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Functional Studies on Gene 2.5 Protein of Bacteriophage T7 : Protein Interactions of Replicative Proteins

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Abstract

Bacteriophage T7 gene 2.5 protein, a single-stranded DNA binding protein, is required for T7 DNA replication, recombination, and repair. T7 gene 2.5 protein has two distinctive domains, DNA binding domain and C-terminal domain, directly involved in protein-protein interaction. Gene 2.5 protein participates in the DNA replication of Bacteriophage T7, which makes this protein essential for the T7 growth and DNA replication. What gene 2.5 protein makes important at T7 growth and DNA replication is its binding affinity to single-stranded DNA and the protein-protein interaction with T7 DNA replication proteins which are essential for the T7 DNA synthesis. We have constructed pGST2.5(WT) encoding the wild-type gene 2.5 protein and pGST2.5Δ21C lacking C-terminal 21 amino acid residues. The purified GST-fusion proteins, GST2.5(WT) and GST2.5Δ21C, were used for whether the carboxyl-terminal domain participates in the protein-protein interactions or not. GST2.5(WT) and GST2.5Δ21C showed the difference in the protein-protein interaction. GST2.5(WT) interacted with T7 DNA polymerase and gene 4 protein, but GST2.5 Δ21C did not interact with either protein. Secondly, GST2.5(WT) interacts with gene 4 proteins (helicase/primase) but not GST2.5Δ21C. These results proved the involvement of the carboxyl-terminal domain of gene 2.5 protein in the protein-protein interaction. We clearly conclude that carboxyl-terminal domain of gene 2.5 protein is firmly involved in protein-protein interactions in T7 replication proteins.

Key words : T7 gene 2.5 protein, replication, site-directed mutagenesis, polymerase chain reaction, protein-protein interaction

Introduction

Four proteins, T7 gene 5 protein, T7 helicase/primase, T7 gene 2.5 protein, and *E. coli* thioredoxin, account for the fundamental reactions that occur at the replication fork of bacteriophage T7.[1,2] The product of gene 5 (80 kDa) is a DNA polymerase, catalyzing the polymerization of nucleotides with low processivity. *E. coli* thioredoxin confers gene 5 to high processive DNA polymerase. Gene 4 encodes two proteins of 56 kDa and 63 kD that supply helicase and primase functions to allow leading and lagging strand DNA synthesis at a replication fork on duplex DNA.[3-5] The product of gene 2.5 is a single-stranded DNA binding protein that is essential for T7 DNA replication, recombination and repair.[2,6-11] And it has been suggested that gene 2.5 protein interacts with other essential proteins of the T7 DNA replication complex ; T7 DNA polymerase and

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the T7 63- and 56- kDa gene 4 proteins.[2,8,10-11] Gene 2.5 protein stimulates DNA synthesis catalyzed by the T7 DNA polymerase on single-stranded DNA templates and increases the processivity of the reaction.[10-11] The ability of gene 2.5 protein to stimulate synthesis of primers by gene 4 protein, has also been confirmed by affinity chromatography.[8,10-12] Gene 4 proteins exist as heterohexamer of helicase/primase complex.[13] The 56-kDa gene 4 protein is a helicase that catalyzes the unwinding of duplex DNA at the replication fork. The 63-kDa gene 4 protein of an additional 63 amino acids at its amino terminus is a primase catalyzing the synthesis of tetraribonucleotides on single-stranded DNA, which, in turn, serve as primers for T7 DNA polymerase on the lagging strand.[4-5]

