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Functional domains in the bacteriophage ϕ29 terminal protein for interaction with the ϕ29 DNA polymerase and with DNA

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ABSTRACT

Deletion mutants at the amino- and carboxyl-ends of the Ø29 terminal protein, as well as internal deletion and substitution mutants, whose ability to prime the initiation of Ø29 DNA replication was affected to different extent, have been assayed for their capacity to interact with DNA or with the Ø29 DNA polymerase. One DNA binding domain at the amino end of the terminal protein has been mapped. Two regions involved in the binding to the DNA polymerase, an internal region near the amino-terminus and a carboxyl-terminal one, have been also identified. Interaction with both DNA and Ø29 DNA polymerase are required to led to the formation of terminal protein-dAMP initiation complex to start Ø29 DNA replication.

INTRODUCTION

Bacteriophage Ø29, which infects _Bacillus subtilis_, has a linear, double-stranded DNA 19285 bp long (1) with the viral protein p3 covalently linked to the 5′ ends by a phosphoester bond between the OH group of serine residue 232 and dAMP, the terminal nucleotide at both 5′ ends (2-4). The terminal protein p3, 266 amino acids long, primes the initiation of replication at either Ø29 DNA end by formation of a covalent complex with dAMP, which is elongated by a strand-displacement mechanism (5-7). An _in vitro_ replication system with purified proteins has been developed in which the Ø29 DNA polymerase catalyzes the formation of the terminal protein-dAMP covalent complex in the presence of either p3-DNA (8,9) or protein-free templates containing Ø29 DNA replication origins (10,11). The protein p3-dAMP initiation complex is elongated by the Ø29 DNA polymerase to produce full-length Ø29 DNA in a very processive way (12,13). A complex between the terminal protein and the DNA polymerase is formed in the presence of NH_4^+ ions, which stimulate Ø29 DNA replication

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(14). In addition, the viral protein p6, that binds to the ∅29 DNA ends (15,16), stimulates the initiation reaction (17), as well as the transition from initiation to elongation (18).

Deletion mutants at the amino- and carboxyl-ends of the terminal protein, as well as internal deletion and substitution mutants have been constructed and their priming capacity studied (19-21). In this paper we report the correlation of the priming activity of the mutant proteins with the capacity to interact with the ∅29 DNA polymerase or with DNA, defining functional domains in the terminal protein for such interactions.

MATERIALS AND METHODS

a) Protein p3 mutants

Construction of the recombinant plasmids encoding the protein p3 mutants used in this work, nucleotide sequence determination, deduced amino acid sequence and priming capacity of the mutants have been described (see Table I for references). The nomenclature for the protein p3 mutants is based on the suggestions of Wetzel (22), but designating the initiator Met as position 1 of the sequence, the last residue of protein p3 corresponding to position 266. With these rules, the proteins deleted at the amino end (20) will be named as p3(Δ2-n), being residues 2 and n included in the deletion. Residues introduced by the process of plasmid construction, as in the cases of C-terminal (19) and internal (21) deletions, will be indicated with the residue number of the first added amino acid noted; for example, the deleted proteins encoded by plasmids pRMcn385 and pAZci315, in which a glycine residue is added, are named p3(Δ241-261/+241G) and p3(Δ56-71/+56G), respectively. The list of the p3 mutants used is shown in Table I.

b) Protein purification

Overproduction of the wild-type and mutant p3 proteins gives rise to the formation of insoluble aggregates. Purification of the proteins from the latter was done as described by Zaballos et al. (21). Essentially the procedure involves protein extraction with 2 M guanidine hydrochloride (GuHCl), renaturation by dialysis against 1 M NaCl buffer and a single chromatographic step on a phosphocellulose column. In some cases (see Results, section a) DNA was also recovered in the GuHCl extraction, being removed by

