Genome wide, supercoiling-dependent \textit{in vivo} binding of a viral protein involved in DNA replication and transcriptional control

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Received February 2, 2004; Revised March 26, 2004; Accepted April 4, 2004

ABSTRACT

Protein p6 of \textit{Bacillus subtilis} bacteriophage F29 is essential for phage development. \textit{In vitro} it activates the initiation of DNA replication and is involved in the early to late transcriptional switch. These activities require the formation of a nucleoprotein complex in which the DNA forms a right-handed superhelix wrapping around a multimeric protein core. However, there was no evidence of p6 binding to F29 DNA \textit{in vivo}. By crosslinking, chromatin immunoprecipitation and real-time PCR we show that protein p6 binds to most, if not all, the viral genome \textit{in vivo}, although with higher affinity for both DNA ends, which contain the replication origins. In contrast, the affinity for plasmid DNA is negligible, but greatly increases when the negative supercoiling decreases, as shown \textit{in vivo} by treatment of cells with novobiocin and \textit{in vitro} by fluorescence quenching with plasmids with different topology. In conclusion, binding of protein p6 all along the F29 genome strongly suggests that its functions in replication and transcription control could be local outcomes of a more global role as a histone-like protein. The p6 binding dependence on DNA topology could explain its preferential binding to viral with respect to bacterial DNA, whose level of negative supercoiling is presumably higher than that of F29 DNA.

INTRODUCTION

In living cells, genomes are packed and organized by architectural proteins associated with DNA. Randomly folded naked DNA would occupy a much larger volume than that of the nuclear compartment or the bacterial cell. Besides, such DNA would be unable to correctly perform essential functions such as replication, transcription, recombination, repair, segregation, mitosis or meiosis. These processes require the interaction of proteins that modify the conformation of DNA by inducing compaction, bending and/or supercoiling. In Eukarya the main architectural proteins are histones, which compact and scaffold chromosomes into a chromatin fiber formed by nucleosomes. Histones drive structural changes in chromatin to regulate the accessibility and transcriptional state of genes (for a review see 1). Other non-histone, sequence-unspecific DNA-binding proteins, such as HMG, also participate in structuring the DNA by competing with and modulating histone binding and by inducing curvature and distortions in DNA (for a review see 2). Condensins and cohesins are members of the SMC proteins, a family of chromosomal ATPases that play central roles in chromosome condensation and sister chromatid cohesion, respectively (for a review see 3). As purified mitotic 13S condensin induces positive supercoiling in plasmid DNA, it is believed that condensin regularly supercoils interphase chromatin by affecting global writhing (4).

In Eubacteria the DNA architectural role has been allotted to the so-called histone-like proteins (for reviews see 5,6). These proteins are mostly sequence-non-specific and are proposed to distribute along the entire nucleoid, as shown mainly by fluorescence approaches (7–9). In \textit{Escherichia coli} the most abundant ones are HU, Fis, H-NS, IHF, Dps and StpA (10). In \textit{Bacillus subtilis} it has been proposed that other proteins such as HBsu (7) and L24 (11) organize the nucleoid \textit{in vivo} or play a local architectural role like LrpC (12,13). A single SMC protein is also found in \textit{B.subtilis}, but not in \textit{E.coli}, which however contains a protein, MukB, with a similar structure. Like their eukaryotic counterparts, both SMC and MukB are essential for chromosome condensation, supercoiling and correct partitioning (for a review see 14).