Recently, the carboxyl-terminal acidic domain of gene 2.5 protein was thought to play a role in the interaction of this protein with other replication proteins.[9-10] The carboxyl-terminus of gene 2.5 protein is highly acidic. The acidic domain plays an important role in the interactions of this protein with other replication proteins.[14] Kim and Richardson have constructed a truncated form of gene 2.5 lacking the carboxyl-terminal 21 amino acid residues.[11] The deleted mutant of gene 2.5 protein cannot support the growth of T7 phage lacking gene 2.5. To confirm the protein-protein interactions of gene 2.5 protein with T7 DNA polymerase and gene 4 protein (helicase/primase), we have constructed gene 2.5 fusion proteins fused to Glutathione S-transferase (GST) gene, named wild-type gene 2.5 to GST gene as GST-2.5 (WT) and 21 C-terminal amino acids deleted gene 2.5 to GST gene as GST-2.5Δ21C, respectively. GST fusion system is a integrated system for the expression, purification, and detection of fusion protein produced in *E. coli*. In the present study, we describe the purification and characterization of fusion proteins purified from the bacterial lysates by Glutathione-Sepharose-4B affinity chromatography and shows the direct protein-protein interactions of fusion gene 2.5 proteins and T7 replication proteins using Western blot analysis.

Materials and Methods

Bacterial strain, Plasmid, and Bacteriophages

E. coli HB101(*F-Δ(mcrCmrr) leu supE44 ara14 lacY1 galK2 proA2 rpsL20(Str^r) xyl-5 mtl-1 recA13*) was used in this study. The expression vector, pGEX-3X was from Pharmacia. The growth and manipulation of bacterial strain and bacteriophage T7 was described previously.[2,9]

Proteins and Other materials

Antibodies of gene 5 protein plus thioredoxin (T7 DNA polymerase), gene 4 protein (helicase/primase), and gene 2.5 protein were from Harvard medical school. Restriction enzymes were from U. S. Biochemical Corp. and New England Biolabs. Goat anti-rabbit IgG Alkaline phosphatase was purchased from Gibco BRL. Glutathione-Sepharose-4B was obtained from Pharmacia. NBT and BCIP were from Gibco BRL. Protein standards and Coomassie Brilliant Blue were from Bio-Rad. Benzamidine hydrochloride and Phenylmethylsulfonylfluoride were from Sigma.

Construction of pGST2.5 and pGST2.5Δ21C

DNA fragments were prepared and cloned by standard procedures.[15] *E. coli* HB101 was transformed with the designated plasmids. To construct pGST2.5, pGP2.5-1 containing wild-type gene 2.5 was digested with BamHI, filled in, and cleaved with NdeI (Kim and Rchardson, 1993). pGEX-3Xb, a derivative of pGEX-3 X, which was constructed by Polymerase chain reaction using two primers, 5'-AAT TAA CAC AGT CTA TGG CCA-3' and 5'-TAT ACG TGG ATC CCC AGA TCC GAT TTT GGA GGA Tg-3', was digested with EcoRI, filled in with *E. coli* Klenow fragments, digested again with NdeI and then ligated with the insert containing the wild-type gene 2.5(Fig 1A). To construct pGST2.5Δ21C, pGP2.5Δ21C was amplified with 5'-end primer ; 5'-CGT AGG ATC CAT ATG GCT AAG AAG ATT TTC ACC TC-3' containing BamHI site and 3'-end primer