Table I. Protein p3 deletion mutants used in this work

Protein	Plasmid	initiation, %	Reference
p3 wt	pRMn7	100	20
p3(Δ2-6)	pAZa305	90	"
p3(Δ2-9)	pAZa308	40	"
p3(Δ2-10)	pAZa309	50	"
p3(Δ2-12)	pAZa311	60	"
p3(Δ2-14)	pAZa313	30	"
p3(Δ2-18)	pAZa317	<5	"
p3(Δ2-37)	pAZa336	<5	"
p3(Δ2-55)	pAZa354	u[b]	"
p3(Δ1-111)[a]	pRMn7	u	21
p3(Δ30-51/+49GDL)	pAZai319	<5	"
p3(Δ56-71/+56G)	pAZci315	<5	"
p3(Δ56-80/+56G)	pAZci324	u	"
p3(Δ263-266)	pRMn25H3	50	19
p3(Δ241-261/+241G)	pRMcn385	u	"
p3(Δ229-261/+229G)	pRMcn392	u	"

[a] p3(Δ1-111) is a protein from an internal translational start in the gene 3 reading frame, characterized by amino acid sequencing of its N-terminus (21).
[b] u indicates that the initiation complex was undetected.

addition of polyethyleneimine.

c) Protein p3-DNA polymerase interaction assay

Wild-type or mutant protein p3 was incubated, in 0.2 ml, with Ø29 DNA polymerase (0.2-0.6 μg of each protein; molar ratio 2:1) for 1 h at 0°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 44 mM NaCl and 20 mM $(NH_4)_2SO_4$. The samples were layered on top of 5 ml linear 15-30% glycerol gradients in the above buffer and centrifuged for 28 h at 290000 x g at 0°C. Fractions of about 0.2 ml were collected from the bottom and protein p3 and DNA polymerase detected by radioimmunoassay as described (23).

d) Analysis of protein p3-DNA complexes

1) Gel retardation assay

Wild-type or mutant protein p3 (10-40 ng) was incubated with 2 ng of a ^{32}P-labelled DNA fragment 450 bp long (positions 4502-4591 from Ø29 DNA) for 30 min at 0°C in a 25 μl mixture contai-

ning 12 mM Tris-acetate, pH 7.4 and 1 mM EDTA. The protein-DNA complexes were resolved by electrophoresis in 4% polyacrylamide gels at 4°C in the above buffer as described (24) and detected by autoradiography.

2) Glycerol gradient centrifugation

Wild-type or mutant protein p3 (0.25-0.6 μg) was incubated, in 0.2 ml, with 20 μg of proteinase K-treated Ø29 DNA (25) for 1 h at 0°C in a buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 75 mM NaCl. Samples were centrifuged for 4 h as described in c; protein p3 was detected by radioinmunoassay and DNA by agarose gel electrophoresis followed by ethidium bromide staining.

e) In vitro initiation and replication assays

The incubation mixture for the initiation reaction contained, in 25 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$, 5% glycerol, 0.25 μM [α-^{32}P]dATP (400 Ci/mmol), 0.5 μg of Ø29 DNA-protein p3 complex as template, 12.5 ng of purified Ø29 DNA polymerase (8) and 5-10 ng of wild-type or mutant protein p3. When indicated, 2.5 μg of purified protein p6 (17) and NaCl were added. When protein-free templates (11) were used, [α-^{32}P]dATP concentration was raised to 0.5 μM, Ø29 DNA polymerase to 25 ng and protein p3 to 40-80 ng. After 10 min at 30°C, the reaction was stopped by adding EDTA to 10 mM and heating for 10 min at 68°C, and the samples were treated with 25 units of micrococcal nuclease for 45 min at 37°C and filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS. The excluded fractions were subjected to SDS-electrophoresis in 15% polyacrylamide gels (26) and the p3-dAMP initiation complex was detected by autoradiography.

For the replication assays, 20 μM each dNTP was added to the above reaction mixture, the reaction was stopped with SDS to 0.1%, filtered as above and the Cerenkov radiation of the excluded fractions was counted. The samples were subjected to alkaline 0.7% agarose gel electrophoresis as described (27).

