Identification of Residues within Two Regions Involved in Self-association of Viral Histone-like Protein p6 from Phage Ø29*

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Protein p6 of Bacillus subtilis phage Ø29 is involved in the initiation of viral DNA replication and transcription by forming a multimeric nucleoprotein complex with the phage DNA. Based on this, together with its abundance and its capacity to bind to the whole viral genome, it has been proposed to be a viral histone-like protein. Protein p6 is in a monomer-dimer-oligomer equilibrium association. We have identified protein p6 mutants deficient in self-association by testing random mutants obtained by degenerated polymerase chain reaction in an in vivo assay for dimer formation. The mutations were mainly clustered in two regions located at the N terminus, and the central part of the protein. Site-directed single mutants, corresponding to those found in vivo, have been constructed and purified. Mutant p6A44V, located at the central part of the protein, showed an impaired dimer formation ability, and a reduced capacity to bind DNA and to activate the initiation of Ø29 DNA replication. Mutant p6I8T has at least 10-fold reduced self-association capacity, does not bind DNA nor activate Ø29 DNA initiation of replication. C-terminal deletion mutants showed an enhanced dimer formation capacity. The highly acidic tail, removed in these mutants, is proposed to modulate the protein p6 self-association.

DNA transactions, such as DNA replication and transcription, require higher-order DNA-protein complexes, the assembly of which is sometimes facilitated by proteins with an architectural function. This is the case of the nucleoid-associated proteins, of which the ones most extensively studied are HU, H-NS, IHF, and FIS. They all bind and bend DNA, sharing some common characteristics such as being very abundant, having a small size, and playing a pleiotropic role. The function of these proteins requires formation of dimers or oligomers. Thus, HU is a homodimer in most bacteria, although it is a heterodimer in Escherichia coli (1, 2), IHF is a heterodimeric protein encoded by two genes, himA (3) and hip (4), and FIS is a homodimer (5, 6). On the other hand, H-NS monomers undergo self-association to form tetramers (7). The N-terminal domain of H-NS is involved in oligomerization (8, 9), and the oligomeric structure of H-NS is necessary for recognition of intrinsically curved DNA and bending (10).

Protein p6 of Bacillus subtilis phage Ø29 is required in vivo for viral genome replication (11, 12) and repression of transcription from the early promoter C2 (13). In vitro studies have shown that protein p6 is involved both in replication and transcription; it stimulates the initiation and the transition to elongation steps of Ø29 DNA replication (14, 15), represses the early C2 promoter (16), and regulates the switch between early and late transcription (17). These functions are accomplished by the formation of a nucleoprotein complex in which the DNA adopts a right-handed toroidal conformation, and thus wrapping around a multimeric protein p6 core (18).

The number of copies of protein p6 in B. subtilis cells at late times of Ø29 infection has been calculated to be 6.6 × 10^3, enough to bind the entire viral progeny DNA (19). This, together with the ability to bind in vitro to the whole Ø29 genome, led us to propose a structural role in compacting and organizing the viral genome (20). The amounts of other histone-like proteins are cell cycle-dependent; thus, for E. coli cells in logarithmic growth, the most abundant ones are FIS and HU, with 1.2 × 10^4 and 1.5 × 10^5 copies per cell, respectively, whereas at stationary phase the most abundant is IHF with 1.2 × 10^4 copies per cell (21). With these amounts, only a minor part of the bacterial genome would be bound by these proteins. Sedimentation equilibrium studies have shown that protein p6 is in a monomer-dimer equilibrium that shifts to higher association states at the millimolar concentrations found in vivo (19). These oligomeric structures have been observed by transmission electron microscopy (22), and their structure, as deduced by image processing, is compatible with that described for the path followed by the DNA in the protein p6-DNA complex (18). Thus, it has been proposed that protein p6 could act as a scaffold organizing the DNA into the appropriate configuration. Protein p6 binding to DNA is highly cooperative and extends throughout the whole Ø29 DNA forming multiple complexes of very heterogeneous sizes; the minimal size ranges from ~130 base pairs (bp) observed by electron microscopy after psoralen cross-linking, to ~80–90 bp, shown by protection of micrococcal nuclease digestion (20). Thus, multiple protein-DNA and protein-protein interactions are required to stabilize the complex, suggesting that association equilibria among protein p6 oligomers would modulate their interaction with DNA.