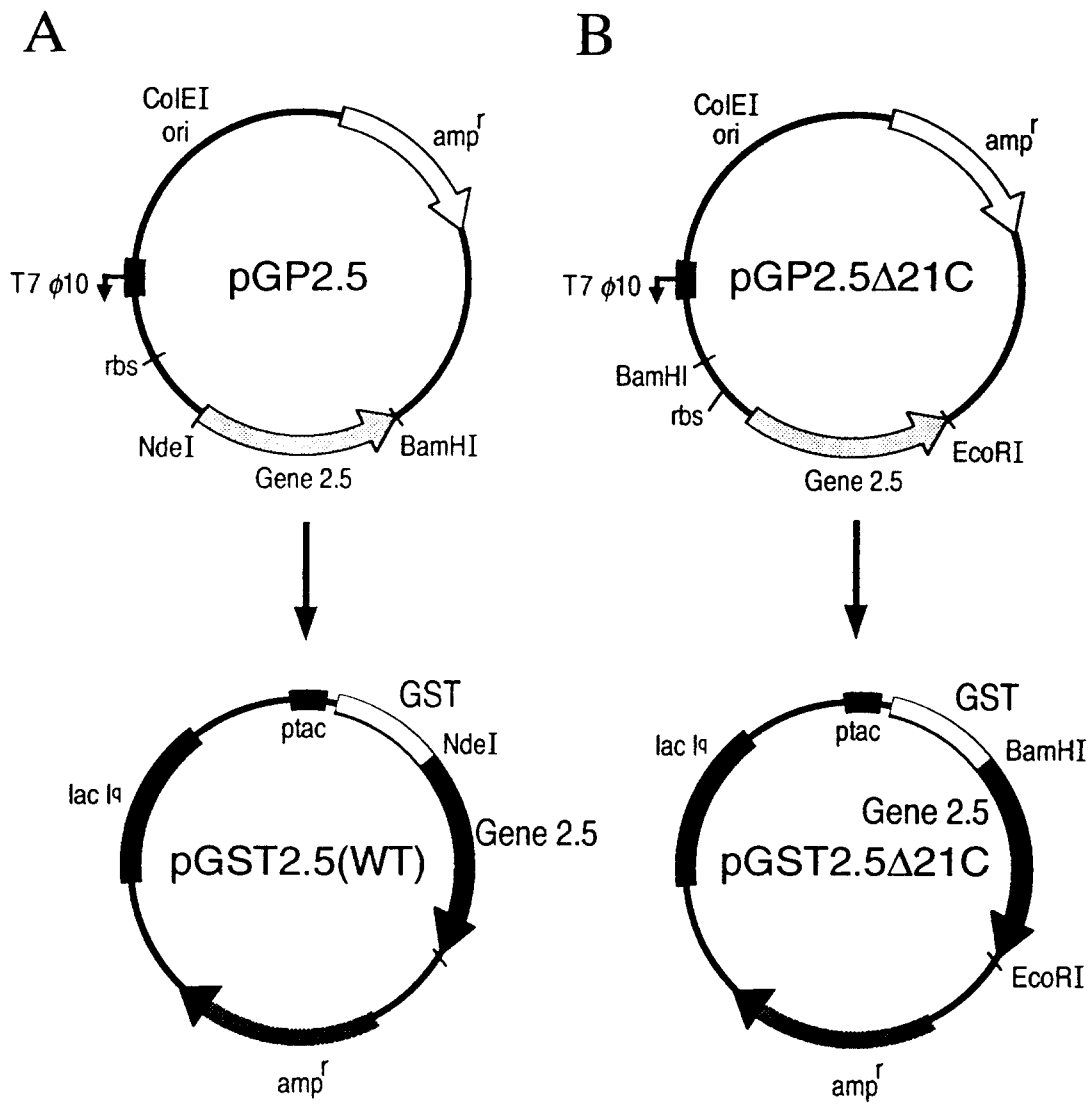

Fig. 1. Construction of pGST-2.5 and pGST-2.5Δ21C.
A. Gene 2.5 was digested from pGP2.5(WT) and ligated into pGEX-3Xb at NdeI and Bam HI sites, a derivative of pGEX-3X as described under "Experimental Procedures". B. Gene 2.5Δ21C was amplified by PCR and ligated into pGEX-3X at BamHI and EcoRI sites.

; 5'-TTT GGT GCG ATT CTT AAG AGC-3' containing EcoRI site. The amplified product was purified, digested with BamHI and EcoRI, and then ligated into pGEX-3X digested with the same enzymes(Fig. 1B).

