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A Mutation in the Gene-encoding Bacteriophage T7 DNA Polymerase That Renders the Phage Temperature-sensitive*

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Gene 5 of bacteriophage T7 encodes a DNA polymerase essential for phage replication. A single point mutation in gene 5 confers temperature sensitivity for phage growth. The mutation results in an alanine to valine substitution at residue 73 in the exonuclease domain. Upon infection of Escherichia coli by the temperature-sensitive phage at 42 °C, there is no detectable T7 DNA synthesis in vivo. DNA polymerase activity in these phage-infected cell extracts is undetectable at assay temperatures of 30 °C or 42 °C. Upon infection at 30 °C, both DNA synthesis in vivo and DNA polymerase activity in cell extracts assayed at 30 °C or 42 °C approach levels observed using wild-type T7 phage. The amount of soluble gene 5 protein produced at 42 °C is comparable to that produced at 30 °C, indicating that the temperature-sensitive phenotype is not due to reduced expression, stability, or solubility. Thus the polymerase induced at elevated temperatures by the temperature-sensitive phage is functionally inactive. Consistent with this observation, biochemical properties and heat inactivation profiles of the genetically altered enzyme over-produced at 30 °C closely resemble that of wild-type T7 DNA polymerase. It is likely that the polymerase produced at elevated temperatures is a misfolded intermediate in its folding pathway.

Protein folding represents an important yet unsolved problem in biology. In particular, the sequence-specific signals that dictate protein folding (1–3), and ultimately, biological function are still not well understood. It is of interest to identify amino acid substitutions that result in the aberrant folding of a protein, as misfolded proteins are implicated in a number of human diseases. For example, protein misfolding and aggregation have been implicated in amyloid-related disease states such as Alzheimer’s disease (4) as well as in cancer via amino acid substitutions in the tumor suppressor p53 (5). Thus, examples of amino acid substitutions that alter the folding, structure, and stability of proteins should provide a framework for the structural analysis of disease-causing mutations and in predicting the effects of some single nucleotide polymorphisms (6).

However, the prediction of protein folding based on sequence remains elusive despite a number of computational models (7–10). A genetic approach to studying folding pathways of globular proteins involves altering specific amino acid residues to determine their role in modulating the activity and structure of a protein (11–14). Temperature-sensitive mutants retain gene function at lower “permissive” temperatures but not at elevated “non-permissive” temperatures. One class of temperature-sensitive mutants are those that alter the folding kinetics of a protein (15).

The replicative DNA polymerase of bacteriophage T7 is the product of gene 5 of the phage. The identity of gene 5 or the structural gene for T7 DNA polymerase was elucidated initially by the use of a temperature-sensitive strain of bacteriophage T7, ts11 (16). The T7ts11 mutant strain was known to carry a mutation in gene 5. Grippo and Richardson (16) first reported that cells infected with T7ts11 phage failed to replicate the phage DNA at elevated temperatures, and extracts of the infected cells contained a DNA polymerase activity that was more heat-labile than that in extracts of cells infected with wild-type T7 phage.

T7 gene 5 protein has become a model DNA polymerase for elucidating mechanisms of DNA replication and fidelity (17, 18). This 80-kDa polymerase with its associated 3′-5′-exonuclease activity is by itself distributive for DNA synthesis and incorporates less than 15 nucleotides before dissociating from a primer terminus (19). However, in a 1:1 complex with a host protein, Escherichia coli thioredoxin, gene 5 protein processively catalyzes the addition of thousands of nucleotides at rates approaching 300 nucleotides per second (19–23). The gene 5 protein-thioredoxin complex will be referred to as T7 DNA polymerase in this manuscript.

Identification of mutations in T7 DNA polymerase that impart temperature sensitivity is of interest not only for structure-function studies but also for locating amino acid residues and specific interactions critical to folding of the polymerase. A crystal structure of T7 DNA polymerase in a catalytically trapped complex with duplex DNA and an incoming nucleotide facilitates the interpretation of such data (24). To this end, the gene 5 region of T7ts11 was sequenced in its entirety. A single mutation that encodes an alanine to valine substitution at position 73 in the exonuclease domain was found to be responsible for the temperature-sensitive phenotype. Ala-73 resides in the exonuclease domain although it is not in the active site (Fig. 1). Such mutations that are spatially removed from the active site and yet effect function are likely to shed light on conformation and folding of the protein. In this paper we describe in vivo studies with temperature-sensitive T7 phage strains that have different mutations at residue 73 of T7 DNA polymerase and biochemical analysis of the purified mutant T7 DNA polymerases.
FIG. 1. Crystal structure of T7 DNA polymerase. A ternary complex of T7 gene 5 protein with its processivity factor, thioredoxin, a primed DNA template, and an incoming 2′-deoxyynucleotide (ddGTP) determined at 2.2 Å resolution (7). Inset, Ala-73 (in red) in the exonuclease domain (helix C) and side chains of the surrounding residues (in green).