\textit{B.subtilis} phage F29 protein p6 is small (103 amino acids) (15) and very abundant, at ~700 000 copies/infected cell, 1.4 times the amount necessary to cover all the F29 DNA molecules present at mid-infection times (16). Comparatively, the most abundant histone-like protein of \textit{E.coli}, Dps, reaches 180 000 copies/cell in late stationary phase, and HU 60 000 copies/cell (10); in \textit{B.subtilis}, HBsu attains 50 000 copies/cell, only 0.2-fold the amount necessary to bind the full F29 DNA (17). \textit{In vitro} protein p6 binds DNA without sequence specificity (18,19), restraining positive supercoils on a covalently closed DNA, similarly to condensins, involved in the high order compaction of DNA needed for the formation of mitotic chromosomes (20). A model has been proposed for the p6–DNA nucleoprotein complex, in which the DNA is
right-handedly wrapped around a multimeric core of protein p6, therefore restraining positive supercoiling (21), with a $\Delta k$ of +0.1 per single protein p6 dimer (18). Finally, protein p6 has a pleiotropic effect, participating in viral DNA replication and transcription control. In vitro p6 binds to $\Phi 29$ DNA termini to enhance the initiation of DNA replication (22) from the origins located at both DNA ends and to repress transcription from the early promoter C2 at the right DNA end (23–25). Protein p6 also binds to the central transcription control region where, together with the viral transcription regulator p4, it triggers the switch of in vitro transcription from the main early promoters A2b and A2c to the late promoter A3 (26–28). However, and in spite of all the wealth of in vitro data, there was no in vivo evidence of p6 binding to DNA.

Consequently, to determine whether protein p6 indeed binds $\Phi 29$ DNA in vivo and, if so, to test its binding specificity, we used formaldehyde-crosslinked chromatin immunoprecipitation (X-ChIP) (29,30). This powerful technique has allowed mapping of the in vivo DNA-binding sites of diverse proteins of many organisms (for a review see 31), including such $B. subtilis$ proteins as SMC (9). To obtain quantitative data on protein p6 binding affinities for different DNA regions we combined the X-ChIP approach with real-time PCR.

In this work we show that protein p6 is able to bind most, if not all, the viral genome, strongly suggesting that its functions in DNA replication and transcriptional control are local outcomes of a more general role as an architectural protein. We also show that binding to the viral DNA is much higher than that to plasmid DNA. However, the affinity for the latter is enhanced when negative supercoiling is decreased, as shown in vivo and in vitro. Protein p6 supercoiling-dependent binding could be the basis for the preferential binding to $\Phi 29$ with respect to plasmid DNA.

### MATERIALS AND METHODS

**Bacteria, plasmids and phages**

*Bacillus subtilis* 110NA (trpC2, spoOA3, su') (32) containing pUB110 derivatives pPR55w6 and pPR55ow6 (33) was used. Figure 1 shows a map of these plasmids, which contain $\Phi 29$ gene 6 in either the correct (w6) or inverted (ow6) orientation. Phage $\Phi 29$ sus 14 (1242), a delayed lysis mutant (34), was used for all infections. Bacteria were grown in LB broth supplemented with 5 mM MgSO$_4$. Phleomycin (Cayla S.A.R.L.) was added to the medium at a final concentration of 0.8 $\mu g$/ml. As a source of plasmid pL259, a BlueScript KS Plus derivative (35), a strain of *E. coli* DH5$\alpha$ was used; in this case, 10 $\mu g$/ml ampicillin was added to the medium.

**Reagents**

Micrococcal nuclease was from Amersham Pharmacia Biotech and proteinase K from Boehringer Mannheim. Wheatgerm topoisomerase I was acquired from Promega and SpeI from New England Biolabs. Protein A–Sepharose CL–4B, lysozyme, RNase A, ampicillin, chloramphenicol, novobiocin, nalidixic acid and chloroquine were from Sigma. Formaldehyde at 37% was purchased from Calbiochem.

DNAs and oligonucleotides

Proteinase K-digested $\Phi 29$ DNA was obtained as described (36); plasmids pPR55w6 and pL259, used for fluorescence measurements, were purified using a Promega Wizard Plus SV miniprep kit. The sequences of the oligonucleotides used for PCR (Isogen) and the coordinates of the amplified DNA sequences are depicted in Table 1.

**Crosslinking, immunoprecipitation and DNA amplification**

The X-ChIP was performed basically as described (37). Analysis of DNA samples was performed by real-time PCR in

![Figure 1. Map of plasmids pPR55w6 and pPR55ow6. These plasmids (4.6 kb) contain $\Phi 29$ gene 6 under the control of the $\lambda$ repU promoter in pPR55w6 or in the inverted orientation in pPR55ow6. Genes encoding the replication protein repU and for phleomycin resistance (ble) are indicated. The sequences selected for PCR amplification are shown in black and the protein p6-binding regions studied, P1 and P2, are shown in gray, as explained in Figure 2B.](image)

