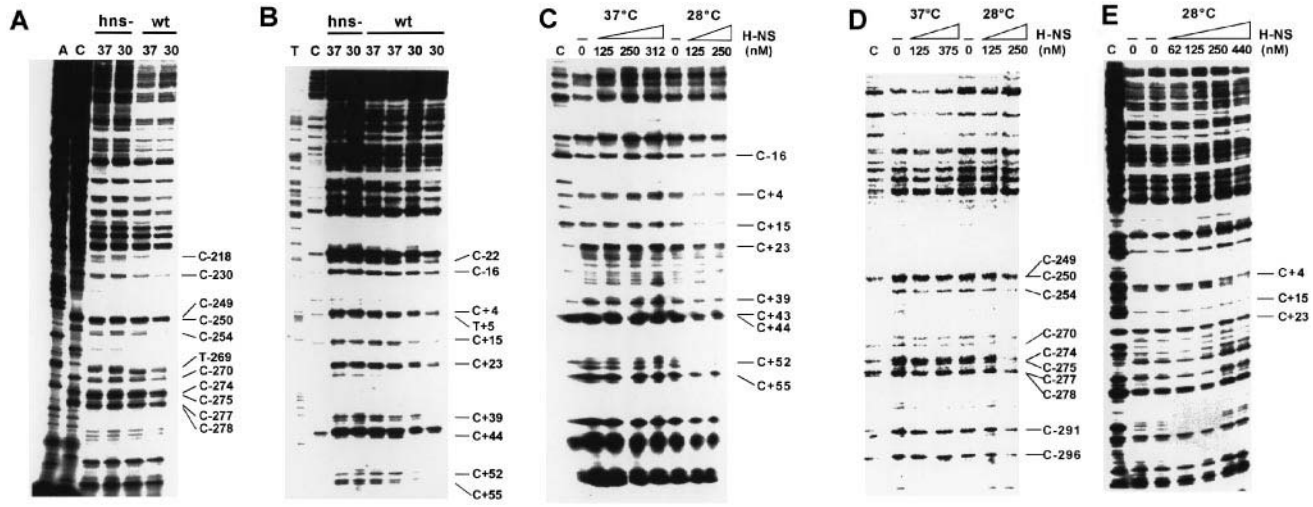

Fig. 3. *In vivo* and *in vitro* DMS probing of the *virF* promoter region. *In vivo* DMS footprinting of pMYSH6504 (**A** and **B**) was carried out as described in Materials and methods using *E.coli* YK4122 (wt) and YK4124 (*hns2*) cells growing at 30 or 37°C as indicated. *In vitro* DMS footprinting was carried out, as described in Materials and methods, at 37 and 28°C, on supercoiled plasmid DNA pMYSH6504 (**C** and **D**) and pF_B41 (**E**), in the absence or presence of the indicated concentrations of H-NS (dimer). For the primer extension by *Taq*I polymerase of the DNA partially cleaved at G and A residues, the ^{32}P-end-labelled primers used were: FO1 (A and D) and BX8 (B, C and E). Lanes A, T and C represent *Taq*I polymerase sequencing reactions using the same primers.

temperature. On the contrary, in the *hns*$^-$ background, there is no difference in the DMS reaction patterns obtained at 37 and 30°C and there is essentially no difference in the reactivity patterns of these DNA samples and the DNA sample extracted from YK4122 (wt) cells grown at 37°C (cf. the appropriate lanes of Figure 3A and B), taking into account that slightly less total DNA was loaded in the case of wild-type lanes. Since the two strains are isogenic except for the presence or absence of a functional *hns* gene and since the regions of reduced reactivity with DMS at 30°C closely correspond to the two H-NS-binding sites identified by DNase I footprinting *in vitro*, these results suggest that *in vivo* there is a temperature-dependent contact between these DNA segments and H-NS.

To confirm this premise, DMS footprinting experiments were also performed *in vitro* in the presence and absence of H-NS (Figure 3C and D). As seen in the figure, most of the bands of site I (i.e. –16, +4, +15, +23, +39, +44, +52 and +55) and of site II (i.e. –249, –250, –254, –270, –274, –275, –277 and –278) which displayed a notable intensity decrease in the DMS probing *in vivo* are also strongly protected from chemical attack *in vitro* at 28°C (cf. Figure 3A and D for site II, and Figure 3B and C for site I). *In vitro* H-NS protection of both sites was again observed only at low (28°C) but not at high (37°C) temperature. Since DMS is known to methylate guanine residues at the N-7 position in the major groove of DNA (Borowiec and Gralla, 1986), the shielding effect of H-NS is compatible with the premise that this protein interacts with the major groove of DNA, as suggested by Tippner *et al*. (1994).

