Residues at the Carboxy Terminus of T_4 DNA Polymerase Are Important Determinants for Interaction with the Polymerase Accessory Proteins†

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Received April 17, 1997; Revised Manuscript Received June 11, 1997⊗

ABSTRACT: Three T_4 DNA polymerase accessory proteins (44P/62P and 45P) stimulate the polymerase (pol) activity and the 3′−5′ exonuclease (exo) activity of T_4 DNA polymerase (43P) on long, double-stranded DNA substrates. The 44P/62P "clamp loader" facilitates the binding of 45P, the "sliding clamp", to DNA that is primed for replication. Using a series of truncated 43P mutants, we identified a region at the extreme carboxy terminus of the DNA polymerase that is required for its interaction with accessory proteins. Truncation mutants of 43P lacking the carboxy-terminal 3, 6, or 11 residues retained full pol and exo activity on short synthetic primer-templates. However, the ability of the accessory proteins to enhance these activities on long double-stranded DNA templates was drastically reduced, and the extent of the reduction in activity was greater as more residues were deleted. One of the truncation mutants (N881), which had 17 residues removed from the carboxy terminus, showed reduced binding affinity and diminished pol activity but enhanced exo activity upon incubation with a small primer-template. The exo activity of the N881 mutant, on short, single-stranded DNA was unchanged, however, compared to the wild-type enzyme. These results are consistent with inferences drawn from the crystal structure of a DNA polymerase from a related T-even phage, RB69, where the carboxy-terminal 12 residues (equivalent to the 11 residues of 43P from phage T_4) protrude from the thumb domain and are free to interact with complementary surfaces of the accessory proteins. The structural integrity of the thumb region in the N881 mutant is probably perturbed and could account for its reduced binding affinity and pol activity when incubated with short, double-stranded DNA substrates.

The bacteriophage T_4 DNA replication complex, one of the simplest and most extensively studied systems of its kind, has served as a model for studying the enzymology of DNA replication [see Nossal (1992) and Young et al. (1992) for reviews]. It is a multiprotein complex consisting of a catalytic subunit (43P) and accessory proteins that modify its properties (Kornberg & Baker, 1992). The catalytic subunit of the holoenzyme is the product of T_4 gene 43 which has a 5′−3′ nucleotidyl transferase (pol)[1] activity as well as a 3′−5′ exonuclease (exo) activity (de Waard et al., 1965; Spicer et al., 1988). In addition, it is an autogenous repressor which binds to a specific site on its mRNA just upstream from the initiation codon, resulting in translational down-regulation (Andrake et al., 1988). Other components of the holoenzyme include 45P (the "sliding clamp") as well as the primase (61P) and helicase (41P) which are required for lagging strand synthesis (Mace & Alberts, 1984a,b; Nossal & Alberts, 1983; Liu & Alberts, 1981; Venkatesan et al., 1982; Hinton et al., 1985). The T4-encoded single-stranded DNA binding protein, 32P, which has affinity for DNA polymerase, can be considered to be part of the holoenzyme complex even though nucleotide addition will occur *in vitro* in its absence (Nossal & Peterlin, 1979; Alberts et al., 1980).

In recent years, a model for the mechanism of T_4 DNA polymerase holoenzyme assembly has been developed, based partly on results obtained with the T_4 *in vitro* DNA replication system and partly by analogy to studies using the corresponding proteins from *Escherichia coli* (Kornberg & Baker, 1992; Onrust et al., 1991; Stukenberg et al., 1991). According to this model, assembly of the DNA replication complex is initiated by binding of the "clamp loader", 44P/62P (Barry & Alberts, 1972), to a primer-template junction (Jarvis et al., 1989a,b; Mace & Alberts, 1984a,b; Sancar & Hearst, 1993; Capson et al., 1991). Using energy released from ATP hydrolysis, 44P/62P then promotes the assembly of a trimeric ring of 45P subunits which encircles the DNA, presumably in a fashion similar to that of the dimeric β protein of the *E. coli* DNA polymerase III holoenzyme (Kong et al., 1992). Finally, the DNA polymerase catalytic subunit, 43P, binds at the primer-template junction, interacting directly with 45P (Kaboord & Benkovic, 1995).

Recently progress has been made toward determining the three-dimensional architecture of the T-even phage DNA replication complex with completion of the crystal structure of the DNA polymerase from bacteriophage RB69, a T-even phage distantly related to T_4 (Wang et al., 1997), the crystal structure of 45P from phage T4 (J. Kuriyan, personal

† This work was supported by USPHS Grant GM54627.

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⊗ Abstract published in *Advance ACS Abstracts,* August 1, 1997.

[1] Abbreviations: pol, polymerase; exo, exonuclease; DEAE, diethylaminoethyl; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA; TBE, Tris buffer−EDTA; EDTA, ethylenediaminetetraacetic acid.

S0006-2960(97)00894-5 CCC: $14.00

communication), and the crystal structure of the gene 32 protein "core" (Shamoo et al., 1995). A striking feature of RB69 DNA polymerase is that it has 12 residues at its C-terminus protruding from the folded domains of the protein. Based on docking experiments with ds DNA and with models of the proposed structure of 45P, we believe that the sliding clamp protein is most likely to interact with this C-terminal region of the phage DNA polymerase. To test this idea, we constructed and expressed a series of deletion mutants with 3, 6, 11, or 17 residues removed from the C-terminus of T_4 DNA polymerase. We first determined the intrinsic pol and exo activities of these altered DNA polymerases, and then examined the ability of 44P/62P and 45P to stimulate these activities on long ds DNA substrates and interpreted the results based on the crystal structure of the homologous DNA polymerase from the T-even phage, RB69.

