Structural and functional analysis of temperature-sensitive mutants of the phage ϕ29 DNA polymerase

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ABSTRACT

The cloning and complete sequencing of gene 2 from four independently isolated temperature-sensitive mutants in the phage ϕ29 DNA polymerase (ts2 mutants) is reported. The results obtained indicate that, *in vivo*, the mutations only affect the initial steps of the replication process. Interestingly, three of these mutations consist in the single amino acid change Ala to Val at position 492 of the protein. The ts2(24) and ts2(98) mutant ϕ29 DNA polymerases were expressed, purified and their thermosensitivity was studied at two different steps of DNA replication: 1) protein-primed initiation and 2) elongation of the DNA chain. Whereas the ts2(24) mutation gave rise to a temperature-sensitive phenotype in both reactions, the ts2(98) mutant protein was rather insensitive to the temperature increase. In addition, the ts2(98) mutant protein showed clear differences in the activation by divalent cations. The relationship of these results with structural and functional domains in the ϕ29 DNA polymerase are discussed.

INTRODUCTION

Temperature-sensitive mutations have long been recognized as a powerful tool in structural and functional studies (1,2). Thus, partial purification and characterization of mutationally altered T4 DNA polymerases (3) was central for demonstrating the postulated 'editing' function of the resident 3′ to 5′ exonuclease activity (4) and for the establishment of structural-functional relationships (5). In the case of *Bacillus subtilis* phage ϕ29 DNA replication the analysis of a collection of temperature-sensitive mutants (6,7) played a crucial role in demonstrating the proposed mechanism of protein-primed initiation (8) and in the identification of the different cistrons involved in viral DNA synthesis (9,10). In this system, a unique viral-encoded DNA polymerase, protein p2, catalyzes both the formation of the initiation complex between the terminal protein, p3, and dAMP, which is the 5′ terminal nucleotide at both DNA ends, and its further elongation (11,12) to produce unit-length ϕ29 DNA (13).In addition, the ϕ29 DNA polymerase has a putative proofreading 3′ to 5′ exonuclease activity (14,15). The ϕ29 DNA polymerase has been included among the group of α-like DNA polymerases because of its sensitivity to several drugs and nucleotide analogs (16,17) and by the presence of amino acid sequences highly conserved in this group of DNA polymerases (17). Further structural-functional relationship analysis, using targeted mutagenesis, has revealed that the ϕ29 DNA polymerase is structurally similar to the Klenow fragment of *E. coli* DNA polymerase I: the 3′ to 5′ exonuclease activity is located in the amino-terminal portion whereas the synthetic activities (initiation and elongation) are associated to the carboxy-terminal part (18;19). In this paper we report the analysis, both *in vivo* and *in vitro*, of several independently isolated ts2 mutants of ϕ29. The results obtained are discussed in terms of structural and functional implications, correlating the position of each mutation with both, reported and new conserved regions.

MATERIALS AND METHODS

Nucleotides, reagents, proteins and templates

Unlabelled nucleotides were purchased from Pharmacia P-L Biochemicals.(α-^{32}P)dNTPs (410 Ci/mmol), (methyl-^{3}H) thymidine (48 Ci/mmol), (^{35}S)methionine (1000 Ci/mmol) and (α-^{35}S)dATP (600 Ci/mmol) were obtained from Amersham International plc. ϕ29 terminal protein and protein p6 were purified as described (20,21). Restriction endonucleases, T4 DNA ligase and Klenow fragment *E. coli* DNA polymerase I were from New England Biolabs. Sequenase 2.0 version of T7 DNA polymerase was from United States Biochemical Corporation. Rabbit anti-p2 serum was obtianed by using highly purified wild-type ϕ29 DNA polymerase (p2) as antigen following conventional methods. The IgG fraction was purified by affinity chromatography on a protein A-Sepharose column. Fungal proteinase K, chromatographically purified, was from Merck. The drug 6(p-hydroxyphenylazo)uracil (HPUra) was a gift from Imperial Chemical Industries. EcoRI-digested ϕ29 DNA was prepared from proteinase K-treated ϕ29 DNA (22).The ϕ29 DNA-protein p3 complex was isolated as described(23).

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Bacteria, plasmids and phages

Bacillus subtilis 110NA try$^-$ spoA$^-$ su$^-$ (24) was used as host bacteria. Wild-type ϕ29 or mutant sus14(1242), which produces a normal burst and delayed lysis of the infected bacteria under restrictive conditions (25), were used. Mutants ts2(24), ts2(98), ts2(99) and ts2(112) were from the collection of Talavera et al.(6). Double mutants with sus14(1242) were generally used to produce a delayed lysis of the infected bacteria. The nomenclature used for the ϕ29 mutants is that described by Mellado et al. (26). Plasmids pBR322 (27) and pT7-4 (28) were used as cloning vectors. The *E. coli* K-12 strain K514 was used as host for transformation. The *E.coli* B lambda lysogens BL21(DE3), BL21(DE3)plysS and BL21(DE3)plysE (29) were used for expression of the mutant proteins.