EXPERIMENTAL PROCEDURES

MATERIALS

DNA—M13 mp18–2 is a 9950-nt1 derivative of vector M13 mp8 that has been described earlier (25). The 24-nt M13 sequencing primer (−47) (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and oligonucleotides for in vitro mutagenesis were from Integrated DNA Technologies. The 24-nt M13 sequencing primer (−47)/H11002

molar ratio (100 nM) in 40 mM Tris-Cl, pH 7.5, and 50 mM NaCl and annealed by heating to 95 °C for 5 min, followed by slow cooling to room temperature. Oligonucleotide concentrations were determined spectrophotometrically. DNA concentrations are expressed in terms of primer 3′-ends unless otherwise specified. Salmon sperm DNA (Type III) was from Sigma. Salmon sperm ssDNA was prepared by alkali denaturation of native salmon sperm DNA by incubation with 50 mM NaOH at 20 °C for 15 min, followed by neutralization with HCl.

Phage and Bacterial Strains—E. coli B and mR180 (F ΔlacU169 araD139 thiA rpsL relA Δde-7 pcm880) have been described (26, 27). E. coli D110 (thyA endA polA1) and C600 (thi-1 thi-1 leuB6 lacY1 tonA21 supE44 mcrB) are from the laboratory collection. E. coli HMS174 (DE3) is from Novagen. Wild-type T7 and mutant T7Δ5 (gene 5 deletion) phage are from the laboratory collection. T7ts11, T7am28 and T7am196 (26) phage were from Dr. F. W. Studier. T7am28 and T7am196 phage contain an amber mutation in gene 5 and have been described (26). Growth and manipulation of bacteriophage T7 and E. coli were carried out as described (26).

Mutagenesis of T7 Gene 5—Plasmids pGP5-A73V, pGP5-A73I, and pGP5-A73F were constructed using standard polymerase chain reaction and cloning techniques. Two oligonucleotide primers, one with a MfeI restriction site and a second with a BamHI restriction site (5′-CGGGATCCCATATGAAGAGATTGTTAAGTCA-3′ and 5′-AATCGTTGCCGATAAGACCAACATGAT-3′), were used to amplify 279 base pairs of the T7 DNA sequence encoding Ala-73. The oligonucleotides used were 5′-CTCGGTTCATATTGCAACATTTACGGTTGGG-3′ (A73V), 5′-CTCGGCTCATATGCAAACCTCAGTTGG-3′ (A73I), and 5′-CTCGGTTCATATGGCAAATGTTATCGTTTTG-3′ (A73F). Sequence changes from wild-type T7 gene 5 have been underlined. Polymerase chain reaction-generated fragments were digested with BamHI and MfeI, and the resulting fragment was ligated into the BamHI and MfeI sites of plasmid pGP5-3. Plasmid pGP5-3 and all mutant derivatives contain gene 5 protein under control of the T7 ϕ10 promoter. The identity of the clones was confirmed by DNA sequencing.

Enzymes and Proteins—Native T7 gene 5 protein and genetically altered gene 5 proteins (gp5-A73V, gp5-A73I) were over-produced in E. coli HMS174 (DE3) cells containing plasmids pGP5–3, pGP5-A73V, and pGP5-A73I, respectively. The 1:1 complex of polymerase and thioredoxin was purified to apparent homogeneity as described (22). Protein concentrations were determined by the method of Bradford (28) and were confirmed by amino acid analysis. Restriction enzymes were from New England Biolabs. ThermoSequenase was from Amersham Pharmacia Biotech. Bovine pancreatic DNase I was from Sigma. Rabbit polyclonal antibodies to gene 5 and gene 4 proteins were from Hazleton Research Products, Inc.

Methods

Purification of T7ts11 DNA—E. coli mR180 cells were grown at 30 °C to a cell density of 6 × 108 cells/ml. The cells were infected with T7ts11 phage at an m.o.i. of 5, and the infected cultures were grown for 3 h. Bovine pancreatic DNase I (0.05 μg/ml) was added, and the incubation continued another 15 min. The culture was then chilled on ice, and NaCl (125 mM) was added. The cell-lysate was centrifuged to remove cell debris. To the supernatant, PEG 8000 (United States Biochemical) was added to a final concentration of 10%, and after the sample was incubated at 4 °C for 2 h the solution was centrifuged. The pellet was redissolved in 40 ml of a 10% polyethylene glycol solution in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0), and the solution centrifuged. The resulting pellet was resuspended in 5 ml of TE and extracted with an equal volume of buffer-saturated phenol (pH 8.0):chloroform:isoamyl alcohol (24:24:1). The aqueous phase was dialyzed against TE. The DNA sample was purified by anion exchange chromatography (QIagen).

Sequencing of T7ts11 Gene 5—Gene 5 of T7ts11 DNA was first amplified by polymerase chain reaction. Two oligonucleotide primers, 5′-ATCGTTGCCGATAAGCACAATGTAT-3′ and 5′-CTCACTACACGGACTACAG-3′, were used to amplify the 5′-end of gene 5. The 3′-end of gene 5 was amplified using primers 5′-CTAAAGGTTGCCGACTAG-3′ and 5′-AGGAGGATCTGTTTTCGT-3′. Sequencing reactions were carried out with 32P-labeled ddNTPs and Thermosequenase using 13 primers. The products of the reaction were separated by electrophoresis on 8% polyacrylamide gels containing 50% urea and then analyzed by autoradiography.

