The EMBO Journal vol.8 no.6 pp.1879 – 1885, 1989

Signals at the bacteriophage ϕ29 DNA replication origins required for protein p6 binding and activity

Manuel Serrano, Julio Gutiérrez[1], Ignacio Prieto[1], José M.Hermoso and Margarita Salas

Centro de Biología Molecular (CSIC-UAM), Universidad Autónoma, Canto Blanco, 28049 Madrid, Spain

[1]Present address: Antibióticos, SA, Bravo Murillo 38, 28003 Madrid, Spain

Communicated by M.Salas

Protein p6 of *Bacillus subtilis* phage ϕ29 binds specifically to the ends of the viral DNA that contain the replication origins, giving rise to a nucleoprotein structure. DNA regions recognized by protein p6 have been mapped by deletion analysis and DNase I footprinting. Main protein p6-recognition signals have been located between nucleotides 62 and 125 at the right ϕ29 DNA end and between nucleotides 46 and 68 at the left end. In addition, recognition signals are also present at other sites within 200–300 bp at each ϕ29 DNA end. Protein p6 does not seem to recognize a specific sequence in the DNA, but rather a structural feature, which could be bendability. The formation of the protein p6–DNA nucleoprotein complex is likely to be the structural basis for the protein p6 activity in the initiation of replication.

Key words: DNA bendability/DNase I footprint/protein–DNA interaction/protein-primed replication

Introduction

The *Bacillus subtilis* phage ϕ29 has a linear, double-stranded DNA with the viral terminal protein p3 covalently linked to both 5′ ends. Replication is initiated at the genome ends by a protein-priming mechanism in which a terminal protein–dAMP complex is formed, catalysed by the ϕ29 DNA polymerase p2. This initiation complex is then elongated by the viral DNA polymerase displacing the non-template strand (reviewed in Salas, 1988).

The viral protein p6, required for ϕ29 DNA replication *in vivo* (Mellado *et al.*, 1980), is very abundant in ϕ29-infected cells (Carrascosa *et al.*, 1976), suggesting that the protein has a structural role. In an *in vitro* minimal replication system in which only terminal protein and ϕ29 DNA polymerase are required to synthesize full-length ϕ29 DNA (Blanco and Salas, 1985), highly purified protein p6 greatly stimulates the formation of the p3–dAMP initiation complex (Pastrana *et al.*, 1985; Blanco *et al.*, 1986) as well as the transition from the initiation complex to the first elongation products (Blanco *et al.*, 1988). By DNase I footprinting it has been shown that protein p6 specifically recognizes terminal ϕ29 DNA fragments and forms a nucleoprotein structure in a co-operative way (Prieto *et al.*, 1988). The specific binding of protein p6 to ϕ29 DNA terminal fragments is stable at 100–125 mM NaCl (Prieto *et al.*, 1988); at this concentration the stimulation of replication by protein p6 is maximal (Blanco *et al.*, 1988). It has been also demonstrated that the binding of protein p6 to closed circular plasmids alters the DNA conformation restraining positive supercoiling (Prieto *et al.*, 1988). Furthermore, the amount of protein p6 needed for the stimulation of replication is similar to that required for DNA binding.

The availability of recombinant plasmids containing the ϕ29 DNA terminal sequences (Gutiérrez *et al.*, 1986, 1988) enabled us to map the DNA regions required for specific binding of protein p6 and to correlate this binding with the function of the protein in the initiation of ϕ29 DNA replication.

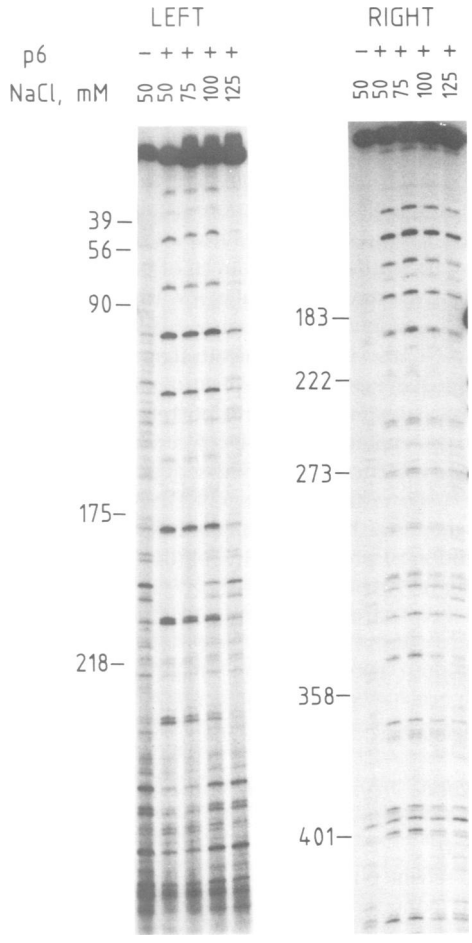

Fig. 1. DNase I footprint of protein p6 to ϕ29 DNA terminal fragments. *Ssp*I D fragment of 651 bp from the right ϕ29 DNA end and *Dra*I K fragment of 468 bp from the left ϕ29 DNA end were labelled at the internal 5′ end with [γ-^{32}P]ATP using polynucleotide kinase. DNase I footprinting was as described in Materials and methods, in the absence or presence of protein p6 and different NaCl concentrations as indicated. Nucleotide positions from either ϕ29 DNA end are also indicated.

Results

Binding of protein p6 to ϕ29 DNA terminal fragments

DNase I footprinting of protein p6 to ϕ29 DNA terminal fragments showed a pattern of hypersensitive bands regularly spaced 24 bp flanking protected regions, indicating the formation of a nucleoprotein structure (Prieto *et al.*, 1988). To determine how far this structure extends inside the genome and its stability along the DNA, we have assayed ϕ29 DNA terminal fragments of 651 bp from the right end and 468 bp from the left end at different salt concentrations. Figure 1 shows that the protein p6 DNase I footprint pattern of hypersensitive bands and protected regions becomes weaker beyond position ~200 from both ϕ29 DNA ends, especially at high salt concentrations.