EXPERIMENTAL PROCEDURES

Cloning and Mutagenesis. All cloning of the DNA fragments was performed according to standard procedures (Sambrook et al., 1989). Plasmid pTL7-g43 (Lin et al., 1994), which contains T_4 gene 43 under the control of a bacteriophage T7 promoter, was used to construct the four truncated gene 43 mutants by introducing stop codons via the Kunkel method (Kunkel, 1985) at appropriate positions to give plasmids pN895, pN892, pN887, and pN881.

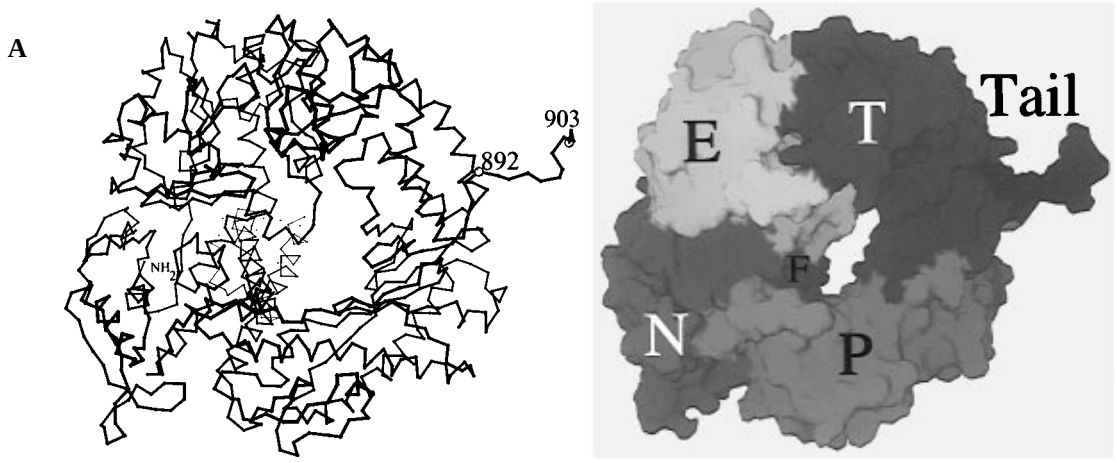
Protein Purification and Characterization. All proteins were purified from *E. coli* BL21/DE3 strains harboring the relevant plasmid vectors. These cells were grown and induced as previously described (Lin et al., 1994). Wild-type and mutant T_4 DNA polymerases were purified by a modification of the method of Nossal (1974) after preparing the crude cell lysates according to Morris *et al.* (1979). Because of the high ratio of 43P or its mutants to intracellular *E. coli* proteins and due to the absence of other T_4-encoded proteins in the crude lysate, it was possible to use just two columns, DEAE-cellulose and phosphocellulose, to purify the DNA polymerases to greater than 90% purity as estimated from Coomassie blue-stained SDS−polyacrylamide gels. The T_4 DNA polymerase accessory proteins 45P and 44P/62P, kindly provided by Dr. Hyacinth Sterling (Yale University), were purified according to established methods (Nossal, 1979; Morris et al., 1979).

Polymerase and Exonuclease Assays Using Short Primer-Templates. Extension of a short primer-template (16/24-mer) substrate was carried out as described by Sattar et al. (1996). Exonuclease assays were performed using the same ds DNA (16/24 mer) substrate as described by Lin et al. (1994).

Assay for Stimulation of Exonuclease Activity by Accessory Proteins. A modification of the procedure of Bedinger and Alberts (1983) was used to assay for stimulation of 3′−5′ exonuclease activity. Briefly, a four-kilobase double-stranded DNA plasmid was linearized with *Eco*RI and labeled with ^{32}P at the 5′ ends. Reactions contained labeled DNA (2.5−10 μg/mL), 33 mM Tris−acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 μg/mL bovine serum albumin, and 500 μM ATP. When present, the 44P/62P protein complex and 45P were used at concentrations of 20 or 40 μg/mL and 15 or 30 μg/mL, respectively. Wild-type or mutant T_4 DNA polymerase was added to a final concentration of 2.5 or 5 μg/mL to start the reactions. After incubation at 37 °C for various times, the reactions were stopped by adding SDS to a final concentration of 1%. The size of the products was determined by electrophoresis on a 1% agarose gel in Tris−acetate buffer, pH 8, with molecular weight markers (bacteriophage λ DNA digested with *Bst*EII and 5′-end-labeled with ^{32}P). The gel was placed onto filter paper, dried, and exposed to Kodak XAR film.