Phage Crosses with Gene 5 Mutations on Plasmids—Crosses were carried out at 30 °C. Plasmids pGP5-A73V, pGP5-A73I, and pGP5-A73F were used to transform E. coli C600 cells, which are su+*. The cells were grown to a density of 3 × 108 cells/ml and then infected with T7am28 phage at an m.o.i. of 5. Recombinant phage (T7tsval, T7tsil, T7tsph) were isolated by plating on E. coli B cells, which are su+*. Temperature sensitivity of the recombinant phage was determined by replica plating at 30 °C and 42 °C. All of the recombinant phage were temperature-sensitive. The gene 5 region of phage DNA was sequenced to confirm the presence of each mutation.

Plating Efficiencies—The efficiencies of plating of T7 wild-type, T7ts11, T7tsil, and T7tsph phage were measured on a lawn of E. coli B cells at various temperatures. E. coli B cells were grown to a density of 2 × 1010 cells/ml. Dilutions of phage (T7, T7ts11, T7tsil, and T7tsph) were mixed with 0.5 ml of cells and 3 ml of top agar (1% tryptone, 0.5% yeast, 0.5% NaCl, 0.7% agar, pH 7.0) and

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1 The abbreviations used are: nt, nucleotide(s); m.o.i., multiplicity of infection; DTT, dithiothreitol; BSA, bovine serum albumin; ss, single stranded; ds, double stranded.
plated on TB (1% tryptone, 0.5% yeast, 0.5% NaCl, 1.5% agar, pH 7.0) plates. The plates were incubated at 25 °C, 30 °C, 37 °C, and 42 °C. The plates were analyzed for plaques after 3–15 h.

Flaoting efficiencies of T7.5 phage were measured on E. coli C600 cells at 30 °C, and 20 °C, respectively. The phage particles were pre-incubated at 37 °C for 2 h, and then the reaction was initiated by addition of Mg2+ for 0.22 °C, 37 °C, or 42 °C, and then the reactions were initiated by the addition of Mg2+ . The pre-incubation mixture (45 μl) contained 1.1 nmol [3H]-labeled M13 ss or dsDNA (in terms of total nucleotides). The reaction was initiated by the addition of 5 μl of 100 mM MgCl2. Final concentrations were 1 nmol [3H]-labeled M13 ss or dsDNA (in terms of total nucleotides), 0.02–0.4 mM DNA polymerase, and 10 mM MgCl2. Reactions also contained 40 μM Tris-Cl, pH 7.5, 5 mM DTT, 50 mM NaCl, and 50 μg/ml BSA. Reaction mixtures incubated at 42 °C also contained 30% glycerol. After incubation at 0 °C for 15 min, precipitated DNA was collected by centrifugation at 12,000 g for 30 min. The acid-soluble radioactivity was quantified by scintillation counting in UltraGold Fluor (Packard).

**RESULTS**

DNA Sequence Analysis of Gene 5 of T7ts11 Phage—T7ts11 is a temperature-sensitive mutant of phage T7 that carries a mutation in gene 5 that renders the DNA polymerase heat-labile relative to wild-type T7 DNA polymerase (16). To identify the mutations in gene 5 responsible for this phenotype, T7ts11 phage DNA was purified, and its gene 5 region sequenced. T7ts11 has two point mutations; a C to T transition at nucleotide 218 of gene 5 (base pair 14,570 in the T7 genome) and a G to A transition at nucleotide 1445 of gene 5 (base pair 15,797 in the T7 genome). These two point mutations result in two amino acid substitutions in T7 DNA polymerase: codon 73, alanine (GCA) to valine (GTA), and codon 482, arginine (CGC) to histidine (CAC), where the mutations are shown in bold.

To assess the contribution of each mutation in conferring temperature sensitivity to T7ts11 phage, the mutations were separated by recombination. The gene 5 mutation in the codon for residue 482 was introduced into the T7 chromosome by recombination between T7am 196 phage and the plasmid encoded gene 5. T7am196 phage carries an amber mutation in gene 5 and consequently does not plate efficiently on su- hosts. The amber mutation is the result of a change in codon 482 from GUC (AUA) to valine (GUA). The amber mutation is the result of a change in codon 482 from GUC (AUA) to valine (GUA) in T7am 196 phage, and the labeled DNA was purified through a Sepharose (CL-6B) column (Amersham Pharmacia Biotech). M13 [3H]ssDNA was prepared by alkali denaturation of [3H]dsDNA by incubation with 50 mM NaOH at 20 °C for 15 min, followed by neutralization with HCl.