Taken together, these experiments demonstrate that the temperature-dependent protection of sites I and II of *virF* detected *in vivo* in a wild-type *hns* background is due to the interaction of these sites with H-NS without the involvement of any other cellular protein. The localization of H-NS-binding sites, identified by the different footprinting techniques used in this study, is summarized in

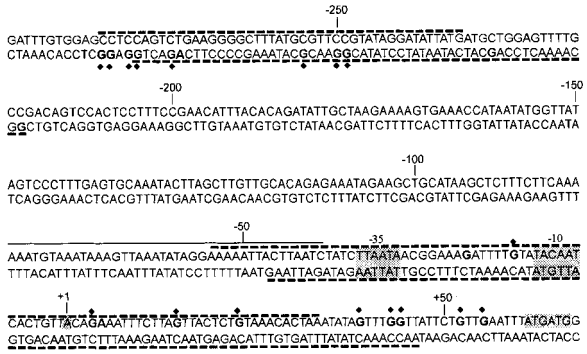

Fig. 4. H-NS-binding sites within the *virF* promoter region. The H-NS-binding sites within the nucleotide sequence (–289 to +67) of the *virF* promoter region are indicated by broken lines (protection from DNase I), by bold letters (protection from DMS *in vivo*) and by diamonds (protection from DMS *in vitro*). The shaded sequences indicate (from bottom right): the translation initiation triplet, the transcriptional start site and the –10 and –35 promoter consensus sequences. The continuous line indicates the predicted DNA curvature.

Figures 4 and 5. As seen in Figure 4, there is excellent correspondence between the sites protected by H-NS from DNase I digestion on both filaments and DMS reaction *in vitro* at 28°C and, in turn, between these sites and those protected *in vivo* by H-NS at the same temperature.

The dimensions of the two sites occupied by H-NS at low temperature and the finding that H-NS has similar affinities for them (as seen in Figure 2A–D, they are protected similarly by comparable amounts of protein) suggest that both sites might be occupied simultaneously and somewhat cooperatively by several H-NS molecules. Moreover, the two sites are not entirely independent of each other, since DMS footprinting carried out on plasmid pF_B41, which unlike pMYSH6504 lacks the promoter-distal H-NS site (Figure 1), revealed only a very weak protection in the region of H-NS-binding site I (cf. Figure 3C and E). These results suggest the existence of long-range protein–protein interactions among H-NS

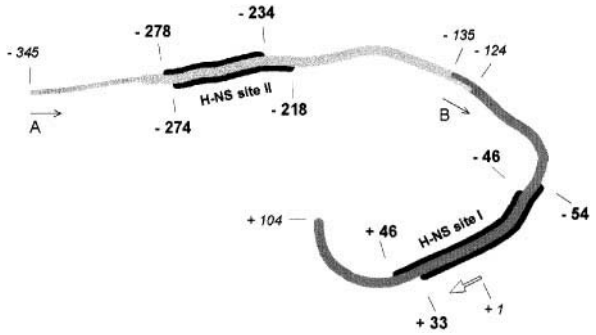

Fig. 5. Computer-generated prediction of intrinsic curvature in the *virF* promoter region. The fragments A (light grey) and B (dark grey) are described in the legend to Figure 1. The transcription start site is indicated by the white arrow. The H-NS-binding sites I and II are positioned according to the footprinting experiments summarized in Figure 4.

molecules occupying sites I and II of *virF*. These interactions could be favoured by the intrinsic DNA curvature predicted between the two H-NS-binding sites (Figure 5) which may contribute to bringing into close proximity these sites which are ~160 bp apart (Wang and Giaever, 1988; Travers, 1989, 1995). Finally, for the interpretation of functional data (see below), it seems relevant to notice that the H-NS promoter-proximal site (site I) includes both –35 and –10 elements of the *virF* promoter. This H-NS-binding model is reminiscent of that proposed for the H-NS–*hns* interaction (Falconi *et al*., 1993) and consistent with the known preference of H-NS for binding curved DNA (Yamada *et al*., 1990, 1991; Owen-Hughes *et al*., 1992; Zuber *et al*., 1994) as well as with the essential role attributed to protein–protein interactions in the correct functioning of this DNA-binding protein (Spurio *et al*., 1997).

In vitro transcription of virF is influenced by H-NS and temperature

If H-NS alone is directly responsible for the thermoregulation of *virF* expression *in vivo*, as suggested by the results presented in Table I and by its temperature-dependent interaction with the *virF* promoter, it might be possible to reproduce this effect in a purified *in vitro* system. That this is indeed the case is shown by the results of experiments in which the *virF* promoter activity was examined in the presence and absence of H-NS as a function of temperature. Thus, the addition of purified H-NS was found to inhibit severely (~80% up to 15 min incubation) transcription of *virF* at 30°C when supercoiled plasmid pMYSH6504 (Figure 1) was used as template (Figure 6A), while transcription at 37°C was only slightly affected by H-NS (Figure 6B). When the transcriptional activity of *virF* in the presence or absence of H-NS was measured as a function of temperature between 26 and 40°C, it was found that the extent of transcriptional inhibition caused by a given amount of H-NS was not constant and did not vary linearly as a function of the incubation temperature (Figure 6C). Instead, below 30°C, H-NS caused a 5-fold inhibition of *virF* transcription but had negligible effects on samples incubated above the critical temperature of 32°C (Figure 6C). The extent of the H-NS repression seen in this experiment at 30°C

