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Interaction of the bacteriophage ϕ29 protein p6 with double-stranded DNA

(DNA–protein interaction/protein-primed replication/DNase I "footprinting")

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ABSTRACT **The *Bacillus subtilis* bacteriophage ϕ29 protein p6 binds to double-stranded DNA, but not to single-stranded DNA, as determined by a gel retardation assay. The nature of the interaction was further studied by DNase I "footprinting" experiments. Protein p6 binds to fragments containing the right or left terminal sequences of ϕ29 DNA, producing a characteristic pattern of hypersensitive bands spaced about 24 nucleotides apart along most of the fragment, flanking protected regions. Binding of protein p6 to an internal ϕ29 DNA fragment was also observed, but the footprint pattern was more salt sensitive than that obtained with the terminal ϕ29 DNA fragments. By electron microscopy, protein p6 was shown to cover the DNA, totally or partially, from one end. In addition, binding of protein p6 to relaxed circular DNA induced positive supercoiling, indicating that a topological change in the DNA occurred.**

The *Bacillus subtilis* phage ϕ29 contains a linear double-stranded DNA 19,285 base pairs (bp) long (1–3) with the viral protein p3 covalently linked to each 5′ end through a phosphodiester bond between the OH group of serine residue 232 and dAMP, the terminal nucleotide at both 5′ ends (4). Phage ϕ29 replicates by a strand displacement mechanism (5–7), starting at either end of the DNA-protein p3 template by formation of a p3-dAMP covalent complex in the presence of free protein p3, viral DNA polymerase p2, and dATP (8, 9). By addition of the remaining dNTPs, the p3-dAMP initiation complex is further elongated by the ϕ29 DNA polymerase, giving rise to full-length ϕ29 DNA molecules (10). The viral protein p6, needed in ϕ29 DNA replication *in vivo* (11, 12), has been purified and shown to stimulate the formation of the p3-dAMP initiation complex from either DNA end (13, 14) and to increase the amount of elongated product in the replication of ϕ29 DNA-protein p3 (14). In addition, protein p6 inhibits transcription from the ϕ29 early promoter C2, at the right end of ϕ29 DNA (15). ("Right" and "left" ends refer to the map in ref. 19.)

In this paper we report the binding of the ϕ29 protein p6 to DNA as determined by gel retardation, DNase I "footprinting," electron microscopy, and supercoiling of relaxed DNA.

MATERIALS AND METHODS

Chemicals and Enzymes. Restriction endonucleases were from New England Biolabs or Boehringer Mannheim; topoisomerase I was from Bethesda Research Laboratories; *Escherichia coli* DNA polymerase I, Klenow fragment, was from New England Biolabs; and DNase I was from Worthington. Chloroquine was obtained from Sigma. [α-^{32}P]dATP (410 Ci/mmol; 1 Ci = 37 GBq) and [γ-^{32}P]ATP (>3000 Ci/mmol) were from Amersham International.

Plasmids and DNA Preparations. The recombinant plasmid pID13, containing the 73-bp *Bcl* I C fragment and the 269-bp *Hin*dIII L fragment, from the left and right ends of ϕ29 DNA, respectively, inserted into plasmid pKK223-3 (16), was as described by Gutiérrez *et al.* (17). The recombinant plasmid p8LIS57 S3.6, 6.5 kilobases (kb) long, containing an African swine fever virus DNA fragment inserted into plasmid pUC8, was a gift from R. Blasco (Centro de Biología Molecular, Madrid, Spain). ϕ29 DNA-protein p3, untreated or treated with proteinase K, was prepared as described by Peñalva and Salas (18) and Inciarte *et al.* (19), respectively. When indicated, the DNA was digested with restriction endonuclease *Cla* I and the two fragments, 13.1 and 6.1 kb long, from the right and left DNA ends of ϕ29, respectively, were isolated by sucrose gradient centrifugation.