RESULTS

a) Purification of the p3 mutant proteins

In the purification of the p3 mutant proteins, differences in two steps of the process were observed: the presence of DNA in the fraction extracted with GuHCl and the strength of binding to

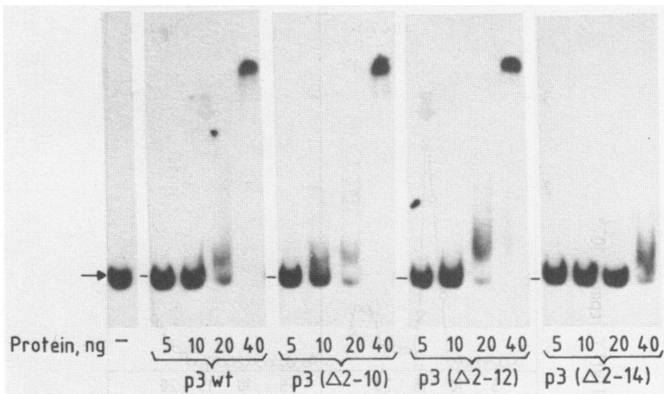

Figure 1. Interaction of the p3 mutant proteins with DNA determined by gel retardation. The indicated amounts of wild-type protein p3 or deletion mutants were incubated with a ^{32}P-labelled DNA fragment as described in Materials and Methods. After electrophoresis the DNA bands were detected by autoradiography. The arrow indicates the position of free DNA.

the phosphocellulose matrix. Wild-type protein p3, C-terminal deletion mutants and N-terminal deletion mutants up to 11 amino acids were coextracted with DNA from the insoluble aggregates and eluted from phosphocellulose at 0.6 M NaCl. N-terminal mutants with deletions higher than 11 amino acids were not coextracted with DNA and showed an inversal correlation between the extent of the deletion and the ionic strength needed for elution from phosphocellulose; thus, p3(Δ2-14) eluted between 0.4 and 0.6 M NaCl, p3(Δ2-18) at 0.4 M NaCl and p3(Δ2-37), p3(Δ2-56) and p3(Δ1-111) were not fixed to the matrix at 0.2 M NaCl. On the other hand, the internal deletion proteins, p3(Δ30-51/ +49GDL), p3(Δ56-71/ +56G) and p3(Δ56-80/+56G), eluted at 0.4 M NaCl (results not shown). Taking into account that phosphocellulose behaves as an affinity matrix for proteins that bind to nucleic acids, the purification of the p3 mutant proteins gave an indirect evidence about the location of a potential DNA-binding domain in the N-terminal portion of protein p3.

b) Interaction of the p3 mutant proteins with double-stranded DNA

Protein p3 interacts with double-stranded DNA (28,29) and this property could be important for the priming function of the protein. To determine the location of the potential DNA-binding domain, the interaction between different p3 deletion mutant proteins and DNA was tested. Figure 1 shows a gel retardation

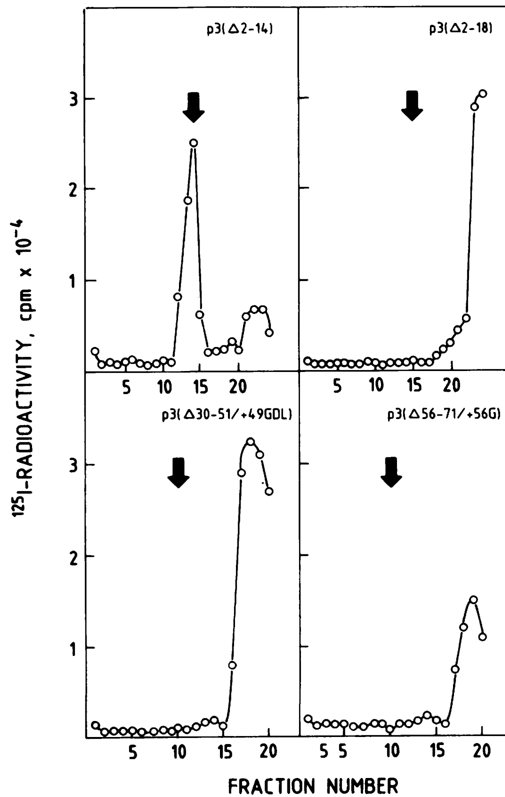

Figure 2. Interaction between the p3 mutant proteins and DNA determined by glycerol gradient centrifugation. Mutant proteins were incubated with ø29 DNA as described in Materials and Methods and, after centrifugation, protein p3 was detected by radio-immunoassay. Vertical arrows mark the position of the DNA peak. Sedimentation was from right to left.