DNA manipulations

Plasmid and phage DNA preparations, purification of DNA fragments, ligation, transformation, antibiotic selection of recombinants and agarose and polyacrylamide gel electrophoresis were as described (30). Plasmid DNA minipreparations (31) were direct substrates for sequencing by the dideoxy chain-termination method (32). A collection of synthetic oligodeoxynucleotides, complementary to ϕ29 gene 2 sequences, were used as sequencing primers. The annealing was at 37°C before incubation with the Klenow fragment of DNA polymerase I at 50°C or with the Sequenase 2.0 version of T7 DNA polymerase at 37°C.

Pulse-labelling of bacteria infected with ϕ29 ts mutants: shift-up experiments

B.subtilis 110NA was grown at 30°C in defined medium (33). When the cell concentration was 10^8/ml the bacteria was concentrated five times in defined medium containing amino acids (0.5 mM) each, uridine (200 μg/ml) and HPUra (100 μg/ml) and infected with the mutant indicated in each case at a multiplicity of infection of 20. After 45 min at 30°C, 0.05 ml of the infected culture was diluted 5-fold into defined medium supplemented with (methyl-^{3}H)thymidine (200 μCi/ml) preheated at 45°C. The mixture was immediately placed at 45°C and, at different times, samples were withdrawn and trichloroacetic acid (TCA)-insoluble radioactivity was determined. To chase the DNA labelled after infection with the different mutants, a 200-fold excess of non-radioactive thymidine was added after 4 min. At different times samples were withdrawn for alkaline sucrose gradient centrifugation and processed essentially as described by Mellado et al.(34). Proteinase K-treated ϕ29 DNA was used as internal unit-length marker.

Induction of cell cultures, analysis of cellular protein and purification of ϕ29 DNA polymerase mutants

The *E.coli* strain BL21(DE3)plysS (29), transformed with the different ϕ29 gene 2-containing recombinant plasmids pMBw2, pMB24 and pMB98, corresponding to the wild-type gene 2 or mutants ts2(24) and ts2(98), respectively, was grown at 30°C in LB medium, in the presence of ampicillin (100 μg/ml) and chloramphenicol (34 μg/ml), until an optical density = 0.45 at 600 nm. The cultures, induced by addition of 1 mM IPTG, were maintained at 30°C for 30 min and then rifampicin (100 μg/ml) was added and the incubation was continued for 40 additional min. When radioactive labelling of induced cultures was carried out, (^{35}S) methionine (10 μCi;1000 Ci/mmol) was added to samples (25 μl) removed at different times from cultures grown in the same conditions described above but in minimal medium (35). The induced ϕ29 DNA polymerase mutant polypeptides were identified by autoradiography and Coomassie-Blue staining of SDS-polyacrylamide gel electrophoretic analysis (36). The overexpressed mutant proteins were purified as described (11) with slight modifications.The protein concentration was determined by radioimmunoassay and SDS-polyacrylamide gel electrophoresis.

Protein p3-dAMP complex formation (initiation assay)

The initiation assay contained, in 25 μl, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 7% glycerol, 50 mM NaCl, 2.5 mg/ml bovine serum albumin (BSA), 0.25 μM (α-^{32}P)dATP (2.5 μCi), 0.5 μg of ϕ29 DNA-protein p3, 20 ng of purified terminal protein, either wild-type ($\approx$1 ng), ts2(24) ($\approx$1 ng) or ts2(98) ($\approx$0.1−0.2 ng) DNA polymerase, and, when indicated, 2 μg of protein p6, which stimulates the initiation of ϕ29 DNA replication (21). After incubation for 10 min at 30°C the reaction was stopped by adding 10 mM EDTA-0.1% SDS, the samples were filtered through Sephadex G-50 spun colums in the presence of 0.1% SDS and the excluded volume was further analyzed by SDS-polyacrylamide gel electrophoresis as described (36). Quantitation was done by excising from the dried gel the radioactive band corresponding to the p3-dAMP complex and counting the Cerenkov radiation, or by densitometric analysis of the exposed films.

When the temperature sensitivity of the ϕ29 DNA polymerase mutants was tested, the corresponding protein fraction was preincubated in 12.5 μl of a mixture containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 20 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 14% glycerol, 100 mM NaCl and 5 mg/ml BSA. After incubation for 3 min at the indicated temperatures, the preincubation mixtures were used for the initiation assay as described above.

Polymerization assay: filling-in reaction

The incubation mixture was as in the initiation assay except that 0.5 μg of EcoRI-digested ϕ29 DNA was used as template and $(NH_4)_2SO_4$ was not added. Either wild-type or mutant ϕ29 DNA polymerases in the amounts indicated in the initiation assay, and 0.1 μM (α-^{32}P)dATP (1 μCi) and 200 μM dGTP and dTTP were added. The molar ratio EcoRI ends: enzyme was 26 for the wild-type and ts(24) DNA polymerases and 130−260 for the ts2(98) DNA polymerase. After incubation for 10 min at 30°C, the reaction was stopped and the samples filtered through Sephadex G-50 spun colums as described above. The excluded volume was counted (Cerenkov radiation) and analyzed by agarose gel electrophoresis and autoradiography. Temperature sensitivity was assayed as described before.