Labeling of DNA Fragments. Restriction fragments, isolated either from proteinase K-treated ϕ29 DNA or from plasmid pID13, were labeled either at the recessed 3′ ends with the Klenow fragment of *E. coli* DNA polymerase I and [α-^{32}P]dATP or at the 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP (20). Since a peptide remains at the 5′ ends of ϕ29 DNA after treatment with proteinase K (4), only the internal 5′ end of the terminal ϕ29 DNA fragments is labeled with the kinase. In some cases, the terminal ϕ29 DNA fragments were treated with piperidine as described by Gutiérrez *et al.* (17) to remove the peptide.

Electrophoresis of Protein-DNA Complexes. Protein samples were incubated for 30 min at 0°C in a final volume of 20 μl of a buffer containing 12 mM Tris·HCl at pH 7.5 and 1 mM EDTA with approximately 1000 cpm (0.4–1 ng) of a ^{32}P-labeled DNA restriction fragment. Protein-DNA complexes were resolved in low ionic strength polyacrylamide gels essentially as described by Carthew *et al.* (21). After electrophoresis, the gels were dried and autoradiographed.

DNase I Footprinting. Footprinting was carried out essentially as described by Galas and Schmitz (22). Protein p6 (2.5–40 μg) was incubated with the DNA fragment (20 ng) labeled at only one 5′ or 3′ end as described before, in 0.1 ml of a buffer containing 50 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, and 50 mM NaCl. After 20 min at 0°C samples were treated with 0.1 μg of DNase I for 2 min at 37°C. Reactions were stopped by addition of 0.15% NaDodSO$_4$/5 mM EDTA/0.15 M NaOAc, pH 6.0, and 50 μg of carrier RNA. After precipitation with ethanol the samples were subjected to electrophoresis in 6% polyacrylamide sequencing gels (23). As DNA sequence controls, the same fragments were digested chemically according to the method of Maxam and Gilbert (23) as modified by Cooke *et al.* (24).

Electron Microscopy of the Protein p6-DNA Complex. Proteinase K-treated ϕ29 DNA (1 μg), *Cla* I ϕ29 DNA restriction fragments A or B, either containing protein p3 or

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treated with proteinase K, or p8LIS57 S3.6 DNA linearized with *Hin*dIII (0.3 μg of each), was incubated with protein p6 (10 μg) or with bovine serum albumin (10 μg) in 0.1 ml of a buffer containing 50 mM Tris·HCl at pH 7.5, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mM EDTA, 50 mM KCl, and 3 μg of bovine serum albumin (buffer A). After 20 min at 0°C the samples were fixed with 0.8% glutaraldehyde by incubation for 5 min at 0°C and then for 10 min at room temperature and filtered through Sepharose 4B-200 (Pharmacia) columns equilibrated in 50 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$. The excluded volume was spread over freshly cleaved mica and washed with triply distilled water for 2 hr (25). The mica was air dried and rotatory shadowed with a platinum–carbon layer at 4° elevation angle and further covered with a 20 Å carbon layer before visualization in a JEOL 100B electron microscope.

Interaction of Protein p6 with Relaxed DNA and Agarose Gel Electrophoresis. Plasmids pID13 or pKK223-3 (1 μg) were relaxed by incubation in buffer A for 1 hr at 37°C with 2 units of topoisomerase I. The reaction was stopped by addition of $NaDodSO_4$ at a final concentration of 1%, and the plasmid DNA was extracted with phenol and chloroform and precipitated with ethanol. The relaxed DNA was incubated with the indicated amounts of protein p6 in 0.1 ml of buffer A. After 20 min at 0°C the samples were incubated with topoisomerase I (2 units) for 25 min at 37°C. The reaction was stopped with 1% $NaDodSO_4$ and the DNA was extracted with phenol and chloroform, precipitated with ethanol, and subjected to 1% agarose gel electrophoresis in 40 mM Tris acetate, pH 8/2 mM EDTA (20). When indicated, chloroquine (0.5 μg/ml) was added to the gels (26), which were run in 89 mM Tris borate, pH 8/2 mM EDTA (20). Gels were stained with ethidium bromide (5 μg/ml).