In this work we have searched regions involved in protein p6 self-interaction. The characterization of deletion mutants has

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2 The abbreviations used are: bp, base pairs(s); OD, oligomerization domain; DBD, DNA binding domain; wt, wild-type; pfu, plaque-forming units; PEI, polyethyleneimine; M̄, apparent weight-average molecular weight; IPTG, isopropyl β-D-thiogalactopyranoside; PAGE, polyacrylamide gel electrophoresis.
Residues Involved in Dimer Formation in Phage Φ29 Protein p6

λ Phage Development—E. coli 71-18 lacI<sup>+</sup> cells transformed with plasmid pBF21 or the derivatives described above, were grown at 37 °C up to an optical density of 0.6 at 620 nm in LB medium containing ampicillin (100 μg/ml). The production of chimeric proteins was induced by addition of 250 μl of 100 mM IPTG to 0.4 ml of culture. After induction, IPTG was still present in the culture essentially as described (10). After 5-min incubation, 5 ml of LB medium containing ampicillin (100 μg/ml) was added to the infected cultures, which were further incubated at 37 °C. At the indicated times, 0.4-ml aliquots were centrifuged and 150 μl of supernatant was mixed with 30 μl of chlororm. The number of plaques forming units per milliliter in the induced lysate was determined using E. coli 71-18 lacI<sup>+</sup> as the recipient strain.

Site-directed Mutagenesis in Gene 6—Site-directed mutants of wt gene 6 were constructed by PCR using plasmid pPR55w6 (23) as template. As primers, we have used four oligonucleotides with degenerated positions, to obtain mutations in amino acids 7, 8, 43, and 44 of protein p6 (5'-CGTTGTCCTGTGTTTTGCTCCGGTA-GC-3', 5'-CCATCGATGCTTTTGCCGTA-3', 5'-GCAATGAGCGGACTATGAGG-3', and 5'-GCACTGACGCCAAGGC-3'). Two oligonucleotides 5'-CGAATGAGCGGACTATGAGG-3' and 5'-CTAACTAGCTAGCTATGAGG-3' were used to generate BamHI and HindIII restriction sites to clone the fragments containing mutated gene 6 into plasmid pBLC28 (28) under the control of λ P<sub>I</sub> promoter. The lysogenic strain E. coli K-12 H1 <sub>1</sub> carrying the cI<sub>A</sub> fusion (cIp6m) was electroporated with the recombinant plasmids to obtain a library of random mutants. The mutations were determined by sequencing the corresponding gene 6 in selected clones from the library.

**Experimental Procedures**

**Materials**—Isopropyl β-D-thiogalactopyranoside (IPTG) and ampicillin were obtained from Sigma. Restriction enzymes (HindIII, EcoRv, BamHI, and DraI), Vent polymerase, and T4-polymerase kinase were obtained from New England BioLabs, Tag polymerase from Perkin-Elmer, oligonucleotides from Genset Oligos, dNTP from Amersham Pharmacia Biotech, (α<sup>32</sup> P)dATP (3000 Ci/mmol) and (γ<sup>32</sup> P)dATP (3000 Ci/mmol) from Amersham International plc. Glutaraldehyde was purchased from Serva.