DNA regions from the right and left ϕ29 DNA ends required for protein p6 binding

To map the DNA regions recognized by protein p6 we have performed DNase I footprinting assays with DNA fragments containing cloned ϕ29 terminal sequences of different lengths from previously obtained recombinant plasmids or replicative forms of M13 derivatives, shown in Figure 2 (Gutiérrez *et al.*, 1986, 1988). Figure 3A shows the results obtained with M13 DNA-derived fragments containing 25, 40 or 125 bp from the right end of ϕ29 DNA. The presence of protein p6 did not change the DNase I digestion pattern with fragments containing 25 or 40 bp from the right ϕ29 DNA end. The same result was obtained with the fragment containing only the M13 sequences. However, protein p6 changed the DNase I digestion pattern of the fragment containing 125 bp from the right ϕ29 DNA end giving rise to the specific salt-resistant pattern of protected regions and hypersensitive bands. This pattern was observed only in the ϕ29 DNA sequences, not being extended into the M13 sequences. These results indicate that the ϕ29 DNA right terminal sequences between positions 40 and 125 contain recognition signals for protein p6 binding.

Fragments containing ϕ29 DNA terminal sequences from the left end attached to pBR322 sequences were also used. Figure 3B shows that with a ϕ29 DNA terminal sequence of 46 bp there was no change in the DNase I digestion pattern after addition of protein p6. In contrast, a similar fragment containing 68 bp produced the specific footprint pattern. In this case the pattern extended through the plasmid sequences along most of the fragment used, though the protections disappeared at 100 mM NaCl. These results indicate that the ϕ29 DNA left terminal sequences between positions 46 and 68 contain recognition signals for protein p6 binding.

DNA regions from the right and left ϕ29 DNA ends required for protein p6 activity in the initiation of replication

We have recently reported that terminal protein-free DNA fragments containing the terminal 12 bp from either ϕ29 DNA end are active templates for *in vitro* protein p3-primed replication (Gutiérrez *et al.*, 1988). To look for a correlation between the DNA binding data and the activity of protein p6 in the initiation of ϕ29 DNA replication, the DNA fragments used to study protein p6 binding were assayed as templates in the *in vitro* replication system. Figure 4A shows that stimulation of p3–dAMP complex formation by protein p6 was obtained with DNA fragments containing 273 or

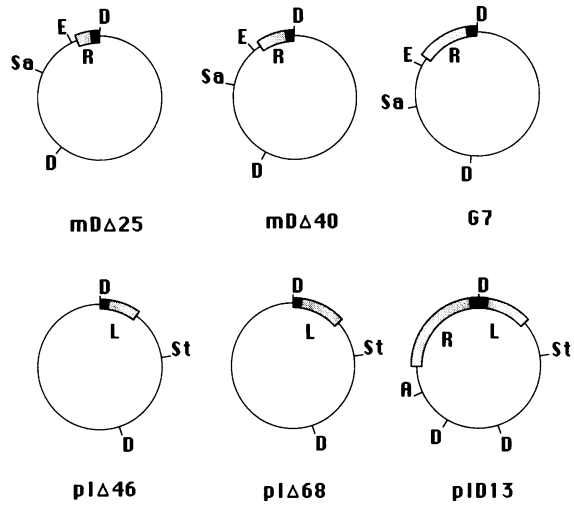

Fig. 2. Recombinant plasmids and replicative forms of M13 derivatives used in this work. M13mp19 derivatives mDΔ25 and mDΔ40 contain 25 and 40 bp from the ϕ29 DNA right end, respectively (Gutiérrez *et al.*, 1988). M13mp8 derivative G7 contains 125 bp from the ϕ29 DNA right end (Gutiérrez *et al.*, 1988). pBR322 derivatives pIΔ46 and pIΔ68 contain 46 and 68 bp from the ϕ29 DNA left end, respectively. pKK223-3 (Brosius and Holy, 1984) derived plasmid pID13 contains 273 and 73 bp from the ϕ29 DNA right and left ends, respectively (Gutiérrez *et al.*, 1986). Grey boxes, terminal sequences from the right (R) or left (L) ϕ29 DNA ends. Black boxes, 12-bp minimal replication origin (Gutiérrez *et al.*, 1988). Restriction enzyme target site abbreviations: A, *Ava*II; D, *Dra*I; E, *Eco*RI; Sa, *Sau*I; St, *Sty*I. Only the relevant sites are indicated. The sizes of the ϕ29 DNA fragments are amplified.

125 bp from the right ϕ29 DNA end, but not with that containing 40 bp, indicating that sequences between positions 40 and 125 at the right end of ϕ29 DNA are required for the protein p6 activity in initiation of replication, as for the specific DNA binding. Figure 4B shows a similar stimulation by protein p6 with fragments containing 73 or 68 bp from the left ϕ29 DNA end, whereas no effect could be seen with a fragment containing 46 bp. Therefore, sequences between positions 46 and 68 from the left end of ϕ29 DNA are also required for the stimulation of the initiation complex formation by protein p6, again correlating with the DNA binding data.