Table 1. Oligonucleotides used in this work

<table>
<thead>
<tr>
<th>Region</th>
<th>Coordinates</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>P1</td>
<td>U-1</td>
<td>5'-AAGTAAGCCCCAACCCTCACATG</td>
</tr>
<tr>
<td></td>
<td>L-259</td>
<td>5'-GCCCACTAATTGTTGAGTGG</td>
</tr>
<tr>
<td>P2</td>
<td>U-4895</td>
<td>5'-GATTTTCCTCTGGCATCATTITGTC</td>
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<tr>
<td></td>
<td>L-5257</td>
<td>5'-CAAAATATCTTCGGTTTCTCTGG</td>
</tr>
<tr>
<td>P3</td>
<td>U-7255</td>
<td>5'-GAGTAGATAGATATTAGGACGCC</td>
</tr>
<tr>
<td></td>
<td>L-7528</td>
<td>5' -CTGACAGAAGACCAAGCACAAGCC</td>
</tr>
<tr>
<td>P4</td>
<td>U-9507</td>
<td>5'-CTGACACATCGGAAATTACAGC</td>
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<td></td>
<td>L-9820</td>
<td>5'-TTGTTGTAACGGTCTCGTGGAC</td>
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<td>P5</td>
<td>U-11567</td>
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<td>L-11778</td>
<td>5'-CCATACACAGGAAACAAGACATC</td>
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<td></td>
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<td>U-155</td>
<td>5'-GGGGCAAGAATGGCAGTCTGGAAC</td>
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<tr>
<td></td>
<td>L-456</td>
<td>5'-TTTCTTGGAGGAGGAGGAGGAGGAGGAG</td>
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</table>

Sequences of the primers used for amplification of $\Phi 29$ (P1–P6) or plasmid (P1 and P2) DNA sequences. The coordinates of the terminal 5' nucleotides are given, indicating whether they correspond to the upper (U) or lower (L) strand.
a Light-Cycler instrument using a Light Cycler-FastStart DNA Master SYBR Green I hot-start reaction mix (Roche). Samples corresponding to total, αp6-immunoprecipitated or preimmune serum (pi)-immunoprecipitated DNA were amplified for each DNA region using the primers depicted in Table 1. The crossing points (the cycle at which amplification enters the exponential phase) were determined and data were interpolated to a standard curve constructed with known amounts of purified, full-length Φ29 or plasmid DNA. The raw data were expressed as pg Φ29 or plasmid DNA per ml culture. Protein p6 binding was expressed as the immunoprecipitation coefficient (IC), the quotient between the specifically immunoprecipitated DNA (αp6 – pi) and the total DNA amount for a given region. The amplification conditions included a preheating step of 20 min at 95°C to activate the polymerase, followed by 30 cycles comprising a denaturation step of 15 s at 95°C for all regions, a hybridization step of 5 s at 53°C for regions Φ1 and Φ5, 5 s at 50°C for Φ6, 10 s at 51°C for Φ3 and Φ4, 10 s at 50°C for P1 and 10 s at 48°C for Φ2 and P2, and an elongation step at 72°C lasting 15 s for Φ1 and Φ5, 30 s for Φ3 and Φ4, 40 s for Φ6 and P1 and 60 s for Φ2 and P2. Finally, a melting analysis was performed by continuous fluorescence measurement from 65 to 95°C, to ensure the presence of a single specific amplification product. Non-specific hybridization of primers to bacterial DNA was previously ruled out in a control with uninfected cells.

**Topoisomer analysis**

For the analysis of plasmid pPR55w6 isolated from novobiocin- or nalidixic acid-treated cells, ~1 µg purified plasmid was treated with 1 U topoisomerase I for 30 s at 30°C in a buffer containing 50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol and 10 mM MgCl2. Reactions were stopped by addition of SDS to 1% and samples applied to a 1.2% agarose gel. Electrophoresis was run in 1× TBE buffer (90 mM Tris–borate and 2 mM EDTA) for 14 h at 35 V and 3 h at 100 V. The gel was soaked in 1 µg/ml chloroquine for 4 h, rotated 90° and electrophoresed at 100 V for 3 h. Finally, the gel was stained with ethidium bromide and photographed with a UviDoc-008.XD apparatus. Image processing and densitometry was performed with ImageQuant software. The median of the topoisomer distribution was calculated as described (38).