RESULTS

Binding of Protein p6 to Double-Stranded DNA. Fig. 1 shows that, after centrifugation of protein p6 through a glycerol gradient, the material binding to φ29 DNA restriction fragments sedimented with the protein p6 peak. A smaller amount of p6 was needed to bind to the terminal *Hin*dIII L fragment than to the fragment lacking the 59 bp at the DNA end (Fig. 1 *Inset a*). The fractions corresponding to the peak of protein p6 did not show binding to single-stranded DNA (results not shown). Binding of protein p6 to DNA was also detected by DNase I footprinting (Fig. 1 *Inset b*) and induction of supercoiling of relaxed circular DNA (Fig. 1 *Inset c*).

DNase I Footprinting. Taking into account that protein p6 stimulates the initiation of φ29 DNA replication (13, 14) that takes place at the φ29 DNA ends, terminal φ29 DNA fragments were used for DNase I footprinting experiments.

(i) Hin*dIII L fragment from the φ29 DNA right end.* Fig. 2*A* shows a longer (lanes a–f) and a shorter (lanes g–l) run of the 269-bp *Hin*dIII L fragment, labeled at the internal 3′ end and digested with DNase I after incubation with increasing amounts of protein p6. Essentially the same pattern of digested bands was obtained in the absence or in the presence of 5 μg of protein p6 (lanes c and d). However, when 10 or 20 μg of protein p6 was added (lanes e and f), a characteristic pattern was obtained all along the fragment, with hypersensitive bands regularly spaced about 24 nucleotides apart, flanking protected regions of about 21 nucleotides. A similar pattern was observed when the complementary strand was used (Fig. 2*B*, lanes c–e). In some cases, in addition to the hypersensitive bands spaced about 24 nucleotides apart, a few bands of lower hypersensitivity or unprotected sites, located in between, could be seen. Fig. 2*E* shows the nucleotide sequence of the φ29 DNA right end, with a summary of the footprint results showing the enhanced sites and protected regions. It can be also seen that the hypersensitive sites of one strand are displaced about 10 and 14 nucleotides from the sites of the other strand. Fig. 2*F* shows a cylindrical projection of the B-DNA with the positions of the hypersensitive sites in the *Hin*dIII L fragment.