The effect of protein p6 on the transition step from initiation to elongation can be analysed by studying replication at high dATP concentration since, under these conditions, the initiation complex formation is not affected by protein p6 (Blanco *et al.*, 1986, 1988). To determine whether the stimulation of the transition step by protein p6 requires similar ϕ29 DNA terminal sequences as the initiation reaction, we have used the set of deletion-containing fragments as templates for replication. Figure 4C shows that replication of a fragment containing the terminal 68 bp from the left ϕ29 DNA end was strongly stimulated by protein p6. As in the initiation reaction experiments, essentially no stimulation by protein p6 was obtained for the replication of a fragment containing 46 bp from the left ϕ29 DNA end. The replication of fragments containing deleted right ϕ29 DNA terminal sequences could not be accurately studied due to the small size of most of them (500–600 bp) (Gutiérrez *et al.*, 1986, 1988). A particular DNA fragment 2122 bp long, containing the right terminal 125 bp from ϕ29 DNA, was assayed as a template for replication, and good stimulation by protein p6 was observed (not shown).

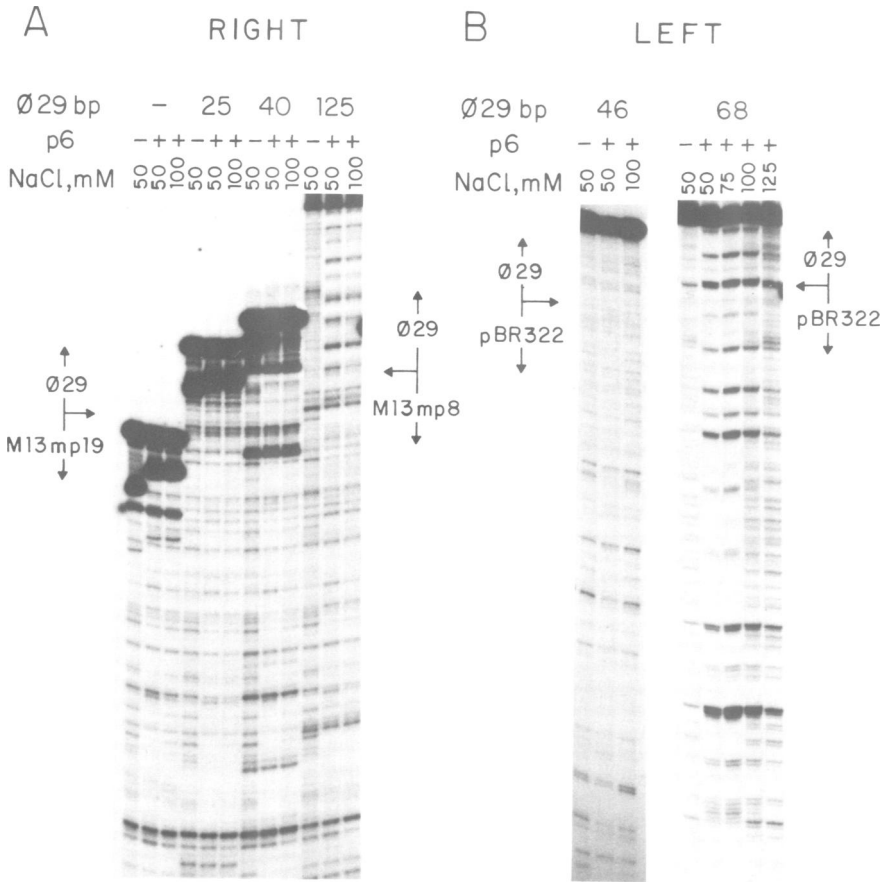

Fig. 3. DNase I footprint of protein p6 to DNA fragments containing ϕ29 DNA terminal sequences. (**A**) DNA fragments containing 25 and 40 bp from the right ϕ29 DNA end were obtained by digestion of mDΔ25 and mDΔ40, respectively with *Dra*I and *Sau*I (see Figure 2), and they also contained 241 bp from M13mp19. The fragment containing 125 bp from the ϕ29 DNA right end was obtained from G7 DNA after digestion with the same enzymes (see Figure 2), and also contained 252 bp from M13mp8. As a control, the fragment *Eco*RI−*Sau*I of 223 bp from M13mp19 was used. (**B**) DNA fragments containing 46 and 68 bp from the left ϕ29 DNA end were obtained from plasmids pIΔ46 and pIΔ68, respectively by digestion with *Dra*I and *Sty*I (see Figure 2), and also contained 345 and 433 bp from pBR322, respectively. All the fragments were labelled at their 3′ recessive ends with [α-^{32}P]dATP and the Klenow fragment. DNase I footprinting was performed as described in Materials and methods, in the absence or presence of protein p6 and different NaCl concentrations as indicated. The location of the M13, pBR322 and ϕ29 DNA sequences is also indicated.