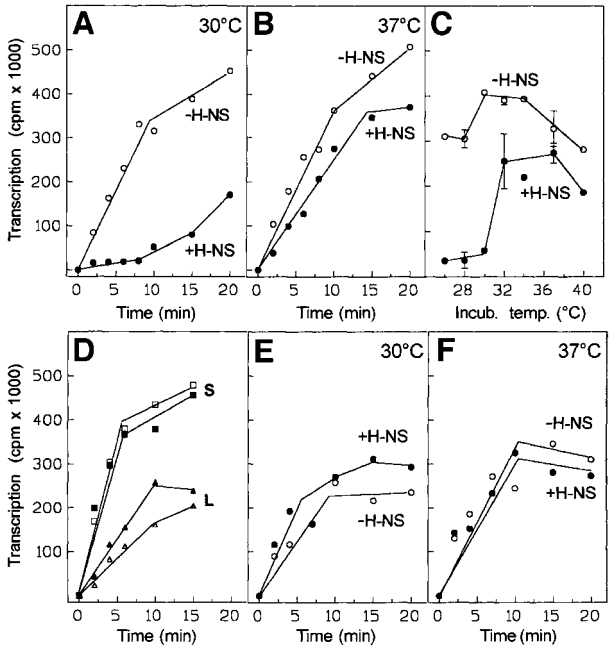

Fig. 6. Effect of H-NS and temperature on the *virF* promoter activity *in vitro*. The figure presents the time course of *in vitro* transcription at 30°C (**A** and **E**) and 37°C (**B** and **F**) in the presence (●) or in the absence (○) of H-NS (150 nM of dimer). Transcription reactions were performed as described (Falconi *et al*.,1993) using supercoiled (A–C) and *Eco*RI-linearized (E and F) pMYSH6504 as DNA template. (**C**) The temperature dependence of the H-NS transcriptional repression of *virF* determined by monitoring transcription after 10 min of incubation with RNA polymerase at the indicated temperatures in the presence or absence of H-NS. (**D**) The levels of *virF* transcription at 30°C (□,△) and 37°C (■,▲) with supercoiled (S) and and *Eco*RI-linearized (L) pMYSH6504 as DNA template. The *virF* transcripts were detected by Northern analysis using the ^{32}P-labelled 750 bp DNA fragment corresponding to the entire *virF* gene as probe; the signal was quantified by Molecular Imager (Bio-Rad Mod.GS-250).

closely corresponds to that found *in vivo* at the same temperature (Table I).

The relevance of DNA supercoiling for the inhibition of *virF* promoter function by H-NS was examined in the following experiment in which the plasmid template pMYSH6504 was linearized by *Eco*RI digestion prior to the transcription test. In a preliminary experiment, the activity of the *virF* promoter on a supercoiled and linearized (L) template was analysed at 30 and 37°C (Figure 6D). As seen from this panel, the supercoiled (S) is transcribed better than the linearized (L) template, and temperature has little and no influence on the activity of the linearized and supercoiled promoter, respectively. Unlike that seen with the supercoiled DNA template, H-NS did not inhibit transcription at either 30 or 37°C (Figure 6E and F), indicating that inhibition of *virF* transcription occurs only on supercoiled DNA at 30°C. The specific requirement for template supercoiling for H-NS inhibition is clearly indicated by the fact that the level of transcription of the supercoiled template at 30°C in the presence of H-NS is way below the level of transcription obtained at either 30 or 37°C on the linearized template in the presence of the same amount of H-NS (cf. Figure 6A, E and F).

As seen above, the upstream region of *virF* contains two H-NS-binding sites designated I and II (Figure 5). Since site I overlaps the conserved elements of the *virF* promoter, it is reasonable to assume that this site is

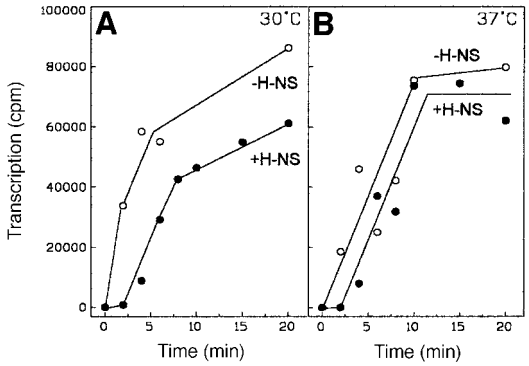

Fig. 7. Effect of H-NS on *in vitro* transcription from a *virF* promoter fragment lacking the upstream H-NS site II. Time course of *in vitro* transcription of supercoiled pFB41 DNA (Figure 1) at 30°C (**A**) and 37°C (**B**) in the presence (●) or absence (○) of 150 nM H-NS (dimer). The other experimental conditions are as described in the legend to Figure 6.

involved in transcriptional repression by H-NS whose interaction with DNA might occlude the access of RNA polymerase to the –35 and –10 consensus sequences.

To determine whether the more upstream H-NS site II is also required for repression, a shorter construct (pF_B41), which lacks ~150 bp of the distal portion of the *virF* promoter region (Figure 1), was used as a transcriptional template. As seen from Figure 7, while essentially no inhibition of transcription by H-NS was seen at 37°C (Figure 7B), at 30°C this protein produced a significant decrease (85%) of *virF* transcription but only for very short incubation times (<4 min) while the inhibition was relieved with longer incubation, dropping to 50 and 25% after 6 and 15 min, respectively (Figure 7A). These data are in full agreement with the observation that *in vivo* there is only a minor difference in the transcriptional activity of *virF* between wild-type and *hns2* strains transformed with the same pF_Blac2 construct (Table I).

Taken together, these transcription results and the aforementioned very weak H-NS footprints on site I in the absence of site II indicate that without the latter site, H-NS forms an unstable nucleoprotein complex which cannot withstand a prolonged competition with the RNA polymerase.

