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Molecular and Biochemical Studies on the DNA Replication of Bacteriophage T7: Functional Analysis of Amino-terminal Region of Gene 2.5 Protein

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Abstract: The product of bacteriophage T7 gene 2.5 is a single-stranded DNA binding protein and plays an important role in T7 DNA replication, recombination, and repair. Genetic analysis of T7 phage defective in gene 2.5 shows that the gene 2.5 protein is essential for T7 DNA replication and growth (Kim and Richardson, 1993). The C-terminal truncated gene 2.5 protein (GP2.5-Δ21C) cannot substitute for wild-type gene 2.5 protein *in vivo*; suggesting that the C-terminal domain of gene 2.5 protein is essential for protein-protein interactions (Kim and Richardson, 1994; *J. Biol. Chem.* **269**, 5070-5078). Truncated gene 2.5 proteins lacking 19 residues (GP2.5-Δ19N) and 39 residues (GP2.5-Δ39N) from the amino-terminal domain were constructed by *in vitro* mutagenesis. GP2.5-Δ19N can support the growth of T7 phage lacking gene 2.5 while GP2.5-Δ39N cannot substitute for wild-type gene 2.5 protein *in vivo*; however, its ability to bind to single-stranded DNA is not affected. These results clearly demonstrate that the 20~39 amino-terminal region of gene 2.5 protein is required for T7 growth *in vivo* but may not be involved in DNA binding activity.
Key words: amino-terminal domain, gene 2.5 protein, mutation, replication.

Bacteriophage T7 has served as a model for understanding the mechanisms involved in the replication of a linear, duplex DNA molecule (Richardson, 1983). Four proteins, T7 DNA polymerase (gene 5 protein), T7 helicase/primase (gene 4 protein), T7 single-stranded DNA binding protein (gene 2.5 protein), and thioredoxin of *Escherichia coli*, account for the fundamental reactions at the DNA replication fork of bacteriophage T7 (Kim and Richardson, 1993). The product of gene 5, a DNA polymerase, forms a tight 1 : 1 complex with the 12 kDa thioredoxin of *E. coli* to achieve processivity of DNA synthesis (Modrich and Richardson, 1975; Tabor *et al.*, 1987). Gene 4 encodes two species of a 63 kDa and a 56 kDa protein, the latter lacking 63 amino acids found at the amino terminus of the 63 kDa protein. The 63 kDa gene 4 protein has both primase and helicase activity, while the 56 kDa protein has helicase activity but is devoid of primase activity (Mendelman and Richardson, 1991; Notarnicola and Richardson, 1993). Gene 2.5 protein is a single-stranded DNA binding protein. The 26 kDa protein exists as a dimer of two identical subunits. Purified gene 2.5 protein physically interacts with the phage encoded gene 5 protein (DNA polymerase) and gene 4 proteins (helicase and primase) and stimulates their activities (Kim *et al.*, 1992a, b; Kim, 1995).

Bacteriophage T7 gene 2.5 protein has been implicated in T7 DNA replication, recombination, and repair (Reuben and Gefter, 1973; Kim *et al.*, 1992a, b). Recently, Kim and Richardson (1993) have shown by genetic analysis that gene 2.5 protein is indeed essential for T7 DNA replication and growth. T7 phage that contains null mutant of gene 2.5 was constructed by homologous recombination. This mutant phage (T7Δ2.5) with a deletion of gene 2.5 does not grow in wild-type *E. coli* strains and have no detectable T7 DNA replication; the T7Δ2.5 phages grow normally in *E. coli* strains expressing wild-type gene 2.5 from a plasmid (Kim and Richardson, 1993).