Comparison of amino acid sequences of DNA polymerases

The DNA polymerase sequences compared were the following: phage T4 (37), phage ϕ29 (38), phage M2 (39), phage PRD1 (40), S1-mitochondrial DNA (41), plasmid pGKL1 (42), plasmid pGKL2 (43),plasmid pClK1 (44), plasmid pAI2 (45), adenovirus type 2 (46), vaccinia virus (47), fowl-poxvirus (48), varicella zoster virus (VZV) (49), herpes simplex virus (HSV-1) (50), Epstein-Barr virus (EBV) (51), human cytomegalovirus (HCMV) (52), baculovirus (AcMNPV) (53), REV-3 from yeast (54), CDC2 from yeast (55), Pol I from yeast (56) and α from human

(57). Multiple alignments were obtained by series of binary (pairwise) alignments followed by shifting by eye to achieve maximal homology. The following conservative amino acids were considered: Y,W,F,I,L and V; D,N,Q and E; K and R; S and T; G and A. Binary alignments were carried out by computer analysis using the programs BESTFIT, PRETTY and GAP from the UWGCG (University of Wisconsin Genetics Computer Group) (58).

RESULTS

DNA synthesis in bacteria infected with φ29 ts2 mutants. Shift-up experiments

Four φ29 ts2 mutants (6,7) were used in shift-up experiments to determine the specific step(s) in DNA synthesis sensitive to temperature. Figure 1A shows that the incorporation of (methyl-^{3}H) thymidine in cells infected with the delayed lysis mutant sus14(1242) continued after the shift to 45°C whereas after infection with the different ts2-sus14(1242) mutants, DNA synthesis stopped slowly after the shift-up, indicating a thermosensitive DNA synthesis. Pulse-chase experiments showed that both in cells infected with the sus14(1242) mutant or with the different ts2-sus14(1242)mutants the pulse-labelled DNA shorter than unit-length (not shown) was chased into mature DNA in 10 minutes after the chase (Fig 1B-D, and not shown). Similar results had been previously obtained with the ts2(98) mutant (34). These results strongly suggest that all the mutants are affected in the initial steps of DNA replication. Therefore, the location of the mutations could be of special interest for defining critical amino acid positions for the protein-priming activity of the φ29 DNA polymerase. The above experiments do not rule out the possiblity that the elongation activity could be protected from thermal inactivation when the DNA polymerase forms part of the replicative complex.

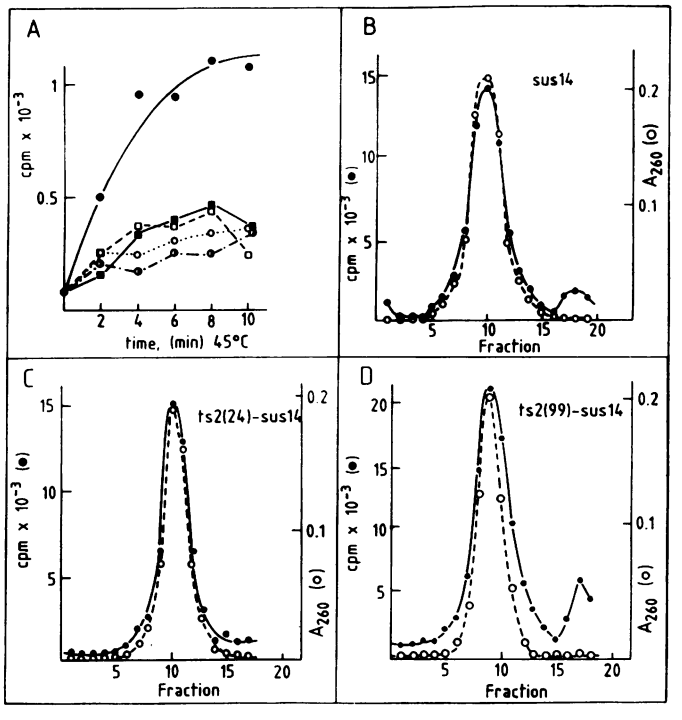

Figure 1. Analysis of the DNA synthesized in bacteria infected with ts2 mutants. A. Incorporation of (^{3}H)thymidine into *B.subtilis* 110NA infected with the φ29 mutants sus14(1242) (closed circles), ts2(98)sus14(1242) (open squares), ts2(99)sus14(1242) (closed squares), ts2(112)sus14(1242) (semi-filled circles) and ts2(24)sus14 (1242) (open circles). *B. subtilis* 110NA was grown and infected at 30°C as indicated in Materials and Methods. After the shift to 45°C and simultaneous addition of (^{3}H)-thymidine, samples of 5 μl were removed at the indicated times to determine TCA-insoluble radioactivity. B-D. Alkaline sucrose gradient of extracts of infected bacteria, pulse-labelled and chased. *B.subtilis* 110NA was grown and infected with the indicated φ29 mutants as described in Materials and Methods, and 10 minutes after the chase (14 minutes after the shift to 45°C and addition of (^{3}H)-thymidine), samples of 0.2 ml were removed, lysed, treated with proteinase K and centrifuged in alkaline sucrose gradients, as described in Materials and Methods, in the presence of 10 μg of φ29 DNA as unit-length marker. The TCA-insoluble radioactivity (closed circles), and the absorbancy at 260nm (open circles) were determined in the different fractions. Sedimentaton was from right to left.

Cloning, sequencing and expression of gene 2 from the ts2 mutant genes

To increase the amount of the ts2-mutant proteins for purification, cloning of the corresponding genes in *E.coli* expression vectors was undertaken. The φ29 DNA HindIII B fragment, containing the whole gene 2 and several other small ORFs (38), was cut with BclI and cloned in pBR322, obtaining the derivative plasmids pBts2 (see Fig.2). The latter were used both for sequencing and subcloning in different expression systems.