The enzymes were preincubated with DNA in the absence of Mg2+ for 0.22 °C, 37 °C, or 42 °C, and then the reactions were initiated by the addition of Mg2+. The pre-incubation mixture (45 μl) contained 1.1 nmol [3H]-labeled M13 ss or dsDNA (in terms of total nucleotides). The reaction was initiated by the addition of 5 μl of 100 mM MgCl2. The reaction was initiated by the addition of 5 μl of 100 mM MgCl2. Final concentrations were 1 nmol [3H]-labeled M13 ss or dsDNA (in terms of total nucleotides), 0.02–0.4 mM DNA polymerase, and 10 mM MgCl2. Reactions also contained 40 μM Tris-Cl, pH 7.5, 5 mM DTT, 50 mM NaCl, and 50 μg/ml BSA. Reaction mixtures incubated at 42 °C also contained 30% glycerol. After incubation at 0 °C for 15 min, precipitated DNA was collected by centrifugation at 12,000 g for 30 min. The acid-soluble radioactivity was quantified by scintillation counting in UltraGold Fluor (Packard).

Nucleotide Turnover Assays—The rate of hydrolysis of nucleoside triphosphates to the corresponding monophosphate (turnover) during DNA synthesis was measured using primed M13 ssDNA and [α-32P]dATP. The reaction mixture for turnover assays was essentially the same as for polymerase assays with primed M13 DNA. Reactions contained 1 μM polymerase and 30% glycerol. Incorporation of [α-32P]dAMP into the primer strand was measured as described for polymerase assays. The amount of [α-32P]dAMP obtained upon exo¬nucleolytic hydrolysis was quantified by TLC. An aliquot of the reaction mixture was applied to a polyethyleneimine plate (JT Baker) and predeveloped with distilled water. The thin layer plate was developed with 0.5 6 LiCl in 1 M formic acid. [α-32P]dAMP formed was measured using phosphorimage analysis with a Fuji BAS 1000 bio-imagining analyzer.

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polymerases that contain substitutions only at codon 73.

Ability of Plasmid-encoded Gene 5 with Mutations at the Codon for Residue 73 to Complement the Growth of T7 Phage Containing a Deletion of Gene 5—To analyze the role of residue 73 in T7 DNA polymerase, we constructed four mutants in which its codon, which normally codes for alanine, was changed to code for either valine, leucine, isoleucine, or phenylalanine. The ability of each of these mutant proteins produced from a plasmid to complement the growth of a T7 phage deleted for gene 5 (T7Δ5) was determined at 25 °C and 42 °C (Table I). In the absence of gene 5, no phage were detected at either 25 °C or 42 °C, since the phage requires T7 DNA polymerase for growth (26). At 25 °C, all four mutants produced plaques at the same efficiency as with the plasmid expressing the wild-type gene 5. When residue 73 is changed to valine, isoleucine or phenylalanine, the plaques were the same size as when residue 73 was alanine (wild-type). In contrast, when residue 73 was leucine, the plaques were significantly smaller than those produced with the wild-type gene 5. At 42 °C, the only gene 5 mutant that complemented the growth of T7 phage is the one in which residue 73 was changed to alanine (wild-type). A likely explanation for this is the higher expression of gene 5 from a plasmid than from the T7 chromosome due to the high copy number of the plasmid. To study the effect of lower concentrations of these mutant proteins on temperature sensitivity of phage growth, we introduced each of these four mutations back into gene 5 on the T7 chromosome.

Introduction of Gene 5 Mutations in the Codon for Residue 73 into T7 Phage—Gene 5 mutations in the codon for residue 73 were introduced into the T7 chromosome by recombination

| Plasmid   | Amino acid substitution | Efficiency of plating |
|-----------|-------------------------|-----------------------|
| pT7–7     | No gene 5               | 10^-6                 |
| pGP5–3    | Wild-type               | 1.0                   |
| pGP5-A73V | Ala-73 to Val           | 1.0                   |
| pGP5-A73L | Ala-73 to Leu           | 1.0                   |
| pGP5-A73F | Ala-73 to Phe           | 1.0                   |

* Plates were incubated overnight.

TABLE II
Effect of Temperature on DNA Synthesis in Vivo by T7 Gene 5 Mutant Phage—T7 wild-type and T7tsval phage were compared for their ability to synthesize T7 DNA during phage growth at 30 °C and 42 °C. E. coli C600 cells were infected with T7 wild-type or T7tsval phage. Rates of in vivo DNA synthesis

| Phage* | Mutation | 25 °C | 30 °C | 37 °C | 42 °C |
|--------|----------|-------|-------|-------|-------|
| T7     | Wild-type| 1.0 (+)| 1.0 (+)| 1.0 (+)| 1.0 (+)|
| ts11   | A73V, R482H| 1.0 (±)| 1.0 (±)| 0.3 (−)| <10^-5 |
| tsval  | A73V     | 1.0 (±)| 1.0 (±)| 0.2 (−)| <10^-5 |
| tsile  | A73I     | 1.0 (±)| 0.6 (±)| 0.2 (−)| <10^-5 |
| tsphe  | A73F     | 1.0 (−)| 0.5 (±)| <10^-7| <10^-7 |

* T7 refers to wild-type T7 phage. ts phage refer to mutant strains of T7 that exhibit a temperature-sensitive phenotype. The substitutions at residue 73 are shown.