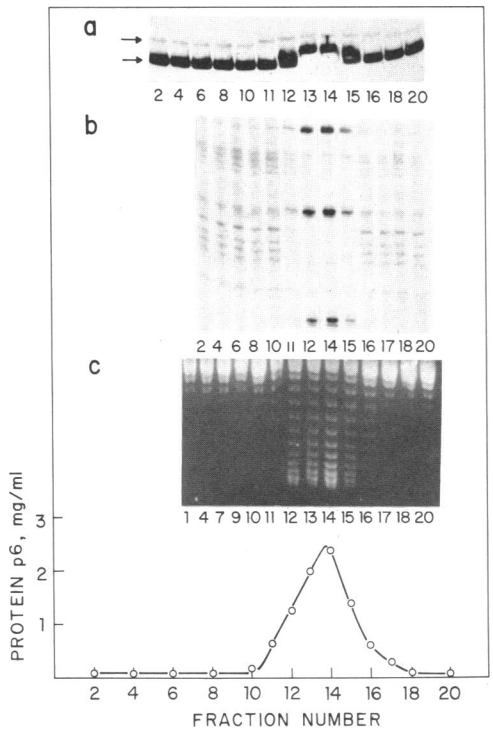

FIG. 1. Sedimentation of protein p6, binding to double-stranded DNA, DNase I footprinting, and supercoiling of relaxed DNA. Purified protein p6 (≈1.9 mg) was subjected to glycerol gradient centrifugation as described by Pastrana *et al.* (13). Fractions were taken and a sample from each was used to determine the amount of protein p6 (○) (27). (*Inset a*) Gel retardation assay. A 4-μl sample from the indicated fractions (in all cases, the numbers below the lanes indicate the number of the fraction used) was used to determine the binding to the ^{32}P-labeled φ29 DNA *Hin*dIII L fragment, 269 bp long, from the right DNA end, or to the latter cut with *Hin*fI to remove 59 bp from the DNA end. The arrows indicate the position of the 210-bp *Hin*fI fragment as well as that of the undigested 269-bp *Hin*dIII L fragment. (*Inset b*) DNase I footprint. Another sample from the indicated fractions (8 μl) was incubated with the φ29 DNA *Hin*dIII L fragment labeled at the internal 3′ end, treated with DNase I, processed, and subjected to polyacrylamide gel electrophoresis. (*Inset c*) Supercoiling of relaxed DNA. Another sample from the indicated fractions (8 μl) was incubated with relaxed DNA, treated with topoisomerase I, and subjected to agarose gel electrophoresis.

(ii) Rsa *I terminal fragment from the φ29 DNA left end.* Fig. 2*C* shows that the pattern obtained with the 306-bp *Rsa* I fragment, from the left end of φ29 DNA, labeled at the internal 5′ end, was very similar to the one obtained with the right end *Hin*dIII L fragment, also labeled at the internal 5′ end, using the same amounts of protein p6, and the location of the hypersensitive sites from each φ29 DNA end were essentially the same in both fragments (Fig. 2*E*).

When the 73-bp *Bcl* I C fragment, from the φ29 DNA left end, was used, the pattern of hypersensitive bands was seen only with 40 μg of protein p6 (not shown). However, when the latter was joined to a 300-bp pBR322 sequence, the characteristic pattern of hypersensitive bands was seen all along the fragment with 15 μg of p6, as indicated in Fig. 2*E*. Note that the positions are very close to those of the right end *Hin*dIII L fragment similarly labeled at the internal 3′ end and that the phasing in the *Bcl* I C fragment is extended