Assay for Stimulation of DNA Polymerase Activity by Accessory Proteins. Stimulation of DNA polymerase activity was measured by a procedure similar to that used by Cha and Alberts (1989). Primer M13PRIM1 was 5′-end-labeled with ^{32}P and annealed to single-stranded, circular M13mp18 DNA as described for the mobility shift experiments (see below) except that the annealing/reaction buffer was 33 mM Tris−acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, and 0.5 mM dithiothreitol. The primer was complementary to nucleotides 4852 through 4876 of M13mp18. Reactions contained this annealing/reaction buffer as well as 100 μg/mL bovine serum albumin, 500 μM ATP, and 200 μM of each of the four deoxyribonucleoside triphosphates. Reactions were initiated by the addition of wild-type or mutant T_4 DNA polymerase to a final concentration of 5 μg/mL and, when indicated, 44P/62P and 45P to final concentrations of 40 μg/mL and 30 μg/mL, respectively. After incubation at 37 °C, 10 μL aliquots were removed at various times, and added to alkaline gel loading buffer (30 mM NaOH, 20 mM EDTA, 10% sucrose, 0.5% bromcresol green, and 0.5% xylene cyanol) which stopped the reactions. Reaction products were analyzed by electrophoresis through a 1% agarose gel in alkaline electrophoresis buffer (30 mM NaOH, 1 mM EDTA) with constant buffer circulation. The gel was neutralized by soaking in TBE buffer (89 mM Tris−borate, 2 mM EDTA) for 1 h, placed on Whatman DE81 filter paper, dried at 60 °C under vacuum, and then exposed to Kodak XAR film for analysis.

Mobility Shift (DNA Binding) Assays. Mobility shift assays were performed according to a procedure suggested by Dr. Michael Reddy (personal communication). A 25-nucleotide synthetic primer, M13PRIM1 (5′-AGCAGCAAATGAAAAATCTAAAGCA-3′), was 5′-end-labeled with [γ-^{32}P]-ATP and T_4 polynucleotide kinase according to standard methods (Sambrook et al., 1989). The labeled primer was annealed to a 45-mer template, M13TEM1A (5′-GAGGTTCAGCAAGGTGATGCTTTAGATTTTTCATTTGCTGTC-3′), by heating to 65 °C for 5 min in buffer A (25 mM Tris−acetate, pH 7.5, 60 mM sodium acetate, and 1 mM EDTA) and cooling slowly to room temperature. Various concentrations of wild-type or mutant T_4 DNA polymerase were added to reaction buffer A containing 20% glycerol and 10 nM annealed primer-template. Reactions were incubated for 15 min at 25 °C and loaded onto a 7.5% polyacrylamide gel cast in buffer A. The samples were electrophoresed for 1 min at 100 mA followed by 15 min at 50 mA. The gel was dried and exposed to Kodak XAR film. The intensity of the bands was estimated by phosphoimaging (Molecular Dynamics) using the ImageQuant data analysis program supplied by Molecular Dynamics. The relative binding affinities were calculated by estimating the ratio of the concentrations of mutant polymerases vs the wild type required to shift 50% of the primer-template from the bottom of the gel to the top (high to low mobility). The relative



B

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                                                  |← - - - - - -  Protruding tail - - - - - - →|
                                                  892
RB69-43P  885 Ser Ala Ala Lys Leu Asp Tyr Glu Lys Lys Ala Ser Leu Leu Asp Met Phe Asp Phe 903
                        |           | | |       |   |   |   |               |
T4-43P    881 Glu Ser Ala Gly Met Asp Tyr Glu Glu Lys Ala Ser Leu Asp Phe Leu Phe Gly 898
                                    887 |             892   |   |   |   |
T4-33P     96 Arg Pro Ser Val Val Arg Cys Glu Lys Thr Asn Thr Leu Asp Phe Leu Leu 112
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FIGURE 1: (A) Left panel: tracing of the α-carbon backbone of RB69 DNA polymerase. The locations of the NH_2- and COOH-termini are indicated. The total length of the RB69 DNA polymerase is 903 residues compared to 898 for T_4 DNA polymerase. The position of residue 892 (11 residues proximal to the C-terminal, residue 903) is indicated. Right panel: space-filling model of TB69 DNA polymerase. The location of four of the five domains and the tail is indicated; E, exonuclear domain; N, NH_2-terminal domain; P, palm domain; and T, thumb domain. (B) Sequence similarity of the carboxy termini of T_4 DNA polymerase, RB69 DNA polymerase, and T_4 gene 33 protein. The carboxy-terminal residues of the truncation mutants N892 and N887, missing 6 and 11 amino acids, respectively, are underlined. The length of the protruding "tail" is shown above the RB69−43P sequence. The vertical lines indicate identities among the three proteins.

binding affinity of wild type was set at 1.0.

RESULTS

Expression and Characterization of Mutant T_4 DNA Polymerases with C-Terminal Deletions. Truncated T_4 DNA polymerases, N895, N892, N887, and N881 lacking the C-terminal 3, 6, 11, and 17 residues, respectively, were expressed and purified to greater than 90% homogeneity as judged by SDS−PAGE (data not shown). The four mutant DNA polymerases and the wild type were assayed for pol and exo activities using a 16/24-mer synthetic primer-template. While the pol and exo activities of N895 and N892 (data not shown) as well as the N887 mutant DNA polymerases were nearly identical to wild-type 43P (Figure 2A), the N881 mutant had greatly reduced pol activity (Figure 2B). However, the exo activity of N881 was about 3-fold higher than that of wild type (Figure 2C) presumably because the 16/24-mer substrate binds mainly in the exo mode since the pol mode binding site was probably perturbed by the C-terminal truncation (see below). In contrast to the results with ds DNA, the exo activity, when ss DNA (16-mer) was used as the substrate, was the same for wild type and all of the mutants (Figure 2D).