assay in which the mutant proteins p3(Δ2-10) and p3(Δ2-12) gave rise to retarded DNA bands in a way similar to that of the wild-type protein, whereas a higher amount of p3(Δ2-14) was needed, suggesting that p3(Δ2-14) has a reduced affinity for DNA. By glycerol gradient analysis (Fig 2), using conditions in which most of the protein p3(Δ2-14) cosedimented with DNA, neither protein p3(Δ2-18) nor the internally deleted proteins, (p3(Δ30-51/+49GDL) and p3(Δ56-71/+56G)), were bound to DNA. On the other hand, the C-terminal deletion mutant proteins, p3(Δ241-261/+241G) and p3(Δ229-261/+229G), behaved as the wild-type protein in their ability to interact with DNA (not shown). Therefore, three

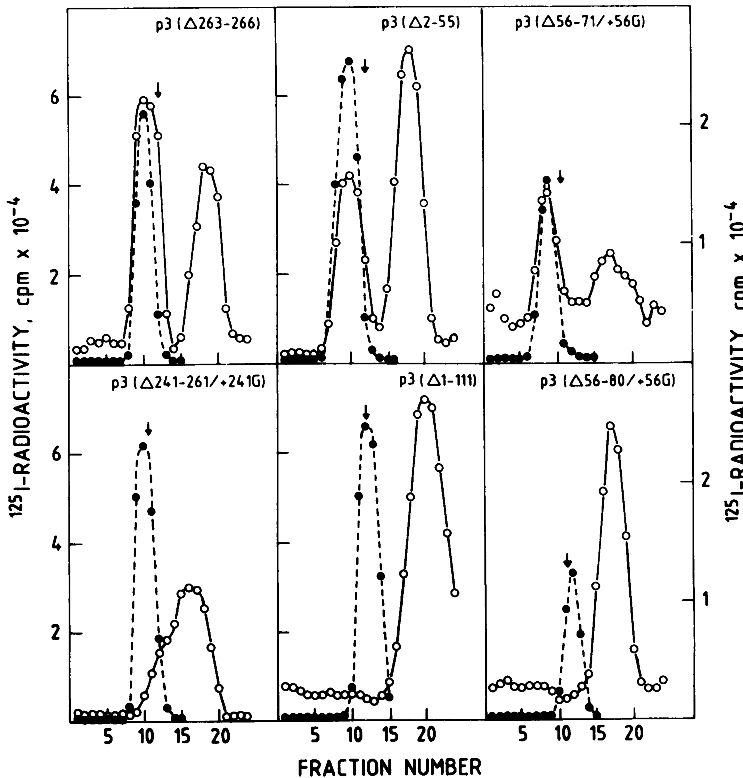

Figure 3. Detection by glycerol gradient centrifugation of the complex between the p3 mutant proteins and the Ø29 DNA polymerase. The wild-type or mutant p3 proteins were incubated with the Ø29 DNA polymerase as described in Materials and Methods and, after centrifugation, the proteins in the different fractions were detected by radioimmunoassay. o——o, protein p3; •--•, DNA polymerase. Vertical arrows mark the position of bovine serum albumin (68 Kda). Sedimentation was from right to left.

regions at the N-terminal part of the p3 molecule comprised between amino acids 13-18, 30-51 and 56-71 are involved in DNA binding.