Overexpression and Purification of GST2.5(WT) and GST2.5Δ21C

The purification of GST fusion proteins could be achieved in a simple one-step procedure from crude cell

lysates. *E. coli* HB101/pGST2.5 or/pGST2.5Δ21C were grown overnight in 2 ml of 1.6% tryptone, 1% yeast extract, 0.5% NaCl, 2% glucose (pH 7.0) and 100μg /ml ampicillin with aeration at 37℃. These cultures were diluted 1 : 10 into fresh pre-warmed 10 ml of 1.6 % tryptone, 1% yeast extract, 0.5% NaCl, 2% glucose (pH 7.0) and 100μml ampicillin. At a cell density corresponding to $A_{600}=1$, 100 mM isopropyl-β-D-thiogalactopyranosid was added to a final concentration of 0.5 mM to induce the expression of GST2.5(WT) and GST 2.5Δ21C fusion proteins. After induction, the cells were incubated for another 2 hr and then harvested by centrifugation at 6,000×g for 10 min in a Sorvall ss-34 rotor. The cell pastes were resuspended in 1 ml of ice-cold 1×PBS (150 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.3)) containing 0.1 mM phenylmethylsulfonylflouoride and 1 mM EDTA, and 250 μml of lysozyme (10mg/ml). The suspensions were lysed and centrifuged for 10 min at 12,000×g at 4℃. The supernatants were collected. One hundred microlitres of the 50% slurry of Glutathione-Sepharose-4B equilibriated with 1×PBS was added to the supernatants, and incubated with gentle agitation at room temperature for 30 min. The suspensions were centrifuged for 5 min at 500×g at 4℃ to sediment the matrix. The supernatants were removed and the Glutathione Sepharose-4B pellets were washed 3 times with 1 ml of 1× PBS. GST2.5(WT) and GST2.5Δ21C proteins bound to the matrix were used as a affinity chromatography.

Preparation of Phage-infected Cells and Cell Extract

This procedure is a slight modification of and Kim and Richardson.[2,9-10] *E. coli* HB101 was grown at 37℃ in LB broth(1% trypton, 0.5% yeast extract and 1% NaCl). At a cell density of 10⁹/ml(A590=2.0), T7 phage were added at a multiplicity of 5. Fifteen minutes after infection, the culture was chilled quickly to 4℃ and then harvested by centrifugation at 6000×g for 10 min. The cell paste were resuspended in 50 mM Tris-HCl, pH 7.5, 25 mM EDTA and 10% sucrose and again harvested by centrifugation. The cell paste were resuspended in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 10% sucrose at a cell density of 8×10^{10} cells/ml and stored at −80℃.

To prepare cell extract, frozen cells were thawed and incubated with 250μml of lysozyme at 0℃ for 15 min in the presence of 100 mM NaCl, 10 mM benzamidine hydrochloride and 0.5 mM phenylmethylsulfonyl fluoride. The suspension was incubated in a 37℃ water bath until the temperature reached to 20℃ and was then quickly cooled on ice to 4℃. The lysate was clarified by centrifugation in Sorvall ss-34 rotor at 20,000 rpm for 30 min.

Reactions between GST2.5(WT) and GST2.5Δ21C, and T7 phage-infected cell extract

GST-fusion proteins, GST2.5(WT) and GST2.5Δ21C, respectively, were incubated with phage-infected cell extract for 15 min at 30℃ with little agitation. The reaction mixture were centrifuged at 500×g for 5 min. The interactive proteins with GST2.5(WT) or GST2.5Δ21C bound to affinity resin were copurified, and washed 10 bed volume of 1×PBS, and treated with loading buffer (60 mM Tris-HCl, pH 6.8, 1% • â-mercaptoethanol, 3 % SDS, 10% glycerol, and 0.001% bromophenolblue). Samples were run on 12% polyacrylamide gel electrophoresis in the presence of 0.1% SDS and stained with Coomassie Brillant Blue.[16]

Western Blot Analysis

The GST-fusion proteins, GST2.5(WT) and GST2.5Δ 21C, and copurified proteins including T7 replicative proteins were fractionated by SDS-PAGE. The proteins were transferred from gel onto the nitrocellulose filters for 2 hrs at 220 mA. The filters were agitated in the blocking buffer 1 hr, then in the diluted antibodies of gene 5 protein plus thioredoxin, gene 4 protein and gene 2.5 protein, respectively, and incubated at room temperature for 1 hr. The filters were washed with PBST, incubated in the goat-anti rabbit-IgG-alkaline phosphatase

conjugate solution, and then developed in the NBT/BCIP solution.