DNA Binding Affinity of T_4 DNA Polymerase Truncation Mutants. To determine whether the reduction of N881 pol activity was due to its decreased affinity for DNA, we estimated the relative binding affinities for all the mutants with a 25/45-mer using a gel shift assay. We found that binding affinities of the wild-type 43P and N887 DNA polymerase mutants were similar, requiring 0.8 and 0.7 μM concentrations of the proteins, respectively, to shift 50% of the oligonucleotides to the higher position in the gel, whereas the binding affinity for N881 needed slightly more than 1.2 μM concentration of the protein to shift 50% of the oligonucleotides to the higher position in the gel, giving a relative binding affinity of 0.67 as shown in Figure 3.

Effect of Accessory Proteins upon the Apparent pol and exo Activities of T_4 DNA Polymerase and Its Truncation Mutants Using Long ds DNA Substrates. Interactions between the wild-type and mutant DNA polymerases and the accessory proteins were examined by measuring their ability to promote DNA synthesis past a pause site in primed M13mp18 single-stranded DNA. In this experiment, a 5′-end-labeled oligonucleotide was annealed to single-stranded, circular M13mp18 DNA and incubated with wild-type or mutant DNA polymerases for various times. The sizes of the extension products were determined after electrophoresis on an alkaline agarose gel. The autoradiogram for wild type and N887 shown in Figure 4 (data for N895 and N892 are not shown) revealed the presence of the expected strong pause site at approximately 1.5 kilobases (kb) from the primer binding site. Each of the polymerases extended nearly all of the available primer to this position; however, none of the polymerases extended the primer beyond 1.5 kb in the absence of accessory proteins (except for a very small amount at 20 min) (Figure 4). In the presence of accessory proteins, wild-type 43P extended all of the primers past the pause site at 1.5 kb, and a considerable portion of the primers were extended all the way around the circular M13mp18 template to yield 8 kb products. In contrast, the N887 mutant did not extend the primer past the 1.5 kb pause site after 5 min, and only a very small amount of extension was observed in the presence of the accessory proteins even after 20 min. The relative pol activity in the presence and absence of accessory proteins was determined quantitatively by comparing the number of primers arrested at the 1.5 kb pause site