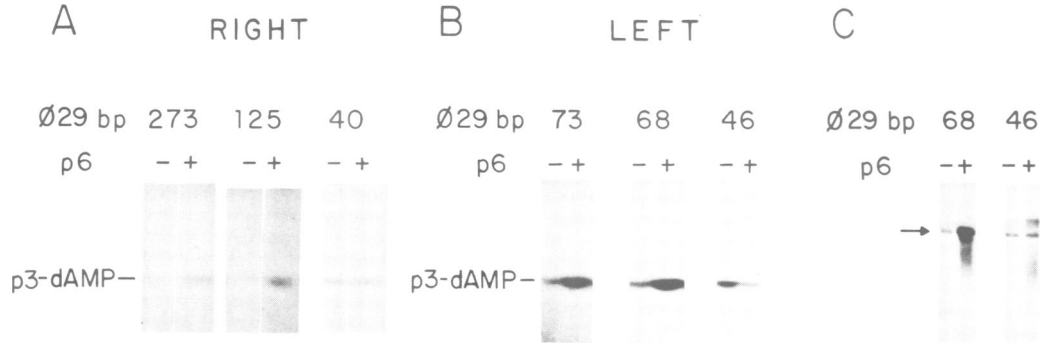

Fig. 4. Template activity of fragments containing ϕ29 DNA terminal sequences. (**A,B**) The protein-primed initiation of replication assay was performed as described in Materials and methods, in the absence or presence of protein p6. The position of the p3-dAMP initiation complex is indicated. The fragments used as templates were as follows. (**A**) DNA fragments containing ϕ29 DNA right terminal sequences of 273, 125 and 40 bp were obtained from pID13, G7 and mDΔ40 DNA, respectively by digestion with *Dra*I (see Figure 2), and also contained 720 bp from the pBR322-derived plasmid pKK223-3 (Brosius and Holy, 1984), 525 bp from M13mp8 and 514 bp from M13mp19, respectively. (**B**) DNA fragments containing ϕ29 left terminal sequences of 73, 68 and 46 bp were obtained from plasmids pID13, pIΔ68 and pIΔ46, respectively by digestion with *Dra*I (see Figure 2), and also contained 2856, 2296 and 2208 bp, respectively from pBR322. (**C**) The terminal protein-primed ϕ29 DNA replication assay was performed as described in Materials and methods, in the absence or presence of protein p6. The arrow shows the position of the input DNA fragments. Label not coinciding with the input fragments is due to replicative intermediates. The fragments used as templates contained ϕ29 DNA left terminal sequences of 68 and 46 bp, obtained as described in (B).

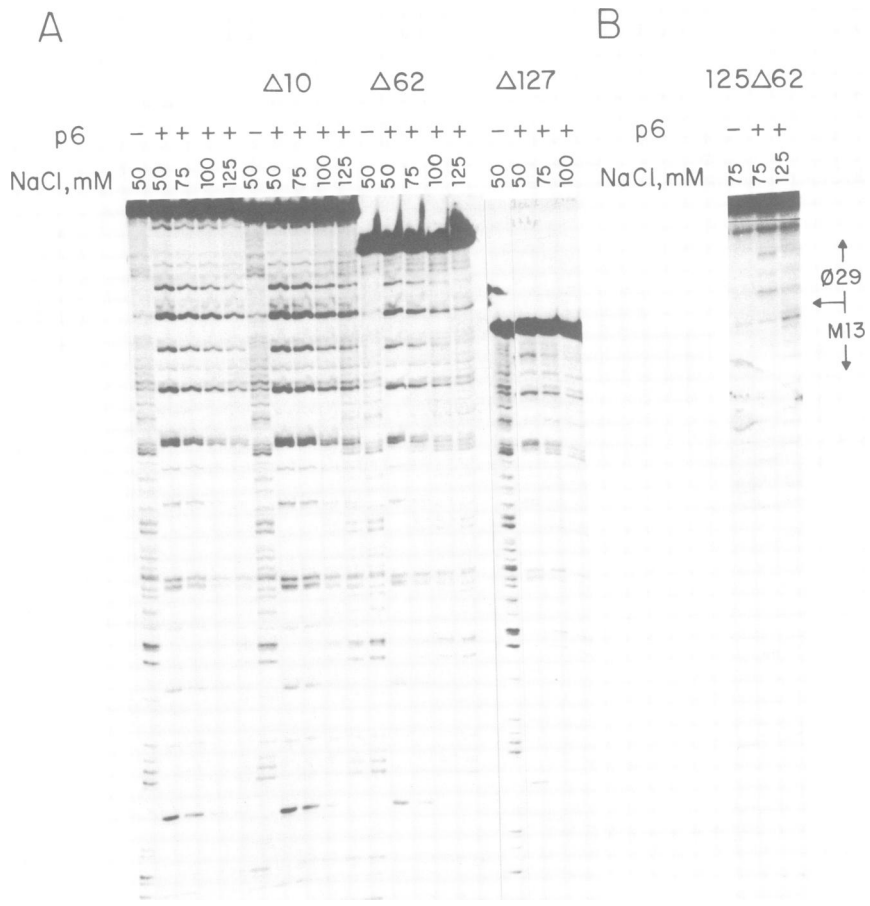

Fig. 5. DNase I footprint of protein p6 to DNA fragments containing deleted ϕ29 DNA right terminal sequences. (**A**) A DNA fragment containing 273 bp from the right ϕ29 DNA end was isolated from the recombinant plasmid pID13 by digestion with *Dra*I and *Ava*II (see Figure 2), and also contained 109 bp from the plasmid vector. The fragment was labelled with Klenow enzyme and [α-^{32}P]dATP at the 3′ recessive end corresponding to the plasmid, and deletions of 10, 62 and 127 bp were made in the ϕ29 sequences by digestion with *Rsa*I, *Hin*fI and *Acc*I respectively. (**B**) The *Dra*I–*Sau*I fragment derived from G7 DNA (see Figure 2), which contains 125 bp from the right ϕ29 DNA end and 252 bp from M13mp8, was digested with *Hin*fI to remove the ϕ29 DNA terminal 62 bp. The junction between ϕ29 and vector sequences is indicated in the figure. The DNA fragments were used for DNase I footprinting as described in Materials and methods, in the absence or presence of protein p6 and different NaCl concentrations as indicated.