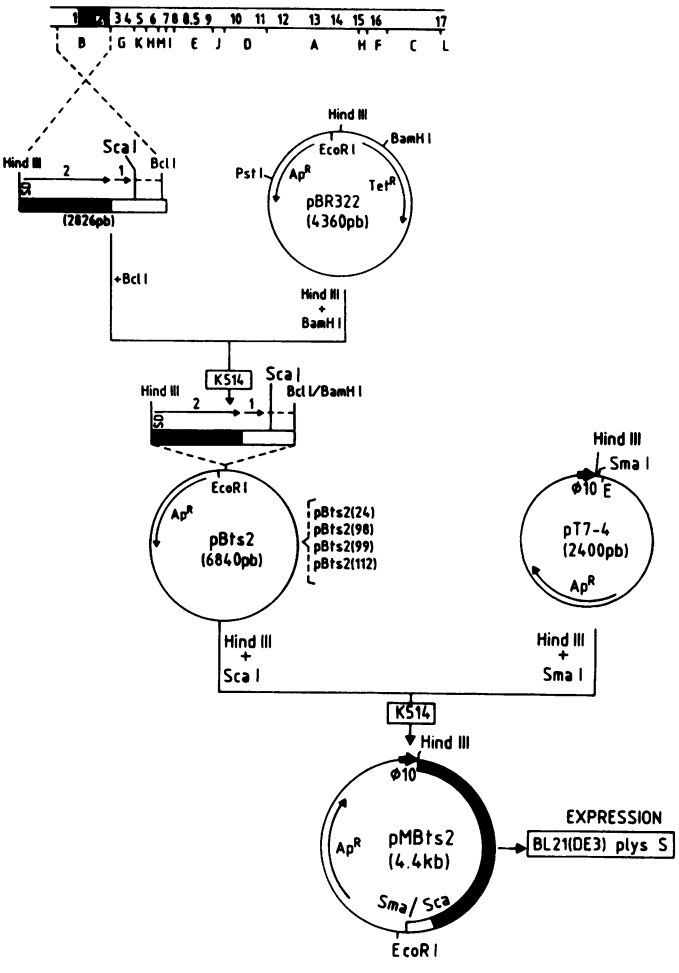

Figure 2. Construction of recombinant plasmids containing gene 2 from φ29 ts mutants. The recombinant plasmids were constructed as described in Results, 2. Ap^R, ampicillin resistance; Tet^R, tetracicline resistance; φ10,φ10 promoter of bacteriophage T7. The top of the figure shows the genetic map and the HindIII restriction map of bacteriophage φ29 DNA. Gene 2 is represented by black boxes. The *E.coli* strains used for selection and for expression are shown in boxes.

Sequencing of the ts2 mutant genes was carried out as described in Materials and Methods. It was found that the ts2(24) phenotype results from a single change (C to T) at nucleotide 1809 from the leftϕ29 DNA end. This transition generates an amino acid change from alanine to valine at position 355 of the ϕ29 DNA polymerase. It was found that mutants ts2(98),ts2(99) and ts2(112) contain the same change (C to T) at position 1398, changing alanine 492 to valine. This is specially remarkable taking into account that these three ts2 mutants were independently isolated; therefore, this result could be related to a high sensitivity of this position to the mutagenic agent used (6) or to a critical role of this position for the structural equilibrium. To rule out possible cloning artifacts, sequencing on the original viral DNA was carried out, confirming the above results. From the whole sequencing of the different gene 2 mutants available (18; this paper), it was found that all of them contained two additional changes. One of these, a T to C transition in position 2093 from the left ϕ29 DNA end, results in a silent mutation (AGT to AGC) in codon 260 of the ϕ29 DNA polymerase, whereas the second, a GC to CG transversion at positions 2346 to 2347, changes codon 176 from alanine to lysine. We found that these changes were also present in our wild-type gene 2, therefore differing from the published sequence (38).

To express in *E. coli* the ts2(24) and ts2(98) ϕ29 DNA polymerase mutants different strategies and expression systems were tested, including the one used for the expression of the wild-type protein (30), based in the P_L promoter of phage lambda. Neither heat nor chemical induction (59) with this system nor other systems based in the P_{tac} or P_{lac} promoters were successful. The low number of clones obtained were able to express the products encoded in the small ORFs contained in the cloned fragment, but no ϕ29 DNA polymerase was expressed. Since all these problems could be related with a high toxicity level of the mutant proteins we used one of the most regulated *E.coli* expression systems, based on the high specificity of the T7 RNA polymerase for its own promoters (28,29). Figure 2 shows the successful subcloning scheme in which the HindIII/ScaI fragment from plasmids pBts2 was put under the control of the ϕ10 promoter of bacteriophage T7, contained in the plasmid pT7−4 (28).The selection for the ampicillin resistant recombinant plasmids pMBts2 was carried out in the *E.coli* strain K514, which cannot express T7 RNA polymerase. The recombinant plasmids were used to transform the expression strain BL21(DE3) and its derivatives BL21(DE3)plysS and BL21(DE3)plysE, which can express the cloned genes after induction by IPTG (29) as indicated in Materials and Methods. No expression of the ts DNA polymerase mutants was obtained when BL21(DE3) was used. However, using the derivative strains that allow a lower basal expression in the absence of induction, clones expressing in a regulated fashion the ts2(24) and ts2(98) mutant proteins were obtained. The overexpressed mutant proteins were purified as described in Materials and Methods to assay the thermosensitivity of the different activities.