**Construction of Phage λ cI Repressor Fusions**—The plasmid pBF21, containing the λ cI gene under the control of a tandem lacUV5 promoter-operator region (10), was digested with HindIII and EcoRv to remove the cI gene fragment encoding the oligomerization domain (OD) (Fig. 1). Plasmids pAcLP6 and pAcLP6m were made by a polymerase chain reaction (PCR) step cloning procedure. Wild-type (wt) gene 6, cloned in plasmid p6N<sub>13</sub>, and mutant gene 6R6A, from plasmid pPR55R6A (23), were obtained by PCR using primers designed to introduce HindIII and EcoRv restriction sites 5'-GAAAGTGGAGAAGGCTTATGCGA-3' and 5'-GCTTACTGCTGTGATCATCATCATTGGACG-3', respectively. PCR products were used as templates to amplify, 0.2 μg of pB5556 or pB5556A as templates, 100 μl dNTP, and 2 units of Vent polymerase on its reaction buffer. PCR fragments were cloned into digested pBF21, to generate plasmids pAcLP6 and pAcLP6m, containing chimeric genes encoding for the DNA binding domain of the cI repressor, cI(DBD), fused to those encoding for p6wt protein or to mutant protein p6R6A, respectively (Fig. 1). The wt and mutant gene 6 were sequenced from the plasmids.

**Random Mutagenesis in Gene 6**—Random mutagenesis of R6A single mutant gene 6 was performed on plasmid pAcLP6m. The oligonucleotides used generated HindIII and EcoRv restriction sites (5'-GGCTC-CAAGGCAGACTTTTAG-3' and 5'-GGAGAATGGCAGATCTAC-3'). To obtain random mutations, PCR was carried out under two different conditions: one had limited DAP (20 μl) and the other had addition of 0.5 mM MnCl<sub>2</sub> (24, 25). Thus, the reactions contained 1 μl oligonucleotides; 0.2 μg of plasmid; 1.25 mM MnCl<sub>2</sub>; 5 units of Taq polymerase; 100 μM each of dATP, dCTP, dGTP, and dTTP; and 0.5 μg of terminal DNA polymerase in the absence or in the presence of 0.5 mM MnCl<sub>2</sub> in Taq polymerase buffer. The mutated fragments were digested with HindIII and EcoRv and cloned into plasmid pBF21 digested with the same enzymes. E. coli 71-18 lacI<sup>+</sup> cells (26) were transformed with these constructions and tested for λ phage development as described below. The mutated gene 6 was sequenced in those clones showing a lytic phenotype.

**Initiation of DNA Replication Assay**—The reaction mixture contained (in 25 μl) 50 mM Tris-HCl, pH 7.5; 10 mM MgCl<sub>2</sub>; 20 mM ammonium sulfate; 1 mM dithiothreitol; 10% glycerol; 0.25 μM (α<sup>32</sup> P)dATP; 0.1 g of DNA; 25 μl of λ phage DNA (42, 43); and 2 mg/ml terminal DNA polymerase, as described (31); 20 ng of terminal protein; 20 ng of λ DNA polymerase; and the indicated amounts of either protein p6wt or protein p6 mutants. After incubation for 15 min at 15 °C, the reaction was stopped by adding up to 10 μl EDTA and 0.1% SDS. The samples were filtered through Sephadex G-50 spin columns in the presence of 0.1% SDS. The initiation complex formed was analyzed by SDS-PAGE as described (31).
RESULTS

Self-association of Protein p6 Deletion Mutants—It has been reported that N-terminal deletion mutants of protein p6 lacking 5 or 13 amino acids (p6NΔ5 and p6NΔ13, respectively) were not able to interact with a Ø29 DNA terminal fragment as detected by DNase I footprinting (29). Because DNA binding is highly cooperative and requires the formation of large oligomers, the lack of DNA binding could be due to a deficiency in protein p6 self-association; therefore, we tested by glutaraldehyde cross-linking the capability of both deletion mutants to self-associate. Fig. 2 shows that dimer formation was highly impaired in the p6NΔ5 mutant, being about 10% that of the p6wt protein. In addition, dimers were not detected with the p6NΔ13 mutant (not shown). On the other hand, a 14-amino acid C-terminal deletion mutant of protein p6 (p6CΔ14) bound DNA 2-fold better than the p6wt protein (30). Two C-terminal deletion mutants of 14 and 16 amino acids (p6CΔ14 and p6CΔ16, respectively) were tested for dimer formation by glutaraldehyde cross-linking. As Fig. 2 shows, the ability of dimer formation in the p6CΔ16 mutant protein was about 2-fold that of the p6wt protein; a similar result was obtained with the mutant protein p6CΔ14 (not shown). Therefore, the observed DNA binding properties of p6NΔ5, p6NΔ13, p6CΔ14, and p6CΔ16 mutants could be explained by their self-association capacities. These results indicate that the N-terminal region of protein p6 is required for glutaraldehyde cross-linking, suggesting that this region is involved in dimer formation; however, glutaraldehyde cross-linking is favored when the C-terminal region is removed, suggesting that this region impairs dimer formation.