Influence of temperature on DNA topology of the virF promoter

Since superhelicity of pMYSH6504 was found to be essential for repression of the *virF* promoter activity by H-NS (Figure 6) and since the temperature may influence the supercoiling level of plasmids (Goldstein and Drlica, 1984; Lopez-Garcia and Forterre, 1997), the distribution of pMYSH6504 topoisomers extracted from wild-type (MC4100) and from two *hns* mutant strains (HN4104 and YK4124) grown in LB medium at 30 and 37°C was analysed. No detectable differences in the level of superhelicity were detected when the topoisomers of the plasmid isolated from wild-type or H-NS-deficient cells were resolved by chloroquine agarose gel electrophoresis (Figure 8), a result similar to that previously obtained by Yasuzawa *et al*. (1992) with different reporter plasmids. As to the effect of temperature, our results indicate that going from 30 to 37°C, there is a slight increase in the level of supercoiling. Since this phenomenon occurs in

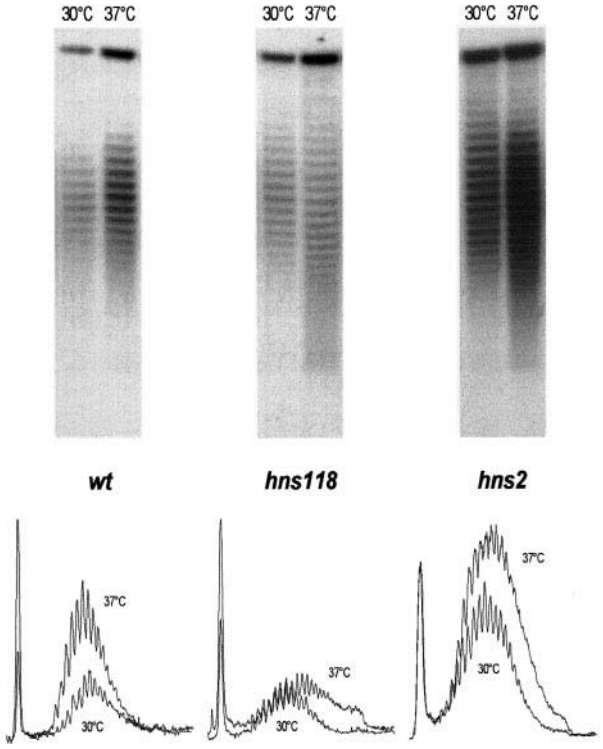

Fig. 8. Analysis of the distribution of pMYSH6504 topoisomers. The experiment was carried out as described in Materials and methods using the indicated *E.coli* strains grown at either 30 or 37°C as indicated. The upper part of the figure presents the electrophoretically resolved (migration is from top to bottom) radioactive bands visualized by the Molecular Imager (Bio-Rad Mod.GS-250), while the lower part presents the densitometric scanning of the same bands. Under the conditions of the experiment, the less negatively supercoiled topoisomers migrate faster.

all the genetic backgrounds assayed, and since its extent is very low, we can conclude that the substantial temperature-dependent change in the level of *virF* expression in the presence of H-NS cannot be attributed to a variation of DNA supercoiling caused by this protein. Furthermore, when *in vitro* DMS footprinting by H-NS was compared using supercoiled and *Eco*RI-linearized pMYSH6504, identical protections were found at 28°C (like that seen in Figure 3C and D) while no protection in either case was found at 37°C (not shown). These results indicate that DNA supercoiling does not modulate the interaction of H-NS with its target but is necessary for successful competition of H-NS with the RNA polymerase.

H-NS binds DNA with low sequence specificity (Rimsky and Spassky, 1990; Lucht *et al*., 1994), but with a definite preference for curved DNA (Yamada *et al*., 1990, 1991). Computer predictions, based on published estimates of dinucleotide wedge angles (Bolshoy *et al*., 1991), suggest the presence of an intrinsic DNA curvature located approximately between –80 and –40 in the *virF* promoter region (Figure 5). Since intrinsic curvature is another structural property of DNA known to be influenced by temperature (Diekmann and Wang, 1985), it could be suspected that changes of curvature within the promoter region might account for the temperature- and H-NS-dependent repression of *virF* expression.

To test this hypothesis, the electrophoretic mobility of three different DNA fragments derived from the *virF*

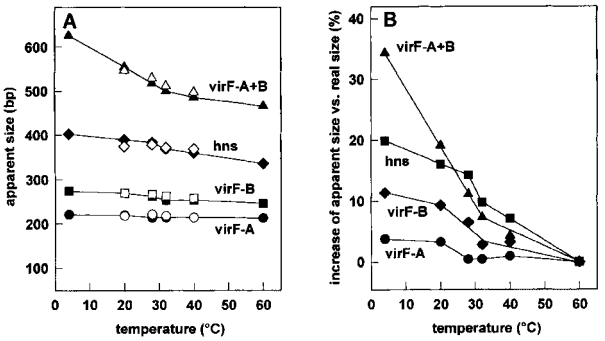

Fig. 9. Temperature dependence of the electrophoretic mobility of DNA fragments derived from the *virF* and *hns* promoter regions. The three DNA fragments derived from *virF*, i.e. virF-A (●), virF-B (■) and virF-A + B (▲), and that derived from *hns* (◆) have been described in Figure 1 and in Materials and methods. These fragments were subjected to electrophoresis on 5% polyacrylamide gels run at different temperatures (4, 20, 28, 32, 40 and 60°C) which were found to remain constant within ± 0.25°C. The apparent sizes were estimated from the electrophoretic mobilities of the fragments in the presence of 10 mM Mg^{2+} (TBM, unfilled symbols) and in the absence of Mg^{2+} (TBE, filled symbols) by comparison with those of non-bent marker fragments electrophoresed in the same experimental conditions. The values plotted in (**A**) represent the average of three independent experiments; within each data set, the difference between minimum and maximum observed values was found to be not higher than 2.01%. The percentage deviation of the apparent size of each fragment compared with its actual size as a function of temperature, relative to TBE gels, is plotted in (**B**).