In addressing the essential role of gene 2.5 protein *in vivo*, one must consider both the ability of the protein to bind to single-stranded DNA and to interact with replication proteins; T7 DNA polymerase and gene 4 proteins (helicase/primase). One approach to determining the specific roles of protein-DNA and protein-protein interactions involving gene 2.5 protein is to identify the domains of gene 2.5 protein involved in the

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interaction and to characterize mutant gene 2.5 proteins altered in these domains. Recently, Kim and Richardson (1994) have constructed a truncated gene 2.5 protein (GP2.5-Δ21C) deleted the 21 carboxyl terminal amino acids by *in vitro* mutagenesis. GP2.5-Δ21 C cannot substitute for wild-type gene 2.5 protein *in vivo*. Purified GP2.5-Δ21C protein retains its ability to bind to single-stranded DNA, but cannot interact with T7 DNA polymerase or T7 gene 4 proteins and no longer stimulates their activities (Kim and Richardson, 1994). The domain responsible for DNA binding and subunit interaction of several single-stranded DNA binding proteins is present in the amino-terminal region (Prasad and Chiu, 1987). A similar separation of this domain for putative DNA binding is present in the amino-terminal region (39~123) of gene 2.5 protein. Furthermore, the amino-terminal 24~54 residues of gene 2.5 protein share some sequence homology with ATP binding sites of known ATPases (Kim *et al.*, 1992a). In order to gain insight into the role of DNA binding, putative ATP binding, and protein-protein interactions of the amino-terminal region of gene 2.5 protein, we have constructed two truncated forms of the gene 2.5 protein lacking the amino-terminal 19 amino and 39 amino acid residues. In this paper we show that the 20~39 amino-terminal region of gene 2.5 protein is critical for DNA replication and the growth of bacteriophage T7.

Materials and Methods

Bacterial strains, and phages

Strains used in this experiment are listed in Table 1. *E. coli* HMS 262, AN1, and JH21 strains lack the chromosomal thioredoxin (*trxA*). T7Δ2.5::*trxA* was constructed by homologous recombination and has a deletion of gene 2.5 with an insertion of the *E. coli trxA* (thioredoxin) gene at the position of gene 2.5 (Kim and Richardson, 1993).

Proteins and other materials

The wild-type gene 2.5 protein was purified to apparent homogeneity from *E. coli* cells overexpressing gene 2.5 protein as described (Kim *et al.*, 1992a). T7 gene 2.5 Δ21C protein (GP2.5-Δ21C) was purified as described (Kim and Richardson, 1994). The T7 gene 2.5 Δ19N and Δ39N proteins (GP2.5-Δ19N and GP2.5-Δ39N) were purified as described (Kim and Richardson, 1994). T7 DNA polymerase (T7 gene 5 protein and *E. coli* thioredoxin complex) was purified from cells overproducing both polypeptides (Tabor *et al.*, 1987). Other enzymes were purchased from United States Biochemical Corp and Promega. T7 [^{3}H] DNA (10 cpm/pmol) (Hinkle and Chamberlin, 1972) and unlabeled T7 DNA (Richardson, 1966) were prepared as previously described. Single-stranded M13mp7 [^{3}H] DNA (45 cpm/pmol) was prepared as described by Matson and Richardson (1983). Unlabeled nucleotides were purchased from Pharmacia. [^{3}H]-labeled nucleoside triphosphates were purchased from Dupont-New England Nuclear. DE81 filter discs were obtained from Whatman (Kent, UK).

Construction of gene 2.5 plasmids

Construction of pGP2.5-WT, which encodes the wild-type gene 2.5 protein, has been described (Kim and Richardson, 1993). Gene 2.5 protein lacking the carboxyl terminal 21 amino acids (GP2.5-Δ21C) has been described (Kim and Richardson, 1994).

Preparation and cloning of DNA fragments were carried out by standard procedures (Sambrook *et al.*, 1989). *E. coli* HB101 and HMS157 cells were transformed with plasmids as previously described (Hanahan, 1985). Two different plasmids lacking the amino-terminal 19 and 39 amino acids were constructed using the polymerase chain reaction (PCR) method (Innis and Gelfand, 1990). To construct the first plasmid, two primers, a 5′-end primer with *Nde*I site (5′-CGTAGGATCCATATGGCCAAGCCGGACTAC-3′) for deletion of the amino-terminal 19 amino acids and a 3′-end primer with a *Bam*HI site (5′-CGTAGGATCCACTTAGAAGTCTCCGTC-3′) were used to amplify the T7 DNA sequences containing the gene 2.5 coding sequence except for the sequence encoding the amino-terminal 19 amino acids. PCR-generated DNA fragments were digested with *Nde*I and *Bam*HI, and then cloned into