Self-interaction of Protein p6 in Vivo—To further investigate the protein p6 regions involved in self-association, we carried out an in vivo assay in which we used the region encoding the N-terminal domain of λ cl repressor as a reporter gene for dimerization (10). Phage λ cl repressor is a two-domain protein that binds DNA as a dimer; the N-terminal part is the DNA binding domain, cl(DBD), whereas the C-terminal one is the oligomerization domain, cl(OD), which is required for efficient binding to the operator. Thus, E. coli cells expressing only cl(DBD) (Δcl) were sensitive to hypervirulent phage λ146 infection, whereas those with intact cl were immune. Replacement of the cl(OD) by another protein provides a self-interaction assay for that protein.

We have used the plasmid pBF21 (10), expressing the λ phage cl repressor under the control of the lac promoter, to construct pΔclp6 plasmid encoding a fusion protein containing the Δcl and the protein p6wt (Fig. 1). The dimerization capacity of protein p6 was assayed by determining the development of...
infecting λ146 phage in E. coli cells harboring the pΔlp6 plasmid upon induction with IPTG. Cells expressing the Δlp6 fusion protein were not immune to λ146 infection (Fig. 3), behaving as the control cells expressing ΔcI. This result was unexpected, because protein p6 self-interacts in vitro. The expression of Δlp6 fusion protein was confirmed by Western blot analysis with protein p6 antiserum (not shown). An explanation for the lytic phenotype could be that the non sequence-specific DNA binding ability of protein p6 (37) prevented the binding of the ΔcI to the operators. To avoid this, plasmid pΔlp6m (see Fig. 1) was constructed, expressing a fusion containing the ΔcI and the protein p6 mutant defective in DNA binding p6R6A (38). Cells harboring the plasmid pΔlp6m showed, upon induction, a time course development of λ146 phage similar to that of the control cells expressing intact repressor. In these cases, the number of pfu/ml was about 10^4-fold lower than that obtained in cells expressing ΔcI. Therefore, we can conclude that the mutant protein p6R6A functionally replaces the cI(OD) allowing dimerization of the ΔcI. This strongly suggests that protein p6 self-associates in vivo.

Random Mutagenesis of Gene 6 and in Vivo Selection of Protein p6 Mutants Defective in Self-interaction—The in vivo assay described above sets up the basis to select mutants in protein p6. Basic and acidic regions of the proteins are pointed out.

Residues Involved in Dimer Formation in Phage Ø29 Protein p6—

The entire nucleotide sequence of the mutated gene 6 was determined from the 35 lytic clones. Four of them had a premature stop codon. Most of the mutants had more than three amino acid changes, and only five carried double or triple mutations as shown in Fig. 5. Amino acid sequence comparison with the p6R6A protein (p6m) showed that the mutations were mainly clustered in two regions: N-terminal, around position 8 (region I, Fig. 5), and a central region around position 44 (region II, Fig. 5), like those in p6m8 and p6m22 mutant proteins, and a central region around position 44 (region II, Fig. 5), like p6m23 and p6m41 mutant proteins. Mutant protein p6m94 carried a single mutation in each region.