Location of the ϕ29 DNA polymerase ts mutations relative to highly conserved regions in DNA polymerases from terminal protein-containing genomes

The ϕ29 DNA polymerase seems to be structured in a similar way to the Klenow fragment of *E.coli* DNA polymerase I (18). The amino-terminal third of the molecule contains the 3'to 5'exonuclease activity whereas the protein-priming and DNA polymerase activities are located in the carboxy-terminal domain (18; 19).Figure 3 shows the location of the ts2 mutations with respect to the three highly conserved carboxy-terminal domains previously described (17). Both mutations map in the DNA polymerase portion involved in the synthetic activities.The ts2(24) mutation is located between the two motifs DVNSLYP and NSLYG (Fig. 3, regions 1 and 2), in a conserved region present only in protein-primed DNA polymerases. The ts2(98) mutation maps near the YCDTDS motif (Fig. 3, region 3), towards the carboxy terminus, in a relatively conserved region (Fig. 3, region 4), present in other subfamilies of DNA polymerases including the Klenow fragment of *E.coli* DNA polymerase I (to be published elsewhere). According with the alignment shown in Figure 3, the T4 DNA polymerase mutant tsL88, with a low mutator phenotype (5), maps at position 694 (60), very close to the equivalent position of the ts2(98) mutation. This relationship and the fact that three independent isolates of ϕ29 map in this region strengthens its functional or structural importance.

Synthetic activities of the ts2 ϕ29 DNA polymerase mutants. Temperature-sensitivity analysis

The ϕ29 DNA polymerase mutants, overproduced and purified as described before, were used to test the temperature-sensitivity of the synthetic activities i.e., initiation and elongation. Figure 4A shows the results obtained when the different mutants were assayed in the initiation reaction after preincubation at the different temperatures as indicated in Materials and Methods. The ts2(24) DNA polymerase was clearly thermosensitive, being its initiation activity after preincubation at 42°C about 4% with respect to that at 30°C. However, the initiation activity of the ts2(98) DNA polymerase was not temperature-sensitive as was the case with the wild-type ϕ29 DNA polymerase. The differences in the absolute values at 30°C between the different DNA polymerases are probably due to the amount of protein available for the assay. The initiation activity of the ts2(24) DNA polymerase was not protected from temperature inactivation by preincubation in the presence of the terminal protein (Fig. 5), with which it forms a tight complex in the presence of NH_4^+ ions (61). The effect of the temperature on the initiation activity of the ts2(24) DNA polymerase seems not only due to a decrease in affinity for the terminal protein because similar inactivation by temperature was observed when higher amounts of protein p3 were added to the preincubated mutant DNA polymerase (not shown). On the other hand, the polymerization activity of the ts2(24) DNA polymerase was clearly thermosensitive, being its activity at 42°C about 6% with respect to that at 30°C (Fig. 4B). The ts2(98) DNA polymerase showed a slight temperature-sensitivity, being its activity after preincubation at 42°C about 35% of that at 30°C. The purification level of these proteins did not allow us to study whether the ts mutations, located in regions involved in synthetic activities, affect the 3′ to 5′ exonuclease activity, situated in the amino-terminal region (18). This is probably not the case because we have shown that synthetic and 3′ to 5′ exonuclease activities seem to be located in independent structural domains (18; 19;unpublished results).

Requirement of activating metal ion for the ts DNA polymerase mutants

All known DNA polymerases have as a strict requirement for activity the presence of divalent activating metal ions (Me^{2+}). In the case of the *E.coli* DNA polymerase I,X-ray crystallographic and targeted mutagenesis studies, have involved