RESULTS

Overexpression and Purification of GST2.5(WT) and GST2.5Δ21C

GST fusion proteins, GST2.5(WT) and GST2.5Δ21C were overexpressed on the plasmids, pGST2.5 and pGST2.5Δ21C, respectively, under the control of *tac promoter*. These proteins were purified by a single step affinity chromatography using Glutathione Sepharose-4 B. The protein-protein interactions were monitored with protein bands by SDS-PAGE and Western blot analysis using antibodies raised against wild-type gene 2.5 protein.

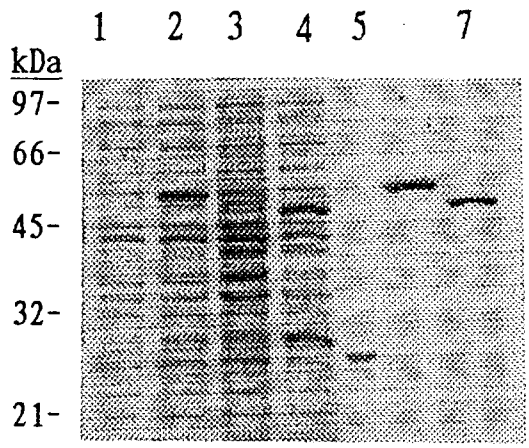

Fig. 2. Purification of GST-fusion proteins.
GST-fusion proteins, GST-2.5(WT) and GST-2.5 Δ21C, were purified by affinity chromatography and resolved by 12% polyacryamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate. Purification of GST-fusion proteins was described under "Experimental Procedures". Lane 1, uninduced cell extract of pGST-2.5 (WT) ; lane 2, induced cell extract of pGST-2. 5 ; lane 3, uninduced cell extract of pGST-2.5Δ 21C ; lane 4, induced cell extract of pGST-2.5Δ 21C ; lane 5, GST protein as control ; lane 6, purified GST2.5(WT) ; lane 7, purified GST-2.5 Δ21C. The molecular mass standards are indicated at the left.

Approximately 30μg of GST-fusion proteins were recovered from 10 ml of induced cells. The purified GST 2.5(WT) and GST2.5Δ21C proteins appear to be homogeneous as judged by SDS-PAGE(Fig. 2).

Protein-protein Interactions

The purified GST2.5(WT) and GST2.5Δ21C were used for affinity chromatography to confirm whether gene 2.5 protein interact with T7 replication proteins ; T7 DNA polymerase and helicase/primase through the acidic carboxyl-terminal domain of gene 2.5 protein. Kim and Richardson have previously shown that purified T7 DNA polymerase interact with gene 2.5 protein as one to one complex and gene 4 proteins also interact with gene 2.5 protein.[10-11] However, gene 2.5 protein lacking 21-arboxyl terminal amino acid residues do not interact with T7 DNA polymerase and gene 4 proteins (helicase/primase).[10-12,17]

Ten micrograms of purified GST2.5(WT) and GST2.5 Δ21C were reacted with T7 phage infected cell extract containing T7 DNA replicative proteins as described in "Materials and Methods." The proteins copurified with GST2.5 (WT) and GST2.5Δ21C were resolved onto a SDS-PAGE, transferred onto nitrocellulose filter. And protein-protein interactions were detected with antibodies raised against T7 DNA polymerase and gene 4 protein, respectively(Fig. 3).