Protein p6 binding to DNA fragments containing deleted ϕ29 DNA terminal sequences

After we had determined the minimal terminal regions of ϕ29 DNA required for protein p6 binding and activity, we addressed the question of the existence of additional protein p6 recognition signals further inside the genome by deletion of such minimal terminal sequences. Figure 5A shows DNase I footprints of protein p6 to the fragment of plasmid pID13 (see Figure 2) that contains the ϕ29 DNA right terminal 273 bp and 109 bp from the vector plasmid, and to the same fragment with the terminal 10, 62 and 127 bp deleted, respectively. The specific DNase I digestion pattern of protected regions and periodic hypersensitive bands was observed in all cases at 50 mM NaCl, and at the same positions as in the intact fragment. However, the binding of protein p6 became more salt sensitive as more terminal sequences were removed. Thus, the protections weakened at 125 mM NaCl in the intact fragment, at 100 mM NaCl in the 10 bp deletion, at 75 mM NaCl in the 62 bp deletion and were very low in the 127 bp deletion even at 50 mM NaCl. These results were also dependent on the amount of protein p6 used. When the protein p6 concentration was further increased, the 127 bp deletion fragment became fully protected at 50 mM NaCl (not shown). The hypersensitive bands were similarly salt resistant in the complete fragment and in the corresponding 10 bp deletion, but they started to fade at 100 mM NaCl in the 62 bp deletion and at 75 mM NaCl in the 127 bp deletion. Protein p6 binding was also analysed in a fragment containing the ϕ29 DNA right terminal sequences from positions 62 to 125. Figure 5B shows that protein p6 footprint to the ϕ29 DNA sequences had about the same salt sensitivity as the 62 bp deletion shown in Figure 5A. These results narrow the right ϕ29 DNA terminal region containing the main protein p6 recognition signals to between positions 62 and 125 bp. We have also tested fragments with longer deletions at the right ϕ29 DNA end with the general finding that the protein p6 interaction was weaker as the deletion increased. Even with a 273 bp deletion, at 50 mM NaCl, although all the DNase I hypersensitive bands were weak, they were located at the same positions as in the intact fragment (not shown).

Similar results were obtained using fragments containing deletions from the left ϕ29 DNA end. Figure 6 shows the protein p6 DNase I footprint to a 307 bp fragment from the left ϕ29 DNA end, and to the same fragment with terminal deletions of 57 and 77 bp respectively, the latter lacking the

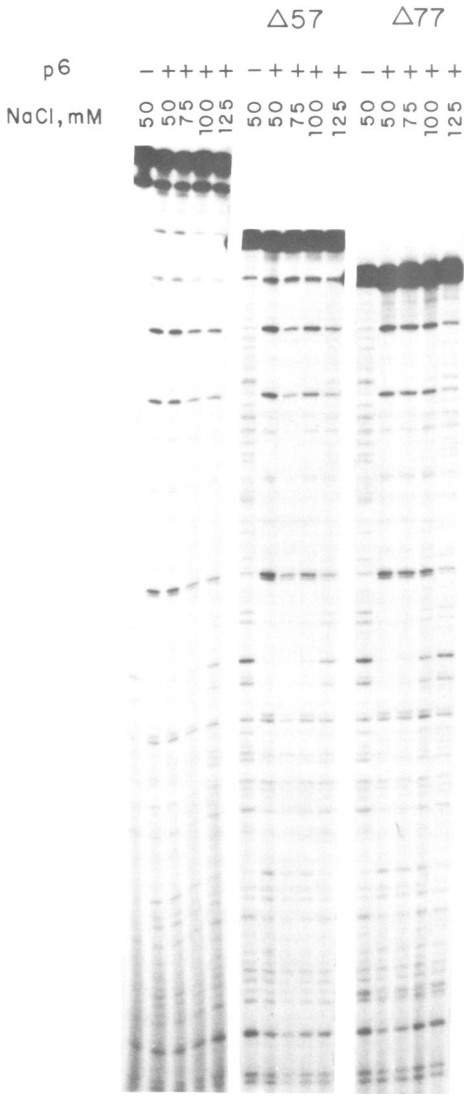

Fig. 6. DNase I footprint of protein p6 to deleted ϕ29 DNA left terminal fragments. The *Rsa*I T fragment of 307 bp from the left ϕ29 DNA end was labelled at the internal 5′ end with [γ-^{32}P]ATP and polynucleotide kinase. Deletions of the terminal 57 and 77 bp were made by digestion with *Taq*I and *Bcl*I respectively. DNase I footprinting was performed as described in Materials and methods, in the absence or presence of protein p6 and different NaCl concentrations as indicated.