Table 1. *Escherichia coli* strains used in this study

Strains	Genetic markers	Source
HMS157	F$^-$ *recB21 recC22 sbcA5 endA gal thi sup*	Kim & Richardson, 1993
HMS174	F$^-$ *hsdR rK12$^+$ mK12$^+$ recA1*	Campbell *et al.*, 1978
HMS262	F$^-$ *hsdR pro leu$^-$ lac$^-$ thi$^-$ supE tonA$^-$ trxA*	Kim & Richardson, 1993
JH21	F$^-$ *pcnB80, ΔtrxA307*	Himawan & Richardson, 1992
AN1	F$^-$ *Δtrx307 metE::Tn10*	Kim & Richardson, 1993
HB101	F$^-$ *Δ(mcrCmrr) leu supE44 ara14 lacY1 galK2 proA2 rpsL20(Str^r) xyl-5 mtl-1 recA13*	Boyer *et al.*, 1969

the *Nde*I and *Bam*HI sites of plasmid pT7-7, generating plasmid pGP2.5-Δ19N. Plasmid pT7-7, constructed by S. Tabor (Harvard Medical School), contains the T7 RNA polymerase promotor ϕ10 as well as a strong translation initiation region prior to the polylinker (Tabor, 1990). To construct the second plasmid, two primers, a 5′-end primer with a *Nde*I site (5′-CGTAGGATCCATATGGTTGACCTGACTATTC-3′) for deletion of the amino-terminal 39 amino acids and a 3′-end primer with the same as above were used to amplify the gene 2.5 coding sequence except for the sequence encoding the amino-terminal 39 amino acids. PCR-generated DNA fragments were digested with *Nde*I and *Bam*HI, and then cloned into the *Nde*I and *Bam*HI sites of plasmid pT7-7, generating plasmid pGP2.5-Δ39 N. The resulting pGP2.5-Δ19N and pGP2.5-Δ39N clones were selected and shown to contain an insert of the correct size and orientation. All the clones generated from the PCR-amplified DNA were sequenced and found to be correct sequences.

Measurements of DNA synthesis

DNA synthesis was measured essentially as previously described (Saito and Richardson, 1981). *E. coli* cells used in Table 2 were grown with shaking at 30℃ in M9 CAA medium. At a cell density of 3×10^8 cells per ml the bacteria were infected with the indicated T7 phages at a multiplicity of infection (m.o.i.) of 7. At the indicated times, aliquots (0.2 ml) of the phage-infected cells were removed and placed in tubes containing 10 μl of [^{3}H] thymidine (50 μCi/ml) in order to measure DNA synthesis. After 90 sec incubation at 30℃, growth was terminated by the addition of 3 ml of cold 5% trichloroacetic acid. The acid-insoluble material was collected on GF/C filters (Whatman) and washed three times with 3 ml of ice-cold 1 M HCl and two times with 3 ml of 95% ethanol. The acid-insoluble radioactivity was measured in a toluene-based solvent in a liquid scintillation counter.

Other methods

Plating efficiencies of T7 wild-type and T7Δ2.7::*trxA* phages on various *E. coli* strains were measured as follows. Bacterial strains were grown at a density of 2×10^8 cells per ml. Various T7 phages in LB were diluted (0.1 ml) and mixed with 0.2 ml bacterial culture and 3 ml of top agar, and plated on LB or LB/ampicillin plates. All plating was carried out at 30℃. The relative binding affinities of T7 gene 2.5 protein, GP2.5-Δ19N and Δ39N proteins for single-stranded M13 mp7 [^{3}H] DNA were compared using the filter binding assay described by Kim *et al.* (1992a). In all other experiments, protein concentrations were determined by measuring the absorbance at 280 nm. DNA concentrations were determined from the specific absorbance at 260 nm, and expressed as moles of PO4. Absorption spectra were recorded Kontron on a (Model UVIKON 922) spectrophotometer.