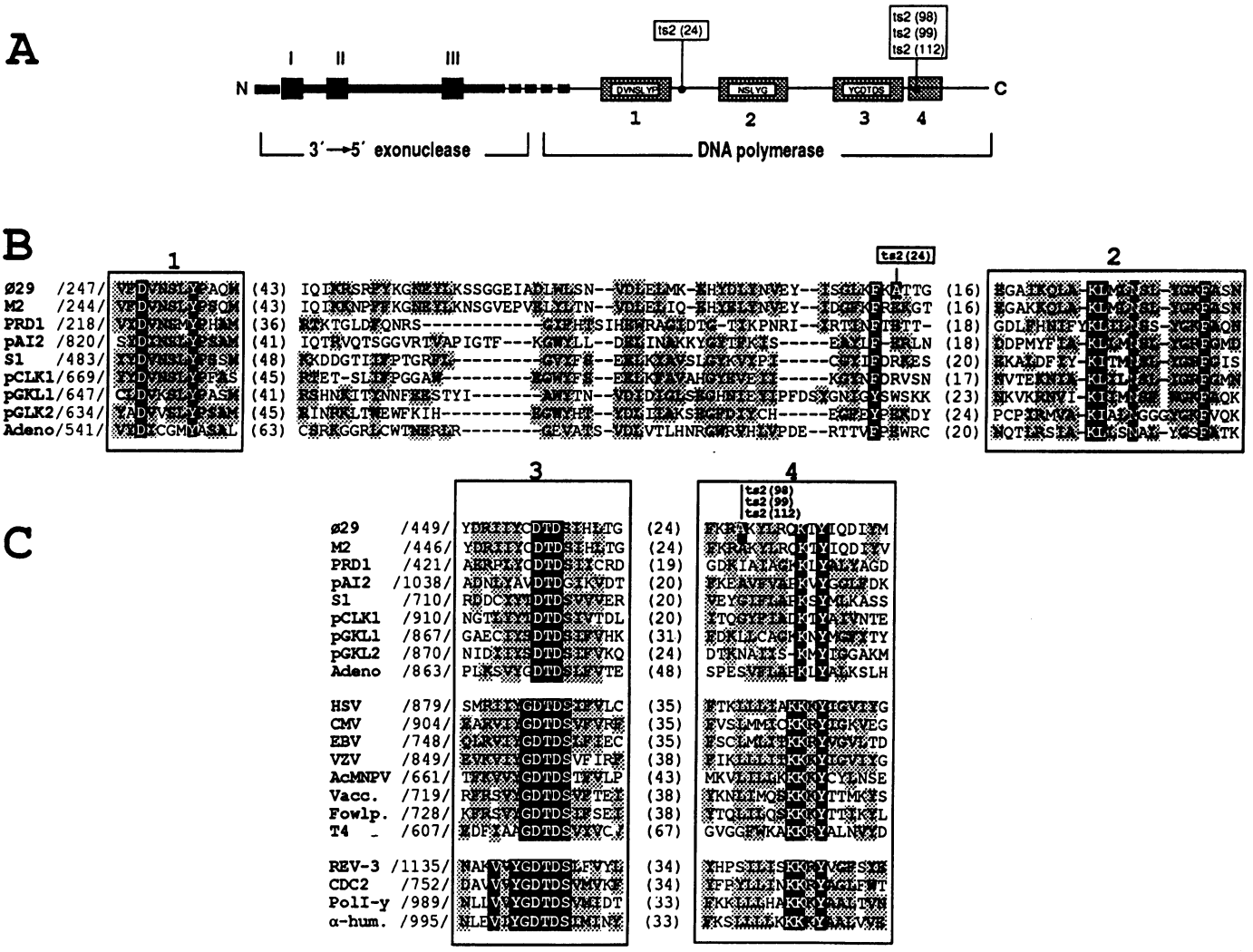

Figure 3. Location of the ϕ29 DNA polymerase ts mutations relative to highly conserved regions in α-like DNA polymerases. The ϕ29 DNA polymerase, showing the main motifs involved in the catalytic activities is represented; I,II and III indicate the three conserved domains shown to be involved in the 3'→5' exonuclease activity (18); 1, 2, 3 and 4 indicate regions involved in protein-primed initiation and polymerization activities. B. Multiple alignment of the amino acid sequences spanning between regions 1 and 2 corresponding to protein-primed DNA polymerases. C. Multiple alignment of regions 3 and 4 corresponding to protein-primed DNA polymerases and eukaryotic (viral and cellular) α-like DNA polymerases. Numbers in brackets indicate the first residue in each sequence, relative to the N-terminal end of each polypeptide. Residues conserved in most of the sequences compared and ϕ29 amino acid residues corresponding to ts mutations are indicated in white letters. Other relevant homology is boxed in grey.

divalent metal ions in both exonuclease (62) and DNA polymerase activities (L.Beese, J.Friedman and T.A.Steitz, personal communication). In general, the divalent activator metal ion used has been Mg^{2+}. The effects observed with other divalent metal ions have been related to a reduction in the replication fidelity (63). The ϕ29 DNA polymerase can use either Mg^{2+} or Mn^{2+} for initiation and polymerization (Esteban et al., in preparation). Using 1 mM $MnCl_2$ versus 10 mM $MgCl_2$ the Mn^{2+}/Mg^{2+} activity ratio for the wild-type ϕ29 DNA polymerase was 3.5 for initiation (Fig. 6A) and 0.2 for polymerization (Fig. 6B). When the effect of Mn^{2+} and Mg^{2+} ions on the activities of the ts DNA polymerase mutants were compared at 30°C, similar ratios as with the wild-type DNA polymerase were obtained with the ts2(24) mutant (Fig.6). However, with the ts2(98)mutant the ratios were greatly increased, 30 for initiation and 9 for polymerization (Fig. 6), being this effect not related with variations in the optimal concentration for each metal. This behaviour could be related with the proximity of the ts2(98) mutation to a region proposed to be involved in Me^{2+} binding, at the polymerization and initiation active site, mainly represented by the central motif YCDTDS (19).