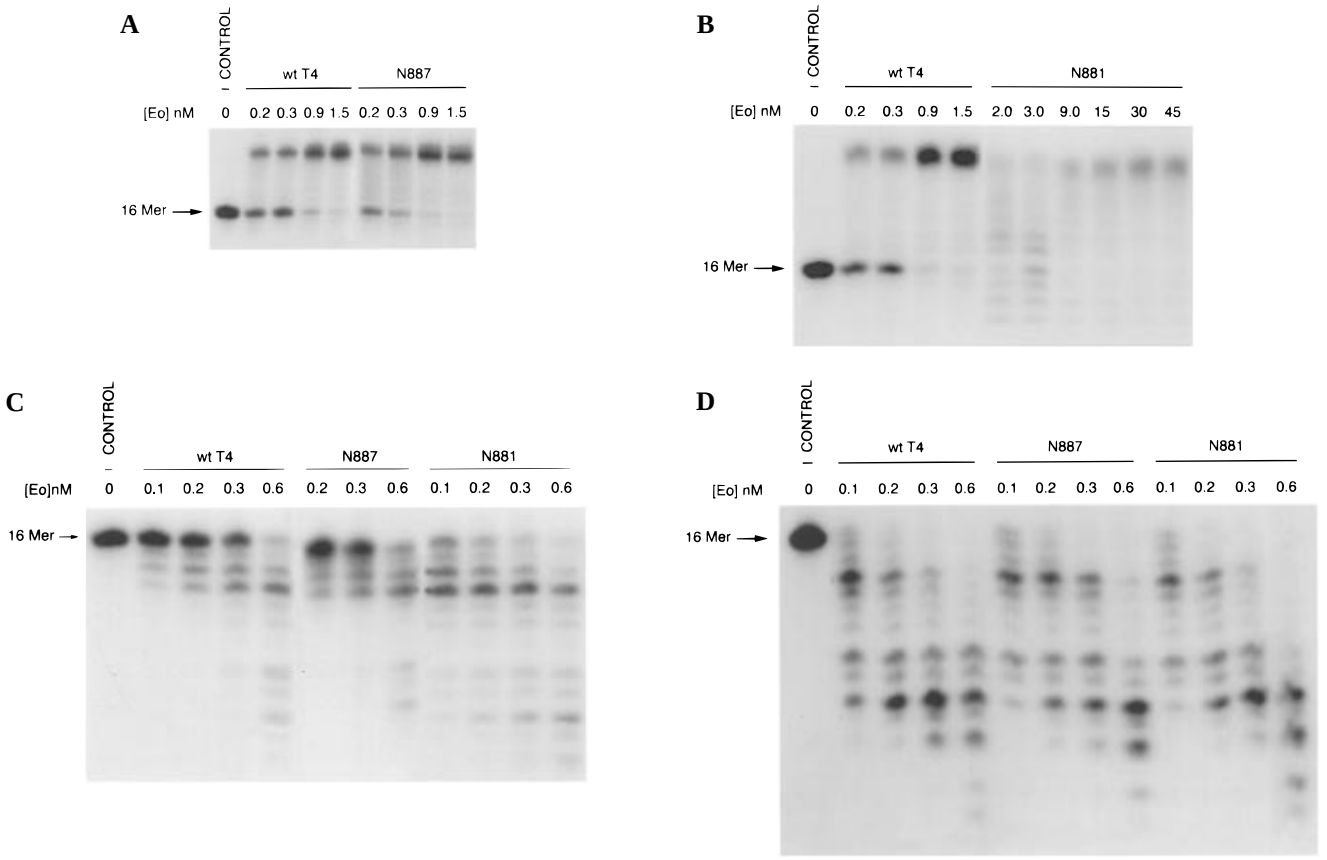

FIGURE 2: Relative polymerase and exonuclease activities of T_4 DNA polymerase truncation mutants. Wild-type and mutant T_4 DNA polymerases were expressed, purified, and assayed for polymerase and exonuclease activities as described under Experimental Procedures. Polymerase activities were determined by estimating the concentration of polymerase required to fully extend 50% of the 16-mer primers annealed to a 24-mer template at a fixed time and temperature. Exonuclease activities were estimated by determining the concentration of polymerase required to reduce the intensity of the 5′-labeled 16-mer by 50%. All assays were performed at 30 °C. [E_0] is the enzyme concentration. (A) Comparison of pol activities of wild-type T_4 DNA polymerase (wt T_4) with mutant N887; (B) comparison of wt T_4 pol activity with mutant N881; (C) comparison of the exo activities of wt T_4 with the N887 and N881 mutants acting on the 16/24-mer primer/template; (D) comparison of the exo activities of wt T_4 with the mutants N887 and N881 acting on the 16-mer alone. The pol activity of N881 in panel B could not be accurately measured because its potent exo activity rapidly degraded the labeled primer. Taking this into account, our best estimate of N881 pol activity was between 10 and 20% of the wild type.

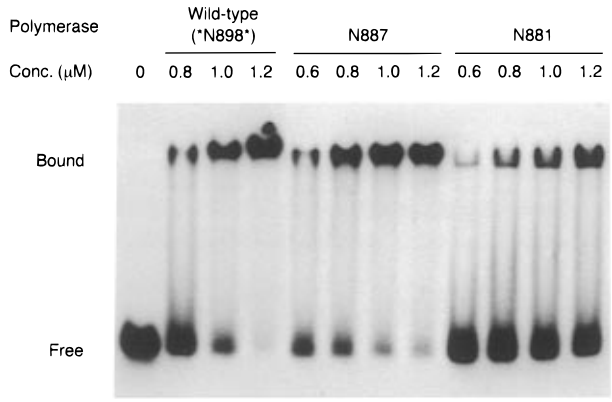

FIGURE 3: Relative affinities of wild-type and mutant T_4 DNA polymerases with a synthetic 25/45-mer primer-template. Primer M13PRIM1 (25-mer) was labeled at the 5′ end with ^{32}P, annealed to template M13TEM1A (45-mer), and incubated at a concentration of 10 nM primer-template with the indicated concentrations of wild-type or mutant T_4 DNA polymerases for 15 min at 25 °C. The complexes were then electrophoresed on a 7.5% polyacrylamide gel, which was subsequently dried and exposed to X-ray film. The type of DNA polymerase and the concentration used are shown at the top of the gel. The positions of bound and free primer-template are indicated on the left.

with the number extended beyond it using a phophorimager. With wild-type 43P, a 54-fold increase in the number of primers extended past the pause site was observed at 5 min in the presence of accessory proteins. It should be noted, however, that this level of stimulation represents only a minimum estimate since almost all the primers had been extended by 5 min. In contrast, the N887 DNA polymerase showed less than a 2-fold stimulation by the accessory proteins at 20 min. If the level of stimulation of wild-type 43P pol activity by the accessory proteins is arbitrarily set at 100%, then the level of stimulation for the N887 protein corresponds to about 4% of that observed with wild type 43P (Table 1). The pol activities of N892 and N895 DNA polymerases were stimulated to 10% and 22%, respectively, relative to the stimulation observed with wild type (Table 1). The N881 DNA polymerase was not included in this experiment because it lacks sufficient pol activity to compete with its very strong exo activity, and, as a consequence, the primer could not be extended to the first pause site even in the presence of high concentrations of dNTPs (data not shown).

The interaction of accessory proteins with mutant DNA polymerases was also tested by measuring exo activities in the presence and absence of accessory proteins. As shown in Figure 5A, the size of the 4 kb plasmid substrate decreased over time in the presence of wild-type DNA polymerase, and enhancement of exo activity was observed when accessory proteins were present. The extent of exonuclease