Table 2. Plating efficiencies of T7 phages on various *E. coli* strains

Strains/Plasmid	Efficiency of Plating[a]	
	T7 (WT)	T7Δ2.5::*trxA*[b]
HMS262 *trxA*$^-$	0	$<10^{-9}$
JH21 *trxA*$^-$	0	$<10^{-9}$
AN1 *trxA*$^-$	0	$<10^{-9}$
HMS262/pGP2.5-WT	$<10^{-9}$	0.95
HMS262/pGP2.5-Δ21C	$<10^{-9}$	$<10^{-8}$
HMS262/pGP2.5-Δ19N	$<10^{-9}$	0.87
HMS262/pGP2.5-Δ39N	$<10^{-9}$	$<10^{-8}$
JH21/pGP2.5-WT	$<10^{-9}$	0.93
JH21/pGP2.5-Δ19N	$<10^{-9}$	0.84
JH21/pGP2.5-Δ39N	$<10^{-9}$	$<10^{-8}$
AN1/pGP2.5-WT	$<10^{-9}$	0.92
AN1/pGP2.5-Δ19N	$<10^{-9}$	0.81
AN1/pGP2.5-Δ39N	$<10^{-9}$	$<10^{-8}$
HMS174	1	$<10^{-8}$

[a]Efficiency of plating was calculated by dividing the number of plaque forming units on a given strain by the number of wild-type T7 plaque forming units on *E. coli* HMS174.
[b]T7Δ2.5::*trxA* was selected and amplified on *E. coli* HMS262/pGP2.5-WT cells.

Results

Construction of gene 2.5 mutants

In order to examine the role of the amino-terminal domain of gene 2.5 protein for T7 DNA replication, two different mutant plasmids (pGP2.5-Δ19N and pGP 2.5-Δ39N) lacking the amino-terminal 19 and 39 amino acids from the coding sequences of the T7 gene 2.5 were constructed using the polymerase chain reaction method. Details of the constructions of the amino-terminal deletion mutants of gene 2.5 were described under "Materials and Methods". The gene 2.5 sequences in the expression vector, pGP2.5 (see Fig. 1) were exclusively expressed and the gene 2.5 proteins were purified using the methods as described (Kim *et al.*, 1992a; Kim and Richardson, 1994).

Growth of phage T7

To determine whether mutant gene 2.5 proteins (GP 2.5-Δ19N and GP2.5-Δ39N) could support the growth of phage T7, we examined the ability of T7Δ2.5::*trxA* to grow on strains in which GP2.5-Δ19N or GP2.5-Δ39N is expressed. T7Δ2.5::*trxA* has the entire gene