Interaction of GST-gene 2.5 fusion proteins and T7 DNA polymerase

T7 DNA polymerase was copurified with GST2.5 (WT) as a complex form and detected with antibodies raised against T7 DNA polymerase, indicating that GST 2.5(WT) interacted with T7 DNA Polymerase(Fig. 3 A). While GST2.5Δ21C containing C-terminal deletion gene 2.5 protein less effectively interacted with T7

DNA polymerase, indicating that the carboxyl-terminal domain of gene 2.5 protein responsible for protein-protein interaction between T7 DNA polymerase and gene 2.5 protein(Fig. 3A). GST2.5Δ21C could not form the complex with T7 DNA polymerase. These result obtained here strongly support the report that carboxyl-terminus of gene 2.5 protein plays a crucial role in the growth and replication of T7, and interaction with T7 DNA polymerase.[2,11]

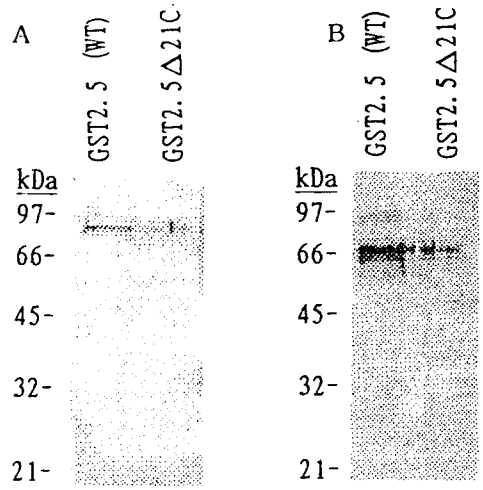

Fig. 3. Western Blot of T7 DNA replication proteins. Detection of a interactive proteins with GST-fusion proteins as described under "Experimental Procedures". The proteins from the SDS-polyacylamide gel were transferred to a nitrocellulose membrane. T7 DNA polymerase and gene 4 proteins were detected using the antibodies raised against each protein. The Western blot was developed using the chromogenic substrate 5-bromo-4-chlorl-3-indolyl phosphate/nitrobluetetrazolium (BCIP/NBT). The detected proteins were as follows ; A. T7 DNA polymerase. B. gene 4 proteins (helicase/primase).

Interaction of GST-gene 2.5 fusion proteins and T7 helicase/primase

T7 gene 2.5 protein increases the frequency of initiation of lagging strand DNA synthesis by greater than 10 fold, suggesting specific interaction of gene 2.5 protein with gene 4 proteins.[8,10-11,15-16] It plays an regulatory role at the DNA replication fork for the coordination of leading and lagging strand DNA synthesis of bacteriophage T7.[13] As shown in Fig. 3B, gene 4 protein interacted with GST2.5(WT), not with GST2.5Δ21C. GST 2.5(WT) containing the carboxyl- terminus could interact with gene 4 proteins (helicase/primase). This data also confirmed that carboxyl-terminal domain plays an essential role in gene 2.5 protein-gene 4 protein interaction.

DISCUSSION

Gene 2.5 protein is essential for T7 DNA replication and growth.[2] GP2.5Δ21C protein lacking 21 carboxyl-terminal domain cannot support the growth and replication of bateriophage T7, indicating that it cannot interact with T7 DNA replication proteins.[11] This data means that the carboxyl-terminus of gene 2.5 protein plays an essential role in the T7 DNA replication through the protein-protein interaction with T7 DNA polymerase.

T7 gene 2.5 protein increases the frequency of initiation of lagging strand DNA synthesis by greater than 10 fold, suggesting specific interaction of gene 2.5 protein with gene 4 proteins.[2,10,17] Gene 4 protein plays an regulatory role in the DNA replication fork for the coordination of leading and lagging strand DNA synthesis of bacteriophage T7.[13] At the T7 DNA replication fork, leading and lagging strand synthesis are coupled and the replication proteins are recycled. Taken together, the protein-protein interaction at the replication fork of T7 plays an essential role in the DNA synthesis.