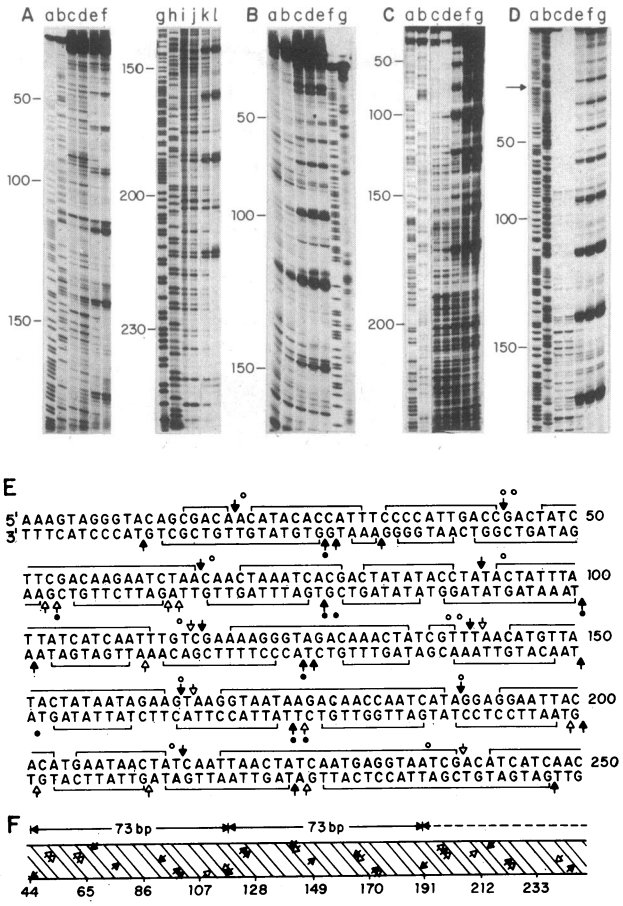

FIG. 2. DNase I footprints of protein p6 on terminal fragments from the right and left ends of ϕ29 DNA. The *Hin*dIII L fragment, 269 bp long, from the ϕ29 DNA right end, labeled at the internal 3′ end (*A*) or at the internal 5′ end (*B*), or the 306-bp *Rsa* I fragment, from the ϕ29 DNA left end, labeled at the internal 3′ end (*C*), was incubated with 0 (lanes c and i), 5 (d, j), 10 (e, k), or 20 (f, l) μg of protein p6 in *A*; 0 (a), 5 (b), 10 (c), 20 (d), or 40 (e) μg of p6 in *B*; or 0 (c), 5 (d), 10 (e), 15 (f), or 20 (g) μg of p6 in *C*; and the samples were treated with DNase I, processed, and subjected to polyacrylamide gel electrophoresis. (*D*) The recombinant plasmid pID13 was cut with *Ava* II, producing a 876-bp fragment that was digested with *Hin*dIII and *Hpa* II, and a 354-bp fragment, containing the 73-bp *Bcl* I C and 269-bp *Hin*dIII L fragments from the ϕ29 DNA left and right ends, plus a 12-bp sequence of pBR322 joined to the *Bcl* I C fragment. The fragment containing the ϕ29 DNA terminal sequences was labeled at the *Hin*dIII cut with the Klenow enzyme and [α-^{32}P]dATP. After incubation with 0 (c), 5 (d), 10 (e), 15 (f), or 20 (g) μg of p6 the samples were processed as described above. Products of a purine-specific or pyrimidine-specific chemical chain degradation reaction of the corresponding fragment were run in parallel in each case: lanes a and b and g and h in *A*, f and g in *B*, and a and b in *C* and *D*. The long run in *A* was for 9.5 hr at 60 W and the short run was for 4 hr at 60 W. The numbers at the left are the nucleotide position starting from the corresponding ϕ29 DNA end (*A–C*) or from the ϕ29 DNA right end (*D*); in this case, the arrow indicates the position where the left and right ϕ29 DNA sequences are joined. (*E*) Sequence of the *Hin*dIII L fragment up to position 250. The strongly hypersensitive sites are indicated by filled arrows and the sites of lower hypersensitivity are indicated by empty arrows. The protected regions are in brackets. The positions of hypersensitive bands in the left ϕ29 DNA fragment, corresponding to the bottom strand, are indicated by filled circles and those in the 73-bp left ϕ29 DNA fragment joined to a pBR322 sequence, corresponding to the top strand, are indicated by empty circles. (*F*) Cylindrical projection of B-DNA, showing the hypersensitive sites of the *Hin*dIII L fragment from position 43 to 250. Filled and empty arrows are as in *E*.

into most of the pBR322 fragment. The pBR322 fragment alone did not produce such a pattern (results not shown).

(iii) *Location at internal positions of the ϕ29 DNA right and left terminal sequences.* To find out whether the terminal ϕ29 DNA sequences have to be located at the DNA ends for the binding of protein p6, as is the case for the formation of the p3-dAMP complex in the initiation of replication (17), a fragment from the recombinant plasmid pID13 was used, containing the 269-bp *Hin*dIII L fragment and the 73-bp *Bcl* I C fragment, with the ϕ29 DNA right and left ends joined at an internal position. The fragment was labeled at the 3′ end of the *Hin*dIII cut and subjected to DNase I footprint analysis. Fig. 2*D* shows that, in the presence of 10–20 μg of protein p6 (lanes e–g), a clear pattern of hypersensitive bands located at the same positions as in the *Hin*dIII L fragment was obtained. The phasing of the hypersensitive sites of the *Hin*dIII L fragment was extended into the *Bcl* I C fragment with the characteristic spacing of 24 nucleotides, and protected regions were also seen. A similar result was obtained when the terminal sequence from the *Hin*dIII L fragment was located at an internal position preceded by a 73-bp pBR322 DNA sequence (results not shown).