DISCUSSION

The analysis of *B.subtilis* infected with the ϕ29 ts2(24) and the ts2(98)-group mutants clearly demonstrated that the viral DNA synthesis was temperature-sentitive, being the initial step(s) of replication the one(s) affected by the shift to the non-permissive temperature. These results could be explained if different active sites in the ϕ29 DNA polymerase (p2) are involved in the initiation and elongation reactions. Then, all the isolated ts2 mutants would be exclusively affected in the initiation domain. Recently, targeted mutagenesis studies have demonstrated that both activities, initiation and elongation, share discrete domains in the enzyme (19; unpublished results) indicating that there is not a physical separation of the two activities. The fact that both

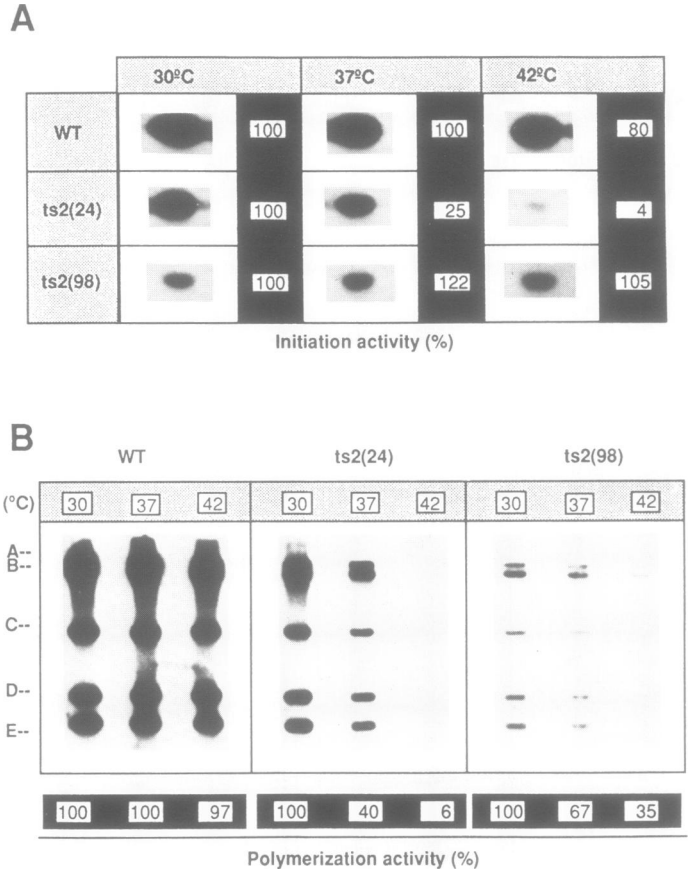

Figure 4. Temperature-sensitivity analysis of the initiation and polymerization activities of the ϕ29 DNA polymerase ts mutants. A. The initiation assay was carried out as described in Materials and Methods, using 2 μg of protein p6. After preincubation at the indicated temperatures, the protein fractions were assayed in the initiation reaction at 30°C, and the p3-dAMP initiation complex formed was analyzed by SDS-polyacrylamide gel electrophopresis. The figure shows the p3-dAMP band and the numbers in the black columns indicate the relative initiation activity of each DNA polymerase, obtained after preincubation at 37° and 42°C, with respect to the activity obtained after preincubation at 30°C. B. The polymerization assay (filling-in reaction) was as described in Materials and Methods using EcoRI-digested ϕ29 DNA as template. (α-^{32}P)dAMP incorporation was quantitated and analyzed by agarose gel electrophoresis, as described in Materials and Methods. The positions of the different EcoRI ϕ29 DNA fragments are indicated in the left side. Numbers at the bottom indicate the relative DNA polymerase activity, obtained after preincubation at 37° and 42°C, respect to the activity obtained after preincubation at 30°C.

mutations, ts2(24) and ts2(98), map 138 amino acids apart and the structural organization model proposed for the ϕ29 DNA polymerase (18), do not support the existence of two different active sites, for initiation and polymerization. Other possiblity is that the ϕ29 DNA polymerase, when forms part of the elongation complex, is less sensitive to a temperature shift than when it is initiating replication. This explanation could be also related with the behaviour of another ϕ29 mutant, ts2(35)(64). In this case, parental phage DNA in infected bacteria rapidly dissociates from the cell membrane after the shift to 45°C, whereas DNA synthesis does not stop immediately. Both experimental results could be reconciled assuming that a temperature-mediated conformational change still allows elongation to occur but avoids interaction with the cell membrane and subsequent reinitiation.

The *in vitro* temperature-sensitivity of the ts2(24) DNA polymerase, both in the initiation and elongation reactions, suggests that the *in vivo* resistance of elongation to a temperature shift must be due to protection by formation of a replicative complex. On the other hand, the slight temperature-sensitivity in elongation and the resistance in initiation activity of the ts2(98) DNA polymerase cannot account for the *in vivo* ts phenotype. This is not the first example reported with a similar behaviour; in the case of the T4 DNA polymerase mutants tsL141 and tsS9, the polymerase activity *in vitro* was similar at 37°C and at 25°C (65). TsS9 mutation maps at position 671 in the T4 DNA polymerase and its phenotype is the consequence of a change of methionine to isoleucine. Protein tsL141 also has a single amino acid change, alanine to valine, at position 737 (60), and it is considered the archetype of the antimutator mutant (5). Taking into account the alignment presented in Figure 3, it can be concluded that both T4 DNA polymerase mutations map near the related region of ϕ29 DNA polymerase where the ts2(98) mutation is located. This apparent relationship between the functional phenotype of different ts DNA polymerases and the physical mapping supports the possible functional relevance of this new homologous region (Fig. 3, region 4). The homology detected with the *E. coli* DNA polymerase I-like enzymes (Blanco et al., in preparation) indicates that segment 4 in Figure 3 could be the homologous one to the segment identified in the *E. coli* enzyme as directly involved in dNTP binding by labelling with pyridoxal 5'-phosphate (66) or azido-dATP (67). The presence in this region of antagonist phenotypes in T4 DNA polymerase mutants (tsL88 is a weak mutator and tsL141, which has the alaline to valine change, is the strongest antimutator described), probably indicates that this region could play a crucial role for