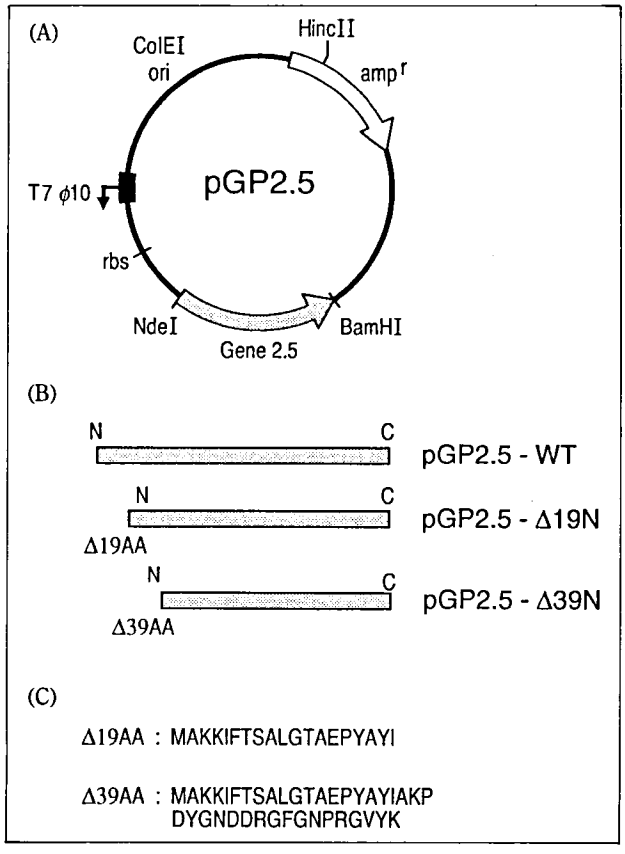

Fig. 1. Construction of Gene 2.5 Plasmids. (A) Diagram of the expression vector of gene 2.5 proteins (pGP2.5-WT, -Δ19N, and -Δ39N). Three different types of T7 gene 2.5 were inserted into a vector (pT7-7) carrying the gene for ampicillin resistance. pT7-7 contains the T7 RNA polymerase promoter φ10 as well as a strong translation initiation region prior to the polylinker (Tabor and Richardson, 1985). (B) Wild-type gene 2.5 coding sequence was amplified as described in Kim and Richardson (1993). Amino-terminal deletion mutants (19 aa and 39 aa deletions) of gene 2.5 were amplified as described under "Materials and Methods". These PCR fragments were cloned into the *Nde*I and *Bam*HI sites of pT7-7 vector, generating pGP2.5-WT, pGP2.5-Δ19N, and pGP2.5-Δ39N. (C) Deleted amino acid sequences of Δ19N and Δ39N.

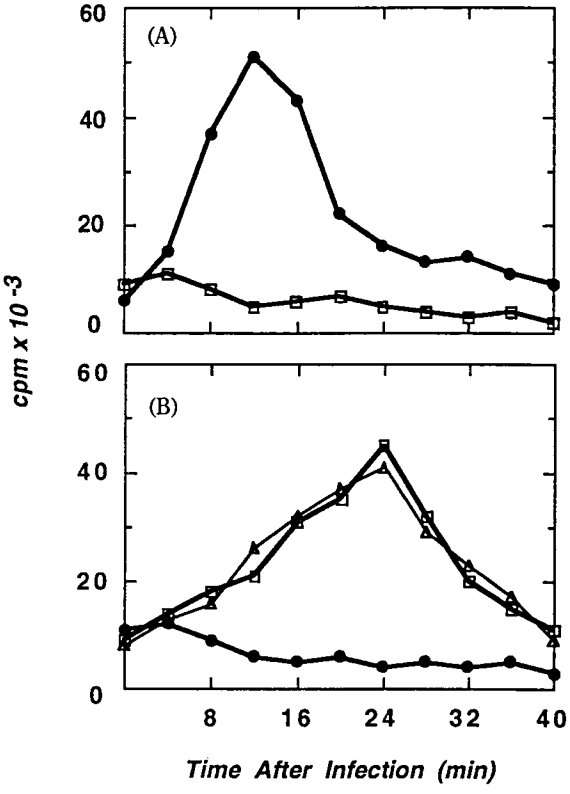

Fig. 2. Time courses of rates of DNA synthesis after infection. The rates of DNA synthesis were measured (see text) at intervals after (A) infection of *E. coli* HMS174 cells with T7 wild-type (●) or T7Δ2.5::*trxA* (□) phages, (B) infection of *E. coli* HMS174/pGP 2.5-WT (□) or HMS174/pGP2.5-Δ19N (△), and HMS174/pGP2. 5-Δ39N (●) with T7Δ2.5::*trxA* phages at a multiplicity of infection of 7 at 30℃. The values of each point are an average of data from two experiments.