promoter region was studied as a function of temperature between 4 and 60°C. The DNA fragments examined in this experiment are designated in Figures 1 and 5 as: A (222 bp), corresponding to the most upstream portion (–345/–124) of *virF*, which contains the H-NS-binding site II; B (239 bp), corresponding to the proximal portion of *virF* (–135/+104), which contains the predicted curvature, the H-NS-binding site I and the conserved elements of the promoter; and A + B (449 bp), corresponding to the entire promoter region (–345/+104). A 317 bp fragment derived from the *hns* promoter region, known to be curved (Falconi *et al*., 1993), was also tested. These fragments, obtained by Pwo PCR amplification, were subjected to polyacrylamide gel electrophoresis at different temperatures which were monitored during the run by a temperature probe polymerized within the gel. The electrophoresis carried out at 60°C yielded mobility values closely corresponding to the known sizes of the fragments, while the mobilties determined at the lowest temperature (4°C) yielded mobility values corresponding to apparent sizes deviating, to different extents, from the actual sizes of the fragments; the maximum and minimum deviations were observed for the A + B (>35%) and for the A (<3%) fragment, respectively (Figure 9A and B). With increasing temperature from 4 to 60°C, each fragment exhibited a different rate of change in its apparent size towards the real value. The A + B fragment of *virF* displays a steep decrease in apparent size between 4 and 32°C, followed by a shallower decline between 32 and 60°C, while the other three fragments display the steepest reduction of their apparent size in a single step between 20 and 28°C.

The abrupt change of the electrophoretic mobility displayed at 32°C by the fragment which corresponds to the entire *virF* promoter region suggests that this DNA segment undergoes a specific and temperature-dependent structural transition which is complete just above 32°C. This modification is probably responsible for modulating the access of H-NS to the *virF* promoter and the consequent capacity of this protein to repress its activity. This premise is entirely consistent with the evidence presented above that this DNA fragment is endowed with unique, temperature-dependent structural and functional characteristics. In fact, it has been shown that: (i) H-NS can stably bind to sites I and II of the *virF* promoter and inhibit transcription *in vivo* and *in vitro* only below 32°C (Figure 6A–C); (ii) essentially no protection by H-NS of either site II or site I was obtained at any temperature when *virF* promoter fragments A and B were used in footprinting experiments (Figure 3E and not shown); and (iii) when the *virF* promoter was deprived of its most upstream region corresponding to fragment A, transcriptional inhibition by H-NS, though still somewhat temperature dependent, became very inefficient, both *in vitro* (Figure 7A and B) and *in vivo* (Table I). The functional relevance of the temperature-dependent conformational transition responsible for the abrupt change in electrophoretic mobility of the A + B fragment seen in Figure 9 is supported further by the finding that a similar transition could also occur under conditions similar to those in which temperature-dependent repression of the *virF* promoter is observed. In fact, as seen in Figure 9A (open symbols), Mg^{2+}, at the same concentration (10 mM) present in the transcription reactions (Figures 6 and 7), did not significantly influence the electrophoretic mobility at different temperatures of any of the DNA fragments examined.

Discussion

The ability of bacteria to infect hosts depends on prompt adaptation to changing environmental conditions such as temperature, pH, osmolarity, and availability of oxygen and nutrients (Dorman and Ni Bhrian, 1993). Much of the adaptation is known to rely on changes in the transcriptional activity of the bacterial genome, but how these environmental parameters can trigger modulations in gene expression remains a central, yet still unanswered question. The notion that H-NS, together with temperature, pH and osmolarity, plays a crucial role in the regulation of several virulence genes (e.g. *cfa/I*, *sfa*, *pap*, *virB* and *virG*) is supported by the evidence that there is a higher and temperature-independent expression of these operons in strains carrying *hns*-defective alleles (e.g. *drdX* and *virR*) (Maurelli and Sansonetti, 1988; Goransson *et al*., 1990; Dagberg and Uhlin, 1992; Jordi *et al*., 1992; Morschhauser *et al*., 1993). In these processes, the expression of the target operons can be prevented by H-NS either directly or through the repression of their transcriptional activators (Goransson *et al*., 1990; Tobe *et al*., 1991; Jordi *et al*., 1992; Lambert *et al*., 1992; Morschhauser *et al*., 1993); how a DNA-binding protein which plays a role in the structural organization of the nucleoid and controls a fairly large number of genes in a temperature-independent manner can also sense changes in environmental temperature and translate them into the regulation of virulence genes remains a fundamental yet still intriguing issue.

The primary event following the upshift of *Shigella* to the host temperature (37°C) is the synthesis of VirF, which in turn triggers a regulatory cascade involving the

activation of *virB* and *virG* (Adler *et al*., 1989; Tobe *et al*., 1991). Unlike the cases of *virB* and *virG*, for which the thermoregulation by H-NS is well documented, only recently has it been suggested that *virF* also might be thermoregulated by H-NS and that its activation requires only the temperature stimulus (Colonna *et al*., 1995; Porter and Dorman, 1997; Prosseda *et al*., 1998).