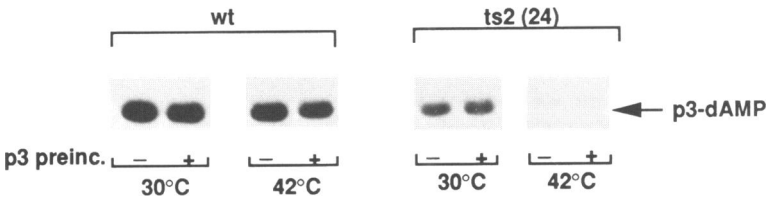

Figure 5. Ineffectiveness of ϕ29 terminal protein to protect ts2(24) DNA polymerase from temperature inactivation. The inhibition assay was carried out as described in Materials and Methods, after preincubation at 30°C or 42°C, in the absence or presence of ϕ29 terminal protein (60 ng). The p3-dAMP initiation complex formed was analyzed by SDS-polyacrylamide gel electrophoresis, autoradiography and densitometry of the exposed film.

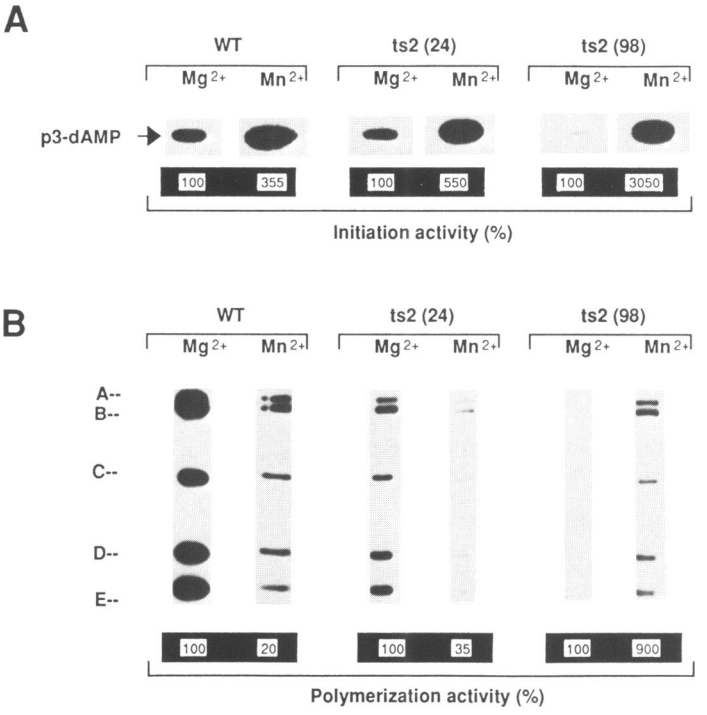

Figure 6. Effect of Mn^{2+} ions on the protein-primed initiation and polymerization activities of the ϕ29 DNA polymerase ts mutants. A.The initiation assay was carried out as described in Materials and Methods, using either 10 mM $MgCl_2$ or 1 mM $MnCl_2$. After incubation at 30°C, the p3-dAMP initiation complex formed was analyzed as described in the legend to Fig. 5. B.The DNA polymerase assay (filling-in reaction) was as described in Materials and Methods. After incubation for 10 min at 30°C using as divalent activating metal ion either 10 mM $MgCl_2$ or 1 mM $MnCl_2$ the [α-^{32}P]dAMP incorporation was quantitated and analyzed by agarose gel electrophoresis as described in Fig.5. Numbers in black indicate the relative initiation and DNA polymerase activities, when Mn^{2+} ions were used instead of Mg^{2+} ions.

insertion fidelity. Another interesting point, relevant for the possible analogy between the ϕ29 ts2(98) and the T4 tsL141 DNA polymerase mutants is the fact that the latter is other example in which five independent isolates have the same amino acid change in the same position (60). This remarkable clustering of ts mutations in few 'hot spots' has been also detected in other systems, such as the SV40 large antigen (68) and bacteriophage T4 lysozyme (2), suggesting that these amino acids could play crucial roles in organizing the structure of one or more functional domains. Another feature inferred from these and other studies (2,65) is that, among the possible changes in alanine, only the ones to valine or threonine were found to produce the ts phenotype, independently of the mutagen used for their generation. Studies on the EcoRI endonuclease (69) have also rendered similar results, the changes of alanine to valine or threonine are the ones destroying the endonuclease activity. Another example of the structural importance of the alanine to valine change is the fact that the point mutation of Ala 31 to Val prohibits the folding of reduced chicken lysozyme (67).

The high activity of the ts2(98) DNA polymerase in the presence of Mn^{2+} (see Fig.6) is particularly interesting. As far as we know this is the first example described for a mutant DNA polymerase that presents alterations in the use of divalent activating ions. This phenotype could be related with the location of the ts2(98) mutation in a region proposed to be involved in dNTP binding (Blanco et al., in preparation) and close to the proposed Me^{2+} binding site, mainly represented by the YCDTDS motif (19).

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