(iv) *Specificity of protein p6 footprinting.* Fig. 3*A* shows that, when the 269-bp fragment from the ϕ29 DNA right end was used, a clear pattern of hypersensitive bands was seen with NaCl at concentrations up to 125 mM. A similar result was obtained with a 306-bp fragment, from the ϕ29 DNA left end (results not shown). However, when an internal fragment 341 bp long, located 845 bp from the left end of ϕ29 DNA, was used, the hypersensitive bands seen in the presence of protein p6 at 50 mM NaCl essentially disappeared at 100 mM NaCl (Fig. 3*B*), indicating that protein p6 does not bind to this fragment under these conditions and suggesting specificity in the binding of protein p6 to the terminal ϕ29 DNA fragments. In addition, the binding of protein p6 to DNA fragments from pBR322, either A+T- or

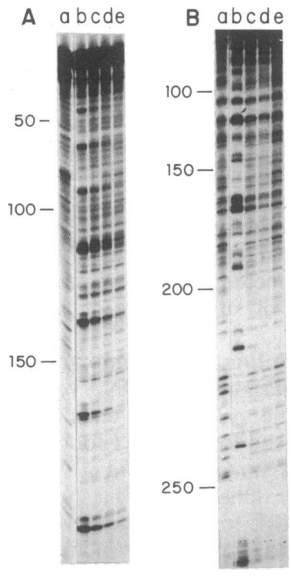

FIG. 3. Effect of salt concentration on the footprint of protein p6. The 269-bp *Hin*dIII L fragment, from the ϕ29 DNA right end, labeled at the internal 3′ end (*A*) or an internal ϕ29 DNA fragment, 341 bp long, located 845 bp from the ϕ29 DNA left end, labeled with polynucleotide kinase at position 1186 (*B*), was incubated with 10 μg of protein p6 in 50 (lane b), 100 (c), 125 (d), or 150 (e) mM NaCl. As a control, the DNA fragments were incubated in 50 mM NaCl in the absence of protein p6 (a). The pattern of DNase I digestion in the absence of p6 was shown not to be affected by the salt concentration. After incubation, the samples were treated with DNase I, processed, and subjected to polyacrylamide gel electrophoresis. Products of a chemical chain degradation reaction were run in parallel (not shown). The numbers at the left are the nucleotide position, starting from the DNA end (*A*) or from position 845 (*B*).

G+C-rich, did not show the characteristic pattern of hypersensitive bands (not shown).

Electron Microscopy of the Protein p6-DNA Complex. When the binding of protein p6 to ϕ29 DNA was analyzed by electron microscopy it was observed that the protein covered the DNA, totally or partially, from one of the ends in a uniform way (results not shown). To determine whether binding was occurring from either ϕ29 DNA end, the interaction of protein p6 with the *Cla* I A or B fragments, from the right and left ends of ϕ29 DNA, respectively, was analyzed. Fig. 4 shows *Cla* I B DNA fragments uncovered (*A*), partially covered (*B* and *C*), or completely covered (*D*) by protein p6. The protein seemed to cover the DNA from one end and the binding produced an increase in the thickness of the DNA but did not result in appreciable compactness of the DNA. Similar results were obtained with the *Cla* I A fragments. To find out whether some DNA sequence specificity in the binding of protein p6 could be detected by electron microscopy, the linearized plasmid p8LIS57 S3.6, of approximately the same size as the ϕ29 DNA *Cla* I B fragment, was used. Essentially similar binding of protein p6 to one DNA end was observed (results not shown).