Plating efficiencies of each phage on E. coli B is given relative to the number of plaque-forming units at 25 °C for that phage and represent an average of three experiments. Signs in parentheses (+, ±, or −) indicate large, medium, or small plaques, respectively.

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infection, the cells were pulse labeled with \[3H\]thymidine for 90 s. The labeling was terminated by the addition of EDTA and SDS, and the incorporation of \[3H\]thymidine was measured on DE81 filter discs as described under “Experimental Procedures”. The plots represent \[3H\]thymidine incorporated (cpm) as a function of time after infection.

were measured by monitoring the incorporation of \[3H\]thymidine into DNA after phage infection (Fig. 2). Fig. 2, (left panel) shows a plot of the incorporation of \[3H\]thymidine as a function of the time of phage infection at 30°C. T7 DNA synthesis in infected cells started ~10 min after infection, after the shutdown of host DNA synthesis (26). In T7tsval-infected cells, DNA synthesis also started to increase 10 min after infection, and at 30 min the level of DNA synthesis was about half that with wild-type phage. The kinetics of DNA synthesis by T7tsval phage at 30°C was in dramatic contrast to the kinetics of its DNA synthesis at 42°C (right panel). Upon infection of cells with wild-type T7 phage at 42°C, T7 DNA synthesis started ~4 min after infection and reached a maximum at 10 min. In contrast, with T7tsval phage at 42°C, DNA synthesis declined immediately upon infection, and there was no detectable DNA synthesis even after 40 min, at which time the cells have lysed.

**Effect of Temperature on the Production of T7 Gene 5 Protein**—To determine whether the failure of T7tsval phage to synthesize T7 DNA at 42°C is due to a failure to synthesize T7 DNA polymerase, decreased solubility of the protein, increased proteolysis of the protein, or the synthesis of an inactive enzyme, we examined the amount of soluble gene 5 protein produced after T7tsval infection at both permissive and non-permissive temperatures. We also determined the polymerase activity in extracts prepared from the infected cells. T7 wild-type or T7tsval phage were used to infect E. coli D110 cells growing at 30°C or 42°C at an m.o.i. of 5. After 15 min, the cells were lysed, and the cell debris removed by centrifugation. The amount of gene 5 protein present in the supernatant was measured by immunoblot analysis using polyclonal antibodies against gene 5 protein (Fig. 3). As a control, the amount of gene 4 protein present in each extract was also measured using a separate antibody specific for this protein. As judged from densitometry of the blots, the amount of gene 5 protein is constant in wild-type T7 and T7tsval-infected cell extracts prepared at either permissive or non-permissive temperatures. Thus the temperature-sensitive phenotype is not due to differential expression, solubility, or susceptibility to proteolysis.

To determine whether the temperature-sensitive phenotype is due to the production of a catalytically inactive gene 5 protein at the non-permissive temperature, we measured the DNA polymerase activity in extracts prepared from T7 phage-infected D110 cells (Table III). D110 cells are deficient in the host DNA polymerase I activity and thus have low levels of host DNA polymerase activity in extracts (32). When the cells were infected at 30°C, DNA polymerase activity in extracts prepared from wild-type T7-infected cells was 2.5-fold higher than the activity in extracts prepared from T7tsval-infected cells whether the assay was carried out at 30°C or 42°C. In contrast, when the extracts were prepared from cells infected at 42°C, there was no detectable DNA polymerase activity in the extracts prepared from T7tsval, regardless of whether the assay was carried out at 30°C or 42°C. As a control, with extracts prepared from cells infected with wild-type T7 phage at 42°C, the activity was ~60% of the amount observed when the cells were infected at 30°C, irrespective of the assay temperature. Thus gene 5 protein with residue 73 mutated to a valine is inactive when it is synthesized at the non-permissive temperature, even when assayed at the permissive temperature. However, it is not detectably more temperature-sensitive when it is synthesized at the permissive temperature and assayed at the non-permissive temperature.

**DNA Synthesis In Vitro by Purified Gene 5 Proteins**—To characterize the temperature-sensitivity of the gene 5 proteins with mutations at residue 73 in greater detail, gp5-A73V and gp5-A73L were over-produced from cloned genes induced at 30°C and purified. We chose these two proteins to characterize biochemically as the change from alanine to valine is the relevant mutation in T7ts11 phage, while in vivo analysis implicated that substitution of residue 73 with leucine had the most drastic effect of the four mutations.

We compared the polymerase activity of wild-type T7 DNA polymerase to gp5-A73V and gp5-A73L on primed M13 ssDNA at varying temperatures (Figs. 4 and 5). The polymerase activity of gp5-A73V is within a factor of two of the activity of wild-type T7 DNA polymerase at 22°C (Fig. 5), 37°C (Figs. 4 and 5), and 42°C (Fig. 4). The polymerase activity of wild-type T7 DNA polymerase is also 2-fold higher than that of gp5-A73L at 22°C and 37°C (Fig. 5).