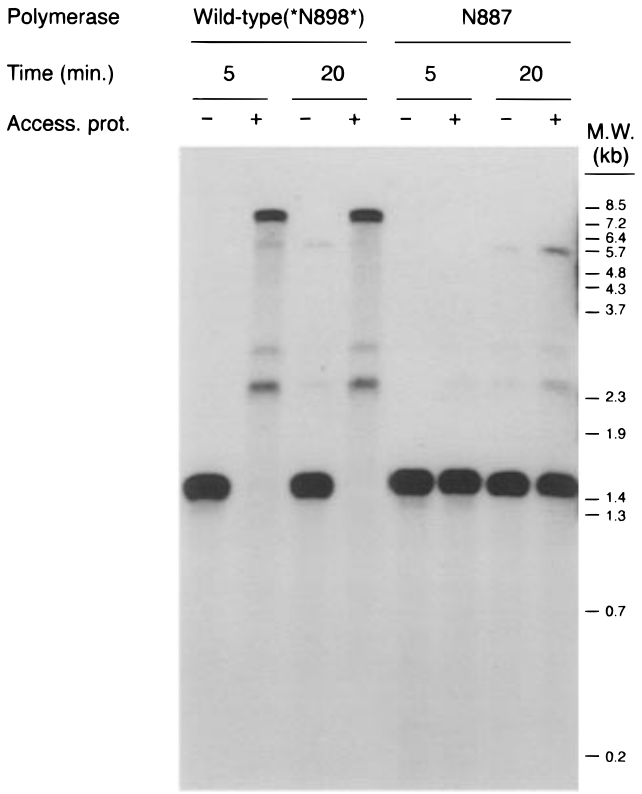

FIGURE 4: Stimulation of the polymerase activity of wild-type and N887 mutant T_4 DNA polymerases by the accessory proteins. Primer M13PRIM1 was labeled at the 5′ end with ^{32}P, annealed to single-stranded M13mp18 DNA, and incubated with wild-type or N887 mutant T_4 DNA polymerases (5 μg/mL) in the absence (−) or presence (+) of the T_4 DNA polymerase accessory proteins 44P/62P (40 μg/mL) and 45P (30 μg/mL) for either 5 or 20 min at 37 °C. The reaction products were electrophoresed on an alkaline agarose gel along with labeled DNA size markers (bacteriophage λDNA digested with *Bst*EII); the gel was dried and exposed to X-ray film. The position of the molecular weight markers is indicated on the right.

digestion for each sample at a given time was estimated by measuring the length (in kb) of the major DNA species. This allowed us to estimate the rate of digestion which is expressed as the number of nucleotides excised per minute. The results show that the exo activity of wild-type 43P doubled in the presence of the accessory proteins, whereas the exo activities of the N887 DNA polymerase increased by only 7% in the presence of 44P/62P and 45P. If the degree of stimulation of wild-type 43P is normalized to 100%, the values for the mutant correspond to barely 3% of wild-type levels (Figure 5A, Table 1). When a similar experiment was done with the N881 DNA polymerase, no stimulation of exo activity by accessory proteins could be detected (Figure 5B, Table 1). Thus, it appears that progressive removal of residues from the C-terminus diminishes the extent to which the polymerase can interact with accessory proteins that enhance processivity during exonucleolytic digestion of long ds DNA.

DISCUSSION

In this paper, we have shown that deletions of small portions of the C-terminus of T_4 DNA polymerase greatly reduce the ability of accessory proteins to stimulate the pol and exo activities of 43P on long ds DNA templates. As the length of the deletion is increased from 3 to 6 and then to 11 residues, the stimulatory effect is correspondingly reduced (Table 1). Using short primer-templates, in the absence of accessory proteins, the pol and exo activities and binding affinities were unaffected until the C-terminal truncation exceeded 11 residues, indicating that deletions up to this length did not perturb the catalytic center and substrate binding sites. Deletion of 17 residues to give the N881 mutant, however, resulted in severe reduction of pol activity and lower primer/template binding affinity. These results reinforce the proposal that the C-terminal residues could interact directly with the sliding clamp (45P) in the holoenzyme and are consistent with similar observations on C-terminal truncation mutants of herpes simplex virus DNA polymerase (HSV pol) (Hernandez & Lehman, 1991; Digard et al., 1993; Stow, 1993) and a pseudorabies polymerase (Berthomme et al., 1995). Although there is significant amino acid sequence similarity between several regions of T_4 DNA polymerase HSV pol and pseudorabies pol (Spicer et al., 1988), there is no similarity when comparing the C-terminal 20 residues of the 3 proteins. This is not entirely unexpected since, although there is a selective pressure for each DNA polymerase to maintain interactions with its own set of accessory proteins, there is freedom for each to coevolve with its own set of accessory proteins to form distinct binding interfaces. In fact, while there is an overall sequence identity of 74% between T_4 and RB69 DNA polymerase, there is only 50% identity at the C-terminus of the two proteins (Wang et al., 1996). The accessory proteins from RB69 also have similarly clustered regions of identity with the corresponding proteins from T_4, and the residues involved in polymerase−accessory protein interactions probably are not identical, since it has also been found that complementation does not always occur in a completely reciprocal fashion between accessory proteins from RB69 or T_4 and their DNA polymerases (J. Karam, personnel communication).

Table 1: Stimulation of pol and exo Activities of Wild-Type and Mutant T_4 DNA Polymerases by Accessory Proteins[a,b]

	T_4 DNA polymerase (%)				
activity	wild type	N895	N892	N887	N881
pol	100	22	∼10[c]	∼4[c]	ND[d]
exo	100	23	∼10[c]	∼3[c]	∼0[c]

[a] Details of the assays are described under Experimental Procedures. [b] Stimulation of pol activity was calculated by comparing the amount of primer extended through the strong pause site in the presence and absence of accessory proteins. The fractional increase in the amount of primer extended beyond the strong pause site in the presence of the accessory proteins. The stimulation of wild-type pol activity was normalized to 100% and used as a standard for comparison with the mutants. Stimulation of exo activity was calculated by comparing the fractional increase in the exonuclease rate (nt/min) for the wild-type enzyme in the presence and absence of accessory proteins. The stimulation of wild-type exo activity was normalized to 100% and used as a standard for comparison with the mutants. In the case of wild-type polymerase without accessory proteins, the excision rate was 16 nt/min, and with accessory proteins, the excision rate was 55 nt/min. The stimulation factor for wild type was $(55 - 16)/16 = 2.44$. This factor was normalized to 100% as shown in the table. Similarly, the stimulation by accessory protein for the N887 mutant was 0.075 so that the stimulation relative to wild type was $0.075/2.44 = 0.03$ or 3% as shown in the table. [c] When stimulation was weak or absent, accurate measurement of the level of stimulation by accessory proteins was difficult. Values given are estimated with a precision of ±50%. [d] Not done. This was due to its low efficiency in extending primers to the strong pause site.