For the fluorescence measurements, stocks of plasmid pL259 with different topoisomer distributions were obtained by treatment for different times with 10 U topoisomerase I and, where indicated, with protein p6 to induce positive supercoiling. Samples were digested with proteinase K and purified by phenol extraction and ethanol precipitation. Aliquots were taken and subjected to 2-dimensional agarose gel electrophoresis as described above. The linearized form of plasmid pL259 was obtained by digestion with SpeI.

**Fluorescence measurements**

Fluorescence measurements were performed in a Varian Cary Eclipse spectrofluorometer and monitored in a 2 mm path length cell at a temperature of 15°C. The tryptophan residue of protein p6 was excited at a wavelength of 290 nm and fluorescence measured at 350 nm.

The value of maximal quenching of fluorescence ($Q_{max} = 0.84$) upon DNA binding was determined by reverse titration experiments in which increasing amounts of linearized plasmid pl259 were added to a fixed amount of protein p6. The experiments were done in 50 mM Tris–HCl, pH 7.5, 10 mM MgCl2, at a protein p6 concentration of 1, 3, 7 and 12 µM to ensure that the point of complete saturation was reached (39). The fluorescence emission constant ($f_{em} = 290/(µM)$) of free protein p6 was determined by measuring the fluorescence of increasing amounts of p6 in the absence of DNA.

To determine the effective binding constant ($K_{eff}$) of protein p6 to the different topological forms of the plasmid, direct titration experiments were performed (40,41). Protein p6 was added to plasmid DNA (20 µM) in 50 mM Tris–HCl, pH 7.5, 10 mM MgCl2, measuring fluorescence after mixing the sample by gentle shaking and incubating for 30–60 s. To calculate $K_{eff}$, we carried out a fitting procedure based on previously published expressions (40) and on the theory of McGhee and von Hippel (42) for the binding of proteins to polynucleotides. The fitting procedure was started by fixing the values of $Q_{max}$, $f_{em}$ and the binding site size of a protein p6 monomer ($n = 12$) and setting $K$ and $\omega$ as the fitting parameters, and continued by an iteration algorithm designed in our laboratory (43). We also performed a graphical approximation to $K_{eff}$ as described (41).

In a plot of the saturation fraction versus $[p6]_{free}$, assuming that $\omega > n$, the value of $[p6]_{free}$ corresponding to half saturation yields $1/K_{eff}$ (39,42).

**RESULTS**

**Protein p6 binds preferentially to Φ29 DNA in vivo**

To determine whether p6 binds Φ29 DNA in vivo we used the X-ChIP technique. After treatment with formaldehyde DNA was sonicated and immunoprecipitated with αp6 antibodies. A control immunoprecipitation was performed in parallel with preimmune serum. Selected sequences, 300 bp on average, were amplified and quantified by real-time PCR. Protein p6 binding is expressed as IC, in which the value obtained with preimmune (pi) serum was subtracted from that obtained with αp6 serum and normalized to the total amount of DNA (T) of that region ($IC = [(\alpha p6 – pi)/T] \times 10^6$). αp6, pi and T values were calculated by real-time PCR and expressed as pg DNA/ml culture. Therefore, protein p6 binding to different DNA regions of plasmid and viral DNA can be directly compared, since it is not dependent on their amount in the cell nor on their amplification efficiency. Figure 2A indicates the genomic position of the six Φ29 DNA regions analyzed, scattered along the viral chromosome and termed Φ1–Φ6. As illustrated in Figure 2B, these regions comprise not only the amplified sequence (~300 bp, black), but also ~450 additional base pairs on each side (gray), as the average size of sonicated DNA was ~750 bp. Regions Φ1 and Φ6 correspond to the left and right Φ29 DNA ends, respectively, and contain the replication origins; Φ6 also comprises the early promoter C2. Region Φ2 includes the central transcriptional control region, with the early promoters A2b and A2c and the late promoter A3. Regions Φ3, Φ4 and Φ5 correspond to late genes.
Figure 3 shows protein p6 binding to the six \(\Phi 29\) DNA regions, \(\Phi 1-\Phi 6\), as well as to a region, termed P1, from plasmid pPR55ow6, a pUB110 derivative (33), in \(\Phi 29\)-infected \textit{B. subtilis} cells. We measured protein p6 binding at 20 (Fig. 3A) and 35 (Fig. 3B) min post-infection. At 20 min the p6/\(\Phi 29\) DNA ratio is higher than at 35 min, since, as measured by real-time PCR, DNA replication had just started (M. Alcorlo, personal communication) and the intracellular amount of protein p6 had not still leveled off (16). In contrast, at 35 min the DNA concentration had increased more than that of protein p6 and consequently the IC values were lower. At both times we observed that protein p6 binds to all six \(\Phi 29\) DNA regions studied, which are scattered and comprise \(\sim 30\%\) of the total \(\Phi 29\) DNA (see Fig. 2A). The highest p6 binding corresponds to regions \(\Phi 1\) and \(\Phi 6\) at both DNA ends, whereas the affinity for the internal regions is lower, especially for region \(\Phi 2\), which contains the transcription control region. Finally, binding to plasmid DNA is significantly lower than that to any of the \(\Phi 29\) DNA regions analyzed. This is especially clear at 35 min, when the IC for \(\Phi 1\) is 15-fold lower than that for region \(\Phi 2\), the lowest affinity \(\Phi 29\) DNA region.