T7 gene 5 protein is extremely non-processive by itself. As a result, the rate of DNA synthesis on long templates like M13 DNA is only a few percent of processive DNA synthesis observed when it is in a complex with thioredoxin (19). The rates of DNA synthesis on M13 DNA observed with gp5-A73V and gp5-A73L indicate normal processivity. The processivity of DNA synthesis catalyzed by gp5-A73V at 22°C and 42°C was also measured directly by measuring the length of products formed by gel electrophoresis. M13 ssDNA annealed to a 5′-32P-labeled 24-nt oligonucleotide was used as the primer-template. To measure the length of products formed from single primer-template-DNA binding events, the concentration of the primer-template was maintained in excess over the polymerase (33). Aliquots of the reaction mixture were removed at different times and were subjected to electrophoresis in an agarose gel. The processivity of gp5-A73V is in the order of thousands of nucleotides, similar to that of T7 wild-type DNA polymerase (data not shown). This result provides strong evidence that a
normal complex is formed between the polymerase and thioredoxin.

**Heat Inactivation of Mutant Gene 5 Proteins**—To determine whether replacing the side chain of residue 73 with sterically larger substituents disrupts the stability of the enzyme, we compared the heat inactivation profiles of gp5-A73V and gp5-A73L to that of wild-type T7 DNA polymerase (Fig. 6). In this experiment, the stabilities of the enzymes have been extrapolated from residual polymerase activity measured after incubation at elevated temperatures. Each polymerase was incubated at the elevated temperature, aliquots were withdrawn at the indicated periods of time, and the residual polymerase activity measured at 37 °C. Wild-type T7 DNA polymerase decays with a half-life of 4.6 min at 42 °C that increases to 5.2 min in the presence of 75% glycerol (data not shown). However, since the presence of glycerol may mask the differential temperature sensitivity of the mutant gene 5 proteins compared with wild-type T7 DNA polymerase, we also carried out this assay by incubating the enzymes at 37 °C in the absence of glycerol. At 37 °C, while wild-type T7 DNA polymerase decays with a half-life of 4.6 min, and gp5-A73L and gp5-A73V decay slightly more rapidly with half-lives of 2.3 min and 3.3 min, respectively (Fig. 6B).

**3'-5'-Exonuclease Activity of Mutant Gene 5 Proteins**—Gene 5 protein has a 3'-5'-exonuclease activity that is active on both ds and ssDNA. Thioredoxin greatly stimulates the 3'-5'-exonuclease activity on dsDNA (34). Since residue 73 is located in the

### Table III

**DNA polymerase activity in phage-infected cell extracts**

| Phage | Assay at 30 °C | Assay at 42 °C |
|-------|---------------|---------------|
| T7    | 120           | 68            |
| tsval | 49            | 49            |

*E. coli* D110 cells were infected with T7 wild-type and tsval phage at 30 °C and 42 °C at an m.o.i. of 5. The cells were lysed 15 min after infection, and the polymerase activity in the supernatant was measured at 30 °C and 42 °C as described under “Experimental Procedures.” Values are expressed as [3H]dTMP incorporated (pmol/min/mg of protein).
exonuclease domain of the T7 DNA polymerase, we measured the 3′-5′-exonuclease activity of the altered polymerases on both dsDNA and ssDNA at 37 °C and 22 °C (Fig. 7). The fraction of DNA degraded by the exonuclease activity was measured as a function of enzyme concentration. The 3′-5′-exonuclease activities on dsDNA of gp5-A73V and gp5-A73L are within 1.5-fold of the activities of wild-type T7 DNA polymerase at both 37 °C and 22 °C (Fig. 7A). The 3′-5′-exonuclease activities on ssDNA of both gp5-A73V and gp5-A73L are lower than wild-type T7 DNA polymerase by a factor of 2 at 22 °C and a factor of 4 at 37 °C (Fig. 7B).

Nucleotide Turnover by Mutant Gene 5 Proteins—Nucleotide turnover is the DNA-dependent conversion of deoxynucleoside triphosphates into their corresponding monophosphates under conditions of DNA synthesis. The polymerization of nucleotides into a primer followed by exonucleolytic degradation by the 3′-5′-exonuclease activity associated with the polymerase results in nucleotide turnover. Thus, it reflects the amount of “editing” that takes place in a reaction (35, 36). To determine whether the substitution of alanine with valine at residue 73 alters the amount of editing, the nucleotide turnover associated with gp5-A73V was compared with that of wild-type T7 DNA polymerase (Table IV). The rate of hydrolysis of deoxynucleoside triphosphate to the corresponding nucleoside monophosphate (turnover) during DNA synthesis was measured using primed M13 ssDNA and [α-32P]dATP at 22 °C, 37 °C, and 42 °C. Rates of DNA synthesis were measured by the incorporation of [α-32P]dAMP. To measure the exonucleolytic release of [α-32P]dAMP, an aliquot of the reaction mixture was removed at the indicated time and analyzed by TLC. The turnover percentage of dAMP was similar for both enzymes at all three assay temperatures.