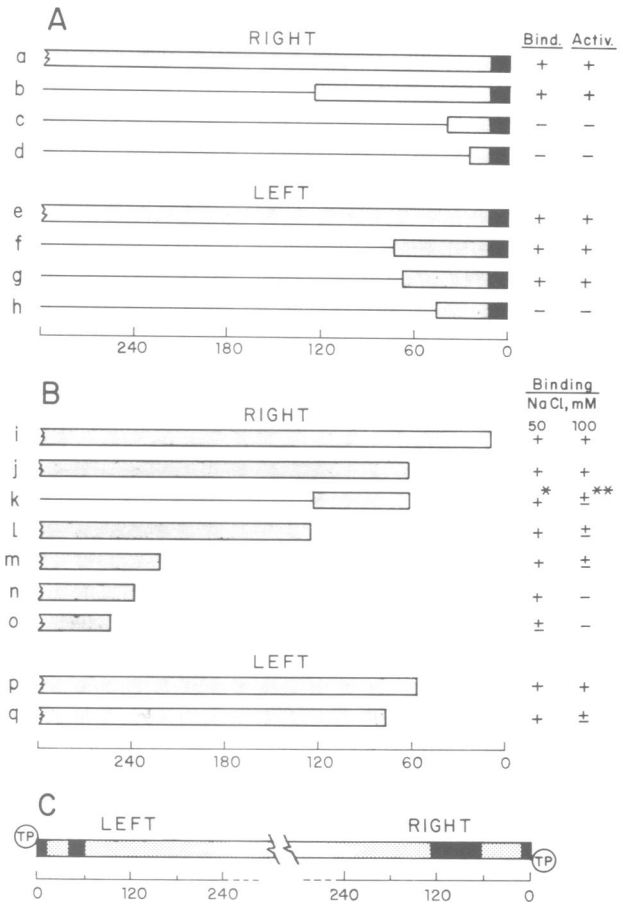

Fig. 7. Summary of protein p6 binding and activity with fragments containing ϕ29 DNA terminal sequences of different length. (**A**) Fragments containing right (**a**–**d**) or left (**e**–**h**) ϕ29 DNA sequences deleted from within the genome. The fragments contained the following ϕ29 DNA terminal sequences (grey boxes): (**a**) 273 bp; (**b**) 125 bp; (**c**) 40 bp; (**d**) 25 bp; (**e**) 307 bp; (**f**) 73 bp; (**g**) 68 bp; (**h**) 46 bp. Bind, protein p6 binding; Activ., protein p6 stimulation of the initiation reaction. Black boxes, 12 bp minimal replication origin (Gutiérrez *et al.*, 1988). Numbers indicate bp from the ϕ29 DNA ends. (**B**) Fragments containing right (**i**–**o**) or left (**p** and **q**) ϕ29 DNA terminal sequences deleted from without the genome. The fragments contained ϕ29 DNA terminal sequences (grey boxes) between the following positions: (**i**) 10–273 bp; (**j**) 62–273 bp; (**k**) 62–125 bp; (**l**) 127–273 bp; (**m**) 222–651 bp; (**n**) 239–651 bp; (**o**) 273–651 bp; (**p**) 57–307 bp; (**q**) 77–307 bp. *, **, binding at 75 and 125 mM NaCl respectively. Numbers indicate bp from the ϕ29 DNA ends. (**C**) Signals at the ϕ29 DNA ends involved in the initiation of replication. TP, terminal protein. Black boxes, minimal replication origins. Dotted, protein p6 binding region. Cross-hatched boxes, regions containing the main protein p6 recognition signals.

68 bp minimal region of protein p6 recognition previously mapped (Figure 4B). Both deleted fragments behaved as the control fragment, with hypersensitive bands located at the same positions, except that some of the protections disappeared at 125 mM NaCl in the 57 bp deletion and at 100 mM NaCl in the 77 bp deletion.

The above results indicate that, beyond the main recognition regions at both ϕ29 DNA ends, additional protein p6 recognition signals are present. Since the hypersensitive sites remain at the same positions in fragments containing the main recognition regions and in those with these regions deleted, all the recognition signals must be in phase.

Discussion

Protein p6 binds specifically to the phage ϕ29 DNA ends forming a nucleoprotein complex. We show here that this structure is extended through a region of ~200–300 bp, much longer than the terminal 12 bp of the minimal replication origins (Gutiérrez *et al.*, 1988). By deletion analysis we have determined the ϕ29 DNA terminal regions required for protein p6 recognition. The ability of the deleted fragments from both ϕ29 DNA ends to form the protein p6–DNA complex, as tested by DNase I footprinting, is summarized in Figure 7. Protein p6 does not bind to the terminal 40 or 46 bp from the right or left ϕ29 DNA ends, respectively (see Figure 7A), suggesting either the lack of recognition signals or an insufficient number of them if multiple signals are required for stable binding. Since protein p6 binds to fragments containing 125 or 68 bp from the right or left ϕ29 DNA ends, respectively, and to a 62 bp terminal

deletion of the first one (see Figure 7A and B), main recognition signals must be present between positions 62 and 125 from the right ϕ29 DNA end and between 46 and 68 from the left one. This is in agreement with previous results showing that the replication of a terminal protein-containing fragment of 59 bp from the right ϕ29 DNA end was not stimulated by protein p6, whereas the protein was active with a fragment 125 bp long (Blanco *et al.*, 1988).

Figure 7A also shows that there is a good correlation between the protein p6–DNA binding results and the protein p6 activity in the initiation of replication. This suggests that the protein p6–DNA nucleoprotein complex most probably constitutes the structural basis for the function of protein p6 in the initiation of ϕ29 DNA replication.

The possibility that a unique protein p6 recognition region near each ϕ29 DNA end could act as a nucleation site for the co-operative binding of protien p6 was ruled out by the results summarized in Figure 7B. Protein p6 binds to fragments lacking the terminal 127 or 77 bp from the right or left ϕ29 DNA ends, respectively, and, although rather weakly, to a fragment lacking the terminal 273 bp from the right end. These results indicate the presence of multiple recognition signals extending over a fairly long region, at least through 273 bp from the right ϕ29 DNA end. The strongest signals would be located closer to the ϕ29 DNA ends, as indicated by the stability of the protein p6 interaction with different fragments at high salt concentration (see Figures 3A and 5). These conclusions are summarized in Figure 7C. Another interesting finding is that the set of signals present at each ϕ29 DNA terminus must be phased, since the position of the DNase I hypersensitive sites in a terminal fragment is conserved in the fragments with sequences deleted from within or from without the genome ends. Furthermore, the phase is the same in the two termini, since the DNase I hypersensitive sites lie in the same relative positions at each ϕ29 DNA end (Prieto *et al.*, 1988).

The regular pattern of DNase I hypersensitive sites every ~24 bp is displaced in one strand with respect to the other (Prieto *et al.*, 1988), suggesting the location of protein p6 contact points every ~12 bp. In fact, by hydroxyl radical footprinting we have determined that protein p6 binds to regions between the DNase I hypersensitive sites with a periodicity of 12 (to be published elsewhere). We have performed a computer search for nucleotide sequence homology at the ϕ29 DNA terminal sequences (Escarmís and Salas, 1981; Yoshikawa *et al.*, 1981). Multiple alignments of 12 bp segments, in which the protein p6 contact points were centred, did not indicate the existence of even a degenerated repeated sequence. Thus, protein p6 probably does not recognize a specific sequence but rather a DNA structural feature. One of the best-characterized DNA structural features known to play an important role in protein–DNA interactions is bendability. DNA bendability has been studied particularly in regard to nucleosome formation (Satchwell *et al.*, 1986) and modulation of protein CAP binding (Gartenberg and Crothers, 1988), and algorithms have been developed that evaluate the tendency of each di- or trinucleotide to widen or narrow the DNA minor groove. We have used the fractional variations of occurrence values obtained by Satchwell *et al.* (1986) to estimate the local facility of the terminal 250 bp from each ϕ29 DNA end to widen (positive values) or narrow (negative values) the DNA minor groove. Figure 8 shows the patterns obtained with DNA sequences from positions 28 to 210 at