In this study, after confirming that below 32°C and throughout the cellular growth cycle, the activity of *Shigella virF* promoter is indeed repressed by H-NS, we reproduced a similar temperature-dependent regulation in *in vitro* transcriptional tests. With these premises, we considered the activation of *virF* an ideal model to study the molecular mechanism of H-NS-dependent thermoregulation of virulence expression. Indeed, this is the first report showing that in a thermoregulated virulence system, H-NS is able to bind to and repress the promoter of a primary regulator as a function of temperature. In fact, through the reductionist approach used in this study, we have been able to show that the low temperature and the presence of H-NS are necessary and sufficient conditions for repression and that transition from repression to derepression occurs at ~32°C, within a very narrow temperature range. This finding, the excellent quantitative correspondence between the H-NS transcriptional repression observed *in vivo* and *in vitro* and the clear-cut evidence that *in vivo* and *in vitro* DMS footprinting of the *virF* promoter occurs exclusively at low temperature in the presence of H-NS indicate that thermoregulation of *virF* expression is solely under H-NS control and does not depend on other factors, such as σ^S, which is required for the expression of other virulence genes (Loewen and Hengge-Aronis, 1994) and is expressed preferentially at low temperature (Sledjeski *et al*., 1996). As mentioned in the Introduction, it has been suggested that variations of DNA supercoiling may represent the key to the control of bacterial virulence. Our results have indeed shown that a supercoiled DNA template is a necessary condition for H-NS repression. We have shown, however, that within the critical temperature range there is no H-NS-dependent change in DNA topology *in vivo* of the very same plasmid carrying *virF*. Furthermore, it was found that DNA supercoiling does not influence the interaction of H-NS with the *virF* promoter region since identical H-NS footprints were obtained on supercoiled and relaxed DNA. Thus, our results (Figure 6D) suggest that RNA polymerase interacts with the promoter in different ways depending on whether the template is supercoiled or relaxed, to give complexes which may or may not be inhibited successfully by H-NS. Another, not mutually exclusive, explanation for the requirement for a supercoiled template to elicit H-NS and temperature-dependent repression is that the temperature-dependent conformational transition of DNA which we suggest to control H-NS repression (see below) could be facilitated by supercoiling.

Since the quaternary structure of H-NS was found to be essential for its biological activity (Spurio *et al*., 1997), we considered the possibility, suggested by Hromockyj *et al*. (1992), that temperature-dependent changes of H-NS quaternary structure might result in temperature-dependent gene repression. Our results have shown, however, that the aggregation state of H-NS is not influenced, either *in vivo* or *in vitro*, by temperature changes within the range (30–34°C) critical for *virF* derepression (R.Spurio, M.Falconi, C.O.Gualerzi and C.L.Pon, unpublished observation). On the other hand, the present data indicate that the temperature sensor within the *virF* system is the DNA structure which, within a very narrow temperature range, allows a more or less efficient interaction with the H-NS repressor. In fact, the DNase I and DMS footprinting data have shown that H-NS binds, only below 32°C, to two sites separated by a fairly long (160 bp) stretch of DNA in which our computer simulation predicts an intrinsic curvature. Our data have allowed us to correlate the simultaneous occupation of these two sites with the temperature-dependent *virF* repression by H-NS. Indeed, the binding of H-NS to its promoter-proximal site (site I) is very unstable in the absence of the upstream site (site II) so as to give only a very weak protection from DNase I and DMS and to cause only a transient and very weak inhibition of transcription both *in vitro* and *in vivo*.

Unlike the case of the protein repressor, the electrophoretic analysis carried out at different temperatures has shown that the structure of the *virF* promoter region is highly sensitive to temperature changes within the relevant temperature range. In fact, four different DNA fragments, each containing one or two H-NS-binding sites, were found to display, at low temperature (4°C), abnormally low mobilities which increased towards the theoretically expected values with increasing temperature. This change in the electrophoretic mobility occurs in different ways for the fragment (A + B) containing both H-NS site I and II of *virF*, for the fragment containing only the binding sites I (fragment B) or II (fragment A) of *virF* and for the fragment containing the two H-NS-binding sites present in the upstream region of the *hns* promoter. In fact, the mobility increase is gradual, with a sharper step at ~30°C for the latter three fragments, while for the first fragment it is fairly sharp between 4 and 32°C and more gradual between 32 and 60°C. The different behaviour indicates that this fragment is endowed with a unique conformation compared with the other three, and its biphasic mobility change suggests that it exists in two different conformations, one, amenable to H-NS repression below 32°C and the other, insensitive to H-NS above this temperature. The nature of the conformational transition occurring in this fragment around 32°C is not known at present. The correlation between the *in vivo* and *in vitro* transcriptional activity of the *virF* promoter, the demonstrated requirement for binding of H-NS to both sites for temperature-dependent repression and the results of the footprinting experiments carried out below and above 32°C suggest, however, that a temperature-sensitive 'hinge' is present on the DNA spacer between H-NS site I and site II. Rotation of the helical axis about this hinge would place in or out of phase the H-NS molecules weakly bound to the two distant sites. The low-temperature-repressible DNA conformation would be that allowing a cooperative stabilizing interaction between the H-NS molecules bound to two sites. The DNA conformational transition monitored in Figure 9 is observed on a linearized DNA fragment on which H-NS is unable to repress transcription (Figure 6E and F); however, it is possible that negative supercoiling might be necessary to stabilize or favour this transition.