DISCUSSION

The replicative DNA polymerase of bacteriophage T7 is encoded by T7 gene 5. A temperature-sensitive mutant of bacteriophage T7, T7ts11 was used by Grippo and Richardson (16) to demonstrate the link between gene 5 and T7 DNA polymerase. In this report, the mutation that imparts temperature sensitivity to T7ts11 phage has been identified, and the biochemical basis for the temperature sensitivity examined. Sequencing of gene 5 of T7ts11 phage revealed two point mutations from the wild-type gene. One of the mutations, a C to T transition, encodes for an alanine to valine substitution at residue 73 in T7 DNA polymerase. This single mutation confers the temperature sensitivity for T7 phage growth observed with T7ts11. The other mutation most likely arose during mutagenesis and has no demonstrable effect on phage growth.

T7 DNA polymerase, like most polymerases of the Pol I family, has a bipartite architecture with a distinct C-terminal polymerase and an N-terminal exonuclease domain (37–41). From crystallographic data, the alanine at residue 73 resides in helix C of the exonuclease domain of the polymerase (Fig. 1). Sequence alignment shows that this alanine is conserved in E. coli DNA polymerase I (Ala-928) and in the DNA polymerases from Bacillus stearothermophilus (Ala-121) and Thermus aquaticus (Ala-129). The alanine at residue 73 is buried in a hydrophobic environment and has low thermal factors and solvent accessibility. It does not appear to be close to the exonuclease active site. Mutations that are spatially removed from the active site and yet affect function could be informative about the conformation and folding of a protein. Previous work has shown that relatively large deletions (42) as well as chemical modifications (43) in the exonuclease domain of T7 DNA polymerase do not affect the polymerase activity. Thus it is especially interesting that the conservative change of alanine to valine in the exonuclease domain of T7 DNA polymerase has such a severe affect on polymerase activity.

In vivo, T7tsval phage cannot replicate its DNA at 42 °C, whereas at 30 °C, the amount of DNA synthesis approaches that of wild-type T7 phage. There are a number of possible explanations for this deficiency at the non-permissive temperature: 1) the polymerase could be poorly induced, 2) the polymerase could be insoluble, 3) the polymerase could be susceptible to proteolysis, 4) the polymerase and/or exonuclease activity could be inactive, or 5) the polymerase could be defective in interacting with another essential replication protein. To determine which of these mechanisms explains the temperature sensitivity of T7tsval, we examined the amount of soluble gene 5 protein produced by the wild-type and T7tsval phage at the non-permissive temperature. The levels of the two proteins were comparable, ruling out differences in induction, solubility, or proteolysis accounting for the temperature sensitivity.

To determine whether the altered polymerase was temperature-sensitive for polymerase activity, we measured polymerase activity at both 30 °C and 42 °C in extracts of cells infected with either wild-type or T7tsval phage at either 30 °C or 42 °C. The level of DNA synthesis activity in extracts that were infected at 30 °C with T7tsval phage was within 50% of that of cells infected with wild-type T7 phage, whether the assay was carried out at 30 °C or 42 °C. This result implies that the polymerase synthesized at 30 °C is as thermally stable as wild-type T7 DNA polymerase. Biochemical data with the purified mutant enzymes, gp5-A73V and gp5-A73L, over-produced at the lower temperatures permissive for T7 phage growth support this model. The thermal stabilities of gp5-A73V and gp5-A73L are within 2-fold of the wild-type polymerase as extrapolated from the kinetics of heat inactivation. The rate of DNA synthesis on primed M13 ssDNA, exonuclease activity on ss
and dsDNA, and nucleotide turnover during DNA synthesis catalyzed by the mutant proteins are also within 2–4-fold of the wild-type polymerase. These data suggest that the mutant enzymes synthesized at lower temperatures are folded in an active conformation that is similar to the native state of the protein. The consistent lower activity of the altered enzymes in biochemical assays may reflect a local perturbation in structure, with the Ala-73 to leucine substitution being more severe than the valine substitution.

There was no detectable polymerase activity in extracts prepared from cells infected with T7tsval phage at 42 °C, irrespective of the assay temperature. This lack of activity presumably is the consequence of misfolding of the mutant gene 5 protein when it is synthesized at the non-permissive temperature. Since DNA polymerase activity in such extracts using salmon sperm DNA as a template is dependent only on T7 DNA polymerase, the principal reason for the temperature-sensitive phenotype cannot be a defect in the ability of the misfolded polymerase to interact with other essential replication proteins. Nevertheless, to examine whether the interaction of the polymerase with another replication protein such as the helicase/primase is disrupted at elevated temperatures, interactions with the primase-helicase of bacteriophage T7 encoded by gene 4 (gp4) were examined in assays with purified proteins. T7 DNA polymerase does not catalyze strand displacement DNA synthesis at a preformed replication fork by itself; it requires the helicase activity of gp4 to catalyze rapid and extensive DNA synthesis via a rolling circle mechanism. This coupling of activity is used as a measure of a functional interaction between the two proteins (44, 45). Gp5-A73V catalyzes strand displacement DNA synthesis in complex with gp4 at elevated temperatures, implying that it interacts with gp4 in a functional mode.3