As an additional control, immunoprecipitation was also performed with antibodies against \(\Phi 29\) protein p17, another viral early protein that does not bind DNA \textit{in vitro} and whose intracellular concentration is close to that of protein p6 (44). No protein p17 binding was detected for the \(\Phi 29\) DNA regions analyzed (not shown), therefore, the possibility that the results are due to random protein–DNA collisions rather than to a genuine interaction can be ruled out.

**Protein p6 binding to plasmid DNA is enhanced by decreasing negative supercoiling**

\textit{In vitro} experiments suggested that p6 restrains DNA positive supercoiling (18), therefore we studied the effect of topology on p6 binding to plasmid DNA. For these experiments we used plasmids pPR55 w6/ow6 (see Fig. 1) and the gyrase inhibitors novobiocin, which decreases negative supercoiling, and as a control nalidixic acid, which does not change superhelicity (45). Figure 4 shows the topoisomer distribution of plasmids after novobiocin or nalidixic acid treatment, with the respective medians indicated by arrows. Thus, as expected, under our \textit{in vitro} conditions negative supercoiling of plasmid DNA is clearly decreased by novobiocin, but essentially unchanged by nalidixic acid.

Therefore, we studied protein p6 binding to plasmid DNA in the absence or presence of novobiocin or nalidixic acid. Cells hosting plasmid pPR55ow6 were infected with \(\Phi 29\), grown for an additional 20 min and then crosslinked after addition or not of novobiocin or nalidixic acid. Uninfected cells harboring plasmid pPR55w6, which express protein p6
constitutively, were also crosslinked in the absence or presence of novobiocin and nalidixic acid as described. Protein p6 binding, expressed as IC, was determined by real-time PCR for region P1 of plasmid pPR55w6 in ϕ29-infected cells (P1ϕ) and for regions P1 and P2 of plasmid pPR55w6 in protein p6-producing cells (P1p and P2p) (see Fig. 1). Figure 5 shows that addition of novobiocin results in a significant increase in protein p6 binding to plasmid DNA in both ϕ29-infected and uninfected cells. Binding to region P1 in ϕ29-infected cells (P1ϕ) increased 23-fold (IC = 1645) and for regions P1 and P2 in protein p6-producing uninfected cells (P1p and P2p) the increases were 34- (IC = 4546) and 27-fold (IC = 2780). In contrast, nalidixic acid, which does not change DNA topology, produced only slight changes in protein p6 binding. The higher protein p6 binding to region P1 in p6-producing cells with respect to infected cells could be due to titration of p6 by ϕ29 DNA. In summary, these results clearly indicate that protein p6 binding to DNA is dependent on superhelicity.