c) Interaction of the p3 mutant proteins with the Ø29 DNA polymerase

It has been suggested that a tight association between protein p3 and the Ø29 DNA polymerase occurs in Ø29-infected B.subtilis cells (30). In addition, in the presence of NH_4^+ ions, these proteins interact in vitro to form an stable complex (14) and stimulation of ø29 DNA replication in vitro by NH_4^+ ions has

been observed (12,14), suggesting a biological role for this association. The p3 mutant proteins were incubated with the Ø29 DNA polymerase as indicated in Materials and Methods, section c, and the relevant results are shown in Figure 3. At least, two distant regions of protein p3 are involved in the interaction with the Ø29 DNA polymerase. One of them is located in the C-terminal portion of the molecule, defined by the interaction of p3(Δ263-266) and the lack of interaction of p3(Δ241-261/+241G). The other corresponds to an internal position near the N-terminus, defined by the interaction of p3(Δ2-55) and p3(Δ56-71/+56G) and the negative result obtained with p3(Δ1-111) and p3(Δ56-80/+56G) mutant proteins.

d) Activity of the p3 mutant proteins

We had previously shown the priming ability of the p3 mutant proteins using either cell-free extracts or partially purified proteins (see Table I). Those data correlate well with the results presented here on the interaction with DNA or with the Ø29 DNA polymerase. The proteins which do not bind to DNA, the N-deleted proteins beyond the 17th residue and the internally deleted proteins, primed the initiation reaction at a level less than 5% that of the wild-type protein, and the proteins which do not interact with the Ø29 DNA polymerase, p3(Δ1-111), p3(Δ56-80/+56G) and p3(Δ241-261/+241G), were unable to form the initiation complex p3-dAMP. No significant differences in priming ability were found when the mutant proteins purified by GuHCl extraction were tested in similar conditions of low salt (Table II and not shown). However, the activity of the N-deleted mutant proteins, p3(Δ2-6), p3(Δ2-9), p3(Δ2-10) and p3(Δ2-14), with a priming ability similar to that of the wild-type protein at low salt, decreased drastically when the *in vitro* reactions were carried out at high salt (Table II). Interestingly, addition of the Ø29 protein p6, which changes the DNA conformation at the Ø29 replication origins (15), restored the amount of replication at 0.1 M NaCl nearly to that obtained at low salt (Table II). On the other hand, differences between the mutant proteins p3(Δ2-10), p3(Δ2-12) and p3(Δ2-14) were observed when protein-free templates were used in the initiation reaction (Fig. 4). Whereas the priming ability of p3(Δ2-10) and p3(Δ2-12) relative to the wild-type protein did not differ significantly using either protein-

Table II. Effect of high salt and protein p6 on the ability of p3 mutant proteins to prime ∅29 DNA replication.

Protein	Activity, %[a]		
	4 mM NaCl -protein p6	100 mM NaCl -protein p6	100 mM NaCl +protein p6
p3 wild-type	100[c]	100[d]	100[e]
p3(Δ2-6)	69	6	66
p3(Δ2-9)	80	9	90
p3(Δ2-10)	90	17	84
p3(Δ2-12)	76	8	82
p3(Δ2-14)	67	4	69

[a]The ability of the mutant proteins to prime ∅29 DNA-protein p3 replication was assayed as described in Materials and Methods (section e, replication assay) using 10 ng of wild-type or mutant p3 proteins.

[c,d,e] 100% activity corresponds to 100, 5 and 75 pmols of dAMP incorporated, respectively.

free templates or p3-DNA, that of p3(Δ2-14) was greatly reduced on protein-free templates.

DISCUSSION

a) DNA binding domain

From the results obtained, which are summarized in figure 5, it can be concluded that the amino acid regions of protein p3 from positions 13 to 18, 30 to 51 and 56 to 71, deleted in the mutant proteins p3(Δ2-18), p3(Δ30-51/+49GDL) and p3(Δ56-71/+56G), respectively, are important for DNA binding and should contain residues interacting with DNA or affecting the conformation of the domain. However, the fact that this conclusion is constrained by the availability of mutants and that all the mutants deleted at the N-terminus have the DNA binding capacity affected in some extent, suggests that all the above amino region is involved in the interaction. Thus, N-deleted proteins up to residue 12, although did not show difference in DNA binding compared with the wild-type protein, were more salt-sensitive in the initiation