The most update experiments that were performed at the minimal replication fork have revealed that at the replication fork of bacteriophage T7, the T7 replication proteins are regulated to couple the leading and lagging

strand DNA synthesis through the recycling of replication proteins. The recycling mechanism, not yet known well though, is thought to be controlled by the gene 4 proteins and the gene 4 proteins is the powerful candidate for coordinating events on the leading and lagging strand. This coordination can be described in terms of coupling and recycling. Coupling can be defined as the interaction between proteins responsible for leading and lagging strand synthesis, an interaction that results in interdependent synthesis of both strands. Recycling can be defined as the repeated use of the same lagging strand replication proteins in Okazaki fragment synthesis through interactions with the leading strand replication proteins. In the experiment performed by Debyser et al., 1994, the processivity of leading and lagging strand DNA synthesis dramatically increased in the presence of gene 2.5 protein.[13] It has been interpreted that the complex of gene 4 proteins requires the T7 gene 2.5 protein to remain bound to the replication fork during DNA synthesis. The previous results strongly suggested that the protein-protein interactions at the T7 DNA replication fork are important for those coordination. The results obtained in this study demonstrate that T7 DNA replication proteins interact one another.

The precise role of gene 2.5 protein *in vivo* is not known well so far, but this protein interacts strongly with T7 DNA replication proteins. This protein-protein interactions make gene 2.5 protein essential for and make possible the recycling and coupling of DNA synthesis in Bacteriophage T7.

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초록 : 박테리오파지 T7의 기능에 관한 연구 : 복제단백질간의 단백질 상호작용
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박테리오파지 T7 gene 2.5 단백질은 single-stranded DNA 결합 단백질로 박테리오파지 T7의 DNA 복제, 재조합, 및 수선에 필수적으로 요구된다. Gene 2.5 protein은 T7의 DNA 합성과 성장에 필수적인 단백질이다. Gene 2.5 protein이 중요시 되는 이유는 이 단백질이 T7의 다른 복제 필수단백질인 T7 DNA polymerase 와 gene 4 proteins(helicase/primase)와 서로 상호작용할 것으로 제안되었기 때문이다(Kim and Richardson, J. Biol. Chem., 1992 ; 1994). 이 단백질의 단백질-단백질 상호작용을 가능하게 하는 domain은 carboxyl-terminal domain일 것으로 여러 실험에서 대두되었기에, 이 domain의 특성을 파악하기 위해 야생형과 변이체 gene 2.5 단백질들을 각각 GST에 융합한 후 fusion 단백질을 정제하였다. 정제된 이 융합 단백질들의 carboxyl-terminal domain이 T7 복제 단백질들과 상호작용을 조사하는지를 조사하기 위해 affinity chromatography로 이용하였다. 실험 결과, 야생형 GST-gene 2.5 융합단백질(GST-2.5 (WT))는 T7 DNA polymerase 와 상호작용을 하였지만, 변이형 융합단백질(GST-2.5Δ21C)는 interaction을 하지 못했다. 이 결과는 carboxyl-terminal domain이 단백질-단백질 상호작용을 하는데 직접적으로 관여하는 것을 증명하였다. 또한, GST2.5(WT) 는 gene 4 protein(helicase/primase)와 직접 상호작용을 하나. GST2.5Δ21C는 상호작용을 하지 못하는 것으로 나타났다. 따라서 gene 4 proteins와의 상호작용에도 gene 2.5 protein의 carboxyl-terminal domain이 직접 관여 한다는 것이 증명되었다. 이상의 결과에서 gene 2.5 protein은 박테리오파지 T7의 유전자 복제 시 단백질-단백질 상호작용에 관여하며, 특히, gene 2.5 protein의 carboxyl-terminal domain이 이러한 상호작용에 직접적으로 관여하는 domain이라는 것을 알 수가 있었다.