Supercoiling-dependent protein p6 binding to plasmid DNA in vitro

We studied the effect of topology on p6 binding to plasmid DNA in vitro. Protein p6 has a single tryptophan residue at position 46 whose fluorescence is quenched upon DNA binding. Therefore, we measured p6 binding by fluorescence quenching with different topological forms of plasmid pL259, which allows calculation of the respective binding constants of protein p6. We also analyzed the linearized form of the plasmid (L) as a control. Figure 6A shows the native form of the plasmid, designated supercoiled form I (sc I), and the topoisomer distributions of topoisomerase I-treated plasmids, termed sc II, sc III and sc IV.

As a previous step, we calculated the maximal quenching value ($Q_{max}$) of protein p6 by reverse titration experiments (as described in 39), in which increasing amounts of the linearized plasmid were added to a fixed amount of protein p6, measuring the maximal fluorescence quenching that was reached. We assayed four concentrations of p6, 1, 3, 7 and 12 µM, finding an increase in the apparent $Q_{max}$ from 0.60 at 1 µM p6 to 0.80 at 3 µM and 0.84 at 7 µM. This value of 0.84 remained constant at 12 µM p6.

To determine the $K_{eff}$ values for the different topological forms of pL259 we performed direct titration experiments in which increasing amounts of protein were added to a fixed amount of DNA (40,41), as described in Materials and Methods. As shown in Figure 6B, in all cases the initial slope of the curves decreased, indicating binding to DNA. The turning point and the slope of the curves during the DNA binding phase indicate different affinities for protein p6, being highest for the linear DNA and lowest for the sc I form. The slopes of the curves of the sc II, sc III and sc IV forms are proportional to their degree of negative supercoiling. When a certain protein concentration is reached, the slope increases parallel to that of the free protein. For the linear DNA sample this is shown in Figure 6B as a dotted line. The point where this line intercepts the abscissa corresponds to a protein concentration [p6] = $Q_{max}$ [DNA]/$n$ (40). For the [DNA] used, we obtained a value of $Q_{max}/n = 0.072$, which gives $Q_{max} = 0.86$ for $n = 12$, very close to that of 0.84 obtained by reverse titration; for $Q_{max} = 0.84$, a value of $n = 11.7$ is obtained, close to that of 12 obtained by footprinting (21). In the corresponding lines for the supercoiled samples, the slope also increases parallel to that of the free protein, but intercepts the abscissa at a lower protein p6 concentration. In Figure 6B we show the slope of sc I as a dashed line for which $Q_{max}/n = 0.038$, which would give $Q_{max} = 0.46$ for $n = 12$ or $n = 22$ for $Q_{max} = 0.84$. This significant variation with respect to the values obtained for the linear DNA would indicate that for a topologically restricted DNA, saturation cannot be reached: as every protein p6 molecule that binds restrains positive supercoiling (18) and produces a compensatory negative supercoiling, this would further impair the next molecule binding. Eventually, the superhelical tension would prevent saturation of DNA by protein p6. The amount of protein p6 allowed to bind DNA would be inversely proportional to the degree of negative supercoiling of the sample. The $K_{eff}$ values for p6 binding to the DNA samples were calculated by computer fitting of direct
titrations fluorescence data as described in Materials and Methods. They were found to be $6.7 \times 10^5$ for sc I, $1.0 \times 10^6$ for sc II, $1.4 \times 10^6$ for sc III, $1.7 \times 10^6$ for sc IV and $2.7 \times 10^6$ per M for the linear sample. Here it is not possible to
determine $K$ and $\omega$ independently due to the inability of supercoiled samples to reach saturation, as explained above.

Figure 6C shows a plot of the saturation fraction ($q$) against $[p6]_{\text{free}}$ in order to obtain a graphical estimation of $K_{\text{eff}}$ values, defined as the reciprocal of $[p6]_{\text{free}}$ when 50% of the DNA is bound to protein (41). The $K_{\text{eff}}$ values obtained, $7.5 \times 10^5$ for sc I, $1.1 \times 10^6$ for sc II, $1.3 \times 10^6$ for sc III, $1.6 \times 10^6$ for sc IV and $2.6 \times 10^6$ per M for the linear sample, are nearly identical to those obtained from the fit of direct titration data and together confirm the dependence of p6 binding on the superhelical state of the DNA. Direct titrations were also performed with linearized and supercoiled native pPR55ow6 plasmid, used in the in vivo studies, obtaining analogous results (not shown).