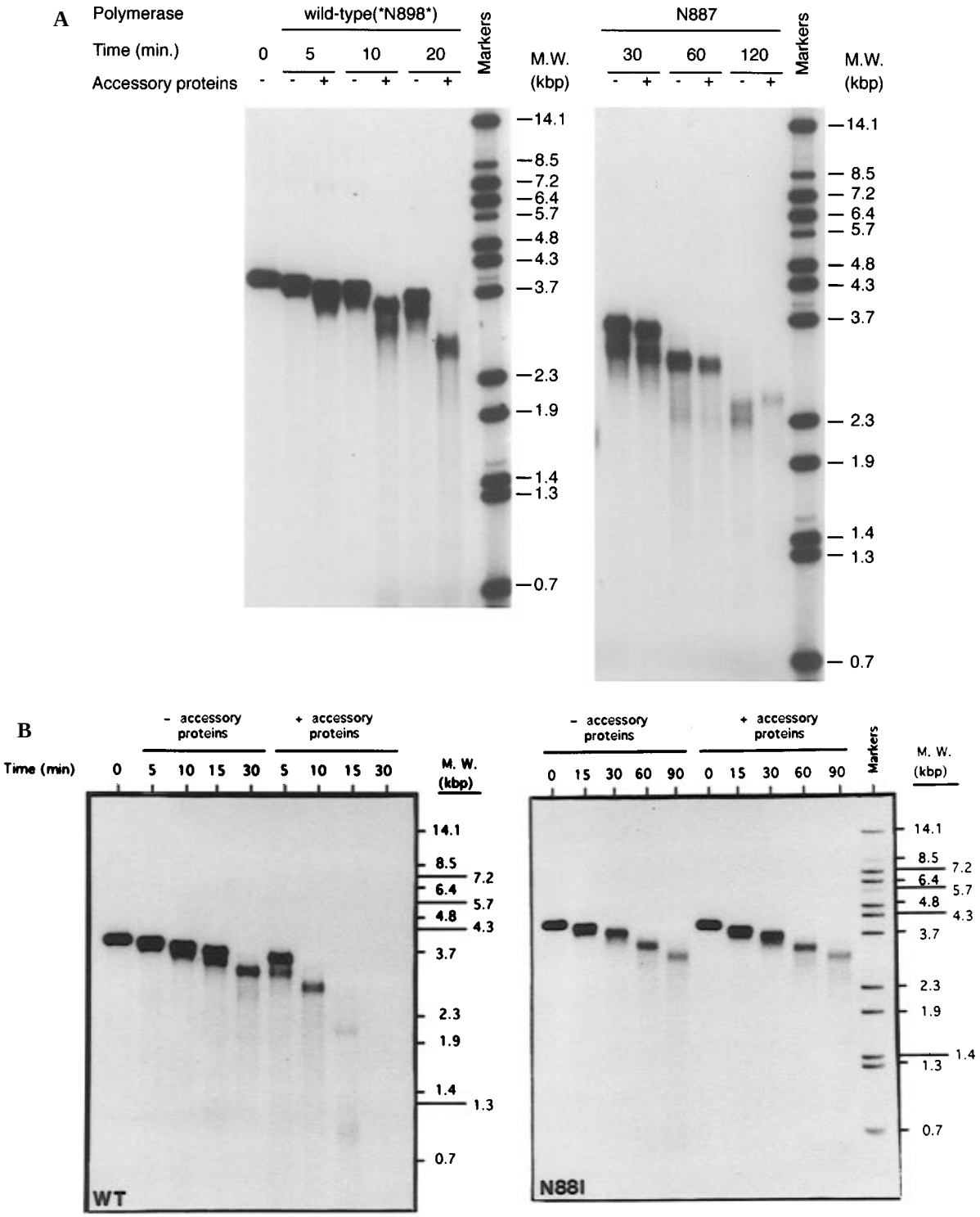

FIGURE 5: Effect of the T_4 DNA polymerase accessory proteins upon the 3′−5′ exonuclease activity of wild-type N887 and N881 mutant T_4 DNA polymerases. A linearized 4.0 kbp plasmid was 5′-end-labeled with ^{32}P and incubated with wild-type or N881 T_4 DNA polymerase (2.5 μg/mL) in the presence or absence of the T_4 DNA polymerase accessory proteins [44/62 protein (20 μg/mL) and 45 protein (15 μg/mL)] and ATP (500 μM). After incubation at 37 °C for the indicated times, the reactions were stopped by adding SDS to a final concentration of 1% and electrophoresed on a 1% agarose gel with DNA molecular weight markers (bacteriophage λDNA digested with *Bst*EII). The gels were dried and exposed to X-ray film to give the resulting autoradiograms: (A) Left panel, wt T_4 DNA polymerase using DNA concentrations of 10 μg/mL; right panel, N887 mutant T_4 DNA polymerase using DNA concentrations of 5 μg/mL. (B) Left panel, wild type (wt); right panel, mutant N881 DNA polymerase. The concentration of the DNA polymerase was 5 μg/mL, the DNA concentration was 2.5 μg/mL, the concentrations of 44P/62P and 45P were 40 μg/mL and 30 μg/mL, respectively, and the reactions were carried out for the times indicated above the gel.