Topological Change of DNA by Protein p6 Interaction. The effect of the interaction of protein p6 on the topology of relaxed plasmid DNA was studied. pID13 plasmid DNA, containing terminal ϕ29 DNA sequences corresponding to the two replication origins, was relaxed with topoisomerase I, incubated with increasing amounts of protein p6, and further treated with topoisomerase I to release any torsional tension in the DNA induced by the protein p6 binding. After deproteinization, the DNA was analyzed by agarose gel electrophoresis. Fig. 5*A* shows that addition of 2.5 μg of protein p6 essentially did not change the topology of the DNA (lanes b and c), whereas 5 μg of p6 gave rise to supercoiling of the DNA (lane d). Very little additional change was obtained with higher amounts of protein p6 (lanes e–h). When the relaxed DNA, after incubation with 10 μg of p6, was not treated with topoisomerase I, no supercoiling of the DNA was observed (lane i). Similar results were obtained with supercoiled pID13 DNA or with plasmid pKK223-3, which lacks the ϕ29 replication origins. The salt concentration affected the supercoiling induced by p6 in both plasmids similarly, and the p6 effect was abolished at 150 mM NaCl (results not shown).

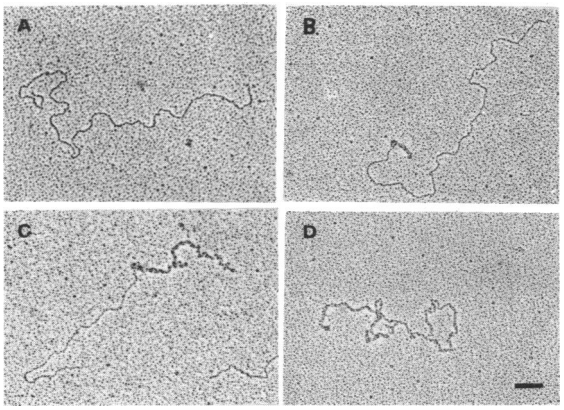

FIG. 4. Electron microscopy of protein p6 bound to *Cla* I B fragments of ϕ29 DNA. Protein p3-containing *Cla* I B fragments (0.3 μg) from the left end of ϕ29 DNA were incubated with 10 μg of protein p6. After 20 min at 0°C the samples were fixed with glutaraldehyde, filtered through a Sepharose 4B-200 column, and processed as described in the text. Panels show *Cla* I B fragments uncovered (*A*), partially covered (*B* and *C*), or totally covered (*D*) by protein p6. The bar represents 0.11 μm.

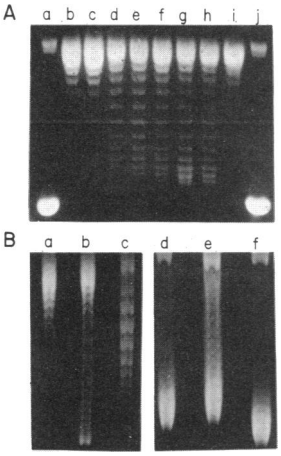

FIG. 5. Agarose gel electrophoresis of relaxed DNA after incubation with protein p6. (*A*) Plasmid pID13 (1 μg) was relaxed with topoisomerase I, incubated with different amounts of protein p6, further treated with topoisomerase I, and subjected to agarose gel electrophoresis. Lanes: a and j, untreated DNA; b–i, treated with topoisomerase I; lanes b, c, d, e, f, g, and h have 0, 2.5, 5, 7.5, 10, 15, and 20 μg of protein p6, respectively; lane i has 10 μg of p6, but the sample was not treated with topoisomerase I after incubation with p6. (*B*) Plasmid pID13 (2 μg) was relaxed with 4 units of topoisomerase I for either 60 min (a, c, d, and f) or 4 min (b and e) at 37°C. Protein p6 (10 μg) was added in lanes c and f and the mixture was further treated with topoisomerase I. Agarose gel electrophoresis was carried out in Tris borate buffer in the absence (a–c) or presence (d–f) of chloroquine (0.5 μg/ml).

To determine whether the supercoils induced by protein p6 are due to an increase or decrease in the linking number after topoisomerase I treatment, relaxed pID13 DNA was incubated with 10 μg of protein p6 and subjected to agarose gel electrophoresis in the absence or presence of chloroquine. Fig. 5*B* shows the control pID13 DNA relaxed with topoisomerase I and run in an agarose gel in the absence (lane a) or presence (lane d) of chloroquine to decrease the twisting number and therefore supercoil the plasmid positively. As another control, the pID13 supercoiled DNA was partially relaxed with topoisomerase I (lane b) and, when run in the chloroquine-containing gel (lane e), the negatively supercoiled DNA was relaxed, whereas the relaxed DNA was positively supercoiled. When the topoisomers induced by protein p6 (lane c) were analyzed in the chloroquine-containing gel, they showed a higher degree of supercoiling (lane f) than the relaxed DNA run in the same gel (lane d), indicating that protein p6 induces positive supercoiling.