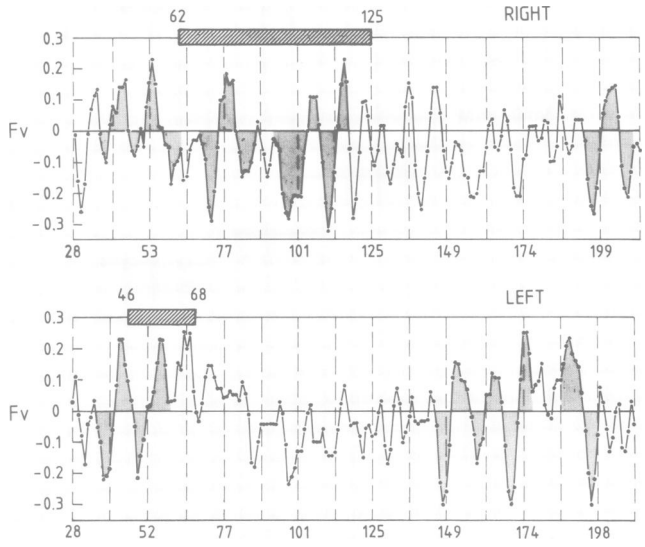

Fig. 8. Local bendability of the bacteriophage ϕ29 DNA ends. Fractional variation in occurrence values (Fv) (Satchwell *et al.*, 1986) corresponding to the average of three overlapped trinucleotides is represented against the bp position with respect to the ϕ29 DNA ends. Terminal sequences are divided by dashed lines into sets of 12-nucleotide segments in which the protein p6 contact points are centred. Cross-hatched boxes correspond to the main protein p6 recognition regions. Shadowed areas represent regions of alternating positive maxima and negative minima Fv values spaced 6 ± 1 nucleotides apart in which positive maxima lie in the left half and negative minima in the right half of the 12-nucleotide segments respectively.

both genome ends. It is noteworthy that the pattern within the major p6-recognition regions mainly consists of alternating positive maxima and negative minima spaced 6 ± 1 bp apart and lying with positive maxima in the first half of the 12 bp segments and negative minima in the second half. Because of the DNA double helix repeat, this motif implies a marked facility of the DNA to be bent every 12 bp, favouring the formation of a right-handed nucleoprotein solenoid, in which the DNA wraps around a protein core, in agreement with the restraining of positive supercoils when protein p6 binds to closed circular DNA (Prieto *et al.*, 1988). The pattern of phased alternating positive maxima and negative minima spaced 6 ± 1 bp apart is also present at more internal sequences; a particularly strong one is present between positions 145 and 178 from the left ϕ29 DNA end. Similar results were obtained when the bendability values of Gartenberg and Crothers (1988) were used. Therefore, bendability fulfils the main characteristics of the signal recognized by protein p6 on DNA: the existence of multiple recognition sites in phase and the absence of a repeated sequence. The structural restrictions imposed by protein p6 binding would account for the observed differences in the co-operative extension of the protein p6 footprint pattern from the ϕ29 sequences to the vector ones (see Figure 3).

The results presented here provide another example of the importance of conformational changes induced in DNA by proteins involved in the initiation of DNA replication (Mukherjee *et al.*, 1985; Borowiec and Hurwitz, 1988; Bramhill and Kornberg, 1988a; Schnos *et al.*, 1988). However, in contrast to other proteins involved in the initiation of DNA replication that recognize specific DNA sequences (Bramhill and Kornberg, 1988b), protein p6 seems to recognize a DNA structural property repeated in phase.

We propose that bendability could be the signal in the DNA recognized by protein p6.

Materials and methods

Chemicals and enzymes

Restriction endonucleases were from New England Biolabs or Boehringer Mannheim and were used according to the suppliers' instructions; polynucleotide kinase was from Boehringer Mannheim; *Escherichia coli* DNA polymerase I, Klenow fragment, was from New England Biolabs, DNase I was from Worthington and proteinase K was from Merck. [α-^{32}P]dATP (410 Ci/mmol) and [γ-^{32}P]ATP (>3000 Ci/mmol) were from Amersham International. Deoxynucleoside triphosphates were from P-L Biochemicals.

Recombinant plasmids and isolation of DNA fragments

Proteinase K-digested φ29 DNA was prepared as described (Inciarte *et al.*, 1976). The recombinant plasmid pID13, containing 73 and 273 bp from the left and right φ29 DNA ends, respectively, was as described (Gutiérrez *et al.*, 1986). The recombinant plasmids or replicative forms of M13 derivatives pIΔ68 and pIΔ46 containing 68 and 46 bp from the left φ29 DNA end, and G7, mDΔ40 and mDΔ25 containing 125, 40 and 25 bp from the right end, were as described (Gutiérrez *et al.*, 1988). A map of these plasmids is shown in Figure 2.

After the appropriate restriction endonuclease digestions of φ29 or plasmid DNA, the fragments to be tested in the protein p6 binding and replication assays were isolated by electrophoresis in gels containing 3.5% polyacrylamide in 0.1 M Tris−borate, pH 8.3, 1 mM EDTA, extracted from the gel and purified as described (Maniatis *et al.*, 1982). For DNase I footprinting experiments the purified DNA fragments were labelled with [α-^{32}P]dATP and Klenow enzyme or with [γ-^{32}P]ATP and polynucleotide kinase (Maniatis *et al.*, 1982).