Residues Involved in Self-association of Protein p6 in Vitro—

The non-random distribution of in vivo selected mutants strongly suggested the involvement of regions I and II in protein p6 self-interaction. To assess directly the involvement of individual residues in protein-protein interaction, we designed site-directed mutations on positions 7, 8, 43, and 44, where wt residues were replaced by those found in mutants selected in vivo (Fig. 5), namely E7V, I8T, Q43R, and A44V. The site-directed mutagenesis was performed by PCR on plasmid pPR55w6, encoding wt gene 6 (23). The mutated genes were cloned into an expression vector under the control of the bacteriophage λ P_{L} promoter. Mutants were overproduced and purified up to at least 90% homogeneity. Protein p6 mutants were tested for activation of initiation of phage Ø29 DNA replication in an in vitro assay with purified proteins (14). Fig. 6 shows the results obtained with the mutant proteins p6E7V, p6I8T, p6Q43R, and p6A44V. Although the mutants p6E7V and p6Q43R showed an activation similar to that of the p6wt protein, the p6A44V mutant protein exhibited a 6-fold reduced capacity to activate the initiation reaction. No activation was detected by the p6I8T mutant. The deficiency observed in the activation of the initiation reaction in p6A44V and p6I8T mutant proteins could be due to an impaired self-association and/or DNA binding. Because we have selected these mutants in an in vivo self-association assay, we first studied the dimerization properties of the protein p6 mutants. The effect of the p6A44V and p6I8T mutations in self-interaction was tested, as a first approach, by glutaraldehyde cross-linking. The amount of dimers formed by the p6A44V and p6I8T mutant proteins was 50% and 7%, respectively, of that found in the p6wt protein (Fig. 7).

![Fig. 4. In vivo dimerization assay for random mutants of protein p6R6A.](image-url)

![Fig. 5. Amino acid sequence of random mutant p6 proteins.](image-url)
These results prompted us to further study the self-interaction capability of the protein p6 mutants by analytical ultracentrifugation. Sedimentation equilibrium studies had shown that protein p6, in the 1–100 µM range, is in a monomer-dimer equilibrium with a dimerization constant ($K_d$) of $2 \times 10^5$ M$^{-1}$ that shifts to higher association states at higher protein concentration (19). Fig. 8 shows that, at 100 µM and 4 °C, the $M_w,a$ of protein p6wt was 25,400, whereas those of p6A44V and p6I8T mutant proteins were 20,600 and 15,500, respectively, the latter being close to the theoretical value for the wt protein p6 monomer (11,900). Assuming that mutant proteins have the same self-association behavior as p6wt (19), the dimerization constant of p6A44V is slightly lower than that of the p6wt$^3$; however, the $K_d$ of p6I8T is, at least, 10-fold lower than that of the p6wt protein.$^3$ Therefore, we can conclude that A44V mutation slightly affects protein dimerization, which is almost abolished by the mutation I8T.

Protein p6 forms a nucleoprotein complex with phage Ø29 DNA terminal fragments, in which the DNA wraps around a multimeric protein core (39). The formation of the complex can be detected by a characteristic DNase I footprint pattern, in which strong hypersensitivities, with a periodicity of 24 bp, are located in between protected regions (36). As shown in Fig. 9, protein p6A44V required a 2.5-fold higher protein amount (0.5 µg) than protein p6wt (0.2 µg) to form the complex; this may reflect the reduced capacity to form dimers of p6A44V mutant. In addition, we can observe that the digestion pattern formed with p6A44V is slightly different than that observed with p6wt. As it could be expected from the previous results, p6I8T mutant protein failed to bind DNA even at a 20-fold higher protein concentration than that of the p6wt (Fig. 9).

**DISCUSSION**

Protein p6 of *B. subtilis* phage Ø29 binds DNA forming a multimeric nucleoprotein complex (39) that is required for activation of Ø29 DNA initiation of replication (37, 38), repression of transcription from early C2 promoter (13, 16), and regulation of viral switch between early and late transcription (17). Protein p6 in solution forms elongated oligomers from preformed dimers, at the *in vivo* estimated protein concentration (19). Oligomeric structures have been proposed to provide the scaffold on which the DNA folds into the appropriate configuration. Protein p6 binding to DNA requires multiple protein-DNA and protein-protein interactions. Thus, failure to bind DNA of N-terminal deletion mutants of 5 or 13 amino acids in protein p6 (29), can be explained by their impaired or lost capacity, respectively, in dimer formation.