DISCUSSION

The viral protein p6, involved in ϕ29 DNA replication, binds to double-stranded DNA but not to single-stranded DNA. DNase I footprint experiments showed that protein p6 binds all along the ϕ29 DNA fragments assayed, producing a characteristic pattern with hypersensitive bands located about 24 nucleotides apart, flanking protected regions. In addition, the hypersensitive sites in the left and right ϕ29 DNA terminal fragments were located at essentially the same positions from the DNA ends. The location of the hypersensitive sites in a cylindrical projection as depicted in Fig. 2*F* shows a helical disposition with a periodicity of about 73 nucleotides, suggesting a helical distribution of protein p6 around the DNA.

The pattern of hypersensitive bands of the two terminal ϕ29 DNA fragments was not drastically affected at 100 mM NaCl, whereas it completely disappeared when an internal ϕ29 DNA fragment was used. These results, together with

the fact that pBR322 fragments did not show the characteristic pattern, suggest the existence of some sequences or structural features present at the ϕ29 DNA ends that determine the orderly, salt-resistant protein p6 binding. The 6-bp inverted terminal repeats present at the ϕ29 DNA ends (28, 29) are not the signals recognized by protein p6, since a fragment lacking these sequences behaved in a similar way as the *Hin*dIII L fragment (results not shown). The possibility that the signals had to be located at the free ϕ29 DNA ends to determine the phasing was ruled out since, when a fragment containing the two ϕ29 DNA terminal sequences located at an internal position was used, the phasing of the hypersensitive sites did not change. In a fragment containing the 73-bp left terminal sequence joined to a pBR322 sequence, the phasing of the hypersensitive sites from the ϕ29 DNA left end was extended into the pBR322 sequence. These results suggest that protein p6 binds to DNA in an orderly fashion from a region present at the ϕ29 DNA terminal sequences. The fact that, even in the presence of a mass excess of protein over DNA of about 300, 5 μg of protein p6 does not produce any footprint pattern, whereas 10 μg of the protein gives rise to the characteristic one, together with the electron microscopy results, suggests that protein p6 covers the DNA in a cooperative way.

The positive supercoiling of relaxed DNA induced by protein p6 suggests a torsional increase in the DNA upon binding of protein p6. The fact that protein p6 induces similar supercoiling of relaxed plasmid DNA containing or lacking ϕ29 DNA terminal sequences, even at 100 mM NaCl, could be explained by the presence in the latter of the structural features suggested to be present at the ϕ29 DNA ends, taking into account the larger size of the plasmids (4.6 kb) with respect to the DNA fragments used in the footprinting assays.

We point out that the amount of protein p6 that stimulates ϕ29 DNA replication *in vitro* (13, 14) is similar to the amount that is required for the binding to DNA. In addition, we have recently observed that the stimulation of ϕ29 DNA replication by protein p6 is higher in 100–125 mM NaCl than in lower salt concentrations (L. Blanco, A. Bernad, and M. Salas, unpublished observation). As already discussed, at 100 mM NaCl there is specific interaction of protein p6 at the ϕ29 DNA ends. On the other hand, the interaction of protein p6 with the ϕ29 DNA ends does not require the presence of the parental terminal protein. This is in agreement with the fact that protein p6 stimulates replication of protein p3-free ϕ29 DNA terminal fragments (14).

The results shown in this paper indicate that protein p6 binds to DNA in a way different from other known DNA-binding proteins. The biological significance of this binding is likely to be related to the stimulation of ϕ29 DNA replication.

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