DNase I footprinting

DNase I footprinting was carried out essentially as described by Galas and Schmitz (1978). Purified protein p6 (Pastrana *et al.*, 1985) was incubated with the labelled DNA fragment (~10 fmol) in 20 μl of a buffer containing 50 mM Tris−HCl, pH 7.5, 20 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 1 mM DTT, 1 mM spermidine and NaCl as indicated. Purified protein p6 (3.2 μg) was used in all the experiments, except that shown in Figure 2B in which a different purification stock of protein p6 was used that only required 1.9 μg of protein to give the same binding activity. 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% SDS, 5 mM EDTA, 0.15 M sodium acetate, pH 6.0, and 50 μg of carrier RNA. After ethanol precipitation the samples were subjected to electrophoresis in 6% polyacrylamide sequencing gels (Maxam and Gilbert, 1980) and autoradiographed.

Assays for the formation of protein p3−dAMP initiation complex and for protein p3-primed DNA replication

The incubation mixture for the initiation reaction contained, in 25 μl, 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 21 mM NaCl, 1 mM DTT, 1 mM spermidine, 0.25 μM [α-^{32}P]dATP (2.5 μCi), purified proteins p2, 25 ng (Blanco and Salas, 1984) and p3, 15 ng (Prieto *et al.*, 1984) and, when indicated, protein p6, 2.5 μg (Pastrana *et al.*, 1985). The DNA fragments used as templates (~6 fmol) are indicated in each case. After incubation for 10 min at 30°C, the reaction was stopped by addition of EDTA to 10 mM and heating for 10 min at 68°C, and the samples were treated with micrococcal nuclease as described (Peñalva and Salas, 1982). The unincorporated [α-^{32}P]dATP was removed by filtration through a Sephadex G-50 spun column in the presence of 0.1% SDS and the samples were subjected to SDS−PAGE as described (Blanco and Salas, 1985). The ^{32}P-labelled p3-dAMP was detected by autoradiography of the dried gel.

For the DNA replication reaction the incubation mixture was as described above except that it contained 78 mM NaCl and 20 μM each dGTP, dCTP, dTTP and [α-^{32}P]dATP (2.5 μCi). After incubation for 10 min at 30°C, the reaction was stopped and the unincorporated [α-^{32}P]dATP was removed from the samples as described for the initiation assay. The samples were subjected to 1% agarose gel electrophoresis after digestion with proteinase K; the gels were dried and autoradiographed.

Acknowledgements

We are grateful to J.M.Lázaro for the purification of proteins and to Dr G.Martín for help with the computer analysis. This investigation has been aided by Research Grant 5 R01 GM27242-09 from the National Institutes of Health, by Grant no. PB0323 from the Dirección General de Investigación Científica y Técnica, and by grants from Fondo de Investigaciones Santiarias and Fundación Ramón Areces. M.S. was the recipient of a fellowship from Fondo de Investigaciones Sanitarias.

References

Blanco,L. and Salas,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1639−1643.
Blanco,L. and Salas,M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6404−6408.
Blanco,L., Gutiérrez,J., Lázaro,J.M., Bernad,A. and Salas,M. (1986) *Nucleic Acids Res.*, **14**, 4923−4937.
Blanco,L., Bernad,A. and Salas,M. (1988) *J. Virol.*, **62**, 4167−4172.
Borowiec,J.A. and Hurwitz,J. (1988) *EMBO J.*, **7**, 3149−3158.
Bramhill,D. and Kornberg,A. (1988a) *Cell*, **52**, 743−755.
Bramhill,D. and Kornberg,A. (1988b) *Cell*, **54**, 915−918.
Brosius,J. and Holy,A. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6929−6933.
Carrascosa,J.L., Camacho,A., Moreno,F., Jiménez,F., Mellado,R.P., Viñuela,E. and Salas,M. (1976) *Eur. J. Biochem.*, **31**, 367−371.
Escarmís,C. and Salas,M. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1446−1450.
Galas,D.J. and Schmitz,A. (1978) *Nucleic Acids Res.*, **9**, 3157−3170.
Gartenberg,M.R. and Crothers,D.M. (1988) *Nature*, **323**, 824−829.
Gutiérrez,J., García,J.A., Blanco,L. and Salas,M. (1986) *Gene*, **43**, 1−11.
Gutiérrez,J., Garmendia,C. and Salas,M. (1988) *Nucleic Acids Res.*, **13**, 5895−5914.
Inciarte,M.R., Lázaro,J.M., Salas,M. and Viñuela,E. (1976) *Virology*, **74**, 314−323.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Maxam,A. and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499−560.
Mellado,R.P., Peñalva,M.A., Inciarte,M.R. and Salas,M. (1980) *Virology*, **104**, 84−96.
Mukherjee,S., Patel,I. and Bastia,D. (1985) *Cell*, **43**, 189−197.
Pastrana,R., Lázaro,J.M., Blanco,L., García,J.A., Méndez,E. and Salas,M. (1985) *Nucleic Acids Res.*, **13**, 3083−3100.
Peñalva,M.A. and Salas,M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5522−5526.
Prieto,I., Lázaro,J.M., García,J.A., Hermoso,J.M. and Salas,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 1639−1643.
Prieto,I., Serrano,M., Lázaro,J.M., Salas,M. and Hermoso,J.M. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 314−318.
Salas,M. (1988) *Curr. Top. Microbiol. Immunol.*, **136**, 71−88.
Satchwell,S.C., Drew,H.R. and Travers,A.A. (1986) *J. Mol. Biol.*, **191**, 659−675.
Schnos,M., Zahn,K., Inman,R.B. and Blattner,F.R. (1988) *Cell*, **52**, 385−395.
Yoshikawa,H., Friedman,T. and Ito,J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 1336−1340.

Received on December 21, 1988; revised on February 28, 1989