Materials and methods

Bacterial strains and general procedures

Escherichia coli K12 strains used in this study were MC4100 [F– *araD*139 Δ (*argF-lac*) $_{U169}$ rps*L150 relA flbB5301 deoC*1 *pstF*25 *rbsR*] (Casadaban *et al*., 1980), HN4104 (MC4100 *hns118*) (Colonna *et al*., 1995), HN4122 [F– *ara* Δ(*lac pro*) *mal thi*], YK4122 (W3110 *trp*$^+$) and YK4124 (YK4122 *hns2*) (Yasuzawa *et al*., 1992). HN4124 (HN4122 *hns2*) was constructed by transduction with P1*vir* grown on YK4124. The presence of *hns*-defective alleles was monitored by the appearance of red colonies on MacConkey base agar containing 0.2% salicine. When appropriate, the following antibiotics/chemicals were added to the media at the following concentrations: ampicillin 50 μg/ml; kanamycin, 30 μg/ml; tetracycline, 5 μg/ml; hygromycin 75 μg/ml; X-gal 20 μg/ml. Isolation of plasmids, restriction digestions, cloning, electrophoresis and purification of DNA fragments, Southern and Northern blotting were carried out according to Sambrook *et al*. (1989). DNA probes were labelled with [α-^{32}P]dATP by the random priming method (Amersham Kit). Purification of H-NS was performed as described (Falconi *et al*., 1988). β-galactosidase assays were performed as described by Miller (1992) on SDS–chloroform-permeabilized cells grown in LB broth (pH 7) supplemented with ampicillin. PCR was performed as previously described (Colonna *et al*., 1995). The sequence of the PCR-generated fragments was checked by the dideoxy chain-terminating method (Sambrook *et al*., 1989).

Plasmid constructions

pMYSH6504 is a pBR322-derived vector containing the *virF* gene of *S.flexneri* 2a virulence plasmid pMYSH6000 (Sakai *et al*., 1986). Plasmids containing the *virF–lacZ* fusions were constructed by cloning PCR-generated fragments of the *virF* promoter region carried by the pMYSH6504 into the multicloning site of the '*lacZYA* translational fusion vector pMC1403 (Casadaban *et al*., 1980) (Figure 1). The forward primers used for amplification were designed with an *Eco*RI site at the 5′ end and correspond, for pF$_{AB}$lac112, to a sequence (–356 to –339) internal to the vector (QH8 5′-CTA CGA ATT CCG CTT CCT TTA GCA GC-3′) and, for pF$_B$lac2, to nucleotides –143 to-121 (BX71 5′-TTG AAT TCA AAT ACT TAG CTT G-3′); the reverse primer designed with a *Bam*HI site corresponds to nucleotides +90 to +115 (QH7 5′-TAG GGA TCC AAG CGA ACC TTT ATA TC-3′) in both constructs. The two fragments (QH8–QH7 = 458 bp and BX71–QH7 = 246 bp) containing the *virF* transcriptional and translational signals and the first 16 codons of *virF* were fused to the *lac*Z gene of pMC1403 digested with *Eco*RI and *Bam*HI enzymes. To construct pF$_B$41, a 997 bp fragment containing the *virF* gene was obtained by amplification of pMYSH6504 (Sakai *et al*., 1986) using BX71 and CF4 (5′-CGGGATCCAAAT-TTTTTATGATA-3′ corresponding to +831 to +852) as primers and cloned into the *Eco*RI–*Bam*HI sites of pBR322.

DNase I footprinting

Supercoiled plasmid DNA (200 ng per sample) was pre-incubated for 20 min at the indicated temperature with increasing amounts of purified H-NS in 30 μl of BB buffer containing 40 mM HEPES-HCl pH 8, 100 mM KCl, 10 mM Mg acetate and 0.5 mM dithiothreitol (DTT). After addition of 30 ng of DNase I, incubation was continued for 40 s, the reactions were stopped by placing the samples on ice and by addition of 1.5 μl of 0.5 M EDTA, 10 μl of Na acetate 3 M. Each DNA sample (100 μl), precipitated with 2.5 vols of ethanol in the presence of 1 μg of tRNA carrier, was resuspended in 10 μl of Polymed PCR buffer supplemented with 3 mM $MgCl_2$, 100 μM of each NTP, 4 pmol of 5′-^{32}P-end-labelled primer and 0.5 U of *Taq*I polymerase (Polymed) and subjected to 25 cycles of linear PCR (denaturation 1 min at 95°C, primer annealing 1 min at 46°C, except for F322 whose T_m was 48°C, and primer extension 1 min at 72°C). The extension products were separated on a 7% sequencing gel. The following pairs of convergent oligonucleotides BX8 (5′-GCG AAC CTT TAT ATC T-3′, coordinates +104 to +89) and F322 (5′-AGA AGC TGC ATA AGC TC-3′, coordinates –106 to –90), and FO1 (5′-CGC TTC CTT TAG CAG C-3′, coordinates –345 to – 330) and F321 (5′-ACT TTT CTT AGC AAT ATC TG-3′, coordinates –169 to –188) were used to detect H-NS protections, on both DNA strands, at sites I and II, respectively.