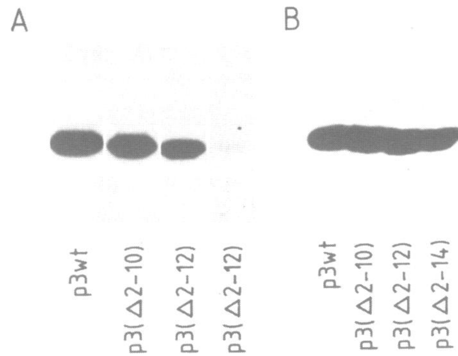

Figure 4. Priming ability of the p3 mutant proteins with protein-free Ø29 DNA templates. A. The reaction for the formation of the p3-dAMP complex was as described in Materials and Methods using 29 ng of the 2364 bp long DraI A fragment from plasmid pIΔ68 (11), containing 68 bp from the left Ø29 DNA end, with 80 ng of wild-type protein p3 or the indicated deletion mutant proteins. After electrophoresis the p3-dAMP complex was detected by autoradiography. B. As in A except that 80 ng of Ø29 DNA-protein p3 was used as template and the amount of wild-type or mutant protein p3 was lowered to 10 ng.

reaction. Moreover, the Ø29 protein p6, that modifies the DNA conformation at the replication origins, restored the activity, suggesting that the binding of the mutant proteins to DNA at high salt is somehow different to that of the wild-type p3, and proper interactions may only take place when protein p6 is present. Mutant protein p3(Δ2-14) binds to DNA with diminished affinity relative to p3(Δ2-12), and this could be reflected in the reduced ability to prime replication with protein-free templates, whereas the priming with the terminal protein-containing DNA is similar to that of p3(Δ2-12). Protein p3 mutants having deletions of 17 amino acids or longer at the amino end and the internally deleted proteins had drastically reduced both binding to DNA and ability to prime p3-DNA replication.

None of the typical structural motifs considered to be responsible for sequence-specific DNA binding, helix-turn-helix (31), zinc finger (32) and leucine zipper (33), are found in protein p3. Since the interaction of protein p3 with DNA does not seem to be sequence-specific (28), it is unlikely that hydrogen bonding to bases plays a major role in such interaction, and electrostatic bonds between phosphate groups and positively

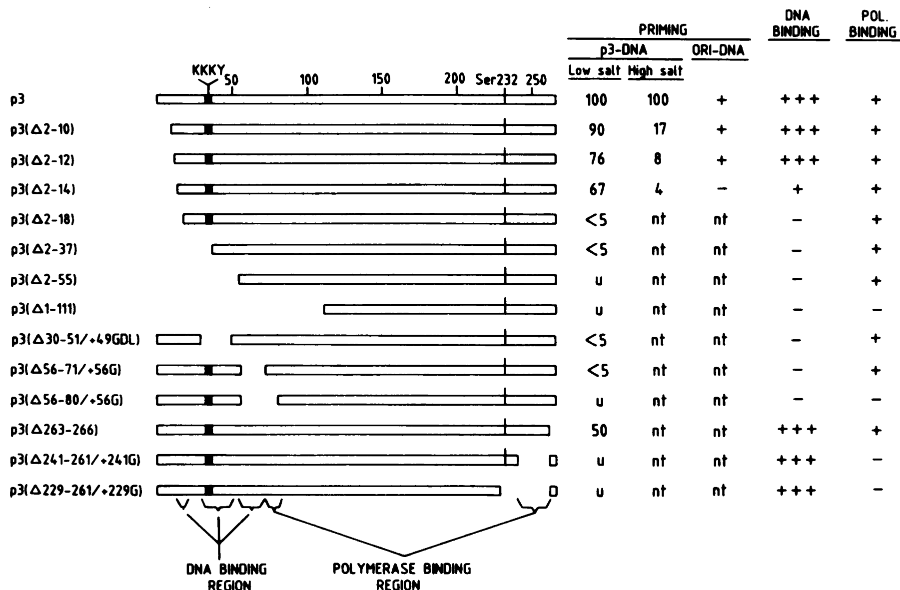

Figure 5. Summary of the properties of the p3 mutant proteins. Boxes show the size of the different mutant proteins relative to the wild-type protein p3. Location of the KKKY sequence and of the Ser232 are indicated. Priming activity values were from Table I, except for p3(Δ2-10), p3(Δ2-12) and p3(Δ2-14) (this paper). ORI-DNA refers to the protein-free template containing the ∅29 left replication origin; u, undetected; nt, not tested.