It is clear that the purified mutant gene 5 proteins synthesized at the permissive temperature interact normally with thioredoxin at all assay temperatures. This conclusion is based on the fact that the rates of extensive DNA synthesis on M13 DNA and exonuclease activity on dsDNA, activities that both require thioredoxin, of the purified mutant polymerases were within a factor of three of wild-type T7 DNA polymerase at all assay temperatures. In addition, the processivity of DNA synthesis catalyzed by the genetically altered polymerase (gp5-A73V) in complex with thioredoxin is on the order of thousands of nucleotides, similar to that of wild-type T7 DNA polymerase. We attempted to purify the mutant DNA polymerases that were over-produced at the non-permissive temperature. However, when over-produced the solubility of the mutant polymerases decreased sharply with increasing temperature. This observation is consistent with protein misfolding and aggregation at higher temperatures. The inactive T7 DNA polymerase produced at elevated temperatures from phage-infected cells was soluble (Fig. 3) presumably because it is present at much lower levels in the cell.

It seems likely that the protein synthesized at elevated temperatures is a kinetically trapped intermediate in the folding pathway of T7 DNA polymerase that does not attain the native conformation, rather than a mutant with reduced stability. We hypothesize that Ala-73 is at a site critical for correct folding of T7 DNA polymerase. In summary, biochemical data with purified proteins and in vivo studies with mutant phage strains suggest that the Ala-73 to Val substitution in T7 DNA polymerase results in a temperature-sensitive folding (tsf) phenotype, similar to that of the temperature-sensitive mutants of the P22 tail spike protein (15). Mutants that display temperature-sensitive folding defects have also been found in interleukin 1β (46), enterotoxin B (47), and EcoRI endonuclease (48). This mutant of T7 DNA polymerase differs from the well-characterized temperature-sensitive mutants of T4 lysozyme (14) that have decreased thermal stability for the native protein formed at permissive temperatures. Among temperature-sensitive mutants of DNA polymerases, to our knowledge, there are only a few well-characterized examples of DNA polymerase mutants, such as adenovirus DNA polymerase (Adls36), that have such folding defects (49). The T4 DNA polymerase mutant, tsL56 has been reported to be inactive when synthesized at elevated temperatures (50, 51) and may in fact have similar properties to the T7 DNA polymerase mutant described here. The extensively studied tsL141 and tsCB120 mutants of bacteriophage T4 also are the result of an alanine to valine mutation (at residue 737) in T4 DNA polymerase (35, 52); however this mutant retains polymerase activity even when synthesized at the restrictive temperature.

Since T7 DNA polymerase is known to tolerate extensive amino acid deletions as well as chemical modifications in its exonuclease domain, it is striking that a single amino acid substitution in the exonuclease domain disrupts polymerase activity. It will be of interest to identify suppressors to this mutation. Such suppressors should not only shed light on the mechanism of temperature sensitivity of T7 DNA polymerase, but may provide insight into improving the yield of recombinant proteins by reducing the extent of aggregation.

The prediction of critical amino acid residues that affect the structure and folding of a protein is an important but elusive task. Using a genetic approach, we have identified a residue that is crucial for the folding of T7 DNA polymerase into its biologically active form. Experimental data on model systems such as T7 DNA polymerase are invaluable in understanding protein folding and such data can be incorporated in the design of computer-based folding algorithms. Since a number of disease states are caused by protein misfolding, examples of amino acid substitutions that alter the folding of a protein are important for providing a framework for understanding the structural basis of misfolding mutations.

3 J. K. Kumar, S. Tabor, and C. C. Richardson, unpublished observation.

| Polymerase | Temperature | dAMP incorporated pmol/min (a) | dAMP formed pmol/min (b) | % Turnover (b/(b + a) × 100) |
|------------|-------------|--------------------------------|-------------------------|-----------------------------|
| Wild-type  | 22 °C       | 5.2                            | 0.15                    | 2.8                         |
| gp5-A73V   | 28 °C       | 3.9                            | 0.07                    | 2.1                         |
| Wild-type  | 37 °C       | 5.6                            | 1.9                     | 3.2                         |
| gp5-A73V   | 28 °C       | 1.1                            |                         | 3.8                         |
| Wild-type  | 42 °C       | 1.4                            | 3.1                     | 3.7                         |
| gp5-A73V   | 48 °C       | 1.8                            |                         | 3.6                         |

TABLE IV
Incorporation and turnover of dAMP during DNA synthesis on primed M13 DNA

Reaction mixtures were incubated with [α-32P]dATP and primed M13 ssDNA template. DNA synthesis was measured by the incorporation of [α-32P]dAMP as described under “Experimental Procedures.” The hydrolysis of [α-32P]dAMP to [33P]dAMP was monitored by TLC analysis of the reaction mixture.
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A Mutation in the Gene-encoding Bacteriophage T7 DNA Polymerase That Renders the Phage Temperature-sensitive

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