2.5 replaced by the thioredoxin gene (*trxA*$^-$) of *E. coli*; T7Δ2.5::*trxA* phage is not viable and is defective in DNA synthesis (Kim and Richardson, 1993). Wild-type T7 and T7Δ2.5::*trxA* phages were plated on *E. coli* HMS262 (*trxA*$^-$) that contains plasmids producing wild-type gene 2.5 protein, GP2.5-Δ19N, and GP2.5-Δ39N, separately (Table 2). Growth of T7 phage in *E. coli* HMS262 depends on the expression of the phage encoded *E. coli* thioredoxin gene since T7 requires thioredoxin and *E. coli* HMS262 lacks the chromosomal thioredoxin gene (*trxA*). As shown in Table 2, GP2.5-Δ39N does not complement T7Δ2.5::*trxA* phage (efficiency of plating $<10^{-8}$), whereas the plating efficiency of T7Δ2.5::*trxA* phage on cells containing wild-type gene 2.5 protein or GP2.5-Δ19N is normal. Other thioredoxin mutant strains, AN1 and JH21, also give similar results as shown in Table 2. These results strongly suggest that the 20~39 amino-terminal region of gene 2.5 protein is required for T7 growth *in vivo*.

DNA synthesis

Kim and Richardson (1993) have shown that T7 gene 2.5 protein is essential for T7 DNA replication; T7 phages in the absence of gene 2.5 protein (T7Δ2.5 phages) do not grow in wild-type *E. coli* and have no detectable DNA replication but the T7Δ2.5 phages grow normally in *E. coli* strains expressing wild-type gene 2.5 protein from a plasmid. The kinetics of DNA synthesis were measured after infection with wild-type phages and T7Δ2.5::*trxA* phages of *E. coli* HMS174 (Fig. 2A). The kinetics of DNA synthesis were compared after infection of *E. coli* HMS174/pGP2.5-WT, *E. coli* HMS174/pGP2.5-Δ19N, and HMS174/pGP2.5-Δ39N with T7Δ2.5::*trxA* (Fig. 2B). As shown previously (Kim and Richardson, 1993), there was a decrease in DNA synthesis in HMS174 cells after infection with T7Δ2.5::*trxA*, presumably due to the shut-off of host

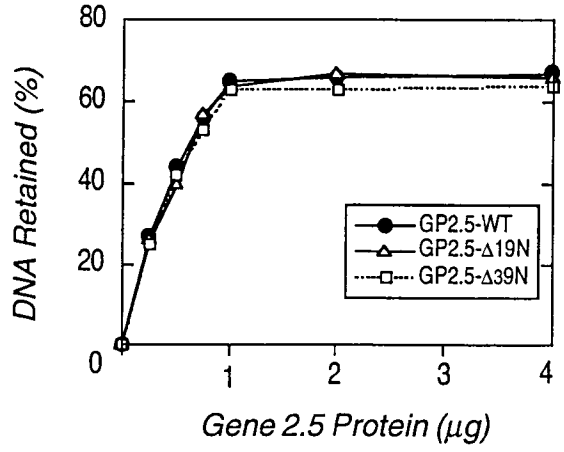

Fig. 3. Analysis of T7 gene 2.5 proteins binding to single-stranded DNA by nitrocellulose filter binding assay. T7 wild-type gene 2.5 protein (●), GP2.5-Δ19N protein (△), and GP2.5-Δ39N (□) were incubated in each reaction mixture (25 μl) at various concentrations and with 80 ng of M13mp7 [^{3}H]DNA. After incubation for 10 min at 30℃, each mixture was filtered through nitrocellulose and the amount of radioactivity retained was determined as described (Kim *et al.*, 1992a).

DNA synthesis and there was no detectable DNA synthesis observed at any time after infection. The defect in DNA synthesis could be overcome by complementation with a plasmid encoding wild-type gene 2.5 protein or GP2.5-Δ19N (Fig. 2B). In striking contrast, however, the 39 amino-terminal deleted gene 2.5 protein (GP2.5-Δ39N) could not restore any detectable DNA synthesis in T7Δ2.5::*trxA* phage infected cells (Fig. 2B).