charged amino acids may be more relevant. Looking at the charge distribution along protein p3, there is a positive cluster at the amino end (net charge +8 in the first 80 residues), being slightly positive in the middle of the molecule and negatively charged at the carboxyl region (34). In the positive cluster at the amino end of protein p3, the sequence KKKY flanked by aromatic and hydrophobic residues is present. Interestingly, a similar amino acid region is shared by the α-like DNA polymerases (35), being located at the C-terminus of these proteins, where the DNA binding domain could reside (36). This kind of amino acid side chains would allow both ionic and hydrophobic interactions with the phosphate backbone and base rings of the DNA, respectively. Our experimental data are in agreement with the idea that the above region could contain the amino acids that interact with DNA: on the one hand, the sequence KKKY is deleted in p3(Δ30-51/

+49GDL) with a loss of DNA binding capacity (see Fig. 5), and on the other, four positively charged amino acids have been removed in mutant p3(Δ2-12) and the binding to DNA is only slightly affected. Moreover, structural predictions showed that the N-part of the proposed DNA binding region could be ordered in protein p3 as an amphipatic α-helix that would not be formed in mutant p3(Δ2-18) (20), explaining the drop in affinity for DNA and priming ability of this mutant relative to p3(Δ2-14). The internal deletion of mutant p3(Δ56-71/+56G) might also affect the folding of the DNA binding domain, although direct interaction of the deleted residues with DNA cannot be discarded.

b) The DNA polymerase binding domain

The unability to prime Ø29 DNA replication of terminal protein mutants which do not interact with the Ø29 DNA polymerase suggests that physical association of both proteins is needed to initiate DNA replication. The detection of at least two separate regions in protein p3 involved in the interaction, comprised between amino acids 72-80 and 242-262, could indicate the existence of either two different domains interacting with the DNA polymerase or a single domain composed of two or more parts of the molecule. The fact that both regions are needed for the interaction supports the latter possibility, unless these regions are affecting the structure of some other part of the molecule. The C-terminal region interacting with the DNA polymerase is located next to the linking site, where the DNA polymerase catalyzes the incorporation of dAMP. Point mutations at this site do not affect the binding to the DNA polymerase (29; Garmendia, C., Hermoso, J.M. and Salas, M., submitted for publication), suggesting that the linking site and the DNA polymerase binding domain are physically separated.

c) New aspects of the protein-priming mechanism

There are several reasons to support the idea that the first step in the initiation of Ø29 DNA replication is the association of the terminal protein and the DNA polymerase: i) their genes are located close to each other and probably transcribed into a polycistronic mRNA (37,38), ii) a partially purified terminal protein-DNA polymerase complex can be isolated from Ø29-infected cells (30) and iii) the two proteins interact *in vitro* in the absence of the p3-DNA template (14). Once the complex is formed, recognition of the replication origins should take place. On the

template counterpart the main component which determines the replication origin is the parental protein attached to the DNA ends (39). Taking into account that linear p3-DNA is able to circularize and to form concatemers due to protein-protein interaction (2,40) it is likely that the interaction between the terminal protein in the complex with the DNA polymerase and the parental terminal protein attached to the DNA is needed for the recognition. Here we show that the interaction of the terminal protein with DNA is important for its priming function. In agreement with the DNA sequence-independent interaction of protein p3, it has been suggested that the replication origin is recognized as a whole structure rather than trough interaction with specific bases (11). It is likely that binding of the terminal protein to DNA is needed to recognize the replication origins or, at least, to locate precisely the terminal protein-DNA polymerase complex at the replication origin.

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