In summary, these data demonstrate that p6 binding is inversely proportional to the degree of negative supercoiling of DNA, in agreement with the observed effect of novobiocin in p6 binding to plasmid DNA in vivo and with the ability of p6 to restrain positive supercoils in vitro (18). This supercoiling-dependent binding most probably constitutes the basis of the p6 specificity for viral DNA observed in vivo, as the non-covalently closed $\Phi 29$ DNA is presumably less negatively supercoiled than the bacterial DNA.

**DISCUSSION**

Proteins involved in organization and compaction of chromosomes have been described in virtually every organism. These architectural proteins share basic features such as abundance, sequence-non-specificity and pleiotropic effects, involved in diverse DNA transactions. In bacteria these proteins are commonly known as ‘histone-like’ (5–6) and a number of them have been co-localized in vivo with the nucleoid (7–9).
Using X-ChIP and real-time PCR we here provide direct evidence that protein p6 of *B. subtilis* bacteriophage Φ29 binds *in vivo* all along the viral genome in the manner of a histone-like protein. We have studied p6 binding to six regions of Φ29 DNA *in vivo*, scattered along and comprising ~30% of the viral genome, demonstrating that protein p6 binds to all of
them. This strongly suggests that protein p6 is likely to form nucleoprotein complexes along most, if not all, the viral DNA. However, binding to Φ29 DNA is not the same in all the regions, but highest at the two DNA ends, which could be due to the presence of sequences preferentially binding p6 (46). This high binding is probably related to a requirement for protein p6 in viral DNA replication (47) and for repression of the early promoter C2 (23–25). Interestingly, binding to internal regions Φ3, Φ4 and Φ5, where no role for p6 has ever been described, is higher than to region Φ2, where p6 is involved in the early–late transcriptional switch (26–28). In summary, p6 binding along Φ29 DNA strongly supports an architectural role, in agreement with other p6 features, such as its abundance, lack of sequence specificity and effects in replication and transcription, which could be envisaged as local outcomes of a more global role as a histone-like protein.

We also show that protein p6 binds with high preference to viral with respect to plasmid DNA and that it is superhelicality which provides this specificity, as the level of negative supercoiling of bacterial (plasmid and chromosomal) DNA is presumably lower than that of non-covalently closed Φ29 DNA. In vivo, we have shown that when the negative superhelicity of the plasmid is decreased by novobiocin, protein p6 binding is significantly increased in infected and uninfected cells; on the other hand, binding was essentially unchanged with nalidixic acid, which inhibits gyrase but does not alter the topological state of the plasmid. We have confirmed these results in vitro by fluorescence quenching of protein p6 with plasmids of different degrees of supercoiling, the highest affinity corresponding to the linearized, topologically unconstrained form. Therefore, protein p6 binding increases when negative supercoiling is lowered, strongly suggesting that, as shown in vitro (18), protein p6 restrains positive supercoiling in vivo. Supercoring-dependent DNA binding is a smart strategy to prevent titration of a sequence-non-specific protein such as p6 by bacterial DNA, with a much higher mass than that of viral DNA.

In conclusion, we report the first in vivo direct evidence of a bacteriophage DNA architectural protein. We have demonstrated that: (i) phage Φ29 protein p6 binds all along the Φ29 DNA; (ii) this binding is specific to viral DNA; (iii) p6 binding depends on DNA superhelicity so that the specificity could be based on a lower negative superhelicity of viral DNA. Together with all the previous evidence in vitro, we conclude that protein p6 globally organizes and compacts the viral genome, with local effects on replication and transcription.

ACKNOWLEDGEMENTS

We are indebted to Dr M.García-Mateu for support with the fluorescence experiments. We are grateful to Dr A. Bravo for providing B.subtilis strain 110NA pPR55 w6/ow6 and to L.Villar for purified Φ29 DNA. This work was supported by research grants 2R01 GM27242-24 from the National Institutes of Health, BMC2002-03818 from Ministerio de Ciencia y Tecnología and by an Institutional grant from the Fundación Ramón Areces to the Centro de Biología Molecular ‘Severo Ochoa’. V.G.-H. is the recipient of a postdoctoral fellowship of Comunidad Autónoma de Madrid.

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