DNA binding properties

Gene 2.5 protein binds selectively to single-stranded DNA (Scherzinger *et al.*, 1973; Reuben and Gefter, 1974; Kim *et al.*, 1992a). The relative binding affinities of T7 gene 2.5 protein, GP2.5-Δ19N, and GP2.5-Δ39 N proteins for single-stranded M13mp7 [^{3}H]DNA were compared using the nitrocellulose filter binding assay (Fig. 3). The DNA binding curves obtained with GP2.5-Δ19N and GP2.5-Δ39N proteins were essentially identical to that observed with wild-type gene 2.5 protein. For each protein, the association constant was calculated by comparing the amount of DNA retained on the filter in the presence of varying amounts of protein with the maximum amount of DNA retained in the presence of saturating levels of the protein. T7 gene 2.5 protein, GP2.5-Δ19N, and GP2.5-Δ39N proteins have a binding constant of $3.2\pm0.5\times10^6$ M^{-1}, indicating that the amino-terminal deletions did not significantly alter the DNA binding properties of gene 2.5 protein. This result clearly demonstrates that the amino-terminal region (1 to 39 amino acid residues) of gene 2.5 protein is not involved in DNA binding.

Discussion

Kim and Richardson (1993) have recently shown that T7 phages with a deletion of gene 2.5 do not grow in *E. coli* and they are defective in DNA replication. The essential nature of gene 2.5 protein for T7 growth and DNA synthesis could be explained for its specific interactions with phage encoded replication proteins, and for its role in recombination and repair. Biochemical studies on the purified gene 2.5 protein have previously demonstrated its physical interactions with T7 DNA polymerase and T7 primase/helicase (Kim *et al.*, 1992b; Kim and Richardson, 1994; Kim, 1995).

Gene 2.5 protein shares some general structural similarities with other single-stranded DNA binding proteins (SSBs), such as *E. coli* SSB and T4 gene 32 protein, which are essential components of DNA metabolism (Chase and Williams, 1986). These proteins possess the amino-terminal domains thought to play a role in DNA binding and the acidic C-terminal domains involved in the interactions of these proteins with other replication proteins (Burke *et al.*, 1980). Our recent studies show that the C-terminal acidic domain of gene 2.5 protein is essential for gene 2.5 protein function *in vivo*. In addition, the purified truncated gene 2.5 protein can no longer form dimers nor can it physically interact with T7 DNA polymerase; however, its ability to bind to single-stranded DNA is not affected.

In the present study, the role of the DNA-protein interactions of gene 2.5 protein has been evaluated by a genetic approach. The amino-terminal domains likely to be involved in these interactions were deleted. Nineteen and thirty-nine amino acids from the amino-terminal of gene 2.5 protein were deleted by *in vitro* mutagenesis. The truncated GP2.5-Δ39N protein could not substitute for wild-type gene 2.5 protein *in vivo* while GP2.5-Δ19N protein could support the growth of T7Δ2.5 phage. However, both GP2.5-Δ19N and GP2.5-Δ39N proteins bind to single-stranded DNA with the same affinities as wild-type gene 2.5 protein. The retention of DNA binding activity is surprising in the reasons that the domain putatively responsible for DNA binding in several single-stranded DNA binding proteins (Prasad and Chiu, 1987) is present in the amino terminal regions. For instance, this domain in *E. coli* SSB protein (29~117 aa) and T4 gene 32 protein (32~125 aa) has been reported (Sancar *et al.*, 1981; Lonberg *et al.*, 1981; Williams *et al.*, 1983; Shamoo *et al.*, 1989). The first 15 amino acids are essential for gene 32 protein-protein interactions in T4 (Casa-Finet, 1989). Furthermore, the amino-terminal 24~54 residues of gene 2.5 protein share some se-

quence homology with ATP binding sites of known ATPases (Kim *et al.*, 1992a). Other single-stranded DNA binding proteins, such as *E. coli* SSB and T4 gene 32 protein do not have any regions of homology to this site. Although the amino-terminal domain (20~39 aa) of gene 2.5 protein is not responsible for DNA binding according to the present studies, it is likely that this putative ATP binding site of gene 2.5 protein may play an important role in the replication of bacteriophage T7. In conclusion, the 20~39 amino-terminal region of gene 2.5 protein is essential for T7 DNA replication.

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