We have looked for other regions involved in protein p6 self-association by using an *in vivo* system based on the immunity of cells expressing λ cl repressor to λ phage infection. If the λ cl(OD) is removed, immunity is lost and the infecting phage can develop lytic cycle. Thus, replacement of the cl(OD) by another protein provides a self-interaction test for this protein. When Δcl was fused to the gene encoding p6R6A mutant protein, immunity was achieved, suggesting that the protein self-interacts *in vivo*. Thus, random mutants of protein p6R6A, obtained by degenerated PCR, were tested for self-association. Although the proteins contained multiple mutations, they were not randomly arranged, but mainly clustered in two regions located at the N-terminal and the central region of protein p6 (regions I and II in Fig. 5). Therefore, single site-directed mu-

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$^3$ G. Rivas, personal communication.

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**FIG. 6.** Activation of initiation of Ø29 DNA replication by wt protein p6 and site-directed mutants. Formation of the initiation complex (TP-dAMP) in the presence of the indicated amount of wt protein p6 or site-directed mutants p6E7V, p6I8T, p6Q43R, or p6A44V.

**FIG. 7.** Dimer formation of protein p6wt and site-directed mutants. Glutaraldehyde cross-linking of protein p6wt and single mutants p6A44V and p6I8T. Proteins were cross-linked as described under “Experimental Procedures” and run in SDS-Tricine-PAGE, along with untreated samples. The mobilities of molecular mass standards are indicated.

**FIG. 8.** Sedimentation equilibrium analysis of protein p6wt and site-directed mutants. Sedimentation equilibrium profile of 100 µM protein p6wt (○), p6A44V (□), and p6I8T (△) taken at 25,000 rpm and 4 °C as described under “Experimental Procedures.” The symbols represent the experimental data, and the solid lines show the best fit functions for a single solute, at sedimentation equilibrium. The $M_w,a$ for each protein is indicated.
tants of protein p6wt corresponding to some of those found in vivo were constructed and assayed in vitro. Taking into account that the in vivo selected mutant proteins p6m23 and p6m41 (Fig. 5) share the Q43R mutation, we would expect an involvement of Gln43 in protein p6 self-interaction. However, this was not confirmed by in vitro experiments with the corresponding site-directed mutant. The mutant protein p6Q43R did not show any significant difference with the wt protein in the activation of Ø29 DNA initiation of replication or glutaraldehyde cross-linking. A possible explanation for this result could be that the in vivo assay is more restrictive. In addition, the single mutation may not be enough to show an impaired dimerization, and additional changes might be required, like those found in the in vivo selected mutants.

The involvement of region II in dimer formation is shown by p6A44V mutant protein, which has a slightly reduced capacity to form dimers. In agreement with this, DNase I footprinting (Fig. 9) shows that p6A44V has a DNA binding affinity lower than that of the p6wt protein and an impaired activation of Ø29 DNA initiation of replication.

The involvement in dimer formation of region I (see Fig. 5) was expected from the results obtained with protein p6 N-terminal deletion mutants. The p6I8T mutant protein failed to activate the Ø29 DNA initiation of replication, DNA binding was not detected, and dimer formation was reduced at least 10-fold. Mutant p6 proteins are folded, as shown in Fig. 10, where the circular dichroism spectra of the wt and mutant p6 proteins do not show significant differences in their secondary structure. Altogether, these results suggest that Ile8 is involved in the self-interaction of protein p6.

It has been reported that a C-terminal, 14-amino acid truncated p6 protein has a DNA binding activity 2-fold higher than that of the p6wt protein (30). We have now shown that this truncated protein has increased its capacity to form dimers. Protein p6 has a very acidic C terminus, where 10 out of 19 residues are either Asp or Glu (see Fig. 5). Therefore, it seems likely that the C-terminal acidic region modulates protein p6 self-association, interacting with a basic region. There is a cluster of basic residues overlapping region II (see Fig. 5), although interaction with region I, which also contains basic residues, cannot be ruled out. This interaction could contribute to the dynamic nature of the protein p6 binding to DNA (20).

In summary, we have found two regions involved in vivo in self-association of phage Ø29 protein p6, the N-terminal and a central region. Site-directed mutation A44V, located at the central region, impairs the protein p6 self-association. The N-terminal mutation I8T has a more drastic effect and completely abolished dimer formation.

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