In vivo DMS footprinting

The *E.coli* strains YK4122 (wt) and YK4124 (*hns2*), harbouring pMYSH6504, were grown to A_{620} = 0.5 at 30 and 37°C and then incubated for 3 min with 10 mM DMS followed by 'quenching' of DMS by addition of 2 vols of cold phosphate-buffered saline (PBS) buffer (Sambrook *et al*., 1989) containing 3 mM β-mercaptoethanol. The chilled cells were immediately collected, washed once with 2 ml of 0.9% NaCl solution, and plasmid DNA was extracted according to the boiling method (Sambrook *et al*., 1989). Aliquots of DNA, partially cleaved at G and A residues, were subjected to primer extension by *Taq*I polymerase and a ^{32}P-end-labelled oligonucleotide essentially as described above for DNase I footprinting.

In vitro DMS footprinting

Supercoiled (or *Eco*RI-linearized) plasmid DNA (200 ng per sample) was pre-incubated at 28 or 37°C for 15 min with increasing amounts of purified H-NS in 50 μl of BB buffer (see above). After addition of 1 μl of DMS to a final concentration of 200 mM, the incubation was continued for 1 min before adding 200 μl of an ice-cold solution containing 500 mM Na acetate (pH 7), 250 mM β-mercaptoethanol, 5 mM EDTA and 25 μg/ml tRNA. Plasmid DNA, precipitated twice with 3 vols of ethanol, subsequently was processed as described above for DNase I footprinting.

Electrophoretic mobility assay of DNA fragments at different temperatures

The *virF* fragments used for these assays were obtained by PCR amplification of pMYSH6504 using as forward primer FO1 (see above) or BX7 (5′-CAA ATA CTT AGC TTG T-3′, coordinates –135 to –120) and as reverse primers BX8 (see above) or PR8 (5′-GCA CTC AAA GGG ACT A-3′, coordinates –135 to –124). The fragment encompassing the intrinsic curvature of the *hns* promoter region was obtained by PCR amplification of *E.coli* chromosomal DNA using QC2 (5′-GAA GAC TGA AAG GTC G-3′) and HC9 (5′-CGC ACG AAG AGT ACG G-3′) corresponding respectively to positions 593–608 and 894–909 of the *hns* sequence (Pon *et al*., 1988). The electrophoretic mobility of these DNA fragments (FO1-BX8, 449 bp; BX7-BX8, 239 bp; FO1-PR8, 222 bp; and QC2-HC9, 317 bp) and that of non-bent marker fragments (Pharmacia's 100 bp ladder) was analysed by electrophoresis on 0.75 mm thick 5% polyacrylamide gels [29.2:0.8 acryl:bis in TBE (Tris–HCl 90 mM, H_3BO_3 90 mM, 2.5 mM Na_2EDTA, pH 8.6) or in TBM (Tris–HCl 90 mM, H_3BO_3 90 mM, 10 mM $MgCl_2$, pH 8.6)] run at different temperatures (4, 20, 28, 32, 40 and 60°C for TBE gels; 20, 28, 32 and 40°C for TBM gels) at 5 V/cm, with (TBM gels) or without (TBE gels) buffer recirculation.

Curvature predictions

Computer-generated predictions of intrinsic curvature were obtained with WEDGE420, a program developed by one of the authors (G.M.) for the analysis of structural parameters of DNA molecules on ×86 PC platforms running DOS (version 3.2 and up) or Windows (version 3.1 and up) operating systems. Input DNA sequences can be up to 20 kb in size. The bending profile, based on the assumptions of Trifonov's 'wedge' model for bent DNA (Bolshoy *et al*., 1991) and generated using published (and user-editable) estimates of dinucleotide wedge angles, is not meant to be a projection of the double helix path on a plane, but rather a representation of the DNA axis with its curves laid on a single plane. Further details and a copy of the executable code and of the accessory files are freely available from the author (MICHELI@axrma.uniroma1.it).

Analysis of DNA supercoiling

The *E.coli* MC4100, HN4104 and YK4124 strains harbouring pMYSH6504 were grown to A_{600} = 0.5 in LB medium at 30 and 37°C. The plasmid was extracted by alkaline lysis (Birnboim and Doly, 1979) with the following modification: to the DNA pellet, dissolved in TE, 0.5 vol. of NH_4 acetate (7.5 M) was added and, after centrifugation, the DNA present in the supernatant was ethanol precipitated. Topoisomers of pMYSH6504 were resolved on 1% agarose gels in the presence 30 μg/ml chloroquine run for 30 h at 2 V/cm at room temperature in TAE buffer (Tris–HCl 40 mM, Na acetate 25 mM, Na_2EDTA 1 mM, pH 8.3). After electrophoresis, the chloroquine was removed by soaking the gel for several hours in distilled water. Plasmid DNA was transferred onto a nitrocellulose filter by Southern blotting and hybridized with a probe consisting of ^{32}P-labelled, *Eco*RI-linearized pMYSH6504. The radioactive bands were detected and quantified by Molecular Imager (Bio-Rad).

Acknowledgements

We thank Drs C.Sasakawa for plasmid pMYSH6504, M.J.Casadaban for plasmid pMC1403, Y.Kano for strains YK4122 and YK4124, and

P.Lejeune for strain TP504. We are also grateful to Professor Cynthia L.Pon and Dr Roberto Spurio for invaluable help and for critical discussion. This work was supported by grants from the Italian Consiglio Nazionale delle Ricerche, MURST (PRIN 'Protein–nucleic acid interactions'), Università Roma La Sapienza (GPA 1996–1998) and in part from the 'Istituto Pasteur–Fondazione Cenci Bolognetti' Foundation.

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Received July 2, 1998; revised October 14, 1998; accepted October 15, 1998