The crystal structure of RB69 DNA polymerase has a disordered carboxy-terminal region of 12 residues emerging from an α-helix that is part of the thumb domain. These last 12 residues protrude as a "tail", in a direction parallel to the groove proposed for the ds DNA binding site, and at a location which is likely to serve as a binding interface for 45P (Figure 1A) (Wang et al., 1997). Based on sequence homology with the RB69 DNA polymerase, the C-terminal 11 residues of T_4 DNA polymerase would be expected to form an equivalent "tail". Four of the last six C-terminal

residues of 43P are hydrophobic (Figure 1B), and it is possible that this six residue "hydrophobic tail" could fit into a hydrophobic pocket in the gene 45 protein trimer and mediate 43P−45P interactions. This is reminiscent of the interaction observed for calmodulin with a calmodulin-binding domain peptide (Ikura et al., 1992). The T_4 gene 41 helicase protein may also have a hydrophobic "tail" capable of interacting with the accessory proteins since a truncated gene 41 protein where the C-terminal 20 residues had been removed after trypsin digestion was not stimulated by the DNA polymerase accessory proteins (Richardson & Nossal, 1989). In the case of HSV DNA polymerase, Digard et al. (1993) have suggested that the C-terminal 35 residues interact directly with its processivity factor UL42, and that these residues have a high probability of forming an α-helix with one side of the surface rich in hydrophobic residues.

To lend further support to the idea that the C-terminal residues of 43P play a direct role in functionally important binding to 45P, it should be noted that there is a strong sequence similarity between the C-terminal 11 residues of T_4 gene 33 protein and the corresponding residues in T_4 43P (Figure 1B). Winkelman et al. (1994) have suggested that the C-terminus of T_4 33P is necessary for functional interactions with 45P which also acts as a mobile enhancer during late transcription of T_4 genes (Herendeen et al., 1989). Thus, there seems to be an emerging pattern in which the C-termini of proteins involved in replication and transcription are used as anchoring links to other proteins that also participate in these events.

As shown in Table 1, the extent of stimulation of pol and exo activities of 43P by its accessory proteins decreased as the length of truncation increased from 3 to 6 to 11 residues. The N887 mutant, with 11 residues deleted, is of particular interest since this is equivalent to the exact length of the "tail" that protrudes in the crystal structure of RB69 DNA pol (Wang et al., 1997). Although the degree of stimulation by accessory proteins was difficult to determine accurately, we nevertheless did observe a low level of stimulation when the accessory proteins were mixed with the N887 mutant DNA polymerase, suggesting that the interaction between 45P and 43P is not restricted entirely to its 11 residue tail. Other regions of 43P, such as part of the thumb domain, may also participate in 43P−45P interactions but less extensively than the tail. In the case of the N881 mutant DNA polymerase, we could not detect any stimulation of exo activity by accessory proteins. The structural integrity of the N881 mutant could have been perturbed as a consequence of deleting the additional six amino acids proximal to the tail as these residues are an integral part of the thumb domain. Interestingly, this putative structural perturbation decreases the binding affinity of the N881 mutant to the 16/24-mer, which could partly explain its greatly diminished pol activity. Note that the exo activity is increased on ds but not on ss DNA (Figure 2B,C). This result can be rationalized if the presumed structural alteration in the N881 DNA polymerase reduces the probability of ds DNA binding in the pol mode, which predominates in wild-type 43P, but increases the probability of ds DNA binding in the exo mode in the N881 mutant. The putative structural perturbation probably does not affect its affinity for ss DNA since the rates of exonucleolytic digestion of ss DNA by the N881 mutant and wild-type DNA polymerase are nearly identical.

Although our discussion assumes a direct interaction between the 43P tail and 45P, we realize that our results could also be explained if the C-terminal 43P tail has a surface complementary to 44P/62P and that this interaction was essential for the rapid assembly of the 43P−45P−DNA primer-template complex.

After completion of this paper, Berdis et al. (1996) published a paper showing that deletion of six residues from the C-terminus of an exo minus T4 DNA polymerase mutant results in its failure to form a stable holoenzyme even though the truncated DNA polymerase had nearly the same kinetic constants as the wild-type enzyme. Both their results and ours, obtained with the N892 mutant, are essentially in agreement even though different experimental strategies were used. Berdis et al. (1996) also showed that a synthetic polypeptide comprised of the C-terminal seven residues of 43P was able to inhibit interaction of the 44P/62P with 45P, resulting in inefficient formation of the holoenzyme. These authors suggest that 45P uses the same surface as the interface that mediates the binding to 44P/62P and to 43P. Further studies are needed to delineate the contribution of individual residues in the 43P 11 residue tail to the specificity and binding affinity of these DNA polymerases to their respective sliding clamps.

ACKNOWLEDGMENT

We thank Dr. Hyacinth Sterling for the gift of purified 44P/62P and 45P T_4 DNA replication accessory proteins.

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BI9708949